ORIGINAL RESEARCH

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The Effects of Pomegranate and Carvacrol on Methotrexate-Induced Bone Marrow Toxicity in Rats

Abstract

Purpose: The aim of this study was to evaluate the effects of pomegranate (PMG) extract and carvacrol (CARV) on methotrexate (MTX)-induced oxidative stress and bone marrow toxicity.

Methods: Wistar albino rats (32 rats) were divided into four groups (n=8): Group 1 was control; Group 2 was given a single intraperitoneal injection of methotrexate (20 mg/kg); Group 3 was treated with carvacrol (73 mg/kg i.p.) one day before MTX (20 mg/kg i.p.) injection; and, Group 4 received a single dose of MTX (20 mg/kg i.p) while PMG was administered orally for seven days at 225 mg/kg. After animals were euthanized, blood samples were taken to evaluate hematological parameters and oxidative stress. In addition, the femur was cropped and bone marrow was extracted for examination.

Results: White blood cell count, hemoglobin, hematocrit and platelet count were found to be decreased in the MTX group, but these changes were prevented in the groups that received CARV and PMG. Furthermore, decreased bone marrow cellularity was found in the groups treated with MTX, whereas the PMG and CARV groups had cellularity similar to controls. Strikingly, oxidative stress increased in the MTX group, but was ultimately decreased in the rats that received the antioxidants PMG and CARV.

Conclusion: Carvacrol and PMG were found to be protective against methotrexate-induced oxidative bone marrow damage. Use of these antioxidants, in combination with chemotherapeutics, may help to reduce some adverse effects of methotrexate.

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Methotrexate (MTX) is a folic acid (FA) antagonist and chemotherapeutic agent in tumor treatment and is one of the most commonly used antimetabolite agents for childhood cancers. It is also used for the treatment of inflammatory diseases such as idiopathic juvenile arthritis, psoriasis and dermatomyositis [1-4]. MTX decreases the production of rheumatoid factor and the interaction between leukocytes. It can be used at an intermittent low dose for the treatment of refractory rheumatic diseases [5] and also for the treatment of ectopic pregnancy [6, 7].

MTX restricts thymidylate synthesis and, ultimately, purine and pyrimidine nucleotides (the building blocks of DNA and RNA) by inhibiting the dihydrofolate reductase enzyme that converts dihydrofolate to tetrahydrofolate [8, 9]. Both therapeutic and toxic effects of MTX have emerged as a result of the changes made by MTX on many metabolic pathways, including DNA synthesis [10]. In addition, MTX treatment is known to cause bone marrow toxicity [11].

Punica granatum, commonly known as pomegranate (PMG), is a fruit that grows in tropical and subtropical areas. The juice extract of PMG contains approximately 40% ellagic acid [12]. Ellagic acid is a polyphenol compound with antioxidant and anti-proliferative properties that also exists in many other fruits and plants such as raspberries, pecan nuts and strawberries. These components demonstrate anti-inflammatory and antioxidant effects by inhibiting the expression of pro-inflammatory enzymes and cytokines [13, 14]. The anti-carcinogenic [15], antioxidant [16, 17], anti-inflammatory [18], antimicrobial [19, 20], antihypertensive and angiogenic effects of PMG extract are well established.

Carvacrol (CARV) is a common ingredient found in the widely used herb, oregano. Oregano is generally accepted as a safe food [21, 22]. Many different features and effects have been reported for oregano, which has been used for epidemics since ancient times, as a result of contemporary studies. In animal experiments, it was observed that CARV extract from oregano has anti-inflammatory [23], antimicrobial [24], anti-tumor, anti-spasmodic [25, 26] and antioxidant [28] properties. In addition, it was reported that it had a protective effect against liver injury [27].

Oxidative stress causes cellular damage as a result of the imbalance between reactive oxygen species and the biological ability of the cell to repair damage or detoxify oxidants [29]. Malondialdehyde (MDA) is an important product of lipid peroxidation and results in some adverse effects, such as ion permeability and change of enzyme activity, by acting on ion exchange through cell membranes. It has been reported that an increase in MDA levels correlates with an increase in free oxygen radicals in a tissue [30]. In the measurement of oxidative

stress, it can be difficult to pinpoint specific oxidants, because an individual measurement for each antioxidant within a biological sample is required. Measurement of total antioxidant capacity (TAC) is useful if one wants to demonstrate total changes in antioxidant status within specific samples [31]; therefore, the measurement of TAC may be an important and useful tool in the prevention of methotrexate-induced oxidative damage/toxicity.

There have been an increasing number of studies about the antioxidant protection of PMG and CARV. This research is the first *in vivo* study investigating the effects of PMG and CARV against the toxicity of MTX on bone marrow in rats. The aim of this study was to investigate whether the adverse effects of MTX can be prevented by these natural molecules.

Materials and Methods

The effects of PMG and CARV on bone marrow toxicity in rats treated with MTX were compared.

Animals

The study protocol was approved by Dicle University Faculty of Medicine, Local Ethics Committee for Animal Experiments. Wistar albino rats were obtained from Dicle University Central Animal House. Male Wistar Albino rats, 8-12 weeks of age with an average weight of 250 grams, were used. Animals were kept under an appropriate moisture, lighting (12 hours of daylight/12 hours of dark) and heat (21 ± 2 °C). Animals were fed standard rat chow and tap water. A wire litter was placed in the cage in order to prevent coprophagy.

Experimental Protocol

Experimental animals (32 rats) were divided randomly into four groups: control, methotrexate (MTX), MTX+CARV, and MTX+PMG groups. The study protocol is summarized in Table 1.

Group 1 (Control): As a basic control group, rats were not given any drug. Normal feeding was provided.

Group 2 (MTX): On the second day methotrexate (MTX, Onco-Tain, Faulding Pharmaceuticals Pic, UK) 20 mg/kg was given intraperitoneally. Rats were fed with normal food for seven days.

Group 3 (MTX+CARV): A 73 mg/kg dose of oregano oil containing 83% CARV was injected intraperitoneally on the first day, followed by a 20 mg/kg dose of MTX injected intraperitoneally on the second day. The plant substance tested in this study, CARV (2-methyl-5-(1-methylethyl)-phenol), was isolated from steam distilled essential oil of *Origanum onites* L.

TABLE 1. Study protocol

	First day	Second day	Third day	Fourth day	Fifth day	Sixth day	Seventh day	Eighth day
Group 1	*	*	*	*	*	*	*	Ex
Group 2	*	MTX	*	*	*	*	*	Ex
Group 3	CARV (73 mg/kg)	MTX	*	*	*	*	*	Ex
Group 4	PMG (225 mg/kg)	MTX+PMG	PMG	PMG	PMG	PMG	PMG	Ex

MTX: methotrexate, CARV: carvacrol, PMG: pomegranate, Ex: animals sacrificed, *: standard rat feeding

collected from West Anatolia as described by Canbek *et al.* [32].

Group 4 (MTX+PMG): 225 mg/kg pomegranate extract (Pomella®, Verdure Sciences, Noblesville, IN, USA; 250 mg/kg/day) was administered orogastrically (with a gavage needle) once each day beginning on the first day and continuing for seven days. Twenty mg/kg of MTX was given intraperitoneally on the second day.

No animal died during the experiment. To obtain blood and bone marrow samples, the animals were sacrificed with general anesthesia: 1 mg/kg xylazine (Rompun®, Bayer AG, Leverkusen, Germany) + 0.5 ml/kg ketamine was injected intraperitoneally for general anesthesia.

Hematology

Blood samples were extracted intracardially for hematological and biochemical tests into separate tubes by opening the abdomen with a median line incision. Heparinized blood samples were used for hematological analysis. The hematological parameters hematocrit (Hct), hemoglobin (Hb), red blood cell count (RBC), total white blood cell count (WBC), mean corpuscular volume (MCV), platelets (PLT) and lymphocytes were measured by automated cell counter (Abbott CELL DYN 3700 model device).

The femur was cropped and the bone marrow cavity was accessed with a needle. Bone marrow samples obtained from the femur were stained using May-Grünwald and Giemsa stainings. Bone marrow aspirates from the femur of each animal were assessed histologically to assess granulocytic cell lineage, erythroid cell lineage, presence of megakaryocyte and density of adipocytes. Microscopic scoring was done by an experienced histologist (Dr. S.S.) blinded to the animal groups. Histological slides were examined with a light microscopic using magnifications of 41, 82 and 164 times. Arbitrary areas were chosen for assessing cell densities. Then count of cell and density of adipocytes per each 10×40 amplification areas (one 10×40 magnification microscopic area was 0.19 mm²) were determined. The average values of five adjacent microscope areas were found, and then cell count and density of adipocytes per 1 mm² were

calculated. Bone marrow cellularity scoring was accepted as follows; (4 points) cell count between 80% and 100%, (3 points) cell count between 60% and 80%, (2 points) cell count between 40% and 60%, and (1 point) count of cells <40%. Density of adipocytes in the bone marrow were scored as follows; (1 point) normal density of adipocytes, (2 points) mild (<25%), (3 points) moderate (<25%-50%) and (4 points) severe intense adipocytes (>50%).

Biochemical measurements

TAC and total oxidant status (TOS) were measured by the Erel method with an Abbott Architect c16000 (Abbott Park, North Chicago, IL, USA) chemical analyzer [31]. Determination of MDA levels was performed by HPLC based on the differentiation with dinitrophenylhydrazine [33].

Statistical analysis

All data are expressed as mean and standard deviation (SD). A one-sample Kolmogorov-Smirnov test was performed in order to determine if the data was normally distributed. Intergroup comparisons were performed using the Kruskal-Wallis and Mann-Whitney U-test. P values <0.05 were considered statistically significant. All data were processed using the statistical package SPSS 18.0 for Windows (IBM Corporation, Armonk, NY).

Results

Significantly lower Hb and Hct values, and higher MCV values were detected in the rats treated with MTX (Group 2) ($p<0.05-0.01$; Table 2). The Hb and Hct values were significantly lower in Group 2 compared with Group 3 (MTX+CARV) and Group 4 (MTX+PMG) ($p<0.05$) (Table 2). The peripheral white blood cell counts were found to be lower in Group 2 when compared with Group 3 and Group 4 ($p<0.05$) (Table 2). Although platelet counts were lower in Group 2, they were closer to normal values in Group 3 and Group 4 (Table 2). MTX-induced changes in the number and percentage of blood cells in the rats treated with PMG were

TABLE 2. The differential values of hematological parameters in the control, methotrexate-treated, CARV+MTX treated and PMG+MTX treated groups

Groups	Hb (g/100 ml)	Htc (%)	MCV (fL)	WBC (cells/ μ l)	RBC ($\times 10^6$ cells/ μ l)	Plt ($\times 10^3$ cells/ μ l)	Lymphocytes (cells/ μ l)
1=Control	11.43 $\pm$ 1.22	34.31 $\pm$ 3.67	55.05 $\pm$ 4.60	8.18 $\pm$ 1.55	5.55 $\pm$ 0.51	386.87 $\pm$ 162.96	64.10 $\pm$ 3.95
2=MTX	7.58 $\pm$ 1.19	22.76 $\pm$ 3.59	61.25 $\pm$ 4.43	3.42 $\pm$ 0.59	3.32 $\pm$ 0.36	88.03 $\pm$ 24.67	76.25 $\pm$ 5.80
3=MTX+CARV	9.27 $\pm$ 0.60	27.82 $\pm$ 1.82	53.12 $\pm$ 3.52	4.30 $\pm$ 1.59	5.02 $\pm$ 0.22	138.92 $\pm$ 26.55	60.37 $\pm$ 2.97
4=MTX+PMG	9.95 $\pm$ 0.47	29.85 $\pm$ 1.41	55.25 $\pm$ 5.14	5.72 $\pm$ 0.99	5.52 $\pm$ 0.45	166.00 $\pm$ 22.27	62.50 $\pm$ 4.24
<i>p-Value between groups</i>							
1-2	0.001	0.001	0.018	0.001	0.001	0.001	0.001
2-3	0.016	0.003	0.005	0.010	0.010	0.003	0.046
2-4	0.005	0.009	0.021	0.001	0.001	0.050	0.001

MTX, Methotrexate; CARV; carvacrol; PMG, pomegranate; TAC, total antioxidant capacity; TOS, total oxidant status; MDA, malondialdehyde

Groups; 1: control group, 2: MTX treatment group, 3: MTX+CARV treatment group, 4: MTX+PMG treatment group

Hb: hemoglobin, Htc: hematocrit, MCV: mean corpuscular volume, WBC: white blood cell counts, RBC: red blood cell count, Plt: platelets

more similar to control groups than rats treated with CARV (Table 2).

Evaluation of bone marrow smears indicated a significant decrease in bone marrow cellularity in Group 2 (median 2.0, range 1.0-2.0) when compared with the control group (median 4.0, range 3.0-4.0) ($p < 0.001$) (Fig. 1a, 1b, 1c).

An increase in bone marrow cellularity was observed in Groups 3 (median 3.0, range 2.0-4.0) ($p = 0.005$) and 4 (median 4.0, range 3.0-4.0) ($p = 0.001$) when compared with Group 2. Additionally, a more significant increase in bone marrow cellularity was detected in Group 3 than in Group 4 ($p = 0.034$) (Fig. 2a, 2b).

Overall evaluation of bone marrow smears showed that side effects of MTX treatment in rats were related to the increase in bone marrow adiposity. The median (minimum-

maximum) values of adiposity scores in groups were as follows; Controls 1.0 (1.0-2.0), MTX 3.0 (2.0-4.0), MTX+CARV 2.0 (1.0-4.0), and MTX+PMG 2.0 (1.0-3.0); however, PMG supplementation was found to be more effective in the suppression of bone marrow adiposity than CARV supplementation ($p = 0.01$) (Fig. 1b, 2a, 2b).

Serum oxidative stress parameters are presented in Table 3. Serum MDA levels were significantly higher in Group 2 than in Groups 3 and 4 as shown in Table 3. Similarly, serum TOS levels were significantly higher in Group 2 compared with Groups 3 and 4 (Table 3). Interestingly, TAC was detected to be significantly lower in Group 2 compared with Group 1 ($p = 0.002$; Table 3). TAC was determined to be significantly lower in the group given only MTX than in the group given MTX+CARV ($p = 0.001$) (Table 3). TAC was detected to be

TABLE 3. Oxidant and antioxidant parameters in rat groups (mean $\pm$ standard deviation)

Groups	TAC (mmol Trolox Eq/g protein)	TOS (mmolH ₂ O ₂ Eq/g protein)	MDA (nmol/g)
1=Control	1.67 $\pm$ 0.08	11.40 $\pm$ 3.38	2.47 $\pm$ 0.55
2=MTX	1.34 $\pm$ 0.15	35.57 $\pm$ 26.45	3.55 $\pm$ 0.34
3=MTX+CARV	1.55 $\pm$ 0.05	12.28 $\pm$ 6.21	2.37 $\pm$ 0.69
4=MTX+PMG	1.65 $\pm$ 0.16	16.18 $\pm$ 5.95	2.47 $\pm$ 0.85
<i>p-Value between groups</i>			
1-2	0.002	0.001	0.002
2-3	0.016	0.003	0.005
2-4	0.005	0.009	0.021

MTX, Methotrexate; CARV, carvacrol; PMG, pomegranate; TAC, total antioxidant capacity; TOS, total oxidant status; MDA, malondialdehyde

1: control group, 2: MTX treatment group, 3: MTX+CARV treatment group, 4: MTX+PMG treatment group

FIGURE 1.

- a. Control group: Normal cellularity and no increase in adiposity (May-Grünwald and Giemsa staining x 40). Arrow: metamyelocyte
- b. MTX treated group: Significant increase in adiposity (May-Grünwald and Giemsa staining x 40). Arrow: lymphocyte
- c. MTX treated group: Decrease in cellularity and increase in adiposity (May-Grünwald & Giemsa staining x 40). Arrow: lymphocyte

FIGURE 2.

- a. MTX+CARV treated group: A slight decrease in cellularity and a slight increase in adiposity (May-Grünwald and Giemsa staining x 40). Arrow: adipocyte
- b. MTX+PMG treated group: Normal cellularity and a slight increase in adiposity (May-Grünwald and Giemsa staining x 40). Arrow: metamyelocyte

lower in Group 2 than in Group 3 (MTX+PMG) ($p = 0.005$) (Table 3).

Discussion

MTX, one of the FA antagonists, is commonly used with an intermittent low dose in the treatment of juvenile idiopathic arthritis [34], autoimmune diseases [35, 36], inflammatory myopathies [37] and panuveitis [38]. In addition, it is also used as a basic chemotherapeutic agent for childhood leukemia and for some other types of cancer. MTX affects highly proliferative cells, and malignant cells are not specifically targeted; thus, other cells, such as hematopoietic cells within the bone marrow, are adversely affected [39]. Many potential side effects have been detected in both clinical observations and experimental animal studies due to short and long term use of MTX. The aim of this study was to investigate the effects of PMG and CARV against MTX bone marrow toxicity in experimental animals. For this reason, the hematological parameters, including cell number and percentage, and biochemical parameters, such as TAC, TOS and MDA, were evaluated. MTX was administered to rats at a dose of 20 mg/kg intraperitoneally, as defined previously in tissue toxicity studies [40, 41].

In recent years, there have been many studies aiming to take advantage of natural products with antioxidant activity in order to prevent tissue damage due to oxidative stress. It has been shown that foods and drinks with high levels of phenolic components reduce the risk for some diseases such as cardiovascular diseases and cancer [42]. Pomegranate is a valuable plant that contains polyphenolic components. In particular, ellagic acid is found in high concentrations in PMG and has functional and medical effects [43]. This component has been linked to the release of antioxidant compounds and the reduction of lipid peroxidation [43]. In addition, ellagic acid displays anti-inflammatory and antioxidant effects by inhibiting the expression of pro-inflammatory enzymes and cytokines.

To date, the complex mechanism(s) of the effect of MTX on bone marrow toxicity has not been elucidated [4]. It has been suggested that oxidative stress is a consequential side effect of chemotherapeutic drugs [44, 45]. For this reason, the effect of PMG and CARV on MTX bone marrow toxicity was investigated by evaluating the oxidative stress parameters MDA, TAC, and TOS [46]. Although PMG and CARV reduced the bone marrow toxicity of MTX, it is not clear whether the reduced toxicity is a result of PMG and CARV directly interfering with the folic acid antagonism or via another mechanism. A PMG- and CARV-induced reduction in oxidative stress and reduced marrow toxicity might also reduce

the desired effects of MTX therapy, though this was not determined.

In previous studies, it has been reported that MTX increases MDA levels and addition of antioxidant substances can decrease MDA levels [47]. Jahovic and colleagues demonstrated that a single 20 mg/kg intraperitoneal dose of MTX significantly increases blood MDA levels in rats [48]. In our study, increased serum MDA levels in the MTX group compared with the control group was detected. Strikingly, the serum MDA levels were significantly lower in MTX+PMG and MTX+CARV groups compared with the MTX group. These results suggest that the antioxidant properties of PMG and CARV are protective against the oxidative side effects of MTX.

TAC and TOS are important biochemical markers for determining oxidative status. In this study, while TAC levels decreased in rats injected with MTX, TAC levels increased in the rats injected with MTX+PMG and MTX+CARV. An increase in TOS levels in rats receiving MTX, and a decrease in TOS levels in MTX+PMG and MTX+CARV rat groups, were observed; probably due to the protective effects of PMG and CARV. Collectively, these results suggest that PMG and CARV exert a protective effect on MTX-treated rats and may be important in the reduction of side effects associated with the use of this chemotherapeutic agent.

Kojima and colleagues demonstrated the significant decrease in red serial cells in rats treated with MTX [49]. In our study, anemia due to disruption of hematopoiesis in the bone marrow in the Wistar albino rats treated with MTX was detected. Lower hemoglobin and hematocrit values and higher MCV values of MTX group may be secondary to folic acid antagonist effect of MTX; however, with the addition of both CARV and PMG, significantly decreased MCV and increased hemoglobin and hematocrit levels were observed (Table 2). In addition, it was also observed that the number of red blood cells was higher in rats with MTX+CARV and MTX+PMG than in the rats treated only with MTX. These anti-anemic effects of CARV and PMG supplementation indicate that CARV and PMG may antagonize folic acid inhibitor effects of MTX.

In a study conducted by McEwen and colleagues, it was reported that MTX treatment causes leukocytopenia in patients [50]. Cohen and colleagues reported that development of pancytopenia after single dose MTX treatment [51]. In our study, MTX-treated rats also had leukocytopenia and the leukocyte count was higher in the rats treated with MTX+CARV and MTX+PMG than in the rats treated with only MTX. Çetiner and colleagues linked the toxic effect of MTX on neu-

trophils and lymphocytes to oxidative tissue damage [40]. Based on this, PMG and CARV appears to decrease the oxidative damage and help to protect the number and percentage of white blood cells. Murakami and colleagues reported that they obtained both thrombocytopenia and thrombocytosis in rats treated with MTX [52]. It was demonstrated in our study, as well as in other previous studies, that MTX causes thrombocytopenia. More importantly, it was observed that PMG and CARV supplementation was effective in preventing thrombocytopenia.

It has been reported that MTX affects hematopoiesis in both erythroid and myeloid cells lineages [50]. Consistent with this observation, our study showed a significant decrease in bone marrow cellularity in rats treated with MTX. The increase in bone marrow cellularity in the group with MTX+PMG was determined to be more significant than the group with MTX+ CARV (Fig. 1b, 1c, 2a, 2b).

Xian and colleagues reported in their recent studies that fat-containing cells increase in bone marrow in rats injected with MTX, and thus bone marrow adiposity increases [53, 54]. In this study, only MTX treatment increased the bone marrow adiposity, and MTX+PMG treatment was more effective in suppression of bone marrow adiposity than MTX+CARV treatment.

In this study, the MTX-related toxicity was assessed but not the MTX-related therapeutic effects; thus, it is not known whether the PMG and CARV interventions would reduce the effectiveness of treatment with MTX.

One of the limitations of our study is the lack of an additional control group in which folic acid was given. Folic acid is commonly used to limit systemic action of MTX in patients receiving treatment with high dose MTX. Giving folic acid is a strategy to augment CNS activity of MTX without causing excessive systemic toxicity. The use of a folic acid group, as additional control group, would help to distinguish between the antioxidant activity versus rescue of the folic acid pathway.

Conclusion

The results of this study are consistent with the previously published studies presenting the adverse effects of MTX on bone marrow. PMG and CARV can achieve protective effects against MTX-induced toxicity and cell death, most likely due to their antioxidant properties. Interestingly, PMG was determined to be more effective in minimizing the adverse effects of MTX than CARV. These two natural products may be more effective in reducing or preventing oxidative tissue damage and toxicity caused by chemotherapeutics. Further experimental

and clinical studies in this area will provide much needed insight into the protective effects of these natural compounds against oxidative stress.

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