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Research Article

IMPACT OF ANTIOXIDANT SUPPLEMENTATION ON TOXICITY OF METHOTREXATE: AN *IN VITRO* STUDY ON ERYTHROCYTES USING VITAMIN E

MANASVEE DHANESHA, KANCHANLATA SINGH, MUSTANSIR BHORI, THANKAMANI MARAR*

School of Biotechnology & Bioinformatics, D. Y. Patil University, Navi Mumbai - 400 614, Maharashtra, India.
Email: dr.marar@yahoo.com

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ABSTRACT

Objective: Methotrexate (MTX) is an antimetabolite used widely in cancer and autoimmune diseases as an inhibitor of the enzyme dihydrofolate reductase. Treatment regimens with MTX lead to the formation of reactive oxygen species and are involved in drug-induced toxicity presenting severe side effects. In the present investigation, red blood cells were used as a model to understand drug-mediated hemolysis and alterations in antioxidant defense system stimulated by MTX. This effort attempts to evaluate, whether pre-supplementation of vitamin E can modulate drug induced toxicity.

Methods: Blood samples from healthy subjects were collected and processed to obtain 10% RBC solution in saline. This solution was further treated with 80μM MTX in presence and absence of 90μM vitamin E respectively. Treated and control RBC solutions were used to assess percent hemolysis, levels of lipid peroxides, GSH, lactate dehydrogenase and activities of specific antioxidant enzymes.

Results: Our study reveals a significant increase in lipid peroxides, reduced glutathione GSH reductase activity after incubation with MTX, lactate dehydrogenase, and a considerable decline in catalase, superoxide dismutase, GSH peroxidase, GSH S-transferase, and GSH reductase after incubation with MTX.

Conclusion: Ameliorative effect of vitamin E supplementation reduces oxidative stress and restores the activities of these antioxidant enzymes, thereby demonstrating the protection rendered by vitamin E. Our data indicates that vitamin E administration during chemotherapy is effective in modulating the chemotherapy-induced side effects thus stressing on the importance of vitamin E supplementation in combination with chemotherapy during cancer treatments.

Keywords: Antioxidant, Lipid peroxides, Superoxide dismutase, Glutathione, Catalase, Red blood cell.

INTRODUCTION

There is incomplete evidence whether antioxidant supplementation alters or interferes with the efficacy of cancer chemotherapy. There are arguments that antioxidants scavenge the reactive oxygen species (ROS) integral to the activity of certain chemotherapy drugs, thereby diminishing treatment efficacy. Counter arguments suggest antioxidants may mitigate drug-induced toxicity and thus allow for uninterrupted treatment schedules and a reduced need for lowering chemotherapy doses.

Methotrexate (MTX) (4-amino-10-methylpteroylglutamic acid) is a potent antineoplastic agent used to treat choriocarcinoma, leukemia, osteosarcoma, non-Hodgkins lymphoma, breast cancer, and lung cancer [1]. It is also involved in the treatment of non-cancerous conditions such as rheumatoid arthritis, psoriasis, immunological abnormalities, and systemic inflammation [2-4]. The primary mechanism of action is the depletion of reduced folates as a consequence of dihydrofolate reductase inhibition, an enzyme involved in DNA synthesis and DNA repair [5,6]. Low-moderate to high doses of MTX causes various side effects and may lead to conditions such as liver cirrhosis or fibrosis [7]. It has also been shown that MTX administration has severe side-effects on the hematopoietic system [8]. MTX induces oxidative stress by increasing ROS production which is implicated in tissue injury. Further, associated increase in oxidative stress may play an important role in the pathophysiology of drug-induced side effects [9,10].

α-Tocopherol (vitamin E) is a potent lipid-soluble chain-breaking antioxidant preventing the propagation of free radical reactions in cell membranes [11]. Increased serum vitamin E levels have been reported to decrease lipid peroxidation (LPX), inhibit protein kinase C, 5-lipoxygenase, smooth muscle cell proliferation, platelet aggregation, and the oxygen burst in neutrophils [12,13]. Pre-treatment with vitamin E has been reported to prevent many changes in serum enzymes and protect increase in hematocrit, fall in leukocyte count, hemoglobin level, mean osmotic fragility of erythrocytes [14]. Vitamin E is also been reported to induce apoptosis in experimental tumor lines, increase the survival time in a terminal cancer patient, and is shown to increase the activity certain drugs *in vivo* [15].

Our study is an attempt to modulate MTX induced side effects by the use of vitamin E as an antioxidant and in addition promoting supplementation of vitamin E in chemotherapy thereby aiding to diminish the consequences of drug toxicity by restoring the activities of antioxidant enzymes, levels of non-enzymatic antioxidants and inhibiting LPX in normal cells using red blood cells (RBCs) as a model system (*in-vitro* studies).

METHODS

MTX, glutathione (GSH) reduced, GSH reductase (GRD), oxidized GSH, nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from Sigma, St. Louis U.S.A. The other chemicals used were of high analytical grade and solvents were of Qualigen grade.

Preparation of RBC

A volume of 10 ml blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes from healthy volunteers after informed consent. The blood was centrifuged at 3000 rpm for 10 minutes and the plasma along with a buffy coat was removed. The RBCs were washed thrice with phosphate buffered saline (PBS), pH 7.4 and the tubes were centrifuged at 3000 rpm for 10 minutes. The RBC pellet obtained after the removal of the supernatant was diluted to 10% using PBS and was used to set up the experiments.

Experimental setup

The 10% solution of RBC pellet was further divided into four groups. The samples treated with vitamin E were kept for pre-incubation an hour prior to the addition of drug and then all samples were incubated for 24 hrs.

1. Control - 10% RBC + PBS (pH 7.4) + blood level glucose (5 mM)
2. Test - 10% RBC+ PBS (pH 7.4) + glucose (5 mM) + MTX (80 µM)
3. Vitamin E control - 10% RBC+ PBS (pH 7.4) + glucose (5 mM) + vitamin E (90 µM)
4. Test + vitamin E - 10% RBC+ PBS (pH 7.4) + glucose (5 mM) + MTX (80 µM) + vitamin E (90 µM)
5. 100% hemolysis - 10% RBC+ autoclaved D/W.

The above tubes were mixed well and incubated at room temperature for 24 hrs on a shaker at 30 rpm.

Estimation of percent hemolysis

The hemolysis was studied in the samples after 24 hrs. All the samples were centrifuged at 3000 rpm for 10 minutes. The absorbance of the supernatant was read at 540 nm and expressed as percent hemolysis. Complete hemolysis was induced by addition of distilled water to the pellet. This hemolysate was used for all further enzyme assays.

Estimation of lipid peroxidation (LPX)

LPX was determined by the method of Ohkawa *et al.* [16]. Proteins of the sample were precipitated by trichloroacetic acid (TCA). To this thiobarbituric acid (TBA) was added and heated at 80°C for 1 hr which forms a pink color adduct that is measured at 532 nm against blank. Tetramethoxy propane was used as a standard. Levels of LPX were expressed as µM of malondialdehyde/mg of protein in the sample.

Assay of superoxide dismutase (SOD) activity

SOD was assayed by monitoring the oxidation of epinephrine according to the procedure of Mishra and Fridovich [17]. The reaction mixture contained carbonate buffer (pH 10.2) and EDTA solution. Aliquots of the hemolysate were added and the absorbance was monitored after adding of epinephrine at 420 nm for 3 minutes. Auto oxidation of epinephrine was also monitored in the reaction mixture without adding the hemolysate. The final activity was expressed in terms of units/mg protein.

Determination of catalase (CAT) activity

CAT activity was determined according to the method Claiborne [18]. Hemolysate in phosphate buffer (pH 7.0) was added to 30 mM hydrogen peroxide (H_2O_2) and the decrease in absorbance due to decomposition of H_2O_2 was monitored at 240 nm. The enzyme activity was expressed as µmoles of H_2O_2 decomposed/minute/mg protein.

Assay of GSH-S-transferase (GST) activity

GST activity was assayed by the method of Habig *et al.* [19]. The reaction of conjugation of 1-chloro, 2, 4-dinitrobenzene (CDNB) with reduced GSH was monitored as an increase in absorbance at 340 nm. The reaction mixture contained phosphate buffer (pH 6.5), 30 mM CDNB and hemolysate. Tubes were incubated at 37°C for 5 minutes, 30 mM GSH was added, and the change in absorbance was recorded. The enzyme activity was expressed as nanomoles of CDNB conjugated/minute/mg protein.

Determination of GSH peroxidase [GPX] activity

GPX activity was assayed by the method of Paglia and Valentine, by monitoring NADPH oxidation in the presence of GRD, which reduces GSSG using hydroperoxide as substrate [20]. To the hemolysate phosphate buffer (pH 7.0), 1 mM EDTA, 2.4 U GRD, 10 mM GSH, 1 mM sodium azide were added and the tubes were incubated at 37°C. 1.5 mM NADPH and 1.5 mM H_2O_2 were added and the absorbance was monitored at 340 nm for 3 minutes. Activity was expressed as µmoles of NADPH oxidized/minute/mg protein.

Assay of GRD activity

GRD catalyzes the NADPH-dependent reduction of GSH disulfide (GSSG) to GSH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm, thus providing a spectrophotometric means of detection by Carlberg and Mannervik method [21]. The reaction mixture consisting of phosphate buffer (pH 7.5), 80 mM EDTA, 7.5 mM GSSG, and hemolysate were incubated at 37°C for 10 minutes. 2 mM NADPH was added to the tubes and change in absorbance was read immediately at recorded at 340 nm for 3 minutes. Activity was expressed as nanomoles of NADPH oxidized/minute/mg protein.

Assessment of GSH levels

Free endogenous total GSH was assayed by the method of Moron *et al*. using 5, 5' dithiobis-2- nitrobenzoic acid (DTNB) as the coloring reagent [22]. Hemolysate proteins were precipitated by the addition of TCA and centrifuged at 3000 rpm for 10 minutes. DTNB reagent and phosphate buffer were added to an aliquot of clear supernatant. The absorbance was read at 412 nm against a blank containing TCA. The amount of GSH was expressed as µmoles of GSH/mg protein.

Lactate dehydrogenase (LDH) assay

Quantitative analysis of LDH in supernatant was estimated by standard IFCC/UV kinetic method using commercial kit (Span Diagnostics Ltd.) and Robonik auto biochemistry analyzer. LDH catalyses the reduction of pyruvate by NADH and the rate of decrease in concentration of NADH, measured photometrically is proportional to the catalytic concentration of LDH present in the sample. The absorbance was read at 340 nm and enzyme activity was expressed international units/ml.

Protein estimation

Protein was estimated by Folin-Lowry method [23]. This reaction produces strong blue color, which predominantly depends upon tyrosine and tryptophan content of protein and to a lesser extent cysteine and other residues in the protein. This color is read at 560 nm and concentration of protein is expressed as mg protein.

Data analysis

Data are represented as a mean±standard deviation. The significance of difference among the groups were assessed using one-way analysis of variance test followed by Tukey's HSD *post-hoc* test of the difference between all group means.

RESULTS AND DISCUSSION

MTX is widely used as a cytotoxic chemotherapeutic agent in the treatment of various malignancies as well as various inflammatory diseases [24]. The efficacy of this agent is often limited by its toxicity which causes severe side-effects affecting the hematopoietic system and hepatocytes [8]. Our work highlights the importance of vitamin E in combination with chemotherapy aiding in reducing the MTX induced side effects in cancer patients by maintaining a balance between oxidant and antioxidant levels.

MTX has been reported to be pro-oxidant in nature. Cell damage is observed due to increased oxidative stress in the cell resulting from a chain of events called LPX. Vitamin E which is a lipid soluble antioxidant acts by breaking this chain of events thus preventing the cell from peroxidative damage. It is a free radical scavenger playing an important role in re-establishing the levels of antioxidant enzymes thus enhancing the efficacy of cancer treatment [25-28].

Hemolysis is a sign of leakage of hemoglobin from erythrocytes indicating injury to the membrane. This study shows a significant increase in the hemolytic activity on MTX treatment when compared to the control group (Fig. 1) illustrating cell rupture or damage due to oxidative stress. Other researchers have also reported an increased hemolysis in erythrocytes subjected to free radical attack and in vitamin E deficient erythrocytes when subjected to oxidative insult [29,30]. Furthermore, a significant increase in hemolysis was observed in hemorrhagic stroke cases, which is also a known cause of free radical production [31]. In

our studies, vitamin E supplementation showed a negligible effect in ameliorating the hemolysis, though many researchers have reported that vitamin E maintains the erythrocyte membrane integrity and decreases hemolysis due to oxidative stress [32,33].

The present study revealed that MTX administration caused a significant increase ($p<0.001$) in the TBA reactive substances, an important marker of LPX indicating MTX is pro-oxidant (Fig. 2). An increase in lipid peroxide levels causes RBC's to lose their ability to change shape and squeeze through the smallest capillaries eventually leading to intravascular hemolysis. Lipid peroxide levels serves as an excellent index of oxidative stress and antioxidant status [34]. The increase in the LPX activity might result from increased production of free radicals or due to a significant decrease in antioxidant machinery represented by GSH, GPX, GST, GRD, SOD, CAT [6,35]. Vitamin E acts as an antioxidant and helps to suppress the elevated levels of lipid peroxide demonstrating its ameliorative effect against MTX thereby minimizing LPX and drug-induced toxicity (Fig. 2) [36,37].

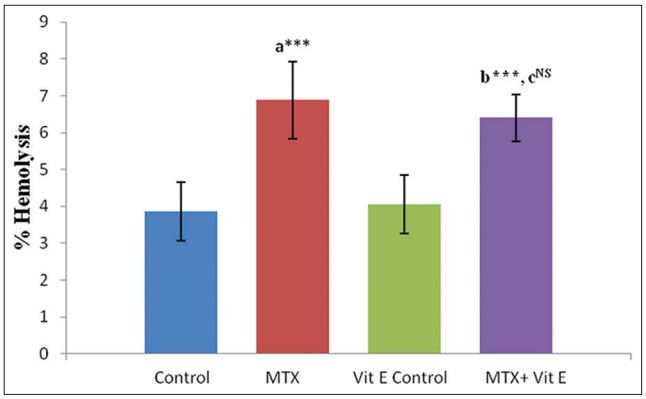

Fig. 1: Effect of vitamin E on methotrexate-induced hemolysis of red blood cells. Values are expressed as mean±standard deviation. Comparisons are made between, (a) Group I and II (b) Group I and IV and (c) Group II and IV. Statistical significance: ***p<0.001, NS: Non-significant

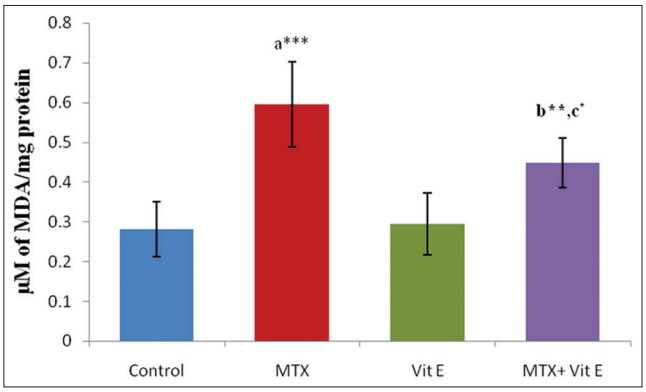

Fig. 2: Effect of vitamin E against methotrexate-induced lipid peroxide content of red blood cells. Values are expressed as mean±standard deviation. Comparisons are made between (a) Group I and II (b) Group I and IV and (c) Group II and IV. Statistical significance*p<0.001, **p<0.01, *p<0.05**

LDH is a cytoplasmic enzyme and catalyzes the reversible oxidation of lactate to pyruvate in the last step of glycolysis. In the present study, LDH activity was significantly increased up to 93% in the supernatant of the MTX treated samples when compared with control (Table 1). According to studies by Jovanovic *et al*. the increased activity of LDH is expected during oxidative stress or cell damage [38]. LDH is an intracellular enzymes and damage to the cell membrane can cause its release in the supernatant. Increase in LDH levels can also be correlated with increased hemolysis. It was observed that supplementation of vitamin E proved to be effective in correcting the levels of LDH hence facilitating in reducing the pro-oxidant effect of MTX and to a great extent would reduce associated tissue damage and hemolysis (Table 1).

The present work shows that the changes in LPX are accompanied by a concomitant decrease in the activities of antioxidant enzymes namely SOD, CAT, and GPX as well as GSH metabolizing enzymes like GST and GRD. CAT acts as a pre-emptive antioxidant and functions by protecting cell against the deleterious effects of H_2O_2 by reducing it to water thus preventing oxidative stress [39]. In the present study, a significant decrease ($p<0.001$) in CAT activity was seen subsequent to treatment with MTX, which shows that cells become susceptible to oxidative stress. SOD constitutes an important link in the biological defense mechanism through dismutation of endogenous cytotoxic superoxide radicals to H_2O_2 and molecular oxygen that are deleterious to polyunsaturated fatty acids and proteins [40]. There was a significant decrease ($p<0.01$) in SOD levels when incubated with MTX (Table 1), this decrease can be attributed to feedback inhibition or oxidative inactivation of the enzyme protein due to an excess of ROS [41]. GPX catalyzes the breakdown of inorganic and organic peroxides and prevents LPX hence maintaining the functional integrity of cell membrane [42]. MTX addition caused significant decline ($p<0.01$) in the activity of the enzyme.In the presence of inadequate CAT and GPX activity to degrade H_2O_2, more H_2O_2 could be converted to toxic hydroxyl radicals that may contribute to the MTX induced oxidative toxicity. The antioxidant enzymes CAT and GPX protect SOD against inactivation by H_2O_2. Reciprocally, SOD protects CAT and GPX against superoxide anions. Thus, the balance of this enzyme system is essential to dispose the superoxide anion and peroxides from the system. The reduction in the activities of these enzymes and increase in LPX could reflect the adverse effect of MTX on this finely balanced antioxidant system [43]. There is improvement in enzyme activity of CAT, SOD, and GPX in MTX and vitamin E treated group (in combination) probably due to decrease in ROS levels resulted from the scavenging activity of vitamin E. Recently, Halani *et al.* [44] investigated the role of vitamin E on activity of SOD and reported increase in levels of SOD after incubation with vitamin E.

Table 1: Effect of vitamin E against MTX induced alterations in the activities of antioxidant enzymes in erythrocytes

Enzymes	Control	MTX	Vitamin E control	MTX+Vitamin E
CAT	0.164±0.014	$0.075±0.021^{a***}$	0.16±0.012	$0.167±0.0\ 37^{bNS,c***}$
SOD	0.297±0.052	$0.164±0.016^{a**}$	0.307±0.044	$0.254±0.016^{bNS,c*}$
GPX	0.465±0.059	$0.294±0.061^{a**}$	0.493±0.051	$0.415±0.038^{bNS,c*}$
GST	1.95±0.42	$0.875±0.171^{a**}$	1.9±0.44	$1.775±0.33^{bNS,c*}$
GRD	7.5±0.90	$4.7±1.03^{a**}$	7.9±0.97	$5.65±0.92^{bNS,cNS}$
LDH	73.55±7.5	$141.5±9.4^{a***}$	74.275±5.9	$99.15±8.5^{b**,c***}$

Values are expressed as mean±SD for four samples in a group. Enzyme activities are expressed as follows: CAT: µmoles of H_2O_2 consumed/minute/mg protein, SOD: Units/mg protein, 1U: Amount of enzyme that inhibit the auto-oxidation of epinephrine by 50%, GPX: µmoles of NADPH oxidized/minute/mg protein, GST: Nanomoles of CDNB conjugated/minute/mg protein, GRD: Nanomoles of NADPH oxidized/minute/mg protein, LDH: IU/ml. Comparisons are made between, (a) Control and MTX, (b) control and MTX+vitamin E, (c) MTX and MTX+vitamin E. Statistical significance: ***p<0.001, **p<0.01, *p<0.05, NS: Non-significant. MTX: Methotrexate, SD: Standard deviation, CAT: Catalase, SOD: Superoxide dismutase, GST: Glutathione S-transferase, CDNB: 1-chloro, 2, 4-dinitrobenzene, GRD: Glutathione reductase, LDH: Lactate dehydrogenase, NADPH: Nicotinamide adenine dinucleotide phosphate, SD: Standard deviation

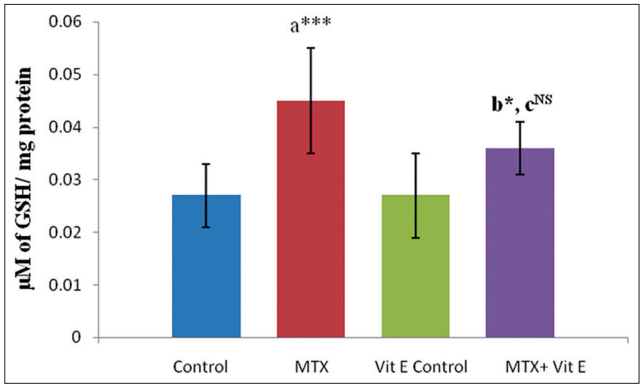

Fig. 3: Effect of vitamin E on methotrexate mediated change in GSH levels of red blood cells. Values are expressed as mean±standard deviation. Comparisons are made between, (a) Group I and II (b) Group I and IV and (c) Group II and IV. Statistical significance: ***p<0.001, *p<0.05, NS: Non significant

GSH is a tripeptide containing cysteine that has a reactive SH group with reductive potency and is the most abundant thiol present in mammalian cells which performs a number of vital cell functions. It can act as a non-enzymic antioxidant by direct interaction of –SH group with ROS or it can be involved in the enzymatic detoxification reaction for ROS, as a cofactor or co enzyme [43]. GSH might assist SOD and exert its effect by scavenging the free radicals [45]. In our study elevated level of GSH was observed in MTX treated groups (Fig. 3) which could correspond with higher resistance to the oxidative stress or may be due to the adaptive response to counter regulate oxidative stress [46,47]. Other researchers have observed a decrease in the level of GSH to about the normal level on treatment with vitamin E and depicted the importance of vitamin E in maintaining the levels of GSH when exposed to stress-inducing agent [48,49].

GSH dependent enzyme like GST is a detoxifying enzyme that catalyzes the conjugation of a variety of electrophilic substrates to the thiol group of GSH, producing less toxic form [50]. A significant reduction ($p<0.01$) in activity of the enzyme was observed after MTX incubation which may be because of oxidative modification of its protein structure (Table 1). Significant increase ($p<0.05$) in the levels of GST was observed on treatment with vitamin E when compared to only MTX treated groups suggesting its protective role in maintaining balance of antioxidants in the cell which could then counterattack increased ROS levels.

The activity of GRD, the enzyme crucial for regeneration of GSH was found to be significantly less ($p<0.05$) when compared with control (Table 1). This enzyme contains one or more −SH group residues essential for its catalytic activity [51]. The reduced GRD activity could be correlated to this enzyme inactivation caused due to the generation of ROS. This can be supported by the fact that under high oxidative stress, GSSG can be effluxed out of the cell or they form mixed disulfides with the cellular proteins and hence GSSG/GSH ratio is an index of oxidative stress in the cell [52-54]. Under normal conditions, NADPH is utilized by GRD to maintain the reduced state of cellular GSH. It has been shown earlier that the cytosolic NADP-dependent dehydrogenase and malic enzyme are inhibited by MTX, indicating that the drug could decrease the availability of NADPH by inhibiting the pentose cycle enzymes. The reduced availability of NADPH may be responsible for the decreased activity of GRD [55,56]. The supplementation of vitamin E elevates the level of GRD to almost that of the control sample. Evident work has been emphasized by other researchers showing a marked increase in level of GRD activity of Vitamin E treated tissues aiding to maintain the levels of GSH since GSH can act as direct scavenger of free radicals [28,27].

CONCLUSION

MTX is a widely used chemotherapeutic agent, however, its efficacy is often limited by its toxicity which is the cause of various side effects. This work is to highlight the importance of vitamin E supplementation as an antioxidant in chemotherapy to re-establish the levels of antioxidant defense enzymes and to strike a balance between the oxidant and antioxidant levels thus preventing the enormous toxicity observed due to this drug. Combinational chemotherapy gives an insight for an effective treatment to cancer patients helping them exhibit minimum levels of the deleterious drug-induced side effects by the use of an efficient antioxidant such as vitamin E.

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