Induction of Erythrocyte Membrane Blebbing by Methotrexate-Induced Oxidative Stress

Tayyba Sattar1,#, Kashif Jilani1,#, Khalida Parveen2, Zahid Mushataq1, Haq Nawaz3, and Maham Abdul Bari Khan1

Abstract
Methotrexate (MTX) is a common chemotherapeutical 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. 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. 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. Common toxic effects of methotrexate include liver and renal injury. Similarly, methotrexate treatment may lead to the development of megaloblastic anemia.

Erythrocytes are hemoglobin carrying cells with normal life span of 100 to 120 days. 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. 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.
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$^+$ channels with an outcome of intracellular KCl and water loss and shrinkage of the cell. Also, blebbing in erythrocyte membrane and phosphatidylserine exposure on cell membrane of the erythrocytes are dependent completely on the increased entry of calcium. Antioxidant defensive system consists of different enzymes and vitamins. This system controls the production of free radicals through enzymatic reactions.

In previous studies, it was observed that different xenobiotics including certain drugs have potent eryptosis triggering activities. In the present research work, isolated human erythrocytes were treated with therapeutical 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 ± 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

![Figure 1A](image1.png)  
**Figure 1A.** Effect of methotrexate on superoxide dismutase activity (U/g Hb). Values are mean ± SEM (n = 20) of erythrocytes following 48 hours incubation with ringer solution, without drug (white bars) and with (10, 15 μM) methotrexate (black bars). Asterisks above the bars indicate statistically significant differences * (p < 0.05), ** (p < 0.001) from untreated cells (ANOVA).

![Figure 1B](image2.png)  
**Figure 1B.** Effect of methotrexate on catalase activity (K/gHb). Bars represent the mean ± 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 μM) 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).

![Figure 1C](image3.png)  
**Figure 1C.** Effect of methotrexate on glutathione peroxidase activity (U/gHb). Vertical bars indicate the mean ± 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 μM) methotrexate.*** (P < 0.001) indicates statistically significant difference from the untreated samples (ANOVA).
Discussion

The presented research work was designed to rule out the oxidative, eryptotic and hemolytic effects of methotrexate on human erythrocytes. Therapeutical doses (10–15 μM) of methotrexate used in the study have already been reported to treat nucleated cells. 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. Superoxide dismutase is an antioxidant enzyme that speedup O2-based free radicals dismutation, and increased reactive oxygen species generation may lead to the reduced activity of superoxide dismutase disturbing the mitochondrial functions. Another antioxidant enzyme, catalase, is a key player in decomposition of H2O2 into H2O and O2. 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. The enzyme glutathione peroxidase prevents the deposition of oxidized lipids in mitochondrial cell membrane and triggers hydrogen peroxide decomposition into water and oxygen. 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.

Erythrocytes membrane blebbing (protrusion) is a confirmed feature of eryptosis. Membrane blebbing is stimulated by the activation of calcium-activated cysteine endopeptidase calpain, that leads to the breakdown of the cytoskeleton of erythrocyte. 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. Oxidative stress stimulates the non-selective cation channels. In published research work related to eryptosis, similar effects would be observed by removing intracellular and extracellular calcium.

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

In this study, therapeutical 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.

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 μM).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 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 H2O2 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 μM) 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 μM) in calcium-free ringer solution, prepared by replacement of CaCl2 (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 d.H2O was considered as 100% hemolysis.40

Statistical Analysis
All results were presented as mean ± 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.

Acknowledgments
Authors are thankful to the Department of Biochemistry, University of Agriculture, and Faisalabad for support in conducting this research study.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iD
Kashif Jilani  https://orcid.org/0000-0002-1761-4100
References

1. Bayram M, Ozogul C, Dursan A, Ercan ZS, Isik I, Dilekoez E. Light and electron microscope examination of the effects of methotrexate on the endosalpinx. *Eur J Obstet Gynecol Reprod Biol.* 2005;120(1):96-103.

2. Rajagopalan PTR, Zhang Z, McCourt L, Dwyer M, Benkovic SJ, Hammers GG. Interaction of dihydrofolate reductase with methotrexate: ensemble and single-molecule kinetics. *Proc Natl Acad Sci Unit States Am.* 2002;99(21):13481-13486.

3. Herfarth HH, Long MD, Isaacs KL. Methotrexate: underused and ignored?. *Dig Dis* 2012;30(3):112-118.

4. Howard SC, McCormick J, Pui C-H, Buddington RK, Harvey RD. Preventing and Managing Toxicities of High-Dose Methotrexate. *Oncol.* 2016;21(12):1471-1482.

5. Casterle CM, Stange KC, Chren M-M. Severe megaloblastic anemia in a patient receiving low-dose methotrexate for psoriasis. *J Am Acad Dermatol.* 1993;29(3):477-480.

6. Bosman GJ, Willekens FL, Werre JM. Erythrocyte aging: a more than superficial resemblance to apoptosis? *Cell Physiol Biochem: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology.* 2005;16(1-3):1-8.

7. Jilani K, Abed M, Zelenak C, Lang E, Qadri SM, Lang F. Triggering of erythrocyte cell membrane scrambling by ursolic acid. *J Nat Prod.* 2011;74(10):2181-2186.

8. Lang F, Lang KS, Lang PA, Huber SM, Wieder T. Mechanisms and significance of eryptosis. *Antioxidants Redox Signal.* 2006;8(7-8):1183-1192.

9. Brugnara C, de Franceschi L, Alper SL. Inhibition of Ca(2+)-dependent K+ transport and cell dehydration in sickle erythrocytes by clotrimazole and other imidazole derivatives. *J Clin Invest.* 1993;92(1):520-526.

10. Lang KS, Duranton C, Poehlmann H, et al. Cation channels trigger apoptotic death of erythrocytes. *Cell Death Differ.* 2003;10(2):249-256.

11. Jilani K, Lang F. Carmustine-induced phosphatidylserine translocation in the erythrocyte membrane. *Toxins.* 2013;5(4):703-716.

12. Adam-Vizi V, Chinopoulos C. Bioenergetics and the formation of mitochondrial reactive oxygen species. *Trends Pharmacol Sci.* 2006;27(12):639-645.

13. Shaik N, Lupescu A, Lang F. Sunitinib-sensitive suicidal erythrocyte death. *Cell Physiol Biochem.* 2012;30(3):512-522.

14. Liang G-T, Chou P-L, Hung Y-T, et al. Vitamin C enhances anticancer activity in methotrexate-treated Hep3B hepatocellular carcinoma cells. *Oncol Rep.* 2014;32(3):1057-1063.

15. Riaz M, Shahid M, Jamil A, Saqib M. In vitro antioxidant potential of selected aphrodisiac medicinal plants. *J Biol Regul Homeost Agents.* 2017;31(2):419-424.

16. Shahid M, Riaz M, Talpur MM, Pirzada T. Phytopharmacology of Tribulus terrestris. *J Biol Regul Homeost Agents.* 2016;30(3):785-788.

17. Kausar A, Shah SMA, Iqbal N, et al. In vitro antioxidant and cytotoxic potential of methanolic extracts of selected indigenous medicinal plants. *Prog Nutr.* 2018;20(4):706-712.

18. Jaleel CA, Gopi R, Manivannan P, Panneerselvam R. Exogenous application of triadimefon affects the antioxidant defense system of Withania somnifera Dunal. *Pestic Biochem Physiol.* 2008;91(3):170-174.

19. Vijayaraghavan R, Panneerselvam C. Erythrocyte Antioxidant Enzymes in Multibacillary Leprosy Patients; 2011.

20. Mladenov M, Gokik M, Hadzi-petrushev N, Gjorgoski I, Jankulovski N. The relationship between antioxidant enzymes and lipid peroxidation in senescent rat erythrocytes. *Physiol Res.* 2015;64(6):891-896.

21. Hassan MQ, Hadi RA, Al-Rawi ZS, Padron VA, Stohs SJ. The glutathione defense system in the pathogenesis of rheumatoid arthritis. *J Appl Toxicol.* 2001;21(1):69-73.

22. Lucero G-MA, Marcela G-M, Sandra G-M, Manuel G-OL, Celene R-E. Naproxen-enriched artificial sediment induces oxidative stress and genotoxicity in hyalella azteca. *Water, Air, Soil Pollut.* 2015;226(6):195.

23. Charras GT, Coughlin M, Mitchison TJ, Mahadevan L. Life and times of a cellular bleb. *Biophys J.* 2008;94(5):1836-1853.

24. Berg CP, Engels IH, Rothbart A, et al. Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. *Cell Death Differ.* 2001;8(12):1197-1206.

25. Calabro S, Alzoubi K, Bissinger R, Jilani K, Faggio C, Lang F. Enhanced eryptosis following juglone exposure. *Basic Clin Pharmacol Toxicol.* 2015;116(6):460-467.

26. Lang F, Jilani K, Lang E. Therapeutic potential of manipulating suicidal erythrocyte death. Expert Opin Ther Targets. 2015;19(9):1219-1227.

27. Duranton C, Huber SM, Lang F. Oxidation induces a Cl–-dependent cation conductance in human red blood cells. *J Physiol.* 2002;539(3):847-855.

28. Lang F, Qadri SM, Lang F. Killing me softly - Suicidal erythrocyte death. *Int J Biochem Cell Biol.* 2012;44(8):1236-1243.

29. Malik A, Bissinger R, Liu G, Liu G, Lang F. Enhanced eryptosis following gramicidin inclusion. *Toxins.* 2015;7(5):1396-1410.

30. Rapido F. The potential adverse effects of haemolysis. *Blood transfusion = Trasfusione del sangue.* 2017;15(3):218-221.

31. Zbidah M, Lupescu A, Jilani K, et al. Apigenin-induced suicidal erythrocyte death. *J Agric Food Chem.* 2012;60(1):533-538.

32. Lupescu A, Bissinger R, Jilani K, Lang F. In vitro induction of erythrocyte phosphatidylserine translocation by the natural naphthoquinone shikonin. *Toxins.* 2014;6(5):1559-1574.

33. Nomura Y, Bhawal UK, Nishikiori R, Sawajiri M, Maeda T, Okazaki M. Effects of high-dose major components in oral disinfectants on the cell cycle and apoptosis in primary human gingival fibroblasts in vitro. *Dent Mater J.* 2010;29(1):75-83.

34. Cabral S, Leis S, Bover L, Nembrot M, Mordoh J. Dipyridamole increases drug uptake in Sarcoma 180 cells. *Proc Natl Acad Sci Unit States Am.* 1984;81(10):3200-3203.

35. Rana RB, Jilani K, Shahid M, et al. Atorvastatin induced erythrocytes membrane blebbing. *Dose-response : A Publication of International Hormesis Society.* 2019;17(3):1559325819869076.
36. Giannopolitis CN, Ries SK. Superoxide dismutases. *Plant Physiol.*, 1977;59(2):315-318.

37. Ilyas S, Jilani K, Sikandar M, et al. Stimulation of erythrocyte membrane blebbing by naproxen sodium. *Dose-response: A Publication of International Hormesis Society*. 2020;18(1):155935819899259.

38. d’Onofrio G, Chirillo R, Zini G, Caenaro G, Tommasi M, Micciulli G. Simultaneous measurement of reticulocyte and red blood cell indices in healthy subjects and patients with microcytic and macrocytic anemia. *Blood*. 1995;85(3):818-823.

39. Malik A, Bissinger R, Calabrò S, Faggio C, Jilani K, Lang F. Aristolochic acid induced suicidal erythrocyte death. *Kidney Blood Press Res*. 2014;39(5):408-419.

40. Lang E, Jilani K, Zelenak C, et al. Stimulation of suicidal erythrocyte death by benzethonium. *Cell Physiol Biochem*. 2011;28(2):347-354.