Stimulation of Erythrocyte Membrane Blebbing by Naproxen Sodium

Sajida Ilyas¹, Kashif Jilani¹, Muhammad Sikandar², Saba Siddiq², Muhammad Riaz³, Ayesha Naveed¹, Ismat Bibi⁴, Haq Nawaz⁵, Muhammad Irfan¹ and Asma Asghar¹

Abstract
Naproxen sodium is a nonsteroidal anti-inflammatory drug (NSAID) having antipyretic and analgesic properties, mainly used for the treatment of rheumatoid arthritis and osteoarthritis. Eryptosis is an alternative term used for suicidal erythrocyte death. In the current study, eryptotic effect of naproxen sodium characterized by membrane blebbing was investigated in erythrocytes after 48 hours of treatment with different concentrations (1-25 μM). The experimental work related to investigation of eryptosis was done by cell size measurement and confirmation of calcium role in the induction of membrane blebbing. As a possible mechanism of eryptosis, oxidative stress induced by naproxen sodium was determined by catalase, glutathione peroxidase, and superoxide dismutase activities. Similarly, hemolytic effect of naproxen sodium was also determined by hemolysis measurement. Results of our study illustrated that the therapeutic doses (10-25 μM) of naproxen sodium induce oxidative stress, confirmed by significant decrease in superoxide dismutase, catalase, and glutathione peroxidase activities that lead to the triggering of cell death by eryptosis and hemolysis.

Keywords
oxidative stress, calcium, cell size, eryptosis

Introduction
Naproxen sodium [(S)-6-methoxy-alpha-methyl-2-naphthaleneacetic acid sodium salt], a nonsteroidal anti-inflammatory drug, poses antipyretic and analgesic properties.¹ The mode of action of this drug include COX inhibition and blockage of prostaglandin synthesis.² Different inflammatory conditions, especially rheumatoid arthritis and osteoarthritis, are frequently treated with naproxen sodium.³ Gastrointestinal toxicity, hepatotoxicity, nephrotoxicity, and jaundice are among the reported side effects of this drug.⁴ Similarly, naproxen sodium also confirmed to induce oxidative stress by the production of reactive oxygen species.⁵

Characteristics of eryptosis include shrinkage of cells, membrane blebbing,⁶ and cell membrane scrambling which leads toward phosphatidylserine translocation in the membrane.⁷ Splenic macrophages recognize, engulf, and decompose erythrocytes with exposed phosphatidylserine on outer leaflet.⁸ Oxidative stress, osmotic shock, and energy-depleted environment may trigger Ca²⁺-[Ca²⁺⁺]ₜ-permeable cation channels that result in the stimulation of Ca²⁺⁺ entry and subsequently different events of eryptosis. High cytosolic Ca²⁺ leads to Ca²⁺⁺-sensitive K⁺ channels activation⁹ that causes cell shrinkage due to KCl loss with water.⁶ Similarly, membrane blebbing due to the breakdown of cytoskeleton and phosphatidylserine translocation in erythrocyte’s cell membrane is completely dependent on high calcium influx.⁵ It is reported that

¹ Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan
² Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan
³ Department of Allied Health Sciences, Faculty of Medical and Health Sciences, University of Sargodha, Sargodha, Pakistan
⁴ Department of Chemistry, The Islamia University of Bahawalpur, Bahawalpur, Pakistan
⁵ Department of Chemistry, University of Agriculture, Faisalabad, Pakistan

Received 28 July 2019; received revised 24 October 2019; accepted 10 December 2019

Corresponding Author:
Kashif Jilani, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan.
Email: kashif.jilani@uaf.edu.pk
stimulated eryptosis may lead to anemia that participates in pathophysiology of several clinical issues.8

Different xenobiotic compounds were investigated and found to have strong eryptotic effect7,10 In the present work, therapeutic doses of naproxen sodium were used to determine their role in the induction of oxidative stress, eryptosis, and hemolysis in erythrocytes.

Methods

For experimental work, fresh and screened blood samples were obtained from different blood banks of Faisalabad. The work was conducted after the approval of directorate of graduate studies and IBC, University of Agriculture Faisalabad, Pakistan.

Leukocyte-depleted cells were prepared by following protocol explained by Fink et al.11 Isolated erythrocytes were stored in separate micro-centrifuge tubes. In vitro incubations of erythrocytes were performed at a hematocrit of 0.4% in Ringer solution (pH 7.4) that contain (in mM) MgSO4 1, NaCl (MCV) and forward scatter (FSC).

Changes in cell size were observed by mean cell volume (MCV) and mean cell volume.

Oxidative Stress Measurement

To determine the oxidative stress in naproxen sodium–exposed erythrocytes, antioxidant enzyme (superoxide dismutase [SOD], catalase, and glutathione peroxidase) assays were performed.

Superoxide Dismutase

Superoxide dismutase’s activity was measured by following the protocol of Rana et al.9 The reaction solution contained methionine 0.222 g in 15 mL H2O, NBT 0.015 g in 17.5 mL H2O, Triton-X 0.0375 mL in 17.5 mL H2O, riboflavin 0.0132 g in 17.5 mL H2O, and buffer 0.2 M.

Glutathione Peroxidase

Phosphate buffer (pH 5) 50 mM, guaiacol 20 mM, H2O2 40 mM, and enzyme extract 0.1 mL were added in reaction mixture by following the protocol of Ullah et al.14 and activity was measured at 470 nm after every 20 seconds.

Catalase

Catalase activity was determined following the method described by Ullah et al.14 Phosphate buffer (pH7) 50 mM, H2O2 5.9 mM, and enzyme extract 0.1 mL were added in reaction mixture, and absorbance was read at 240 nm.

Cell Size Measurement

Changes in cell size were observed by mean cell volume (MCV) and forward scatter (FSC).
demonstrates that the 48-hour exposure of erythrocytes with naproxen sodium (1-25 μM) resulted in apparent increase of erythrocytes MCV, which may be due to membrane blebbing. Additional confirmation of naproxen sodium–induced membrane blebbing was done by FSC measurement of erythrocytes, shown in Figure 4B. Further investigations were done for the confirmation of calcium role in induction of membrane blebbing, so the naproxen sodium–exposed cells were subsequently treated with calcium channel inhibitor. Figure 5 shows the cell size measurement of erythrocytes after 48-hour exposure to naproxen sodium (25 μM) in the absence and presence of calcium channel inhibitor amlodipine. In the presence of 10-μM amlodipine, a significant decrease in cell size in comparison to the cells treated in the absence of amlodipine was observed which might be due to inhibition of calcium entry.

The necrotic effect of naproxen sodium on erythrocytes was investigated by hemolysis measurement. Significant increase in hemolysis percentage after 48-hour incubation of erythrocytes with naproxen sodium was observed when compared to the percentage of hemolysis of control cells as given in Table 1 and shown in Figure 6.

### Discussion

This study was designed to explore the effect of naproxen sodium on antioxidant’s enzymatic activities, erythrocyte’s size, confirmation of calcium role in the induction of suicidal death of erythrocytes, and hemolytic activity. The naproxen sodium doses used in the study were lower than concentrations used to treat nucleated cells.17

### Table 1. Effect of Naproxen Sodium on Superoxide Dismutase, Catalase, Glutathione Peroxidase, and Hemolysis in Erythrocytes.a

| Parameters                      | Concentration of Naproxen Sodium, μM |
|---------------------------------|-------------------------------------|
|                                 | 0     | 1     | 10    | 25    |
| Superoxide dismutase, U/g Hb    | 889.25±132.12 | 809.6±121.65 | 698.7±97.34 | 531.1±63.05 |
| Catalase, U/g Hb                | 34.90±0.21  | 34.65±0.22  | 34.04±0.23  | 33.73±0.28  |
| Glutathione peroxidase, U/g Hb  | 402.65±17.79 | 344.6±16.19 | 294.7±16.48 | 231.55±13.22 |
| Hemolysis, %                    | 1.45±0.12  | 1.8±0.28  | 2.48±0.58  | 3.13±0.37   |

Abbreviations: μM; concentration in micro mole, U/g Hb; unit per gram hemoglobin; SEM, standard error of mean.

aValues are mean ± SEM, where SEM is the standard error of mean.
Antioxidants are the biological substances with capability of scavenging the free radicals such as reactive oxygen species produced during oxidation process that may lead to oxidative stress. Superoxide dismutase catalyzes the dismutation of O₂ free radicals, and high ROS production may lead to low-ering of SOD level and disturb mitochondrial functions. Catalase is a major antioxidant enzyme that decomposes H₂O₂ into H₂O and O₂. Hydrogen peroxide’s accumulation may result in decreased level of catalase, and its overproduction showed a protective effect against oxidants in the cells. Similarly, glutathione peroxidase in mitochondrial cell membrane prevents the accumulation of oxidized lipids and decomposes hydrogen peroxide into water. Our experiments related to enzyme activities clearly indicate the oxidative stress in naproxen sodium–treated cells as SOD, catalase, and glutathione peroxidase activities reduced. Previous studies related to oxidative effects of naproxen sodium depicts that the lowering of the antioxidants enzyme activities due to overproduction of oxidants in the cell is the reflection of oxidation.

Erythrocyte membrane blebbing, that is, swelling or protrusions, is a reported marker of eryptosis. Membrane blebbing in the cell is due to the activation of calcium-dependent cysteine endopeptidase calpain, which is involved in the breakdown of the erythrocyte’s cytoskeleton. The happening of oxidation and membrane blebbing in naproxen sodium–treated cells confirming its eryptotic effect.

Antioxidants are the biological substances with capability of scavenging the free radicals such as reactive oxygen species produced during oxidation process that may lead to oxidative stress. Superoxide dismutase catalyzes the dismutation of O₂ free radicals, and high ROS production may lead to lowering of SOD level and disturb mitochondrial functions. Catalase is a major antioxidant enzyme that decomposes H₂O₂ into H₂O and O₂. Hydrogen peroxide’s accumulation may result in decreased level of catalase, and its overproduction showed a protective effect against oxidants in the cells. Similarly, glutathione peroxidase in mitochondrial cell membrane prevents the accumulation of oxidized lipids and decomposes hydrogen peroxide into water. Our experiments related to enzyme activities clearly indicate the oxidative stress in naproxen sodium–treated cells as SOD, catalase, and glutathione peroxidase activities reduced. Previous studies related to oxidative effects of naproxen sodium depicts that the lowering of the antioxidants enzyme activities due to overproduction of oxidants in the cell is the reflection of oxidation.

Erythrocyte membrane blebbing, that is, swelling or protrusions, is a reported marker of eryptosis. Membrane blebbing in the cell is due to the activation of calcium-dependent cysteine endopeptidase calpain, which is involved in the breakdown of the erythrocyte’s cytoskeleton. The happening of oxidation and membrane blebbing in naproxen sodium–treated cells confirming its eryptotic effect.

It is reported that the intracellular Ca\(^{++}\) has key role in the triggering of oxidative stress-induced eryptosis. For the confirmation of its role in the stimulation of membrane blebbing by naproxen sodium, calcium channel blocker amlodipine (10 μM) was used. Nonselective cation channels are triggered by oxidative stress. Amlodipine nonselectively inhibits cation channels and stops the Ca\(^{++}\) entry in to the cell. By removing intracellular and extracellular Ca\(^{++}\), similar effects would be observed in previous studies.
Disposing of defective erythrocytes before hemolysis is an important physiological role of eryptosis.29 Hemoglobin is released through hemolized erythrocytes that may be filtered through kidney or precipitate in the lumen of renal tubules.30 The release of erythrocytes content, especially hemoglobin during hemolysis, resulted in less nitric oxide bioavailability and promotes serious clinical issues, including systemic vasoconstriction, vasomotor instability, and endothelial dysfunction.31

Conclusion
It is concluded that the used therapeutic doses (10-25 μM) of naproxen sodium may trigger the erythrocyte death rate by increased eryptosis and hemolysis due to the induction of oxidative stress and subsequent calcium influx.

Authors’ Note
Sajida Ilyas and Kashif Jilani contributed equally and thus shares first authorship.

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
Muhammad Riaz https://orcid.org/0000-0002-5524-7735

References
1. Capone ML, Tacconelli S, Sciulli MG, et al. Human pharmacology of naproxen sodium. J Pharmacol Exp Ther. 2007;322(2):453-460.
2. Della Rocca G, Di Salvo A, Cagnardi P, Marchesi M, Conti M. Naproxen in the horse: pharmacokinetics and side effects in the elderly. Res Vet Sci. 2014;96(1):147-152.
3. Grenni P, Patrolecco L, Ademollo N, Tolomei A, Caracciolo AB. Degradation of gemfibrozil and naproxen in a river water ecosystem. Microchem J. 2013;107:158-164.
4. Patiño-Camacho SI, Campos MD, Beltrán-Villalobos K, Castro-Vidal DA, Montiel-Ruiz RM, Flores-Marrieta FJ. Low doses of tizanidine synergize the anti-nociceptive and anti-inflammatory effects of ketorolac or naproxen while reducing of side effects. Eur J Pharmacol. 2017;805:51-57.
5. Sehonova P, Phalova L, Blahova J, et al. Toxicity of naproxen sodium and its mixture with tramadol hydrochloride on fish early life stages. Chemosphere. 2017;188:414-423.
6. Lang K, Duranton C, Poehlmann H, et al. Cation channels trigger apoptotic death of erythrocytes. Cell Death Differ. 2003;10(2):249.
7. Shaik N, Lupescu A, Lang F. Sunitinib-sensitive suicidal erythrocyte death. Cell Physiol Biochem. 2012;30(3):512-522.
8. Lang F, Lang KS, Lang PA, Huber SM, Wieder T. Mechanisms and significance of eryptosis. Antioxid Redox Signal. 2006;8(7-8):1183-1192.
9. Rana RB, Jilani K, Shahid M, et al. Atorvastatin induced erythrocytes membrane blebbing. Dose Response. 2019;17(3):1559325819869076.
10. Jilani K, Lang F. Carmustine-induced phosphatidylserine translocation in the erythrocyte membrane. Toxins. 2013;5(4):703-716.
11. Fink M, Bhuyan AM, Zacharopoulou N, Lang F. Stimulation of eryptosis, the suicidal erythrocyte death, by costunolide. Cell Physiol Biochem. 2018;50(6):2283-2295.
12. 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.
13. Jilani K, Qadri SM, Lanf F. Geldamycin-induced phosphatidylserine translocation in the erythrocyte membrane. Cell Physiol Biochem. 2013;32(6):1600-1609.
14. Ullah S, Li Z, Hasan Z, Khan SU, Fahad S. Malathion induced oxidative stress leads to histopathological and biochemical toxicity in the liver of rohu (Labeo rohita, Hamilton) at acute concentration. Ecotoxicol Environ Saf. 2018;161:270-280.
15. Khan S, Norville KJ, Khan I, Siddiqui F, Karki A. Calcium channel blocker overdose treated with calcium resulting in pancreatitis: a case report. Cureus. 2019;11(4):e4493.
16. Bissinger R, Malik A, Jilani K, Lang F. Triggering of erythrocyte cell membrane scrambling by salinomycin. Basic clin pharmacol toxicol. 2014;115(5):396-402.
17. Wätjen W, Debbab A, Hohlfeld A, et al. Enniatins A1, B and B1 from an endophytic strain of Fusarium tricinctum induce apoptotic cell death in H4IIE hepatoma cells accompanied by inhibition of ERK phosphorylation. Mol Nutr Food Res. 2009;53(4):431-440.
18. 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.
19. Shahid M, Riaz M, Talpur M, Pirzada T. Phytopharmacology of Tribulus terrestris. J Biol Regul Homeost Agents. 2014;28(5):785-788.
20. Kauser 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.
21. Jaleel CA, Gopi R, Manivannan P, Paneerselvam R. Exogenous application of triadimefon affects the antioxidant defense system of Withania somnifera Dunal. Pestic Biochem Physiol. 2008;91(3):170-174.
22. Vijayaraghavan R, Paneerselvam C. Erythrocyte antioxidant enzymes in Multibacillary leprosy patients. IJABPT. 2011;2(2):409-412.
23. 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.
24. Hassan M, Hadi R, Al-Rawi Z, Padron V, Stohs S. The glutathione defense system in the pathogenesis of rheumatoid arthritis. J Appl Toxicol. 2001;21(1):69-73.
25. Lucero GMA, Marcela GM, Sandra GM, Manuel GOL, Celene RE. Naproxen-enriched artificial sediment induces oxidative stress and genotoxicity in Hyalella azteca. *Water Air Soil Poll.* 2015;226(6):195.

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

27. Berg C, Engels I, 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.

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

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

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

31. Rapido F. The potential adverse effects of haemolysis. *Blood Transfus.* 2017;15(3):218-221.