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Amino acid supplementation confers protection to red blood cells prior to Plasmodium falciparum bystander stress

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Abstract:
Malaria is a highly oxidative parasitic disease in which anemia is the most common clinical symptom. A major contributor to malarial anemia pathogenesis is the destruction of bystander, uninfected red blood cells (RBCs). Metabolic fluctuations are known to occur in the plasma of individuals with acute malaria, emphasizing the role of metabolic changes in disease progression and severity. Here, we report that conditioned media from Plasmodium falciparum culture induces oxidative stress in uninfected, catalase-depleted RBCs. As cell permeable precursors to glutathione, we show a benefit of pre-exposure to exogenous glutamine, cysteine, and glycine (QCG) amino acids for RBCs and that this pre-treatment intrinsically prepares RBCs to mitigate oxidative stress.

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Amino acid supplementation confers protection to red blood cells prior to *Plasmodium falciparum* bystander stress

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Abstract

Malaria is a highly oxidative parasitic disease in which anemia is the most common clinical symptom. A major contributor to malarial anemia pathogenesis is the destruction of bystander, uninfected red blood cells (RBCs). Metabolic fluctuations are known to occur in the plasma of individuals with acute malaria, emphasizing the role of metabolic changes in disease progression and severity. Here, we report that conditioned media from *Plasmodium falciparum* culture induces oxidative stress in uninfected, catalase-depleted RBCs. As cell permeable precursors to glutathione, we show a benefit of pre-exposure to exogenous glutamine, cysteine, and glycine (QCG) amino acids for RBCs and that this pre-treatment intrinsically prepares RBCs to mitigate oxidative stress.

Key points

- Intracellular reactive oxygen species (ROS) is acquired in red blood cells (RBCs) incubated with Plasmodium falciparum conditioned media
- Glutamine, cysteine, and glycine amino acid supplementation led to increased glutathione biosynthesis and reduced ROS levels within stressed RBCs

Introduction

Anemia is the most common clinical consequence of human malaria, a parasitic disease with nearly 250 million cases annually[1]. Pathogenesis of malarial anemia is multifaceted consisting of a loss of both infected and uninfected red blood cells (RBCs) as well as dysregulation of new RBC production [2]. In malaria infections caused by *P. falciparum*, uninfected RBCs are lost at a much greater rate than infected RBCs. This so called "bystander effect" occurs prior to adaptive immune system activation[3], contributing

greatly to the development of malarial anemia. The mechanisms of the malarial bystander effect on uninfected RBCs are unclear, although a loss in membrane deformability is known to contribute to the removal of bystander RBCs from circulation[4,5]. Bystander RBCs have also been shown to undergo cell surface changes promoting erythrophagocytosis through both complement-mediated activation[6] and phosphatidylserine antibody-mediated removal[7]. *In vivo* models for malaria demonstrate that over 75% of the RBCs that are lost are uninfected during naïve infection, while only ~5% of all RBCs destroyed during infection are due to direct parasite infection with inhibition of erythropoiesis making up the remaining portion[8]. Culture medium from *P. falciparum*-infected RBCs was shown to impact biological function of nucleated erythroid cells[9]. In addition, parasite-derived mitochondrial DNA within the media of *P. falciparum* parasite culture was found to elicit toll-like receptor 9 (TLR9) binding, thereby altering the membranes of healthy RBCs[10]. Interrupted glycolysis also increases RBC susceptibility to senescence and oxidative damage and further highlights the importance of the exogenous metabolic environment for RBCs[11]. These previous findings indicate multiple mechanisms contributing to the bystander effect in malaria.

While mature RBCs have limited metabolic activity due to a lack of membrane-bound organelles, these cells have multiple active antioxidant components to counter oxidative stress in their environment. These intracellular defenses include reduced glutathione (GSH), catalase, peroxiredoxins, and glutathione peroxidase[12,13]. Oxidative stress plays a major role in many anemia-inducing conditions, such as malaria and sickle cell disease (SCD)[14,15]. In malaria, RBCs from patients have reduced levels of intracellular catalase[16], indicating these cells are deficient in their ability of fully combatting oxidative stress and rendering avenues of antioxidant therapy as a viable treatment to lessen disease severity. Exogenous GSH, a potent antioxidant, is structurally unable to freely permeate

cellular membranes; therefore, individual amino acid building blocks of the tripeptide GSH (glutamine, cysteine, and glycine) are taken up by RBCs which perform *de novo* GSH biosynthesis inside the cell[17]. Additionally, significant host metabolic alterations occur during malaria. This includes markedly reduced levels of plasma free amino acids such as glutamine and arginine[18–25], suggesting amino acid supplementation could perhaps provide therapeutic benefit in malaria.

Oral treatment with L-glutamine (Gln) is a recently approved therapy for sickle cell disease[26]. While the exact cellular mechanism remains unclear, a potential role for Gln is to improve the nicotinamide adenine dinucleotide phosphate (NADPH) stores in sickled RBCs[27], lessening the oxidative stress. Gln also serves as a precursor for arginine, an amino acid that has been inversely associated with mortality in cerebral malaria[22] and proposed to be beneficial in both malaria[28] and sickle cell disease[29]. Gln is also implicated in malarial anemia where low plasma Gln levels were found to be associated with severe pediatric malarial anemia[30]. Here, we explore the oxidative impact of the *P. falciparum* culture environment on uninfected, catalase-inhibited RBCs as a proxy for *in vivo* severe malaria bystander effect. Furthermore, we investigate the role of exogenous amino acid supplementation on bystander RBCs and show that RBCs pretreated with precursor antioxidant amino acids glutamine, cysteine, and glycine concomitantly have increased intracellular glutathione synthesis and thus these amino acids together confer protection from oxidative stress.

Methods

Blood washing and perturbations

Heparinized venous blood, Type O+, was collected from healthy adult participants with informed consent and used with approval from Wake Forest University Institutional Review Board (Study ID: IRB00024199) or was commercially purchased from BioIVT Inc and received within 24 hours of collection. Briefly, RBCs were washed three times in excess 1X Phosphate Buffered Saline (PBS), pH 7.4 (Gibco™) and centrifuged at 900 x g to remove plasma and buffy coat layers with each wash. Washed RBCs were stored in 1X PBS supplemented with 5 mM D-glucose (PBS+Gluc) and used the same day. All RBC incubation experiments were performed at 1% hematocrit (hct) while rocking at 37°C. Overnight 24-hour incubations were performed for amino acid supplementation and *P. falciparum* conditioned medium (*Pf*CM) stress. For amino acid supplementation experiments, indicated concentrations of amino acids were thoroughly dissolved in PBS+Gluc and incubated overnight. The amino acid concentrations were selected to be 1000 μM to approximate the average glutamine concentration in plasma of patients with SCD treated with L-glutamine[27] which is also in the realm of levels of glutamine in Kenyan children with malaria but without SMA (mean of 1361 μM for those without SMA as compared to 484 μM for those with SMA).[30] Cells were then washed twice in PBS to remove amino acids prior to stress incubations. All hydrogen peroxide perturbations were performed in the presence of 1 mM sodium azide (NaN_3) to block intracellular catalase activity and incubated for 15-minutes while rocking at 37°C, as described previously[31–33]. *Pf*CM perturbations were also performed with 1 mM NaN_3 to block intracellular catalase activity. Control media conditions for *Pf*CM perturbations included RPMI 1640 (Gibco™) supplemented with 1 mM NaN_3.

Plasmodium falciparum culture and conditioned media

Plasmodium falciparum of the 3D7 laboratory strain (MRA-102; BEI Resources) was used in the generation of *Pf*CM. RBCs were combined from two O+ donors at 2% hct and maintained in standard culture media consisting of RPMI 1640 (Gibco™) supplemented with sodium bicarbonate, HEPES buffer, hypoxanthine, gentamicin, and 0.5% (wt/vol) Albumax II[34] in a gas environment of 1% O_2, 5% CO_2, and 94% N_2. Parasites were synchronized using a 5% Sorbitol solution, seeded at 0.4% parasitemia, and allowed to grow with no media change, but one gas exchange, for 72 hours. The culture was then centrifuged at 600 x g for 15 minutes, then 1600 x g, then 3600 x g, and then filtered with a 0.45 µm filter, before storing at 4°C, as described previously[35]. For glucose supplementation experiments, 5.5 mM D-glucose was dissolved into *PfCM* and RPMI 1640 base media, respectively. The value of 5.5 mM was selected as being on the high end of the human fasting glucose range (3.9.- 5.6 mM) and approximately 50% of the concentration of D-glucose in fresh RPMI (11.1 mM).

Intracellular ROS detection

Intracellular ROS was measured using a Cellular ROS Assay Kit (ab113851; Abcam). Washed RBCs were incubated with a 2 µM working concentration of 2',7' – dichlorofluorescin diacetate (DCFDA) for 30 minutes. RBCs were then washed twice with 1X PBS and used for either flow cytometry analysis or live cell imaging.

Flow cytometry

RBCs were analyzed by either a BD LSRFortessa™ X-20 Flow Cytometer (Becton Dickinson) or a CytoFLEX V0-B3-R1 Flow Cytometer (Beckman Coulter). RBCs were passed at a flow rate of about 5,000 cells per second. Samples ran on the BD LSRFortessa™ X-20 Flow Cytometer (Becton Dickinson) utilized a 60 milliwatt 488 nm laser at 550 volts for excitation and a green (505 longpass and 530/30 bandpass),

fluorescein isothiocyanate (FITC) emission filter for detection. Samples ran on the CytoFLEX V0-B3-R1 Flow Cytometer (Beckman Coulter) utilized a 50 milliwatt 488 nm laser for excitation and a 525/40 band pass emission filter for detection. For data analysis, mean fluorescent intensity was measured for 100,000 events, gated on doublet discrimination (FSC-H vs FSC-A), and analyzed using FCS Express 7 Research software (De Novo).

Live cell imaging

Experiments were carried out using a Leica Thunder Live Cell Imaging System and LasX acquisition software. Images were acquired as 16-bit data with a Leica K8 sCMOS camera (2048 X 2048 pixels), using a 63X Plan Apo oil immersion lens (1.4NA). RBCs were plated onto 35 mm optical coverglass dishes (Ibidi) immediately before imaging. For time lapse experiments, images were acquired every 15 seconds. All excitation/acquisition parameters were held constant across imaging experiments including light-emitting diode excitation level and camera exposure time. All analyses were performed using Leica instantaneous computation clearing intensity (ICC) values. For analysis, stationery cells were chosen using Differential Interference Contrast images and isolated using region of interest selection using FIJI[36]. Fluorescence in regions of interest was quantified from ICC adjusted images at each timeframe image. Data was normalized to average fluorescence of first three timepoints, prior to treatment. For visualization purposes, images were adjusted to maximize for brightness contrast using FIJI[36].

Scanning electron microscopy

Perturbed RBCs were washed and fixed in an osmotic-controlled glutaraldehyde solution, as previously reported . Briefly, glutaraldehyde-fixed RBCs were washed and

resuspended in distilled H_2O at 0.5% hct before air drying overnight on 12mm round coverslips at 60°C. Images were collected with Everhart-Thornley secondary electron detection using either an AMRAY 1810 or a Phenom XL scanning electron microscope at 10Kv accelerating voltage. Typical magnifications employed were 2000X to allow for high resolution of RBCs while maintaining a reasonable field size. Samples were prepared for imaging by first dehydrating on carbon tab aluminum stubs, then gold sputter coating under argon gas conditions to a thickness of about 7-10nm. Echinocyte morphology stages were defined as previously described[38–40] and total number of cells in two fields of view were imaged and quantified for morphology scoring. Each field of view had varying total cells on slide (range: 131-384 cells) and so the morphology scores and stages were reported per 100 cells to account for this variation per independently imaged field of view.

Osmotic gradient ektacytometry

A Technicon osmotic gradient ektacytometer (Technicon Instrument Corp.) facilitated the deformability measurements of the RBCs. Thirty-one (31) g/L of polyvinylpyrrolidone (PVP) polymer (Sigma, 437190) mixed with 0.9 g/L of sodium phosphate dibasic anhydrous Na_2HPO_4 (Fisher, 7558-79-4), 0.24 g/L of sodium phosphate monobasic NaH_2PO_4 (Fisher Biotech, 10049-21-5), and 0.544 g/L of sodium chloride NaCl (Sigma, S7653) was prepared in Milli-Q ultrapure water with the pH of 7.4. The “low” solution (40 mOsm) was used to make the “high” (750 mOsm) and “sample” solutions, by dissolving 11.25 g of NaCl in 500 mL of the low solution, and 1.9782 g of NaCl in 250 mL of the low solution (290 mOsm), respectively. After calibration using known osmolality mixtures and finding the required parameters as well as laser alignment of the ektacytometer, 150 µL of the blood samples (40% hematocrit) was diluted into 4 mL of sample solution. The population of RBCs suspended in high viscous media was directed into the gap between

two coaxial cylinders. The outer cylinder stayed motionless while the inner cylinder rotated with distinct angular velocity to apply the defined shear stress of 159 dynes/cm^2 (~16 pascals) at a controlled temperature. During operation, the focused laser beam passed through the suspension and generated elliptical diffraction patterns of the flowing RBCs projected on a detector. LabVIEW software recorded the diffraction patterns and the corresponding osmoscans and quantitative statistics. Data was fit using Origin 2016 software and the averages of three separate scans from each blood sample were analyzed. GraphPad Prism was used for the graphing of the recorded result.

DNA extraction and amplification

Following *Pf*CM exposure, RBCs were washed in 1X PBS (Gibco) and DNA was extracted from samples using a commercially available kit (K0781; Thermo Fisher Scientific). Quantitative PCR amplification of a 500bp fragment of the *P.falciparum* mitochondrial cytochrome c oxidase subunit III (coxIII) gene using primers previously developed[10] was performed using PowerTrack™ SYBR green Master Mix (A46012; Thermo Fisher Scientific) and a Roche LightCycler 480. Cycle threshold (Ct) and baseline threshold values were calculated using the Roche LightCycler 480 software.

Metabolite extraction

Following perturbation experiments, 1x10^7 RBCs were pelleted and the supernatant was carefully removed. Pelleted cells were resuspended in 1X PBS with 100 mM N-ethylmaleimide (NEM) (Cat#128-53-0, Millipore Sigma) for 15 minutes at room temperature[31]. Following NEM incubation, cells were washed twice with 1X PBS. After thorough removal of NEM from supernatant, PBS was added with a final cell concentration of 20% hct and cell concentration was recorded using a hemocytometer. Methanol was added (4:1) followed by vortexing and storing cells on ice for 30 minutes.

Sample tubes were then centrifuged at 18,000 x g and supernatant was carefully removed and stored in -80°C for mass spectrometry analysis.

Targeted Mass Spectrometry

Targeted LC-MS/MS analysis was performed at the Proteomics and Metabolomics Shared Resource (Wake Forest University School of Medicine, Winston Salem, NC). Briefly, extracted samples were dehydrated and reconstituted in H_2O followed by mass spectrometry (Sciex 7500 MS) analysis[41] for relative quantification of reduced glutathione (GSH alkylated by NEM) and oxidized glutathione (GSSG) metabolites, without any further derivatization[42]. Separation was performed on a Thermo Scientific Hypersil GOLD aQ reverse phase column (2.1 x 150 mm, 3µm) with a gradient mobile phase system consisting of an aqueous phase of 0.1% formic acid (A) and an organic phase of acetonitrile (B) at a flow rate of 0.5 mL/min (0 - 0.5 min, 0.5-5% B; 0.5 - 6.5 min, 5-98% B; 6.5 min-9 min, 98% B). The mass spectrometer used the following source parameters: Ion source gas 1: 35 psi, Ion source gas 2: 70 psi, Curtain gas: 40 psi, CAD gas: 9 psi, Source temperature: 250°C, Spray voltage: 5500 V. Transition masses for targeted analysis were 433.00 > 304.00 m/z (NEM-labeled GSH), and 613.20 > 355.25, 613.20 > 484.20 and 613.20 > 231.05 m/z for GSSG. Relative peak intensity values were normalized to 10^6 cells per sample. Total glutathione levels were determined by adding intensities of GSH-NEM and GSSG with correction for ionization efficiency. Heparinized venous blood, Type O+, was collected from healthy adult participants with informed consent and used with approval from Wake Forest University Institutional Review Board (Study ID: IRB00024199) or commercially purchased from BioIVT Inc.

Results

Exogenous Gln alone is not sufficient but exogenous amino acid cocktail reduces oxidative stress acquired from H_2O_2

Gln's relevance in both malaria and sickle cell anemia along with it being a cell-permeable precursor to GSH, lead us to first to explore the impact exogenous Gln supplementation on oxidative stress in RBCs. For these studies, we used the intracellular fluorescent ROS indicator, DCFDA. To verify that measured changes are indeed due to ROS and not the result of nonspecific effects from the DCFDA probe, we (a) tested and verified that mean fluorescent intensity increases linearly with hydrogen peroxide concentrations (**Supplemental Figure 1**) and (b) tested and verified that cells pretreated with amino acids in the absence of H_2O_2 stress have no observable change in mean fluorescence intensity compared to the cells incubated with PBS + gluc (**Supplemental Figure 2**). Despite a clear significant difference in intracellular ROS levels upon H_2O_2 treatment (fold change of 1.0 for 0 µM H_2O_2 vs 4.1 for 50 µM H_2O_2, p = 0.036), we found no difference between H_2O_2-stressed RBCs pre-exposed to control null media or media supplemented with Gln overnight (**Figure 1A**).

As H_2O_2 is known to impair RBC function through reduced RBC membrane deformability and cellular dehydration[32,33,43], we assessed the osmotic effect[44] of Gln supplementation and saw an expected decrease in membrane deformability, as measured by DI_{max}, in response to H_2O_2 (0.385 DI_{max} for 0 µM H_2O_2 vs 0.360 DI_{max} 50 µM H_2O_2, p = 0.035). However, RBCs supplemented with Gln had a similar loss in deformability when stressed with H_2O_2, suggesting no impact of Gln on that parameter (**Figure 1B and 1C**).

In terms of cell hydration, we observed slight dehydration in RBCs stressed by H_2O_2 that bordered on significance (347 mOsm/kg for 0 µM H_2O_2 vs 340 mOsm/kg for 50 µM H_2O_2,

p = 0.061), but more interestingly, we found that Gln supplementation significantly improved hydration status compared to RBCs not supplemented with Gln (340 mOsm/kg for 0 µM Gln vs 344 mOsm/kg 1000 µM Gln, p = 0.031) (**Figure 1D**). These data demonstrate that Gln supplementation prior to oxidative stress is osmotically advantageous for RBCs hydration but does not improve RBC membrane deformability nor reduce intracellular ROS generation in RBCs.

Although Gln supplementation alone did not seem to protect RBCs from oxidative stress, Gln is a precursor for glutamate which is required, in addition to cysteine and glycine, for *de novo* GSH synthesis inside the RBC to combat high levels of oxidative stress[45,46]. We incorporated those additional amino acids into our approach and found that RBCs exogenously pretreated with Gln (Q), cysteine (C), and glycine (G) together, hereby referred to as QCG, incurred significantly less intracellular ROS when stressed with H_2O_2 (mean fold change of 5.0 without QCG vs 4.0 with 1000 µM QCG pre-treatment, p = 0.009) (**Figure 2A**). This oxidative protection was most notably conferred from 1000 µM QCG supplementation. We also found RBCs pre-exposed to QCG had a significant reduction in loss of membrane deformability once stressed with H_2O_2 (**Figures 2B-C**), although the 1000 µM QCG concentration of pretreatment did not reach significance (p = 0.068). Similar to Gln supplementation, QCG pretreatment resulted in significantly higher hydration status in RBCs (340 mOsm/kg without QCG vs 350 mOsm/kg with QCG, p=0.021), as measured by O_{hyp} (**Figures 2B and 2D**). Reduction in oxidative stress in RBCs pre-exposed to QCG was synergistic compared to effects from individual amino acid pre-treatment alone (**Supplemental Figure 2**). As a comparison with QCG, we also tested the impact of arginine amino acid supplementation as it has been reported to be beneficial in both sickle cell anemia and malaria studies; however, exogenous arginine

supplementation did not provide a significant intracellular oxidative advantage to RBCs (**Supplemental Figure 2**),

***P. falciparum*-conditioned medium induces stress in human RBCs**

To determine the oxidative impact of *P. falciparum* conditioned medium (*Pf*CM) on uninfected, catalase-depleted RBCs, we measured RBC echinocytosis, a morphological change associated with oxidative stress[47]. We observed that RBCs exposed to *Pf*CM had significantly higher morphology scores (mean score of 116 for control vs. 138 for *Pf*CM, p = 0.0002) **(Figure 3A)** and a higher percentage of echinocytes (mean of 10.5% for control vs. 24.9% for *Pf*CM, p = 0.002) **(Figure 3B),** as compared to RBCs in control RPMI media**.** Although overall RBC morphology stages showed slight variations between participants, more severe echinocyte stages were present in RBCs from all participants exposed to *Pf*CM **(Supplemental Figure 3)**. Next, we aimed to determine whether redox state was affected in mature RBCs alongside the change in morphology. RBCs incubated with *Pf*CM had a significant increase in intracellular ROS as detected by DCFDA, compared to RBCs from the same donor exposed to control media (fold change of 1.0 for control vs. 1.8 for *Pf*CM, p = 0.011) **(Figure 3C-D)**. To confirm the *Pf*CM-induced oxidative stress was not due to known depleted levels of glucose during *P. falciparum in vitro* cultivation[48], we supplemented *Pf*CM and control media with excess glucose and found that although oxidative stress was slightly improved when *Pf*CM was supplemented with excess glucose as compared to *Pf*CM without excess glucose, there was still a significant increase in intracellular ROS (**Supplemental Figure 4A**). As recent work has demonstrated that free mitochondrial DNA can stimulate TLR9 responses in RBCs and induce morphological changes (e.g., echinocytosis and increased rigidity)[10], we wanted to know whether our *Pf*CM also had elevated mitochondrial DNA and whether this coincided with the increase in intracellular ROS.

Indeed, when we performed quantitative PCR for CPG-containing mitochondrial DNA in the *Pf*CM exposed RBCs, we corroborated recently published findings by Lam *et al.* and we did see an association between the conditions with elevated mitochondrial DNA and elevated intracellular ROS[10].

QCG supplementation lessens *Pf*CM-induced oxidative stress in RBCs

We next aimed to determine whether QCG supplementation also conferred protection to RBCs exposed to *Pf*CM. Indeed, RBCs pretreated with QCG amino acids were found to have a significant reduction in intracellular ROS following *Pf*CM incubation (mean fold change of 1.72 for 0 µM QCG vs. 1.15 for 1000 µM QCG, p = 0.027) (**Figure 4A**). Morphologically, we observed RBCs pretreated with QCG had overall improved morphology scores after *Pf*CM stress (mean score of 130 for 0 µM QCG vs. 122 for 1000 µM QCG, p = 0.040) (**Figure 4B**). Therefore, we found that QCG supplementation confers protection to *Pf*CM-induced oxidative stress in RBCs and that decreased intracellular ROS levels coincided with improved RBC morphology. We did not however find that QCG supplementation had any effect on lessening the amount of mtDNA, suggesting that QCG supplementation does not work through depleting the amount of mtDNA stressor in the media (**Supplemental Figure 4B**).

QCG supplementation induces intracellular RBC glutathione synthesis

We hypothesized that QCG protection occurs via intracellular GSH metabolic pathways given that each amino acid is a known precursor for *de novo* GSH synthesis (**Figure 5A**). We found increased levels of total glutathione in QCG-supplemented RBCs (**Figures 5B-D, Supplementary Figures 5A-C**). This activity was evident from QCG supplementation, with or without induced oxidative stress. We also observed an increased level of oxidized glutathione (GSSG) in QCG supplemented RBCs after

exposure to either *Pf*CM (1525 mean peak area intensity for 0 µM QCG vs 6725 for 1000 µM QCG, p = 0.009) (**Figure 5C**) or H_2O_2 (111,650 mean peak area intensity for 0 µM QCG vs 211,245 for 1000 µM QCG, p = 0.103) (**Supplemental Figure 5B)** compared to RBCs without QCG supplementation or exposure to oxidative stress. These results indicate that QCG supplemented RBCs have increased intracellular glutathione biosynthesis.

Supplementation with QCG promotes RBC intrinsic antioxidant properties

Given the aforementioned findings, it remained unclear whether QCG-mediated protection was a generalized RBC response or if QCG protection occurred specifically under oxidative stress exposure. To determine this, we analyzed the kinetic response of QCG supplemented RBCs to oxidative stress. RBCs pre-exposed to QCG showed lower levels of intracellular ROS within 2.5 min of H_2O_2-induced oxidative stress while RBCs pre-exposed to either PBS or Gln had higher levels of ROS that developed quicker within the cells (**Figure 6A**). Cellular response to oxidative stress among all preincubation conditions appeared to be heterogenous across cells (**Figures 6B-D**) and interestingly, we observed punctate-like areas of increased fluorescence in each preincubation condition with or without H_2O_2-induced oxidative stress, suggesting internal organization of intracellular ROS with morphology consistent with that of Heinz-Ehrlich bodies. This phenomenon was observed in both H_2O_2 and *Pf*CM-stressed RBCs (**Figure 6E**). Together, these data indicate QCG preincubation intrinsically prepares RBCs to counter oxidative stress prior to any oxidative stress exposure rather than mounting an antioxidant response directly following oxidative stress.

Discussion

In this study, we investigated the metabolic role of amino acid supplementation on oxidatively stressed RBCs with an overall goal of alleviating RBC oxidative burden. We first explored Gln supplementation on RBCs stressed by H_2O_2 and observed that while Gln supplementation alone was not sufficient to reduce oxidative stress levels within RBCs, Gln supplementation promoted RBC hydration (**Figure 1**). In contrast, we identified a significant decrease in intracellular ROS in RBCs that had been supplemented with QCG amino acids (**Figure 2**). These data suggest that observed ROS protection is conferred through RBC GSH biosynthesis pathway, as total glutathione was increased in RBCs supplemented with QCG (**Figure 5**) and this protection from intracellular ROS development was found to be rapid (2.5 min) upon induction of oxidative stress (**Figure 6**). Interestingly, single amino acid supplementation did not reduce intracellular ROS, an effect we only saw with simultaneous supplementation with QCG (**Supplemental Figure 2).** Post-exposure of QCG did not have a comparable protection to RBCs that were oxidatively stressed first (data not shown), suggesting that utilization of QCG is done to equip RBCs to defend themselves against any oxidative stress they may incur in the future. Here, we also report for the first time to our knowledge that conditioned media from *P. falciparum* culture increases intracellular ROS in uninfected, catalase-depleted RBCs (**Figure 3**), and is a likely contributor to malaria bystander effect. Additionally, we found that QCG supplementation does indeed confer protection in the form of reduced intracellular ROS and improved echinocyte morphology to *Pf*CM stressed RBCs (**Figure 4**) despite the inhibition of catalase in these RBCs, highlighting a therapeutic prospect for malarial anemia.

Recently *Pf*CM was shown to increase oxidative stress in erythroid precursor cells[9] and alter membrane structure and binding in uninfected mature RBCs[10]. However, it was unclear if *Pf*CM also perturbed the oxidative status of uninfected mature RBCs. We

417 confirmed one mode of action that *Pf*CM has on mature, catalase-depleted RBCs is
418 through oxidative stress as measured by the induction of intracellular ROS. In general,
419 elevated ROS reduces RBC survivability *in vivo*[49], suggesting this could be an additional
420 contributor to pathogenesis of bystander effect in malaria.
421
422 Gln is implicated in both sickle cell anemia and malarial anemia, highlighting a possible
423 role for metabolic intervention in anemic conditions. Lower plasma Gln levels are
424 associated with pediatric malarial anemia[30] while oral Gln supplementation is an
425 approved treatment for sickle cell anemia, although cellular mechanisms of this therapy
426 are still under investigation. Here, we show that Gln supplementation improves RBC
427 hydration status, a potential mechanistic role that warrants more investigation
428 specifically in sickle RBCs. It is long appreciated that RBCs respond to exogenous
429 metabolites and that the lack of necessary exogenous metabolites negatively impacts
430 RBC lifespan[50,51].Prior to this study, it was unknown how pre-exposure to exogenous
431 amino acids impacted RBCs in the context of oxidative stress and malaria bystander
432 effect. Our study design of amino acid supplementation was focused on recapitulating
433 the plasma environment if key metabolites were supplemented in advance of infection or
434 oxidative stress. We found that RBCs supplemented with QCG amino acids are
435 equipped to counter oxidative stress from both H_2O_2 and *Pf*CM. We showed that this
436 benefit was intrinsic to RBCs and that ROS development was mitigated within minutes in
437 the cell. As intracellular GSH synthesis occurs in the order of hours within RBCs[17,52], this
438 suggests that GSH stores increase in response to QCG pre-incubation, and are not a
439 combative cellular response to oxidative stress. Glutamine is an important precursor to
440 arginine *in vivo* via citric acid cycle and mitochondrial pathways and supplementation
441 with arginine is reported to be beneficial in both malaria[28] and sickle cell anemia[29]. Within
442 the context of RBCs as reported in this study, arginine supplementation did not confer

443 oxidative protection intracellularly, suggesting that the beneficial role of arginine, and
444 therefore glutamine as an arginine precursor, requires pathways that are active in
445 nucleated cells rather than in RBCs. Collectively, our results highlight a beneficial role for
446 exogenous QCG to RBCs prior to oxidative stress. These findings may suggest a
447 prophylactic or therapeutic role of amino acid supplementation in oxidative anemias,
448 including in malaria.
449
450 Our study had limitations. First, these experiments were performed *in vitro* with only
451 RBCs present However, in an *in vivo* context, RBC exogenous amino acid availability
452 and utilization would be complicated by other cells present which also can utilize these
453 substrates, Secondly, we induced oxidative stress in RBCs under the presence of
454 sodium azide, a known inhibitor of the catalase enzyme, to model stressed uninfected
455 RBCs from *P. falciparum* culture and malaria infected patients that have been shown to
456 have significantly reduced catalase levels[16,53], it is important to acknowledge that
457 complete ablation using sodium azide is a non-physiological model and is more severe
458 than what is experienced by RBCs under physiological conditions. Third, we are unable
459 to comment on the exogenous bioavailability and utilization of QCG by RBCs in an *in*
460 *vivo* context, as these studies focused on cellular, *in vitro* effects of QCG
461 supplementation. More studies would need to be performed in *in vivo* systems. In terms
462 of our measurement of ROS, we performed one assay, DCFDA, which provides a
463 general measure of intracellular ROS, but further studies using additional probes to
464 better confirm and define the type(s) of ROS in the cell would be valuable. It is also
465 challenging to determine the extent of clinical significance through measuring
466 deformability. While effects may be small, any amount of reduced deformability reflects
467 rheological deficiencies that could be tied to increased blood viscosity and propensity for
468 hemolysis. The fact that QCG supplementation shows a significant improvement after an

acute insult with H_2O_2 coupled with catalase inhibition may translate to clinical improvements in contexts in which insults are prolonged. For example, in severe malarial anemia, prolonged exposure to oxidative stress could reduce RBCs' endogenous antioxidant capacity (e.g., intracellular glutathione stores) and exogenous amino acids could serve as essential building blocks for build back up those internal stores. A strength but also a limitation of our study is that we use different human participants in the various experiments shown. On one hand, this shows the broad applicability of these findings to different humans, but on the other hand, we do not have one set of human participants that we have analyzed side-by-side in all experiments. As such, there is a certain amount of human-to-human variability that we have as part of this study. Finally, while we find an association between intracellular ROS and altered RBC morphology as have others[54], we cannot definitively conclude that this is a causative relationship. Future work must be done to fully decipher the mechanistic processes underlying RBC morphology change in the context of exogenous stressors.

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Author Contributions

R.J.C. and H.C.B. designed and conceptualized the project; H.C.B. performed and analyzed the results of the experiments, designed the figures, and wrote the initial manuscript. E.A. performed and analyzed ektacytometry experiments. C.E.S. performed flow cytometry experiments. D.S.N. performed parasite culture and conditioned media generation. J.F.W. contributed technical expertise for flow cytometry experiments. A.O.C. performed mass spectrometry experiments. C.M.F. supervised mass spectrometry and contributed redox biology expertise. G.S.M. assisted with microscopy experiments and contributed technical expertise for fluorescent and scanning electron microscopy imaging. D.B.K-S. supervised ektacytometry, contributed red cell biophysics expertise, and contributed to the overall experimental design. R.J.C. supervised the experiments, data analysis, and revising of the manuscript. All authors contributed to and endorsed the final version of the manuscript.

Disclosure of Conflicts of Interest

The authors do not have any conflicts of interest to disclose.

Figure Legends

Figure 1: Glutamine pre-treatment benefitted oxidatively stressed RBCs through osmotic protection

(**A**) Fold change of intracellular ROS detected by DCFDA staining and flow cytometry in RBCs. (**B**) Representative ektacytometry curve as cells pass through osmotic gradient at a constant shear stress of 16 Pa. (**C**) Maximum deformability index (DI_{max}) values and (**D**) RBC hydration graphed as O_{hyper}.; n=3. Mean ± standard error of the mean denoted by error bars. One-way paired t-test; ns = not significant, *$p < 0.05$

Figure 2: QCG preincubation reduces oxidative impact of H_2O_2 induced stress

(**A**) Fold change of intracellular ROS detected by DCFDA staining and flow cytometry in RBCs; n=6 (**B**) Representative ektacytometry curve as cells pass through osmotic gradient at a constant shear stress of 16 Pa. (**C**) Maximum deformability and (**D**) hydration level of RBCs from ektacytometry curves**;** n=3. Mean ± standard error of the mean denoted by error bars. One-way paired t-test; ns = not significant, *$p < 0.05$, ** $p < 0.005$, *** $p<0.0005$.

Figure 3: *Pf*CM increases RBC echinocytosis and intracellular ROS

RBCs incubated in control media or *Pf*CM overnight were imaged with Scanning Electron Microscopy (AMRAY 1810, 2000X total magnification) and assessed based on (**A**) morphology scores and (**B**) percentage echinocytes (n=7). RBCs incubated in control media or *Pf*CM overnight were washed after incubations and stained with DCFDA to quantify intracellular ROS with flow cytometry. Representative flow cytometry histogram shown in (**C**) and bar graph of ROS fold change compared to control in (**D**);

n=4. Mean ± standard error of the mean denoted by error bars. One-way paired t-test; *p < 0.05, ** p < 0.005, *** p<0.0005.

Figure 4: QCG supplementation protects RBCs from *Pf*CM induced oxidative stress

(**A**) Fold change of intracellular ROS detected by DCFDA staining and flow cytometry in RBCs, n=3. (**B**) RBC morphology score assessed from scanning electron microscopy images, n=5. Mean ± standard error of the mean denoted by error bars. One-way paired t-test; ns = not significant, *$*p < 0.05$*

Figure 5: Glutathione metabolic changes within RBCs supplemented with QCG followed by *Pf*CM stress (**A**) Schematic of metabolic processes known to occur within RBCs from QCG amino acids. Peak area intensity of (**B**) reduced glutathione (GSH), (**C**) oxidized glutathione (GSSG), and (**D**) total glutathione as measured by LC-MS/MS, n=3. One-way paired t-test; ns = not significant, *$*p < 0.05$, ** $p < 0.005$.*

Figure 6: QCG supplementation rapidly protects RBCs from intracellular ROS formation during oxidative stress

RBCs pretreated with either PBS, Gln (Q), or QCG were labeled with DCFDA and placed in a glass bottom petri dish per sample condition and allowed to settle for 45 seconds before given a bolus (red arrow, **A**) of PBS (grey line, **B-D**) or 50 μM H_2O_2 (red line, **B-D**). Stationary cells (total 8 cells per condition) were quantified (**A**) and imaged (**B-D**) per condition at each 15 second time point for five minutes. Images were taken with Leica Thunder at 63X oil immersion (**A-D**). Representative images of DCFDA

labeled RBCs stressed with 50 μM H_2O_2 (left) or *Pf*CM (right) (**E**). Images taken with Echo Revolve Fluorescent Microscope at 100X oil immersion (**E**).

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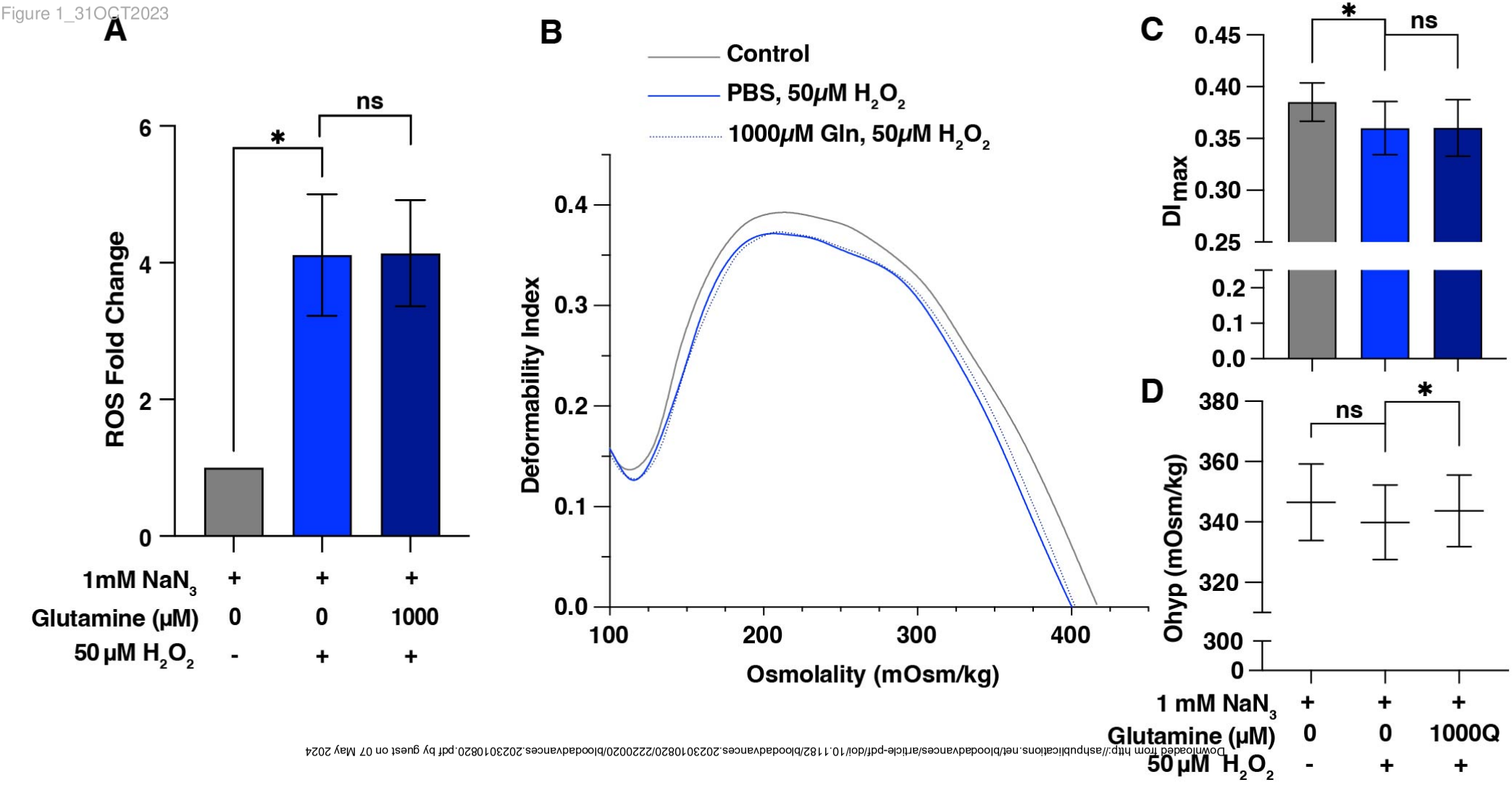
Figure 1_31OCT2023



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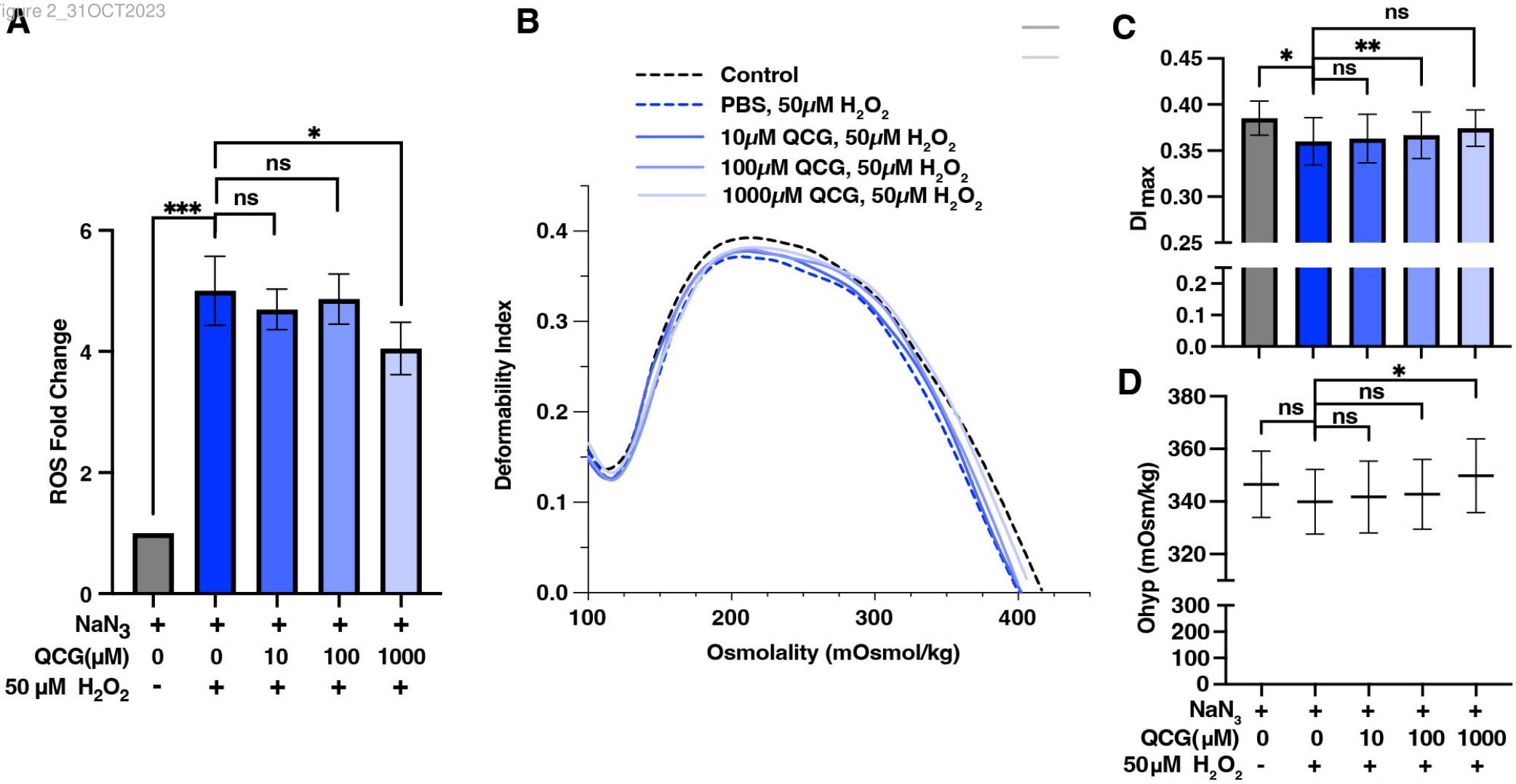

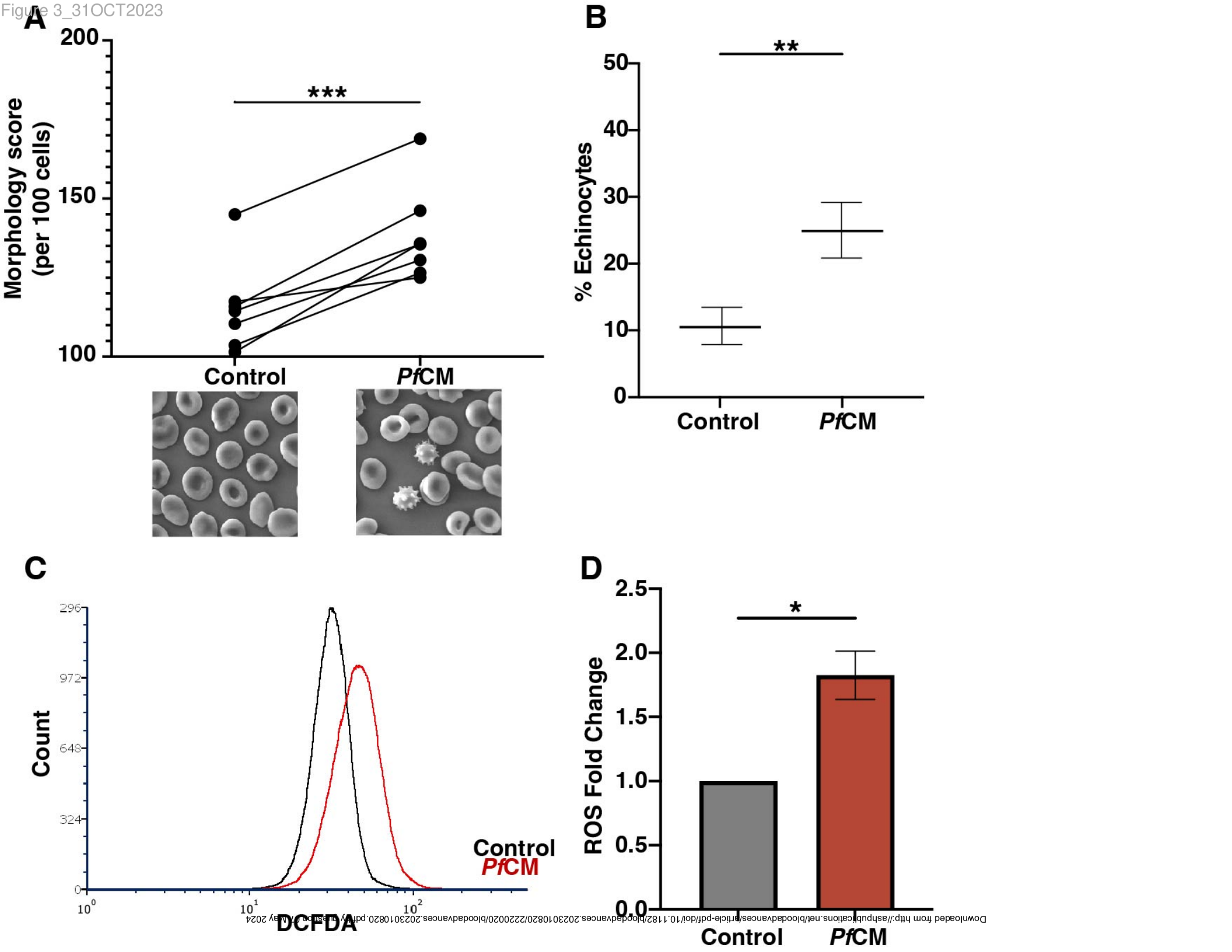

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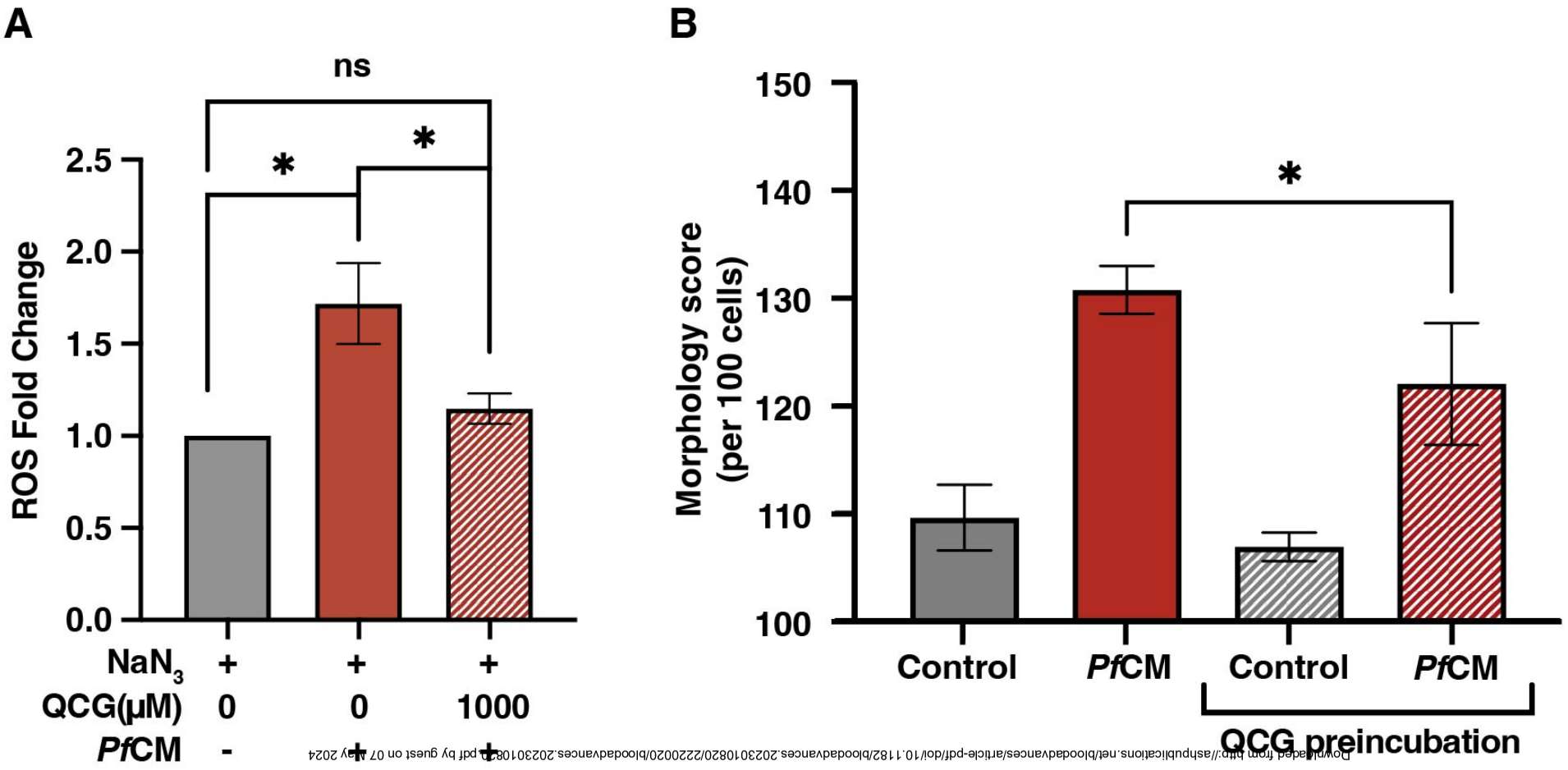

Figure 5