CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES
http://doi.org/10.5281/zenodo.3843877

Available online at: http://www.iajps.com *Research Article*

COMPARATIVE STUDY ON THE PROTECTIVE EFFICACY OF BETA-CAROTENE AND RESVERATROL (3 4 5 TRI_HYDROXYSTILLBENE) ON METHOTREXATE INDUCED HEPATOTOXICITY IN EXPERIMENTAL ANIMALS

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Article Received: March 2020 **Accepted:** April 2020 **Published:** May 2020

Abstract:

Background: *Methotrexate is an antimetabolite and antifolate drug. It is used in the therapy of leukemia, lymphoma and several solid tumors. . Beta-carotene can be stored in body and converted to retinol as needed, thus making it a form of vitamin A for humans and other animals. Resveratrol gets extensively metabolized in the body. Liver and Gut are the major sites of metabolism.*

Aim: *The Aim of the study is to compare and evaluate the protective efficacy of Beta-Carotene on Methotrexate induced hepatic damage in rat liver and to compare and evaluate the protective efficacy of Resveratrol on Methotrexate induced hepatic damage in rat liver.*

Study Design: *This is an experimental study. The study was conducted on 48 healthy Wister Albino rats obtained from animal house – BMSI JPMC, for 25 days from 13 August, 2018 to 05 September, 2018.*

Material And Methods: *For present study, forty eight (n = 48) Wister Albino rats, either sex, weighing 210–310 Gm. were utilized. Rats were divided into six (6) groups randomly, each group including eight (n = 8) animals, grouped as Control (Group I), B-carotene only (Group II), Resveratrol only (Group III), MTX only (Group IV), B-carotene + MTX (group V) and Resveratrol + MTX (group VI).*

Results: *(SPSS 17.0) was used for statistical analysis. The results were compared with paired t-test with p< 0.05 considered as statically significant. All results were expressed as means ± standard error (SEM). The mean value of initial body weight in control group I was 239.33±0.882 Gm and final body weight was 248.50±0.764 Gm.*

Conclusion: *Based on present study it is concluded that methotrexate severely causes hepatic damage in rats, which can be protected by β-carotene and Resveratrol.*

Keywords: *Methotrexate, Beta-carotene, Resveratrol.*

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Please cite this article in press Sarah Sughra Asghar et al, ***Comparative study on the protective efficacy of beta-carotene and resveratrol (3 4 5 tri_hydroxystillbene) on methotrexate induced hepatotoxicity in experimental animals.,*** *Indo Am. J. P. Sci, 2020; 07(05).*

INTRODUCTION:

Methotrexate is an antimetabolite and antifolate drug. It is used in the therapy of leukemia, lymphoma and several solid tumors.[1] It also has potent activity against psoriasis and has immune-modulatory effects against inflammatory bowel disease and inflammatory arthritis. It is used in systematic lupus erythematous, graft versus host disease and in combination with other drugs, neoplastic diseases.[2] It may stimulate increased release of adenosine; an endogenous anti-inflammatory autacoid. Methotrexate may also stimulate apoptosis and death of activated T lymphocytes[3].

Methotrexate because of its potent immune-modulatory effect it is used in treatment of eczema[4]. It is used as a chemotherapeutic agent for decades; methotrexate has been adopted for use as a medical therapy for un-ruptured ectopic pregnancies[5]. Methotrexate has been used for demyelinating polyneuropathy[6]. It is a potent abortificant and kills embryonic tissue (Kahn et al., 2000). Studies have shown that it is effective for relapse of acute Lymphoblastic leukemia. Methotrexate is historically and currently indicated for use in pediatric populations with various neoplasm such as meningeal leukemia, osteosarcoma, some brain tumors and non – Hodgkin's lymphoma[7].

B-carotene is an unsaturated hydrocarbon which is synthesized by plants and not by animals. B-Carotene is composed of two retinal groups, and is broken down in the mucosa of human small intestine by β-carotene 15, 15' monoxygenase to retinol, a form of vitamin. Beta-carotene can be stored in body and converted to retinol as needed, thus making it a form of vitamin A for humans and other animals [8].

B-Carotene is the predominant source of the essential nutrients, vitamin A (retinol and its esters. B-Carotene can be regenerate by the antioxidant form of vitamin E (Niki, 1995), of great importance are the data that showed the synergetic action of beta carotene and vitamin E in protection of lipids in membranes. B-Carotene also enhances lymphocyte proliferation. It has been reported that beta-carotene fed to mice reduced the rate of skin cancer developments. In some studies it was found that high plasma level of beta carotene was associated with higher forced vital capacity (FVC) [9].

Resveratrol interferes with all three stages of carcinogenesis –initiation, promotion, and progression. These mechanisms include modulation of transcription nuclear factor. Resveratrol has been shown to induce fast ligand mediated apoptosis and gene p53. Resveratrol has inhibitory effect on cardiac fibroblast and inhibits progression of cardiac fibrosis. Resveratrol also increases natural testosterone production as being an aromatase inhibitor.

Resveratrol gets extensively metabolized in the body. Liver and Gut are the major sites of metabolism. It is excreted in urine[10]. The strongest evidence of anticancer action of Resveratrol exists for tumors it can contact directly, such as skin and gastrointestinal tract tumors. Resveratrol appeared to prevent development of mammary tumors in animal models. When injected in high doses, Resveratrol slowed the growth of neuroblastoma[11]. Resveratrol has been effective for photo protection induced by ultraviolet radiation which is one of major cause of premature aging. Resveratrol inhibits platelet aggregation.

Liver is the largest organ of body weighing approximately 1500 Gm. The organ is wedge-shaped. It is located in right hypochondrium and epigastrium and extending into left hypochondrium[12]. It is protected by rib cage and maintains its position through peritoneal reflections, referred as ligamentous attachments.
Various factors responsible for the maintenance of liver position include tone of abdominal muscle, intrahepatic position of hepatic veins where they enter the inferior vena cava, as well as the ligamentous attachment to diaphragm and anterior abdominal wall.

Liver has extensive range of functions which are interrelated to one another. This becomes obvious during pathological conditions of liver when more than one function is affected.

MATERIAL AND METHODS:

This is an experimental study. The study was conducted on 48 healthy Wister Albino rats obtained from animal house – BMSI JPMC, for 25 days from 13 August, 2018 to 05 September, 2018. Animal weight was between 210 – 310 Gm.
Rats were divided into six (6) groups randomly, each group including eight (n = 8) animals, grouped as Control (Group I), B-carotene only (Group II), Resveratrol only (Group III), MTX only (Group IV), B-carotene + MTX (group V) and Resveratrol + MTX (group VI). The doses of methotrexate, and B-carotene used in present study were selected on the basis of dosages reported in earlier study[13].

- Group I (control) rats received equivalent volumes of saline `
- Group II (B-carotene) rats received B-carotene (10mg/kg/ day intra peritoneal) for 24 days.

- Group III (Resveratrol) rats received resveratrol (10mg/kg/ day intra peritoneal) for 24 days.
- Group IV (MTX) rats were given MTX as a single intra peritoneal dose (in saline, 20mg/kg) on day 21 of the experiment.
- Group V (β-carotene + MTX) rats were given β-carotene by intra peritoneal injection in vehicle (saline) 10 mg/kg/day for 24 days and then further administered MTX at a dose level of 20 mg/kg on day 21 of the experiment.
- Group VI (Resveratrol + MTX) rats were given intra peritoneal injection in vehicle (saline) 10mg/kg/day for 24 days and were further administered MTX at a dose level of 20 mg/kg on day 21 of the experiment.

At the end of the experimental period i.e. 25th day, the rats were weighed and sacrificed by giving prolonged ether anesthesia in a glass container and then fixed on the dissecting board. A midline longitudinal incision was made in upper trunk extending from manubrium stern to lower abdomen to expose the organs. Histological analysis was performed at department of pathology, BMSI, JPMC. Biochemical determination of components in tissue homogenates was performed at Biochemistry laboratory of Liaquat National hospital, Karachi.

The liver was exposed and gross appearance was observed for any change in color, shape, size, consistency and contour. The liver of each animal was exposed and removed. The absolute weight of each liver was recorded on Sartorius balance and relative weight was calculated by the following formula (Sohrabi *et al*., 2010)

$$\frac{\text{Weight of the liver}}{\text{Final body weight}} \times 100$$

After weighing and washing in normal saline, the liver was cut into small pieces and fixed into 10% buffered neutral formalin and in alcoholic formalin for PAS at least 36 hrs.
The liver tissues were fixed in 10% formalin for 24 hours. From fixed liver tissue 2mm square pieces were cut and fixed in ascending strength of alcohol, cleared in xylene, infiltrated and embedded with paraffin.

5 µm thick sections were cut with the help of rotatory microtome. Then paraffin sections were floated on water bath at 42ºC, the tissues sections were taken on albumenized glass slides and fixed on hot plate at 60ºC.
Biochemical analysis in all groups was done as per standards protocols. Liver homogenate was used to analyze catalase (CAT), glutathione peroxidase (GP-x) and melondialdehyde (MDA) activities.

RESULTS:

(SPSS 17.0) was used for statistical analysis. The results were compared with paired t-test with $p< 0.05$ considered as statically significant. All results were expressed as means ± standard error (SEM).

Observation on Body Weight (Gm)
Observation on control group I
The mean value of initial body weight in control group I was 239.33±0.882 Gm and final body weight was 248.50±0.764 Gm.

Table 1: Comparative study of animals Weight (Gm) of various Groups I to VI by paired T-test

Groups	Mean (Gm) ± Standard Error Mean (SE)	
	Initial Weight	Final Weight
Group I (Saline Control)	239.33 ± 0.882	248.50 ± 0.764
Group II (β -Carotene alone)	233.17 ± 1.641	246.00 ± 1.528
Group III (Resveratrol alone)	226.67 ± 1.994	236.83 ± 1.815
Group IV (Methotrexate alone)	235.50 ± 1.335	210.17 ± 2.088
Group V (β -carotene + MTX)	232.17 ± 0.601	217.00 ± 2.191
Group VI (Resveratrol + MTX)	239.83 ± 1.302	231.50 ±0.671

Observation on β-carotene group II
The mean initial body weight in β-carotene group II was 233.17±1.641 Gm and final body weight was 246.00±1.528 Gm

Observation on Resveratrol group III
The mean initial body weight in group III was 226.67±1.994 Gm and final body weight was 236.83±1.815 Gm
Observation on methotrexate group IV

The mean value of initial body weight group IV was 235.50±1.335Gm and final body weight 210.17±2.088 Gm. The data showed that there was significant decrease in (p≤0.05) final body in group IV when compared to group I and group II and group III.

Table II: Comparative study of animals Weight of various Groups I to VI by paired T-test

Groups	**IW vs FW Sig. (2-tailed) (<0.05)**
Group I (Saline Control)	.000**
Group II (β -Carotene alone)	.000**
Group III (Resveratrol alone)	.003**
Group IV (Methotrexate alone)	.000**
Group V (β -carotene + MTX)	.003**
Group VI (Resveratrol + MTX)	.005**

Observation on β-carotene with methotrexate group V

The mean value of initial body weight in group V was 232.17±0.601Gm and final body weight was 217.00±2.191 Gm. The data showed that there was significant decrease in (p≤0.001) final body weight in group IV when compared to group V.

Observation on Resveratrol with methotrexate group VI

The mean value of initial body weight in group VI was 239.83±1.302 Gm and final body weight was 231.50±0.671 Gm. The data showed that there was significant decrease (p≤0.05) in final body in group IV when compared to group VI.

Observation on Absolute Weight of Liver

Observation on control group I, β-carotene group II and Resveratrol group III

The mean value of absolute weight of liver in control group I was 8.99±0.37 Gm, in β-carotene group II was 8.97±0.36 Gm and in Resveratrol group III was 8.98±0.36 Gm.

Table III: Mean Absolute Weight of Liver (Gm) of Group I – VI

Groups	**Mean ± Standard Error Mean (SE) Gm**
Group I = Saline (control)	8.99 ± 0.37
Group II = β -Carotene alone	8.97 ± 0.36
Group III = Resveratrol alone	8.98 ± 0.36
Group IV = Methotrexate alone	10.30 ± 0.43
Group V = β – Carotene + MTX	9.56 ± 0.42
Group VI = Resveratrol + MTX	9.42 ± 0.39

Observation on methotrexate group IV

The mean value of absolute weight of liver in methotrexate group IV was 10.30±0.43 Gm. The data showed that there was significant increase in (p≤0.0001) absolute weight of liver when group IV was compared with group I. The data also showed that there was significant increase in (p≤ 0.01) absolute weight of liver when group IV was compared to group II and group III.

Table IV: Statistical Comparison using Paired T-test of mean absolute weight among Various Groups I to VI

Paired Samples Statistics (95% Confidence Interval of the Difference)	
Paired Comparison Groups	**Sig. (2-tailed) (P< 0.05)**
Group I = Saline vs. Group IV = Methotrexate	0.0001**
Group II = Carotene vs Group IV = Methotrexate	0.01**
Group III = Resveratrol vs Group IV = Methotrexate	0.01**
Group V = β – Carotene + MTX vs Group IV = MTX	0.03**
Group VI = Resveratrol + MTX vs Group IV = MTX	0.03**
Group V = β – Carotene + MTX vs Group VI = Resveratrol + MTX	0.21*

Results were considered significant when P < 0.05

MTX = Methotrexate

KEY: ** = Significant

* = Non Significant

Observation on β-carotene with methotrexate group V.
The mean value of absolute weight of liver in group V was 9.56±0.42 Gm. The data showed that there was significant increase in (p≤ 0.03) absolute weight of liver when group IV was compared with group V.
Observation on Resveratrol with methotrexate group VI
The mean value of absolute weight of liver in group VI was 9.42±0.39 Gm. The data showed that there was significant (p≤0.05) increase in absolute weight of liver when group IV was compared with group VI.

Observation on Mean Relative Weight of Liver (Gm)
Observation on control group I, β-carotene group II, Resveratrol group III.
The mean value of relative weight of liver in control group I was 4.495±0.45 Gm. The mean relative weight of liver in β-carotene group II was 4.485±0.51 Gm. The mean value of relative weight of liver in Resveratrol group III was 4.490±0.65 Gm.

Table V: Mean relative weight of liver (Gm %) in Various Groups I – VI

Groups	**Mean ± Standard Error Mean (SE)**
Group I = Saline (Control)	4.495 ± 0.45
Group II = β -Carotene	4.485 ± 0.51
Group III = Resveratrol	4.490 ± 0.65
Group IV = Methotrexate	5.150 ± 0.34
Group V = β – Carotene + MTX	4.780 ± 0.45
Group VI = Resveratrol + MTX	4.720 ± 0.41

Results are expressed as Mean ± SE in Gm % (Standard Error Mean = SE) MTX = Methotrexate,

Observation on methotrexate group IV
The mean value of relative weight of liver in methotrexate group IV was 5.150±0.34 Gm. The data showed that there was significant increase in (p≤0.001) mean relative weight of liver when group IV was compared with group I. The data also showed there was significant increase in (p≤0.01) in relative weight of liver when group IV was compared with groups II and III.

Table VI: Statistical Comparison of mean relative weight of liver among Various Groups I to VI by Paired T-Tests

Paired Samples Statistics (95% Confidence Interval of the Difference)	
Paired Comparison Groups	**Sig. (2-tailed) (P< 0.05)**
Gorup I = Saline vs Group IV = Methotrexate	0.001**
Group II = β Carotene vs Group IV = Methotrexate	0.01**
Group III = Resveratrol vs Group IV = Methotrexate	0.01**
Group V = β – Carotene + MTX vs Group IV = Methotrexate	0.03**
Group VI = Resveratrol + MTX vs Group IV = Methotrexate	0.03**
Group V = β – Carotene + MTX vs Group VI = Resveratrol + MTX	0.21*

Results were considered significant when P < 0.05
MTX = Methotrexate
KEY:
** = Significant
* = Non Significant

Observation on β-carotene with methotrexate group V
The mean value of relative liver weight in group V was 4.780±0.45 Gm. The data showed that there was significant decrease in (p≤ 0.01) relative weight of liver when group V was compared with group IV.
Observation on Resveratrol with methotrexate group VI
The mean value of relative weight of liver in Resveratrol with methotrexate group VI was 4.720±o.41 Gm. The data showed that there was significant (p≥0.05) decrease in relative weight of liver when group VI was compared with group IV animals.
The mean hepatocyte size in group II was 23.07±0.69 µm.

Table VII: Determination of Hepatocytes Sizes (µm) in Various Groups I – VI

Groups	Mean ± Standard Error Mean (SE)
Group I (Saline)	22.2150 ± 0.38087
Group II (β-Carotene)	23.0700 ± 0.69963
Group III (Resveratrol)	22.5783 ± 0.54981
Group IV (Methotrexate)	28.8050 ± 0.37599
Group V (β – Carotene + MTX)	25.7233 ± 0.44670
Group VI (Resveratrol + MTX)	25.2783 ± 0.66156

Results are expressed as Mean ± SE (Standard Error Mean = SE)
MTX = Methotrexate

DISCUSSION:

Methotrexate is an antifolate, anti-metabolite drug. It is used in the therapy of leukemia, lymphoma and several solid tumors[1]. β-carotene is one of carotenoid found in plants. It is a potent anti-oxidant. Resveratrol is both free radical scavenger and a potent antioxidant, for its ability to promote activities of a variety of antioxidant enzyme[14]. The present study was designed to observe the protective effect of β-carotene and Resveratrol on methotrexate induced hepatic damage in rats.

Before recording the finding on liver, the effects of Methotrexate and β-carotene, and Resveratrol on the animal's body weight and liver weight were recorded.

In present study, methotrexate was used at a dose of 20 mg/kg of body weight, intraperitoneal which was according to protocols suggested by Vardi et al (2010). In the present study there was a reduction of body weight of group IV which could be due to altered hepatic metabolism[15]. This reduction in body weight could be because of alteration of physiological factors by methotrexate.
In present study the animals of group V and group VI had increase in the body weight, more or less same as compared to control rats which was in accordance with the study reported by Vardi et al (2010). Weight gained could be explained due to increase in appetite caused by Resveratrol which reduces oxidative stress. Similarly, β-carotene also improves appetite by decreasing oxidative stress as reported by Kheir-Eldin et al (2001) and Morakinyo et al (2012)[16] who also observed increased body weight after co-administration of β-carotene, in acetaminophen induced hepatoxicity.

In present study in methotrexate group IV there was increase in absolute and relative weight of liver, this is also in agreement with Vardi et al (2010) observed increase in absolute and relative liver weight after treatment with methotrexate.

The increase in absolute and relative liver weight in group IV was due to methotrexate toxicity, which caused cellular hypertrophy, sinusoidal congestion and infiltration of inflammatory cells as suggested by Vardi et al (2010).

In the present study after post treatment with β-carotene plus methotrexate group and Resveratrol plus methotrexate treated group animals the absolute and relative liver weight was decreased, this is in agreement with Tunali-Akbay et al (2010)[15].

Increase in size of hepatocyte and its nuclei was observed in current study which could be attributed to injury caused by methotrexate which may be explained on the basis of distortion and disintegration of hepatocytes architecture as reported by Tunali-Akbay et al (2010)[15]. It was also reported by Vaghasiya et al (2009)[17] who also showed ballooning of hepatocytes. It was also in agreement with work done by Rubin (2001). It may be due to accumulation of toxic metabolites which caused damaged cell membrane resulting in ballooning of hepatocytes as suggested by Rubin (2001)[18].

In present study, in methotrexate treated group IV animals, the level of hepatic enzymes ALT, AST, LDH, and γGT were also increased in correspondence to histological findings. This was probably due to hepatocytes destruction resulting in increase in membrane permeability and leakage of enzymes into sinusoids and then into circulation as suggested by Vardi et al (2010). This correlates with findings of Kuvandik et al (2008) who reported that the levels of AST, ALT, LDH and γGT were increased significantly in acetaminophen hepatoxicity. In our study after co-administration of β-carotene in group V the levels of AST, ALT, LDH and γGT were lower as β-carotene restored the hepatocellular destruction. This correlates with the result of Sugiura et al (2006)[19] who showed that carotenoid may prevent increase serum hepatic enzymes. In our study Resveratrol given to group VI normalized the level of hepatic enzymes as shown by Tunali-Akbay et al (2010)[15]. This is in

agreement with Pervaiz (2003)[20] who had showed that Resveratrol is both free radical scavenger and potent antioxidant and its ability to promote activities of variety of antioxidant enzymes.

The results of the present study suggested that methotrexate caused direct toxic insult to liver tissue and co-administration of β-carotene and resveratrol resulted in normalizing the hepatic lobular architecture appreciably.

CONCLUSION:

From this study it is concluded that methotrexate severely causes hepatic damage in rats, which can be protected by β-carotene and Resveratrol. It is suggested that results could be considered promising enough in humans who are on methotrexate that lead to hepatotoxicity, which can be prevented by use of β-carotene and Resveratrol.

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