Original Paper

Vitamin C Inhibits Aggravated Eryptosis by Hydrogen Peroxide in Glucose-6-Phosphated Dehydrogenase Deficiency

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Key Words

G6PD deficiency • Eryptosis • Hydrogen peroxide • Vitamin C • Anemia • Malaria

Abstract

Background/Aims: The study was aimed to investigate if vitamin C could exert protective effects on development of eryptosis caused by glucose-6-phosphate dehydrogenase (G6PD) deficiency and hydrogen peroxide. **Methods:** Isolated erythrocytes with different G6PD activity (normal or deficient) were divided into various groups treated by either Vitamin C or H₂O₂. Phosphatidylserine (PS) extroversion rate was detected by Annexin V binding. The intracellular Ca²⁺ concentration was detected by Fluo3-fluorescence, and western blot was used to detect the expression of apoptosis factor caspase 3. **Results:** Compared with the blank group, the PS extroversion rate ($P < 0.001$), intracellular Ca²⁺ concentration ($P < 0.001$) and active caspase 3 expression level ($P < 0.05$) of erythrocytes significantly increased after treatment of 0.05% H₂O₂. Then the index of eryptosis significantly decreased after erythrocytes were treated with Vitamin C (1 mg/ml) for 30 min (all $P < 0.05$). The decline in erythrocytes with G6PD normal activity was more significant than those with G6PD deficiency. **Conclusion:** Vitamin C could effectively inhibit the eryptosis contributed by H₂O₂ oxidative stress, and the suppression on eryptosis with G6PD normal activity was more effective than that with G6PD deficiency.

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Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency (OMIM 305900) is a hereditary disorder featured by hemolytic erythrocyte enzyme defect, leading to around 400 million cases worldwide [1]. G6PD is a crucial rate-limiting enzyme in the pentose phosphate pathway which contributes much to maintaining intracellular reducing state [2]. The

underlying mechanism lies in its facilitating catalyzation of glucose-6-phosphate (G6P) into G6P lactone and biosynthesis of nicotinamide adenine dinucleotide phosphate (NADPH) [2]. G6PD deficiency is primarily caused by genetic mutations and totally 206 mutation sites have been discovered (<http://www.hgmd.org>), such as G6PD Canton (1376G>T), G6PD Kaiping (1388G>A), G6PD Gaohe (95A>G), G6PD Quing Yuan (392G>T), “Chinese-5” (1024C>T) and G6PD Viangchan (1311C>T). Besides, the above mutations were prevalent in south of China such as Guangdong, Guangxi and Yunnan Province [3, 4].

Since erythrocytes differ from nucleus cells in its shortage of cell nucleus and certain organelles (e.g. mitochondria), the pentose phosphate pathway becomes its only way to produce NADPH [5]. As a result, compared with normal nucleus cells, erythrocytes seem to be more susceptible to oxidative stress, and eryptosis, the molecular programming process characterized by cell suicide, could be more readily induced [6]. Virtually, erythrocytes with G6PD deficiency that can hardly synthesize NADPH would accelerate the progress of eryptosis [7]. The morphological features of eryptosis include shrinking of red blood cell (RBC) membrane and phosphatidylserine (PS) exposure on the surface of RBCs [8]. Of note, eryptosis has been confirmed to be associated with inherent mechanisms as follows: overload of intracellular Ca^{2+} concentration, formation of ceramide, activation of caspase and excitation of calpain [9, 10]. Thus, it is hypothesized that the eryptosis stimulated by oxidative stress might be correlated with the above actions. Furthermore, as excessive eryptosis is likely to cause such disorders as anemia, diabetes mellitus, renal insufficiency, and hemolytic-uremi syndrome, exploration of the potential treatments becomes increasingly urgent [11]. It has been demonstrated that pharmacological doses of Vitamin C would relieve hemolysis within patients that suffered from G6PD deficiency [12-14]. Nonetheless, it was undeniable that over-dose Vitamin C could also contribute to aggravated haemolysis of G6PD-deficient erythrocytes, as reported in both case reports and mechanism researches [15-17]. Thus, this study was intended to investigate the eryptosis conditions among the population with G6PD deficiency or normal activity habituated in Daidehong prefecture of Yunnan province and to explore the inherent mechanisms with in-vitro erythrocytes that were treated with proper concentrations of Vitamin C and hydrogen peroxide.

Materials and Methods

Clinical specimens

Blood samples used in this study were collected from the First People’s Hospital of Xianyang City. This study was approved by the Ethics Committee of the First People’s Hospital of Xianyang City and all methods conducted were complied with relevant guidelines. Written informed consent was obtained from each participant before blood collection. EDTA anticoagulative tubes (BD, USA) were used to collect peripheral vein blood samples from 22 participants, and the samples were stored at 4°C.

Enzyme assay

The WHO standard method [18, 19] along with the improved G6PD/6PGD ratio assay [20] was considered as the benchmark for G6PD activity assessment. Specifically, optical densities (ODs) of G6P (S1) and 6PG (S2) were determined with application of UV-2100 ultraviolet spectrophotometer (Shimadzu, Japan) at the wavelength of 450 nm. G6PD activity was calculated as the following formula: $S1/S2$. The reagents of β -NADP, G6P-Na and 6PG-Na were all purchased from Sigma Corporation.

Genotyping

TIANamp Blood DNA Kit (TIANGEN, Beijing, China) was used to extract genome DNA. As suggested by the G6PD genome sequence data, the primers for amplification of 11 regions on G6PD exon and intron fragments were designed and synthesized by Primer 5.0 software [3, 21, 22]. The 25 μ l reaction system is comprised of 1.25 U Taq Polymerase (TaKaRa), 2 $\times$ Premix buffer (TaKaRa), 0.2 μ M primers (Sangon Biotech) and 200 ng genomic DNA. The reaction was carried out under the following conditions: predegeneration at 94°C for 3 min; degeneration at 94°C for 30s, annealing at 60°C for 30s, extending at 72°C for 1 min,

30 cycles; extending at 72°C for 10 min. The PCR amplification products were detected by bidirectional DNA sequencing (Sangon Biotech). The sequencing results were compared with the sequence data in the database and analyzed with Chromas and Clustalx.

Erythrocytes isolation and chemical treatment

A total of 22 samples were divided into G6PD normal group (male 11) and G6PD deficiency group (male 9, female 2). Flesh blood was centrifuged at 1000g for 20 min at room temperature and plasma containing blood platelets and white cells was carefully removed. Isolated erythrocytes were washed twice by Ringer solution and the erythrocytes hematocrit was set for 40%. Ringer solution (in mM) was prepared in the laboratory according to the formula: 125 NaCl, 5 KCl, 1 MgSO₄, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl₂, pH 7.4 at 37°C [23]. Subsequently, the mixture was maintained at pH 7.4 and at 37°C. Erythrocytes in the two groups were pretreated with Ringer solution (Blank group) or Vitamin C (1 mg/ml, VC group), and then incubated at 37°C for 30 min. Since vitamin C affects erythrocytes in a concentration-dependent manner, previous documents were referred to and preparing experiments were conducted so that the protective effects of vitamin C on erythrocytes could be ensured [19]. Erythrocytes were washed twice by Ringer solution again. The erythrocytes in the treatments groups above were treated with Ringer solution or hydrogen peroxide (H₂O₂, 0.5%) for 30 min at 37°C. Finally, erythrocytes samples were randomly divided into 8 groups (Fig. 1): G6PD normal/Blank group (only treated with Ringer solution), G6PD normal/VC group (treated with Ringer solution and Vitamin C), G6PD normal/H₂O₂ group (treated with Ringer solution and H₂O₂), G6PD normal/VC+H₂O₂ group (treated with Vitamin C and H₂O₂), G6PD deficiency/Blank group (only treated with Ringer solution), G6PD deficiency/VC group (treated with Ringer solution and Vitamin C), G6PD deficiency/H₂O₂ group (treated with Ringer solution and H₂O₂), G6PD deficiency/VC+H₂O₂ group (treated with Vitamin C and H₂O₂).

Ascorbic acid (AA) detection

About 1 ml methanol (volume fraction: 70%) with addition of 1 mmol/L ethylene diamine tetraacetic acid (EDTA) was prepared to treat cells for 10 min. Subsequently, the extraction liquid containing AA was determined with application of ascorbic acid assay kit (BioVision corporation, America).

PS extroversion rate detection

Erythrocytes were suspended in 100µl Annexin-V binding buffer and cell concentration was adjusted to the level of 1×10⁶ per ml. Then, 5µl FITC-Annexin-V was added to cell suspension, gently mixed and stored in dark at room temperature for 15 min. After that, 400µl Annexin-V binding buffer was added to the mixture so that the cells resuspended and then the mixture was analyzed using flow cytometer (BECKMAN-COULTER). The light path was calibrated with fluorescent microsphere to ensure the variable coefficient (CV) < 2% in the flow cytometer. FL-1 fluorescence signal (excitation wavelength at 488 nm, emission wavelength at 525 nm) of 10,000 erythrocytes was detected in the cell suspension. FL-1 fluorescence signal indicated the PS extroversion rate.

Fig. 1. Treatment-group assignment of erythrocytes samples.

Intracellular Ca^{2+} concentration detection

Erythrocytes were suspended in 1 ml Ringer solution which adjusts the concentration to the level of 1×10^6 /ml. 5 μ l Fluo-3/AM buffer was added to cell suspension, mixed gently and stored in dark for 25 min at 37°C. After the supernatants were removed by centrifugation, erythrocytes were washed twice with Ringer solution and 1 ml Ringer solution was applied to suspend cells for detection. The light path was calibrated with fluorescent microsphere to ensure variable coefficient (CV) < 2% in the flow cytometer. The FL-1 fluorescence signal of 10,000 erythrocytes was detected.

Western blot analysis

A total of 1×10^6 cells were collected from each group, and proteins were extracted using RIPA Lysis Buffer. The proteins expression levels were detected using the BCA method. Equal quantity of proteins in each group was resolved on 10% SDS PAGE (polyacrylamide gel electrophoresis) and then transferred to PVDF (polyvinylidene fluoride) membrane. Moreover, 5% skim milk was used for blocking for 1 h at room temperature. Subsequently, membrane was incubated with primary antibody rabbit anti-caspase-3 (1:1000) at 4°C overnight. After the membrane was washed by TBST, the HRP (horse radish peroxidase) labeled as the second goat-anti-rabbit antibody (1:5000) was used as probe for combination. The signal was normalized to the internal reference GAPDH (1:5000) and was semi-quantitatively analyzed through Gel-Pro Analyzer 4.0 software.

Statistical Analysis

Data was presented as mean $\pm$ standard deviation (SD), and SPSS 18.0 software was used to perform the statistical analysis. The comparison between two groups was assessed by the t-test, while the difference among multiple groups was tested by one-way ANOVA (statistical significant: P value < 0.05). R language software (qq norm) was carried out to figure out if relevant values were in accordance with normal distribution. If variances of values were not homogeneous despite their conforming to normal distribution, the results of student's t test would be corrected with welch method. Besides, the results that did not conform to the normal distribution were compared with usage of nonparametric rank sum test.

Results

G6PD mutation detection

The WHO standard were adopted as the classification criteria: a zero enzyme activity with chronic non-spherocytic hemolytic anemia was identified as clinical class I; an enzyme activity between 0% and 10% with hemolytic anemia and occasional favism or medicines intake was defined as clinical class II; an enzyme activity between 10% and 60% without hemolytic anemia was defined as clinical class III; an enzyme activity between 60% and 100% without hemolytic anemia was identified as clinical class IVa; an enzyme activity between 100% and 150% without hemolytic anemia was identified as clinical class IVb; an enzyme activity above 200% without hemolytic anemia was defined as clinical class V. Six point variants were discovered in 11 G6PD-deficient patients. Some nucleotide changes were consistent with previous study [19] including 392G→T, 1376G→T, while new changes like 487G→A were discovered. Details about the correlation between genotype and phenotype correlation in 11 G6PD-deficient patients were shown in Table 1.

PS extroversion rate of erythrocytes

The PS extroversion rates of suspended erythrocytes were detected using flow cytometry (Fig. 2) and whether the determined figures met normal distribution was judged as shown in Fig. 3. The PS extroversion rate of erythrocytes in the blank or VC group with G6PD normal activity or G6PD deficiency were extremely low without significant difference. The addition of 0.5% H_2O_2 significantly increased the PS extroversion rate compared with the blank group (both $P < 0.001$). However, the PS extroversion rate of G6PD deficiency erythrocytes was significantly higher than that of G6PD normal erythrocytes ($P < 0.001$). H_2O_2 could contribute to PS extroversion on the erythrocytes membrane and erythrocytes

Table 1. Genotype and phenotype correlations in 11 G6PD-deficient patients. The WHO standard (WHO Scientific Groups 1967) were adopted as the classification criteria: a zero enzyme activity with chronic non-spherocytic hemolytic anemia was identified as clinical class I; an enzyme activity between 0% and 10% with hemolytic anemia and occasional favism or medicines intake was defined as clinical class II; an enzyme activity between 10% and 60% without hemolytic anemia was defined as clinical class III; an enzyme activity between 60% and 100% without hemolytic anemia was identified as clinical class IVa; an enzyme activity between 100% and 150% without hemolytic anemia was identified as clinical class IVb; an enzyme activity above 200% without hemolytic anemia was defined as clinical class V. F, female; M, male

Exon	cDNA nucleotide change	Name	Amino acid change	Class I	Class II	Class III	Class IV	Class V	Total
5	392G→T	G6PD Quing Yuan	p.131Gly→Val	0	0	1 (M)	0	0	1
6	487G→A	G6PD Mahidol	p.163Gly→Ser	0	0	2 (M)	0	0	2
11	1311C→T	G6PD Viangchan		0	0	0	1 (F)	0	1
12	1376G→T	G6PD Canton	p.459Arg→Leu	0	3 (1M, 2F)	0	0	0	3
12	1388G→A	G6PD Kaiping	p.463Arg→His	0	3 (M)	0	0	0	3
Mutant allele unknown				0	1 (M)	0	0	0	1
Total				0	2	8	1	0	11

Fig. 2. Phosphatidylserine (PS) extroversion rate (%) of erythrocytes with normal G6PD activity or G6PD deficiency in different treatment groups detected by flow cytometry. G6PD+ represented G6PD normal activity, G6PD- represented G6PD deficiency. *** $P < 0.001$.

Fig. 3. Normal distribution results regarding PS extroversion rate of 8 treatment groups.

with G6PD deficiency may be more sensitive to H₂O₂ than erythrocytes with normal G6PD. Moreover, the PS extroversion rate in the VC+H₂O₂ group was significantly higher than that in the VC group ($P < 0.001$), while it was significantly lower than that in the H₂O₂ group ($P < 0.01$), suggesting that Vitamin C could reverse the effect of oxidation stress on erythrocytes

Fig. 4. The concentration of intracellular Ca^{2+} in erythrocytes with normal G6PD activity or G6PD deficiency in different treatment groups detected by flow cytometry. G6PD+ represented G6PD normal activity, G6PD- represented G6PD deficiency. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 5. Normal distribution results regarding intracellular Ca^{2+} concentration of 8 treatment groups.

triggered by H_2O_2 . Interestingly, the PS extroversion rate of G6PD deficiency erythrocytes was significantly higher than that of G6PD normal erythrocytes in the $\text{VC}+\text{H}_2\text{O}_2$ group ($P < 0.001$).

Intracellular Ca^{2+} concentration in erythrocytes

As shown by Fig. 4, the concentration of intracellular Ca^{2+} in the H_2O_2 group was significantly increased compared to the blank group ($P < 0.001$). No significant difference was detected between the blank and VC group. The intracellular Ca^{2+} concentration in the $\text{VC}+\text{H}_2\text{O}_2$ group had a declining tendency in comparison to the H_2O_2 group with no statistical significance ($P > 0.05$), suggesting that the intracellular Ca^{2+} concentration was not only associated with the treatment of H_2O_2 or Vitamin C but also related to the G6PD activity. Furthermore, the intracellular Ca^{2+} concentration of erythrocytes with G6PD deficiency was significantly higher than those with G6PD normal activity in the blank ($P < 0.01$), H_2O_2 ($P < 0.05$) and VC group ($P < 0.05$) after adjustment (Fig. 5).

Caspase 3 expression detected by western blot

As suggested by Fig. 6, results from western blot indicated that inactive caspase 3 expression remained stable in all groups. In contrast, the active caspase 3 expression levels significantly increased in the H_2O_2 group compared to the blank group ($P < 0.05$) (Fig. 7). And the active caspase 3 expression levels in the $\text{VC}+\text{H}_2\text{O}_2$ group were significantly lower than those in the H_2O_2 group ($P < 0.05$). No significant difference was found between the blank and VC group. The active caspase 3 expression level of erythrocytes with G6PD deficiency was significantly higher than that of erythrocytes with G6PD normal activity

Fig. 6. The expression level of active caspase 3 in erythrocytes of different groups detected by western blot. iCaspase 3 represents inactive Caspase 3, weighing for 32 kD; aCaspase3 represents active caspase 3, weighing for 20 kD; GAPDH, 37 kD, used as internal reference. G6PD+ represented G6PD normal activity, G6PD- represented G6PD deficiency.

Fig. 7. The expression levels of active caspase 3 (aCaspase3) relative to GAPDH in erythrocytes of different groups detected by western blot. **P* < 0.05, comparison with blank group; #*P* < 0.05, comparison with H₂O₂ group.

especially in the H₂O₂ and VC+H₂O₂ group (both *P* < 0.05). As a result, H₂O₂ could contribute to eryptosis and erythrocytes with G6PD deficiency were more prone to oxidative stress in comparison to erythrocytes with normal G6PD. Vitamin C could effectively inhibit the eryptosis contributed by H₂O₂.

Discussion

Since the pentose phosphate pathway was the only source which produced NADPH in erythrocytes, the defense system that tackled oxidative stress in erythrocytes was highly dependent on the activity of G6PD enzyme and erythrocytes in G6PD deficient patients could be more prone to oxidative stress. Specifically, NADPH has been considered as a necessary substrate within the anti-oxidant system for translating oxidized glutathione into reduced glutathione and activating catalase through allosteric effects [24]. Hence, G6PD activity deficiency was suspected to inhibit the antioxidant effect of erythrocytes by attenuating the level of NADPH. The hypothesis has partly been confirmed by our study results that G6PD+ carriers were less susceptible to H₂O₂ than G6PD- carriers, and G6PD- carriers were harder to recover from damage of H₂O₂ after treatment of vitamin C.

In fact, whether the eryptosis induced by H₂O₂ in erythrocytes was intrinsic or extrinsic still remained inconclusive. We considered this eryptosis to be mostly extrinsic if “intrinsic” was defined as eryptosis caused by mutations of DNAs and “extrinsic” was defined as eryptosis induced by damage of cellular components. Specifically, H₂O₂ belonged to reactive oxygen species (ROS) that were produced by erythrocytes in a continuous manner [25]. The ROS could contribute to lipid peroxidation, protein oxidation and rise of met-hemoglobin, malondialdehyde and protein carbonyls within erythrocytes [26]. Besides, hydroxyl radicals transformed from H₂O₂ also served to initiate annexin binding in erythrocytes [27]. The above reactions might finally lead to loss of membrane integrity and the so-called hemolysis [28]. Undeniably, ROS might initiate destruction of hereditary materials, since that H₂O₂ has been documented to induce DNA fragmentation in fish erythrocytes [27]. Nonetheless, distinct from fish erythrocytes, mature erythrocytes of mammals no longer retained their nuclei [29]. Therefore, whether H₂O₂ would lead to DNA degradation within cytoplasm or not was still a challenging issue.

Since erythrocytes did not include nuclei, the eryptosis pathway might be dependent on Ca^{2+} permeability cation pathway, rather than mitochondria-specific NF- κ B, p53 and STAT3/NFAT pathways [30, 31]. The overload of intracellular Ca^{2+} concentration activated K^+ outflow from the potassium channel which further contributed to hyperpolarization of cytomembrane and outflow of Cl^- . In consequence, cell volumes significantly shrink due to osmotic pressure change and the forward scatter decreases [32]. Besides intracellular Ca^{2+} , activations of caspase and calpain were also involved in eryptosis which modified the structures of cell skeleton [33]. Moreover, eryptosis was also characteristic of micro-circulative damages which were contributed by PS eversion of erythrocytes [11]. The extroversion of PS on RBC membranes could also contribute to blood coagulation and thrombogenesis [11]. Then eryptosis would take place if the erythrocytes with PS eversion could not be degraded by phagocytes or liver Kupffer cells with PS receptor [34].

Vitamin C, the could-be treatment for G6PD deficiency, was predominantly taken into erythrocytes in two forms, namely, ascorbic acid (AA) and dehydroascorbic acid (DHA). AA mainly relies on Na^+ -dependent transporter, while DHA was mediated by glucose transporter (GLUT) [35, 36]. Besides, DHA has been reported to be reduced to AA at first inside the cell, and it is AA that played key anti-oxidant roles [37, 38]. It was indicated in Fig. 2 that PS extroversion rates of G6PD+ carriers in vitamin C + H_2O_2 group were significantly ameliorated than those in H_2O_2 group, while the trend was not so remarkable with regard to the carriers with G6PD deficiency. It could be explicated by the fact that vitamin C can promote formation of NADPH through strengthening the reducing characteristics of NADPH-dependent methemoglobin reductase [39, 40]. Furthermore, the anti-oxidative ability of vitamin C cannot be overlooked even though its action concentration is far beyond the natural concentration. For instance, vitamin C could scavenge excessive oxygen free radicals arising from G6PD deficiency or damage of hydrogen peroxide [39, 41]. Besides, vitamin C could also aid in recycling of oxidized vitamin E in indirect manners [42].

Interestingly, Vitamin C did not bring down the increased Ca^{2+} concentration within erythrocytes that was caused by oxidative stress. The distinct tendencies between indicators of PS extroversion rate and Ca^{2+} concentration could probably be explained by their involvement with dissimilar signaling pathways that were relevant to eryptosis. In particular, oxidative stress was correlated with protein kinase activation, while intracellular Ca^{2+} concentration was related with phosphatase activation [43]. Vitamin C was a reagent that was applied to antagonize oxidative stress and could more directly affect protein kinase activation, suggesting that the indirect effects of vitamin C on intracellular Ca^{2+} concentration might not be larger than those on actions related with oxidative stress. Besides, caspase levels also displayed remarkable changes between treatments of H_2O_2 and vitamin C + H_2O_2 , which appeared distinct from Ca^{2+} concentration. The phenomena could be suspected that vitamin C might deactivate caspase that was independent of Ca^{2+} entry [44].

Despite the above achievements, several limitations remain present. Firstly, the sample size in this study is not large enough to draw unbiased conclusions. Secondly, although this study validated the effects of H_2O_2 and vitamin C on eryptosis among samples with G6PD deficiency or G6PD normal activity *in vitro*, the conclusion still needs to be confirmed *in vivo*. Finally, more specific and precise technologies are still required to qualify and quantify the process of eryptosis.

Disclosure Statement

The authors declare no commercial or financial conflict of interest.

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