

A study of the effect of *Nigella sativa* (black seeds) on methotrexate-induced hepatotoxicity in rabbits

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ABSTRACT

Background: To evaluate the effectiveness of *Nigella sativa* (NS) in the prevention of hepatotoxicity of large doses of methotrexate (MTX) (IP) in rabbits.

Methods: Three groups of male rabbits, six in each were used. Oral dosing was administered as a paste; formula 1 was prepared by mixing 2 g flour with water; formula 2 contained flour and NS and water. Group 1 was fed with formula 1 daily and injected with 2 ml/kg normal saline IP. Group 2 was given formula 1 daily with 20 mg/kg MTX IP. Group 3 was fed with formula 2 daily + MTX 20 mg/kg IP. Injections were given weekly for 5 weeks, and then the animals were sacrificed at day 39. Liver enzymes, malondialdehyde (MDA), glutathione (GSH), and histopathology of the liver were evaluated.

Results: Liver enzymes, serum, and liver MDA were significantly increased by MTX. MTX + NS treatment significantly reduced the rise in liver enzymes, MDA in serum with little effect on liver MDA. Serum aspartate aminotransferase, alkaline phosphatase, and bilirubin were reduced from 82.8±18.04 U/L, 4.9±2.0 kind and king unit/100 ml and 0.74±0.1 mg/dl to 56.1±7.5, 2.0±0.6 and 0.27±0.1 respectively. Unexpectedly, serum and liver GSH were slightly increased by MTX. Treatment with MTX + NS further increased these levels. Histologically, portal and lobular sinusoidal dilatation, lymphocytic infiltration, and hepatocyte hydropic degeneration were seen in all rabbits on MTX, which disappeared in three rabbits on NS + MTX.

Conclusion: NS is hepatoprotective against MTX induced hepatotoxicity.

Keywords: Methotrexate, Liver toxicity, Antioxidant, *Nigella sativa*

INTRODUCTION

Methotrexate (MTX), an antimetabolite drug possess an indispensable place in the treatment of cancer, and many other common chronic inflammatory diseases such as rheumatoid arthritis and psoriasis.¹ In these diseases, MTX was used in low doses and described as having low profile of toxicities,^{2,3} however, at these doses, liver toxicity is still a major concern.⁴ On the contrary, chronic use of MTX may result in serious toxic effects on many organs such as the gastrointestinal tract, liver, kidney, lung, and bone marrow.⁵ *Nigella sativa* (NS) was found in many studies protective to the liver against many drugs such as INH⁶ or chemicals as CCL₄.⁷ NS, in addition has been used topically and orally and found beneficial in ameliorating psoriatic lesions.^{8,9} The

study was, therefore, designed to investigate the protective effect of NS against MTX-induced hepatotoxicity in rabbits.

METHODS

Preparation of NS

NS seeds were purchased from a local market for medicinal plants in Basrah, authenticated by an expert herbalist and a voucher specimen was kept in the Department of Pharmacology for future reference. Viability of the seeds was guaranteed by cultivating 100 seeds, implanted, a number of plants were counted and their growth was followed up. The seeds were crushed by electric grinder into a fine powder

and used for preparation of a paste for oral dosing (dietary admixture).¹⁰ Two types of paste formulas were prepared; formula 1 (placebo paste) as follow: for each 1 kg of animal's body weight, 2 g flour +0.7 ml distal water. The final weight of the paste was 2.7 g and the total volume is about 2 ml; this volume was measured by compressing the paste in a graduated cylinder; formula 2 was prepared by mixing 1 g NS powder +1 g flour +0.7 ml distal water. Before dosing, each rabbit was isolated in a special restriction cage which inhibited the rabbit from approaching its dropping to ensure fasting condition. After a 12 hrs fasting, the animal was transferred to a cage containing the paste only; these cages did not contain sawdust or any material that may contaminate the paste. After ensuring the paste has been completely eaten, the rabbits were returned back to their original cages and kept on a restricted diet containing clover and lettuce.

Animal handling

The experiment was carried out on 18 sexually mature male rabbits. The body weight ranged from 1 to 2 kg. The animals were housed in the main animal house at the College of Medicine. They were kept in wooden cages for acclimatization with a 12:12-hrs light/dark cycle and free access to food and drinking water.

Study design

The study protocol was approved by a local institutional ethical committee and carried out between November 2013 and May 2014.

The rabbits were randomly divided into three groups, six animals in each group. Group 1 (control group) was fed with formula 1 (placebo paste) daily and injected with 2 ml/kg normal saline IP at days 11, 18, 25, and 32 of the experiment then sacrificed at day 39. Group 2 (MTX group); rabbits were fed with formula 1 daily and injected with 20 mg/kg MTX IP for a time scales as previously described then sacrificed at day 39. Group 3 (NS + MTX); the animals were fed with a paste containing NS (formula 2) daily and injected with 20 mg/kg MTX IP for the described time scale then sacrificed at day 39.

Blood sampling and tissue handling

At the morning of day 39, the rabbits were anesthetized with inhalation of chloroform, a sample of blood was directly taken from the heart by cardiac puncture immediately before sacrificing. 5-10 ml of blood was obtained, transferred into a non-heparinized tube, allowed for 30 mins to clot. Serum was separated by centrifugation at 3000 rpm for 20 mins. 1 ml of fresh serum was used for measurement of serum malondialdehyde (S. MDA), the rest of the serum was frozen at -20°C for measuring liver enzymes and serum glutathione (GSH).

After sacrificing the animal, liver specimens were immediately removed, rinsed in freshly prepared phosphate-

buffered saline pH=7.4, stored in 10% neutral buffered formalin and sent to the Department of Pathology and Forensic Medicine at Basrah College of Medicine for histopathological examination. Another piece of 2 g of the liver was divided and homogenized mechanically using electric homogenizer (using Heidolph Electrical Homogenizer, Korea) to obtain 10% homogenate for measurement of liver MDA and GSH.

Histopathological examination

The slides were coded to ensure blindness. Histopathological examination was performed by a senior histopathologist.

Laboratory measurements

Estimation of S. MDA

S. MDA levels were estimated by thiobarbituric acid assay as described by Buege and Aust (1978).¹¹

Estimation of MDA in liver homogenates

MDA levels in liver homogenates were estimated as described by Ohkawa et al., 1979.¹²

Estimation of GSH in serum and liver homogenate

GSH measurements were performed by ELIZA (HumaReader HS, Germany) using a kit specific for rabbits (Cusabio reagents, Cusabio Laboratories, Daxueyuan Road, Donghu Hi-Tech Development Area, Wuhan, China).

Estimation of liver enzymes

The activities of serum aspartate aminotransferase (S. AST), serum alanine aminotransferase (S. ALT), serum alkaline phosphatase (S. ALP) and serum total bilirubin (S. Bil) were determined by commercially available kits (Biolabo Reagents, Biolabo SA, France).

Statistical analysis

The results were expressed as mean±standard deviation, data were computerized for statistical evaluation by using SPSS computer program, version 19 (SPSS Inc. Chicago; USA; available form: <http://www.spss.com>). T-test for unpaired data were used for statistical analysis, $p < 0.05$ is considered significant.

RESULTS

Effect on S. ALT

There was a significant increase in S. ALT by 83% from 8.75±2.13 U/L in the control group to 16.08±5.3 U/L in

the MTX group, $p=0.008$, mean difference of 7.33 U/L (95% CI -12.5 to -2.1). Treatment with NS combined with MTX did not blunt the rise in S. ALT produced by MTX. On the contrary, there was a small and insignificant increase by (2%) in S. ALT in the group of rabbits treated with the combination NS + MTX. The level of S. AST was 16.08 ± 5.3 in the MTX treated group and became 16.5 ± 7.2 U/L in the group treated with the combination NS and MTX with a mean difference of 0.42 U/L (95% CI -7.7 to 8.5), $p=0.81$ (Table 1).

Effect on S. AST

S. AST was significantly increased by 241% from 24.25 ± 5.3 U/L in the control group to 82.8 ± 18.04 U/L in the MTX group, $p=0.004$, mean difference of 58.55 U/L (95% CI -75.7 to -41.4).

The rise in S. AST by MTX was significantly decreased by 32% to 56.1 ± 7.5 U/L following treatment with NS with a mean difference of 26.7 U/L (95% CI -44.4 to -8.8), $p=0.006$ (Table 1).

The effect on S. Bil

S. Bil was significantly increased by 89% from 0.39 ± 0.05 mg/dl in the control group to 0.74 ± 0.1 mg/dl in the MTX group, $p=0.004$, mean difference of 0.35 mg/dl (95% CI -0.46 to -0.23).

The rise in S. Bil by MTX was significantly decreased by 62% to 0.27 ± 0.1 mg/dl in the group treated with NS with a mean difference of 0.47 mg/dl (95% CI -0.6 to -0.3), $p=0.004$ (Table 1).

Effect on S. ALP

S. ALP was significantly increased by 97% from 2.5 ± 0.1 kind and king unit/100 ml in the control group to 4.9 ± 2.0 kind and king unit/100 ml in the MTX group, $p=0.008$, mean difference of 2.4, (95% CI -4.3 to -0.5). The rise in S. ALP by MTX was significantly decreased by 58% to 2.0 ± 0.6 kind and king unit/100 ml following treatment with NS with a mean difference of 2.9, (95% CI -4.8 to -0.9), $p=0.006$ (Table 1).

Effect on S. MDA

S. MDA was significantly increased by 117% from 0.17 ± 0.03 $\mu\text{mol/L}$ in the control group to 0.37 ± 0.14 $\mu\text{mol/L}$ in the group treated with MTX, $p=0.006$, mean difference of 0.2 $\mu\text{mol/L}$ (95% CI -0.3 to -0.06).

The rise in S. MDA by MTX was significantly decreased by 59% to 0.15 ± 0.05 $\mu\text{mol/L}$ following treatment with NS with a mean difference of 0.22 $\mu\text{mol/L}$ (95% CI -0.3 to -0.08), $p=0.006$ (Table 2).

Effect on S. GSH

Unexpectedly, S. GSH was increased by MTX from 14.05 ± 4 nmol/ml in the control group to 20.7 ± 6.7 nmol/ml, however, this did not achieve statistical significance, $p=0.14$, mean difference of 7.65 nmol/ml (95% CI -13.9 to 0.7).

In the group of rabbits treated with the combination NS + MTX, S. GSH level was further increased to 24.0 ± 7.6 nmol/ml with a mean difference from MTX value of 3.3 nmol/ml (95% CI -5.9 to 12.5), $p=0.52$ (Table 2).

Table 1: Effect of MTX and NS on liver enzymes in the serum of the rabbit.

Parameters	Control	MTX	Percentage change from control	p value	MTX+NS	Percentage change from MTX	p value
S. ALT	8.75 ± 2.13	16.08 ± 5.3	+83	0.008	16.5 ± 7.2	+2	0.81
S. AST	24.25 ± 5.3	82.8 ± 18.04	+241	0.004	56.1 ± 7.5	-32	0.006
S. Bil	0.39 ± 0.05	0.74 ± 0.1	+89	0.004	0.27 ± 0.1	-62	0.004
S. ALP	2.5 ± 0.1	4.9 ± 2.0	+97	0.008	2.0 ± 0.6	-58	0.006

MTX: Methotrexate, NS: *Nigella sativa*, S. ALT: Serum alanine aminotransferase (U/L), S. AST: Serum aspartate aminotransferase (U/L), S. BIL: Serum total bilirubin (mg/dl), S. ALP: Serum alkaline phosphatase (kind and king unit/100 ml)

Table 2: Effect of MTX and NS on GSH and MDA in the serum and liver homogenate of rabbits.

Parameters	Control	MTX	Percentage change from control	p value	NS+MTX	Percentage change from MTX	p value
S. MDA ($\mu\text{mol/L}$)	0.17 ± 0.03	0.37 ± 0.14	+117	0.006	0.15 ± 0.05	-59	0.006
S. GSH (nmol/ml)	14.05 ± 4	20.7 ± 6.7	+46	0.14	24.0 ± 7.6	+13	0.52
Liver MDA ($\mu\text{mol/L}$)	385.83 ± 88.79	1688.81 ± 811.37	+337	0.004	972.95 ± 336.15	-42	0.055
Liver GSH (nmol/ml)	5.42 ± 2.4	8.23 ± 2.4	+51	0.054	8.61 ± 3.14	+4	0.873

MTX: Methotrexate, MDA: Malondialdehyde, GSH: Glutathione, S: Serum, NS: *Nigella sativa*

Effect on liver MDA

MDA level in liver homogenate was significantly increased to $1688.81 \pm 811.37 \mu\text{mol/l}$ in the MTX treated group compared with $385.83 \pm 88.79 \mu\text{mol/l}$ in the control group, $p=0.004$, mean difference of $1302.98 \mu\text{mol/L}$ (95% CI -2045 to -560).

The rise in liver MDA by MTX was insignificantly decreased in the group treated with NS + MTX to $972.95 \pm 336.15 \mu\text{mol/L}$, mean difference of $715.05 \mu\text{mol/L}$ (95% CI -1514 to 83), $p=0.055$ (Table 2).

Effect on liver GSH

Similar to S. GSH, liver GSH was increased from $5.42 \pm 2.4 \text{ nmol/ml}$ in the control group to $8.23 \pm 2.4 \text{ nmol/ml}$ in the MTX group. This increase was not small (51%) but just approached borderline statistical significance, $p=0.054$, mean difference of 2.81 nmol/ml (95% CI -5.9 to 0.3). Treatment with the combination NS + MTX increased GSH in liver by 4% compared with MTX alone. This change was statistically not significant (Table 2).

Histopathological examination

Histopathological findings were presented as a score from 0 to 3 according to morphological findings. There were three histopathological manifestations; no changes were labeled as zero and ascribed to normal; scores 1, 2, and 3 were ascribed to histopathological features of mild, moderate, and severe, respectively.

Control group

All liver samples of the control group ($n=6$), revealed no histopathological changes and obtained a zero score. A representative histopathological slide is presented in Figure 1a.

MTX group

The following histopathological features were observed in liver samples ($n=6$) treated with MTX:

1. Portal and lobular sinusoidal dilatation
2. Lymphocytic infiltration
3. Hepatocyte hydropic degeneration.

This group obtained score 3 and ascribed to severe hepatic damage (Figure 1b).

NS + MTX treatment

In this group, NS was not able to revert MTX induced histopathological changes in liver specimens of three rabbits. These samples were labeled as score 3 (Figure 1c).

While the other three rabbits treated with NS + MTX, NS produced partial improvement in histopathological features in MTX induced liver changes. Portal and lobular sinusoidal dilatation and lymphocytic infiltration were still seen. These samples were described as having moderate changes (Score 2) (Figure 1d).

DISCUSSION

MTX is a cytotoxic drug used for cancer chemotherapy and for a variety of chronic inflammatory diseases such as rheumatoid arthritis and psoriasis. Hepatotoxicity including fibrosis and cirrhosis is a commonly reported side effect particularly when prolonged high doses are used.¹³

MTX associated toxicities are usually challenged with administration of folic acid.¹⁴ In the present study, NS has been investigated to counteract hepatotoxicity induced by MTX in a rabbit model.

NS powder was prepared, at first, as a suspension by mixing 1 g of the powder in 5 ml drinking water. It was noticed that the suspension precipitated in the container and can easily occlude the nasogastric tube used for dosing and ultimately a proper dose of NS cannot be guaranteed.

NS was then decided to be given orally as a paste (dietary admixture). The rabbit was isolated in a special cage to ensure fasting for overnight before dosing and closely observed

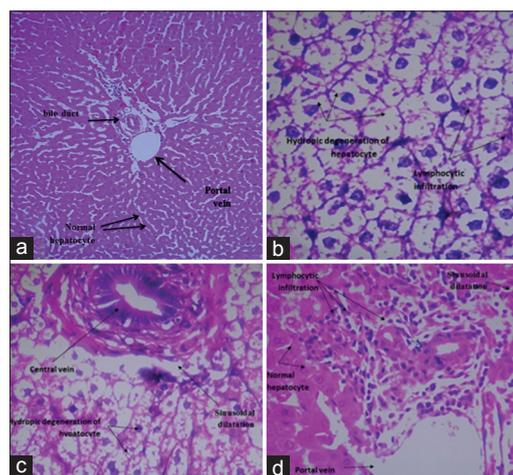


Figure 1: (a) A healthy liver tissue from the control group with normal hepatocyte (hematoxylin-eosin stain, power $\times 10$), (b) liver tissue from a rabbit receiving methotrexate. Lymphocytic infiltration and hepatocyte hydropic degeneration is seen (hematoxylin-eosin stain, power $\times 40$), (c) liver tissue from a rabbit received methotrexate and *Nigella sativa* showing sinusoidal dilatation and hepatocyte hydropic degeneration (hematoxylin-eosin stain, power $\times 40$), (d) liver tissue from a rabbit received methotrexate and *Nigella sativa* showing sinusoidal dilatation, lymphocytic infiltration with no hepatocyte hydropic degeneration.

on the next day of the experiment to ensure the full amount of the paste has been eaten by the rabbit. It has been described that dosing as dietary admixture is relatively reliable and generally accepted as a method of dosing, however, it is not as precise as gavage, but has the advantage of less traumatic to the animal as esophageal rupture, minimal or no risk of aspiration and less stressful to the animal due to frequent handling.¹⁰

In the control group, liver enzymes such as S. AST, S. ALP, S. Bil, S. MDA, and liver MDA were within normal range and were comparable with studies of others.^{6,15} Treatment with MTX raised liver enzyme levels, which was significantly reduced when MTX and NS used in combination with an exception of the effect on S. ALT, which was increased with MTX treatment but remained high with the combination of MTX + NS. Such behavior warrants further evaluation.

Surprisingly, GSH level, both in serum and liver showed elevation in the group treated with MTX, an effect unexpected from a cytotoxic drug and attributed to interference with GSH metabolism.^{16,17} Regardless to the observed rise in GSH level, this did not stop MTX from damaging the liver; which may suggest mechanisms other than oxidant/antioxidant involved in MTX induced hepatotoxicity that cannot be opposed by MTX induced rise in GSH, and that all MTX treated rabbits showed hydropic degeneration on histopathological examination.

Hydropic degeneration is a reversible swelling of hepatocyte,³ which may occur as a result of failure of sodium - potassium pump leading to increased osmotic pressure within the cells.¹⁸ Correction of MTX induced hydropic degeneration by NS, though in three rabbits out of six treated with MTX, may indicate an activity of NS in maintaining cellular integrity by stabilizing sodium - potassium ATPase and restoring its activity.^{19,20} The observed incomplete protection of MTX hepatotoxicity by NS may in part be attributed to reduced absorption of NS from rabbits gastrointestinal tract as for complete absorption, empty stomach is required which is difficult to accomplish with absolute certainty because of a habit rooted in the rabbit of eating its dropping (autocoprotophagia) when fasted for a period of time. Interaction between MTX and NS with reduced absorption of NS is unlikely since MTX and NS were administered by a different route.

Elevated MDA and other hepatic enzyme in MTX treated group indicate a state of oxidative stress that can be opposed by NS; this effect could be attributed to antioxidant potential of NS. In conclusion, NS has some protective effect against MTX induced hepatotoxicity. The result of the present study is useful for patients with psoriasis since NS has been shown to have antipsoriatic effects,^{8,9} thus a combination with MTX could have an additive antipsoriatic effect and from the other hand may help prevent hepatotoxicity of MTX.

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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