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Curative and Protective Effect of Resveratrol against Methotrexate Induced Iiver Injury in Rats

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Abstract
The present study was carried out to evaluate the hepatic changes, and antioxidant levels after using the methotrexate and investigate the protective and curative effect of resveratrol against these effects. This study was performed by determined the evaluate the liver function (AST,ALT,ALP and bilirubin) and antioxidant levels (catalase, GSH) and MDA. Twenty eight male rats were randomly divided into four equal groups. First group (Control) received distilled water only, second group treated with methotrexate (0.45 mg/kg.Bwt), third group treated with resveratrol (40 mg/kg.Bwt) 2 hrs before methotrexate (0.45 mg/kg.Bwt).fourth group treated with resveratrol (40 mg/kg.Bwt) 2 hrs after methotrexate (0.45 mg/kg.Bwt). The drugs were administrated orally for 28 days. The results showed that the animals that treated with methotrexate were suffered from liver damages by significant increasing in the liver enzymes and bilirubin and also, significant decreasing in the antioxidants (catalase and GSH), and significant increasing in the MDA.While resveratrol (before and after) methotrexate showed decreasing in the level of liver enzyme (ALT, AST and ALP) as compared with methotrexate alone. The administration of reseveratrol (before and after) methotrexate administration led to decrease in the levels of bilirubin as compared with methotrexate alone and significant increasing in the antioxidant parameters (catalase and GSH), while there were significant decreasing in MDA. In this study methotrexate induced alteration in the liver function and decreased the antioxidants levels, while the resveratrol have the ability to maintain liver function, and to increase antioxidant levels.
Key words: Resveratrol, Methotrexate, Liver Injury

Introduction

Methotrexate (MTX), being a folic acid antagonist, is reckoned a cytotoxic agent that effectively inhibits cellular growth. It is used in many clinical indications such as lymphomas, leukemia, and other malignancies; autoimmune diseases, psoriasis and rheumatoid arthritis; and ectopic pregnancy [**1].**

In spite of the cytotoxic function of MTX which causes adverse effects on pulmonary, hepatic, renal and hematopoietic tissues, yet patients can endure them [**2].**

Rat studies have indicated that the side effect of MTX is possibly related to the increasing levels of reactive oxygen species (ROS), hydroxyl radicals and hydrogen peroxide as a result of oxidative stress and lipid peroxidation [**3]** along with decreased levels of antioxidant defense molecules**.** Moreover, it has been reported that MTX reduces cellular levels of glutathione (GSH), an important cytosolic antioxidant and free radical scavenger, and it increases the levels of malondialdehyde (MDA) and myeloperoxidase (MPO) **[5]**.

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[2] Have affirmed that upregulation of p53 and p21 plays an important role in the induction of hepatocyte apoptosis following administration of a single dose of MTX in rats. Hence the reduction of MTX side effects is a hot topic in medicine although very few drugs are discovered, that reverse the MTX-induced oxidative damage in liver tissue. The liver plays a significant role in transforming and clearing chemicals and is susceptible to the toxicity from certain agents. When certain medicinal agents are taken in overdoses or introduced within therapeutic ranges, the liver may be injured. Other chemical agents as those used in laboratories and industries, natural chemicals and herbal remedies can cause hepatotoxicity. So those chemicals which cause liver injury are called hepatotoxins. As such toxic hepatitis can be caused by toxic substances and certain drugs [**2].**

In addition to the drug-induced liver injury, a substantial number of compound failures are attributed to hepatotoxicity highlighting the need for toxicity prediction models and drug screening assays, such as stem cell- derived hepatocyte-like cells, which are capable of detecting toxicity early in the drug development process. Subclinical injury to the liver which is often caused by Chemicals manifests only as abnormal liver enzyme tests **[4]**.

Resveratrol (RSV) is a polyphenol molecule which exists in different concentrations of many various plant sources. RSV is also discovered to have important anti-inflammatory, anti-mutation, antoxidant and DNA protective actions, when consumed by humans and animals. Most of the active research with RSV has been conducted in neuro and cardioprotection, yet several studies have referred to RSV's use for arthritic joint pain [**6]**.

[7] have observed the RSV intra-articular injection to animals saves cartilage and reduces the inflammatory reaction in simulated knee osteoarthritis. The anti-inflammatory properties of RSV have also been observed in experimental animal models with paw edema attributed to the suppression of inflammatory prostaglandin synthesis **[7]**. RSV is also found as a potent and specific inhibitor of TNF-α– and IL-1b–induced NF-kB activation. RSV manifests the anti-inflammatory properties as it suppresses COX-2 by blocking NF-kB activation **[8]**. Recent studies have illustrared that RSV posses many therapeutic effects on liver disorders. RSV has significantly increased survival after liver transplantation, necrosis, apoptosis and decreased fat deposition, induced by ischemia in wistar rats. It provides liver protection against cholestatic, chemical, and alcohol injury. RSV can improve glucose metabolism, lipid profile, steatosis and reduces liver fibrosis and is being able to change hepatic cell fatty acid composition **[9]**.Hence, this study aimedto evaluating the protective and curative effect of resveratrol as antioxidant and reduces toxic hepatitis in rats treated with methotrexate in rat model.

Materials and Methods

Animals:

Twenty eight (28) male rats, their age ranged between 8-10 weeks, and their weight ranged between 200 - 250 grams obtained from Samarra Drugs Industries. The animals were kept for 2 weeks for adaptation before beginning the experiment. The rats were randomly divided into six groups each group have seven animals and housed in stainless steel wire mesh cages on a bedding of wood shavings. Ambient temperature was controlled at (25 ± 3 C)with relative humidity of 50% ± 15% and a light/ dark cycle of 12 hrs. /12 hrs. Food and water were provided at all times except before drugs administration (1/2 hrs. before administration).

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Drugs Materials

The methotrexate (made by Ebewe pharma company in Austria), and Resveratrol (made by Asquared nutrition company in U.S.A.) were obtained from Samarra district pharmacies,Iraq.

Experimental Design:

Twenty eight (28) male rats divided into four equal groups and treated orally for 28 days as the following:

1-First group: was served as control and treated with distilled water.

2-Second group: was treated with 0.45 mg/kg of methotrexate orally **[5]**.

5-Third group: (Protection): was be received 40 mg/kg of resveratrol **[10]** 2 hrs before administration of methotrexate orally.

6- Fourth group: (Curative): was received 40 mg/kg of resveratrol 2 hrs after administration of methotrexate orally.

Blood Serum Samples

Blood samples were collected after the last dose from rat heart directly by (insulin syringe) in a dry, clean and sterile centrifuge tubes (gel tubes), and then left few minutes allowed to be clotted at room temperature before circulation by centrifuge at (3000) rpm for 10 minutes to separate the clear sera which were put in eppendorf tube by micropipette till performing the biochemical analysis **[11]**.

Alanine Transaminase (ALT):

Alanine Transaminase or Alanine aminotransferase (ALT or GPT) catalyzes the transfer of the amino group from alanine to 2-oxoglutarate, forming pyruvate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm, by means of the lactate dehydrogenase (LDH) coupled reaction **[12]** and **[13].**

Aspartate Transaminase (AST):

Aspartate Transaminase or Aspartate aminotransferase (AST or GOT) catalyzes the transfer of the amino group from aspartate to 2-oxoglutarate, forming oxalacetate and glutamate. The catalytic concentration is determined from the rate of decrease of NADH, measured at 340 nm, by means of the malate dehydrogenase (MDH) coupled reaction **[12]** and **[14]**.

Alkaline Phosphatase (ALP):

Alkaline phosphatase (ALP) catalyzes in alkaline medium the transfer of the phosphate group from 4-nitrophenylphosphate to diethanolamine (DEA), liberating 4-nitrophenol. The catalytic concentration is determined from the rate of 4-nitrophenol formation, measured at 405 nm [**15]**.

Bilirubin (Total and Direct)

Direct bilirubin in the sample reacts with diazotized sulfanilic acid forming a coloured complex that can be measured by spectrophotometry. Both direct and indirect bilirubin couple with diazo in the presence of cetrimide **[16]**. The terms “direct” and “total” refer to the reaction characteristics of serum bilirubin in the absence or presence of solubilizing (accelerating) reagents. The “direct” and “indirect” bilirubin is only approximately equivalent to the conjugated and unconjugated fractions.

Glutathione(GSH) in Serum

The level of glutathione in the serum is measured by using the Ellman detector method, where the sulfahydral free spectrum of the triptychione peptide interact with the Ellman detector in a basal medium to

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give a yellow-colored complex a combination of a two-sulfur compound and a nitro benzoic acid (TNB), the color intensity depend on the concentration of glutathione found in the sample [**17]**.

Malondialdehyde (MDA)

The concentration of MDA in serum was determined according to Buege and Aust method **[18]**. Malondialdehyde formed from breakdown of polyunsaturated fatty acids serves as a convenient index of peroxidation reaction. The thiobarbituric acid method was used to estimate the MDA, which reacts with thiobarbituric acid (TBA) giving pink color read at λ max 535 nm [**19]** .

Catalase Activity Assay Kit (Colorimetric/ Fluorometric)

Catalase Activity Assay Kit (Abcam, England)(Colorimetric/Fluorometric) (ab83464) is a highly sensitive, simple and direct assay for measuring catalase activity in a variety of biological samples such as cell and tissue lysates or biological fluids. In this assay, the catalase present in the sample reacts with (H_2O_2) to produce water and oxygen. The unconverted H_2O_2 reacts with probe to produce a fluorometrically at Excitation/Emission= 535/587 nm.

Therefore, the catalase activity present in the sample is reversely proportional to the signal obtained. The kit can detect as little as 1 μU of catalase activity.

Statistical Analysis

Statistical analysis of data was performed using SAS (Statistical Analysis System - version 25). One-way ANOVA and Least significant differences (LSD) post hoc test was performed to assess significant difference among means. $P < 0.05$ was considered statistically significant **[20]**.
(SAS. 2010. SAS/STAT Users Guide for Personal Computer. Release 9. 1. SAS Institute, Inc., Cary, N. C., USA.).

Ethics

The handling of animals and the experimental protocol are performed for being sure that animals do not suffer at any stage of the experiments.

Results & Discussion

The Effects of Resveratrol on Serum Liver Enzymes in Rats Treated with Methotrexate.

The rats that treated with methotrexate alone showed significant increasing ($P< 0.05$) of the serum liver enzymes included (AST, ALT and ALP) as compared with negative control group; these results are in agreement with those obtained by [**21]**. The hepatic injury induced by methotrexate led to an increasing in the serum ALT and AST in comparison with normal negative control, as indicator for marked hepatocellular injury with cholestasis. The livers enzymes normally present in the cytosol and release into the blood stream when the hepatocellular plasma membraneis damaged **[22]**. ALP is excreted normally via bile by the liver. The liver injury due to drugs can result in defective excretion of bile by hepatocytes which are reflected as their increased levels in serum **[23]**. While resveratrol (before and after) methotrexate administration showed decreasing in the level of liver enzyme (ALT) no significantly as compared with administration of methotrexate alone, but there was significant decreasing ($P< 0.05$) in the levels of (AST and ALP) as compared with administration of methotrexate alone and become near to control levels. These results are in agreement with those obtained by **[24]**. **[25]** showed revealed that RSV pretreatment provides

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protection against MTX induced hepatic damage by reducing TBARS content, as an index of lipid peroxidation, RSV treatment significantly prevented MTX-induced hepatotoxicity, as indicated by AST, ALT, and ALP levels.

In other hand, the administration of resveratrol after methotrexate led to significant decreasing (P< 0.05) in level of liver enzyme (ALP and AST) as compared with administration of methotrexate alone (Table: 1), These results are in agreement with those obtained **by [26]**.

Table (1): Show the Effects of Resveratrol on Serum Liver Enzymes in Rats Treated with Methotrexate.

Test / Group	ALT (U/L)	ALP(U/L)	AST(U/L)
Negative Control	23.2±0.37 B	49.40±0.51 B	56.20±1.62 B
Methotrexate (Positive control)	30.4±1.21 A	69.0±6.81 A	72.0±4.77 A
Resveratrol before	28.40±0.81 A	53.0±1.05 AB	67.0±2.09 AB
Resveratrol after	27.60±1.72 A	50.60±0.87 B	61.2±1.06 B
LSD	5.35	18.5	15.9

N=7

The different capital letters refer to significant differences between different treated groups at (P< 0.05).

The Effects of Resveratrol on Serum Bilirubin in Rats Treated with Methotrexate.

The rats that treated with methotrexate administration alone showed significant increasing (P<0.05) in serum bilirubin levelas compared with negative control group, this result is in agreement with that obtained by **[27]**. Increasing in the serum bilirubin has been associated with hepatocellular damage and with intra and extra-hepatic biliary tract obstruction **[5]**.

In the groups that treated with resveratrol (before and after) methotrexate administration there were significant decreasing (P<0.05) in the levels of bilirubin as compared with administration of methotrexate alone (Table:2), these results are in agreement with those obtained by **[26].**

Table (2): Show the Effects of Resveratrol on Serum Bilirubin in Rats Treated with Methotrexate

Groups	**Bilirubin**(mg/dl)
Negative Control	0.38±0.066 B
Methotrexate (Positive control)	0.62±0.086 A
Resveratrol before	0.40±0.10 B
Resveratrol after	0.35±0.06 B
LSD	0.22

N=7

The different capital letters refer to significant differences between different treated groups at (P<0.05).

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The Effects of Resveratrol on Serum Malondialdehyde (MDA), Enzymatic Antioxidant (Catalase) and non-Enzymatic Antioxidant (GSH)in Rats Treated with Methotrexate.

The group of rats that treated with administration of methotrexate alone showed significant increasing ($P<0.05$) of MDA levels, significant decreasing ($P<0.05$) of catalase and GSH levels as compared with negative control group, these results are in agreement with those obtained by [**5]**. In liver, the conversion of MTX to its major extracellular metabolite, 7-hydroxy methotrexate, takes place where it is oxidized by a soluble enzymatic system. GSH is one of the most common biologic non-enzymatic antioxidant. Its function includes removal of free radicals such as H2O2 and superoxide anions [**28] and [29]**. The increasing in the MDA levels was attributed to enhanced lipid peroxidation, leading to tissue damage and failure of antioxidant defense mechanisms to prevent excessive free radicals formation [**5]**. Catalase action was detoxified H_2O_2, when present at high concentration, accumulatingH_2O_2 arisen from the decreasing of catalase activities. In the treated animals the increasing of H_2O_2concentration inactivated superoxide dismutase (SOD) activity and this might render the liver more susceptible to H_2O_2 and hydroxyl-radical induced oxidative stress **[30**].

While in the groups that treated with resveratrol (before and after) methotrexate administration showed increasing ($P<0.05$) in the levels of GSH and catalase as compared with methotrexate alone, these results are in agreement with those obtained by **[24]**. RSV could improve the antioxidant defense system by modulating antioxidant enzymes through down regulation of extracellular signal-regulated kinase (ERK) activated by reactive oxygen species (ROS) **[31]**. The first line of defence against free radicals is SOD, which catalyzes the dismutation of superoxide anion radical into hydrogen peroxide (H2O2). Subsequently, H2O2 is transformed into water and oxygen by catalase. Glutathione peroxidase enzyme removes H2O2 by using it to oxidize reduced glutathione into oxidized glutathione **[24]**.

The groups that treated with resveratrol (before and after) methotrexate administration showed decreasing (but not significant) ($P<0.05$) in the levels of MDA as compared with administration of methotrexate alone (Table : 3), these results are in agreement with those obtained by **[24]** they showed MDA is an end product and remnant of lipid peroxidation which acts as a cytotoxic messenger for primary reactions. It can escape from cells to initiate attack in other parts of the body. **[32]** suggested that GSH plays a key role in detoxifying the reactive toxic metabolites of hepatotoxic substances. Failure to detoxify the metabolites promotes liver necrosis. GSH form adducts with the toxic metabolites and contributes to the detoxification of toxic materials. It has been suggested that one of the principal causes of liver injury is lipid peroxidation caused by its free radical derivatives.

Table (3): Shows the Effects of Resveratrol on Serum Malondialdehyde (MDA), Enzymatic Antioxidant (Catalase) and non-Enzymatic Antioxidant (GSH)in Rats Treated with Methotrexate.

Group / **Test**	**GSH** 10^{*-5} µM	**MDA** (µmol/l)	**Catalase** (nmol/ml)
Control negative (-)	0.100± 0.009	1.02±0.5 C	0.936±0.036 A
Methotrexate positive (+)	0.058±0.017	2.01±0.37 A	0.451±0.087 C

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Resveratrol before	0.076±0.012	1.27±0.25 BC	0.755±0.029 B
Resveratrol after	0.101±0.033	1.91±0.23 AB	0.680±0.037 B
LSD	0.089	0.95	0.14

N=7

The different capital letters refer to significant differences between different treated groups at (P<0.05).

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