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Study of Role for *Allium Sativum L.* and Melatonin in Reduce Methotrexate Side Effect on Normal Male Rabbets

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Abstract

This study was to evaluate the role of garlic and melatonin in the reduction of the toxic effect of the drug methotrexate with concentrations 0.52 mg/ kg of body weight of rabbits on total count of white blood cells and enzymes liver functions, used in this study (90) of the male rabbits, were randomly divided into five groups, six rabbits in each group, given all the animals normal food during the experimental period of 30 days, explained to the animals at the end of each period of experience and took blood samples to count white blood cells, measure liver function enzymes like Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), was reached the following conclusions: (1) The treatment of male rabbits with methotrexate reduced the rate of white blood cells, compared with a negative control and other experimental groups in the first week, but for the observed improvement in the levels of the number of white blood cells (WBCS) in the aggregates experimental treatment in addition to methotrexate, garlic and melatonin. (2) The treatment of animals by methotrexate alone showed an increase in enzyme levels (AST), (ALT) and (ALP) while there was a decrease in enzyme levels in treated experimental animals by methotrexate addition to both the garlic and melatonin levels with high spirits. (3) Thus, it can be said that the injection of garlic extract with dose 6 mg/kg of body weight of rabbits, hormone melatonin had a preventive role in reducing the side effects of methotrexate in the number of total white blood cells and activity of hepatic enzymes.

Keywords: Methotrexate, Garlic, Melatonin, Rabbets

دراسة عن دور الثوم والميلاتونين في اختزال التأثير الجانبي لعقار الميثوتريكسيت في ذكور الأرانب السليمة

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الخلاصة:

أجريت هذه الدراسة لتقييم دور الثوم والميلاتونين في اختزال التأثير السمي لعقار الميثوتريكسيت بتركيز 0.52 مغ/كغم من وزن الجسم على العدد الكلي لخلايا الدم البيض وإنزيمات وظائف الكبد حيث استخدم في الدراسة (90) من ذكور الأرانب جلبت من السوق المحلية قسمت عشوائياً إلى خمس مجاميع ستة أرناب في كل مجموعته، أعطيت جميع الحيوانات الغذاء الطبيعي لمدة 30 يوماً، أخذت عينات الدم لعد خلايا الدم البيض ولقياس إنزيمات وظائف الكبد (AST, ALT, ALP)، وتم التوصل إلى النتائج التالية: (1) إن معاملة ذكور الأرانب بعقار الميثوتريكسيت أدى إلى خفض معدل خلايا الدم البيض بالمقارنة مع مجموعة السيطرة

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السالبة والمجاميع التجريبية الأخرى في الأسبوع الأول، ولكن لوحظ تحسن في مستويات عدد خلايا الدم البيض في المجاميع التجريبية المعاملة بالاضافة الى الميثوتريكسيت بالثوم والميلاتونين. (2) إن الحيوانات المعاملة بالميثوتريكسيت لوحده اظهر ارتفاع في مستويات إنزيم (AST), (ALT) (ALP) بينما لوحظ انخفاض في مستويات الإنزيم في الحيوانات التجريبية المعاملة بالميثوتريكسيت بالإضافة إلى كلا من الثوم والميلاتونين وبمستويات معنوية مرتفعه. (3) يمكن القول بان حقن مستخلص الثوم بتركيز 6 مغ/كغم من وزن جسم ذكور الارانب وهرمون الميلاتونين كان لهما دور وقائي في خفض التأثير الجانبي للميثوتريكسيت على العدد الكلي لخلايا الدم البيض وفعالية ونشاط انزيمات خلايا الكبد.

Introduction

Methotrexate (MTX), a folic acid antagonist, is widely used as a treatment for malignancies as well as in the treatment of various inflammatory and autoimmune disorders. MTX is classified as an anti metabolite drug, which means that it is capable of blocking the metabolism of cells. The efficacy of MTX is often limited by severe side effects and toxic sequel, such as intestinal injury hepatotoxicity and suppression of bone marrow, In fact, MTX depletes folate species and the lack of folate affects several biochemical pathways, including purine metabolism. These metabolic alterations are responsible for both the therapeutic and the toxic effects of MTX [1,2]. Furthermore, Methotrexate-induced hepatotoxicity is well recognized in the treatment of leukemia, psoriasis and rheumatoid arthritis. The pathological lesions are non-specific, consisting of fatty change, nuclear pleomorphic, hepatocyte necrosis, portal chronic inflammatory infiltrate, fibrosis and cirrhosis. The mechanism of liver injury is poorly understood; intracellular accumulation of methotrexate polyglutamate and consequent folate depletion are suspected to play a role. Early studies in psoriasis clearly established a relationship of the hepatic injury with the frequency of methotrexate administration [3], Severe MTX poisoning with acute hepato-renal dysfunction has been treated with plasma exchange and hemodialysis¹⁰. MTX-induced toxicity appears to be a consequence of the interaction of many factors. Garlic, *Allium sativum L.* is a member of the Alliaceae family, has been widely recognized as a valuable spice and a popular remedy for various ailments and physiological disorders. The name garlic may have originated from the Celtic word 'all' meaning pungent [4], Garlic contains at least 33 sulfur compounds, several enzymes, 17 amino acids, and minerals such as selenium. It contains a higher concentration of sulfur compounds than any other *Allium* species. The sulfur compounds are responsible both for garlic's pungent odor and many of its medicinal effects. Dried, powdered garlic contains approximately 1% *alliin* (S-allyl cysteine sulfoxide). One of the most biologically active compounds, *allicin* (diallyl thiosulfinate or diallyl disulfide) does not exist in garlic until it is crushed or cut; injury to the garlic bulb activates the enzyme allinase, which metabolizes alliin to allicin. Allicin is further metabolized to vinyl dithiines. This breakdown occurs within hours at room temperature and within minutes during cooking. Allicin, which was first chemically isolated in the 1940's, has antimicrobial effects against many viruses, bacteria, fungi and parasites. Garlic oil, aged garlic and steam-distilled garlic do not contain significant amounts of aliin or allicin, but instead contain various products of allicin transformation; none appears to have as much physiologic activity as fresh garlic or garlic powder, Oral administration of raw garlic protects tissue damage by increasing the antioxidant status against oxidative stress like acute hepatotoxicity or mild hepatocellular injury, [5-7].

Oxidative stress has been implicated a common link between chronic liver damage and hepatic fibrosis, free radicals may play a role in MTX-induced hepatic toxicity. Lipid peroxidation, mediated by oxygen free radicals, is believed to be an important cause of destruction and damage to cell membranes and has been suggested a contributing factor to the development of MTX mediated tissue damage. Attention has been focused on the role of reactive oxygen species in mediating the micro vascular disturbances that precede tissue damage induced by various chemicals MTX-induced oxidative stress and melatonin. Besides their direct damaging effects on tissues, free radicals seem to trigger the accumulation of leukocytes in the tissues involved, and thus aggravate tissue injury indirectly through activated neutrophils. It has been shown that activated neutrophils secrete enzymes (e.g. elastase and proteases) and liberate oxygen radicals [8]. Herein, elevated MPO levels in both liver and kidney tissues indicate that neutrophil accumulation contributes to MTX-induced oxidative organ injury. Furthermore, the results also suggest that melatonin has a preventive effect through the inhibition of neutrophil infiltration. The severity of MTX-associated liver injury is related to both the

dose and duration of the treatment. In some experimental studies, liver injury was not observed with high dose MTX, presumably because the duration of exposure to the drug was limited by the systemic toxicity (e.g. bone marrow and gastrointestinal injury) that necessitated killing the animals [9]. Our study aimed to evaluate treatment role for Garlic extract, melatonin to reduce effect of a single dose of 0.25 mg/kg/day MTX caused toxic effects on the liver tissue and affect on it is enzymes that was suitable for studying the oxidative injury without systemic toxicity.

Methods of Research

Preparation of drugs and Solution

Methotrexate: Methotrexate (EBWE pharmaceuticals, Australia) was used by intramuscular administering with concentrations 0.52 mg/ kg of body weight of rabbits by using an insulin needle [10, 11] and [12]

Garlic extract: Garlic cloves were obtained from the local market. These were then cleared of any adhering dried material. Peeled garlic (30 g) portion was homogenized in 70 ml of distilled water and crushed with distilled water in a mortar. The mixture was strained through cheese cloth and the filtrate was kept for subsequent studies, was used by intraperitoneally (I .p), administering with dose 6 mg /kg of body weight of rabbits by using an insulin needle [13, 14].

Hormone melatonin :Melatonin was dissolved in (9 g/L) saline with absolute ethanol and stored at - 20°C until used, was used by (s.c.) administering with concentrations MEL (5 mg/ kg/ s.c) mg / kg of body weight of rabbits in the dorsal region near the clavicle .by using an insulin [15, 16].

Animal housing and mating: The rabbits were obtained from the local market, bred and housed in the animal house. Animals were (3-4) months of age and mature sexually with an average weight of 1100-1500 kg .They were kept in plastic cages, furnished by wood shavings, cleaned every three days and given water and food according to need (*ad libitum*); the weather is of 12 hours light and 12 hours darkness, also the temperature $24^{\circ} \pm 2$.after that they male rabbits isolated randomly to cages according to the designing of experiment.

Animals grouping:In this study (90) male rabbits were classified into five groups, as follows:

Control group negative (18 male rabbits) received normal saline 0.25 mg/ kg/ day body weight by intramuscular injection, were divided into three different periods of the study for a period of 10, 20, 30 days.

1. Control group positive (18 male rabbits) Received MTX 0.25mg/ kg/ day body weight by intramuscular injection.
2. Experimental group 1 (18 male rabbits) Received MTX 0.25 mg/ kg/ day body weight by intramuscular injection, and garlic extract Received 6 mg/ kg/ day body weight by intraperitoneally injection.
3. Experimental group 2 (18 male rabbits) Received MTX 0.25 mg/ kg/ day body weight by intramuscular injection, and melatonin Received 5 mg/ kg/ day body weight by (s.c) injection.
4. Experimental group 3 (18 male rabbits) Received MTX 0.25 mg/ kg/ day body weight by intramuscular injection, and garlic extract Received 6 mg/ kg/ day body weight by intraperitoneally injection, an melatonin Received 5 mg/ kg/ day body weight by (s.c) injection.

Rabbits slaughter test: After the termination of the experiment, 10, 20, and 30 days from beginning of treatment, (6 rabbits) from each experimental group were randomly chosen for slaughter test. And then they were weighed individually as pre-slaughter weight. Rabbits were slaughtered by cutting the jugular veins. After complete bleeding, during slaughtering of rabbits, individual blood samples were collected into tubes from each rabbit within each treatment and divided into two samples. Heparin was used as an anticoagulant. Plasma samples were obtained by centrifugation at 3,000 rpm for 20 min. Stored until being used for analysis (17). The Plasma samples testing enzymatic it is divided into two parts, the first enzymes liver function (AST, ALT and ALP).

Examinations study

a. The total number of white blood cells (WBCs): Used the Eliza device (Medonic M-Series) to all count white blood cells.

b. Examinations of liver function:

1. Measure of AST and ALT enzymes level to measure of AST enzyme level in the serum: of blood according to instructions of Manufacturer Company of the Eliza kit (Spectrum - Germany).

2. Measure of ALP enzyme level: to measure of ALP enzyme level in the serum of blood according to instructions of Manufacturer Company of the Eliza kit (Bio System - Spain).

Results and Discussions

1. Count of white blood cells WBC:

The results show in the Table-1 decrease in the number of white blood cells in the animals injected Methotrexate (5.46 ± 2.28) for a period of (10) days and decrease was at a level indicative a significant probability ($P \leq 0.01$) compared to the negative group control. Show increase in the number of white blood cells in the blood of animals injected Methotrexate and garlic (9.35 ± 2.71), animals injected Methotrexate, melatonin and garlic (9.43 ± 1.55), for a period of (10) the increase indicative statistically significant at the level of probability ($P \leq 0.01$), ($P \leq 0.05$) respectively, compared with the positive group control. Show increase in the number of white blood cells in the blood of animals injected Methotrexate and garlic (10.98 ± 2.91) for a period of (20) the increase indicative statistically significant at the level of probability ($P \leq 0.05$) compared with the positive group control.

Table 1- Shows the average number of white blood cells in experimental animals and control.

Groups	Mean($\text{cell} \times 10^3/\text{mlu}^3/\text{blood}$) \pm Stander Deviation		
	10 th day N6	20 th day N6	30 th day N6
Control	9.25 ± 2.88	9.73 ± 3.45	7.81 ± 2.58
Methotrexate only	5.46 ± 2.28^B	7.61 ± 0.69	8.68 ± 1.62
Methotrexate + Garlic	9.35 ± 2.71^b	10.98 ± 2.91^a	10.55 ± 3.98
Methotrexate + Melatonin	7.01 ± 1.83	9.43 ± 1.55	10.06 ± 3.89
Methotrexate + Melatonin + Garlic	8.50 ± 1.10^a	9.05 ± 0.98	8.01 ± 2.19

- **Capital Letters:** significant differences between the experimental and negative control.
- **Small Letters:** significant difference between the experimental and Methotrexate only (positive control).

Present study showed results has been different compared with previous studies, they showed to Methotrexate administration to male rats with single dose for 5 days caused product of reactive oxygen induced inflammatory changes in liver tissues were evaluated by measuring myeloperoxidase activity and index for neutrophil infiltration [18], Methotrexate can rarely cause a drop in the number of white blood cells, which are needed to fight infection, whereas in other studies came currently with our study which showed administration of MTX caused decrease in WBCs according to its normal effect as treatment role, it is regard one of the chemotherapy drug use to treat many different cancers. In rheumatoid arthritis methotrexate is thought to work by suppressing the excessive activity of the immune system that causes the inflammation, while the present study show increase in the number of white blood cells in the blood of animals injected Methotrexate and garlic came currently with [19], Those rats given higher doses of garlic had a majority of neutrophil over lymphocyte in respective of duration. Also, a higher dose of 200mg/day garlic given for a longer period was observed to cause a significant increase in total white blood cell (TWBCs). These data therefore support the earlier reports by that garlic extracts stimulate immune functions. Garlic treated rats showed a significant decrease in total white blood cells (TWBCs) and neutrophils but an increase in lymphocytes [20], Melatonin secretion was significantly and inversely associated with TWBC and Platelets counts in the general elderly population. The associations were independent of several major causes of systemic inflammation, including aging, obesity, smoking, hypertension, diabetes, and physical inactivity. [21].

2. The Activity of Alanine Aminotransferase (ALT)

The results show in the Table-2 decrease in the level of the enzyme ALT in animals injected Methotrexate, melatonin and garlic (6.66 ± 1.50) for a period of (10) days, and this decrease was statistically significant at the level of indicative probability ($P \leq 0.01$), compared with the negative and positive group control. And show decrease in the level of enzyme the ALT in animals injected Methotrexate and melatonin (12.83 ± 0.93) and animals injected Methotrexate, melatonin and garlic (5.83 ± 1.47), for a period of (20) day, was a decrease indicative statistically significant at the level of probability ($P \leq 0.01$), ($P \leq 0.001$) respectively, compared with negative and positive group control. Show decrease in the level of the enzyme ALT in animals injected Methotrexate and garlic (14.16 ± 2.78), animals injected Methotrexate and melatonin (5.00 ± 2.75), animals injected Methotrexate, melatonin and garlic (3.00 ± 1.26) for a period of (30) days, this decrease was statistically significant at the level of indicative probability ($P \leq 0.05$), ($P \leq 0.001$), ($P \leq 0.001$), respectively, compared with negative and positive group control.

Table 2- Show the level enzyme ALT in experimental animals and control groups.

Groups	Mean (IU/L) ± Stander Deviation		
	10 th day N6	20 th day N6	30 th day N6
Control	23.83±6.43	29.83±5.45	28.16±34.77
Methotrexate only	24.50±7.50	24.33± 9.70	21.33±4.03
Methotrexate + Garlic	33.16±14.63	27.50±10.69	14.16±2.78 ^A
Methotrexate + Melatonin	16.66±2.50	12.83±0.93 ^B	5.00±2.75 ^{Db}
Methotrexate + Melatonin + Garlic	6.66±1.50 ^{Bb}	5.83±1.47 ^{Dc}	3.00±1.26 ^{Dc}

- **Capital Letters:** significant differences between the experimental and negative control.
- **Small Letters:** significant difference between the experimental and Methotrexate only (positive control)

3. Activity of Aspartate Aminotransferase (AST)

Show results in Table-3 increase in the level of activity of the enzyme AST in animals injected Methotrexate (14.50 ± 9.87), animals injected Methotrexate and garlic (14.66 ± 2.06), animals injected Methotrexate and melatonin (13.50 ± 1.97) for a period of (10) days, this increase was statistically significant at a level indicative the probability ($P \leq 0.05$), ($P \leq 0.01$), ($P \leq 0.05$) respectively, compared with negative group control. Also decrease in the level of activity of the enzyme AST in animals injected Methotrexate, melatonin and garlic (7.50 ± 4.03) for a period of (10) day, this was decrease indicative statistically significant at the level of probability ($P \leq 0.01$), compared with positive group control. And a decrease in the level of activity of the enzyme AST in animals injected Methotrexate and melatonin (5.00 ± 1.54), animals injected Methotrexate, melatonin and garlic (4.00 ± 2.09) for a period of (20) days respectively this was decrease indicative statistically significant at the level of probability ($P \leq 0.01$), ($P \leq 0.01$) respectively compared with negative and positive group control. Show decrease in the level of enzyme activity of AST in animals injected Methotrexate and melatonin (3.66 ± 1.21), animals injected Methotrexate, melatonin and garlic (3.16 ± 1.83) for a period of (30) day, this was decrease indicative statistically when level of probability ($P \leq 0.01$), ($P \leq 0.01$) respectively, compared with negative group control.

Table 3- Show the levels enzyme AST in experimental animals and control groups.

Groups	Mean(U/L) ± Stander Deviation		
	10 th day N6	20 th day N6	30 th day N6
Control	8.16±2.63	11.50±3.67	10.83±3.16
Methotrexate only	14.50±9.87 ^A	11.50±12.14	6.83±1.94
Methotrexate + Garlic	14.66±2.06 ^B	11.16±3.31	9.66± 2.58
Methotrexate + Melatonin	13.50±1.97 ^A	5.00±1.54 ^{Bb}	3.66±1.21 ^B
Methotrexate + Melatonin + Garlic	7.50±4.03 ^b	4.00±2.09 ^{Bb}	3.16±1.83 ^B

- **Capital Letters:** significant differences between the experimental and negative control.
- **Small Letters:** significant difference between the experimental and Methotrexate only (positive control).

4. Activity of Alkaline Phosphatase (ALP)

Show the results in the Table-4 increase in the rate of enzyme activity in the basal phosphate animals injected Methotrexate (181.83 ± 29.58) for a period of (10) days, this increase was statistically indicative when the level of probability ($P \leq 0.001$) compared with the negative group control. Show decrease in the level of activity of the enzyme ALP in all experimental groups of periods (10) day, this decrease was statistically significant at the level of indicative probability ($P \leq 0.001$) compared with positive control a group. As well as Show decrease in the level of activity the enzyme ALP in animals injected Methotrexate and garlic (49.66 ± 20.51), animals injected Methotrexate and melatonin (27.16 ± 5.98), animals injected Methotrexate, melatonin and garlic (13.00 ± 2.60) for a period of (20) days, the decrease statistically significant at the level of probability ($P \leq 0.001$), compared with negative and positive group control.

And Show decrease in the level of activity of the enzyme ALP in animals injected Methotrexate and melatonin (12.83 ± 3.71), animals injected Methotrexate, melatonin and garlic (5.00 ± 2.60) for a period of (30) days, this decrease is statistically significant at the level of probability ($P \leq 0.05$), ($P \leq 0.005$) respectively, compared with negative group control.

Also Show decrease in the level of activity of the enzyme ALP in animals injected Methotrexate and melatonin (12.83 ± 3.71), animals injected Methotrexate, melatonin and garlic (5.00 ± 2.60) for a period of (30) days, this decrease is statistically significant at the level of probability ($P \leq 0.001$) compared with positive group control.

Table 4- Show the level of the enzyme ALP in control experimental animals and control groups

Groups	Mean(U/L) \pm Stander Deviation		
	10 th day N6	20 th day N6	30 th day N6
Control	31.83 \pm 10.87	76.33 \pm 20.75	30.66 \pm 10.78
Methotrexate only	181.83 \pm 29.58 ^D	71.16 \pm 15.61	39.50 \pm 5.28
Methotrexate + Garlic	30.33 \pm 6.83 ^d	49.66 \pm 20.51 ^{Db}	33.33 \pm 14.81
Methotrexate + Melatonin	43.00 \pm 10.33 ^d	27.16 \pm 5.98 ^{Dd}	12.83 \pm 3.71 ^{Ad}
Methotrexate + Melatonin + Garlic	20.83 \pm 10.49 ^d	13.00 \pm 2.60 ^{Dd}	5.00 \pm 2.60 ^{Cd}

- **Capital Letters:** significant differences between the experimental and negative control.
- **Small Letters:** significant difference between the experimental and Methotrexate only (positive control).

Oxidative stress produced by free radicals has been implicated in the pathogenesis of acute liver injury. The aim of our study was to investigate whether Garlic and melatonin, a potent antioxidant, could diminish hepatic injury in rabbits induced by Methotrexate; Liver enzymes such as ALT, AST and ALP are marker enzymes for liver function and integrity. These enzymes are usually raised in acute hepatotoxicity or mild hepatocellular injury, but tend to decrease with prolonged intoxication due to damage to the liver, so oral administration of raw garlic protects tissue damage by increasing the antioxidant status against oxidative stress. Hence, garlic plays a promising role in antioxidant and it can be considered as a potent drug for the treatment of liver disorders. *In vivo* antioxidant effects of several organic-sulfur compounds derived from garlic have been studied, researchers reported that these antioxidant effects were due to the activation and modification of several enzymes such as 3-hydroxy-3 methylglutaryl- Co A reductase, glutathione-s-transferase and catalase [22 , 23].

The current study were analogous with the finding of the researcher and his follows [24] ,they were indicated that exogenously administered melatonin exhibits a potent hepatoprotective effect against acetaminophen (APAP)induced hepatic damage ,they show treatment of mice with melatonin (50 or 100 mg/kg) 8 or 4 hr before APAP administration (750 mg/kg, p.o.) suppressed the increase in plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in a dose- and a time-dependent manner, Melatonin treatment (100 mg/kg, p.o.) 4 hr before APAP administration remarkably inhibited hepatic necrosis. In the same line other studies find the Protective effects of melatonin against carbon tetrachloride which induced hepatotoxicity in male Wistar rats ,they show in their study to serum biochemical parameters like Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) were significantly higher in animals treated with CCl₄ than in the control grop, addition to that In liver sections of the CCl₄-injected group, necrosis, fibrosis, mononuclear cell infiltration, hemorrhage, fatty degeneration and formation of regenerative, when rats injected with CCl₄ were treated with melatonin, significantly reduced elevations in serum biochemical parameters were found [25], The increased serum levels of AST and ALT are due to the damage to the structural integrity of the liver, since these enzymes are normally located in the cytoplasm and released into the circulation after cellular injury [26] Also Hajovsky *et al.* [27] reported that thioacetamide produces free radicals, which affect the cellular permeability of hepatocytes leading to elevated levels of serum biochemical parameters like ALT, and AST, When Ramadan *et al.* 2013 reported melatonin may be utilized to reduce liver injury associated with oxidative stress. [28]

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