


Induction of Erythrocyte Membrane Blebbing by Methotrexate-Induced Oxidative Stress

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Abstract

Methotrexate (MTX) is a common chemotherapeutic agent and folate antagonist with reported apoptotic activity in nucleated cells. The presented research work was planned to investigate the eryptotic effects of methotrexate after the exposure of erythrocytes to therapeutical doses (10–15 μM) of methotrexate. Eryptosis and the role of calcium in the stimulation of membrane blebbing were evaluated through the determination of mean cell volume. Oxidative stress induced by methotrexate (10–15 μM) was determined by antioxidative enzyme activities. Cytotoxic activity against human erythrocytes was examined through hemolysis assay. Exposure of erythrocytes to methotrexate results in significant reduction of superoxide dismutase, catalase, and superoxide dismutase activities at 10 and 15 μM in comparison to the untreated cells. Erythrocytes mean cell volume (MCV) was increased after 48 hours exposure of erythrocytes to methotrexate (10 μM). Significantly increased hemolysis percentage was observed at 10 μM after 48 hours incubation of erythrocytes with methotrexate. The results of the study suggested that the therapeutical doses (10–15 μM) of methotrexate may lead to increase in eryptotic and hemolytic activity of erythrocytes through free radical generation and subsequent calcium entry.

Keywords

oxidative stress, membrane blebbing, hemolysis, eryptosis

Introduction

Malignant and non-malignant cells are sometimes treated with folate antagonist methotrexate that inhibits the activity of folate-dependent enzymes.¹ Methotrexate mainly inhibits the enzyme dihydrofolate reductase which is crucial to uphold cellular tetrahydrofolate pool during purine and thymidine production, so, involved in the inhibition of DNA, RNA, thymidylates, and different proteins synthesis.² Methotrexate is also used in treating a number of autoimmune disorders, such as lupus erythematosus, Crohn's disease, sarcoidosis, psoriatic arthritis, psoriasis, juvenile dermatomyositis, and rheumatoid arthritis.³ Common toxic effects of methotrexate include liver and renal injury.⁴ Similarly, methotrexate treatment may lead to the development of megaloblastic anemia.⁵

Erythrocytes are hemoglobin carrying cells with normal life span of 100 to 120 days.⁶ Eryptosis is programmed or suicidal erythrocytes death, which is principally featured

by cell membrane blebbing, cell shrinkage, and phosphatidylserine translocation from inner to outer leaflet.⁷ The major inducers of eryptosis are energy depletion, oxidative stress, hyper osmotic shock, high calcium levels, and activation of different kinases. It is also confirmed that the uncontrolled eryptosis leads to anemia that is related to the pathophysiology of different clinical problems.⁸

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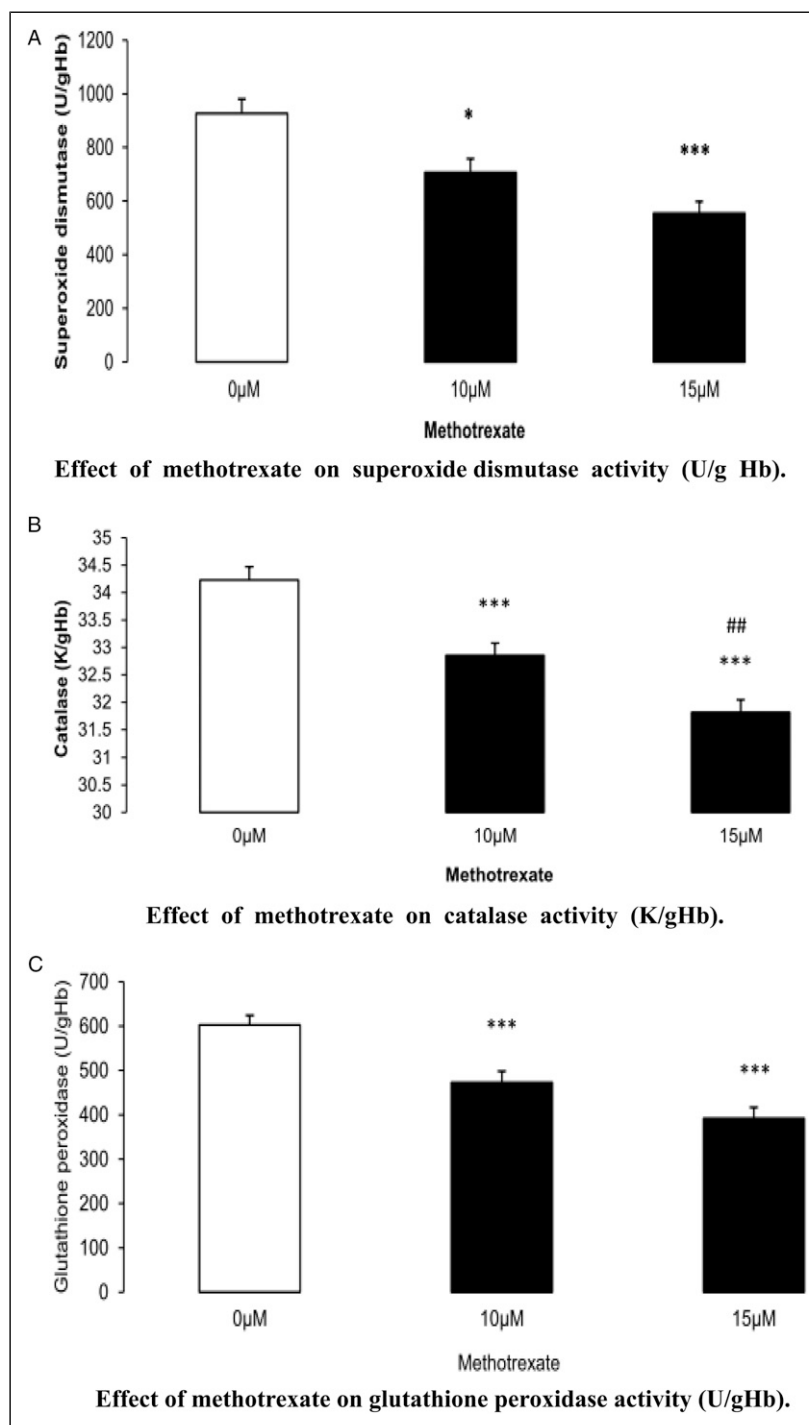


Figure I. (A) Effect of methotrexate on superoxide dismutase activity (U/g Hb). Values are mean \pm SEM ($n = 20$) of erythrocytes following 48 hours incubation with ringer solution, without drug (white bars) and with (10, 15 μ M) methotrexate (black bars). Asterisks above the bars indicate statistically significant differences *, *** ($p < 0.05$), ($p < 0.001$) from un-treated cells (ANOVA). (B) Effect of methotrexate on catalase activity (K/gHb). Bars represent the mean \pm SEM values ($n = 20$). Erythrocytes treated with methotrexate and incubated for 48 hours with ringer solution without (white bar) or with (black bars) (10, 15 μ M) methotrexate. Asterisks above the bars indicate standard error mean (SEM). *** ($P < 0.001$) shows significant difference from control and ### ($p < 0.01$) shows difference among both treatments (ANOVA). (C) Effect of methotrexate on glutathione peroxidase activity (U/gHb). Vertical bars indicate the mean \pm SEM ($n = 20$) of glutathione peroxidase activity in erythrocytes. White bar shows the enzyme activity of cells incubated in ringer solution without drug while black bars show the enzyme activity of cells incubated in ringer solution with (10, 15 μ M) methotrexate.*** ($P < 0.001$) indicates statistically significant difference from the untreated samples (ANOVA).

Energy depletion, oxidation, and osmotic shock may stimulate calcium permeable cation channels and lead to the enhanced calcium entry in the cell and subsequently provoke different steps of eryptosis. High intracellular calcium triggers Ca^{2+} sensitive K^+ channels⁹ with an outcome of intracellular KCl and water loss and shrinkage of the cell.¹⁰ Also, blebbing in erythrocyte membrane and phosphatidylserine exposure on cell membrane of the erythrocytes are dependent completely on the increased entry of calcium.¹¹ Antioxidant defensive system consists of different enzymes and vitamins. This system controls the production of free radicals through enzymatic reactions.¹²

In previous studies, it was observed that different xenobiotics including certain drugs have potent eryptosis triggering activities.^{11,13} In the present research work, isolated human erythrocytes were treated with therapeutic doses of methotrexate, to investigate its oxidative, eryptotic, and hemolytic effects on erythrocytes.

Results

Results of the study are presented in figures prepared with mean \pm SEM values with indication of statistical significance. Oxidation is among the inducers of eryptosis, and the effects of possible oxidation induced by methotrexate on red cells were evaluated through enzyme assays by measuring the antioxidant enzyme activities in treated and control cells. **Figure 1A** is illustrating that methotrexate (10–15 μM) exposure to erythrocytes for 48 hour. resulted in a mild but significant reduction in the activity of superoxide dismutase (SOD) at 10 μM while highly significant reduction in SOD activity was observed at 15 μM of methotrexate when compared to control cells. **Figure 1B** is depicting the moderate but significant decline in the activity of catalase enzyme following 48 hr. methotrexate (10–15 μM) exposure to erythrocytes compared to the control cells. Likewise, **Figure 1C** is demonstrating the significant reduction in glutathione peroxidase activity in red cells at 10 and 15 μM concentrations of methotrexate.

Eryptotic effect of methotrexate was confirmed by mean cell volume (MCV) estimation. Exposure of erythrocytes to methotrexate (10 μM) for 48 hours resulted in a significant increase in MCV of red cells possibly because of membrane blebbing (**Figure 2**). Calcium role in triggering the membrane blebbing was confirmed by MCV measurement of control and methotrexate-treated red cells in the absence and presence of calcium. MCV was found to be much lower in red cells treated with methotrexate in the absence of calcium compared to red cells treated with methotrexate in the presence of calcium, possibly due to the lack of calcium for the production of blebbing (**Figure 3**).

The hemolytic effects of methotrexate on erythrocytes were determined by hemolysis % measurement. **Figure 4** is demonstrating the significantly increased hemolysis % at 10 μM

after 48 hours incubation of red cells with methotrexate than control cells.

Discussion

The presented research work was designed to rule out the oxidative, eryptotic and hemolytic effects of methotrexate on human erythrocytes. Therapeutic doses (10–15 μM) of methotrexate used in the study have already been reported to treat nucleated cells.¹⁴ In biological systems, antioxidants are the compounds with the potential to scavenge the free radicals produced during oxidation, which may cause stress to the cell.^{15–17} Superoxide dismutase is an antioxidant enzyme that speedup O_2 -based free radicals dismutation, and increased reactive oxygen species generation may lead to the reduced activity of superoxide dismutase disturbing the mitochondrial functions.¹⁸ Another antioxidant enzyme, catalase, is a key player in decomposition of H_2O_2 into H_2O and O_2 .¹⁹ It is confirmed that the accumulation of hydrogen peroxide may result in the reduction of catalase activity and its excessive production provides protection against oxidants in cells.²⁰ The enzyme glutathione peroxidase prevents the deposition of oxidized lipids in mitochondrial cell membrane and triggers hydrogen peroxide decomposition into water and oxygen.²¹ The experimental results related to enzymes activity confirming the induction of oxidative stress in methotrexate-treated cells as all enzyme activities are reduced. Published studies related to the investigation of induced oxidative stress demonstrated that the reduction in the antioxidant enzymes activity as a result of increased production of oxidants in the cell is the confirmation of oxidation.²²

Erythrocytes membrane blebbing (protrusion) is a confirmed feature of eryptosis.²³ Membrane blebbing is stimulated by the activation of calcium-activated cysteine endopeptidase calpain, that leads to the breakdown of the cytoskeleton of erythrocyte.²⁴ The induction of oxidative stress and membrane blebbing in cells treated with methotrexate is confirming its eryptotic effect. Intracellular calcium is a key player in stimulating the eryptosis induced by oxidative stress.^{25,26} Oxidative stress stimulates the non-selective cation channels.¹¹ In published research work related to eryptosis, similar effects would be observed by removing intracellular and extracellular calcium.²⁷

Physiological process for the removal of old or defective erythrocyte from the circulation prior to the lysis is known as eryptosis.²⁸ In hemolysis, released hemoglobin may precipitate in the renal tubules lumen after passing through the kidney cells.²⁹ The release of hemoglobin during hemolysis promotes a serious clinical problem including less NO bio-availability, systemic vasoconstriction, endothelial dysfunction, and vasomotor instability.³⁰

In this study, therapeutic doses (10–15 μM) of methotrexate which are higher than physiological doses were used to confirm its oxidative as well as eryptotic effect. It is clear from

the results that the high dose of methotrexate may induce erythrocytes removal from the circulation through increased eryptosis and red cells lysis. Similarly, the enhanced death rate due to methotrexate exposure may lead to the development of anemia in patients.

Method

Freshly collected non-infectious blood samples were obtained from various blood banks and hospitals of Faisalabad, Pakistan. The study was approved by the Institutional Bioethics Committee (IBC) and Directorate Graduate Studies (DGS), University of Agriculture, Faisalabad, Pakistan.

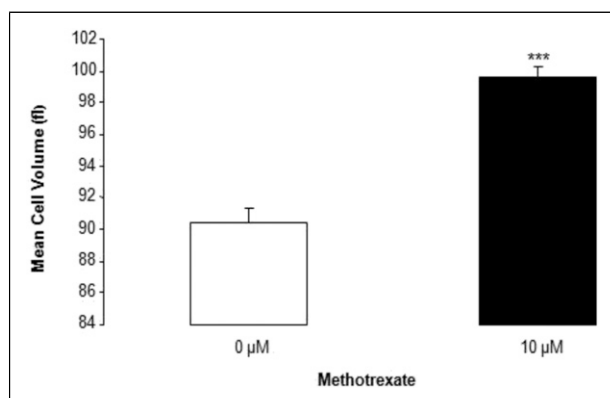


Figure 2. Effect of methotrexate on erythrocytes mean cell volume. Bars represent the mean \pm SEM ($n = 10$) values. Treated blood was incubated for 48 hours with ringer solution, without drug (white bar) and with drug (black bars) (0, 10 μ M) methotrexate. *** ($P < 0.0001$) indicates statistically significant difference from the untreated samples (t-test).

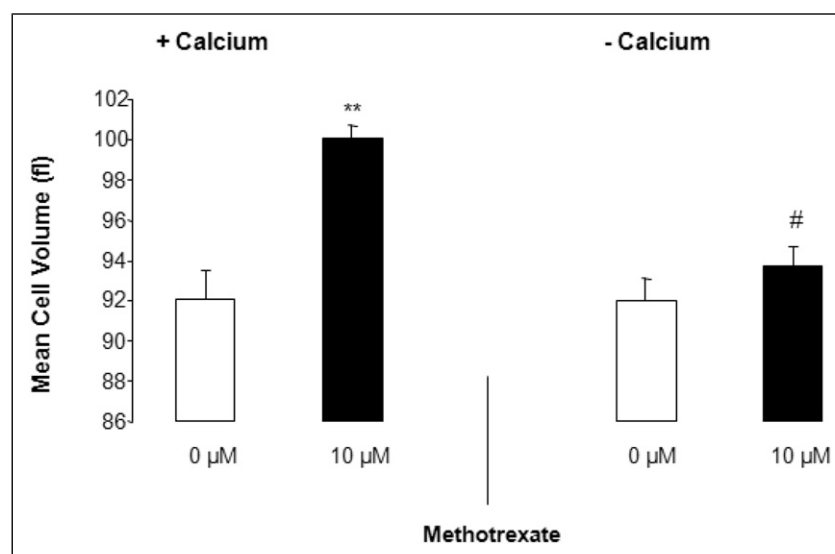


Figure 3. Cell size measurement of methotrexate exposed erythrocytes in the presence and absence of calcium. Bars represent the means \pm SEM ($n = 10$). White bars (without methotrexate), black bars (with 10 μ M methotrexate). ** ($P < 0.01$) 0–10 μ M methotrexate-treated erythrocytes size measurement activity. # ($P < 0.05$) presence of calcium. (ANOVA).

Erythrocytes were isolated following the protocol as described by Zbidah.³¹ Leucocytes and platelets depleted red cells were placed in micro centrifuge tubes. *In-vitro* incubation of red cells was carried out at a hematocrit of .4% using ringer solution (pH 7.4) at 37°C for 48 h. The ringer solution contains NaCl 125 mM, MgSO₄ 1 mM, glucose 5 mM, KCl 5 mM, CaCl₂ 1 mM, and 32 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES).³² Methotrexate was purchased from Sigma-Aldrich, USA. Erythrocytes were then exposed to the indicated doses of methotrexate (10–15 μ M).^{33,34}

Oxidative Stress Determination

Oxidative stress induced by methotrexate in treated erythrocytes was evaluated by measuring the activity of antioxidation enzymes (catalase, superoxide dismutase, and glutathione peroxidase) by spectrophotometer.

Superoxide Dismutase

The activity of superoxide dismutase was determined by the method described by Rana et al³⁵ and Giannopolitis.³⁶ The reaction mixture contained .222 g methionine in 15 mL of H₂O, .015 g NBT in 17.5 mL of H₂O, .0132 g riboflavin in 17.5 mL of H₂O, .0375 mL Triton X-100 in 17.5 mL of H₂O, and phosphate buffer (0.2 M).

Glutathione Peroxidase

The activity of glutathione peroxidase was determined by adding 50 mM phosphate buffer (pH 5), 20 mM guaiacol, 0.1 mL enzyme extract, and 40 mM H₂O₂ in the reaction

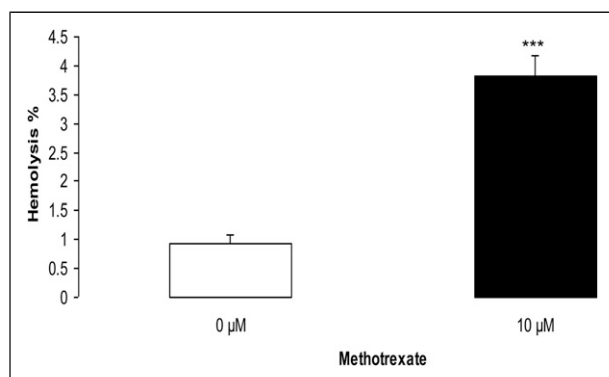


Figure 4. Methotrexate-induced hemolysis in erythrocytes. Vertical bars represent mean \pm SEM values ($n = 05$). Erythrocytes exposure to ringer solution without methotrexate (white bar) and with $10\mu\text{M}$ methotrexate (black bars). *** ($P < 0.0001$) highly significant difference (t-test).

solution according to the method used by Ilyas et al.³⁷ Enzyme activity was measured at 470 nm after every 20Sec.

Catalase

Phosphate buffer (pH7) 50 mM, enzyme extract .1 mL, and H_2O_2 5.9 nM were mixed in reaction solution and catalase activity was determined at 240 nm.³⁵

Measurement of Cell Size

Erythrocyte volume was determined by measuring the mean cell volume (MCV) in control and treated cells with effective dose ($10\mu\text{M}$) of methotrexate. MCV was estimated by using automated hematology analyzer KX-21 Sysmex, Japan.^{37,38}

Confirmation of the Role of Calcium in Membrane Blebbing

The role of calcium in the stimulation of cell size variation was confirmed by treating erythrocytes with methotrexate ($10\mu\text{M}$) in calcium-free ringer solution, prepared by replacement of CaCl_2 (1 mM) by glycol-bis(2-aminoethylether)-N,N,N',N'-tetra acetic acid (1 mM).³⁹ MCV measurement was also carried out to confirm the inhibition of blebbing.

Measurement of Hemolysis

To confirm the hemolytic effect of methotrexate, erythrocyte samples were centrifuged at 400g for 3 min at room temperature. Hemoglobin concentration in supernatants was measured at 405 nm wavelength. The absorption of red cells lysed in $\text{d.H}_2\text{O}$ was considered as 100% hemolysis.⁴⁰

Statistical Analysis

All results were presented as mean \pm SEM (standard error of mean). Statistical analysis was conducted by applying Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test as post and or t test, as appropriate.³⁵ * $P < .05$, ** $P < .01$, and *** $P < .001$ confirm the significant differences in the absence of methotrexate.

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Declaration of Conflicting Interests

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