EFFECTS OF METHOTREXATE ON ANTIOXIDANT ENZYME STATUS IN A RODENT MODEL

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Abstract
Methotrexate (MTX), a folic acid antagonist is widely used as a cytotoxic chemotherapeutic agent, however its associated hepatotoxicity is considered to be a major clinical side-effect. The aim of this study was to investigate the status of antioxidant enzymes during oxidative stress in liver homogenates of rats subjected to oral methotrexate administration. A total of forty two, 7-week old male Wistar rats with mean weight of 172 g, divided into two groups were used. The first group, control (n = 6), were fed only standard rat chow as their diet and water ad libitum. The second group (n = 36, subdivided into six sub-groups), fed on the same rat chow diet, received orally administered methotrexate at a dose of 13.4 mg/kg at weekly intervals for 6 consecutive weeks. Thiobarbituric acid reactive substance (TBARS) levels and the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) were subsequently determined spectrophotometrically on liver homogenates of all animals. It was found that methotrexate caused a significant increase in TBARS levels (an important marker of lipid peroxidation) in the methotrexate administered groups when compared with the control group (P < 0.05). The activities of superoxide dismutase, catalase, and glutathione reductase were significantly decreased in the methotrexate groups at weeks 2, 3, 4, 5 and 6 when compared with the control group (P < 0.05). The results therefore indicate that methotrexate causes oxidative stress by reducing the activities and consequently the effectiveness of the antioxidant enzyme defense system.

Keywords
Methotrexate, oxidative stress, superoxide dismutase, catalase, glutathione reductase, liver

Introduction
Methotrexate (MTX), a folic acid antagonist, is widely used as a cytotoxic chemotherapeutic agent in the treatment of various malignancies such as acute lymphoblastic leukaemia as well as in the treatment of various inflammatory diseases.[1, 2] The efficacy of this agent is often limited by its toxicity which causes severe side-effects and may lead to conditions such as liver cirrhosis, fibrosis of the liver, hypertrophy of the hepatocytes, hepatitis, hepato-cellular necrosis and death.[3, 4] It has also been shown that MTX toxicity has severe side-effects on the haematopoietic system[5] and liver enzymes in general.[6] The exact mechanisms of methotrexate-induced toxicity have to date not yet been elucidated.[6, 7]

Gressier, et al.[8] demonstrated that MTX increases the amount of hydrogen peroxide and other free radicals which are released by stimulated polymorphonuclear neutrophils (PMNs), which may lead to toxicity by accelerating the rate of cellular damage. It is known that MTX strongly interferes with the metabolism of homocysteine by reducing the levels of 5-methyltetrahydrofolate and as an indirect result, the levels of homocysteine, S-adenosylmethionine (SAM) were also found to decrease.[9, 10] MTX has been shown to lead to a reduction in methionine synthesis, antioxidant enzymes such as catalase, glutathione peroxidase, superoxide dismutase and a decrease of SAM (SAM acts as an antioxidant) in cerebrospinal fluid of patients on MTX treatment.[11, 12, 13] Due to its antioxidant effects, a deficiency of SAM caused by MTX may be a reason for increased reactive oxygen species (ROS) and it was shown by Villalobos et al.[14] that administration of SAM in fact caused an inhibition of lipid peroxidation in a rat model. MTX has also been reported to affect DNA and RNA synthesis by interfering with the biosynthesis of thymidine and purines.[15] It is thought that the detrimental effects of MTX is partly due to its direct toxic action by increasing ROS production. It has further been reported that MTX administration induces oxidative stress and significantly reduces antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in liver, intestinal mucosa and spinal cord tissues of rats.[15, 16] It is however known that cells are protected against oxidative stress by the action of certain enzymes, vitamins, and other substances, collectively known as antioxidants.[17] As a consequence of the constant oxidative onslaught, cells have developed antioxidant systems to counter the pro-oxidant fluxes. Included among these antioxidant mechanisms are enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR). These enzymes react directly with the oxidizing radicals to yield non-radical products. When the balance between ROS production and antioxidant defenses are altered, “oxidative stress” results, which through a series of events deregulates the cellular functions and could possibly lead to various pathological conditions.[18]

Few studies have demonstrated that methotrexate administration causes cell injury and reduction in the activities of antioxidant enzymes[19] however, the exact duration of administration as well as its resultant effects on antioxidant enzymes in the liver has not been well investigated.

This study was undertaken to investigate the antioxidant enzyme status in liver homogenates of rats subjected to oral methotrexate administration.

Materials and Methods
Methotrexate (oral) was obtained from the pharmaceutical depot at the Lagos State University Teaching Hospital (LASUTH), Ikeja, Lagos, Nigeria.
Animals
Forty-two male Wistar rats (aged 7 weeks) with a mean weight of 172g were obtained from the Laboratory Animal Production Unit of the Nigeria Institute of Medical Research (NIMR), Yaba, Lagos. All animals used in this study were treated in accordance with the principles of laboratory animal care as indicated in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. Animals were housed in a temperature controlled room (25±2°C) in which a 12 hour light : 12 hour dark cycle was maintained for one week to condition the animals prior to the start of the experiment. Standard rat chow and tap water were provided ad libitum.

Experimental design
The animals were divided into two main groups (control group and experimental group). The experimental group was further subdivided into six subgroups, making a total of seven subgroups:

- group i – control group,
- group ii – methotrexate treated rats (13.4 mg/kg body weight (recommended dosage as prescribed by the manufacturer) for one week,
- group iii – methotrexate treated rats (13.4mg/kg body weight recommended for two weeks),
- group iv – methotrexate treated rats (13.4mg/kg body weight for three weeks),
- group v – methotrexate treated rats (13.4mg/kg body weight for four weeks),
- group vi – methotrexate treated rats (13.4 mg/kg body weight for five weeks and
- group vii – methotrexate treated rats (13.4mg/kg body weight for six weeks).

Animals in the control group (group i) received standard rat chow and water but no methotrexate for six weeks. Methotrexate (13.4mg/ml) was administered orally to the animals in the experimental group for one, two, three, four, five and six weeks respectively at one interval in that order. At the end of the respective experimental periods of 1, 2, 3 4, 5 and 6 weeks, the rats were anaesthetised and sacrificed by cervical decapitation. The control animals were sacrificed at the sixth week. The livers were removed, blotted dry and immediately transferred to an ice bath.

Preparation of liver homogenates
Livers were homogenized in 0.25M sucrose solution, diluted with 0.9% saline whilst being kept in an ice bath. The homogenates were then centrifuged at 5000 x g for 30 minutes and the supernatant collected. TBARS, SOD, CAT and GR levels were determined in the supernatants.

Determination of lipid peroxidation biomarker
Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) was determined according to the method of Niehaus and Samuelsson [20] and is expressed as µmol/L.

Determination of catalase (CAT)
Catalase activity was determined according to the method of Beers and Sizer [18] by measuring the decrease in the H₂O₂ concentration and reading the absorbance at 249 nm. The amount of enzyme activity that decomposed 1 µmol of H₂O₂ per min is expressed as IU.

Determination of superoxide dismutase (SOD)
Estimation of superoxide dismutase was done by an improved spectrophotometric assay based on epinephrine auto-oxidation [29] and expressed as I U.

Determination of glutathione reductase (GR)
Activity of GR was determined according to the method of Golderg and Spooner. [20] The difference in absorbance per minute was used to calculate the enzyme activity, using a molar absorptivity of NADPH at 6.22 x 10⁵ L mol⁻¹ cm⁻¹. The activity of the enzyme is expressed as µmol of NADPH oxidized/min.

Statistical analysis
Results were expressed as mean ± SD (standard deviation). Using Student’s t-test, the level of statistical significance was set at P<0.05.

Results
This study analyzed the effect of administration of methotrexate (MTX) on the antioxidant enzyme status in the livers of rodents to whom it was given at different time intervals (in weeks).

The results showed that MTX caused a significant progressive increase in the level of TBARS (Table 1; groups i-vi) over the duration of administration.

In the MTX treated groups, the catalase activity was found to show a decrease for group iv (week 3), followed by group v (week 4), group vi (week 5), and group vii (week 6) in comparison to group i (control). However, its activity increased in group ii (week 1) followed by a progressive decrease as can be seen in group iii to group vii. (Table 2)

Methotrexate administration was shown to cause a significant decrease in superoxide dismutase activity (groups iv, v, vi, and vii i.e. at week 2, 3, 4, 5 and 6, respectively) (Table 3) and also indicated a significant decrease in glutathione reductase activity (groups v, vi and vii) in the livers as compared to the control specimens (Table 4).

Discussion
The study examined the role of oxidative stress in an attempt to suggest a possible mechanism of MTX-induced hepato-cellular damage or toxicity in a rodent model. In this study we demonstrated that MTX not only caused a significant increase in an oxidative stress biomarker (TBARS) but also a significant decrease in the status of antioxidant enzymes (SOD, CAT and GR) in the liver following administration of the drug.

It has been suggested that an increase in oxidative stress (caused by ROS) is linked to the effects of MTX and that MTX-induced toxicity increases lipid peroxidation in different tissues in rats. [21, 22] The ROS thus formed may lead to cellular damage by peroxidation of membrane lipids, sulfhydryl enzyme inactivation, protein cross-linking and DNA breakdown. It has been shown that treatment with MTX leads to a reduction in the effectiveness of antioxidant defence systems and that cellular levels of glutathione are reduced by MTX. [21] TBARS is the breakdown product of the most important chain reactions which lead to the oxidation of polyunsaturated fatty acids and is believed to serve as a reliable marker of oxidative stress-mediated lipid peroxidation. [23] Jahovic et al. [6] demonstrated in a rat model that MTX administration can result in increased levels of malondialdehyde. We believe that increased TBARS levels as observed in our study can be modulated by the likelihood that lipid peroxidation can be induced by MTX itself or as a result of a possible increase in ROS induced by MTX. It may also be considered that MTX can inhibit some antioxidant enzymes which in turn may cause lipid peroxidation to increase due to a reduction in the activities of protective antioxidant enzymes such as SOD and CAT. Gressier and
### Table 1: Effect of administration of methotrexate on TBARS in liver homogenates

<table>
<thead>
<tr>
<th>Group</th>
<th>TBARS (μmol/L)</th>
<th>T-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>i (control)</td>
<td>2.13 ± 0.61</td>
<td>1.8125</td>
<td></td>
</tr>
<tr>
<td>ii (week 1)</td>
<td>2.26 ± 0.92</td>
<td>0.2885</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>iii (week 2)</td>
<td>2.30 ± 0.04</td>
<td>0.7739</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>iv (week 3)</td>
<td>4.05 ± 1.47*</td>
<td>6.7541</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>v (week 4)</td>
<td>4.45 ± 0.54*</td>
<td>6.9755</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>vi (week 5)</td>
<td>5.11 ± 0.08*</td>
<td>11.8648</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>vii (week 6)</td>
<td>5.83 ± 0.06*</td>
<td>14.7862</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD of 6 rats in each group.
* indicates P < 0.05 i.e. mean values being significantly different from those of the control group.

### Table 2: Effect of administration of methotrexate on catalase activity in liver homogenates

<table>
<thead>
<tr>
<th>Group</th>
<th>Catalase (IU)</th>
<th>T-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>i (control)</td>
<td>39.61 ± 1.14</td>
<td>1.8125</td>
<td></td>
</tr>
<tr>
<td>ii (week 1)</td>
<td>41.11 ± 1.35*</td>
<td>0.8210</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>iii (week 2)</td>
<td>40.37 ± 1.96</td>
<td>2.0794</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>iv (week 3)</td>
<td>38.23 ± 1.44*</td>
<td>1.8405</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>v (week 4)</td>
<td>32.61 ± 1.25*</td>
<td>10.1352</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>vi (week 5)</td>
<td>32.15 ± 1.13*</td>
<td>11.3841</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>vii (week 6)</td>
<td>31.25 ± 1.15*</td>
<td>12.7575</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD of 6 rats in each group.
* indicates P < 0.05 i.e. mean values being significantly different from those of the control group.

### Table 3: Effect of administration of methotrexate on superoxide dismutase activity in liver homogenates

<table>
<thead>
<tr>
<th>Group</th>
<th>Superoxide dismutase (IU)</th>
<th>T-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>i (control)</td>
<td>6.43 ± 0.41</td>
<td>1.8125</td>
<td></td>
</tr>
<tr>
<td>ii (week 1)</td>
<td>6.14 ± 1.49</td>
<td>0.4597</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>iii (week 2)</td>
<td>6.94 ± 0.36*</td>
<td>2.2898</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>iv (week 3)</td>
<td>5.38 ± 0.59*</td>
<td>3.5798</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>v (week 4)</td>
<td>4.32 ± 0.26*</td>
<td>10.6458</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>vi (week 5)</td>
<td>4.27 ± 0.31*</td>
<td>10.2335</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>vii (week 6)</td>
<td>3.82 ± 0.81*</td>
<td>7.0421</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD of 6 rats in each group.
* indicates P < 0.05 i.e. mean values being significantly different from those of the normal healthy control group.

### Table 4: Effect of administration of methotrexate on glutathione reductase activity in liver homogenates

<table>
<thead>
<tr>
<th>Group</th>
<th>Glutathione reductase (μmol)</th>
<th>T-test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>i (control)</td>
<td>54.02 ± 2.32</td>
<td>1.8125</td>
<td></td>
</tr>
<tr>
<td>ii (week 1)</td>
<td>54.16 ± 1.81</td>
<td>0.1165</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>iii (week 2)</td>
<td>52.47 ± 2.30</td>
<td>1.1621</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>iv (week 3)</td>
<td>52.41 ± 1.64</td>
<td>1.3881</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>v (week 4)</td>
<td>43.28 ± 1.69*</td>
<td>9.1655</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>vi (week 5)</td>
<td>39.64 ± 1.46*</td>
<td>12.8500</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>vii (week 6)</td>
<td>35.88 ± 3.86*</td>
<td>9.8664</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD of 6 rats in each group.
* indicates P < 0.05 i.e. mean values being significantly different from those of the normal healthy control group (1).
co-workers have also indicated that free radicals such as hydrogen peroxide (H$_2$O$_2$) are implicated in methotrexate-induced lipid peroxidation.

Catalase, which acts as a preventative antioxidant plays an important role in protection against the deleterious effects of lipid peroxidation. In the present study, catalase activity increased in the first 2 weeks of MTX administration, but on continuous administration, catalase activity decreased significantly when compared to controls as shown in Table 2, suggesting that peroxidative damage to tissues probably commenced after 2 weeks of MTX administration.

Superoxide dismutase (SOD) catalyses the dismutation of oxygen (O$_2^-$) radical anions to hydrogen peroxide (H$_2$O$_2$) and oxygen radicals (O-). Huang et al. reported on the importance of SOD in protecting cells against oxidative stress. Our study showed a progressive decrease in SOD activity after the first week of MTX administration (Table 3) and this decrease could possibly be attributed to feedback inhibition or oxidative inactivation of enzyme proteins due to an excess of reactive oxygen species (ROS) generation. Hydrogen peroxide is known to slowly but irreversibly inactivate the enzyme SOD.

The reduction in hepatic glutathione reductase activity from the second week onwards (as shown in Table 4) indicates that the reduction of oxidized glutathione to reduced glutathione is impaired.

The findings of this study suggest that oxidative stress commences approximately after one (1) week of MTX administration and increases progressively. It has further been shown that free radical mediated damage is aggravated by a reduction in protective antioxidant enzymes caused by the MTX.

MTX therapy/administration perturbs the antioxidant system in a time dependent manner. The net result appears to show that the biological situation resulting from treatment with MTX leads to a reduction in the effectiveness of the antioxidant enzyme defense system.

It is proposed that future studies should include the determination of homocysteine, S-adenosylmethionine, caspases and other liver enzymes such as alanine amino transferase and aspartate amino transferase. It is also suggested that a histological examination of the liver should be performed. It is further proposed that it is necessary to investigate whether the negative effects of MTX on liver cells can be reversed by supplementation with antioxidants.

**Limitations of the study**

Our study focused only on the antioxidant enzyme status in the livers of rodents following methotrexate administration. Further studies on determining homocysteine and S-adenosylmethionine concentrations including caspases, alanine amino transferase and aspartate amino transferase are envisaged.

**References**


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