ECRONICON OPEN ACCESS

EC PHARMACOLOGY AND TOXICOLOGY

Research Article

Hydroxytyrosol Abrogates Methotrexate-Induced Pulmonary Toxicity in Rats Via Modulation of NF-kB and Caspase 3 Pathways

Mohamed F Manie[1], Hala M Fawzy[1] and El-Sayed M El-Sayed[2]*

[1]*Pharmacology Department, Egyptian Drug Authority (EDA), Formerly Known as National Organization for Drug Control and Research, Giza, Egypt*

[2]*Pharmacology and Toxicology Department, Faculty of Pharmacy, Al-Azhar University, Nasr-City, Cairo, Egypt*

***Corresponding Author:** El-Sayed M El-Sayed, Pharmacology and Toxicology Department, Faculty of Pharmacy, Al-Azhar University, Nasr-City, Cairo, Egypt. E-mail: elsayed200_1956@hotmail.com

Received: October 28, 2022; **Published:** November 05, 2022

Abstract

Methotrexate (MTX) is an antineoplastic agent widely used in cancer treatment. It has been reported that MTX causes toxicity in many organs, including kidney, liver and lungs. Numerous studies showed that some antioxidants have possible protective effects against MTX's side effects. This study aimed to investigate the protective effect of hydroxytyrosol (HT) on MTX-induced lung toxicity in rats via illustrating histologic and biochemical methods. In the study, 48 male Wistar rats weighing 160 - 180g were used and randomly allocated in 6 groups. Group 1 is the control group, that received saline orally for 14 days. Group 2 were given HT (40 mg/kg, orally) once daily for 14 days. Groups 3 received MTX (14 mg/kg, orally) once weekly for two successive weeks and served as the positive control. Groups 4, 5 and 6 received HT-orally- in a dose of 10, 20 or 40 mg/kg/day, respectively, concomitantly with MTX (14 mg/kg/week, orally) for 14 days. Administration of MTX significantly increased oxidative stress as manifested by a marked increase in MDA, and significant decrease in GSH lung content. In addition, MTX induced inflammatory and apoptotic response as manifested by a marked increase in NF-kB and active caspase 3 in pulmonary tissue. Moreover, MTX-induced lung damage appeared as histopathological alterations. On the contrary, HT administration attenuated MTX toxicity in the rats' lungs through restoration of oxidative status. Besides, HT alleviated NF-kB and active caspase 3 expression in lung tissue. In histologic analysis, HT prevented the MTX-mediated tissue damage and other histopathological alterations in the rats' lung tissues. The present study has revealed that HT provides a possible protective effect via inhibiting ROS, NF-kB and active caspase 3 as well as histopathological changes in MTX-induced lung damage.

Keywords: *Lung Toxicity; Hydroxytyrosol; Methotrexate; Oxidative Stress; NF-kB; Caspase 3*

Introduction

Methotrexate (MTX), a folic acid antagonist, is a chemotherapeutic drug used to treat a variety of cancers. It has also been used to inhibit the immune system [1]. Methotrexate is a chemotherapeutic drug that is used to treat chronic and acute leukaemia, multiple myeloma, lymphoma, and rheumatoid arthritis [2,3]. The underlying cause of MTX toxicity is unknown, although according to a prior study, MTX inhibits NADP malic enzymes, which may lower the amount of NADPH accessible to cells [4,5]. The NADPH is known to be used by glutathione (GSH) reductase to maintain stable GSH levels. As a result of the lower GSH level in the cells, the cells may be more sensitive to oxidative stress [6]. Other possible mechanism of MTX-induced lung damage is the inflammatory reaction of MTX. Administration of MTX

increases the levels of interleukin 1beta (IL-1β), and tumor necrosis factor alpha (TNF-α), which are indicators of inflammatory response [7]. Many antioxidants have been found to provide health advantages in the fight against MTX toxicity in lung tissue. Antioxidants defend against oxidative stress and pro-inflammatory effects induced by MTX [7,8]. The HT is a naturally occurring polyphenol found in a wide range of fruits and vegetables [9]. Several epidemiological and experimental studies have discovered that these chemicals are good anti-oxidants, anti-inflammatories, antiapoptotic, anti-mutagenic, anticancer, anti-angiogenic, and anti-proliferative agents [10].

Purpose of the Study

The purpose of this study was to evaluate the pulmonary toxicity after MTX treatment. The potential underlying processes of lung affection were investigated. The possible modulating effects of HT in such affection was further investigated.

Materials and Methods

Animals

Forty-eight Wistar adult albino rats were used. The rats were housed in a temperature of 22 ± 2°C, relative humidity of 55 ± 5%, and 12/12h light/dark cycle. The study was conducted in Experimental Animal Centre, Egyptian Drug Authority. This study was conducted in agreement with the ethical guidelines for investigations in laboratory animals and got approval from the Research Ethics Committee of NODCAR, (NODCAR/I/51/19).

Experimental design

The rats were divided into three groups. Each group consisted of 8 rats:

- Group 1: Rats were given saline (p.o.) as a normal control.
- Group 2: As a positive control, rats were given MTX (14 mg/kg, p.o.) once weekly for two weeks [11].
- Group 3: Rats were given HT (40 mg/kg, p.o.) once daily for two consecutive weeks [12].
- Group 4: For two weeks, rats were given MTX (14 mg/kg/week, p.o.) and HT (10 mg/kg/day, p.o.) concurrently [13].
- Group 5: For two weeks, rats were given MTX (14 mg/kg/week, p.o.) and HT (20 mg/kg/day, p.o.) concurrently [13].
- Group 6: For two weeks, rats were given MTX (14 mg/kg/week, p.o.) and HT (40 mg/kg/day, p.o.) concurrently [12].

Sample preparation

At the completion of the experiment, rats were decapitated under ether anesthesia, and their lungs were immediately excised, cleaned with ice-cold saline, and blotted dry. A portion of each lung was homogenized in PBS (10% w/v), centrifuged for 15 minutes (4000 rpm, 4°C), and supernatants were frozen at -80°C for subsequent analysis of oxidative stress biomarkers (GSH and MDA), NF-kB, and caspase 3. Another section of the lung was fixed in 10% formalin-saline and processed for histological analysis using H&E stain.

Biochemical investigations

Assessment of oxidative stress in lung

Commercial kits were used to determine the GSH and MDA contents (Biodiagnostic®,Egypt; Catalog No: GR2511and MyBioSource®, USA; Catalog No: MBS268427,respectively). All operations were carried out in accordance with the manufacturer's instructions.

Assessment of active caspase-3 in lung

The active caspase-3 was evaluated using active caspase-3 ELISA Kit (MyBioSource®, USA; Catalog No: MBS261814) in accordance with the manufacturer's instructions.

Assessment of nuclear factor-kappa B (NF-kB) in lung

Rat Nuclear factor-kappa B (NF-kB) ELISA Kit (MyBioSource®, USA; Catalog No: MBS287521) was used to assess NF-kB in line with the manufacturer's specifications.

Histopathological examination

Lung autopsies were taken from rats in separate groups and preserved in 10% formalin saline for 24h. Washing was done using tap water, followed by dehydration with successive dilutions of alcohol (methyl, ethyl, and 100% ethyl). Specimens were cleaned with xylene before being embedded in paraffin for 24h in a hot air oven at 56°C. A sledge microtome was used to cut paraffin beeswax tissue blocks at a thickness of four microns. The resulting tissue slices were collected on glass slides, deparaffinized, and stained with hematoxylin and eosin stain using an electric light microscope [14]. The severity of histological abnormalities in lung tissue was denoted by the following symbols: -: none, +: mild, ++: moderate, +++: severe.

Statistical analysis

We utilized one-way analysis of variance (ANOVA) with Tukey post hoc test to assess multiple comparisons among groups. Graph Pad prism 5.0 Software (GraphPad Software Inc.) was used for graph construction and statistical analysis. The data were presented as mean ± standard deviation (SD).

At $p \leq 0.05$, differences were considered significant.

Results

Effect of hydroxytyrosol on GSH content in lung tissue

MTX caused a significant decrease in GSH content by 70% as compared to the normal control group. On the contrary, HT (10, 20, and 40 mg/kg/day), significantly increased GSH content approximately by 27.5%, 79.4%, and 209%, respectively, as compared to MTX group (Figure 1).

Effect of hydroxytyrosol on MDA content in lung tissue

Administration of MTX significantly induced 6-folds elevation in MDA lung content. In contrast, HT (10, 20, and 40 mg/kg/day), significantly reduced MDA level (27.3%, 39%, and 83.4%, respectively) as compared to MTX group (Figure 2).

Effect of hydroxytyrosol on NF-kB content in lung tissue

The NF-kB content increased four-fold in MTX-treated group when compared to the control group. A marked improvement was noticed in HT-treated group, where there was a significant reduction of NFkB content by 26.4%, 44%, and 67%, respectively as compared to MTX group (Figure 3).

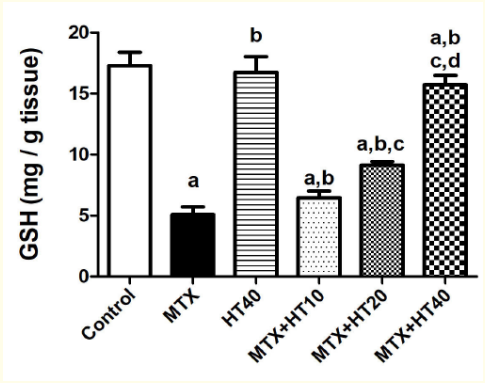

Figure 1: *Effect of HT (10, 20 and 40 mg/kg) on GSH content in MTX-induced lung toxicity in rats.*

Pulmonary toxicity was induced by oral administration of MTX (14 mg/kg/week, p.o., for 2 weeks), then rats were treated with HT (10, 20 or 40 mg/kg/day, p.o., for 2 weeks). At the end of experiment, rats were scarified by decapitation; the lungs were isolated, homogenized in phosphate buffer saline (10% homogenate), and used for the estimation of GSH content.

Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Each bar represents the mean of 8 animals ± SD. a: Significantly different from control group; b: Significantly different from MTX group; c: Significantly different from MTX + HT10 group; d: Significantly different from MTX + HT20 group at P < 0.05.

HT: Hydroxytyrosol; GSH: Glutathione Reduced; MTX: Methotrexate.

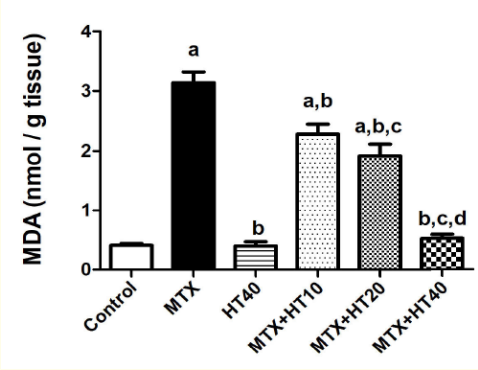

Figure 2: *Effect of HT (10, 20 and 40 mg/kg) on MDA content in MTX-induced lung toxicity in rats.*

Pulmonary toxicity was induced by oral administration of MTX (14 mg/kg/week, p.o., for 2 weeks), then rats were treated with HT (10, 20 or 40 mg/kg/day, p.o., for 2 weeks). At the end of experiment, rats were scarified by decapitation; the lungs were isolated, homogenized in phosphate buffer saline (10% homogenate), and used for the estimation of MDA content.

Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Each bar represents the mean of 8 animals ± SD. a: Significantly different from control group; b: Significantly different from MTX group; c: Significantly different from MTX + HT10 group; d: Significantly different from MTX + HT20 group at P < 0.05.

HT: Hydroxytyrosol; MDA: Malondialdehyde; MTX: Methotrexate.

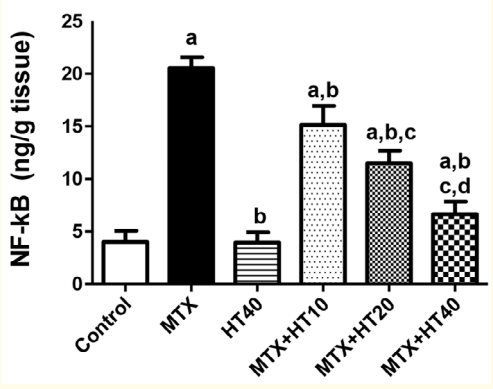

Figure 3: *Effect of HT (10, 20 and 40 mg/kg) on NF-kB content in MTX-induced lung toxicity in rats -Pulmonary toxicity was induced by oral administration of MTX (14 mg/kg/week, p.o., for 2 weeks), then rats were treated with HT (10, 20 or 40 mg/kg/day, p.o., for 2 weeks). At the end of experiment, rats were scarified by decapitation; the lungs were isolated, homogenized in phosphate buffer saline (10% homogenate), and used for the estimation of NF-kB content.*

Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Each bar represents the mean of 8 animals ± SD. a: Significantly different from control group; b: Significantly different from MTX group; c: Significantly different from MTX + HT10 group; d: Significantly different from MTX + HT20 group at P < 0.05.

HT: Hydroxytyrosol; MTX: Methotrexate; NF-kB: Nuclear Factor-Kappa B.

Effect of hydroxytyrosol on active caspase 3 content in lung tissue

Treatment with MTX markedly increased active caspase 3 by around 2-folds in MTX group. On the other hand, HT (10, 20, and 40 mg/kg/day) significantly reduced active caspase 3 by 17%, 37% and 71.4%, respectively as compared to MTX group (Figure 4).

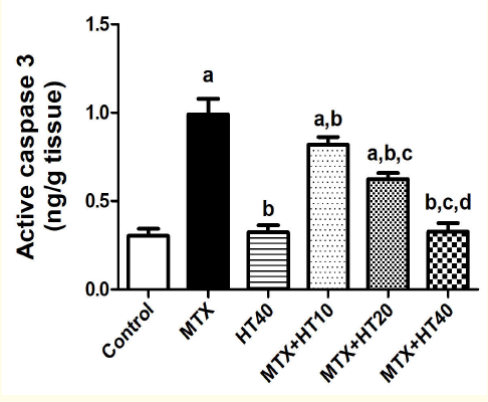

Figure 4: *Effect of HT (10, 20 and 40 mg/kg) on active caspase 3 content in MTX-induced lung toxicity in rats.*

Pulmonary toxicity was induced by oral administration of MTX (14 mg/kg/week, p.o., for 2 weeks), then rats were treated with HT (10, 20 or 40 mg/kg/day, p.o., for 2 weeks). At the end of experiment, rats were scarified by decapitation; the lungs were isolated, homogenized in phosphate buffer saline (10% homogenate), and used for the estimation of active caspase 3 content.

Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.

Each bar represents the mean of 8 animals ± SD. a: Significantly different from control group; b: Significantly different from MTX group; c: Significantly different from MTX + HT10 group; d: Significantly different from MTX + HT20 group at P < 0.05.

HT: Hydroxytyrosol; MTX: Methotrexate.

Effects of hydroxytyrosol on lung histopathology

Table 1 and figure 5 showed histopathological changes in lung tissues. The histopathological examination of control group lung sections (H&E; 40) revealed normal histological structure of the bronchioles and surrounding air alveoli (Figure 5A). However, MTX treatment resulted in worsening alterations such as localized lymphoid cell aggregation in the peribronchiolar tissue and emphysema in the air alveoli as well as inflammatory cell infiltration, which replaced the air alveoli (Figure 5B and 5C). Treatment with HT (10, 20, or 40 mg/kg/day) (Figure 5D) significantly reduced the histopathological changes caused by MTX.

Histopathological Alteration	Groups	Control	MTX	MTX+HT40
Focal peribronchiolar lymphoid cells proliferation		-	+++	+
Peribronchiolar collagen and fibroblastic cells proliferation		-	+++	-
Emphysema of air alveoli		-	+++	+
Bronchiolar epithelium hyperplasia		-	+++	-

Table 1: *Effect of HT (10, 20, and 40 mg/kg) on histopathological findings of lung tissues of MTX-treated rats.*

-: None; +: Mild; ++: Moderate; +++: Severe; HT: Hydroxytyrosol; MTX: Methotrexate.

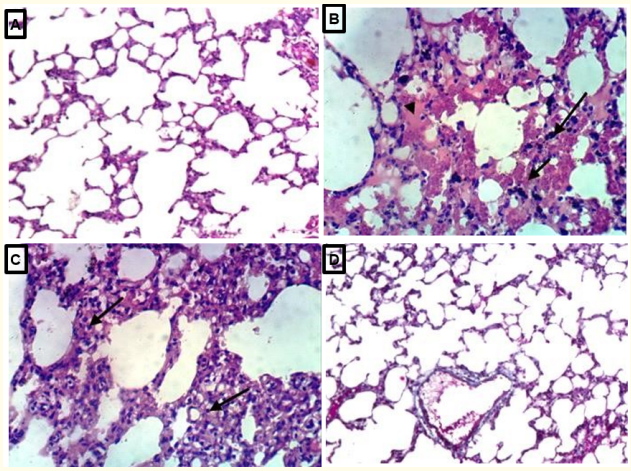

Figure 5: *Effect of HT and DEX treatment on histopathological changes in the lung tissues of MTX-treated rats using hematoxylin and eosin (H&E). Treatment with HT40 declines MTX-induced histopathological changes in rats' lungs. Histological sections of lung tissue were stained with H&E (A-D). (A) Control animal lung tissue images displays the bronchioles' and air alveoli's normal histological structure: well-organized alveolar space with thin lined interalveolar septa; (B) MTX-treated animal lung section shows focal pulmonary haemorrhage, haemosiderosis, oedema; (C) MTX-treated animal lung section shows interstitial pneumonia; (D) MTX+HT40-treated animal lung section exhibits normal histological structure. The images were taken under a light microscope with original magnification, ×40.*

Discussion

Methotrexate is a commonly used antineoplastic and immunosuppressive drug that causes organ toxicity in humans and animals [15]. The MTX-induced biochemical and histological changes in experimental studies are similar to those seen in humans. Free-radical scaveng-

ing enzymes such as superoxide dismutase (SOD), catalase (CAT), and GSH are inhibited by oxidative stress-mediated cellular damage [16]. Previous research found that MTX might produce excessive generation of ROS and inflammatory mediators, as well as lung tissue damage to pulmonary endothelial cells and alveolar capillaries [17]. Our investigation showed that there was an increase in MDA lung content and a decrease in GSH content in the MTX group.

Hydroxytyrosol has been reported in previous studies to be a potent antioxidant and anti-inflammatory [18]. Treatment with HT alleviated oxidative-stress-mediated cellular damage in MTX-induced lung tissue damage. This was manifested by a marked decrease in MDA pulmonary content as well as significant elevation in GSH level in pulmonary tissue.

Conclusion

In the current study, histopathological investigation confirmed the biochemical results where MTX administration induced a significant increase in inflammatory and apoptotic markers. These findings might occur due to activation of the caspase cascade. These results are in agreement with Chauhan [19] and Zaki [20] who reported that MTX displays inflammatory lung affection through induction of oxidative stress, apoptosis and inflammation. The increased expression of pulmonary NF-kB and caspase-3 was consistent with the raised levels of pro-inflammatory cytokines, further confirming the organ damage. These results are in agreement with a prior research that found that MTX intoxication increased interleukin levels [21]. In conclusion, HT possesses anti-inflammatory, antioxidant, and anti-apoptotic properties. The protective effect of HT against MTX-induced lung damage is demonstrated by the downregulation of inflammatory cytokines as well as tissue expression of NF-kB and caspase-3. These actions might explain why HT can prevent MTX-induced lung toxicity in rats.

Acknowledgement

We are deeply thankful to Prof. Dr. Adel B. Khelosy, Department of Pathology, Faculty of Veterinary Medicine, Cairo university, and Mohamed G. Ewees, assistant lecturer, Department of Pharmacology & Toxicology, Al Azhar Faculty of Pharmacy, Cairo; for their kind help in histopathological and immunofluorescence investigation, respectively.

Conflict of Interest

The authors declare that they have no conflict of interest.

Bibliography

1. Ohbayashi M., *et al*. "Induction of pulmonary fibrosis by methotrexate treatment in mice lung *in vivo* and *in vitro*". *The Journal of Toxicological Sciences* 35.5 (2010): 653-661.

2. Choi G., *et al*. "Emerging nanomaterials with advanced drug delivery functions; focused on methotrexate delivery". *Coordination Chemistry Reviews* 359 (2018): 32-51.

3. Sueda TÜRK., *et al*. "Rutin Attenuates Methotrexate-Induced Hepatic Oxidative Stress in Rats". *Atatürk Üniversitesi Veteriner Bilimleri Dergisi* 16.3 (2021): 291-297.

4. Hemeida RA and OM Mohafez. "Curcumin attenuates methotraxate-induced hepatic oxidative damage in rats". *Journal of the Egyptian National Cancer Institute* 20.2 (2008): 141-148.

5. Arpag H., *et al*. "Protective effects of alpha-lipoic acid on methotrexate-induced oxidative lung injury in rats". *Journal of Investigative Surgery* 31.2 (2018): 107-113.

6. Moreno-Sánchez R., *et al*. "Control of the NADPH supply and GSH recycling for oxidative stress management in hepatoma and liver mitochondria". *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 1859.10 (2018): 1138-1150.

7. Shalaby YM., *et al*. "Involvement of Nrf2/HO-1 antioxidant signaling and NF-κB inflammatory response in the potential protective effects of vincamine against methotrexate-induced nephrotoxicity in rats: cross talk between nephrotoxicity and neurotoxicity". *Archives of Toxicology* 93.5 (2019): 1417-1431.

8. Khudhair DH., *et al*. "Combination of Vitamin C and Curcumin Safeguards against Methotrexate-Induced Acute Liver Injury in Mice by Synergistic Antioxidant Effects". *Frontiers in Medicine* 9 (2022): 866343.

9. Bertelli M., *et al*. "Hydroxytyrosol: A natural compound with promising pharmacological activities". *Journal of Biotechnology* 309 (2020): 29-33.

10. Fernández-Mar MI., *et al*. "Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review". *Food Chemistry* 130.4 (2012): 797-813.

11. Fikry EM., *et al*. "Bone marrow and adipose-derived mesenchymal stem cells alleviate methotrexateinduced pulmonary fibrosis in rat: comparison with dexamethasone". *Journal of Biochemical and Molecular Toxicology* 29.7 (2015): 321-329.

12. Fuccelli R., *et al*. "Hydroxytyrosol exerts anti-inflammatory and anti-oxidant activities in a mouse model of systemic inflammation". *Molecules* 23.12 (2018): 3212.

13. De La Cruz JP., *et al*. "Differences in the neuroprotective effect of orally administered virgin olive oil (Olea europaea) polyphenols tyrosol and hydroxytyrosol in rats". *Journal of Agricultural and Food Chemistry* 63.25 (2015): 5957-5963.

14. Bancroft JD and M Gamble. "Theory and practice of histological techniques". Elsevier health sciences (2008).

15. Ali YA., *et al*. "Polydatin combats methotrexate-induced pulmonary fibrosis in rats: Involvement of biochemical and histopathological assessment". *Journal of Biochemical and Molecular Toxicology* 36.5 (2022): e23019.

16. Heidari R., *et al*. "Mitochondrial dysfunction and oxidative stress are involved in the mechanism of methotrexate-induced renal injury and electrolytes imbalance". *Biomedicine and Pharmacotherapy* 107 (2018): 834-840.

17. Heidari Khoei H., *et al*. "Astaxanthin prevents the methotrexate-induced reproductive toxicity by targeting oxidative stress in male mice". *Toxin Reviews* 38.3 (2019): 248-254.

18. Fusco R., *et al*. "Biochemical evaluation of the antioxidant effects of hydroxytyrosol on pancreatitis associated gut injury". *Antioxidants* 9.9 (2020): 781.

19. Chauhan P., *et al*. "Protective effects of Glycyrrhiza glabra supplementation against methotrexate induced hepato-renal damage in rats: An experimental approach". *Journal of Ethnopharmacology* 263 (2020): 113209.

20. Zaki SM., *et al*. "Febuxostat ameliorates methotrexate-induced lung damage". *Folia Morphologica* 80.2 (2021): 392-402.

21. Abdel-Daim MM., *et al*. "Diosmin attenuates methotrexate-induced hepatic, renal, and cardiac injury: a biochemical and histopathological study in mice". *Oxidative Medicine and Cellular Longevity* (2017).

Volume 10 Issue 12 December 2022