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RESEARCH ARTICLE

In vitro regulation of reactive oxygen species formation in red blood cells of homozygous sickle cell patients using Vitamin C

[version 1; peer review: 2 approved with reservations]

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Abstract

Background: Sickle cell patients produce more reactive oxygen species (ROS) than healthy individuals, leading to increased cell membrane damage. Theoretically, reducing ROS formation would preserve red cell membranes of sickle cell patients. Vitamin C is a powerful anti-oxidant capable of inhibiting ROS formation in a variety of situations, by functioning as an electron donor to reduce molecular oxygen. This study aimed to determine whether Vitamin C reduced ROS formation in sickle red cells.

Methods: 27 homozygous (HbSS) patients were recruited from the outpatient clinics of Lagos University Teaching Hospital, Nigeria, and annex at the Sickle Cell Foundation, Lagos, Nigeria. Demographic information and EDTA patient blood samples were collected. The test group were red cells preincubated in 80uM and 100uM Vitamin C concentrations before stressing with tertbutylhydroperoxide. These were compared to stressed matched controls preincubated in phosphate buffered saline. Cell staining was done with CellRox Orange followed by flow cytometry to quantify ROS.

Results: ROS count for Vitamin C pre-treated red cells was significantly lower than matched controls ($p < 0.001$). Average ROS count for 80uM test samples was 27.5/ul (95% CI, 17.5 to 72.5) and for 100uM 3.9/ul (95% CI, 1.9 to 5.9). Male gender was significantly associated with elevated baseline ROS count ($p = 0.03$).

Conclusion: Vitamin C reduced ROS formation in HbSS cells. Future studies should focus on a role for Vitamin C as a safe, cheap addition to maintenance therapy of sickle cell patients.

Keywords

Regulation, Reactive oxygen species, HbSS patients, Vitamin C

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Introduction

Sickle cell anemia is a single-nucleotide polymorphism due to substitution of glutamic acid by valine at position 6 of the B-globin chain¹. In the homozygous (HbSS) state, this occurs on both B-globin chains leading to severe disease. Paulson *et al.* demonstrated a direct quantitative effect of the sickle gene pair on sufferers². The HbS molecule polymerizes easily under stress conditions with formation of insoluble polymers, which distort red cell shape, leading to membrane damage and eventual red cell breakdown¹. Conditions implicated in sickle red cell stress include hypoxia, infections and dehydration. These cause formation of reactive oxygen species (ROS), which oxidatively damage cell membranes³. ROS has a multiplier effect leading to more ROS generation, establishing a positive feedback system, the end-result of which is damage to red cell membranes⁴. Sickle red cells produce greater amounts of ROS than normal cells and are also more susceptible to oxidation⁵.

ROS formation is maximal during the reperfusion state when there is increased formation of methemoglobin and superoxide ion due to electron transfers that occur between the heme iron and oxygen⁶. Comparatively, HbS molecules auto-oxidize more than the HbA molecules and also have a poor mechanism to clear generated superoxide ion⁷. Consequently, the lifespan of the sickle red cell is about 1/5th that of the normal red cell⁸.

Theoretically, reducing the ROS formation would ameliorate HbS molecule and lipid membrane polymerization and improve red cell life span. In a flow cytometric comparison of ROS production in normal vs thalassaemic red cells, significantly higher ROS generation in thalassaemic red cells was noted⁵. In addition, pretreatment of the red cells with N-acetyl-L-cysteine ameliorated the generation of ROS in both the normal and thalassaemic cells after the cells were stressed with 2',7'-dichlorofluorescein diacetate, a potent inducer of ROS formation. Furthermore, Furman *et al.* found that sickle red cell pretreatment with Ginkgo biloba extract prior to cell stressing also ameliorated ROS, and reduced membrane polymerization and formation of methemoglobin⁹.

Vitamin C is a powerful anti-oxidant, working as an electron donor capable of reducing molecular oxygen^{10,11}, making it important in a variety of physiological processes and reactions in the human body, including, fatty acid transport¹², synthesis of collagen¹³, prostaglandin metabolism¹⁴ and neurotransmitter synthesis¹⁵. Vitamin C is almost completely absorbed in the distal small intestine via energy-dependent processes¹⁶ and exhibits a steady-state concentration at oral doses of 200–400mg/day, corresponding to plasma concentrations between 60–100µmol/L¹⁷. Vitamin C is cheap, well-studied and readily available, making it attractive to study as a regulator of ROS formation in sickle red cells.

Statement of the problem

Nigeria has the highest number of people with sickle cell disease (SCD) in the world, with an estimated 91,000 babies born with the condition yearly¹⁸. This number is expected to grow. This means that Nigeria and other countries with a high disease burden will continue to require research to improve policies for prevention and

management of sickle cell patients. Catastrophic financial burden has occurred in families of sickle cell patients evaluated in a study in Ekiti State, Nigeria¹⁹. Many of these families spent over 10% of family income to cover hospital admissions of an SCD patient and greater than 90% of these families had no health insurance and had to borrow to meet their financial needs. This study investigated a possible role for Vitamin C, a cheap drug, in reducing ROS formation in sickle cell patients.

Methods

Subject recruitment and collection of samples

Institutional approval was given by the Lagos University Teaching Hospital Health Research Ethics Committee (Assigned number: ADM/DCST/HREC/APP/1533). 27 HbSS patients were recruited from the Sickle Cell Clinic of Lagos University Teaching Hospital (LUTH) and LUTH clinic annex at Sickle Cell Foundation of Nigeria, Lagos Office between May 22–25, 2017. These patients were known HbSS patients documented on file and previously confirmed by hemoglobin electrophoresis. Every 3rd HbSS patient who presented in the clinic and met the inclusion criteria was enrolled in the study after written informed consent was obtained in accordance with the Declaration of Helsinki. Afterwards, a screening questionnaire was applied to obtain each candidate's demographics followed by weight and height measurements. Four volunteers declined height and weight assessment. 4mls of fresh blood was then drawn from the antecubital vein of each enrolled candidate into EDTA tubes. All samples were analyzed within 4 hours of collection.

Inclusion criteria. Patients of all ages who did not receive blood transfusion within 3 months and had not taken Vitamin C or multivitamin supplements within 3 months of the study were recruited. Patients taking hydroxyurea were included if they had been on a stable dose for at least 3 months.

Exclusion criteria. Blood transfusion within 3 months of study; acutely-ill patients; use of Vitamin C or multivitamin supplements within 3 months.

Materials and reagents

Gibco Phosphate-buffered saline (PBS; catalog number 20012043; Life Technologies, Grand Island, New York). CellRox Orange Flow Cytometry Assay kit (catalog number: C10493; Life Technologies). The kit includes the fluorophore, CellRox Orange reagent; N-acetyl cysteine (NAC; an antioxidant) and tert-butyl hydroperoxide solution (TBHP; to induce ROS). CellRox Orange reagent localizes to the cell cytoplasm and has absorption/emission maxima of 545/565 nm respectively²⁰. L-Ascorbic acid powder (CSPC Weisheng Pharmaceutical, China); a 200µM solution of Vitamin C was prepared in cold PBS and protected from light.

Sample preparation

Sample preparation and analysis were done at the Nigerian Institute of Medical Research - Human Virology Lab (NIMR-HVL), an ISO-certified lab in Yaba, Lagos. Pre-wash red cell counts for all samples were obtained from the hematology analyzer before centrifugation at 500xg for 10 minutes and supernatant removal. Cells were washed thrice in cold PBS and finally re-suspended in 5mls

of PBS followed by determination of post-wash red cell count. This step was an adaptation of a previous process²¹.

Cell treatment

The protocol for this step was adapted from Life Technologies standardized protocol²⁰. For each sample, volume corresponding to 5×10^5 cells was pipetted into four different micro-centrifuge tubes. One was immediately incubated with CellRox orange stain at a final concentration of 500nM and ROS quantified on the Partec Cyflow Counter Version 2.4 to determine basal ROS present per sample. Another tube was incubated with PBS and then stressed at final concentration of 200uM TBHP to serve as control. Two other tubes were pre-treated with 80uM and 100uM concentrations of Vitamin C respectively, before stressing with 200uM TBHP to represent the test samples. In a supplemental study, nine samples were non-randomly selected and incubated with 200 uM NAC followed by TBHP stressing. Incubation time per added reagent was 30 minutes.

Cell staining and flow cytometry

CellRox Orange was prepared with DMSO and then added to all the samples described above at a final concentration of 500nM. After 30 minutes of incubation, 800uL of the solution was transferred into Rohren tubes for immediate analysis on the flow cytometer. A total of 5×10^5 cells were analysed and pulsed gating was used to exclude doublets.

Statistical analysis

The primary outcome measure was a comparison of the quantity of ROS formed following stressing of test vs control cells. Secondary analyses evaluated the relationship between gender, age, BMI and hydroxyurea use on basal ROS in sickle red cells. Microsoft Excel Version 14 was used for analysis. Analysis of variance (ANOVA) was used for statistical comparisons (significance defined by P values ≤ 0.05).

Results

Characteristics of the participants

27 participants were recruited into the study. The sample was equally distributed between males and females (Table 1). Blood transfusion rate in this population was lower than among Congolese sickle cell patients²², but higher than for U.S Medicaid patients seen between 2007–2012²³. Hydroxyurea utilization was higher than among Florida Medicaid patients²⁴ and improved over a previous report from a major Nigerian Teaching Hospital²⁵.

Red cell count

Average pre-wash red cell count was $2.89 \times 10^{12}/L$. The average male red cell count was higher than females (Table 2). Post-wash red cell count was calculated after final cell suspension. Volume equivalent of 5×10^5 red cells was calculated from the post-wash red cell count (Table 2).

Table 1. Baseline characteristics of study participants.

Characteristic	N	%
Age		
<10	2	7.4
10–19	3	11.1
20–29	15	55.6
30–39	4	14.8
40–49	3	11.1
Gender		
Male	14	51.9
Female	13	48.1
BMI		
Underweight (<18.5)	10	33.3
Normal (18.5–24.9)	11	40.7
Overweight (≥ 25)	2	7.4
Data missing	4	18.5
Employment status		
Employed	6	22.2
Unemployed	21	77.8
History of blood transfusion		
Transfused*	13	48.1
Never transfused	14	51.9
Hydroxyurea status		
Currently on hydroxyurea	9	33.3
Not on hydroxyurea	18	66.7

* refers to patients transfused at a time earlier than 3 months prior to study – refer to exclusion criteria.

Relationship between baseline ROS count and patient characteristics

Baseline ROS count (BRC) refers to number of ROS per ul before any red cell intervention. Average total BRC was 441.9/ul (Table 2). When BRC was matched in terms of gender, men were found to have a significantly higher count than women, with average ROS count of 583.1/ul (95% CI, 373.1 to 793.1; p-value = 0.03; Table 3). Average BRC for those ≥ 20 years was higher than those <20 years; those with BMI ≥ 18.5 had higher average ROS than those <18.5; and those on hydroxyurea also had higher average basal ROS than those not taking hydroxyurea.

Table 2. Baseline characteristics, red cell count and reactive oxygen species formation per sample for each reaction.

Samples	Gender	Age	BMI	HS	Red cell count ($\times 10^{12}/L$)		ROS count per ul			
					Prewash	Postwash	Baseline	Control	80uM Vit. C	100uM Vit. C
1	M	0	N/D	0	2.97	2.05	348	670	2	2
2	M	0	1	0	2.80	1.92	668	793	1	1
3	M	0	0	0	2.90	1.96	339	1342	3	1
4	M	1	0	1	2.64	1.81	318	1768	3	3
5	F	0	1	0	3.12	2.19	401	1360	2	2
6	F	0	1	0	2.46	1.72	359	1171	627	3
7	M	0	0	0	3.19	2.04	183	339	4	3
8	F	0	1	0	2.43	1.72	205	470	4	2
9	M	1	N/D	1	2.81	1.78	441	561	1	1
10	F	0	1	0	3.11	1.84	257	729	6	1
11	F	1	N/D	1	2.38	1.63	330	562	18	0
12	F	0	0	0	2.78	1.97	272	907	3	2
13	M	0	1	0	3.66	2.61	197	678	5	3
14	F	1	N/D	1	2.85	1.87	305	844	2	1
15	F	0	0	0	2.69	1.74	192	626	2	3
16	F	0	1	0	3.04	1.49	332	1373	5	2
17	M	0	1	0	3.01	1.88	169	714	3	3
18	F	1	1	1	1.88	0.78	47	186	5	5
19	F	0	0	0	2.76	1.69	82	363	3	3
20	F	0	0	0	2.81	1.12	95	250	3	28
21	M	0	0	0	5.62	2.37	464	578	5	4
22	M	0	1	0	2.62	1.40	735	1747	15	6
23	M	1	0	1	1.93	1.35	1539	1322	5	3
24	M	0	1	0	2.52	1.39	1163	1473	4	11
25	M	1	0	1	2.67	1.52	863	1345	5	4
26	M	1	1	1	3.17	1.69	737	836	3	4
27	F	1	1	1	3.45	2.14	890	1029	3	4
Average					2.89	1.76	441.9	890.2	27.5	3.9

M – male; F – female; HS – Hydroxyurea Status; N/D – not determined; Vit.C – Vitamin C. Scoring system – Age: 0 means <20 years and 1 means ≥ 20 years; BMI: 0 means <18.5 and 1 means ≥ 18.5 ; hydroxyurea status: 0 refers to those not taking and 1 to those taking hydroxyurea.

Baseline ROS prediction score

A score of 1 each was given to the male subjects, subjects on hydroxyurea at time of recruitment, age ≥ 20 years and for BMI ≥ 18.5 . A score of 0 was applied in each case for women, those not on hydroxyurea, aged <20 years and BMI < 18.5 (Table 2). Average BRC for subjects with scores ≥ 3 were higher than those with scores 2, though not significant (p-value = 0.11). When each score cohort was matched in terms of gender, the average ROS production for men remained higher than for women without statistical significance (Table 3).

Vitamin C regulation of ROS formation in stressed red cells

ROS count after TBHP stressing of test and matched controls were compared for each sample (Figure 1). The controls had a higher ROS count than test cells. As shown in Figure 2, at both concentrations of Vitamin C, there were significantly less ROS formation than controls (p-value<0.001). Average ROS count for 80uM test samples was 27.5/ul (95% CI, 17.5 to 72.5) and for 100uM test group, it was 3.9/ul (95% CI, 1.9 to 5.9). No statistical difference existed between Vitamin C pretreatment at 80uM and at 100uM (p-value = 0.31).

Table 3. Average total and gender-matched baseline ROS count.

Gender	Average total ROS per ul	P-value	Average total ROS per ul for score ≥ 3	P-value	Average total ROS per ul for score 2	P-value
Male	583.1 (95% CI, 373.1 to 793.1)	0.03	719.0	0.55	463.4	0.37
Female	289.8 (95% CI, 169.8 to 409.8)		468.5		305.5

Figure 1. ROS count per sample matched for control and at 80uM and 100uM Vitamin C pretreatment. **Figure 2. Average ROS count for control and following 80uM and 100uM Vitamin C pretreatment.**

NAC regulation of ROS formation – a supplemental analysis

We compared a subset of controls with NAC-pretreated cells. Samples 1 – 9 were pre-treated with 200uM NAC and the results compared with matched controls pretreated with PBS (Table 4 and Figure 3). NAC at 200uM final concentration reduced ROS formation compared to control. However, no statistical significance was noted (p-value = 0.12).

Dataset 1. Baseline characteristics, measured heights, weights and calculated BMI, red cell count and reactive oxygen species formation per sample

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M – male; F – female; HS – Hydroxyurea Status; Y/N – Yes/No answers for hydroxyurea use and blood transfusion history; Vit.C – Vitamin C; N/D – not determined.

Table 4. Sample cohort comparing positive control with 200um NAC.

Samples	Control (per ul)	NAC-treated (per ul)
1	670	816
2	793	734
3	1342	590
4	1768	571
5	1360	329
6	1171	1221
7	339	633
8	470	581
9	561	263
Average	941.6	637.6
P-value = 0.12

Figure 3. Average ROS count for control and following 200uM NAC pretreatment.

Discussion

Individual characteristics and baseline reactive oxygen species count

Individually, age ≥ 20 years, BMI ≥ 18.5 , hydroxyurea use and the male gender appear to be associated with increased baseline ROS count (BRC). In normal subjects, a statistically significant correlation was described by Kukovetz *et al.* between increasing age and generation of ROS²⁶. However, even though our study did not show a statistical significance between increasing age and increased BRC, an appropriate path would be to investigate this relationship further with larger patient cohorts. Furthermore, this study has revealed an association between BMI ≥ 18.5 and increased BRC. Previously, a relationship between increasing obesity and generation of serum ROS Japanese students was noted²⁷, while increased ROS production has been described in obese diabetics, particularly with abdominal obesity²⁸. We also found that patients on hydroxyurea had a higher average BRC than those not taking hydroxyurea. It has

been shown that hydroxyurea mediated *E. coli* cell death by inducing envelope ROS formation leads to membrane damage and cell death^{29,30}. Elevated ROS formation has also been seen in hydroxyurea-treated yeast cell cytoplasm³¹. Hydroxyurea inhibits ribonucleotide reductase leading to a replication fork arrest³² and accumulation of ROS. Male gender is an independent factor statistically associated with increased BRC ($p < 0.05$) in this study. Previous studies have shown that men have increased oxidative stress levels, more biomarkers of stress, elevated ROS production and lower antioxidant levels^{33–36}. Among sickle cell patients in a Camerounian Hospital, women had a greater total anti-oxidant capacity compared with males³⁷. Our data showed that, on average, men aged ≥ 20 years whose BMI were ≥ 18.5 and who were also on hydroxyurea produced more ROS. This is equivalent to Score 3 on the baseline ROS score described in the Results section. This group might be a target for an ROS-reducing agent. Further studies are required here.

Vitamin C regulation of ROS formation in stressed cells

A significant reduction in ROS formation for all test samples pretreated with 80uM and 100uM concentrations of Vitamin C prior to tert-butyl hydroperoxide stressing vs matched controls (P-values < 0.05) was noted. It is evident that Vitamin C inhibited formation of ROS formation in test cells and may have served to protect the red cell membrane from damage. Guaiquil *et al.* found that increased sensitivity of glutathione-depleted human myeloid cells to membrane damage was significantly reversed by preloading with Vitamin C³⁸. As noted earlier, there is a reduction in ROS formation in murine models with pulmonary contusion treated with Vitamin C¹¹. This finding is significant because if we can reduce ROS formation in sickle cell patients by simply administering Vitamin C, we would theoretically reduce cell injury and improve disease-free intervals for patients. Our study did not find a significant difference in ROS formation between cell cohorts pretreated at 80uM vs 100uM concentrations of Vitamin C. One sample pretreated with 80uM Vitamin C did not show as much reduction in ROS in the degrees seen with other samples (Figure 1). This effect was not noted at incubation with 100uM Vitamin C. This might be due to individual variation in response rates at 80uM concentration. It is possible that extending Vitamin C incubation time beyond 30 minutes might have yielded larger changes.

Rate of cell lysis between treated vs control was not compared in this study. It will be interesting to see if the treated cells were more resistant to lysis than matched controls. Additionally, controlled studies to examine the effect on basal ROS formation of administering Vitamin C to sickle cell patient cohorts over time would be appropriate.

Supplemental information

N-acetylcysteine did not significantly reduce ROS. When compared to Vitamin C, there was a significant difference in efficacy, with Vitamin C showing superiority over NAC. It may help to consider other concentrations of NAC in future experiments.

Conclusion

Vitamin C significantly decreased ROS formation in stressed red cells of sickle cell patients. Future studies are required to evaluate the effect of Vitamin C administration on sickle cell patients.

Data availability

Dataset 1. Baseline characteristics, measured heights, weights and calculated BMI, red cell count and reactive oxygen species formation per sample. M – male; F – female; HS – Hydroxyurea Status; Y/N – Yes/No answers for hydroxyurea use and blood transfusion history; Vit.C – Vitamin C; N/D - not determined. doi, [10.5256/f1000research.12126.d169449](https://doi.org/10.5256/f1000research.12126.d169449)³⁹

Competing interests

No competing interests were disclosed.

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Edeghonghon Olayemi

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The authors set out to investigate a possible in vitro role for Vitamin C in reducing Reactive Oxygen Species (ROS) formation in sickle cell disease patients.

Comments

Abstract

Were there matched controls? How were they selected?

Introduction

The study ignored the role of glucose 6 phosphate dehydrogenase status which plays an important role in protecting red cells against oxidative stress; especially since a significant proportion of the population at the study site will be G6PD deficient. G6PD plays an important role in the production and removal of ROS.

Methods

The sample size is rather small, how was this determined? What was the study design? The inclusion / exclusion criteria did not state if the patients were in steady state or not and if so how was this determined?

The calculation of the baseline ROS prediction score is not clear.

Results

It is known that obesity is associated with increased ROS generation, thus, addition of data from overweight patients and the 4 patients with 'unknown' BMI to those of patients with normal BMI could have adversely affected the results. These patients should be excluded in calculating the association between BMI and ROS.

The finding that men produced more ROS may be related to their G6PD status.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Benign haematology specifically sickle cell disease and coagulation

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 29 August 2017

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Eitan Fibach

Department of Hematology, Hadassah University Hospital, Ein-Kerem, Jerusalem, Israel

The authors measured the in vitro effect of vitamin C on reactive oxygen species generation in RBC of patients with Sickle Cell Disease.

Comments

The data are not entirely novel. The authors should cite previous studies on the oxidative status of RBC from patients with sickle cell disease and their response to antioxidants, including Vit. C. For example, Amer et al.¹

The experimental approach is not clear.

For example:

Abstract

"matched controls" – here and elsewhere – what does it mean? RBC of SCA patients not treated with Vit C? what about normal individuals?

Materials and reagents

"Blood transfusion rate" – Provide data. When were the blood samples obtained? Before/after transfusion?

"Cell staining and flow cytometry" – How were the cells analyzed and ROS determined? "number of ROS per ul" – not clear. "per ul" of what? Before or after washing? Why not calculate the ROS per washed RBC?

Relationship between baseline ROS count and patient characteristics

"Baseline ROS count (BRC) refers to number of ROS per ul before any red cell intervention." What does "before any red cell intervention" mean?

"Baseline ROS prediction score" – Explain "prediction score"

Other comments

Introduction

"Paulson *et al.* " – Change to Pauling *et al.*

"Paulson *et al.* demonstrated a direct quantitative effect of the sickle gene pair on sufferers²." – explain.

"...during the reperfusion state..." – explain.

Table 1. Baseline characteristics of study participants. The parameters should be shown per males and females.

Figure 3. - What does "positive control" mean?

References

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Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

No

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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