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Research paper

Potential therapeutic action of nitrite in sickle cell disease

Nadeem Wajih[a,b], Swati Basu[a,b], Anuj Jailwala[a], Hee Won Kim[a], David Ostrowski[a], Andreas Perlegas[a], Crystal A. Bolden[a], Nancy L. Buechler[c], Mark T. Gladwin[d,e], David L. Caudell[f], Elaheh Rahbar[g], Martha A. Alexander-Miller[h], Vidula Vachharajani[b,c,**], Daniel B. Kim-Shapiro[a,b,*]

[a] Department of Physics, Wake Forest University, Winston-Salem, NC 27109, United States
[b] Translational Science Center, Wake Forest University, Winston-Salem, NC 27109, United States
[c] Department of Anesthesiology, Wake Forest University School of Medicine, Winston-Salem, NC 27157, United States
[d] Heart, Lung, Blood and Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA 15213, United States
[e] Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh, Pittsburgh, PA 15213, United States
[f] Department of Pathology-Comparative Medicine, Section on Rheumatology and Immunology, Wake Forest University School of Medicine, Winston-Salem, NC 27157, United States
[g] Department of Biomedical Engineering, Section on Rheumatology and Immunology, Wake Forest University School of Medicine, Winston-Salem, NC 27157, United States
[h] Department of Microbiology and Immunology, Section on Rheumatology and Immunology, Wake Forest University School of Medicine, Winston-Salem, NC 27157, United States

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ABSTRACT

Sickle cell disease is caused by a mutant form of hemoglobin that polymerizes under hypoxic conditions, increasing rigidity, fragility, calcium influx-mediated dehydration, and adhesivity of red blood cells. Increased red cell fragility results in hemolysis, which reduces nitric oxide (NO) bioavailability, and induces platelet activation and inflammation leading to adhesion of circulating blood cells. Nitric Oxide inhibits adhesion and platelet activation. Nitrite has emerged as an attractive therapeutic agent that targets delivery of NO activity to areas of hypoxia through bioactivation by deoxygenated red blood cell hemoglobin. In this study, we demonstrate anti-platelet activity of nitrite at doses achievable through dietary interventions with comparison to similar doses with other NO donating agents. Unlike other NO donating agents, nitrite activity is shown to be potentiated in the presence of red blood cells in hypoxic conditions. We also show that nitrite reduces calcium associated loss of phospholipid asymmetry that is associated with increased red cell adhesion, and that red cell deformability is also improved. We show that nitrite inhibits red cell adhesion in a microfluidic flow-channel assay after endothelial cell activation. In further investigations, we show that leukocyte and platelet adhesion is blunted in nitrite-fed wild type mice compared to control after either lipopolysaccharide- or hemolysis-induced inflammation. Moreover, we demonstrate that nitrite treatment results in a reduction in adhesion of circulating blood cells and reduced red blood cell hemolysis in humanized transgenic sickle cell mice subjected to local hypoxia. These data suggest that nitrite is an effective anti-platelet and anti-adhesion agent that is activated by red blood cells, with enhanced potency under physiological hypoxia and in venous blood that may be useful therapeutically.

1. Introduction

Sickle cell disease (SCD) is the most common genetic disease affecting about 2600 births a year in North America, while over 80% of patients are in Africa [1]. It is caused by a single mutation in the beta subunit of hemoglobin (glu → val) [2] that results in hemoglobin (Hb) polymerization under hypoxic conditions [3]. Polymerization distorts the shape of the red blood cell (RBC), a process known as sickling. It has

Abbreviations: Hb, hemoglobin; SCD, sickle cell disease; eNOS, endothelial nitric oxide synthase; L-NAME, L-NG-monomethyl arginine citrate; GSNO, S-nitrosoglutahthione; PRP, platelet rich plasma; RBC, red blood cell; DEANO, Diethylamine NONOate; HUVEC, human umbilical vein endothelial cells; LPS, lipopolysaccharide; WT, wild type; EPR, electron paramagnetic resonance; PBS, phosphate buffered saline; IVM, intravital microscopy; Hct, hematocrit; CFSE, Carboxyfluorescein succinimidyl ester; DI_{max}, maximum deformability index; Osm_{min}, minimum osmolality; Osm_{max}, maximum osmolality

* Correspondence to: Department of Physics, Wake Forest University, 208 Olin Physical Laboratory, Winston-Salem, NC 27109, United States.
** Corresponding author.
E-mail addresses: vvachhar@wakehealth.edu (V. Vachharajani), shapiro@wfu.edu (D.B. Kim-Shapiro).

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been shown that repeated cycles of sickling and unsickling lead to activation of the Gardos channel ($K_{Ca}3.1$), a calcium-activated potassium efflux channel, which is a major factor in RBC dehydration and reduced deformability and increased fragility [4,5]. Dehydration increases the intracellular concentration of Hb which enhances the rate of polymerization [6–8]. Red blood cell fragility results in hemolysis in SCD and other hemolytic diseases [9,10], diabetes, and transfusion of older stored blood; which in turn can lead directly to inflammation [11–13], and platelet activation [14–20]. There is abundant in vitro and animal data from multiple labs that support the notion that inflammation, cell adhesion, and platelet activation contribute to pathology in SCD [14,20–25], as well as other diseased conditions including atherosclerosis [26,27], diabetes [28], and sepsis [29–33]. Platelet activation can both initiate and accelerate inflammation as well as be induced by inflammation [26,27]. The role of platelet activation and its aggregation with neutrophils, in addition to adhesion of circulating blood cells to the endothelium, play major roles in pathology of SCD [25,34].

Since cell-free hemoglobin, released upon hemolysis, scavenges NO faster than that encapsulated in the RBC, low NO bioavailability contributes to pathology in sickle cell and other diseases [9,10]. Low NO bioavailability has been shown to promote platelet activation [35–37] and to increase cellular adhesion [38–40]. Inhibition of endothelial nitric oxide synthase (eNOS) with L-NG-monomethyl Arginine citrate (L-NAME) increased leukocyte rolling and adhesion [41]. In another study, blocking NO synthase led to an increase in platelet activation [42]. As a result, several different NO donating drugs have been explored as anti-platelet agents including NONOates, S-nitrosothiols, and organic nitrates [43,44]. Whereas organic nitrates have other vascular effects, S-nitrosoglutahthione (GSNO) has been shown to have some platelet specificity that has been demonstrated in clinical trials [45,46]. The potency of S-nitrosothiols and NO itself has been shown to be higher in platelet rich plasma (PRP) than when RBCs are present [20,47–49].

Although once considered to be biologically inert in human physiology in terms of production of NO activity [50], nitrite is now recognized as a storage pool for NO activity that is harnessed under hypoxia [51]. Resting plasma nitrite is at least hundreds times more abundant than steady state plasma NO [52,53] and, preferentially under hypoxia, produces effects characteristic of those attributed to NO through conversion to other NO congeners or NO itself [54]. Unlike other NO donors, nitrite was thought to be ineffective in limiting cytokines associated with circulating blood cell adhesion [55]. It is now widely accepted that nitrite can be bioactivated by red blood cell (RBC) hemoglobin [49,56–58]. Contrary to the case of NONOates and GSNO, Srihirun, Schechter and coworkers showed that in the presence, but not absence, of RBCs nitrite inhibits platelet activation, and this activity is potentiated in hypoxia and inhibited by NO scavenging agents [49]. Antiplatelet effects of nitrite have been demonstrated in rodent [59] and human studies [60,61]. In addition, nitrite in the drinking water of mice reduced leukocyte adhesion that was due to a high cholesterol diet [62]. Nitrite therapeutics has gained increased attention through the nitrate-nitrite-NO cycle where dietary nitrate is partially reduced to nitrite by oral bacteria, nitrate and nitrite are released into blood, and then nitrate is taken back into the mouth by salivary glands [53].

In this paper, we have studied several potentially beneficial effects of nitrite as a therapeutic for SCD. We examined the potency of nitrite in terms of anti-platelet activity compared to other NO donors in vitro and then examined the ability of nitrite to reduce platelet and leukocyte adhesion in inflammation, hemolysis and sickle cell murine models in vivo. We directly compare the anti-platelet activity of 1 μM concentration of each, GSNO, Diethylamine NONOate (DEANO) and nitrite since plasma nitrite levels go up to about 1 μM on average after consumption of high nitrate-containing foods or beverages due to reduction of nitrate to nitrite by commensal oral bacteria [53,63]. Thus, our use of 1 μM concentration tests the effects of nitrite at levels achievable after dietary intervention but at concentrations of NO and GSNO that are about 1000 times greater than normal physiological levels [52,63,64]. Nitrite was also shown to improve RBC properties following calcium leak. We also explored the ability of nitrite to inhibit RBC adhesion in a microchannel assay. Finally, the potency of nitrite observed in vitro is recapitulated in our in vivo murine models where we used intravital microscopy to assess the effect of nitrite feeding on platelet and leukocyte adhesion.

2. Materials and methods

2.1. Materials

Blood was obtained from volunteers after obtaining written informed consent with all procedures having been approved by the Institutional Review Board of Wake Forest University Health Sciences. Pac-1 and CD61 monoclonal antibodies were purchased from Becton Dickinson Immunocytometry systems. FITC Annexin V Apoptosis Detection Kit l catalogue number 556547 was purchased from BD Pharmingen. DEANO was purchased from Cayman Chemicals and prepared in 0.01 N NaOH before dilution into PRP. GSNO was prepared by combination of acidified nitrite and glutathione followed by precipitation and drying under vacuum and stored at −80 °C until use. Concentrations of GSNO were determined spectrophotometrically. The amount of NO released from DEANO was determined by measuring the amount of methemoglobin formed from oxygenated hemoglobin using electron paramagnetic resonance (EPR) spectroscopy in a Bruker EMX X-band spectrophotometer at a temperature of 5 K. Lipopolysaccharide (E coli O111:B4 LPS), sodium nitrite and all other chemicals were purchased from Sigma/Aldrich (St. Louis, MO). Calcium ionophore A23187, acid free catalogue number 1234 was purchased from TOCRIS. Human umbilical endothelial cells (HUVEC) were purchased from LONZA. Micro- slide y-shaped flow chamber slides were purchased from ibidi (Germany). The wild type (WT: C57Bl/6; 6–8 week old) and Townes (B6;129-$Hba^{tm1(HBA)Tow}$ $Hbb^{tm2(HBG1,HBB*)Tow}$/$Hbb^{tm3(HBG1,HBB)Tow}$/J: 6–8 week old) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The animal studies were approved by the Institutional Animal Care and Use Committee of the Wake Forest School of Medicine and experiments were performed according to the NIH guidelines.

2.2. Methods

2.2.1. In vitro platelet assays

Platelet and RBC preparations to measure platelet activation were performed as described previously [57,58]. After discarding the first sodium citrate vacutainer of drawn blood into sodium citrate tubes (a common practice to avoid basal platelet activation), the blood was centrifuged at 120*g* for 10 min to collect PRP. RBCs were washed with phosphate buffered saline (PBS, pH 7.4) and deoxygenated for experiments in hypoxia by gentle rocking under a closed atmosphere with positive nitrogen pressure. The oxygenation state of the RBC hemoglobin (Hb) in all experiments was determined by measuring the near infra-red absorbance using a Cary 100 Varian spectrophotometer equipped with an integrating sphere to collect scattered light and fitting to basis spectra as described previously [65]. PRP was diluted into PBS 1:7; when present RBCs were then added to a final hematocrit of 15%, and the freshly prepared NO donors (all at 1 μM final concentration) were added immediately and incubated for five minutes at 37 °C, after which ADP (1 μM) was added. This mixture was incubated for another six minutes and 10 μl was taken and mixed with Pac-1 and CD61 antibodies for 15 min at room temperature in the dark and then fixed in 1% buffered formalin. Platelet activation was quantified using a BD Bioscience FACS Calibur flow cytometer.

2.2.2. Platelet aggregation assay

Whole venous blood was used to measure platelet aggregation,

diluted 1:1 in isotonic sodium chloride with 3 mM sodium citrate. A Multiplate 5.0 Analyzer from diaPharma was used for platelet aggregation studies. Aggregation was induced by ADP (1 μM) after preincubation with NO donors (all at 1 μM final concentration) for five minutes. All these procedures were carried out at 37 °C.

2.2.3. Red Cell Deformability assay

Freshly drawn whole blood (2 ml) was deoxygenated for 30 min, treated with or without 20 μM calcium ionophore A23187, and with or without nitrite. It should be noted that normal serum already has about 2.5 mM total calcium. The concentrations of calcium and ionophores employed here were arrived at based on previous results and empirical attempts to produce measurable changes in deformability that are comparable to those observed in SCD [66] For samples treated with nitrite, after an initial bolus of 10 μM nitrite incubated for 10 min at 37 °C, 20 μM calcium ionophore A23187 was added, followed by continuous administration of 2 μl/min of a 1 mM nitrite solution using a Harvard syringe pump for 20 min at 37 °C in the dark with gentle rocking. For samples treated without nitrite, 20 μM ionophore A23187 was added and, instead of nitrite, PBS was continuously administered for 20 min at 37 °C in the dark with gentle rocking. Untreated deoxygenated whole blood was used as a control. During the continuous administration of nitrite and PBS, blood was kept degassed under nitrogen. All samples were placed on ice without touching the ice immediately following treatments. Aliquots of 150 μl were used to determine deformability parameters using the Ektacytometer (Technicon Instrument Corp.) as described previously (68).

Steady state concentrations for the nitrite treatments were calculated based on the final nitrite concentration at the end of the continuous administration. Red cells were immediately sedimented and the nitrite concentration was measured using a Sievers Nitric Oxide Analyzer (NOA) employing sodium iodide to reduce nitrite to NO as described by the manufacturer. The average steady-state nitrite concentration was calculated as the final nitrite concentration minus the initial concentration divided by two (thus assuming nitrite accumulation was linear from zero),

2.2.4. Phospholipid asymmetry assay

Calcium treatments for the phospholipid asymmetry assay were performed similarly. Washed RBCs at 35% hematocrit (Hct) in DPBS were deoxygenated for thirty minutes at room temperature. RBCs were pretreated with 10 μM nitrite for fifteen minutes at 37 °C. Calcium ionophore A23187 (10 μM) and 2 mM calcium were added to the RBCs. Nitrite was infused into the RBC suspensions at a rate of 2 μl/minute using the Harvard syringe pump with gentle rocking at 37 °C under dark for sixty minutes. During the continuous administration of nitrite, RBCs were kept degassed under nitrogen. RBCs were kept on ice after the reaction for annexin V FITC staining. Steady state nitrite concentrations were determined as described above for the deformability studies.

The annexin V binding assay was performed using a BD Pharmingen FITC Annexin V Apoptosis detection kit l as described by the manufacturer. Briefly, 50 μl of RBCs were taken out from the reaction mix and suspended in 500 μl of annexin V binding buffer. 10 μl of FITC labeled annexin V was added to the RBCs and incubated for 15 min at room temperature in the dark. The reaction was stopped by adding 480 μl of 2% buffered formalin.

2.2.5. Flow cytometry

Samples were analyzed on a Becton Dickinson FACSCalibur. Data acquisition and analysis were performed using CellQuestsoftware. Fifty thousand events per sample were acquired. The fluorescence and light scatter channels were set to logarithmic gain. RBC populations were gated by forward and side scatter plots. FITC positive RBCs were defined based on unstained RBCs.

2.2.6. In vitro microchannel flow assay

Flow through, μ-slide, y-shaped flow chamber slides were coated with 1 ng/ml human fibronectin. Two hundred μl of a HUVEC cell suspension at 3.5×10^5 cells were plated onto the fibronectin coated y-shaped flow chamber slides. HUVEC cells were grown in EGM-2 complete medium. Confluent HUVEC cells were treated with 10 ng/ml IL-1β for 48 h in EBM-2 medium containing 0.2% serum. After 48 h of IL-1β exposure, the flow chamber slides were removed from the tissue culture incubator and placed in another incubator for flow analysis at 37 °C. Chamber slides were hooked up to a MASTERflex L/S easy load II peristaltic pump. Slides were washed with warm PBS to remove any cell debris. Deoxygenated human RBCs (18% Hct) with and without 50 μM nitrite were flowed through the chamber at 1.5 ml/min for 15 min, corresponding to a shear stress of 3.4 dynes/cm^2. Slides were washed with PBS (two times the volume of RBCs). Washed cells were fixed with 2% buffered formalin. Throughout the experiment, temperature was maintained at 37 °C. Flow medium and RBCs were kept under nitrogen throughout the experiment to maintain deoxygenated conditions. Fixed cells were analyzed using a Nikon eclipse Ti microscope hooked up to a PCO edge CMOS camera and camera software. Captured images were further analyzed for RBC counts using imageJ (NIH) software with the cell counter plugin.

2.2.7. In vivo adhesion assays

2.2.7.1. Animals. This study was approved by the Institutional Animal Care and Use Committee of the Wake Forest School of Medicine and experiments were performed according to NIH guidelines. Mice were fed with either sodium nitrite, 100 mg/liter, for three weeks or tap water as indicated. In addition, mice in the nitrite-treated group also received 6.9 mg/kg intraperitoneal injection of sodium nitrite as a single dose 4 h prior to studying leukocyte/platelet adhesion. To study inflammation, mice were injected with 5 μg LPS or normal saline (vehicle for LPS) intraperitoneally as indicated. Hemolysis was induced with intravenous infusion of 150 μl pyrogen-free sterile water vs. control (normal saline 150 μl intravenous infusion) for 30 min as indicated. Townes (B6;129-Hba$^{tm1(HBA)Tow}$ Hbb$^{tm2(HBG1,HBB*)Tow}$/Hbb$^{tm3(HBG1,HBB)Tow}$/J) sickling, humanized transgenic sickle cell mice were divided into two separate groups, with and without nitrite treatment. The control group received no nitrite while treated mice underwent nitrite treatment as stated above.

2.2.7.2. Intravital microscopy (IVM). Mice were anesthetized with intramuscular administration of Ketamine (150 mg/kg) and Xylazine (7.5 mg/kg). Internal jugular vein and carotid artery were cannulated. Laparotomy was performed and the small intestinal loop was exteriorized. Mice were placed on the platform in a left semi-recumbant position and an exteriorized intestinal loop was placed on the fluid filled (normal saline) chamber; the intestinal loop was covered with a 25 mm round coverslip. In sickle cell mice, the mesentery was continuously superfused with bicarbonate buffered saline (pH 7.4; bubbled with gas mixture containing 5% CO_2 and balance nitrogen). The platform was placed under the fluorescent microscope for IVM. Five post-capillary (10–40 μm diameter; 200 μm length) venules were recorded after intravenous injection of Rhodamine 6 G (to label leukocyte) and Carboxyfluorescein succinimidyl ester (CFSE)-labeled platelets.

The platelet isolation-details are as outlined previously [29,67]. The platelets ($n = 100 \times 10^6$) were infused intravenously over 5 min (yielding < 5% of the total platelet count); allowed to circulate for 5 min prior to video-recording of the venules. These platelet isolation procedures are associated with no effect on the activity or viability of isolated platelets as previously demonstrated (22).

The 60 s recording of each post-capillary venule (n = 3–5/mouse; 4–6 mice per group) was analyzed for leukocyte/ platelet adhesion (quantified off line). A cell (leukocyte/platelet) was considered adherent if remained stationary for at least 30 consecutive seconds of the one

minute recording. The mean of the average values of leukocyte adhesion determined in each mouse were then used to generate a group mean.

2.2.8. Ex vivo measures of RBC nitrite, nitrate, and hemolysis

As described above, In the hemolysis studies, mice were either given a 21-day diet of 100 mg/L nitrite in drinking water in addition to 6.9 mg/Kg nitrite in normal saline 4 h prior to IVM, or just normal saline 4 h prior to IVM. To induce hemolysis, 150 µl sterile water was injected via left jugular cannula 30 min prior to IVM. Blood was collected immediately after the IVM experiment.

We subjected a separate group of WT mice for ex vivo measurement of hemolysis (n = 5 each group) and collected blood after induction of hemolysis vs. control; hemolysis was induced using 150 µl sterile water/normal saline via left jugular cannula 30 min prior to blood collection. These mice were used to measure hemolysis induced by water infusion.

For all blood measures in mice, blood was collected via the right carotid cannula in a heparinized syringe and centrifuged at 3000 rpm for 5 min. Plasma was collected and stored at −80 °C. Stored plasma was thawed and protein was precipitated by treatment with an equal volume of 100% methanol, centrifuged at 13,000 rpm for 10 min and the supernatant was analyzed for nitrite and nitrate concentrations using ENO-20 Nitric Oxide Analyzer (Eicom, USA, San Diego CA). Freshly made nitrite and nitrate standards were also measured.

Hemolysis of plasma samples from mice was determined by absorption spectroscopy using a Cary 50 spectrophotometer (Agilent technologies, CA) in a 1 mm pathlength cuvette. Concentration of free hemoglobin in plasma was calculated using the extinction coefficient $\varepsilon = 14.6\ mM^{-1}\ cm^{-1}$ at A_{577}.

2.2.9. Statistics

Significance of differences between treatments were evaluated using two-tailed *t*-tests. Significance was set at $p < 0.05$.

3. Results

3.1. Nitrite is a potent anti-platelet agent that is bioactivated by RBCs in hypoxia

In all experiments, we used 1 µM ADP to activate platelets and 1 µM of each of the NO donors. Using EPR, we found that 1 µM DEANO produced 0.9 ± 0.5 µM NO (half-life of 2 min). In examining the concentration dependence of GSNO and DEANO on platelet inhibition we found that 1 µM was more effective than lower concentrations tested ($P < 0.01$ compared to 0.1 µM, Fig. 1A,B). Higher concentrations of GSNO and DEANO did not further improve antiplatelet activity ($P > 0.1$ compared to 5 µM, Fig. 1A,B). Effects of each NO donor agent are shown in Fig. 1C-F for conditions in the presence and absence of RBCs (15% Hct, similar to microcirculatory levels). Under normoxic conditions and in the absence of RBCs, GSNO and DEANO both significantly reduced platelet activation whereas nitrite had no effect (Fig. 1C). Similarly, under hypoxia and in the absence of RBCs, nitrite had no effect on platelets while GSNO and DEANO significantly inhibited platelet activation (Fig. 1D). When RBCs were added and platelet inhibition was evaluated under normoxic conditions, nitrite still had no effect, but both GSNO and DEANO still inhibited platelet activation, although to a smaller extent than when RBCs were absent (Fig. 1E and compare to Fig. 1C). However, when platelet inhibition was evaluated under hypoxic conditions, all NO donors had an effect including nitrite (Fig. 1F). In these experiments, RBC deoxygenation initially produced 85 ± 12% deoxygenated Hb that fell to 74 ± 12% at five minutes (after mixing with PRP) and was measured to be 76 ± 5% after 15 min. Platelet inhibition by nitrite in the presence of RBCs under hypoxic conditions was significantly greater than inhibition by GSNO ($p = 0.01$) and similar to that of DEANO ($p = 0.3$). These data show that whereas platelet inhibition by DEANO and GSNO is blunted by RBCs, the presence of RBCs is necessary for nitrite-dependent inhibition of platelet activation; making nitrite potentially more suitable for in vivo physiologic/pathophysiologic conditions (where RBCs are present) in the microcirculation. Moreover, the effect of nitrite is seen here only in hypoxic conditions. These data thus strongly support our original hypothesis that nitrite is bioactivated by deoxygenated Hb [56].

In order to further evaluate the relative efficacy of nitrite among NO donors, we measured its effects on platelet aggregation in whole blood. The percentage deoxygenation of the venous blood used was measured to be 47 ± 12%. Fig. 2A-D show representative raw tracings of platelet aggregation measured by impedance using Multiplate®. The data show a reduction in platelet aggregation in response to ADP (~10–17%) with NO donors compared to control (whole blood). The average total platelet aggregation is illustrated in Fig. 2E. Nitrite is seen to be as effective as GSNO and DEANO in inhibiting platelet aggregation in whole blood.

3.2. Nitrite effects on RBC properties after calcium influx

Cycles of sickling and unsickling lead to calcium leakage which activate the Gardos channel resulting in potassium efflux and cellular dehydration [68]. Dehydration decreases RBC deformability and increases HbS polymerization due to increased mean corpuscular hemoglobin concentration [69]. In addition, calcium leak leads to membrane lipid asymmetry with exposure of phosphatidylserine on the surface of RBCs which contributes to increased adhesivity of sickle RBCs [70,71]. These same phenomena can be recapitulated in normal RBCs using calcium ionophores [66,70,72]. Thus, inhibiting the Gardos channel has been a major therapeutic target in treating SCD as it would improve RBC deformability, decrease RBC adhesion, and decrease sickling [73].

We have previously shown that a single bolus addition of 10 µM nitrite trends to blunt calcium-influx associated loss of RBC deformability during a 3–4 h time-course of cycles of sickling and unsickling [66]. However, a single 10 µM dose is very small compared to what is achievable in vivo through a dietary nitrate intervention where steady-state plasma nitrite reaches about 1 µM over several hours [74]. Assuming an average rate constant of $1\ M^{-1}\ s^{-1}$ for the reaction of nitrite with Hb [75], the half-life of nitrite in blood would only be about two minutes. Thus, for the studies shown in Fig. 3, we administered nitrite at a steady rate with goal of maintaining a steady-state concentration that mimics that achievable through dietary nitrate intervention. The steady state nitrite concentration achieved for these studies was calculated to be 5.7 ± 1.7 µM based on measured values at the end of the experiment (see Section 2.2). Thus, the nitrite concentration was higher than what we were aiming for (1 µM), but only by a factor of about 6 and still below the 10 µM. Fig. 3A shows the deformability profile for RBCs from a healthy volunteer plotted as a function of osmolality. Major parameters [76] are illustrated including the maximum deformability index (DI_{max}) which is related to membrane stiffness, the minimum osmolality (Osm_{min}) which is the osmolality where the minimum deformability on the hypotonic occurs and is related to the initial surface to volume ratio at critical hemolytic volume, the maximum osmolality (Osm_{max}) which is the osmolality where DI_{max} occurs and is related to the cellular hydration, and O′ which is the osmolality where $DI = ½DI_{max}$ on the hypertonic arm and is related to the cellular density. Fig. 3B shows representative deformability profiles using normal RBCs in which calcium and calcium ionophore A23187 is used to mimic calcium-influx mediated RBC dehydration that occurs in SCD and the effects of nitrite treatment. It is seen that nitrite treatment blunts the effects of calcium influx on RBC deformability. Fig. 3C and D show the average DI_{max} and O′ while Fig. 3E and F show the average Osm_{min} and Osm_{max} with and without calcium ionophore ± nitrite treatments (n = 6). In all deformability parameters, there was a significant improvement upon treatment with nitrite except Osm_{max}, which showed a trend towards improvement.

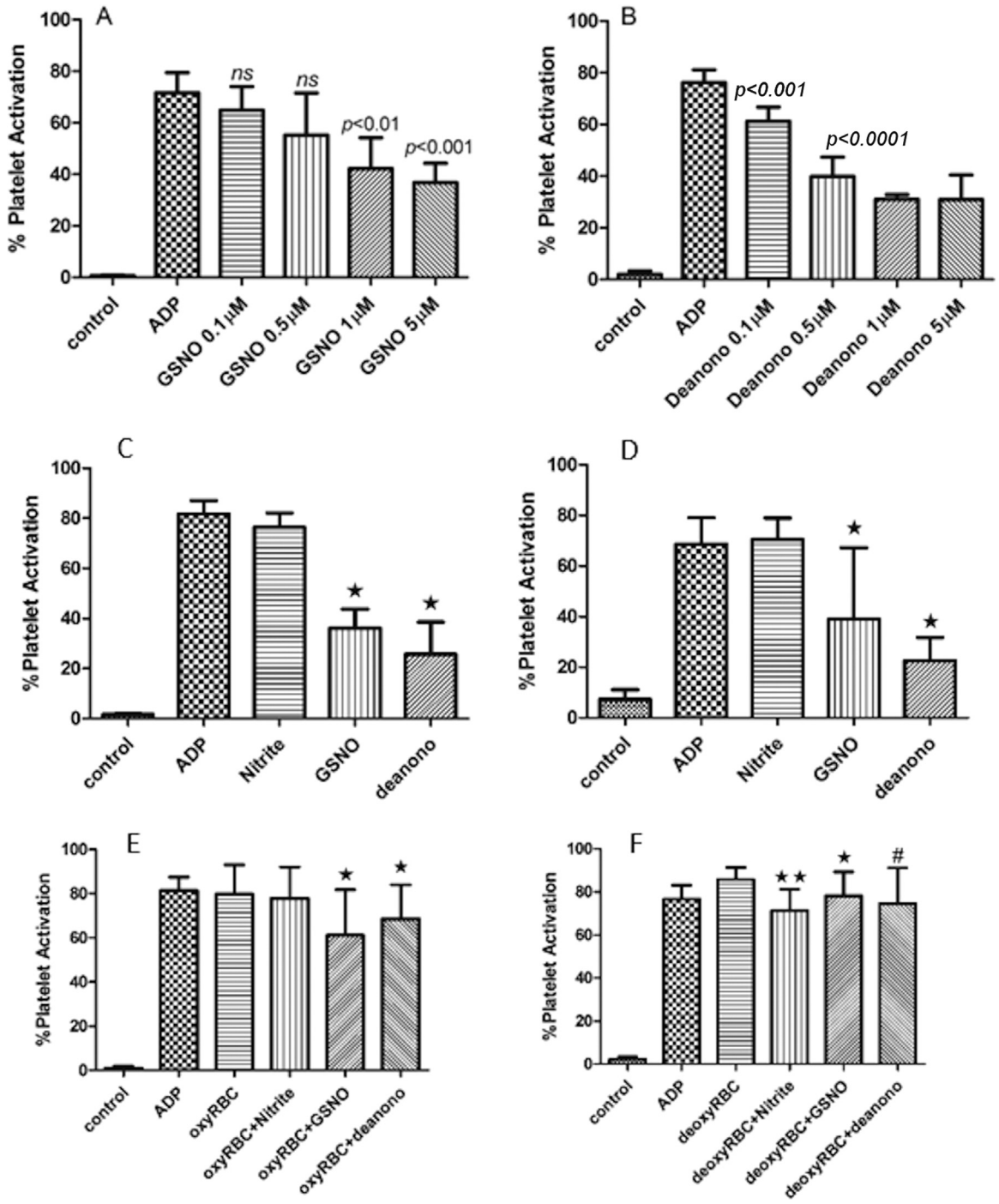

Fig. 1. Comparative analysis of NO donors on platelet activation. All measures were made on samples where ADP was added to induce platelet activation except in the controls. (A) GSNO dose response curve. GSNO at 1 and 5 μM significantly inhibits platelet activation compared to PRP + ADP. p value for 1 μM is $p < 0.01$ and for 5 μM $p < 0.001$ compared to PRP + ADP, n = 3 (B) DEANOate dose response curve. DEANONOate significantly inhibited platelet activation compared to PRP + ADP. Respective p values for DEANOate are 0.1 μM $p = 0.0005$, 0.5 μM $p < 0.0001$, 1 μM p < 0.0001 and for 5 μM $p = 0.0001$, n = 3. In panels C-F, effects of platelet inhibition of 1 μM of each NO donor was evaluated following activation with 1 μM ADP. (C) Normoxia PRP. PRP was equilibrated with air and platelet activation was evaluated in the absence of red blood cells (*$p < 0.05$ vs PRP + ADP, n = 3). (D) Hypoxia PRP. PRP was equilibrated with nitrogen and platelet activation was evaluated in the absence of red blood cells (*$p < 0.05$ vs PRP + ADP, n = 3). (E) Normoxia RBCs with PRP. Red blood cells were equilibrated with air and mixed with normoxic PRP to final Hct of 15% and platelet activation was evaluated (*$p < 0.05$ vs RBC + ADP, n = 4). (F) Hypoxia RBCs with PRP. Red blood cells were deoxygenated (oxygenation between about 15% and 25%, see text for details). These RBCs were mixed with PRP and platelet activation was evaluated. (**$p < 0.008$ vs RBCs + ADP, *$p < 0.02$ vs RBC + ADP and vs RBCs + ADP + nitrite, #$p < 0.05$ vs RBC + ADP, n = 4).

Calcium influx into the cell is also known to induce surface exposure of phosphatidylserine which has been associated with increased adhesivity of sickle RBCs [70,71]. Fig. 4 shows that nitrite treatment reduces calcium-influx mediated loss of phospholipid asymmetry. Panels A-D show representative data from the different treatments. Side and forward scatter gating was used to identify the RBC population. The R2 gate shows positive annexin V labeling. No positive staining cells were observed in the absence of annexin V (Panel A) and only a limited number of cells stained positive at baseline, i.e. in the absence of treatment (Panel B). When the ionophore was present, RBC labeling with annexin V was greatly increased indicating surface exposure of phosphatidylserine (Panel C) which is blunted by nitrite

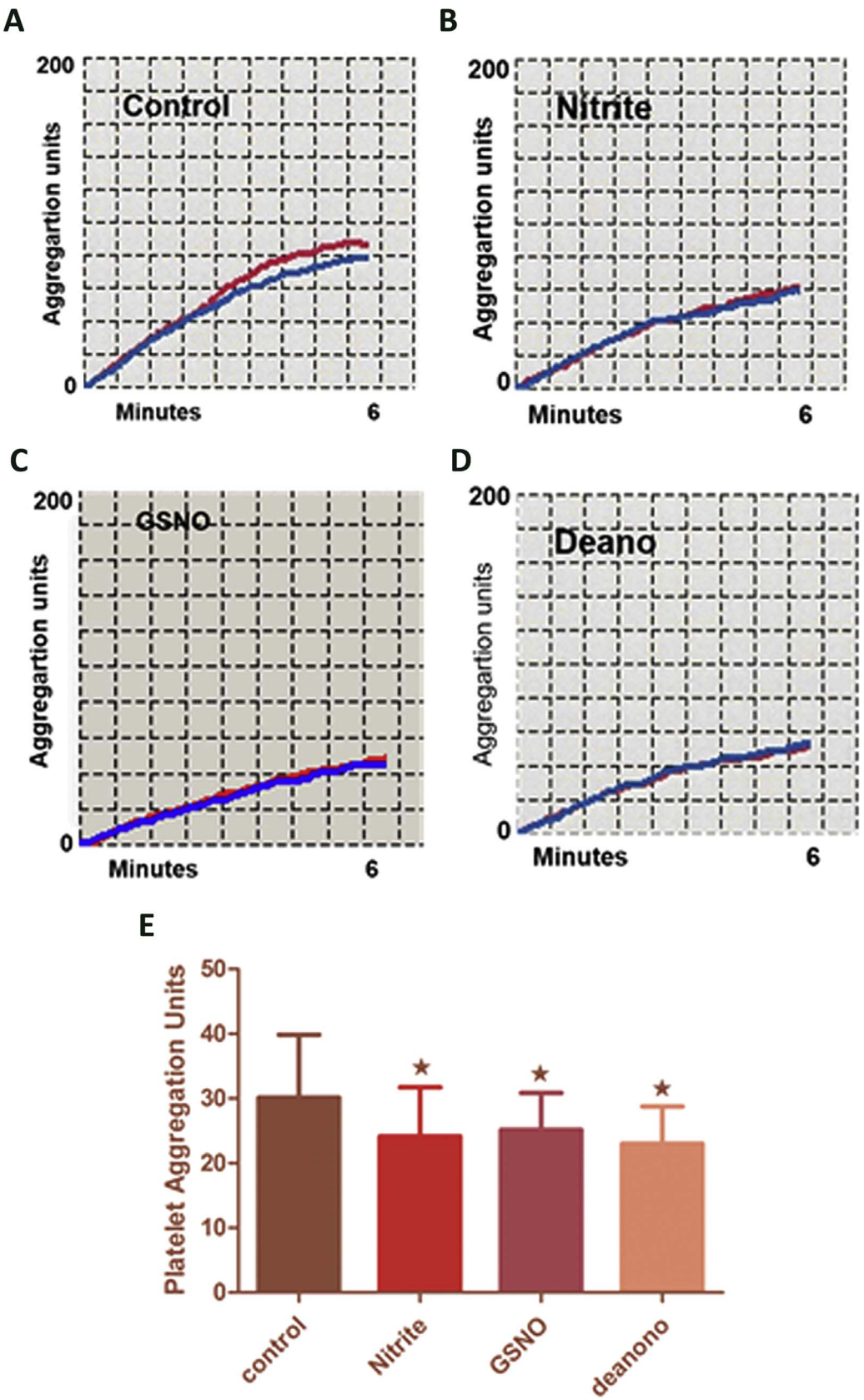

Fig. 2. Comparative analysis of NO donors on whole venous blood. Effects on platelet aggregation of 1 μM of each NO donor was evaluated except in control following aggregation with 1 μM ADP. (A-D) Representative traces from whole the blood aggregation assay plotted in arbitrary units based on impedance. Red and blue are from two separate electrodes. (A) Control; (B) Nitrite; (C) GSNO and (D) Deanonoate. Total aggregation time was six minutes. (E) Average aggregation for each condition calculated as the area under the curve over six minutes. (*$p < 0.05$ compared to control, n = 4). Error bars represents ± SD.

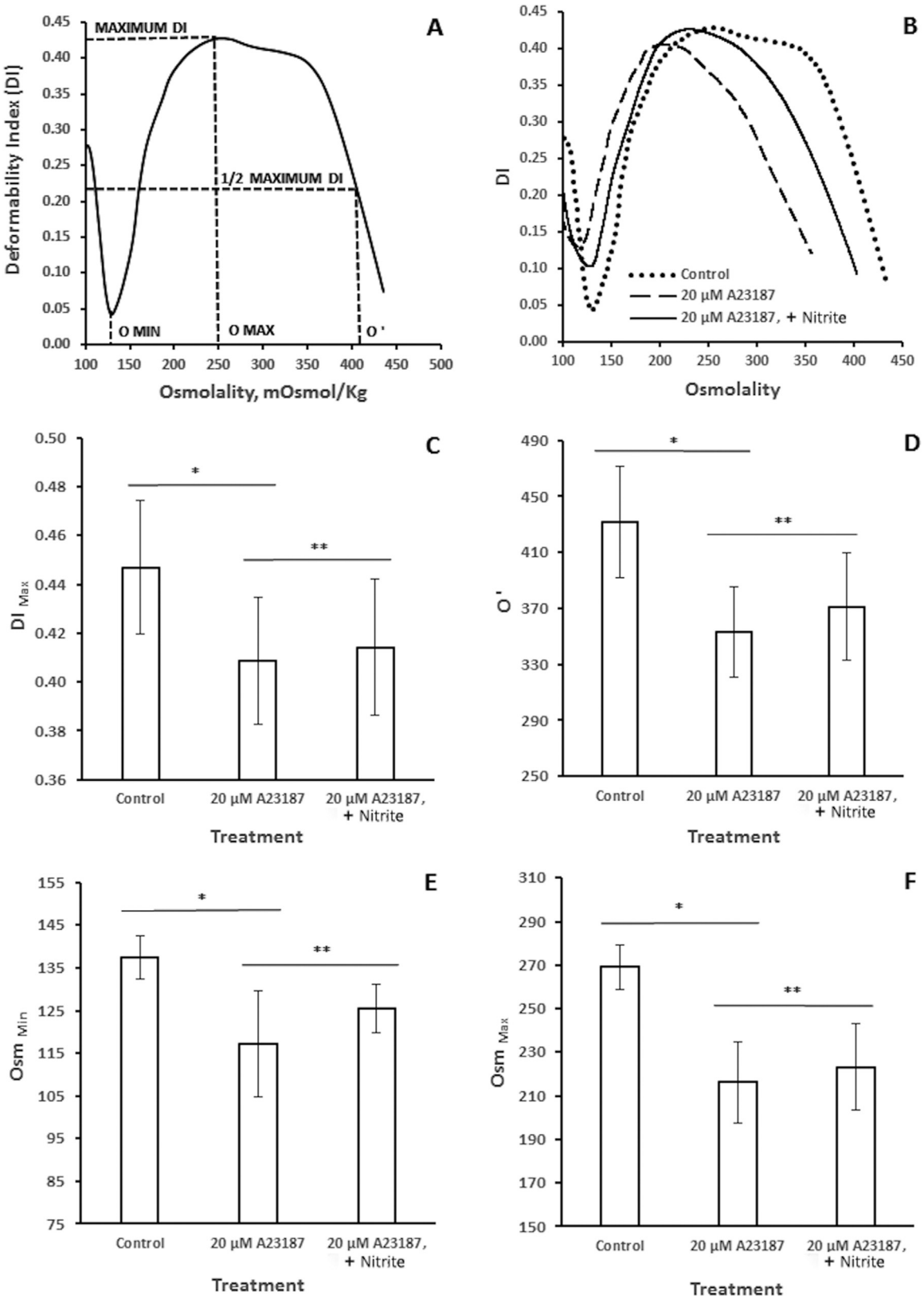

Fig. 3. Effect of nitrite on deformability of red blood cells treated with calcium ionophore with or without nitrite. Whole blood was treated with 20 µM calcium ionophore A23187, followed by continuous administration of PBS with or without nitrite (n = 6). Results are an average of 6 repeats, each repeat was done using blood from a different healthy volunteer. A. Deformability profiles of normal red blood cells, showing parameters, DI_{max}, O′, Osm_{min} and Osm_{max}, measured by ektacytometry. B. Representative traces of deformability of red blood cells from untreated whole blood (Control) and red blood cells of whole blood treated with 20 µM A23187 calcium ionophore with or without infused nitrite. C. Differences in DI_{max} in red blood cells from untreated whole blood or whole blood treated with 20 µM A23187 calcium ionophore with or without infused nitrite (*$p = 0.0003$, ** $p = 0.02$). D Differences in O′ in red blood cells from untreated whole blood or whole blood treated with 20 µM A23187 calcium ionophore with or without infused nitrite (*$p = 0.00004$** $p = 0.004$). E. Differences in Osm_{min} in red blood cells from untreated whole blood or whole blood treated with 20 µM A23187 calcium ionophore with or without infused nitrite (*$p = 0.006$, ** $p = 0.04$). F. Differences in Osm_{max} in red blood cells from untreated whole blood or whole blood treated with 20 µM A23187 calcium ionophore with or without infused nitrite (*$p = 0.0001$, ** $p = 0.08$). Nitrite significantly improved the profiles of deformability parameters as indicated, except for Osm_{max} which trended towards improvement,.

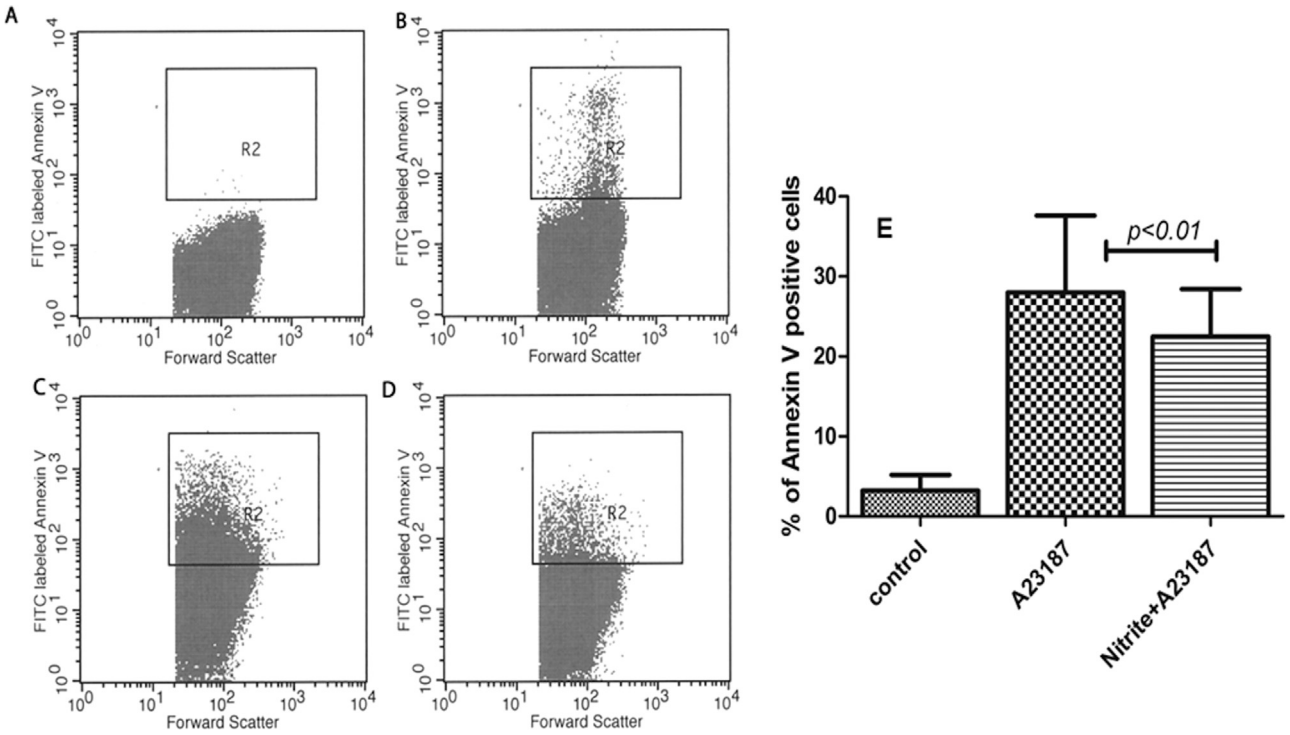

Fig. 4. Effect of nitrite on surface exposure of phosphatidylserine. RBCs were exposed to calcium using a calcium ionophore in the presence or absence of an average steady state nitrite concentration of 3.4 μM. Figures A-D show representative dot plots generated by flow cytometric analysis of FITC labeled annexin V binding: (A) Unstained control RBCs, (B) Annexin V binding to the control RBCs, (C) Annexin V binding of RBCs treated with ionophore A23187 and 2 mM calcium and (D) Annexin V binding of RBCs treated with ionophore A23187, 2 mM calcium and nitrite. (E) Bar graph showing the average percentage of annexin V labeled RBCs when untreated or treated with ionophore in the presence or absence of nitrite. Nitrite significantly decreased phospholipid asymmetry ($p = 0.01$, $n = 4$).

(Panel D). The continuous administration of nitrite resulted in a steady-state nitrite concentration of 3.4 μM. Averaged data is shown in panel E. These data show treatment with nitrite blunted the surface exposure of phosphatidylserine in deoxygenated normal RBCs induced by treatment with calcium and a calcium ionophore for sixty minutes.

3.3. Nitrite attenuates RBC adhesion to activated endothelial cells in a flow channel assay

As an initial step in characterizing the potential effect of nitrite on adhesion of circulating blood cells, we employed a microfluidic channel assay. An inflammatory mediator was used to activate confluent endothelial cells, and partially deoxygenated RBCs were passed through the channel. Adhesion was monitored after fixation using differential interference contrast microscopy. Fig. 5A and B show representative micrographs of adherent RBCs to activated HUVEC cells with and without nitrite treatment (50 μM). Nitrite substantially reduced the number of adherent RBCs as summarized in Fig. 5C.

3.4. Nitrite treatment attenuates cell adhesion with hemolysis and inflammation

Using an intravenous sterile water infusion model of hemolysis in WT mice as reported previously [77], we show significant increases in the leukocyte and platelet adhesion in the mesenteric microcirculation in mice with hemolysis vs. mice without hemolysis (Fig. 6). Water infusion resulted in 123 ± 43 μM plasma Hb (in heme) compared to 17 ± 6 μM Hb with saline infusion ($p < 0.0001$). Our nitrite treatment did not significantly increase plasma nitrite compared to control but nitrate was measured to be significantly higher, 37 ± 9 μM vs 25 ± 9 μM ($p < 0.05$). We studied the effect of nitrite treatment on hemolysis-induced cell adhesion. As shown in Fig. 6, hemolysis increased leukocyte and platelet adhesion in the mesenteric microcirculation of mice; moreover, when treated with nitrite prior to hemolysis, we observed a significant decrease in leukocyte and platelet adhesion vs. hemolysis without treatment (Fig. 6).

We also studied the effect of nitrite treatment on microvascular dysfunction during inflammation. We measured mesenteric leukocyte and platelet adhesion in response to intraperitoneal LPS administration in mice with and without sodium nitrite treatment. Again, plasma nitrite was not significantly higher in the plasma of nitrite treated animals at collection compared to controls (0.37 ± 0.04 μM vs 0.34 ± 0.05 μM ($p = 0.2$)), but plasma nitrate levels were significantly higher (61 ± 17 μM vs 34 ± 12 μM ($p < 0.05$)). As shown in Fig. 7, we demonstrate that the leukocyte/platelet adhesion in mesenteric microcirculation is increased with LPS stimulation vs. control. Importantly, nitrite treatment significantly attenuated leukocyte and platelet adhesion in response to LPS stimulation significantly.

3.5. Nitrite treatment attenuates hemolysis and circulating blood cell adhesion in sickle cell model mice

The sickle cell mouse model developed by Townes and colleagues [78] demonstrates pathology similar to that of patients with SCD including increased inflammation [78,79]. We treated some of these mice with nitrite as described above and compared results using untreated mice. As in other nitrite interventions described here, plasma nitrite only trended to be higher than that in nitrite treated animals (1.4 ± 2.2 μM ($n = 5$) vs 0.17 ± 0.05 μM, $p = 0.22$, $n = 3$) but plasma nitrate was significantly higher in the nitrite treated group (52 ± 4 μM ($n = 5$) vs 11 ± 2 μM, $p < 0.00001$, $n = 3$). We examined leukocyte and platelet adhesion in the mesenteric venules with and without nitrite treatment using IVM (Fig. 8A, B). During the measurements, the mesenteric bed was immersed in a bath that was bubbled with 95% N_2 and 5% CO_2 to create some degree of local hypoxia. Substantial adhesion (comparable to what is observed in the inflammation and hemolysis models discussed above) was observed but this was dramatically reduced by nitrite treatment (Fig. 8A, B). In addition, we

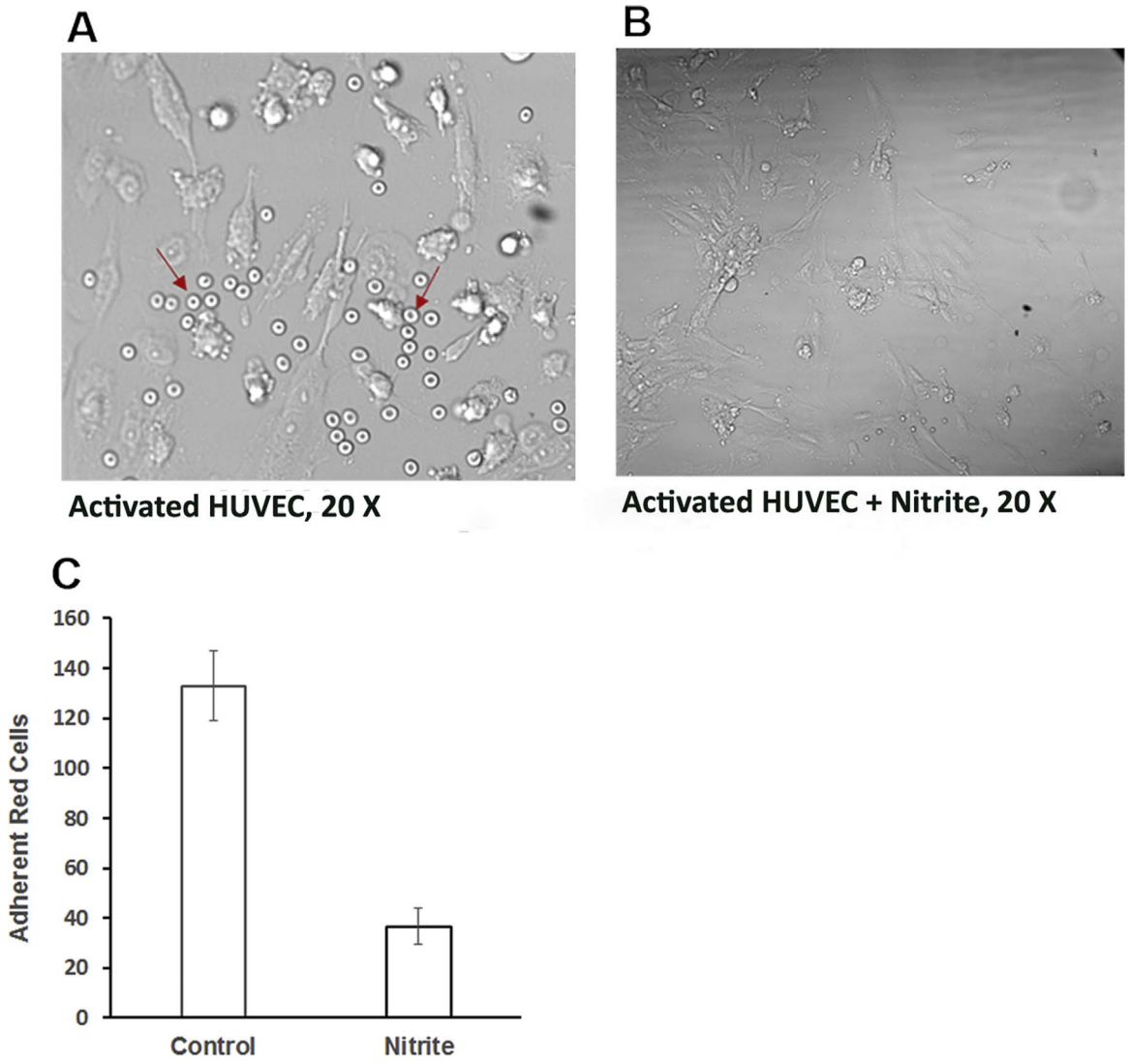

Fig. 5. Microfluidic channel red cell adhesion. Confluent HUVEC cells, grown on a y-shaped flow microchannel were treated with 10 ng/ml ILβ1 for 48 h. Deoxygenated human RBCs (72% deoxy) with and without (control) 50 μM nitrite were flown through the chamber with shear stress of 3.4 dynes/cm^2 for 15 min at 37 °C in a nitrogen atmosphere. Slides were washed and fixed. Captured images in our Nikon microscope were analyzed for RBC counts by imageJ software. (A) Activated HUVEC arrows show the RBCs adhere to the HUVECs. (B) Activated HUVECs exposed to RBC and Nitrite. (C) Average number of RBCs counts from four different fields under the microscope from three different experiments on three different days, P < 0.0001.

collected blood from the mice after the experiments and measured cell-free Hb in the supernatant. Fig. 8C shows representative absorption spectra and Fig. 8D shows the average hemolysis in saline treated vs nitrite treatment, with nitrite treatment resulting in lower hemolysis (0.04 ± 0.01 mM (n = 5) vs 0.018 ± 0.008 mM (n = 3), $p = 0.01$).

4. Discussion

Sickle cell disease is primarily caused by a mutation in Hb that results in Hb polymerization with downstream pathological effects including poor RBC deformability, elevated platelet activity, increased red cell adhesivity and increased adhesion of leukocytes and platelets in the context of inflammation [80–82]. In addition, polymerization leads to increased RBC fragility which releases cell-free Hb that decreases NO bioavailability [9,10]. In 2003, we and others first suggested that nitrite is reduced to NO by RBC deoxygenated Hb leading to downstream NO-related activity [56]. A major question remains regarding how NO itself can escape rapid scavenging by Hb [83]. Nevertheless, the fact that RBCs are needed for nitrite-mediated inhibition of platelet activation, as shown in Fig. 1 and previously [49], and nitrite-mediated inhibition of platelet activation is potentiated in hypoxia and abrogated in the presence of an NO scavenger [49,58] strongly supports the deoxyhemoglobin/nitrite hypothesis. In this paper, we show that, in partially deoxygenated conditions, nitrite is as or even more effective than other known NO-based anti-platelet agents. Nitrite also blunts calcium-induced damage to RBCs. In addition, we show that nitrite bioactivation is observed in terms of blunting platelet and leukocyte adhesion in a mouse model of inflammation and hemolysis. Finally, we demonstrate reduction in adhesion of these blood cells and in RBC hemolysis as a result of nitrite treatment in humanized transgenic sickle cell mice.

Our data show that nitrite inhibits platelet activation preferentially under hypoxic conditions while still being effective in inhibiting platelet aggregation at oxygen pressures of mixed venous blood. Nitrite is seen to be as or more effective as an antiplatelet agent as GSNO and DEANO at a concentration of nitrite achievable after consumption of high nitrate foods and beverages, but 1000 times greater than physiological concentrations of GSNO and NO [52,63,64]. These results are truly remarkable when one considers that, from a simple kinetic analysis, all or most of the NO produced from nitrite should be scavenged in the RBC by oxygenated Hb [83]. Although the effect of nitrite on platelet activation seen in Fig. 1F is statistically significant, it may appear modest from a clinical perspective. It should

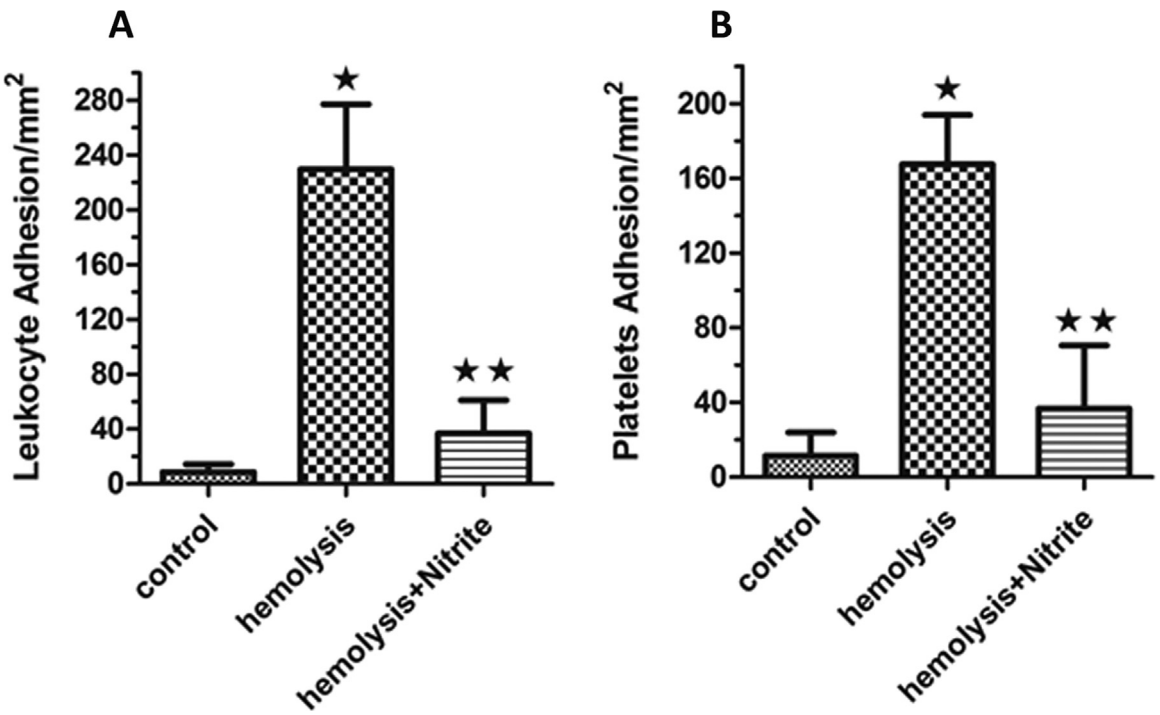

Fig. 6. Nitrite treatment and cell adhesion with hemolysis. Leukocyte and platelet adhesion in mesenteric microcirculation significantly increased in hemolysis vs. control mice; nitrite treatment significantly decreased leukocyte and platelet adhesion with hemolysis. n = 5 for all three groups.

be noted that these experiments were conducted to compare the different agents under the same doses which were chosen to mimic steady state nitrite dose achievable after drinking beet root juice and doses that are at the saturation level of other donors. However, it is important to remember that in vivo, 1 μM is a steady state nitrite concentration – not a bolus one as used in Fig. 1F. Other studies have shown larger effects of nitrite on platelet activity given at higher doses for various platelet activators [49,84–86].

We have previously shown that a single bolus addition of 10 μM nitrite trended to blunt loss of deformability due to cycles of sickling and unsickling [66]. This action was attributed to inhibition of the Gardos channel so that calcium still entered the cell, but potassium was not exported and dehydration was blunted [66]. As described above, a single dose of 10 μM is very low for a three-hour time course of sickling and unsickling due to the fast consumption of nitrite by deoxygenated RBCs. Thus, we explored the effects of nitrite on Gardos channel inhibition at concentrations mimicking those achievable with dietary nitrate intervention, about 1 μM steady state [74]. In this study, results of deformability assays indicate that nitrite continuous administration (maintaining 5.7 ± 1.7 μM nitrite at steady state) after calcium iono-

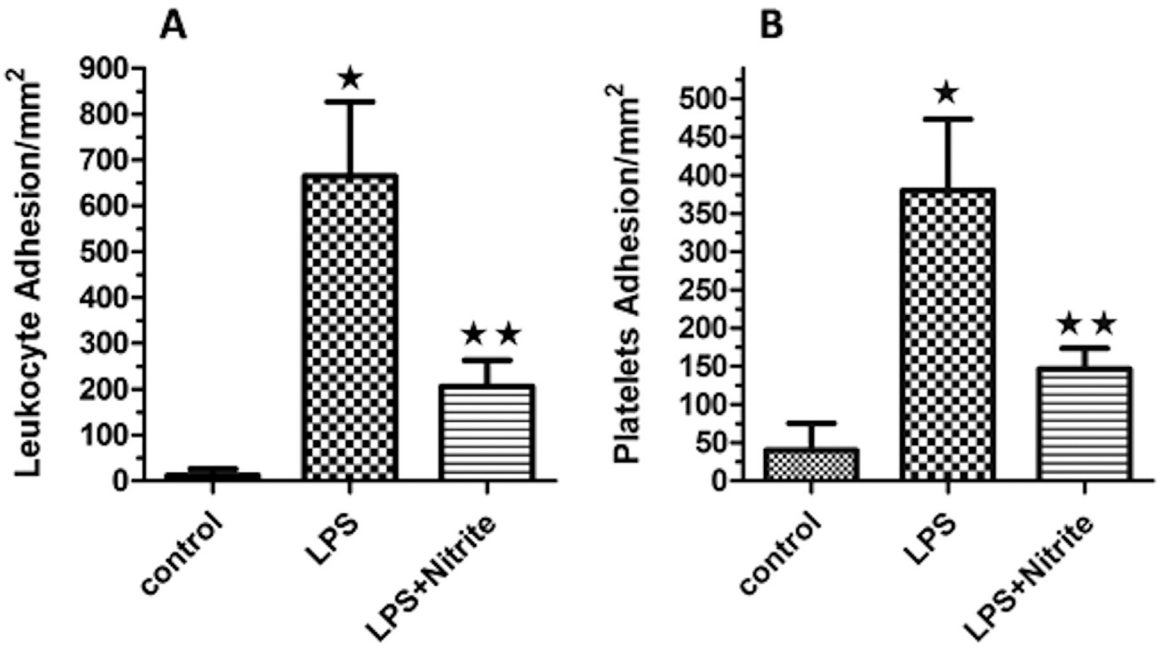

Fig. 7. Nitrite treatment attenuates leukocyte and platelet adhesion in mesenteric microcirculation after LPS-induced inflammation. Leukocyte (left) and platelet (right) adhesion in mesenteric microcirculation were significantly increased with LPS vs. Control. Leukocyte and platelet adhesions in LPS + Nitrite groups were significantly lower vs. LPS alone. n = 5–6 as indicated.

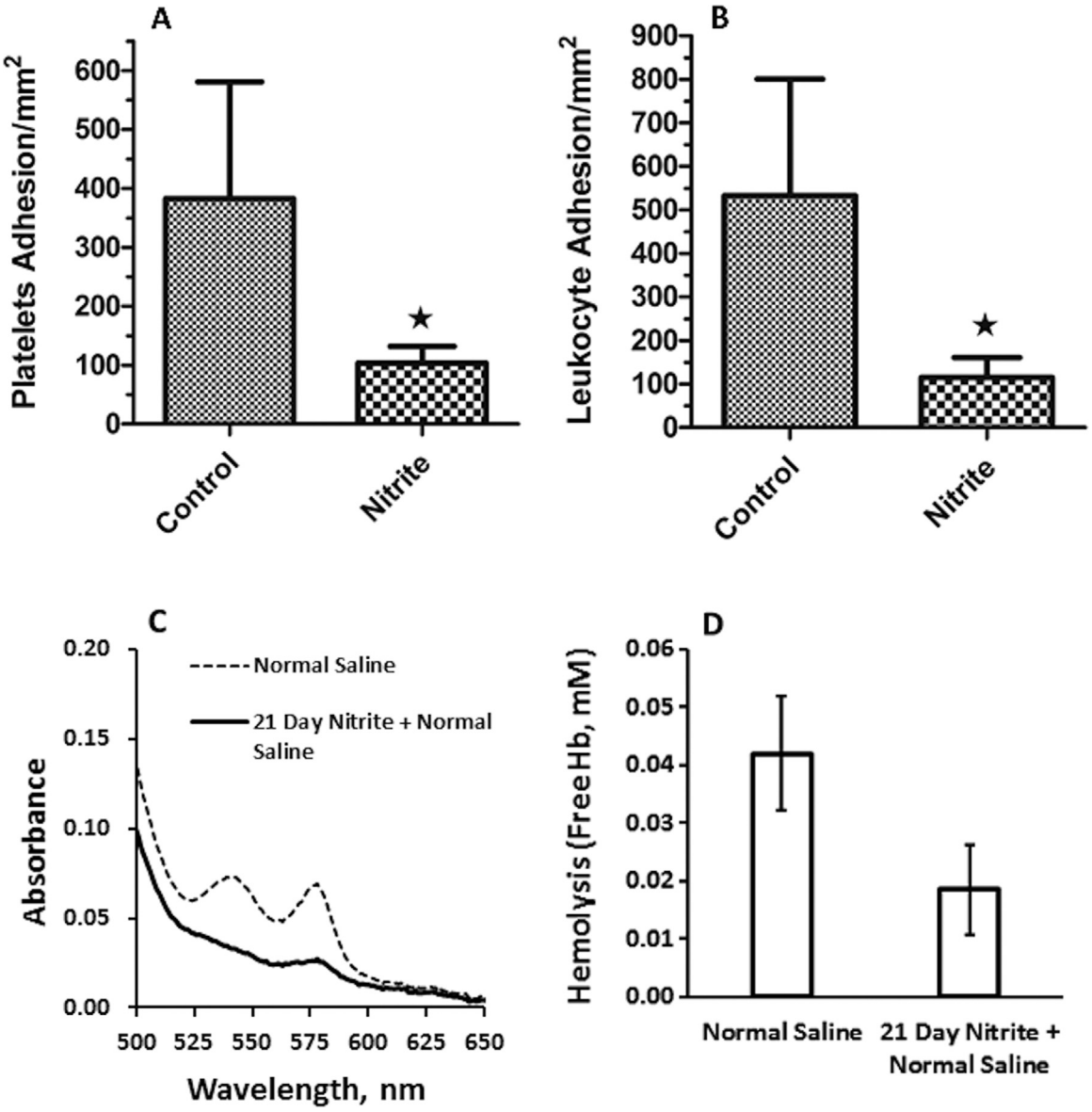

Fig. 8. Nitrite effects in sickle cell model mouse studies. Effect of nitrite on adhesion in sickle cell mice (Townes). Nitrite treatment substantially decreased adhesion of platelets (A) and Leukocytes (B) (n = 4). (C) Representative absorption spectra of plasma (cell-free) Hb used to measure hemolysis. (D) Average hemolysis without nitrite treatment (normal saline) was 0.042 ± 0.01 mM free hemoglobin (n = 3), while with nitrite treatment (21-day nitrite + normal saline) was 0.018 ± 0.01 mM free hemoglobin (n = 5).

phore treatment (to mimic dehydration of red blood cells in SCD) to deoxygenated whole blood of normal volunteers can significantly improve reduction in RBC membrane stiffness, significantly improve initial surface to volume ratio at critical hemolytic volume and cellular density and improve cellular hydration of RBCs, thereby improving overall deformability compared to just ionophore treatment. These data suggest that nitrite may be able to decrease sickling (by increasing cellular hydration) although this needs further investigation.

We also showed that nitrite administration reaching a final concentration of 6.8 μM (average steady state concentration of 3.4 μM assuming a linear increase in nitrite concentration) protected against calcium influx-mediated surface exposure of phosphatidylserine. Surface exposure of phosphatidylserine was assessed by annexin V binding. The percentage of annexin-bound RBCs was lower than that one may expect based on previous studies [70], perhaps due to reagent efficacy and assay conditions, but nitrite treatment clearly reduced the percentage of annexin V-bound RBCs. These effects of nitrite on RBC properties could be beneficial in lessening pathology in SCD.

One weakness in our results examining nitrite effects on calcium-influx induced RBC phospholipid asymmetry and deformability is that although we were aiming to test nitrite concentrations similar to those achievable through dietary nitrite (1 μM), the concentrations administered were higher based on measured concentrations at the end of the continuous administration (3.4 and 5.7 μM). The overshoot was due to the fact that the rate of nitrite consumption by RBCs is complicated by the dependence of the reaction rate on the Hb quaternary structure [75] and hence hard to predict. Thus, we overestimated the proper rate of administration of nitrite. Nevertheless, it should be noted that the duration of our in vitro treatments are much shorter than what would be achievable in a study employing chronic dietary nitrate. In addition, other methods of nitrite administration (such as a slow releasing pill) could be envisaged to maintain larger but still safe nitrite concentrations.

Previous work suggests that nitrite, unlike other NO donors, would not affect circulating blood cell adhesion [55]. However, those experiments showing no effect of nitrite were conducted in the absence of RBCs. Here, we have shown that nitrite attenuates RBC adhesion to endothelial cells in a microchannel assay. It is known that sickle RBCs

adhere much more tightly to cultured endothelial cells than do normal RBCs, but normal RBCs do demonstrate adherence [87]. Several RBC ligands have been identified that are responsible for adhesion and some of these are elevated in sickle RBCs while others are likely simply more activated [21]. Likewise, several adhesion molecules on endothelial cells have been identified that mediate adhesion [21,81]. Previous work has shown that NO decreases sickle RBC adhesion most likely through decreasing the action of endothelial cell P-selectin [88]. We suggest that in our studies, RBCs bioactivate nitrite producing NO activity which decreases adhesion in similar ways. Further work is required to fully elucidate the effects of nitrite on RBC adhesion and to see if concentrations that reflect more common therapeutic dosing (compared to bolus addition of 50 μM nitrite in our microchannel assays) can alleviate RBC adhesion to endothelia.

We tested whether nitrite can attenuate circulating blood cell adhesion in vivo using hemolysis and LPS murine models of inflammation. Induced hemolysis resulted in about 120 μM in heme which is what can be seen in transfusion of older stored blood [89] and several fold higher than what is found in SCD [9]. Our nitrite treatment did not result in elevated measured plasma nitrite upon collection but we attribute this to the fact that nitrite would have a half-life of only several minutes in vivo [75]. Thus, by the time blood was collected, plasma nitrite was likely no longer elevated. On the other hand, nitrate (which is produced from nitrite reacting with Hb) has a half-life of several hours. Thus, the increase observed in plasma nitrate is probably due to increased nitrite. Previous studies in mice observed increases in plasma nitrite/nitrate beginning 4–12 h after LPS injection with a maximum value obtained between 10 and 20 h with variability among studies [90–92]. We collected blood about 4.5 h after LPS injection which is probably too short a time period to observe significant effects of LPS on plasma nitrite/nitrate. In nitrite untreated mice, we did observe a trend in plasma nitrate increasing in LPS infused mice (34 ± 12 μM) compared to saline infused mice (29 ± 10 μM)), $p = 0.15$; but this increase was much smaller than in nitrite-treated mice: 61 ± 17 μM. Thus, nitrite treatment dominated any changes in nitrite/nitrate over any effect of LPS treatment. It is remarkable that the modest nitrite treatments performed here, as evidenced by small but significant elevations in plasma nitrate, were so effective in reducing platelet and leukocyte adhesion in vivo.

Using a sickle cell mouse model, we also observed that nitrite treatment decreased adherence of leukocytes and platelets in the mesenteric venules subjected to local hypoxia. Hypoxia was induced by bathing the exposed mesenteric vascular bed in anoxic gas, but the mice were breathing normal air. In addition, we found that RBC hemolysis, as measured by cell-free hemoglobin in the plasma, was reduced due to nitrite treatment. This reduction in hemolysis suggests that sickle RBC mechanical properties may have been improved in vivo as shown for in vitro experiments in Fig. 3. Decreasing cellular density (indicated by O′) would decrease polymerization and thereby decrease fragility [69]. Alternatively, or in addition, lower hemolysis may be due to reduced inflammation.

Enthusiasm for the use of nitrite in treatment of SCD may be dampened by lack of efficacy observed in a few NO-based trials [93,94]. A recent study using inhaled NO showed promise in reducing pain crises [95], but the study was small. Despite potential of NO to relieve multiple symptoms of SCD (as demonstrated by us and others [36–40,42,66,72,88]), successful translation has been elusive. However, it is important to note that not all NO congeners are the same and nitrite has several properties that make it more promising than other agents: (1) nitrite targets NO activity to hypoxic areas where it is most needed [88] (unlike other agents like NO inhalation that targets the lungs and sGC activators which have systemic activity), (2) nitrite can be delivered to produce NO activity chronically with potential for constantly elevated levels achieved through the nitrate-nitrite-NO cycle [74]. [Importantly – the major inhaled NO trial [93] involved *acute* administration of inhaled NO which (unlike *chronic* administration of nitrite through daily dietary nitrate) would not be expected to affect many pathologies including adhesion and red cell properties], (3) the selective nitrosative chemistry of nitrite is different from other NO donors – for example whereas NO itself and nitrosothiols do not affect sickling induced RBC dehydration and decrease in deformability, we have shown that nitrite does (in this study and [66]). Notably, that sufficient systemic nitrite was not produced in the inhaled NO study was suggested as a possible cause for the observed lack of efficacy [93].

5. Conclusions

In summary, our in vitro data suggest that nitrite may be more suitable than GSNO and DEANO for administration as a platelet-inhibiting agent in vivo pathophysiologic conditions as nitrite gains function in the presence of RBCs (as found in vivo) whereas GSNO and DEANO lose function. However, our in vitro results would have to be verified in vivo. Nitrite also reduces RBC damage due to calcium influx and adhesion of circulating blood cells. We suggest nitrite can be used as an effective, targeted NO delivery agent to areas of low oxygen tension, particularly in the microcirculation and venous system. The targeted nature of nitrite bioactivation can be harnessed in conditions where hypoxia is an issue such as SCD, shock, diabetes and other conditions.

Author contributions

NW, MTG, MAM, VV, and DBK-S designed the research. NW, SB, HWK, CAB, DO, AP, NB, and AJ performed the research, DBK-S, VV, NW, SB, MTG, ER, and DLC analyzed and interpreted data. NW, SB, VV, and DBK-S wrote the manuscript with help from all other authors.

Disclosures and conflicts of interest

Drs. Kim-Shapiro and Gladwin are listed as a co-inventor on a patent related to use of nitrite in cardiovascular conditions. Dr. Kim-Shapiro owns stock in and serves on the scientific advisory board for Beverage Operations LLC which has licensed Wake Forest University intellectual properties and thus has a financial interest in Beverage Operations LLC.

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