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Induction of Erythrocyte Shrinkage by Omeprazole

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Abstract

Omeprazole, a proton pump inhibitor blocks the H^+/K^+-ATPase channels of gastric parietal cells. It is used for the treatment of peptic ulcer. Prolonged use of omeprazole may involve in inducing anemia. The key marker of eryptosis includes membrane blebbing, cell shrinkage and phosphatidylserine (PS) exposure at the cell surface. In current study, the eryptotic, oxidative as well as hemolytic effects of therapeutical doses (0.5, 1 and 1.5 μM) of omeprazole were investigated after exposing erythrocytes for 48 hours. Investigation of eryptosis was done by cell size measurement, PS exposure determination and calcium channel inhibition. As a possible mechanism of omeprazole induced eryptosis, oxidative stress was investigated by determining the catalase, glutathione peroxidase and superoxide dismutase activities. Similarly, necrotic effect of omeprazole on erythrocytes was also evaluated through hemolysis measurement. Results of our study illustrated that 1.5 μM of omeprazole may induce significant decrease in superoxide dismutase, glutathione peroxidase and catalase activities as well as triggered the erythrocytes shrinkage, PS exposure and hemolysis. Role of calcium was also confirmed in inducing erythrocyte shrinkage. It is concluded that the exposure of erythrocytes with 1.5 μM omeprazole may enhance the rate of eryptosis and hemolysis by inducing oxidative stress.

Keywords

oxidative stress, calcium, cell size, eryptosis

Introduction

A proton pump inhibitor omeprazole[1] has a broad spectrum use especially in the treatment of gastroesophageal reflux disease and Zollinger-Ellison syndrome. Peptic ulcers caused by stress, nonsteroidal anti-inflammatory drugs and infection of *Helicobacter pylori* have been treated by using opmeprazole.[2-4] Previous studies related to the adverse effects of omeprazole reflects that its excessive use results in production of oxidative stress which leads to the stimulation of apoptosis in jurkat cells[5] and in the induction of iron deficiency anemia in patients.[6,7]

Characteristics of eryptosis mainly include the shrinkage of cells[8] and cell membrane scrambling which leads toward phosphatidylserine translocation.[9] Splenic macrophages recognize and engulf erythrocytes exposed with phosphatidylserine.[10] Stress condition including oxidation, osmotic shock and energy depletion activates Ca^{2+} $[Ca^{2+}]_i$ permeable cation channels. The increased activity of cytosolic Ca^{2+} $[Ca^{2+}]_i$ results in Ca^{+2} sensitive K^+ channels activation leading toward cell shrinkage[11] by subsequent KCl and water loss from the cell.[12] Phosphatidylserine translocation due to breakdown of phosphatidylserine asymmetry of erythrocyte's cell membrane is also the result of increased $[Ca^{2+}]_i$.[13] In addition, phosphatidylserine-exposed erythrocytes may adhere to the vascular wall and can therefore play a vital role in the

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pathogenesis of thrombosis and thrombo-occlusive diseases.[14] Several xenobiotic compounds are reported as stimulators of eryptosis.[9,13] Uncontrolled eryptosis also contributes in the pathophysiology of several clinical conditions[13] mainly by the induction of anemia in patients.[15]

The current study explores the eryptotic effect of the therapeutical doses of omeprazole possibly through oxidative stress induction.

Material and Method

For experimental work screened blood samples were collected from different blood banks of Faisalabad city. The work has been conducted with the approval of directorate of graduate studies and IBC, University of Agriculture Faisalabad, Pakistan.

Leukocyte depleted erythrocytes were prepared by following the protocol described by Rana et al., 2019.[16] To isolate the cells, the anti-coagulated whole blood was centrifuged at 500 *g* for 10 min at 4°C. Ringer solution was added to the erythrocyte pellet after removing the supernatant plasma followed by centrifugation at 500 *g* at 4°C for 10 min. Again removed the supernatant and ringer solution was added to erythrocyte pellet making the volume of the solution double to the volume of erythrocyte pellet alone. The solution was again centrifuged at same speed for 10 min followed by removing the supernatant. This washing step was repeated 3 times. This washing procedure is adequate to get 70 to 80% hematocrit of human erythrocytes. Isolated erythrocytes were stored in separate micro-centrifuge tubes. *In vitro* incubation of erythrocytes was performed at a hematocrit of 0.4% in ringer solution (pH 7.4) that contain (in mM) $MgSO_4$ 1, NaCl 125, KCl 5, glucose 5, $CaCl_2$ 1, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) 32 at 37 °C for 48 h.[17] Isolated erythrocytes were than exposed to lower concentrations (0.5, 1 and 1.5 μM) of omeprazole (Sigma-Aldrich, USA) as indicated by *Paz* et al., 2019[18] while the control cells were completely untreated. Dimethyl sulfoxide (DMSO) was used as solvent for omeprazole.

Oxidative Stress Measurement

To determine the oxidative stress in omeprazole exposed erythrocytes, antioxidant enzyme's (catalase, glutathione peroxidase, superoxide dismutase) assays were performed.

Superoxide Dismutase

Superoxide dismutase activity was measured by following the protocol of Rana et al. (2019).[16] The reaction solution contained methionine 0.222 g in 15 ml H_2O, NBT 0.015 g in 17.5 ml H_2O, Triton-X100 (0.0375 ml) in 17.5 ml H_2O, riboflavin (0.0132 g) in 17.5 ml H_2O and buffer 0.2 M.

Catalase

Catalase activity was determined by following the protocol of Ullah et al. (2018).[19] Phosphate buffer (pH7) 50 mM, H_2O_2 5.9 mM and enzyme extract 0.1 ml was added in reaction mixture and absorbance was read at 240 nm.

Glutathione Peroxidase

Phosphate buffer (pH 5.0) 50 mM, guaiacol 20 mM, H_2O_2 40 mM and enzyme extract 0.1 ml were added in reaction mixture by following the protocol of Ullah et al. (2018)[19] and the enzyme activity was measured at 470 nm after every 20 Sec.

Cell Size Measurement

Mean cell volume (MCV) was measured to determine the size of control and treated cells with the help of automated hematology analyzer.[20]

FACS Analysis of Annexin-V-Binding

After 48 hr incubation with 1.5 μM omeprazole, 50 μl cell suspension was washed in ringer solution with 5 mM $CaCl_2$ and stained by using Annexin-V-FITC (ImmunoTools, Friesoythe, Germany) for 20 min under protection from light. Annexin-V fluorescence intensity was measured in FL-1 with an excitation wave-length of 488 nm and an emission wavelength of 530 nm on an FACS Calibur (BD, Germany).[13]

Confirmation of Ca^{+2} Role

Amlodipine is a calcium channel blocker. Omeprazole exposed erythrocytes were treated with 10 μM amlodipine to confirm the calcium role in triggering of eryptosis. The inhibition of eryptosis was confirmed by MCV measurement.[16]

Hemolysis Measurement

After incubation, samples were centrifuged (3 min at 400 g at room temperature) and supernatant was collected for the determination of hemolysis. Hemoglobin concentration was measured at 405 nm.[21] The absorption of the supernatant of erythrocytes lysed in d.H_2O was defined as 100% hemolysis.[22]

Statistical Analysis

All data is expressed as arithmetic means ± SEM. Statistical analysis was made by using ANOVA with Tukey's test as post test or *t* test, as appropriate.[13]

Results

The current study was designed to explore primarily the eryptotic effect of omeprazole by adopting the mechanism of oxidative stress induction. To confirm this, antioxidant's enzymatic activities, erythrocyte's size, PS exposure at cell surface, hemolytic activity and confirmation of calcium role

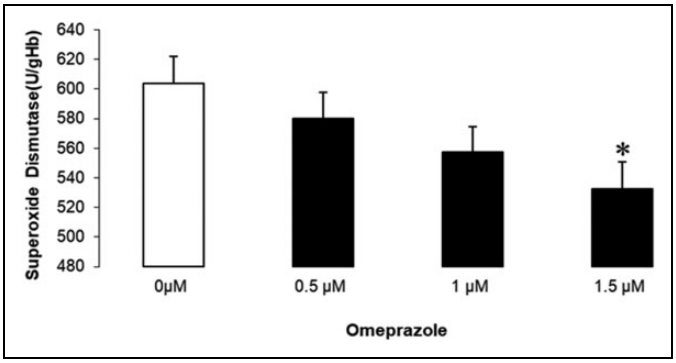

Figure 1. Effect of omeprazole on superoxide dismutase activities (U/g Hb) in erythrocytes. arithmetic means ± SEM (n = 15) of the erythrocytes exposed for 48 hours to ringer solution without (white bar) or with (black bars) 0 to 1.5 μM omeprazole. (*P < 0.05) indicate significant difference from the absence of omeprazole (ANOVA).

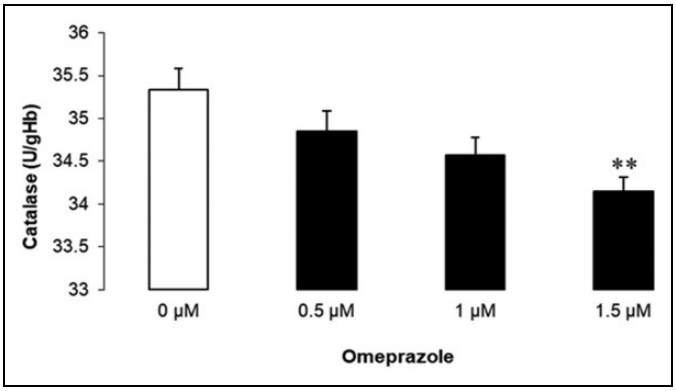

Figure 2. Effect of omeprazole on catalase activities (U/g Hb) in erythrocytes. arithmetic means ± SEM (n = 15) of the erythrocytes exposed for 48 hours to ringer solution without (white bar) or with (black bars) 0 to 1.5 μM omeprazole. (**P < 0.01) indicate very significant difference from the absence of omeprazole (ANOVA).

in the induction of suicidal death of erythrocytes were determined. The omeprazole concentrations used in the study were lower than the concentrations already reported to treat *S. cerevisiae* and S180 cells (18). Figure 1 is illustrating that 48 hour exposure of erythrocytes to omeprazole (0.5, 1 & 1.5 μM) resulted in significant decrease in activities of superoxide dismutase at 1.5 μM with respect to control. Figure 2 is demonstrating the catalase activities after 48 hour omeprazole (0.5 -1 & 1.5 μM) exposure to human erythrocytes. Results of this experiment showed moderate but significant decrease in enzyme's activity at 1.5 μM concentration of omeprazole. Figure 3 is indicating a significant reduction in the activities of glutathione peroxidase after 48 hour exposure of erythrocytes with omeprazole (0.5 -1 & 1.5 μM). The variation in anti oxidative enzymes activities in erythrocytes after omeprazole treatment under *in vitro* condition confirmed the generation of oxidative stress which may be due to generation of reactive oxygen species.

Cell shrinkage is an important marker of eryptosis. The 48-hour exposure of omeprazole (1.5 μM) to erythrocytes resulted

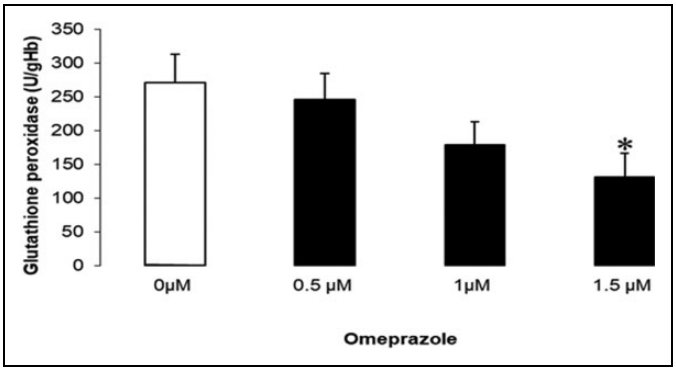

Figure 3. Effect of omeprazole on glutathione peroxidase activities (U/g Hb) in erythrocytes. Arithmetic means ± SEM (n = 15) of the erythrocytes exposed for 48 hours to ringer solution without (white bar) or with (black bars) 0 to 1.5 μM omeprazole. (*P < 0.05) indicate significant difference from the absence of omeprazole (ANOVA).

Table 1. Effect of Omeprazole Exposure on Mean Cell Volume (MCV) and Hemolysis of Erythrocytes.

	Omeprazole exposure		
Parameters	0 μM	1.5 μM	p value
MCV (fl)	90 ± 1.69	74 ± 0.77	<0.001***
Hemolysis (%)	1.35 ± 0.13	3.22 ± 0.38	<0.05*

Values are arithmetic mean ±SEM, where SEM: Standard error of mean. MCV: Mean cell volume. *** highly significant at p < 0.001, * significant at p < 0.05.

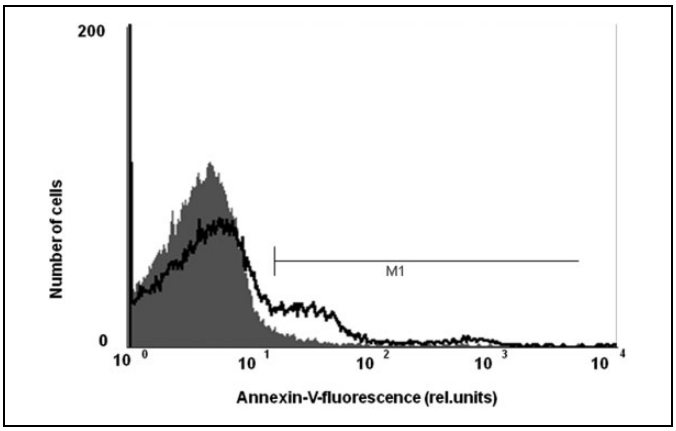

Figure 4. Effect of omeprazole on PS exposure. Original histogram of Annexin V binding of erythrocytes following exposure for 48 h to ringer solution without (gray shadow) and with (black line) presence of 1.5 μM omeprazole.

in lowering the mean cell volume, which may be due to erythrocytes shrinkage (Table 1). For additional confirmation of omeprazole induced eryptosis, PS-exposure in erythrocytes was determined after 48 hr. exposure of 1.5 μM omeprazole. The original histogram in Figure 4 depicting the clear increase of PS exposure in treated cells as compared to the untreated cells. As PS exposure is among the main features of eryptosis,

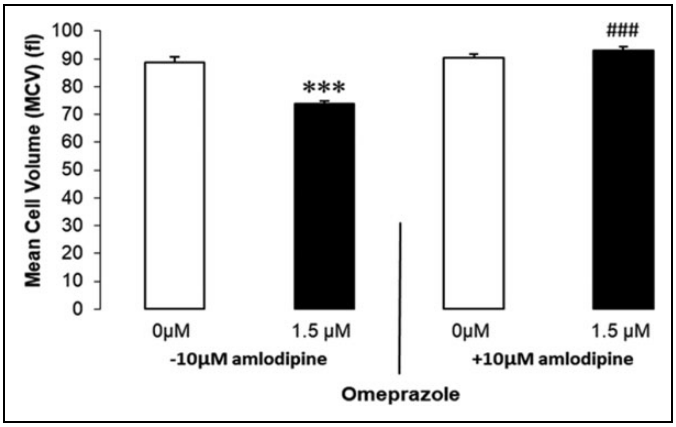

Figure 5. Cell size measurement of omeprazole exposed erythrocytes after Ca2+ inhibition. white bars showing the value of control and black shade shows the 1.5μM omeprazole concentrations in the absence (left bars, minus 10 μM amlodipine) and presence (right bars, plus 10 μM amlodipine) of amlodipine. Arithmetic means ± SEM (n = 10) of the erythrocytes exposed for 48 hours to ringer solution without (white bar) or with (black bars) 1.5 μM omeprazole. (***P < 0.001), (###P < 0.001) indicate extremely significant difference in cell size after amlodipine 10 μM treatment (ANOVA).

so an increased % of PS-exposed cells is the confirmation of stimulated eryptosis. To rule out the hemolytic role of omeprazole, the results of % hemolysis in omeprazole treated cells are shown in Table 1. The 48-hour exposure of erythrocytes with omeprazole (1.5 μM) resulted in negligible but statistically significant increase in hemolysis % as compared to control cells.

For the confirmation of Ca^{+2} role in the stimulation of oxidative stress induced eryptosis, calcium channel blocker amlodipine was used. Figure 5 is illustrating the cell size measurement of erythrocytes after 48-hour exposure to omeprazole (1.5 μM) and amlodipine (10 μM). The results showed significant increase in mean cell volume in amlodipine treated cells that is surely due to the inhibition of calcium entry and subsequent prevention of cell shrinkage. Non-selective cation channels are triggered by oxidative stress.[13] The result of this experiment showed no shrinkage, so confirmed the role of calcium in the omeprazole induced shrinkage of erythrocytes.

Discussion

Oxidative stress is a reported mechanism of eryptosis and variation among anti-oxidative enzymes in an important indicator of oxidative stress.[23] Superoxide dismutase enzyme catalyzes the dismutation of O_2 free radicals that on accumulation may results in lowering the superoxide dismutase's level and mitochondrial dysfunction resulting in oxidative stress.[24] Catalase is an iron-dependent enzyme that may act peroxidatively or catalytically[24] and considered as a major antioxidant enzyme.[25] Hydrogen peroxide's accumulation leads to the reduced catalase activity and oxidative stress. Catalase showed a protective effect against oxidants in cells with its overproduction.[26] Similarly, the inhibition of glutathione peroxidase activity may leads to the accumulation of H_2O_2[27] as it prevents the accumulation of oxidized lipids and promotes the decomposition of hydrogen peroxide into water and oxygen.[28] Previous study reported that omeprazole may induce oxidative stress through the generation of ROS.[29] In current *in vitro* studies, the reduction of all 3 enzyme activities after omeprazole treatment is the confirmation of oxidative stress induction.

Erythrocyte shrinkage and PS exposure on erythrocyte membrane is among the marker of eryptosis.[30] In current studies, the lowering of mean cell volume and increased % of PS-exposed cells is the confirmation of stimulated eryptosis.[22] Disposing ofdefective erythrocytes before hemolysis is an important physiological role of eryptosis.[31] Hemoglobin is released through hemolyzed erythrocytes that may be filtered through kidney or may precipitate in acidic lumen of renal tubules.[32] In performed studies, it is noted that omeprazole treatment slightly but significantly increased the hemolysis %. Non-selective cation channels are triggered by oxidative stress.[13] Amlodipine inhibits non-selective cation channels and blocks the Ca^{+2} entry.[33] As reported before, cell shrinkage is a characteristic of eryptosis.[34] The result of our experiment showed no shrinkage, so confirming of calcium role in the omeprazole induced shrinkage of erythrocytes. By removing intracellular and extracellular Ca^{+2}, similar effects would be observed.[35] As for as, the limitation of this research work is concerned, the used concentration of omeprazole are therapeutical, so the future prospect related to this work is to find out the eryptotic effects of physiological doses of omeprazole.

Conclusion

The performed *in vitro* studies are confirming the oxidative, eryptotic and hemolytic effects of used therapeutical doses of omeprazole. 1.5 μM omeprazole may generate oxidative stress by lowering the antioxidative enzymes activities, enhance the rate of eryptosis by promoting cell shrinkage and PS exposure. Slight increase in hemolysis % was also observed.

Authors' Note

Ayesha Naveed and Kashif Jilani contributed equally and thus shares first authorship.

Declaration of Conflicting Interests

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