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**| RESEARCH ARTICLE**

# Anti-inflammatory and Anti-oxidative effects of Atorvastatin and Roflumilast against Methotrexate induced hepatotoxicity in Rats

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**| ABSTRACT**

Background: Methotrexate (MTX) is an antagonist of folic acid, frequently recommended as a cytotoxic medication to treat a number of illnesses, including leukemia and inflammatory diseases like rheumatoid arthritis and psoriasis. However, because of its severe adverse effects, particularly hepatotoxicity, its usage in clinical practice has been restricted. Members of the Statins category, sometimes referred to as HMG-CoA reductase inhibitors, include atorvastatin (ATR), which is frequently used to treat excessive cholesterol and lower the risk of cardiovascular illnesses. Roflumilast (ROF), a phosophdiestrase 4 inhibitor, assuming in treatment the chronic obstructive lung disease and has been revealed to be necessary antioxidant and anti-inflammatory properties. The current study investigated the potential hepatoprotective benefits associated with ATR and ROF in preventing MTX-induced damage to the liver in rats. Methods: 24 Wistar rats (male) were separated to four groups. Acute liver injury was induced by a dose of 20 mg/kg MTX intraperitoneally (ip.) administration for seven consecutive days . Control group: The usual intravenous saline injections were given daily. Induction group: daily IP injection of MTX (20 mg/kg). Third group (ATR+MTX) once daily (15 mg/kg, oral + 20 mg/kg, IP. Fourth (ROF+MTX) group ; Every day (5 mg/kg, oral plus 20 mg/kg; i.p) was administered to ten days. Treatment groups (third and fourth) began three days with ATR and ROF, orally before MTX injection, and continued for ten days consecutively. Results: When MTX (20 mg/kg, IP) was administered for 7 days consecutively, the levels of hepatic antioxidant enzymes, for instance glutathione [GSH], superoxide dismutase [SOD], and catalase [CAT], were significant reduced. However , the levels of malondialdehyde [MDA] and inflammatory markers; Interleukins (IL-6 and IL-1), tumor necrosis factor-alpha [TNF-a], and the liver enzymes significantly rose; alanine aminotransferase [ALT] and aspartate aminotransferase [AST]. The changes in these measures were considerably reduced after ten days of ATR and ROF treatment. In conclusion, the results suggest that ATR and ROF may be useful therapeutic agents for reducing the effects of MTX-induced liver damage in rats. It look like from our outcomes that the treatment groups appear to alleviate MTX-induced liver damage by moderating inflammation and oxidative stress in the liver.

**| KEYWORDS**

Atorvastatin, Roflumilast, Methotrexate, Hepatotoxicity, Liver enzymes

**| ARTICLE INFORMATION**

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## 1. Introduction

Methotrexate is a widely used antimetabolite and immunosuppressant which becomes particularly hepatotoxic risks when used over a long duration. The described toxicity can present as a range of liver damage including modest, yet temporary increases in liver enzymes culminating to more serious outcomes such as fibrosis and cirrhosis [1]. For patients receiving methotrexate, regular hepatotoxicity evaluation through liver enzyme tests can identify the changes indicative of incipient liver damage and allow timely intervention to minimize expansive liver damage. [1]

Even though low dose is often regarded as safe, the accumulative dosage and length of therapy are essential risks for hepatic problems, as shown in [2]. The use of folic acid supplements and other preventive agents are still under research for controlling

and preventing liver damage from methotrexate [3]. Hepatotoxicity is one enduringly harmful and surprisingly obscure impact of high-dose methotrexate (HDMTX) treatment [4].

MTX buildup in adipose tissue with metabolic dysfunction related steatotic liver disease (MASLD) and/or metabolic associated steatohepatitis (MASH) leads to slow MTX clearance which worsens the risks of hepatic damage [4].

There are numerous interconnected processes that contribute to hepatotoxicity that results from MTX treatment. Methotrexate polyglutamates (MTX-PGs) buildup in hepatocytes is a important component that affects numerous cellular processes [3, 4]. By generating reactive oxygen species (ROS) and harming the already compromised antioxidant apparatus, MTX-PGs exacerbate oxidative stress [2, 5]. The integrity of the cellular membranes and mitochondrial activity are probably negatively impacted by these activities [5].

Furthermore, MTX interferes with folate metabolism which also decreases the availability of tetrahydrofolate (THF), fundamental for DNA and RNA biopolymers [2]. Of note, these changes may trigger the release of TNF-α and IL-6 and other pro-inflammatory cytokines that are known to activate self-amplifying signaling loops, promote inflammation and fibrosis [1, 2]. MTX may actively drive hepatocytes toward apoptosis and perpetuate liver injury [5]. The presence of comorbidities such as non-alcoholic fatty liver disease and genetic predisposition may also increase the severity of these effects [1, 6]. How offset from equilibrium liver damage, which is exhibited from mild enzyme level elevations to advanced fibrosis and cirrhosis, becomes is contingent on the liver damage from mild to severe [5].

Atorvastatin is one of the most commonly recommended statins because it greatly aids in lipid control for the prevention of heart disease. Its most important action is on the hepatic free cholesterol level because atorvastatin is a competitive HMG-CoA reductase inhibitor which is the rate restrictive phase in cholesterol creation in the liver [8].

Lowest inhibition of atherogenic risk and many other cardiovascular risks, mild tendency to increase, low density lipoprotein cholesterol (LDL-C) is greatly inhibited due to this [9].

Atorvastatin as LDL-C reducer has more potent waning effect on HDL-C which is the major specie of reverse cholesterol transport from blood circulation back to the tissues [8]. Some clinical indications include primary and secondary prevention of myocardial ischemia, stroke, angina [9, 10]. A wide range of patients with type 2 diabetes, hypertension, and familial hypercholesterolemia have shown the benefit of the drug's efficacy [8].

Atorvastatin may be an effective therapy to mitigate liver damage induced by methotrexate (MTX), considering that its most valued use is as an anti-cholesterol medication. The pathways are numerous and the mechanisms, as always, are complex. According to one theory, atorvastatin's anti-inflammatory effects are helpful in the inflammation reduction associated with MTX hepatotoxicity[9]. Its antioxidants may also help one of the major culprits of MTX induced liver damage, oxidative stress[11]. It is also possible that atorvastatin might affect a cell signaling network associated with liver damage and liver recovery[11]. Some studies suggest that statins have the ability to alter the progression of MTX liver toxicity through the modulation of apoptosis and fibrosis[12].

Inhaled medications containing selective phosphodiesterase-4 (PDE4) inhibitors, such as roflumilast, are employed for treating chronic obstructive pulmonary disease (COPD) chiefly due to their anti-inflammatory mechanisms. Indication of new studies that roflumilast may lessen the hepatotoxic effects of MTX treatment. This roflumilast's hepatoprotective action stems from multiple mechanisms. Because MTX causes the liver to produce pro-inflammatory mediators such interleukin(IL-6) and TNF-α, roflumilast can potentially reduce the inflammatory response it induces [13].

Moreover, the roflumilast antioxidant effects of have been proved in various models. Roflumilast may help maintain the structure and functions of hepatocytes by reducing oxidative stress, which is the leading cause of MTX-induced liver injury [14]. Some studies postulate that the modification of intracellular signaling mechanisms responsible for apoptosis and fibrosis enables roflumilast to lessen hepatic damage and prevent more advanced liver diseases [15]. As in-vivo studies, roflumilast appears to have a protective effect on hepatic injury by decreasing MTX-related elevation of liver enzymes (ALT, AST) and improving the histology [13,14]. Roflumilast modulation of PDE4 activity may also influence other signaling molecules associated with hepatic homeostasis and repair. Current research is focused on preclinical models, but findings suggest roflumilast may pose potential as an MTX hepatotoxicity mitigation strategy. Further investigation is needed to understand the exact mechanisms as well as to confirm these hepatoprotective effects in the clinical setting [16,17]. The primary objective of this study was to experimentally demonstrate the hepatoprotective effect of ATR and ROF by evaluating their anti-inflammatory properties, taking into consideration the connection between total antioxidant capacity and general oxidative stress, and investigating important liver function enzymes in rabbits with liver injury caused by MTX.

## 2. Materials and Methods

### 2.1. Drugs and chemicals

From EBEWE Pharma (Austria) MTX was acquired of 50mg/ 2ml vial. Atorvastatin 20 mg company of Pfizer (USA) and roflumilast 500 mcg Rofaday- India were purchased from community pharmacy (Thi-Qar, Iraq), We purchased 4% dimethyl sulfoxide (DMSO) from RX Chemicals Company in Mumbai, India. The Company of Nanjing Jiancheng (China) supplied the test kits for the biochemical estimations of catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD). The Company of RX Chemicals, located in Mumbai- India, supplied all extra chemicals utilized by the study.

### 2.2 Animal

The National University of Science and Technology's Medical Research Ethics Committee accorded its approval to the animal study protocols. Twenty-four male Wistar rats weighing between 175 and 215 grams were acquired from the College of Pharmacy's animal house and split up into four groups of six. Every group had unfettered access to food and water while being kept in a cage. The rats were kept in an automated 12-hour light/dark cycle and a temperature range of 26°C to 25°C in a laboratory-like environment prior to the experiment.

### 2.3 Study Design

A liver toxicity model has been developed by giving large dosages of methotrexate (20 mg/kg, ip. injection) for 7 days in a row [18] in order to cause acute liver injury. The biochemical, and liver enzyme alterations were assessed for hepatic toxicity using this model. Four groups of six male Wistar rats each were randomly selected from a total of twenty-four animals and divided into;

- Group I, the control group, was given oral DMSO as the vehicle and an ip. injection of 0.9% NaCl (NS) solution once a day for 7 days in a row.

- Group II: MTX group got DMSO as the vehicle and a once-daily ip. injection of MTX (20 mg/kg) for 7 days in a row[18].

- Group III (ATR + MTX) was administered 20 mg/kg , IP injection of MTX and 15 mg/kg, oral of ATR once daily, three days prior the MTX injection, and this treatment was persisted for seven consecutive days [19].

- Group IV (ROF + MTX) was administered 20 mg/kg , IP injection of MTX and 5 mg/kg, oral of ROF once day a three days before the MTX injection and continued for seven days in a row[16]. On the $11^{th}$ day, all rats were sacrificed through decapitation. Blood samples were collected, and tissue samples from the liver were also extracted for further analysis.

### 2.4 Blood Samples and tissue Preparation

On the 11th day, Rats were put under anesthesia and then decapitated as sacrifices.Blood samples were taken from the heart and centrifuged using a SIGMA SM7000 (SIGMA, UK) at 3000 rpm for 10 minutes. After that, the sera were kept at -80°C for investigations of biochemical markers. Following the collection of liver samples, they were washed using cold normal saline (NS 0.09%) solution, weighed, and kept at -80°C to measure the levels of different tissue homogenate mediators.

### 2.5 Assessment of liver homogenate

Phosphate-buffered saline (PBS) was used to clean 100 mg of liver tissue to get rid of any blood before homogenizing. A polytron homogenizer model PT 3100 was then used to homogenize the tissue of liver in 10 mL PBS for five cycles at 3000 rpm. The tissue was centrifuged for 10 minutes at 3500 rpm after being kept at -20°C for 20 minutes. Prior to analysis, the specimen's supernatant was preserved in an Eppendorf tube at -80°C.

### 2.6 Biochemical Estimation

A cooled phosphate buffer with a pH of 7.4 was mixed with the liver tissues. Inflammatory markers such interleukins (IL-1 and IL-6) and tumor necrosis factor-alpha (TNF-α) were detected in liver samples using commercial kits and the sandwich ELISA technique. Additionally, antioxidants were examined; Sun et al.'s approach [20] was applied for assessing the activity of superoxide dismutase (SOD), and glutathione (GSH) was found and measured using a luminescent-based assay [21]. Similarly, the Ohkawa et al. approach was used to quantify the quantities of malondialdehyde (MDA) [22]. After a heart puncture, A clear serum is produced by collecting blood samples and centrifuging them for ten minutes at 3500 rpm. Quantifying the enzymes of AST and ALT activity in blood samples through a spectrophotometric autoanalyzer device.

### 2.7. Analytical statistics

Here, the numerical variables are displayed as mean ± standard error (SEM). For data analysis, SPSS version 26 (IBM Corp., USA) was applied. After comparing the treatment groups using the one-way analysis of variance (ANOVA), the Tuckey's test was used post hoc, furthermore, a value of less than 0.05 ($P<0.05$) was deemed to be significantly different.

## 3. Results

### 3.1. Treatment group effects on oxidative stress indices

The group that received MTX demonstrated significantly lower levels of liver antioxidant enzymes (high significance, $p<0.001$), as seen by lower levels of GSH, SOD, and CAT activities compared to the control group, with the exception of higher MDA levels. However, compared to the MTX-treated group, the treatment groups that received ATR and ROF displayed a significant ($p<0.05$) increase in these antioxidant agents (GSH, SOD, and CAT), as well as a decrease in MDA levels (table 1).

**Table (1); The effect of treatment groups on rats' oxidative stress assesses concerning MTX-induced hepatotoxicity. Applying the mean ± SEM to reveal the results. (a), showing a statistically significant variance ($p<0.05$) from the control group; (b), which demonstrates a statistically significant change ($p<0.05$) from the MTX group. (#), the significance level is high ($p<0.001$). MTX, methotrexate; ATR, atorvastatin; ROF, roflumilast; GSH, glutathione; SOD, superoxide dismutase; and MDA, malondialdehyde.**

| Groups=6 | GSH (U/mg protein) | SOD (U/mg protein) | CAT (U/mg protein) | MDA (nmol/mg protein) |
|---|---|---|---|---|
| Control | 13.99 ± 2.02 | 510.11 ± 18.89 | 79.99 ± 6.07 | 0.65 ± 0.05 |
| MTX | 5.01 ±0.23$^{a\#}$ | 217.49± 14.64$^{a\#}$ | 29.72± 1.91$^{a\#}$ | 5.01 ± 0.15$^{a\#}$ |
| ATR + MTX | 10.77 ±1.56$^{b}$ | 367.78± 15.98$^{b}$ | 59.42 ± 3.33 $^{ab}$ | 1.21 ± 0.17$^{b}$ |
| ROF + MTX | 9.98 ±1.71$^{b}$ | 356.41± 15.67$^{b}$ | 53.23 ± 2.99$^{ab}$ | 0.96 ± 0.21$^{b}$ |

### 3.2 Effects of treatment groups on inflammatory parameters

In addition, the MTX-treated group's inflammatory hepatic mediators such as (TNF-α), (IL-6), and (IL-1) were high significantly ($p<0.001$) raised when comparison with the control group. On the other hand, ATR and ROF (highly significant) treatment groups revealed greater reduction of these inflammatory mediators (TNF-α, , IL-6 and IL-1) in comparison to the MTX-treated group which showed decreased inflammation in liver tissue as demonstrated in table (2).

**Table (2); Effects of treatment groups on inflammatory parameters in rats exposed to hepatotoxicity caused by MTX. Applying the mean ± SEM to reveal the results. (a), showing a statistically significant variance ($p<0.05$) from the control group; (b), which indicates a statistically significant variance ($p<0.05$) from the MTX group. (#), the significance level is high ($p<0.001$). MTX, methotrexate; ATR, atorvastatin; ROF, roflumilast; tumor necrosis factor-alpha [TNF-a], and interleukins (IL-6 andIL-1).**

| Groups= 6 | TNF- α (ng/g tissue) | IL-6 (ng/g tissue) | IL-1 (ng/g tissue) |
|---|---|---|---|
| Control | 12.65 ± 0.56 | 6.73 ± 0.48 | 15.33 ± 2.09 |
| MTX | 55.07 ± 3.29$^{a\#}$ | 28.77 ± 1.77 $^{a\#}$ | 94.76 ± 8.56 $^{a\#}$ |
| ATR + MTX | 26.87 ± 4.78$^{ab}$ | 11.59 ± 0.85 $^{ab}$ | 29.87 ± 4.78 $^{ab}$ |
| ROF + MTX | 17.43 ± 1.37$^{b\#}$ | 9.50 ± 0.85 $^{b\#}$ | 21.67 ± 3.21 $^{b\#}$ |

### 3.3 Effects of treatment groups on liver enzymes activity

Moreover, the MTX-treated group displays marked increment ($p < 0.001$) in the activity of hepatic enzyme, specially , AST and ALT comparative to control group. Instead, ATR and ROF treated groups outcomes yield a significant decline ($p<0.05$) in these hepatic enzyme activities when compared to the MTX group demonstrating slighter liver tissue damage of rats as shown in table (3).

**Table (3); Effects of treatment groups on the activity of liver enzymes in rats exposed to hepatotoxicity caused by MTX. The mean ± SEM is applied to present the results. Statistical analysis: (a), showing a statistically significant variance ($p<0.05$) from the control group; (b), viewing a statistically significant change ($p<0.05$) from the MTX group; (#), extremely significant ($p<0.001$); [MTX], methotrexate [ATR], atorvastatin; [ROF], roflumilast ; [AST], aspartate aminotransferase and [ALT], alanine aminotransferase.**

| Groups=6 | ALT ( IU/L) serum levels | AST (IU/L ) serum levels |
|---|---|---|
| Control | 19.61±1.93 | 24.00 ± 2.45 |
| MTX | 77.93± 7.34 [a#] | 89.86± 11.99 [a#] |
| ATR + MTX | 29.17 ± 8.80 [ab] | 44.00 ± 7.25 [ab] |
| ROF + MTX | 25.67 ± 7.05 [b] | 31.30 ± 5.05 [b] |

### 4. Discussion

There are multifactorial processes that lie under methotrexate-induced hepatotoxicity such as direct cellular toxicity, oxidative stress as well as the accumulation of methotrexate polyglutamates in hepatocyte cytoplasm [2, 5]. Existing hepatic pathologies, the use of other hepatotoxic medication, and some genetic factors may also intensify the incidence and severity of hepatic injury [6, 7]. According to the current study, MTX induces hepatotoxicity for a variety of reasons. Through all of this, we attempted to demonstrate whether ATR and ROF protect the liver from MTX-induced damage. We discovered that these protective effects are mediated through the inhibition of oxidative stress and inflammatory mediators.

Our study's findings showed that administering MTX raises MDA levels and lowers the activity of antioxidant enzymes (CAT, SOD, and GSH) in liver tissue. Moreover, oxidative stress is known to be a major factor in the hepatic tissue damage brought on by MTX [23]. Hydrogen peroxides and oxygen radicals have been implicated in the emergence of multiple pathological processes linked to the negative effects of MTX on the liver [24]. Intracellular glutathione runs exhausted when MTX lowers intracellular NADPH levels [25]. Cells are more vulnerable to the effects of free radicals when intracellular glutathione, a cytosolic antioxidant, declines [26].

New study has focused on oxidative stress leading to cellular damage as a mechanism of MTX-induced liver toxicity [27–29]. Excessive ROS production from oxidative stress induction can impact intracellular components, namely proteins, lipids, and DNA, and ultimately lead to changes in cell activity and its organelles, particularly mitochondria. Numerous elements that are essential for inflammation and apoptosis are eventually activated as a result of these processes [30].

Pretreatment with ATR and ROF could preserve the integrity of the hepatocyte membrane under current experimental settings by inhibiting a key molecular pathway that causes liver damage and preventing ROS from peroxiding membrane fatty acids[31]. These biochemical alterations were greatly decreased by ATR and ROF pretreatment showing that treatment groups (3 and 4) may effectively reverse MTX-induced liver cell injury and these results in accordance with research[32,33]

The present study showed that MTX caused significantly higher levels of TNF-α, IL-6, and IL-1. Similar findings have been reported in [34,35] that the expression of cytokines was considerably augmented after treatment with MTX. The expression of the inflammatory mediators such as TNF-α, IL-6, and IL-1, are owing to activated NF-κB transfers to the nucleus of the liver cell and binds to their DNA[36,37].

ROF inhibits inflammatory cells' ability to hydrolyze cAMP. [38] Broad-spectrum anti-inflammatory effects, including decreased release of inflammatory mediators from neutrophils, decreased cytokine secretion, downregulation of cell surface markers in different cell types, and a lower incidence of programmed cell death, are consequently brought on by elevated intracellular levels of cAMP. These effects have been demonstrated to be beneficial for patients undergoing exacerbations of their COPD. especially those whose inflammatory biomarkers are higher than baseline values [39]. It has been demonstrated that ROF can lessen

inflammation in response to allergens and assist control the systemic inflammatory response to inflammation generated by lipopolysaccharides [40,41].

However, because of its capacity to reduce cholesterol, atorvastatin is a popular ingredient in statin medications. It also possesses anti-inflammatory properties, which, like all of atorvastatin's other actions, are mediated through a variety of routes. The synthesis of isoprenoids, which are essential building blocks for the activation of pro-inflammatory signaling molecules including Rho and Rac, can be inhibited by atorvastatin [42]. This suppression suppresses a number of downstream inflammatory pathways, including the activation of NF-kappa B (NF-κB), which is known to be a transcriptional regulator for the production of several inflammatory cytokines [43].

Additionally, atorvastatin has been demonstrated to diminish inflammation by altering cytokine systemic inflammation via TNF-α and IL-6 [43]. The reduction of inflammatory mediators in this study due to different mechanisms for ATR and ROF and the results resembling this study and others like it demonstrate how ATR might inhibit inflammatory processes at such critical cascade points suggesting that it could be used to treat a variety of conditions other than hyperlipidemia[44]

The current study's findings demonstrated that rats given MTX had severe liver damage, as proved by a considerable augmentation in enzyme levels of liver. The best way to detect liver necrosis is using these cytosolic enzymes [45]. Hepatocyte mortality is linked to a leak in the cell membrane, which is indicated by an increase in their activities in the serum [46]. In this instance, it has been recognized that hepatotoxicity of MTX was belonged by a noticeable rise in AST and ALT serum levels [47]. In line with previous research, the AST and ALT levels in the animals treated with ATR and ROF in our study were substantially lower than those in the animals treated with MTX [48 ].

ATR is one of the drugs that can decrease the elevation of hepatic enzyme activities in drug-induced hepatotoxicity. for example, studies such as [49] indicate that (AST and ALT) are important liver enzymes that can dramaticly elevate their activity using a range of drugs such as methotrexate (MTX). Such damage leads to the leakage of these enzymes which are indicative of hepatic injury. Nevertheless, ATR therapy is thought to reduce ALT and AST levels. This advocates that ATR be responsible for hepatoprotection against drug-induced liver damage. and constant with research [49] and alike to the current study's findings..

ROF was noted to decrease the indicators of oxidative stress, cytokines responsible for liver damage, and inflammation sequentially [50]. The beneficial characteristics of ROF justified testing higher dosages to curb MTX-induced toxicity in critical organs. The dosage was decided based on previous studies which proved ROF's anti-inflammatory action on the subject animal model [51]. Thus, the results of this study demonstrated that the dosage was adequate to protect the Wistar rats' livers from the acute hepatotoxic effect of methotrexate. The study also noted that MTX indeed resulted in an increase in the ALT and AST levels signifying liver toxicity and this was consistent with previously reported data [52-54]. It was confirmed that the increase in these enzymes due to MTX was eliminated with ROF treatment, thus confirming its hepatoprotective properties.

## 5. Conclusion

The purpose of this research was to determine whether ROF or ATR might protect against mild to moderate liver damage brought on by MTX. Our results suggest that, in addition to the studied drugs' reduction of oxidative and inflammatory processes, the protective effects of ATR or ROF against MTX-induced hepatotoxicity may also be linked to improvements in essential liver function parameters.

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