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Nitric oxide donor 8-bromo-cGMP suppresses cholesterol depleted RBCs band 3 dysfunction

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ABSTRACT

Red blood cells (RBCs) carry large cholesterol fractions and imbalance in them leads to several vascular complications. RBCs band 3 protein plays an important role in maintaining membrane integrity and there are many reports on cholesterol and band 3 protein interaction. Yet, RBCs band 3 protein role in regulating cholesterol homeostasis needs to be investigated. In this study, we induced cholesterol-depletion and band 3 inhibition in RBCs; both of which cause stress by decreasing band 3 channel activity with an increase in RBCs adhesion to endothelial cells (EC) by elevating band 3 phosphorylation (Tyr21), methemoglobin level and decreasing nitric oxide level. We hypothesized that nitric oxide (NO), a prominent determinant for RBC structural stability, would protect RBCs from stressors. To estimate this, we used three NO donors (SpNO, Sildenafil citrate and 8-Bromo-cGMP) and found that all 3 NO donors were able to recover, with 8-Bromo-cGMP being the most effective as it not only increased band 3 channel activity but also decreased RBC-EC adhesiveness and methemoglobin level in both stressors. Whereas NO donor's treatment did not display an ameliorative impact when both stresses were combined. Overall, these findings may shed light on the role of 8-bromo-cGMP in regulating RBC cholesterol homeostasis by maintaining band 3 function. Further studies in this direction might help identify targets for the therapeutic use of NO donors in the treatment of blood disorders.

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Introduction

Alteration in RBC function significantly contributes to various hemorheological disturbances and extreme pathophysiological conditions (1-3). Under normal physiological conditions, RBC exists as single, free-flowing entities, which appear altered under conditions of disease and stress (4,5). One of the most observed changes is RBC aggregability, deformability and adherence to endothelial cells which is known to consequently affect blood viscosity (1,6,7). Recent studies in this field have highlighted the role of nitric oxide (NO), particularly endogenous NO synthesized in RBCs, as a primary modulator of RBC rheology. Kleinbongard et al., 2006 reported the expression of a functional eNOS-like enzyme (RBC NOS) localized in the RBC membrane and cytoplasm that has similar properties to eNOS (8,9). It was also found that subjecting RBCs to shear stress resulted in RBC NOS activation (10). Earlier findings from our lab showed that immobilized RBCs when exposed to fluid shear stress showed an increased amount of eNOS activity and intracellular NO levels. We found that RBCs were more sensitive in their response to physical/mechanical perturbations by synthesizing NO. We hypothesized that mechanical perturbations alter the order of freedom in the RBC membrane that provokes the Band 3 channel activity (11). Extracellular NO treatment has been shown to prevent disease severity (12,13). A recent finding shows that nitric oxide reduces the cross-linking of band 3 protein under conditions of stress (11). But the mechanism by which nitric oxide regulates the RBC membrane through its association with band 3 is not clear. In this direction, we wanted to further investigate the effect of NO on Band 3 protein.

Band 3 protein (i.e., erythrocyte anion exchange AE1, or SLC4A1) is a 911 AA long, anion exchange protein of the bicarbonate transported family and is involved in the transport of CO_2 in blood, maintaining intracellular pH and providing mechano-structural support in RBCs (14). Band 3 function is regulated by its phosphorylation status while band 3-phosphorylation has been associated with senescence

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of RBC, glucose-6-phosphate dehydrogenase deficiency, diabetic RBC and hemoglobinopathies (8). However, the underlying biochemical mechanisms leading to band 3 phosphorylation and its pathophysiological significance have only been partially defined (15).

In RBC, disruption of the normal lipid composition of the outer sheath has been observed in diabetic patients (10). Several biophysical studies have supported the view that cholesterol interacts with Band 3 affects its dynamics, self-association, and transport activity. It has been shown that cholesterol affects the aggregation state of Band 3 (14). Another study indicates that significant lipid loss from the RBC membrane occurs later in the RBC storage period (16). It is known to strongly interact with cholesterol and is proposed to contain a high affinity to inhibiting cholesterol-binding sites (17). Computational studies suggest that negatively charged phospholipids and cholesterol interact strongly with band 3 protein, forming an annulus around the protein (18). Alteration in the levels of membrane cholesterol in the cells modulates cell rigidity and stress resistance. Even though all cells in the body synthesize cholesterol except RBC, the plasma membrane of RBCs contains a high concentration of cholesterol (~50% of lipids) (19). Yet the influence of changes in RBC membrane cholesterol levels on band 3 activity has not yet been explored in detail.

In this study, we assessed the adhesive nature of RBCs, methemoglobin level, band 3 channel activity and band 3 phosphorylation under cholesterol depletion conditions and checked for the ability of nitric oxide donor's compounds, namely Sildenafil citrate, 8-bromo-cGMP and SpNO, to recover NO levels in stressed RBCs.

Materials and methods

Materials

DMEM, FBS, trypsin, and penicillin/streptomycin were purchased from PAN Biotech. Sildenafil citrate (SC), Methyl beta-cyclodextrin (MBCD), 4,4'-diisothiocyanato-2,2'-stilbenedisulfonate (DIDS), and guanosine-3,5-monophosphate 8-bromo-sodium salt (8BrcGMP) were from Sigma Chemical Co., St. Louis, MO. 4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate (DAF-FM-2DA) was purchased from Invitrogen, NY, US. SpNO was obtained from Cayman Chemicals. All other chemicals were of laboratory grade and obtained commercially.

Blood sample collection and Ethics

Blood samples were collected from (4 males and 4 females) healthy volunteers at Lions blood bank, Chennai, India, and informed written consent was obtained. The study protocol was approved by the Institutional Biosafety and Ethical Committee of AU-KBC Research Centre, Chennai, India (Annexure I: Project Number III dated 19 July 2016). As an inclusion criterion, samples were collected from healthy volunteers aged between 20-30 years. During blood collection, subjective feelings of illness with positive results on infectious disease testing and pregnancy were excluded. All screened volunteers who met the inclusion/exclusion criteria were enrolled in the study.

Red Blood cell preparation

Isolation of RBCs from blood samples was done as previously described (20). Briefly, RBCs were separated from leukocytes and platelets by centrifugation (800× g at 4 °C for 10 min) and the RBC pellet was washed thrice in an isotonic buffer solution (300 mOsm; 0.9% NaCl). Cholesterol depletion was induced in RBC to decrease cholesterol levels in the RBC membrane. This was achieved by using methyl-beta cyclodextrin (MBCD) and was prepared as previously described (21). In brief, different (0.5, 1, 2.5, 5 and 10mM) concentrations of MBCD were prepared in physiological salt solution (PSS) and this was used to treat RBCs for 10 minutes at room temperature (RT). After incubation, RBCs were washed twice with PSS. 2.5mM concentration of MBCD was used in all the experiments after determining its stress effect on RBCs. For inhibiting band 3 channel activity, 100µM of band 3 inhibitor-4,4'-diisothiocyanato-2,2'-stilbenedisulfonate (DIDS) was chosen. RBCs were incubated in DIDS for 5 mins at RT. For recovering RBC from stress, NO donors used: 8-bromo-cGMP (500µM), Spermine NONOnate (1µM) and Sildenafil citrate (1µM) in all the experiments.

Endothelial cell

We used an immortalized endothelial cell line (EA.hy926) established through hybridization of

human umbilical vein endothelial cells with the A549/8 human lung carcinoma cell line (generously gifted by Dr. C.J.S. Edgell University of North Carolina, Chapel Hill) (22). The cells were cultivated in DMEM medium (HyClone, Logan, USA) supplemented with 10% Fetal bovine serum (FBS) (v/v) (HyClone, Logan, USA) and 1% penicillin/streptomycin (w/v). The cells were maintained at 37°C in a humidified 5 % CO_2 incubator. For *in-vitro* static adhesion assay, cells were seeded in 24 wells and used when they reached maximum confluency.

Band 3 chloride/bicarbonate exchange (AE1) channel activity measurement

Intracellular (Cl^-) was measured by the Cl^- sensitive fluoroprobe MQAE (23). Samples were incubated with 5mM of N-(6-methoyquinolyl) acetoxy-acetyl-ester, MQAE (5 mM) for 30 minutes in chloride free-buffered solution at 37 °C. Absorbance was measured using Varian Cary Eclipse UV–Vis Fluorescence spectrophotometer at 355/460 nm. The data is accordingly presented as "Band 3 channel activity (arbitrary unit)".

Real-time NO production using DAF-FM fluorescence

RBC samples were diluted at 1:10 in PBS and treated with 5μM DAF-FM 2DA at RT for 30 minutes in the dark and washed twice in PBS (24). Unstained cells served as autofluorescence controls. Samples were mounted in coverslips and smears were analyzed 1 to 5 minutes after preparation. Real-time NO production was observed using a fluorescent microscope Olympus IX71. Images were captured and micrographs were taken at 37°C. Images were processed using ImageJ software.

Methemoglobin (MetHb) level assessment

MetHb levels have been determined according to Morabito and co-authors (25). The method is based on methemoglobin and (oxy)hemoglobin measurement by spectrophotometry at, respectively, 630- and 540-nm wavelengths. Briefly, 25 μL of whole fresh blood (control) or treated samples were lysed in a 1975 μL hypotonic buffer (2.5 mM Na_2HPO_4, pH 7.4, 4 °C). Samples were then centrifuged (Eppendorf, 12,000×g, 10 min, 4 °C) to discard membranes and supernatant spectrophotometrically read (Beckman DU-640). MetHb percentage was calculated as follows: MetHb % = OD630/OD540 × 100 (OD (optical density)).

RBC-EC interaction assay

RBC adhesion assay was performed as previously described with minor modifications (26). Briefly, control and stressed RBC were suspended in Serum-Free medium SFM [Dulbecco's basal modified eagle medium (DMEM) without fetal bovine serum] and centrifuged at 800 ×g and 4 °C for 5 minutes twice, before the adhesion assay. Gradient concentration of dextran treated RBCs was added to the 24 well plates containing confluent endothelial cells (EAhy926 immortalized cell line) and incubated for 30 minutes at 37°C in a CO_2 incubator. Unadhered RBCs were removed by washing twice with a respective concentration of the dextran solution in which each sample and the adherent single and rouleaux RBCs were imaged with an Olympus IX71 inverted fluorescence microscope fitted with an Olympus digital camera (DP71, Tokyo, Japan). Images were captured in 24 random locations of 400μm x 400μm images (20x magnification) in triplicates (i.e., 3 wells for one condition) and the result was acquired from 3 individual donors for the respective RBC stress experiments. For all adhesion assay experiments, RBC samples were suspended in isotonic buffer (control) or in stress inducing chemical to a final concentration of 0.16×10^6 RBC/ml. RBCs adhered to EC was counted was quantified using ImageJ software.

Immunofluorescence microscopy for Tyr-21 phosphorylated band 3

Samples were incubated in 2.5% glutaraldehyde at room temperature for 15 minutes. After several washings in PBS, the samples were incubated for 1.5 hours in PBS containing 3% bovine serum albumin to block nonspecific binding and 0.05% Tween 20 for permeabilization of RBC membranes. RBC was immunolabeled against band 3 and tyr-21 phosphorylated band 3 using primary polyclonal rabbit antibodies at a 1:10 dilution for 3 hrs. The coverslips were then washed with PBS and incubated with secondary goat anti-rabbit antibody conjugated with Fluorescein isocyathiocynate (FITC) at a dilution of 1:2500 for 1 hr. The samples were then mounted on a cover slide and examined under a fluorescent microscope (OlympusIX71) at 60X (11). Fluorescence

intensity was calculated with an image analysis module of Adobe Photoshop ver. 7.0.

Scanning Electron Microscope (SEM) Analysis

SEM was done as per the standard protocol described previously (27). Briefly, chemically treated RBCs were washed with PBS (pH 7.4) and fixed using 2.5% glutaraldehyde for 20 minutes and stored at 4° C. Further, the sample was postfixed with 1% osmium tetroxide (pH 7.4) and dehydrated with graded ethanol followed by isoamyl acetate. Subsequently, the samples were dried, and sputter-coated with a thin gold layer (Quorum Technologies, UK). Samples showing RBC-EC adhesion were analyzed using SEM (TESCAN VEGA3).

Statistical analysis

All the experiments were performed in triplicates. Data were quantified and expressed as mean ± SD, and differences between groups were analyzed using the one-way ANOVA test. Tukey multiple comparison tests was used to analyze significance among the groups in comparison with the control. $p \leq 0.05$ was considered significant in all the analyses. The results were computed with GraphPad Prism version 7.0 software.

Results and discussion

Methemoglobin formation

The formation of methemoglobin is a marker for stressed RBC (28). As a consequence of stress, ferrous ions oxidized to ferric form, converting hemoglobin to methemoglobin in RBC (29). To investigate the effect of stress-induced by these chemicals (MBCD and DIDS), gradient concentration of both chemicals was given to RBC samples. It yielded significant methemoglobin (MetHb) change (Fig.1a, c) in comparison to the control in both the stress conditions. With NO donor's treatment, it preferentially reduced MetHb level in pretreated 2.5mM cholesterol depleting agent and 100µm of band 3 inhibiting agent. These elevated MetHb levels were greatly lowered by 8-bromo-cGMP among the other NO donors (Fig 1b, d).

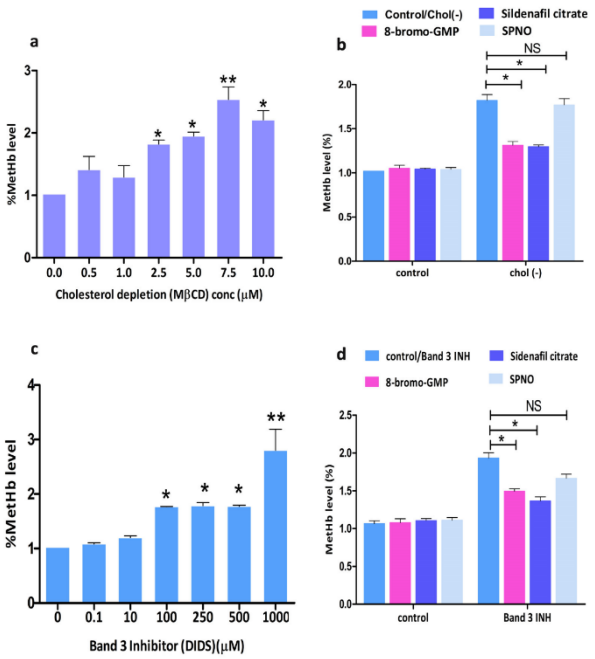

Figure 1. Stressed RBC methemoglobin level: (a)Schematic illustration of MetHb level change in cholesterol depleted RBC." *" indicates comparison with control. (b) Cholesterol depleted and band 3 inhibited RBC) and unstressed RBC samples (control) incubated with 3 different NO donors in which 8-bromo-cGMP and sildenafil citrate depicts a significant reduction in MetHb level in comparison to cholesterol depleted RBC. (c) Schematic illustration of MetHb level change in band 3 inhibiting agent (DIDS) treated RBC."*" indicates comparison with control. (d) Cholesterol depleted and band 3 inhibited RBC) and unstressed RBC samples (control) incubated with 3 different NO donors.A significant reduction in MetHb level with 8-bromo-cGMP and sildenafil citrate was observed. Significance of difference from stressed RBC/control RBC (n=3; *P≤0.05; **P≤0.001); NS- not significant difference from stressed RBCs.

Influence of NO donors in RBC-NO production after stress

To understand if cholesterol depleting agent, MBCD (2.5mM) and band 3 inhibitor (100µM) agent DIDS has a NO-inhibitory potential in RBC, intracellular NO was measured after exposing it. After exposure to these chemicals, it subsequently decreased ($P<0.001$) nitric oxide production in comparison to control, reflecting its stress effect on RBC. On pretreated stressed samples (i.e., MBCD and DIDS treated sample), it showed recovery in nitric oxide with all the nitric oxide donors. When the recovery effect was compared among the three NO donors, 8-bromo-cGMP was found to be consistently more effective in both the stress condition. (Fig.2).

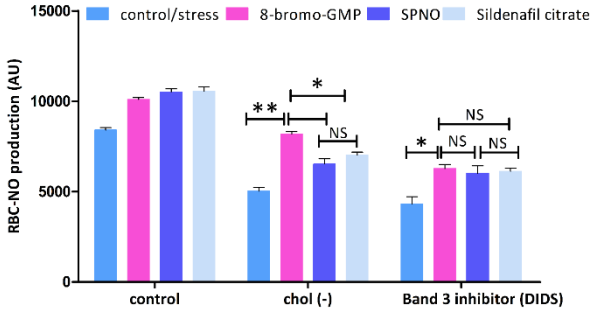

Figure 2. Recovery in RBC-NO production with NO donors after cholesterol depletion and band 3 inhibition stress: Intracellular NO level following incubation with (methyl-beta-cyclodextrin, a cholesterol depleting agent and DIDS, a band 3 inhibiting agent) and without (control), and concurrent incubation with NO donors: SpNO, Sildenafil citrate and 8-bromo-cGMP show a significant increment in NO level in all stressed RBC samples after NO donor's treatment. While incubation with 8-bromo-cGMP significantly increased the intracellular NO in stressed RBC samples more than in other NO donors. Significance of difference from control (n=3;*P≤0.05;**P≤0.001); NS-not significant difference from control.

Attenuation of stressed RBC adhesion to EC with NO donors

In several disease severity cases; RBCs were observed to be adherent to the endothelial cells (27,30). The chemicals used to inhibit band 3 demonstrated its ability to promote RBCs adhesion to EC under in vitro static conditions (Fig 3a). Furthermore, NO donors-Sildenafil citrate, 8-bromo-cGMP and SpNO, contributed to preventing RBC adhesion to EC in cholesterol depleted and band 3 inhibited RBC samples was demonstrated in the RBC-EC adhesion result (Fig 3b, c).

Band 3 channel activity in stress RBC with 8-bromo-cGMP

We previously reported that vortexed RBC activates band 3 anion exchanger and endothelial nitric oxide synthase (eNOS). Importantly, alteration in-band 3 channel anion exchanger directly affects band 3 phosphorylation which critically affects RBC function. Band 3 channel activity was determined to be affected by the higher concentration of cholesterol depletion stress (Fig 4a). Among the 3 different NO donors, 8-bromo-cGMP was found to be significantly reversing cholesterol depleted-RBC band 3 channel activity. Whereas it did not show a difference in band 3 channel activity change with band 3 inhibitor and in combined stress (DIDS and MBCD) (Fig 4b).

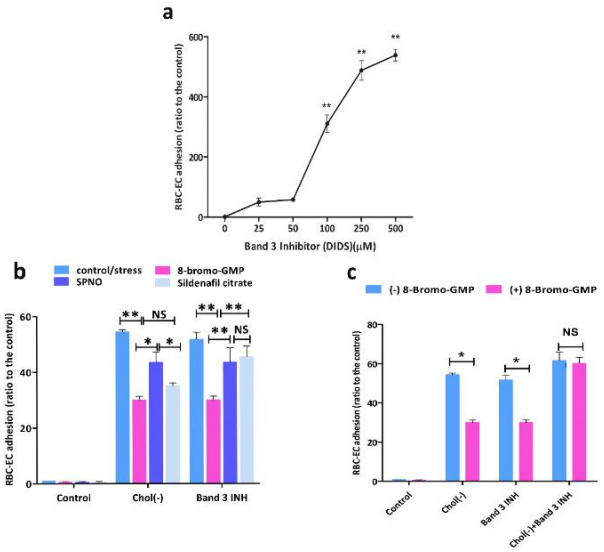

Figure 3. Stressed RBCs adhesion to EC with and without NO donors: (a) RBCs were treated with a gradient concentration of band inhibitor (DIDS) and their adhesiveness to EC was analyzed as described in Materials and Methods.)." *" indicates comparison with control. NS-not significant. (b) Cholesterol depleted and band 3 inhibited RBC) and unstressed RBC samples (control) incubated with 3 different NO donors: 8-bromo-cGMP, SpNO, and sildenafil citrate and were assessed to determine the change in adhesiveness to EC. (c)Among the other three NO donors, 8-bromo cGMP treatment showed a significant recovery in RBC-EC adhesion. Significance of difference from control (n=3; ∗P≤0.05; **P≤0.001).

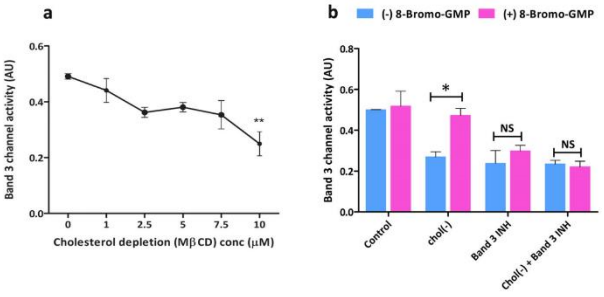

Figure 4. Band 3 channel activity in stressed RBC with and without 8-bromo-cGMP:(a). Band 3 channel activity under cholesterol depletion was determined with gradient concentration of cholesterol depletion agent. With an increase in concentration band 3 channel activity decreased with a significant change in 10μM concentration. (b) Under 8-bromo cGMP treatment, cholesterol-depleted RBC samples regained band 3 channel activity whereas with band 3 inhibition it failed to recover. Further, when both stresses were combined, 8-bromo-cGMP was unable to regain band 3 channel activity in stressed RBC samples. Significance of difference from control (n=3; **P 0.001). NS-not significant.

Immunofluorescence of band 3 phosphorylation in stress RBC with 8-bromo-cGMP

Studies have shown that RBC under stress conditions induced phosphorylation of the band 3 Tyr 21 residue (31). Determination of RBC band 3 phosphorylation tyr21, therefore, represents a reliable readout to examine band 3 phosphorylation after incubation with cholesterol depleting and band 3 inhibiting agents. Our immunofluorescence study result showed a significant reduction in band 3 Tyr 21 phosphorylation in both stress conditions in presence of 8-bromo-cGMP fig 5(a-d).

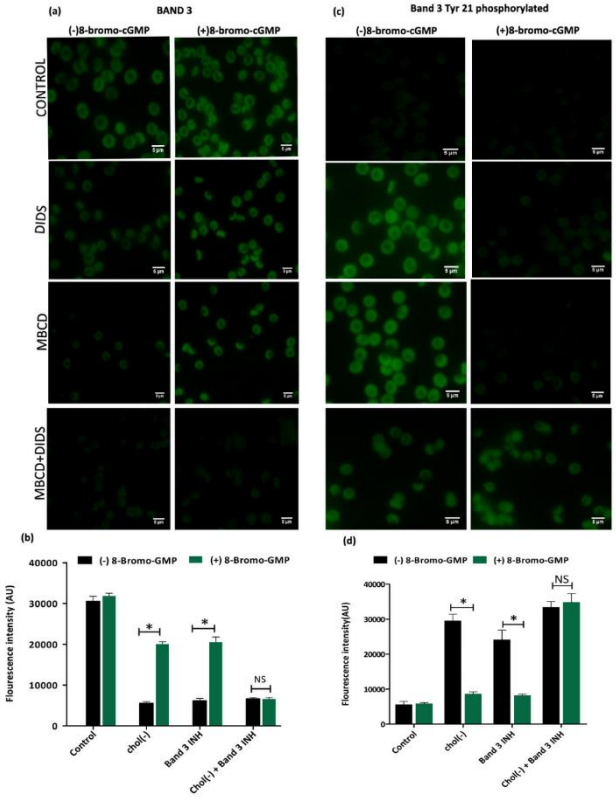

Figure 5. Immunofluorescence expression of band 3 and band 3 phosphorylation RBC: (a) Band 3 protein expression with and without 8-bromo-cGMP to band 3 inhibitor (DIDS), Cholesterol depleting agent (MBCD), a combination of band 3 inhibitor (DIDS) and cholesterol depleting agent (MBCD) treated RBC samples. (b) Tyrosine 21 phosphorylated of Band 3 protein expression with and without 8-bromo-cGMP treatment to control, band 3 inhibitor (DIDS), Cholesterol depleting agent (MBCD), the combination of band 3 inhibitor (DIDS) and cholesterol depleting agent (MBCD). (c)Graphical representation of the increase in band 3 expression in stressed RBC (*P<0.01) with 8-Bromo-cGMP (d). Graphical representation of decrease in Tyrosine 21 phosphorylated band 3 expression in stressed RBC (*P<0.01). On the other hand, when both stresses were combined, 8-bromo-cGMP did not change band 3 and Tyr 21 phosphorylated band 3 expression.

SEM analysis of Stressed RBCs and recovery by 8 bromo-cGMP

SEM analysis of RBCs under individual stress conditions, RBC-EC adhesion pattern, as well as recovered RBCs under the effect of 8-bromo-cGMP, was performed (Fig 6). Cholesterol depleted RBC in SEM micrographs showed a crenated RBC shape (Fig 6g-k). Interestingly at higher magnification, we observed the presence of ruptured pores in cholesterol-depleted RBC (figure 6k). Also, as depicted in figure 6g, h; we observed that RBCs adhere in the EC intercellular space in higher numbers (i.e., in the EC-EC junction). Comparing SEM micrographs of the control sample and stressed RBCs treated with 8-bromo-cGMP, we can ascertain the ability of 8-bromo-cGMP to recover NO and reverse the effect of stress on RBCs. figure (6l & 6m)

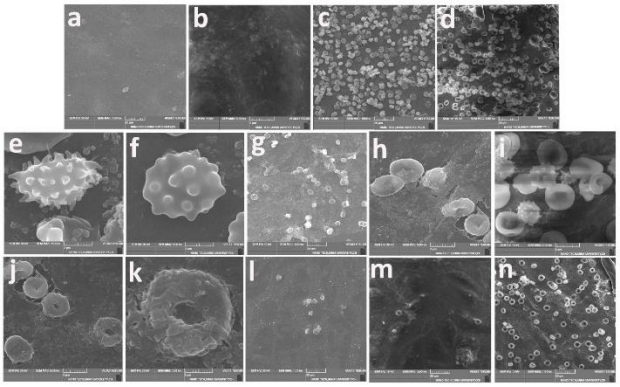

Figure 6. RBC-EC adhesion morphological imaging (Scanning electron microscopy; SEM) (a)-Control, (b)-8-Bromo-cGMP, (c-g)-Cholesterol depleted RBC Adhesion to EC, (h-k)-Band 3 inhibitor exposed RBC adhesion to EC, (l)-8 Bromo cGMP + cholesterol depleted RBC adhesion to EC, (m)-8 Bromo cGMP + Band 3 inhibitor exposed RBC adhesion to EC, (n)-8 Bromo cGMP + cholesterol depletion+ Band 3 inhibitor exposed RBC adhesion to EC.

RBCs are an important source of unesterified cholesterol as their membranes are cholesterol-rich (32,33). Imbalance in cellular cholesterol homeostasis is associated with various metabolic disorders (34,35). A higher concentration of cholesterol in the plaque may contribute to the development of acute coronary syndrome (ACS), while low cholesterol content in the red blood cell membrane may lead to a more gradual progression of the plaque size (36). In addition, an increase in blood cholesterol concentrations is closely associated with decreased RBC dysfunction (37).

There have been many studies highlighting the interaction between cholesterol and band 3 protein. Some reported that their interaction decreased band 3 channel activity (18,38). Similarly, another study revealed that anion transport decreases with cholesterol enrichment and increases with cholesterol depletion. Whereas other studies recorded the decrease in cholesterol promoting aggregation of band 3 which indirectly refers to impairment in band 3 function (39).

Previously we had reported that cholesterol-depleted RBC adheres to EC pertaining to stress caused by the removal of cholesterol in RBC (30). In our present study, we observed similar RBC- EC adhesion abnormality when RBCs were treated with higher concentrations of band 3 inhibiting agent (DIDS). This implicated that the absence of cholesterol and band 3 channel activity, promotes RBC dysfunction. Our results are in concurrence with previously reported findings and we show the reduction of band 3 channel activity in the cholesterol depleted RBC (i.e., in a higher concentration of cholesterol depleting agent (MBCD) as like other chemical treatments like band 3 inhibition and oxidative stress (15). From our investigation, it is evident that band 3 clustering in RBC promotes ferric methemoglobin (MetHb)as both stressors promote methemoglobin level (40), while NO donors were capable of suppressing it when these two stress-induced separately. With the combination, stressors caused irreparable damage in RBCs. Also, SEM analysis of cholesterol-depleted RBC showed a crenated RBC shape which indirectly depicts phosphatidylserine (PS) exposure in RBC and that could lead to increased susceptibility to the clearance of these cells (26,41,42). At higher magnification, we also interestingly noticed ruptured pores in cholesterol depleted RBC, indicating the capability of MBCD in rupturing RBC membrane (figure 6k). We also detected a higher proportion of adhered RBCs in the intercellular space between adjacent Endothelial cells (EC-EC cell junctions).EC junction controls vascular homeostasis by regulating immune cell permeability, extravasation, and infiltration (43). Accumulation of RBCs in this junction could cause serious damage to blood vessels, exacerbating vascular complications.

In earlier studies on the effect of hypertonic, ATP-depletion, sepsis, and oxidative stress on RBCs; RBCs showed an increase in band 3 tyrosine phosphorylation (44-46). The sites of tyrosine phosphorylation have been identified to occur commonly in all stress conditions and were determined to be residue 8 and 21 at the extreme N-terminus of the cytoplasmic tail of band 3 (47). It was also found that this region binds to several cytoplasmic proteins-such as aldolase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (47). Thus, there exists a correlation between the state of band 3 phosphorylation and RBC function. In our present study we found that in both cholesterols depleted and band 3 inhibited RBCs, band 3 protein is tyrosine 21 phosphorylated.

Nitric oxide (NO) is known to play a major role in cardiovascular regulation and has also been found to inhibit RBC aggregation and RBC-EC adhesion (48,49). Increased shear rate resulting from changes in blood flow is known to increase the production of NO in a shear stress-dependent manner (41). NO is highly reactive, and its half-life *in-vivo* is only a few seconds (even less in the bloodstream). However, the availability and significance of NO signaling molecules derived from erythrocytes, have gained attention in recent years (50). RBC was stimulated by NO in cardiovascular regulation but warrants further investigations in future studies. NO donors maintain RBC deformability evoked by external stressors. The lipid composition in the RBC membrane including cholesterol, sphingomyelin and phosphatidylcholine is reduced in diabetes mellitus 2 patients (T2D) (2). It also suppresses tyrosine phosphorylation of band 3 phosphorylation under hypoxic conditions (51).

However, major questions concerning the precise mechanisms by which NO activity modulates band 3 channel activity in RBC and the influence of cholesterol on this interaction, remain unanswered. In this view, we wanted to explore the role of NO donors (Sildenafil citrate, SpNO and 8-bromo-cGMP) in recovering band 3 channel activity and decreasing band 3 tyrosine phosphorylation and RBC-EC adhesion after cholesterol depletion in RBC. Sildenafil citrate, besides being known for its role as a NO donor (Shukla N et al., 2005) was known to affect RBC function by reducing RBC surface roughness and promoting hemolysis in higher concentrations (52). In our present study, it recovers RBC from cholesterol depletion and band 3 inhibition stress. Earlier studies on 8-Bromo-guanosine 3':5'-cyclic monophosphate (8-Br-cGMP), an analogue of cyclic guanosine monophosphate

(cGMP) in regulating RBCs Na+/H+ exchange. While Spermine NONOate (SpNO)belongs to the family of amine-based diazeniumdiolates (NONOate) reported to activate soluble guanylyl cyclase (sGC) and increase the time stored RBC lifespan (53,54). At lower concentrations, SpNO increased RBC deformability and facilitated RBC passage in narrow capillaries (55, 56).

We found that all 3 NO donors showed a similar recovering effect on stressed RBCs (i.e., with cholesterol depleting and band 3 inhibiting agents (DIDS and MBCD)). Yet, among the three NO donors, 8-bromo-cGMP was able to efficiently restore RBC band 3 function and NO production by inhibiting band 3 Tyr 21 phosphorylation and decreasing RBC-EC adhesion, MetHb level and increasing band 3 channel activity. Thus, we postulate that NO may modulate band 3 function by inhibiting its tyrosine 21 phosphorylation, in absence of cholesterol and further helping restore RBC function from stress. Overall, we conclude that NO donors might act as potential therapeutic agents in the treatment of hemolytic disorders associated with RBC dysfunction and vascular complications.

Conclusions

In summary, the current study showed the potential of NO donors, namely sildenafil citrate, 8 bromo-cGMP and SpNO in reversing the effects caused by cholesterol depletion and inhibition of band 3 channel activity in stressed RBCs. RBC deformability is indicative of microvasculature disorder and it is known from studies on Band 3 protein that NO has a compensatory role in improving RBC deformability possibly by reducing the crosslinking of Band 3. We thus tested the potential of the above-mentioned 3 NO donors in effecting the rescue of RBC deformation in RBCs that were exposed to 2 different kinds of stress-(depletion of RBC cholesterol levels, inhibition of band 3 channel activity and a combination of both). Our results indicate that all 3 NO donors were able to reverse the effects of just cholesterol depletion or band-3 channel inhibition, but RBC dysfunction remained irreversible in RBCs that were deformed by the combination of both stresses. Out of the 3 NO donors used in this study, 8-bromo-cGMP was found to be the most effective in recovering RBC dysfunction. The exact mechanism by which NO donors affect this recovery and their potential use as therapeutic agents need to be further validated.

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Interest conflict

The authors declare that they have no conflict of interest.

Author Contributions

Original draft preparation; Writing &Editing, MV-Data analysis, methodology, MV; Conceptualization, Validation, Investigation, Funding Acquisition, Project administration, SC, and TJ. Sample collection, PS. All authors reviewed the manuscript**.**

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Abbreviations

DIDS-4,4'-diisothiocyanato-2,2'-stilbenedisulfonate; DMEM- Dulbecco Modified Eagle medium; PBS-Phosphate buffer saline; FBS- Fetal Bovine Serum; SFM - Serum free medium (DMEM without FBS); SpNO-Spermine NONOnate; 8-Bromo-cGMP- 8-Bromoguanosine 3',5'-cyclic monophosphate; MBCD- Methyl-beta-cyclodextrin, MetHb-Methemoglobin, eNOS- endothelial nitric oxide synthase

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