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Glutathione depletion, Pentose Phosphate Pathway Activation, and Hemolysis in Erythrocytes Protecting Cancer Cells from Vitamin C Induced Oxidative Stress

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Running title: Hemolysis from DHA-induced oxidative stress protection

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ABSTRACT

The discovery that oxidized vitamin C, dehydroascorbate (DHA), can induce oxidative stress and cell death in cancer cells has rekindled interest in the use of high dose vitamin C (VC) as a cancer therapy. However, high dose VC has shown limited efficacy in clinical trials, possibly due to the decreased bioavailability of oral VC. Because human erythrocytes express high levels of Glut1, take up DHA, and reduce it to VC, we tested how erythrocytes might impact high dose VC therapies. Cancer cells are protected from VC-mediated cell death when co-cultured with physiologically relevant numbers of erythrocytes. Pharmacological doses of VC induce oxidative stress, glutathione (GSH) depletion, and increased glucose flux through the oxidative pentose phosphate pathway (PPP) in erythrocytes. Incubation of erythrocytes with VC induced hemolysis, which was exacerbated in erythrocytes from G6PD patients and rescued by antioxidants. Thus, erythrocytes protect cancer cells from VC induced oxidative stress and undergo hemolysis in vitro, despite activation of the PPP. These results have implications on the use of high dose VC in ongoing clinical trials and highlight the importance of the PPP in the response to oxidative stress.

Consistent with its function as a potent reducing agent essential for numerous biological reactions, VC can be readily oxidized to dehydroascorbate (DHA) in aqueous solution. DHA is transported intracellularly by Glut1 (1) after which it is recycled to reduced VC at the expense of cellular antioxidants, such as GSH (2,3). This paradoxical role for VC as a source of oxidative stress has been found to be selectively toxic to some cancer cells, particularly to those overexpressing Glut1 (4,5). While the benefits of VC supplementation as a cancer therapy have been inconsistent (6), higher doses of intravenous VC have

shown more promise, and several clinical trials are ongoing (7).

Like many cancer cells, human erythrocytes (RBCs) express very high levels of Glut1. The abundance of the Glut1 transporter in erythrocyte membrane allows RBCs to mediate the transport of glucose and DHA at rates that far exceeds their capacity to utilize it (3). There is substantial evidence that erythrocytes participate in VC recycling *in vivo* (8,9). In addition, erythrocytes have also been found to protect both tissues and cells from H_2O_2 mediated damage through their high-capacity redox systems (10). We hypothesized that erythrocytes might protect cancer cells from VC-mediated toxicity through similar mechanisms. Indeed, we find that physiologically relevant numbers of erythrocytes protected HCT116, A375, and SK-MEL-28 cells from VC mediated toxicity. Erythrocytes incubated with VC showed higher levels of oxidative stress and shifted glucose metabolism towards the oxidative PPP, which produces NADPH for ROS mitigation. Finally, we find that VC induced hemolysis of erythrocytes, which was exacerbated by chemical or genetic inhibition of G6PD and rescued by incubation with β mercaptoethanol. This study broadens our current understanding of VC metabolism by erythrocytes and has implications on the use and monitoring of VC in the clinical setting.

RESULTS

Erythrocytes Protect Cancer Cells from VC and H_2O_2 Induced Cell Death—The selective toxicity of VC to *KRAS* and *BRAF* mutant colorectal cancer cells is due, in part, to the high expression levels of Glut1 and preferential uptake of oxidized DHA by the facilitative glucose transporter (5). Consistent with their very high expression of Glut1, erythrocytes have also been shown to transport DHA efficiently (11). We compared the kinetics of DHA uptake by erythrocytes and HCT116 at physiological concentrations of glucose and found that erythrocytes accumulated intracellular VC much more rapidly than HCT116 cells (Figure 1A). Consistent with the previous studies on the accumulation of ascorbate in erythrocytes (8), we found that accumulation of VC by RBCs peaked rapidly (~1 minute) and decreased slightly over time consistent with the slow release of recycled ascorbate back into the media. In contrast, the kinetics of DHA uptake by HCT116 cells was delayed and increased slowly over 30 minutes. The rapid uptake and reduction of DHA by erythrocytes is consistent with the undetectable levels of DHA in healthy human serum (12).

Given the rapid kinetics of DHA uptake by erythrocytes, we hypothesized that RBCs might protect cancer cells from VC mediated toxicity. First, we determined the sensitivities of HCT116 and two *BRAFV600E* mutant melanoma cell lines to VC. While HCT116 cells were the most sensitive to VC, both BRAF mutant melanoma cell lines were also sensitive to VC-induced cell death at physiological glucose concentrations (Figure 1B). The intravenous administration of high dose ascorbate has the ability to increase serum ascorbate to >25 mM (7). Thus, all three mutant cells showed sensitivity to VC at pharmacologically relevant doses. Next, these cancer cells were co-cultured with RBCs and then treated with VC. The addition of as few as ten thousand RBCs significantly protected HCT116, A375, and SK-MEL-28 cells from VC-induced toxicity (Figure 1C, 1D). Higher numbers of erythrocytes offered near complete protection of all three cancer cell lines from VC-mediated toxicity. These striking *in vitro* findings suggest circulating erythrocytes might exert modifying effects on VC toxicity in the *in vivo* setting.

The protective effects of RBCs against oxidative stress were not limited to VC. Consistent with reports that RBCs can protect tissues from hydrogen peroxide (H_2O_2)-induced tissue damage (10), we found that RBCs could protect cancer cells from H_2O_2 mediated cell death. After first determining the sensitivity of each of the

cell lines to H_2O_2, we also found that as few as ten thousand RBCs could also protect cancer cells from H_2O_2 toxicity (data not shown). Moreover, the addition of both VC and H_2O_2 resulted in a synergistic toxicity to the cancer cells lines. Co-culture with erythrocytes provided protection against this synergistic oxidative stress (data not shown).

Metabolomic Flux Analysis of RBCs Treated with VC—Despite the high redox capacity of erythrocytes, the uptake and recycling of large amounts of DHA might present an oxidative burden to erythrocytes. To test this, erythrocytes were incubated with the ROS sensitive dye, 2',7'–dichlorofluorescin diacetate (DCFDA). We found that VC increased the intracellular ROS of erythrocytes in a dose dependent manner. The amount of oxidative stress induced by VC was comparable to similar concentrations of H_2O_2 (Fig 2A). Consistent with GSH's function as a the principal cellular antioxidant responsible for the recycling of DHA by erythrocytes (8), we found that erythrocyte GSH levels decreased significantly upon incubation with VC (Figure 2B).

The regeneration of GSH from oxidized glutathione (GSSG) requires NADPH and GSH reductase. Keratinocytes, fibroblasts, and cancer cells can respond rapidly to oxidative stress by routing glucose flux through the oxidative pentose phosphate pathway (PPP) to generate NADPH (13,14). Because erythrocytes lack organelles to mount a transcriptional response to oxidative stress, the reduction of NADP+ to NADPH might constitute an especially important facet of the response against RBC oxidative stress. DHA has previously been shown to stimulate oxidation of glucose through the pentose phosphate pathway (9). To quantitate the impact of oxidative stress on glucose flux in RBCs, we used [1,2-^{13}C]glucose to monitor the labeling of glucose-derived metabolites. In this labeling scheme, m+1 labeled metabolites from the lower half of glycolysis (i.e., dihydroxyacetone phosphate (DHAP), phosphoenolpyruvate (PEP), and pyruvate) arise from flux through the oxidative PPP and m+2 labeled metabolites, from glycolysis (Figure 2C). Importantly, these measurements underestimate total flux through the oxidative PPP as they report only carbons flowing back into the pools of glycolytic intermediates as opposed to supplying ribose-phosphate pools for nucleotide biosynthesis and other branch pathways. In unperturbed erythrocytes, ~4-8% of lower glycolytic intermediates were labeled as m+1. After treatment with VC, the fractional abundance of m+1 labeled products increased significantly, as much as 3-fold for some metabolites (Figure 2D, supplemental data). Similar changes were observed in H_2O_2 treated cells. Analysis of other labeled isotopologues revealed significant decreases in the levels of m+2 labeled isotopologues consistent with decreases of up to 20% in the glycolytic pathway after VC treatment (Figure 2E, supplemental data). Interestingly, changes in glucose flux were actually less pronounced in RBCs treated with 1.0 mM VC or 0.1 mM H_2O_2 than those treated with 0.2 mM VC. High levels of oxidative stress can overwhelm the antioxidant systems of RBCs and compromise RBC membrane integrity and metabolism (15). Thus, we speculate that high levels of oxidant stress may irreparably damage RBC and render them metabolically inactive.

Hemolysis in Erythrocytes Treated with VC—Incubation of RBCs with VC results in an induction of oxidative stress, depletion of GSH, and increased flux through the PPP. These findings are consistent with the PPP's key role in producing NADPH for RBCs, and suggest that inhibition of the PPP could damage VC-treated RBCs. Although several cases of VC-induced hemolysis have been reported in the literature (16-18), the mechanism of this hemolysis has not been described in the literature. Importantly, all patients reported to have significant VC-induced hemolysis were found to possess a

genetic deficiency of glucose-6-phosphate dehydrogenase (G6PD), a key upstream enzyme of the enzyme of the PPP. First, we tested whether VC might induce hemolysis in normal erythrocytes *in vitro*. Indeed, we found that pharmacological concentrations of VC induced significant hemolysis as measured by both hemoglobin release and decreased RBC number, with complete hemolysis noted at 1.6 mM VC (Figure 3A). At lower concentrations of VC, hemolysis was more striking at later time points, likely reflecting the depletion of glucose from the incubation media (Figure 3B). Consistent with an important role for the PPP in maintaining RBC redox, dehydroepiandrosterone (DHEA), a PPP inhibitor, significantly exacerbated VC-induced RBC hemolysis under all conditions (Figure 3A, 3B). Moreover, erythrocytes from a G6PD patient showed increased oxidative stress both at baseline and also in response to VC and H_2O_2 treatments (Figure 3C). G6PD erythrocytes were also significantly more susceptible to VC induced hemolysis (Figure 3D). The incubation of normal RBCs with β-mercaptoethanol (β-ME), an antioxidant, partially rescued RBCs from both VC-induced oxidative stress (Figure 3E) and hemolysis (Figure 3F).

DISCUSSION

Our findings that erythrocytes can protect cancer cells from VC induced oxidative stress extend previous studies suggesting an antioxidant role for erythrocytes. While most clinical trials involving high-dose VC include G6PD deficiency as exclusion criteria, we suggest that all patients at risk of hemolysis be excluded from such trials. Consistent with this suggestion, a recent randomized, double-blind, placebo controlled trial found that oral supplementation of VC significantly worsened hemolytic markers in sickle cell anemia and β-thalassemia patients, contrary to their expectations (19). While the presence of sufficient serum glucose might mitigate hemolysis in ongoing high-dose VC trials, low levels of hemolysis might even be detectable in otherwise healthy patients. To test this possibility, clinical monitoring of hemolytic parameters could be considered in future or ongoing clinical trials. Moreover, our studies suggest that VC alone may have limited utility in blood storage where it has been tested extensively (20,21). Rather, preservation of erythrocytes in solutions with glucose and alternative anti-oxidants might better prevent RBC oxidative stress and hemolysis and promote longer-term erythrocyte stability during storage.

Our results also highlight the importance of glucose flux through the oxidative PPP as a critical component in the response to oxidative stress. While many studies have focused on glucose's roles as an energy source and biosynthetic intermediate, more recent studies have begun to probe the role of glucose flux through the PPP in the response to oxidative stress (13,22). We speculate that the dependence of RBCs on the PPP in the response to oxidative stress might help explain why they have evolved to express extraordinarily high levels of the Glut1 transporter. It will be interesting to test whether other cell types that express high levels of glucose transporters show a similar dependence on glucose in the response to oxidative stress.

EXPERIMENTAL PROCEDURES

Cell lines, Cell culture, RBC Co-culture, and Treatments—A375 and SK-MEL-28 cell lines were cultured in DMEM (Gibco 11965) supplemented with 10% FBS and 1% Penicillin-Streptomycin. HCT116 cells were cultured in the same medium with 5% FBS. For VC and/or H_2O_2 treatment, cells were seeded at a density of 5000 cells/well in a 96-well plate in 100 μl DMEM (Gibco A14430) supplemented with 5 mM glucose and 10% heat inactivated FBS. After 36 hours, 100 μl of fresh medium containing VC and/or H_2O_2 was added to the existing culture, and cells were cultured for an additional 24 hours. For co-culture experiments, human RBCs

(RBCs) were added to the culture just prior to supplementation with VC and/or H_2O_2. After 24 hours, the RBCs were gently washed out before measuring cell viability. Cell viability was determined through the Dimethylthiazolyl Diphenyltetrazolium Bromide MTT (ThermoScientific) assay according to the manufacturer's instructions. Cancer cells cultured in the absence of RBCs and treatments were used as the reference to calculate the relative survival of the different experimental conditions. Cell viability was also separately determined by counting cells that were seeded at a density of 1×10^5 cells/well in a 12-well plate in 1 ml of medium. Cells were trypsinized and counted in the presence of Trypan Blue. For β-mercaptoethanol (β–ME) rescue experiments, 0.5 mM VC was added first to the RBCs, followed quickly by the indicated amount of β–ME. Because the toxicity of VC varied between 96-well and 12-well plates, the concentration of VC used in MTT and cell counting experiments were independently determined.

Patient Enrollment and Preparation of RBCs—Erythrocyte analyses were approved by the institutional review board, and written informed consent was received from participants prior to inclusion in the study. The G6PD patient had a documented G6PD deficiency of 0.8 IU/g Hb (reference: 8.8 to 13.4). Venous blood was collected from volunteers in heparinized tubes; anticoagulated blood was centrifuged at 500g for 10 min at 4°C and the leukocytes were aspirated. The RBC pellet was then washed three times with DPBS under the same conditions. Washed cells were resuspended in PBS at ~80% hematocrit and kept at 4°C until use. RBCs were used on the day of collection.

Intracellular ROS and GSH assay—2×10^7 RBCs were plated in 6 well plate in 2ml DMEM (Gibco A14430) supplemented with 5 mM Glucose and 10% heat inactivated FBS at 37°C in at 5% CO_2. ROS levels in RBCs were quantified by BD FACSCalibur flow cytometer using 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) staining. For H_2O_2 treatment, 5 μM DCFDA was first added to the cells, followed quickly by the addition of different doses of H_2O_2; samples were collected after 30 min. For VC treatment, VC was first added to the RBCs, after a 2 hr incubation, 5μM DCFDA was added and cells were collected after 30 min. For collection, the samples were placed on ice, centrifuged at 1500 rpm for 4min, gently washed once with FACS buffer (DPBS with 2% FBS and 0.2 mM EDTA), and resuspended in FACS buffer for analysis. For cancer cell ROS measurements, cells were seeded at a density of 1×10^5 cells/well in a 12 well plate in the same medium as described for VC and H_2O_2 treatment. After 24 hours, 1×10^6 RBCs were added to indicated wells. Next, H_2O_2 was added to the cells, followed quickly by the addition of DCFDA. Cancer cells were collected after 30 min for FACS analysis. For intracellular GSH measurement, RBCs were treated with VC or H_2O_2 for 120 or 30 min respectively, quickly collected on ice, gently washed once with ice-cold culture medium, and then lysed in 200 μl GSH assay buffer. GSH was measured using the GSH Colorimetric Assay Kit (Biovison) following the manufacturer's instructions.

[1,2-^{13}C]glucose Flux Analysis—2×10^7 RBCs were added to 5ml of medium supplemented with 5 mM [1,2-^{13}C]glucose or unlabeled glucose and 10% dialyzed FBS with or without vitamin C or H_2O_2. RBCs were carefully dispersed after addition to the media. After 6 hours, cells were collected by centrifugation at 1500rpm for 10 min at 4°C. The media was aspirated and the cells frozen in liquid nitrogen, lysed with three freeze thaw cycles in 1 mL of 50% methanol (MeOH), then centrifuged to remove protein debris. The lysates were dried, methoximated and derivatized with 10mg/ml methoxyamine (Fisher PI-45950) at 70°C for 10 min followed by 105 ml MTBSTFA (Sigma 394882) at 70°C for 1 hour. After derivitization, 1 ml of the sample was injected onto an Agilent

6970 gas chromatograph networked to an Agilent 5973 mass selective detector. The abundance of the following ions were monitored: *m/z* 484-487 for DHAP, *m/z* 261-264 for lactate, *m/z* 453-456 for PEP and *m/z* 174-177 for pyruvate.

DHA uptake assay—Transport measurements were performed as previously described with modifications (11,23). Ascorbic acid, L-[1-14C] (ARC 1569) was purchased from American Radiolabeled Chemicals. Labeled ascorbic acid purchased from PerkinElmer did not perform as reported in control uptake assays. HCT116 cells were seeded in triplicate in 12-well plates (250,000 cells/well) for 18-24 hrs, washed twice with PBS, and incubated with serum-free DMEM containing 0.1% BSA for 2 hrs. Cells were then washed and incubated in a Ringer's solution (126 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM HEPES, 2.5 mM glucose, 1 mM $CaCl_2$, 0.1% BSA, pH 6.0). Ascorbate oxidase (10U/ml) was used to convert [^{14}C]-VC to [^{14}C]-DHA, and transport was initiated by the addition of 0.5 μCi of [^{14}C]AA and 100 μM cold AA to each well. Uptake assays were performed for 0, 1, 3, 9, and 27 min at RT. Transport was terminated by the removal of uptake solution followed by 4 washes with ice-cold stop solution containing 100 μM phloretin and 10 μM cytochalasin B in PBS. For DHA uptake in RBCs, 20 μl of erythrocyte suspension containing 2.5×10^6 cells in Ringer's solution was used for each reaction. Uptake was initiated by adding 100 μl of Ringer solution containing 0.5 μCi and 100 μM cold AA to each sample for the indicated time points. Transport was terminated by the addition of ice-cold stop solution. Cells were centrifuged at 16,000x*g* for 1 min followed by three additional washes. Both HCT116 and pelleted erythrocytes were lysed in 0.5ml of 0.2% SDS. 375 μl of the lysate was quantitated by liquid scintillation counting.

RBC hemolysis assay—Washed RBCs were resuspended in Ringer's solution at a cell density of 1×10^7 cells/ml. RBC suspensions were preincubated with 25 μM DHEA or DMSO control at 37°C with shaking for 15 min prior to treatment with VC. For antioxidant rescue experiments, RBCs were preincubated with Ringer's solution supplemented with the indicated amounts of β–ME. Aliquots of 2 ml RBC suspension containing 1×10^7 cells/ml were used in each replicate. RBC suspensions were incubated with various concentrations of freshly prepared VC at 37°C for indicated time points. For time course experiments, 0.6 mM VC was used for all time points. RBC hemolysis was determined by both absorbance at 540 nm and cell count from the same sample. Absorbance at 540 nm from hemoglobin released into RBC supernatants was normalized to the absorbance of RBCs completely hemolysed with 3 mM VC (100% hemolysis).

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Footnote: The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contributions: ZZ, EL, RD, and RW designed the experiments; ZZ, EL, and JS performed experiments; ZZ, EL, JS, and YY acquired data; ZZ, EL, JS, RD, and RW analyzed data; DG and AZ enrolled volunteers; ZZ and RW wrote the manuscript with contributions from EL, JS, and RD.

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Abbreviations: BRAF, Proto-oncogene v-Raf murine sarcoma viral oncogene homolog B; DCFDA, Dichlorofluorescin diacetate; DHA, Dehydroascorbate; DHAP, Dihydroxyacetone phosphate; DHEA, Dehydroepiandrosterone; Glut1, Solute carrier family 2, facilitated glucose transporter member 1; GSSG, Glutathione disulfide; KRAS, Proto-oncogene V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; PEP, Phosphoenolpyruvate; PPP, Pentose Phosphate Pathway; VC, Vitamin C

FIGURE LEGENDS

FIGURE 1. Erythrocytes protect cancer cells from VC and H_2O_2 toxicity. **(A)** ^{14}C-dehydroascorbate (DHA) uptake assay demonstrates that RBCs accumulate DHA more rapidly than HCT116 cells. **(B)** HCT116 and *BRAF* mutant melanoma cells (A375 and SK-MEL-28) show a dose dependent toxicity to VC mediated toxicity as assessed by MTT. **(C)** Photomicrographs of A375 cells reveals that VC treatment (0.6 mM for 24 hrs) caused cytotoxicity. Co-culture with RBCs (indicated by arrows) rescues cell viability. **(D)** The co-culture of RBCs with HCT116, A375, and SK-MEL-28 cancer cells protects them from VC-induced toxicity as assessed by MTT assay. An equal number of cells cultured without VC or RBCs was used as the reference (100%) to calculate relative survival (n=3, error bars = SD; ANOVA with Dunnett's correction; * P≤0.05, ** P≤0.01, ***P≤0.001, ****P≤0.0001). **(E)** The co-culture of RBCs with HCT116, A375, and SK-MEL-28 protects them from VC-induced toxicity as assess by cell number.

FIGURE 2. Erythrocytes show increased oxidative stress, decreased GSH, and increased glucose flux through the oxidative PPP after treatment with VC. **(A)** FACS analysis of erythrocytes treated with increasing amounts of VC (0, 0.1, 0.2, 0.4, 0.6, and 1 mM) or H_2O_2 (0, 0.05, 0.25, 1 mM) show increased levels of ROS as indicated by DCFDA fluorescence. The grey curve represents a control without DCFDA. **(B)** Intracellular GSH decreases after incubation with VC. **(C)** Schematic of [1,2-^{13}C]glucose metabolic tracing experiments. Some of the glucose proceeding through the PPP result in the generation of m+1 labeled products. However, some products of the oxidative PPP will not be detected as m+1 labeled products (dashed arrows). Red boxes highlight the subset of monitored products. **(D)** Quantitation of labeled products in metabolic tracing experiments. Incubation of erythrocytes with VC or H_2O_2 results in significant increases of m+1 labeled products, indicating increased flux through the PPP (top row), and significant decreases of m+2 labeled products, indicating decreased flux through glycolysis (bottom row). (n=3, error bars = SD; ANOVA with Dunnett's correction; * P≤0.05, ** P≤0.01, ***P≤0.001, ****P≤0.0001 when compared to control).

FIGURE 3. VC induced hemolysis is exacerbated by G6PD inhibition. **(A)** Dose dependent hemolysis of RBCs by VC as assessed by hemoglobin absorbance (left) and cell counts (right) after 1.5 hours of incubation. Dehydroepiandrosterone (DHEA) significantly exacerbated hemolysis as indicated by red asterisks (n=3, error bars = SD; t-test with Holm-Sidak correction, alpha=0.05). Data represent replicate measurements that are representative of >3 independent experiments. **(B)** Incubation of RBCs with 0.6 mM VC resulted in extensive hemolysis at 8 hrs. DHEA significantly exacerbated hemolysis at 8 hrs (n=3, error bars = SD; t-test with Holm-Sidak correction, alpha=0.05). **(C)** RBCs from a G6PD patient showed higher levels of ROS at baseline, when treated with VC (0.1 and 0.2 mM), and when treated with H_2O_2 (0.1, 0.2 mM) compared with control RBCs as indicated by DCFDA fluorescence. **(D)** RBCs from a G6PD patient showed significantly higher levels of hemoly-

sis in response to incubation with VC (1.0 mM, 1.5 hrs) compared to control RBCs (n=3, t-test, P≤0.0001). **(E)** Incubation of RBCs with β-mercaptoethanol rescued VC-induced oxidative stress. **(F)** Incubation of RBCs with β-ME resulted in a dose-dependent rescue of hemolysis (right) (n=3, t-test, P≤0.0001).

FIGURE 1

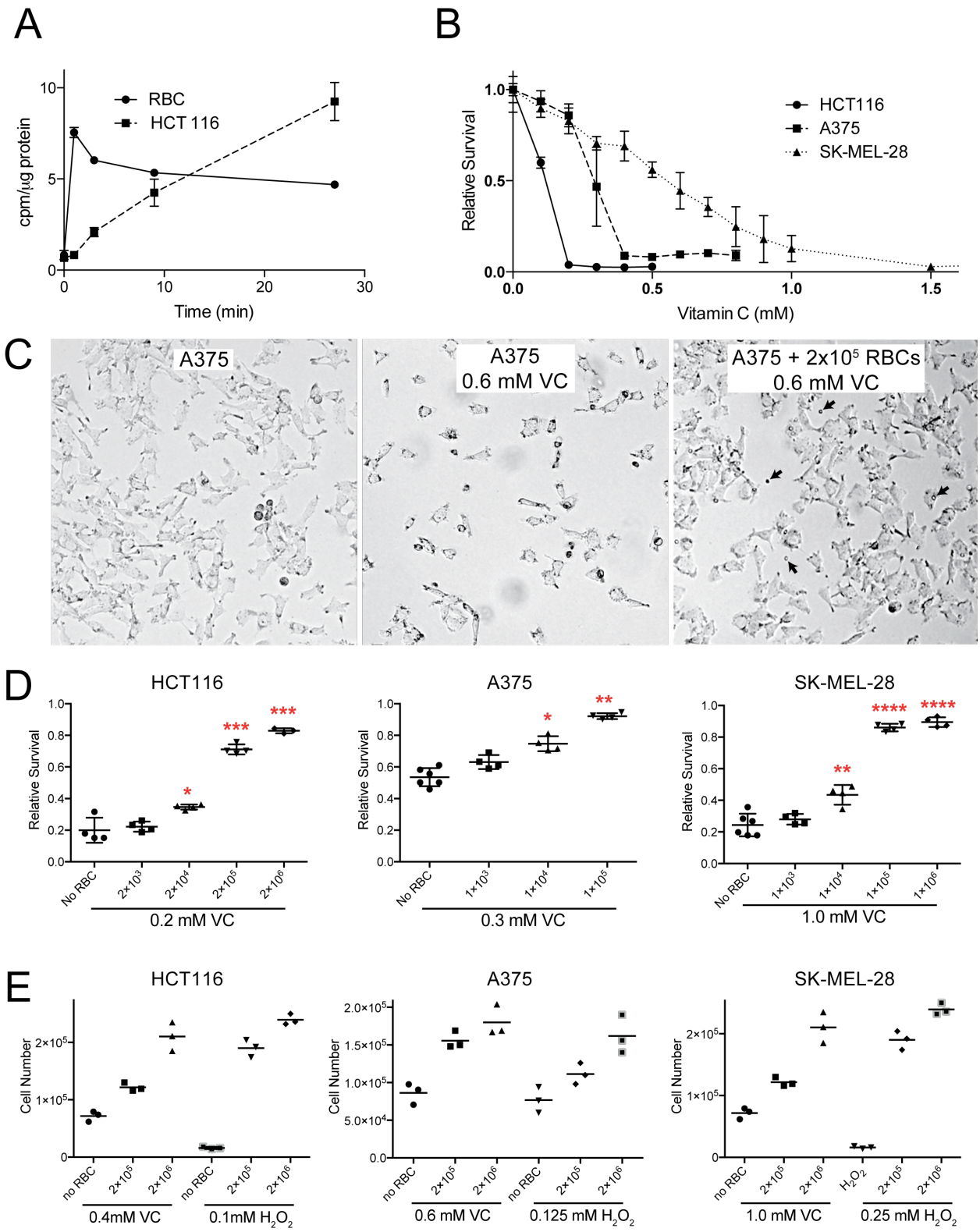


FIGURE 2

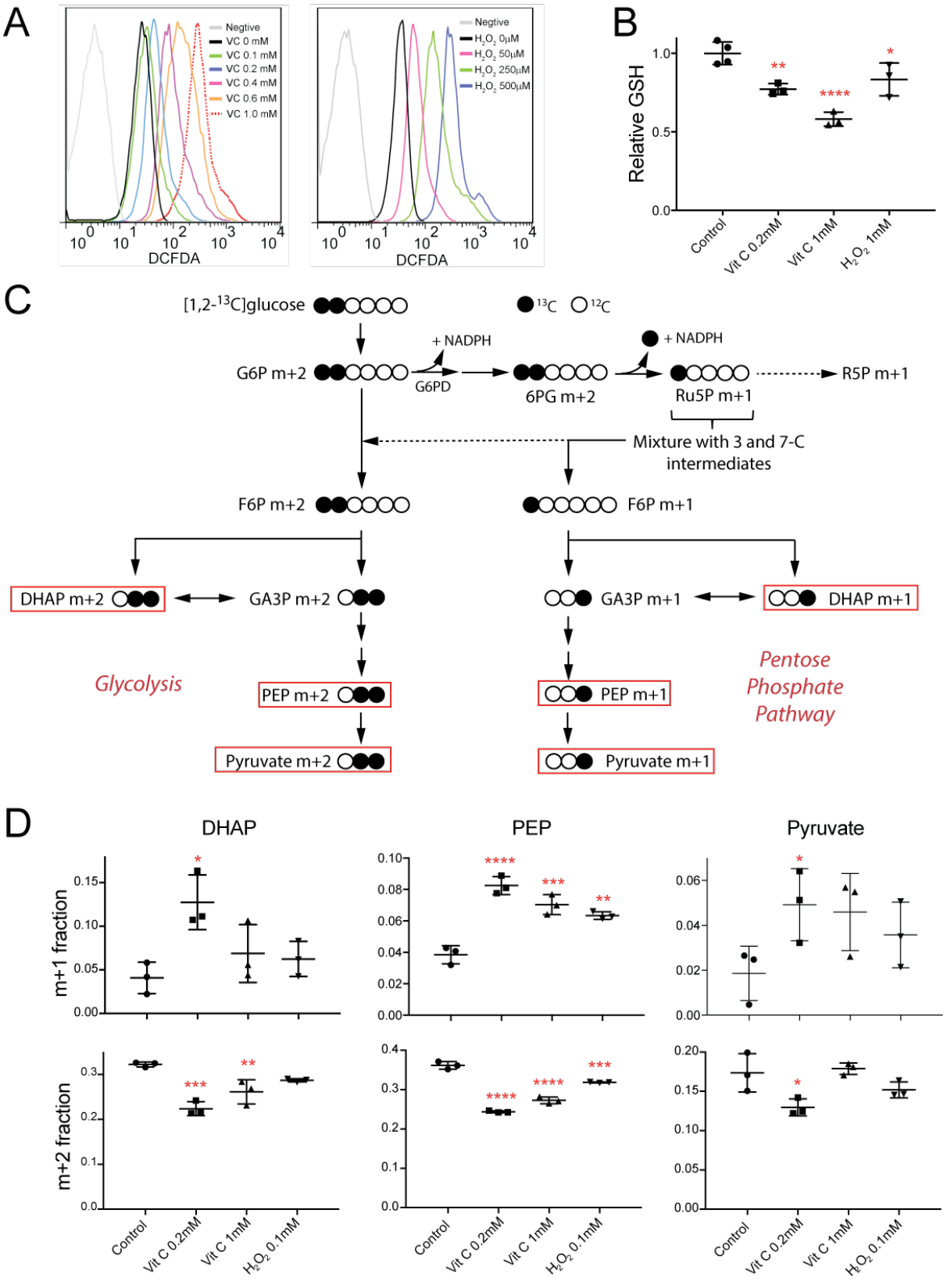
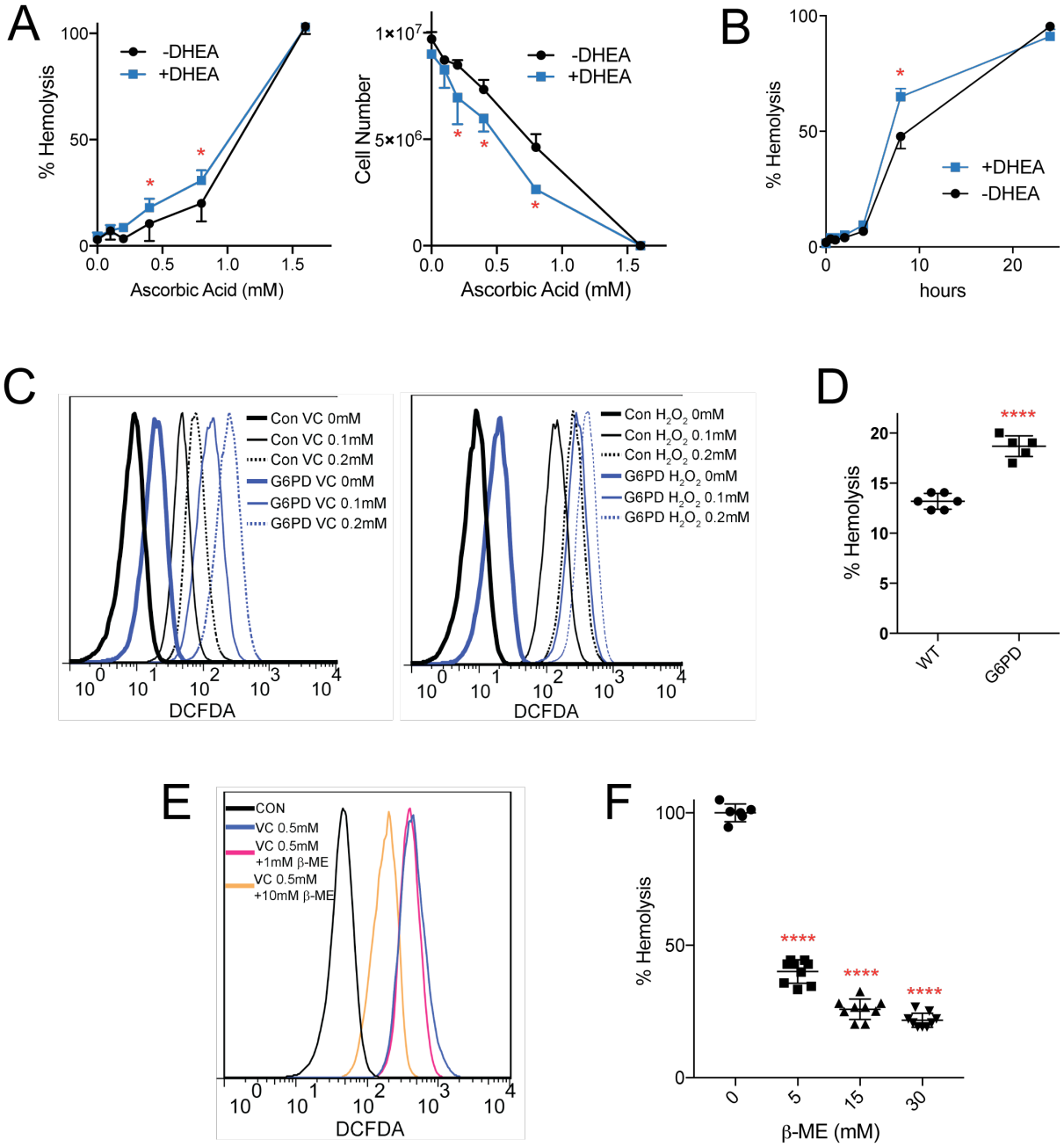


FIGURE 3



Glutathione Depletion, Pentose Phosphate Pathway Activation, and Hemolysis in Erythrocytes Protecting Cancer Cells from Vitamin C Induced Oxidative Stress

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