Extracellular Methemoglobin Mediated Early ROS Spike Triggers Osmotic Fragility and RBC Destruction: An Insight into the Enhanced Hemolysis During Malaria

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Abstract Malaria infection is known to cause severe hemolysis due to production of abnormal RBCs and enhanced RBC destruction through apoptosis. Infected RBC lysis exposes uninfected RBC to the large amount of pro-oxidant molecules such as methemoglobin. Methemoglobin (MetHb) exposure dose dependently makes RBCs susceptible to osmotic stress and causes hemolysis. MetHb mediated oxidative stress in RBC correlated well with osmotic fragility and hemolysis. Interestingly, a reactive oxygen species (ROS) spike at 15 min was responsible for the observed effects on RBC cells. Two natural antioxidants N-acetyl cysteine and mannitol protected the RBC from MetHb-mediated defects, which clearly indicated involvement of oxidative stress in the process. MetHb due to its pseudo-peroxidase activity produces ROS in the external microenvironment. Therefore, classical peroxidase inhibitors were tested to probe peroxidase activity mediated ROS production with defects in RBCs. Clotrimazole (CLT), which irreversibly inactivates the MetHb (CLT-MetHb) and abolishes peroxidase activity, did not produce significant ROS outside RBC and was inefficient to cause osmotic fragility and hemolysis. Hence, initiating a chain reaction, MetHb released from ruptured RBC produces significant ROS in the external microenvironment to make RBC membrane leaky and enhanced hemolysis. Together data presented in the current work explored the role of MetHb in accelerated hemolysis during malaria which could be responsible for severe outcomes of pathological disorders.

Keywords RBC · Methemoglobin · Reactive-oxygen species · Hemolysis · Antioxidant · Malaria

Abbreviations

GSH	Reduced glutathione
NAC	N-acetyl cysteine
ROS	Reactive oxygen species
MetHb	Methemoglobin
CLT-MetHb	Clotrimazole modified methemoglobin

Introduction

Red blood cell (RBC) is a biconcave disc shape cell with an average diameter of 7.5 μm . RBC is produced in bone marrow with an average life span of 120 days. During this period, RBC needs to do several cycles of gaseous exchange (CO_2/O_2), experience shear stress (pass through small blood vessels) and other physiological or non-physiological stresses. As a result, RBC inevitably aged out and express specific cell surface molecule to get cleared by circulating phagocytes [1]. The whole process of RBC destruction within reticulo-endothelial system doesn't cause inflammatory reactions [2]. However during malaria, an accelerated RBC lysis occurs, leading to the development of hemolytic anemia [3]. According to a rough estimate, during malaria every infected RBC (IRBC) causes lysis of ten or more uninfected RBC to exhibit hemolytic anemia [4]. A recent study correlates the enhanced uninfected RBC destruction through apoptosis with the observed development of anemia during malaria [5]. The mechanistic details of apoptosis in uninfected RBC or inducer molecules are not known but results indicate pro-oxidant molecules released from infected RBC might be

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responsible. But accelerated destruction of uninfected RBC correlates well with poor antioxidant protection within RBC and higher rate of hemoglobin conversion to methemoglobin [6].

During hemolysis, hemoglobin present inside IRBC is released into the blood circulation. The hemoglobin in the external microenvironment is oxidized by molecular oxygen to form methemoglobin [Fe^{3+}] or oxidation by reactive oxygen species (ROS) into ferryl hemoglobin ($\text{Fe}[\text{IV}] = \text{O}$) [7, 8]. Ferryl hemoglobin is unstable and eventually gets converted into the methemoglobin through electron transfer. Besides direct oxidation of hemoglobin in cell free micro-environment, a number of extrinsic factors, such as, toxic drug molecules, metabolic by-products of pathogenic organisms, and pro-inflammatory cytokines cause imbalance of the oxidation/reduction system of RBC [6]. As a result, a large amount of intracellular hemoglobin oxidized to form methemoglobin and induce RBC cytoskeleton rearrangement, membrane depolarization, increase in deformity and hemolysis [5, 9]. In most of the pathological situation, rate of RBC destruction is very high and underlying molecular events are not clear.

Methemoglobin, a pro-oxidative molecule has potential to produce large amount of peroxide to accelerate oxidative-mediated tissue damage [10, 11]. Methemoglobinemia [presence of $>1\%$ metHb in serum] is known to produce functional and structural defects in RBCs [12]. During hemolysis, RBC present in the close vicinity will be the first target of methemoglobin directly or free radicals derived from it. Hence, we explored a chain reaction of RBC lysis, where molecules released from initial hemolysis destruct more RBCs and thereby amplifies the lysis reaction. In the current study, we have validated the role of released methemoglobin in amplification of lysis reactions that cause physiological and structural alterations in exposed normal RBC. Our data clearly supported the involvement of methemoglobin release to makes RBC cells vulnerable to osmotic shock and responsible for high level of hemolysis. It caused the development of oxidative stress and a sharp ROS spike was responsible for structural defects in RBCs. Based on these results we propose that MetHb released from RBC is responsible for enhanced hemolysis and severe outcome during malaria.

Materials and Methods

Methemoglobin (M4257), N-acetylcysteine (A2750), mannitol (N9647), 2,7, dichlorofluorescein diacetate (35845), glutaraldehyde (EM grade) 25% (G6257), clotrimazole (C6019), thiobarbituric acid (T550), 1,1',3,3'-tetraethoxy propane (T9889), guanidine hydrochloride (G4505), 5,5'-dithiobis (2-nitrobenzoic acid) (S39637) were

procured from Sigma. Vitamin C, dinitrophenyl hydrazine, ethylacetate, DMSO, hydrogen peroxide were procured from Merck. Other chemicals were analytical grade.

Preparation of RBC from Human Blood

Human blood was collected in EDTA containing tubes. Whole blood was centrifuged at 1,000 rpm and plasma was removed. Wash the RBC pellet with PBS containing glucose (1 mg/ml) and cells from the top layer containing WBCs were removed. This step was repeated three times and finally RBC pellet was re-suspended in glucose containing PBS at 5% hematocrit. A thin smear was made on a glass slide to monitor the purity of RBC preparations. In all of the experiments, we have used RBC preparations with less than 1% other blood cells.

Measurement of RBC Osmotic Fragility and Hemolysis

RBC preparations (5% hematocrit) were treated with different concentration of methemoglobin (0–1,000 μg) and after treatment, RBCs were separated from the reaction mixture by centrifugation at 1,000 rpm and supernatant was read at 540 nm in infinite M200 (Tecon, Austria) microtiter plate reader to measure hemolysis. RBC incubated in PBS was treated as control and 1% v/v triton-100 lysed RBC was considered as 100% hemolysis. For osmotic fragility, RBCs isolated from reaction mixture were exposed to 100 mM NaCl solution for 30 min at room temperature. Cells were centrifuged again and supernatant was read at 540 nm in infinite M200 microtiter plate reader (Tecon, Austria). RBC incubated with PBS was treated as a control. 1% v/v Triton X-100 lysed RBCs were considered as 100% hemolysis. For antioxidant treatment, RBC cells were treated with antioxidants, N-acetyl cysteine (5 μM) and mannitol (2.5%) either before treating with methemoglobin or 1 h post treatment.

Flowcytometric Analysis of ROS Containing RBC Population

RBCs were loaded with 10 μM fluorescent ROS probe 2,7, dichlorofluorescein diacetate (DCFHDA) for 1 h at 37°C in 5% CO_2 incubator. RBCs were washed three times with PBS to remove excess DCFHDA and treated with MetHb (700 μg) for 15 min at 37°C. After treatment all samples were kept on ice and analyzed in FACS Caliber (BD Biosciences) to detect ROS containing RBC population. In similar conditions, control cells were also analyzed.

Fluorescence Microscopy of Methemoglobin Treated RBC

RBCs were loaded with 10 μ M fluorescent probe DCFHDA for 1 h at 37°C in 5% CO₂ incubator and treated with methemoglobin, fixed with 2.5% glutaraldehyde. A thin smear was prepared and microscopy was performed on fluorescence microscope 80Ti (Nikon) under 100 $\times$ oil immersion objective to observe ROS containing fluorescent RBC cells. Control cells were similarly processed and analyzed. Files were opened in Adobe Photoshop 7.0 and Gray levels were adjusted by using the auto level command with black and white clip set to 0%. Images were cropped again and scaled for final display.

Measurement of Oxidative Stress Within RBC

Oxidative stress within methemoglobin treated RBCs was assessed by measuring lipid-peroxidation, Protein carbonyl, reduced glutathione (GSH) and ROS levels as described previously [13]. RBCs exposed to PBS served as control and used to calculate fold change.

Preparation of Clotrimazole Modified Methemoglobin (CLT-MetHb)

Methemoglobin (10 mg/ml), clotrimazole (20 mM) and hydrogen peroxide (1 mM) solutions were mixed together in a glass beaker in a ratio of (120:6:1) with constant shaking for 30 min at 25°C. The reaction volume was dialyzed against PBS (pH 7.4) for 24 h with 4 changes. Concentration of clotrimazole modified Methemoglobin (CLT-MetHb) was adjusted to 7 mg/ml and stored at –80°C in small aliquot for future use.

Measurement of Methemoglobin Peroxidase Activity

Methemoglobin peroxidase activity was measured by guaiacol oxidation as described previously [14].

Measurement of ROS Production in External Microenvironment by Methemoglobin

Measurement of ROS production activity of methemoglobin was performed as described earlier [15]. In a 0.2 ml reaction RBCs and methemoglobin (700 μ g) were mixed together and as indicated after every time point, an aliquot (50 μ l) from reaction mixture was taken out and RBCs were separated by centrifugation. Supernatant was added to the 950 μ l DCFHDA (10 μ M) and fluorescence spectra were recorded in Flumax-3 (λ_{ex} 488 nm and λ_{em} 530 nm). Fluorescence value at 530 nm was taken into account for calculating the amount of ROS and PBS treated RBCs were

used to calculate fold change in ROS level. CLT-MetHb was analyzed under the identical conditions.

Statistical Analysis

Statistical analysis was performed using student *t*-test (Microsoft Excel) and a *P*-value < 0.05 was considered significant.

Results

External Methemoglobin Exposure Makes RBC Susceptible for Hemolysis

Under stress or oxidation–reduction imbalance, membrane polarity is compromised and RBC lysis occurs. To test whether MetHb can affect the ability of RBC to maintain osmotic balance, we exposed the RBC to different concentration of MetHb for 30 min at 37°C. Cells were recovered from reactions and osmotic fragility was assessed by exposing them to 100 mM NaCl (hypotonic) solution at room temperature (RT). MetHb dose dependently causes RBCs susceptible to osmotic stress and at 1,000 μ g more than 50% RBCs were vulnerable to osmotic shock (Fig. 1a). Loss of ability to maintain osmotic balance results into the RBC lysis. As expected, we found RBC lysis with increasing concentration of MetHb complementing osmotic fragility results (Fig. 1b). Together the data indicate that MetHb exposure reduces the ability of RBC to maintain osmotic balance and if MetHb exposure remains high it leads to cell lysis (hemolysis).

Methemoglobin Exposure Causes Oxidative Stress Within RBC

MetHb treated RBCs gives several fold high fluorescence compare to RBCs exposed to MetHb for 0 h and analyzed under identical conditions. Analysis of FACS curves indicated that MetHb exposure causes more than 80% RBCs with high ROS (fluorescence) compared to control cells (Fig. 2a). A thin smear of these cells was also observed under a fluorescence microscope to detect the distribution of ROS (fluorescence) within RBCs. MetHb treated RBCs were exhibiting bright fluorescence throughout cytosol compare to control cells (Fig. 2a, inset). Increased level of ROS in cytosol leads to the development of oxidative stress. Oxidative stress in biological system can be assessed by biochemical parameters such as an increase in lipid peroxidation or protein carbonyl levels and reduction in GSH levels. RBCs treated with methemoglobin (700 μ g) were lysed with 1% v/v Triton X-100 and lipid peroxidation and protein carbonyl levels were measured. Within

Fig. 1 MetHb Exposure causes RBC cells susceptible for hemolysis. RBC hematocrit [5%] prepared in PBS was exposed to different concentration of MetHb (0–1,000 μg) for 30 min at 37°C and **a** Osmotic fragility or **b** hemolysis was measured as described in the method section. A representative test tube picture of **(a)** osmotic

fragility or **(b)** hemolysis measurement of RBC treated with MetHb was shown at the bottom of the curve. Data were from two independent experiments performed in triplicate. All *error bars* indicate standard deviation (SD) and were calculated from triplicate measurements. If not visible, an error is smaller than *symbols*

15 min, Lipid Peroxidation level sharply raised three fold compared to control samples. Afterwards, it sharply scaled down to basal level (Fig. 2b). MetHb treated RBCs also exhibited similar response curve for protein carbonyl levels as well (Fig. 2c). MetHb treatment caused a sharp reduction in GSH levels within 15 min and then increased afterwards to reach levels comparable to control cells (Fig. 2d).

Methemoglobin Mediated Initial ROS Spike Prime the RBCs to Exhibit Membrane Defects

The ROS pattern within RBC had two phases, a sharp increasing phase (spike) and then gradual increasing phase (accumulation) at a later time point. We performed an inverse experiment of removing the ROS by antioxidant treatment to probe spike (first hour) or accumulation part (second hour) of ROS curve. MetHb treated and untreated RBCs were incubated with two natural antioxidant molecules, N-acetyl cysteine (NAC) and mannitol either from the beginning (to remove spike) or after 1 h (to remove accumulation). Both treatments significantly reduced the ROS level within MetHb treated RBC cells (Fig. 3a). Antioxidant treatment during initial spike phase inhibited the MetHb mediated membrane fragility and hemolysis comparable to control level, where as antioxidant treatment after 1 h had no or very little inhibitory effects on MetHb mediated osmotic fragility and hemolysis (Fig. 3b, c). The antioxidant treatment experiment clearly indicates MetHb mediated ROS spike primed the RBCs to exhibit osmotic fragility and ultimately caused hemolysis.

Methemoglobin Associated Peroxidase Activity Causes RBC Defects

MetHb has a very low level of intrinsic pseudo-peroxidase activity which can have the potential to produce free radicals in the micro-environment [16]. But a recent report suggests that peroxidase activity associated with MetHb attenuate H_2O_2 induced oxidative stress [17]. To probe the role of MetHb peroxidase activity with observed RBC defects, we tested many peroxidase inhibitors using guaiacol oxidation assay. In our previous study, we have found that clotrimazole dose dependently inhibits the peroxidase activity of PfHpo, a hemo-peroxidase present in malaria parasite [13]. Clotrimazole mediated inhibition is irreversible in nature and is a suicidal inhibitor [13]. We tested the effect of clotrimazole on MetHb in guaiacol oxidation assay. To our surprise, CLT irreversibly inhibits MetHb associated peroxidase activity, even after its removal by dialysis (Fig. 4a, inset). Exploiting this feature we prepared a clotrimazole modified inactive MetHb (CLT-MetHb) as given in the method section. CLT-MetHb didnot produce significant ROS where as MetHb causes high ROS in the micro-environment (Fig. 4a). In comparison, CLT-MetHb (inactive MetHb) was inefficient to cause osmotic fragility and hemolysis compared to MetHb (Fig. 4b, c). CLT-MetHb even at very high concentration ($\sim 2,000 \mu\text{g}$) for 2 h also failed to cause any osmotic fragility or hemolysis (data not shown). Together our data strongly suggested that peroxidase activity of MetHb is responsible for observed RBC defects and hemolysis.

Fig. 2 MetHb exposure causes development of oxidative stress within RBC Cells. **a** RBCs loaded with fluorescent probe DCFHDA (10 μ M) for 1 h at 37°C, exposed to MetHb causes RBC population with high ROS level. A small aliquot of cells used were taken to prepare a thin smear and observed with fluorescence microscope Nikon 80Ti equipped with DS-Fi1-U-2 CCD camera (**a**, inset). MetHb exposure causes an increase in **b** lipid peroxidation, **c** protein

carbonyl level and **d** decrease in GSH level. RBC cells treated with glucose containing PBS serve as control. Data were from three ($n = 3$) independent experiments performed in triplicate. All error bars indicate standard deviation (SD) and were calculated from triplicate measurements. If not visible, an error is smaller than symbols

Discussion

Toxic substances (external or metabolic derived by-products) appear in blood first and injures RBC in a variety of ways causing its immediate hemolysis. A number of different models have been proposed in the past to explain hemolysis with much emphasis on the steps of RBC lysis [18–21]. The earlier biochemical model proposed that RBC lysis is a two step process, initial “sensitization” and then actual “lysis”. Both steps are catalyzed by two distinct molecules, hence hemolysis model believes that molecules causes severe damage to RBC membrane fragility

[sensitization] but needs additional help to facilitate the efficient lysis (hemolysis) [18–20]. Conversion of hemoglobin to methemoglobin within RBC triggers the development of oxidative stress and causes structural membrane defects (sensitization). An additional physical damage (via passing through thin vessels) to cells causes hemolysis [22]. In this study we explored a unique role of methemoglobin playing a master role to catalyze both the steps of hemolysis. Based on our results in the current study, we observed a “chain reaction” of RBC lysis induced by initially released MetHb from parasitized RBC (Fig. 5). In the proposed model, initial RBC lysis causes release of Hb or

Fig. 3 MetHb mediated ROS spike is responsible for observed RBC osmotic fragility and hemolysis. Two natural antioxidants, N-acetyl cysteine (NAC) and mannitol were used to probe the role of oxidative stress in MetHb treated RBC with osmotic fragility and hemolysis. **a** Both antioxidants were added to the RBC either before the addition of MetHb or after 1h of MetHb treatment. Both treatments were efficient to abolish ROS spike or accumulation phase. ROS levels were determined using fluorescent probe, DCFHDA as described in the method section. RBC cells treated as in (a) and used to determine **b** osmotic fragility or **c** hemolysis. Data were from four independent experiments ($n = 4$) performed in triplicate. All error bars indicate standard deviation (SD) and were calculated from triplicate measurements

MetHb into the blood or local microenvironment. MetHb due to its pseudo-peroxidase activity causes production of large amount of ROS outside (sensitization). External ROS disturbs the interior oxidation/reduction balance of RBC to develop oxidative stress. Oxidative stress induces cytoskeleton changes (protein carbonyl formation) which results in structural membrane defects. RBC cells at this stage are vulnerable for immune-mediated elimination and hemolysis. RBC cell lysis releases Hb/MetHb to start another cycle to amplify RBC damage. Hence, MetHb mediated ROS production is a critical step to amplify the initial lysis signal. Development of oxidative stress and down-stream events prime the RBC to get lysed. Infusion of hemoglobin solution in healthy volunteers as well as a variety of acquired and iatrogenic hemolytic disorders suggest links between hemolysis and hemoglobinemia [23–25].

During malaria, accelerated RBC destruction attributes to induction of apoptosis like process in uninfected RBC and development of oxidative stress [5]. Oxidative stress developed during the first hour (a sharp ROS spike by 15 min) of MetHb treatment was found to be responsible for the observed loss of membrane fragility and hemolysis (Fig. 3). Recent studies indicate a cyto-protective antioxidant like properties of hemoglobin to reduce H_2O_2 mediated toxic effects in endothelial cells [17]. Compare to classical heme dependent peroxidases, free radical originates on MetHb but leaks out into the microenvironment [16]. Hence, the chemistry of hemo-protein in the aqueous system is complex and final biological effects depend on the presence of other substances (free radicals) in the microenvironment.

Hemolysis is a critical parameter to decide the quality of preserved blood. During a period of 42 days of storage, free hemoglobin/methemoglobin level reaches to 200 mg/dl [26]. According to the American association of blood banks (AABB) blood or blood products with 1% hemolysis is safe for transfusion, if it provides 75% RBCs in the next 24 h [27]. It is interesting to note that in our study we have used the MetHb at a concentration of free MetHb in the preserved blood units in a blood bank. Our work clearly

highlights the probable mechanism for the observed destruction of remaining 25% RBC cells within 24 h transfusion of stored blood. In an isolated system MetHb exposure mediated induction of loss in osmotic fragility

Fig. 4 The pseudo-peroxidase activity of MetHb is responsible for ROS accumulation within the microenvironment. **a** MetHb pseudo-peroxidase activity causes production of reactive oxygen species in the microenvironment. CLT-MetHb was inactive in an guaiacol oxidation assay (**a**, *inset*). CLT-MetHb was inefficient in causing production of ROS within the micro-environment as compare to unmodified MetHb. All values were expressed as fold change in

comparison to samples at time 0. RBCs were treated with PBS, MetHb or CLT-MetHb (1 = PBS, 2 = MetHb or 3 = CLT-MetHb) and **b** osmotic fragility or **c** hemolysis were measured as described in the method section. All error bars indicate standard deviation (*SD*) and were calculated from triplicate measurements. If not visible, an error is smaller than symbols

Fig. 5 Scheme of enhanced RBC lysis by Methemoglobin mediated chain reactions. A scheme of methemoglobin mediated chain reactions to cause enhanced RBC destruction where MetHb released from first RBC lysis catalyzes the further RBC destruction to amplify the RBC lysis reactions. A detailed description is given in the text

and hemolysis is never been studied. But under in vivo conditions, contribution of molecules from other blood cells cannot be ruled out. In summary, we have put efforts

to understand the role of MetHb in making RBC prone to osmotic shock and amplifying the RBC destruction to enhance hemolysis.

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Conflict of interest No competing financial interests exist.

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