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Original Article

Sodium pyruvate ameliorates *in vitro* oxidative damage resulting from lethal methemoglobinemia

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Abstract: *Introduction*: The goal of this study was to evaluate the effects of sodium pyruvate (SP) on lethal methemoglobinemia with rat RBCs *in vitro*. *Methods*: Eight mM H_2O_2 was added to Group H_2O_2 to induce a lethal methemoglobinemia model. Group SP was pretreated with SP (5, 10, 20, and 50 mM, respectively) to verify the effects of SP on the lethal methemoglobinemia. Variables regarding oxidative stress, energy metabolism and oxygen delivery were determined. *Results:* The increased levels of MetHb, ROS, and MDA, but decreased contents of ATP and GSH, activities of LDH and Na^+-K^+ATPase and levels of 2,3-DPG and P_{50} are shown in the methemoglobinemia model. Pretreatments with SP significantly reduced MDA generations, increased GSH and ATP levels and enhanced the activities of LDH and Na^+-K^+ATPase. SP also maintained RBC function via inhibiting transformation of Hb to metHb and increasing the 2,3-DPG level and the P_{50} value. *Conclusion:* SP ameliorated lethal methemoglobinemia *in vitro,* through promoting glycolytic pathways, thus enhancing redox potentials and function with rat RBCs. SP may be a prospective protective agent for lethal methemoglobinemia.

Keywords: Erythrocytes, methemoglobinemia, oxidative stress, sodium pyruvate, glycolysis

Introduction

Methemoglobinemia occurs when the hemoglobin is oxidized from the ferrous state (Fe^{2+}) to the ferric state (Fe^{3+}) to form methemoglobin (MetHb) and is characterized by high levels of MetHb in the blood [1]. MetHb is incapable of oxygen transport. The symptoms of patients with methemoglobinemia are often manifested as cyanosis, dyspnea, headache, and dizziness. Especially, when metHb is greater than 70%, patients go into arrhythmias, seizures, lethargy, stupor, and even death [2].

In healthy individuals, the MetHb concentration is kept at 1.5% of total hemoglobin. However, in some patients with hereditary glucose-6-phosphate dehydrogenase (G6PD) deficiency, NADPH diminishes, and MetHb increases, which is called congenital methemoglobinemia. Acquired methemoglobinemia is a complication of ingestion or skin exposure to toxic chemicals or drugs. It is reported that a dark, chocolate brown blood with a raised methemoglobin of 30~50% can be found in patients after local anesthesia with lidocaine/prilocaine [3]. Rasburicase, a drug for the management of tumor lysis syndrome is reported to induce methemoglobinemia [4, 5]. Sodium nitrite, a coloring or preservative agent in foods and an anti-microbial agent in meat products, is a common cause of acquired methemoglobinemia and can elevate the MetHb level to > 90% [6]. The lethal dose of sodium nitrite in adults is just 2.6 g [6]. Severe methemoglobinemia with fatal outcomes following ingestion of sodium nitrite and intentional self-poisoning have been reported [7]. Healthy individuals have few symptoms with MetHb < 15%. When MetHb reaches to 20~30%, the symptoms, such as headache, dizziness, and syncope, begin [8]. However, patients with severe anemia, heart disease, lung disease, sepsis, and sickle cells should be treated even if the MetHb is ~10% [9].

Methylene blue and blood exchange transfusion have been used for treating methemoglobinemia. In general, methylene blue, a cofactor for NADPH MetHb reductase, is the first-line antidote for acute methemoglobinemia and is recommended to use in asymptomatic patients with MetHb levels greater than 30% and in symptomatic patients with levels greater than 20% [9] at a dose of 1-2 mg/kg body weight intravenously over 5 minutes [6]. However, methylene blue is not recommended in patients with G6PD deficiency, since it may worsen methemoglobinemia and induce hemolysis [10]. More importantly, if given in large doses (> 7 mg/kg), methylene blue itself can raise MetHb levels and increase the risk of rebound methemoglobinemia [11, 12]. Methylene blue also can increase the risk of dapsone-induced hemolysis [12]. However, under these conditions or failure to respond to methylene blue, exchange transfusion may be required in the patients [13]. Until now, no standard treatment has been available for acute methemoglobinemia. It is necessary to develop the new drugs for the prevention and treatment of methemoglobinemia.

Besides the elevated MetHb level, damage occurs in RBCs of patients with methemoglobinemia. With oxidation, the cellular membrane is damaged, the enzymes are deactivated and ATP generation decreased [14, 15]. Due to the lack of mitochondria and nuclei, RBCs obtain energy through anaerobic glycolysis, which is inhibited by oxidation and not able to repair itself. Therefore, it is recommended to use drugs with the ability of improving metabolic disturbances for prevention and treatment of methemoglobinemia.

Pyruvate, an endogenous metabolic intermediate, is capable of protecting organs in various models of oxidative stress, such as lens with oxidants [16], diabetic cataracts [17], oxidant- and ischemia-induced neuronal damage [18, 19] and cardiac ischemia-perfusion injury [20]. It also has been widely used in cell culture media to remove H_2O_2 [21]. Additionally, pyruvate improves metabolic and acid-base disturbances [22-25], which facilitates RBCs protection against oxidation. In our previous paper, we demonstrated that addition of sodium pyruvate (SP) to stored RBCs could restore the P_{50} and SOD activity, thus improving the storage lesion [26]. These results suggest that pyruvate may be a potential drug for methemoglobinemia with both anti-oxidant and the metabolic modulation.

Therefore, in the present study, H_2O_2, the metabolite of some drugs and foods, which could penetrate the membrane to oxidize hemoglobin, was used to induce the lethal methemoglobinemia model with rat RBCs (metHb reached to ~70%) *in vitro*. The effect of pyruvate on methemoglobinemia was investigated and the potential underlying mechanism was explored.

Materials and methods

Animals

All animal experiments were carried out with the approval of the Laboratory Animal Center of the Academy of Military Medicine Sciences. Healthy male Wistar rats (270-290 g) were purchased from Vital River Laboratories (Beijing, China) and were given access to food and water *ad libitum* until the start of experiments.

Preparation of RBC samples

Rats were anesthetized with sodium pentobarbital (Peking Chemical Agent Co., Beijing, China) (2.5%, w/v, 0.23 ml per 100 g of body weight) by intraperitoneal injection and placed on a heating pad (TMS-202, Softron, Beijing, China) in a supine position. The left femoral vein was cannulated for the administration of heparin sodium (Chinese Medicine Group Chemical Agent Co.) (0.3%, w/v, 0.15 ml per 100 g of body weight). The carotid artery was cannulated to collect blood. The plasma and buffy coat were removed by aspiration to isolate RBCs. The cells were washed three times with phosphate buffered saline (PBS) (pH 7.4, 295 mOsm/kg) by centrifugation for 5 minutes at 3000 rpm and at 4°C and immediately re-suspended at 10% hematocrit in PBS buffer.

The preparation of methemoglobinemia model and the treatment

RBC suspensions (10% hematocrit in PBS buffer) were divided into three groups: (1) Group control, (2) H_2O_2-induced methemoglobinemia group (Group H_2O_2), (3) pretreated with different concentrations of SP (sodium pyruvate, Sigma, USA) (Group SP). SP was dissolved in

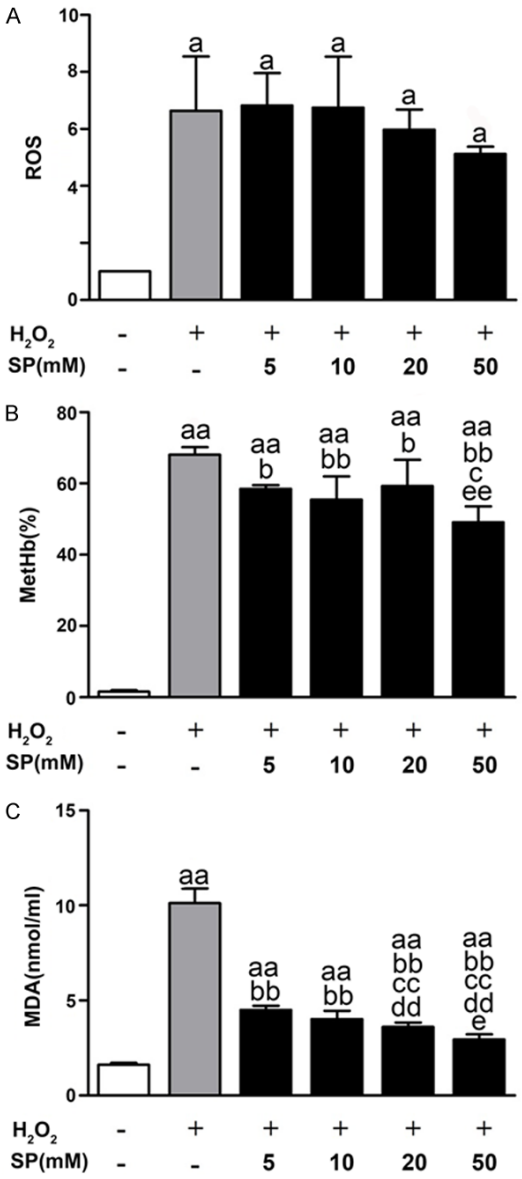

Figure 1. Effects of SP on the content of ROS, MetHb, and MDA in RBCs suffering methemoglobinemia. H_2O_2 induced the significant increase of ROS (A), MetHb (B) and MDA (C). Pretreatment with SP restrain the oxidative damage from H_2O_2. $^{a}P < 0.05$, *vs.* Group Control; $^{aa}P < 0.01$, *vs.* Group Control; $^{b}P < 0.05$, *vs.* Group H_2O_2; $^{bb}P < 0.01$, *vs.* Group H_2O_2; $^{c}P < 0.05$, *vs.* Group SP at 5 mM; $^{cc}P < 0.01$, *vs.* Group SP at 5 mM; $^{dd}P < 0.01$, *vs.* Group SP at 10 mM; $^{e}P < 0.05$, *vs.* Group SP at 20 mM; $^{ee}P < 0.01$, *vs.* Group SP at 20 mM.

PBS buffer and added into the RBC suspensions in the Group SP at final concentrations of 5, 10, 20, and 50 mM. All groups were then incubated at 37°C for 30 minutes. Sodium azide (NaN_3, 0.4 mM final concentration), an inhibitor of catalase, and H_2O_2 were added to the Group H_2O_2 and Group SP. The final concentration of H_2O_2 was 8 mM. Thus, a lethal methemoglobinemia model was induced and the concentration of metHb reached to ~70%, which might lead to vascular collapse, even death *in vivo* [2]. All groups were then incubated at 37°C for another 1 hour. The RBCs in all groups were washed twice with PBS prior to the subsequent tests.

Measurement of ROS levels

The non-fluorescent compound 2,7-dichlorofluorescein diacetate (DCFH-DA, Sigma, USA) diffuses into cells and becomes oxidized in the presence of ROS to form highly fluorescent DCF. To detect ROS production, 100 μL washed RBCs were mixed with 1900 μL of PBS buffer and incubated with DCFH-DA (100 μmol/L) in the dark at 37°C for 30 minutes. The cell suspension was then washed once with PBS and the fluorescence intensity was measured by flow cytometry analysis in a cell count of 50000 at an excitation wavelength of 490 nm and an emission wavelength of 530 nm (BD FACSCalibur, USA).

Measurement of metHb levels

MetHb levels of three groups were measured using a blood analyzer (ABL90 CO-OX; Radimeter Copenhagen, Denmark).

Measurement of oxygen affinity

Oxygen affinity (P_{50}) was determined by recording the oxygen dissociation curve using a TCS Hemox Analyzer (TCS Scientific Corp., New Hope, PA) at 37°C. Briefly, 20 μL RBCs suspension was mixed with 4 mL Hemox buffer, 20 μL bovine serum albumin and 10 μL antifoaming agent. The mixture was saturated to a pO_2 of 150 mmHg using compressed air. Then, the gas stream was switched to pure N_2 to deoxygenate the RBCs suspension. The Hemox-Analyzer recorded the oxygen dissociation curve and calculated the P_{50}.

Measurement of other biochemical indices

GSH, MDA, and ATP levels and Na^+-K^+ATPase activities were measured using assay kits from Jiang Cheng Technology (Nanjing, China). Lactate dehydrogenase (LDH) activity was

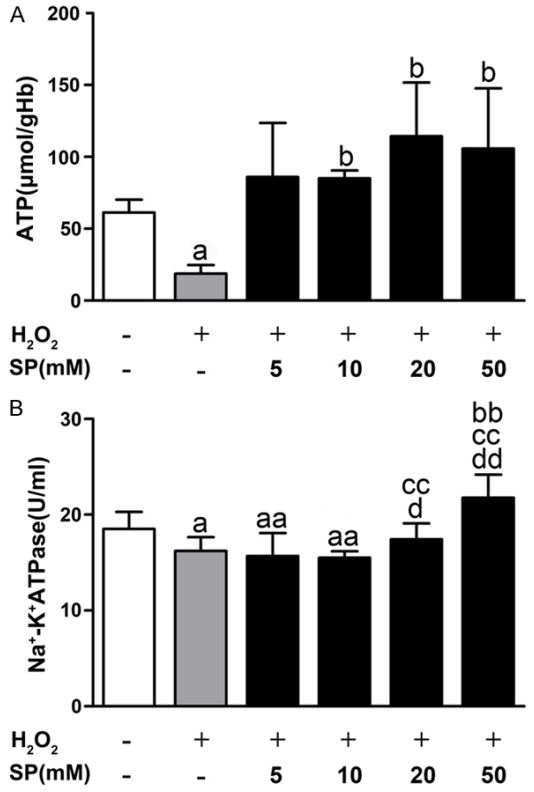

Figure 2. Effects of SP on the content of ATP and the activity of Na^+-K^+ATPase in RBCs suffering methemoglobinemia. H_2O_2 suppressed the ATP content (A) and Na^+-K^+ATPase activity (B). ATP content in SP groups were not suppressed. The activity of Na^+-K^+ATPase showed a significant increase at the SP concentration of 20 mM and 50 mM. $^aP < 0.05$, *vs.* Group Control; $^{aa}P < 0.01$, *vs.* Group Control; $^bP < 0.05$, *vs.* Group H_2O_2; $^{bb}P < 0.01$, *vs.* Group H_2O_2; $^{cc}P < 0.01$, *vs.* Group SP at 5 mM; $^dP < 0.05$, *vs.* Group SP at 10 mM; $^{dd}P < 0.01$, *vs.* Group SP at 10 mM.

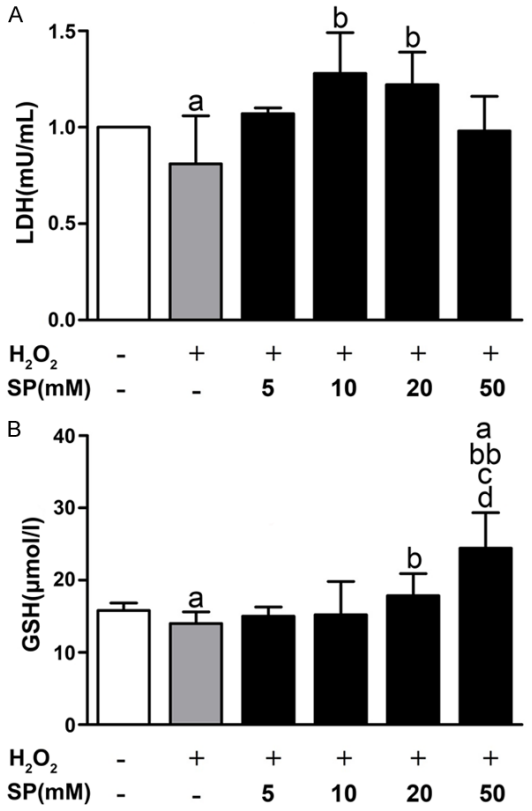

Figure 3. Effects of SP on LDH activity and GSH level in RBCs suffering methemoglobinemia. H_2O_2 suppressed the LDH activity (A) and GSH level (B). Pretreatment with SP resulted in the increased LDH activity. However, LDH activity decreased with the concentration of SP reaching to 50 mM. The GSH level showed increase with the concentration of SP higher than 20 mM. $^aP < 0.05$, *vs.* Group Control; $^bP < 0.05$, *vs.* Group H_2O_2; $^{bb}P < 0.01$, *vs.* Group H_2O_2; $^cP < 0.05$, *vs.* Group SP at 5 mM; $^dP < 0.05$, *vs.* Group SP at 10 mM.

determined with a colorimetric assay kit (BioVision, USA) using a multiscan spectrum (SpectraMax M5, Molecular Devices, USA). Amounts of 2,3-diphosphoglycerate (2,3-DPG) were determined by measuring NADH oxidation using a reagent kit (Roche Applied Science, Germany) conducted with an UV-Visible spectrophotometer (Helios β, Thermo, USA). All measurements were carried out according to the manufacturer's instructions.

Statistical analysis

At least three independent experiments were conducted. Data are reported as mean ± SD. Data were analyzed using Statistical Analysis System software (SAS Version 9.2. SAS Institute, USA). One-way analysis of variance (ANOVA) was used to calculate statistical significance among multiple groups. *t*-test was used to compare the data between two groups (e.g. Group control and Group H_2O_2). When normality and homogeneity of variance assumptions were not satisfied, non-parametric Kruskal-Wallis test was applied. $P < 0.05$ was considered statistically significant.

Results

SP inhibited ROS and decreased MetHb and MDA levels

As shown in **Figure 1A**, H_2O_2 dramatically increased intracellular ROS in Group H_2O_2 by up to about seven times of the basal level ($P < 0.05$). Pretreatments with various concentrations at 5, 10, 20, and 50 mM SP decreased

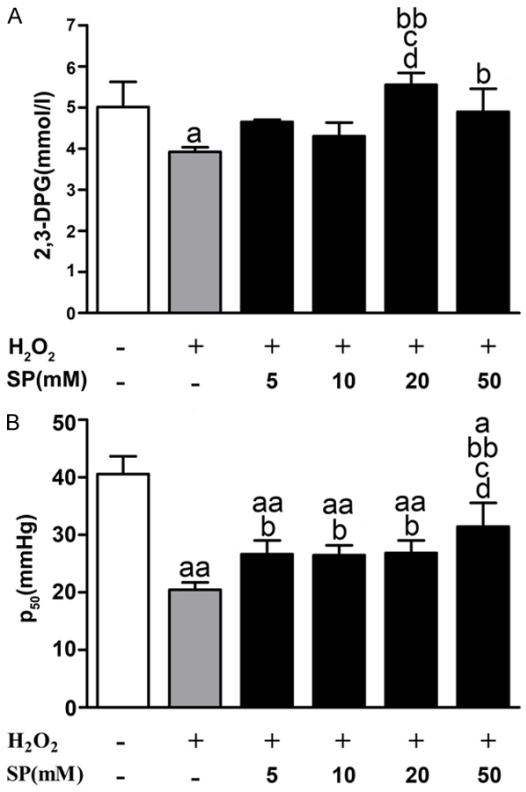

Figure 4. Effects of SP on the content of 2,3-DPG and the value of P_{50} in RBCs suffering methemoglobinemia. H_2O_2 suppressed the content of 2,3-DPG (A) and the value of P_{50} (B). Pretreatment with SP restrained inhibition of H_2O_2 on P_{50} effectively. When the concentration of SP reached to 20 mM, the content of 2,3-DPG in RBCs restored. $^{a}P < 0.05$, *vs.* Group Control; $^{aa}P < 0.01$, *vs.* Group Control; $^{b}P < 0.05$, *vs.* Group H_2O_2; $^{bb}P < 0.01$, *vs.* Group H_2O_2; $^{c}P < 0.05$, *vs.* Group SP at 5 mM; $^{d}P < 0.05$, *vs.* Group SP at 10 mM.

ROS levels gradually. However, the ROS level in the Group SP was still significantly higher than that in the Group control ($P < 0.05$), but showed a slight decrease comparing with that in the Group H_2O_2 ($P > 0.05$).

Treatment of RBCs with H_2O_2 markedly increased levels of MetHb ($P < 0.01$) and MDA ($P < 0.01$) (**Figure 1B** and **1C**), which suggested that the lethal methemoglobinemia model was successfully established. Pretreatment with SP significantly reduced the levels of metHb and MDA raised by H_2O_2 treatment. The MetHb level in Group SP was still significantly higher than that in the Group control ($P < 0.01$), while significantly lower than that in the Group H_2O_2 (5 mM, 20 mM $P < 0.05$; 10 mM, 50 mM $P < 0.01$). Furthermore, the MetHb level in the 50 mM SP group was significantly lower than that in the 5 mM group ($P < 0.05$) and the 20 mM group ($P < 0.01$). Pretreatments with SP dramatically reduced MDA, compared with H_2O_2 treatment alone ($P < 0.01$). Pretreatments with 20 mM and 50 mM SP resulted in significant lower levels of MDA than treatment with 5 mM and 10 mM SP ($P < 0.01$). Furthermore, the MDA level in 50 mM group was significantly lower than that in the 20 mM group ($P < 0.05$). The MDA level showed approximately a dose-response with the increasing SP concentration.

SP increased ATP levels and Na^+-K^+ATPase activity

Levels of ATP in RBCs, mainly derived from anaerobic glycolysis, were decreased significantly after treatment with H_2O_2 ($P < 0.05$) (**Figure 2A**). Notably, pretreatments with various concentrations of SP greatly elevated ATP levels. ATP levels in 10 mM, 20 mM, 50 mM SP groups were significantly higher compared with H_2O_2 treatment ($P < 0.05$). Pretreatment with SP fully reversed H_2O_2-induced inhibition of the ATP production. However, there was no difference among the groups with various SP levels. The activity of Na^+-K^+ATPase, which couples hydrolysis of ATP to Na^+ and K^+ transport and plays a key role in maintaining the ion-gradient across the cell membrane of RBCs, was significantly reduced after treatment with H_2O_2 ($P < 0.05$) (**Figure 2B**). Pretreatment with 5 mM and 10 mM SP didn't increase the Na^+-K^+ATPase activity which was decreased by H_2O_2 treatment. Petreatment with 20 and 50 mM SP restored Na^+-K^+ATPase activities, which were higher comparing with the 5 mM SP group ($P < 0.01$) and the 10 mM SP group (20 mM, $P < 0.05$; 50 mM, $P < 0.01$). Especially, the Na^+-K^+ATPase activity in 50 mM SP group was significantly higher than that in the Group H_2O_2 ($P < 0.01$). The Na^+-K^+ATPase activity showed approximately a dose-response with the increasing SP concentration.

SP restored LDH activity and GSH levels

LDH activity plays an important role in RBCs because it reduces pyruvate to lactate, which couples NADH oxidation to NAD^+, a rate-limiting process in glycolysis. LDH activity in the Group H_2O_2 was reduced comparing with the Group control ($P < 0.05$, **Figure 3A**). Pretreatments

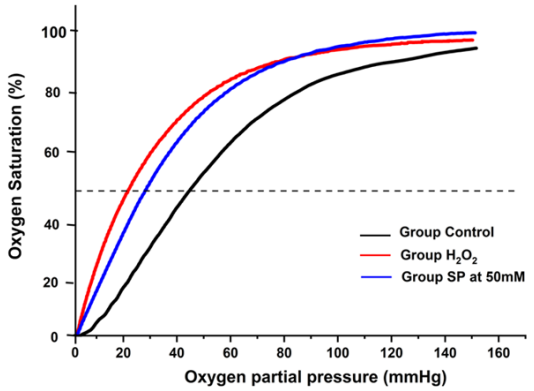

Figure 5. Oxygen dissociation curve of RBCs suffering methemoglobinemia in the Group Control, the Group H_2O_2, and the Group SP at 50 mM. H_2O_2 induced the left-shift of the oxygen dissociation curve. However, pretreatment with SP at the concentration of 50 mM prevented the influence of H_2O_2 on oxygen dissociation curve effectively.

with 10 mM and 20 mM SP showed significantly higher LDH activities, compared with the Group H_2O_2 ($P < 0.05$).

GSH levels in RBCs were significantly declined by H_2O_2 treatment ($P < 0.05$) and were significantly elevated with SP pretreatments gradually. When pretreating with 20 mM SP, the GSH level was higher than that in the Group H_2O_2 ($P < 0.05$) (**Figure 3B**). Additionally, the 50 mM SP group showed a higher GSH level than the Group control ($P < 0.05$), which was also higher than the Group H_2O_2 ($P < 0.01$), the 5 mM SP group ($P < 0.05$), and the 10 mM SP group ($P < 0.05$).

SP preserved 2,3-DPG and P_{50}

P_{50}, which is defined as PO_2 when oxygen saturation of hemoglobin reaches 50%, is used to represent oxygen affinity. 2,3-DPG, an allosteric effector, could influence the value of P_{50}. H_2O_2 markedly decreased 2,3-DPG contents ($P < 0.05$) and P_{50} values ($P < 0.01$) comparing with the Group control (**Figure 4**). Pretreatments with SP increased 2,3-DPG contents to the basal level. Pretreatment with 20 mM SP significantly restored the 2,3-DPG contents decreased by H_2O_2 ($P < 0.01$), which was also significantly higher than that in the 5 mM and 10 mM SP groups ($P < 0.05$). Pretreatment with 50 mM SP also significantly increased 2,3-DPG levels compared with the H_2O_2 treatment ($P < 0.05$). However, P_{50} was markedly enhanced in the Group SP at all concentrations of SP, compared with the Group H_2O_2 (5 mM, 10 mM, 20 mM, $P < 0.05$; 50 mM, $P < 0.01$), although the P_{50} was still lower in all the SP groups than in the Group control (5 mM, 10 mM, 20 mM, $P < 0.01$; 50 mM, $P < 0.05$). The P_{50} in the 50 mM SP group was significantly higher than that in the 5 mM and 10 mM SP groups ($P < 0.05$). Oxygen dissociation curves of the Group control, the Group H_2O_2 and the Group SP at 50 mM are shown in **Figure 5**. RBCs in the group H_2O_2 showed a left-shift oxygen dissociation curve, which shifted right after pretreatment with 50 mM SP.

Discussion

Based on protection of SP against RBCs storage lesion in our previous paper [26], the present study employed an H_2O_2-induced model to verify the effects of SP on RBCs in lethal methemoglobinemia and explore the mechanism. SP pretreatments resulted in the reduction of MDA level raised by H_2O_2 and restoration of ATP generation and Na^+-K^+ATPase activity, which were consistent with previous findings from a variety of cells and tissues *in vitro* and *in vivo* models [19, 22-24, 27]. Additionally, SP also markedly protected RBC function, leading to reduction of MetHb and improvement of P_{50} levels. These results suggest that SP may be a prospective preventative and protective drug for severe methemoglobinemia. We further determined how SP works in the prevention and treatment of methemoglobinemia.

As previously described [28, 29], pyruvate neutralizes H_2O_2 in a non-enzymatic and stoichiometric manner. Our study used H_2O_2-induced methemoglobinemia model and the results show that pretreatments with SP at different concentrations significantly decreased both metHb and MDA levels in a dose-response manner approximatively. Furthermore, ROS levels could be reduced by SP pretreatment slightly. The inconsistent results between individual parameters suggested that the reactions between SP and H_2O_2 in the present model were far more complex than simple direct neutralization, which has been also raised by Long, et al. [21], who found a greater loss of pyruvate in comparison with the generation of H_2O_2 in cell culture media. It has been reported that exogenous pyruvate leads to a significant

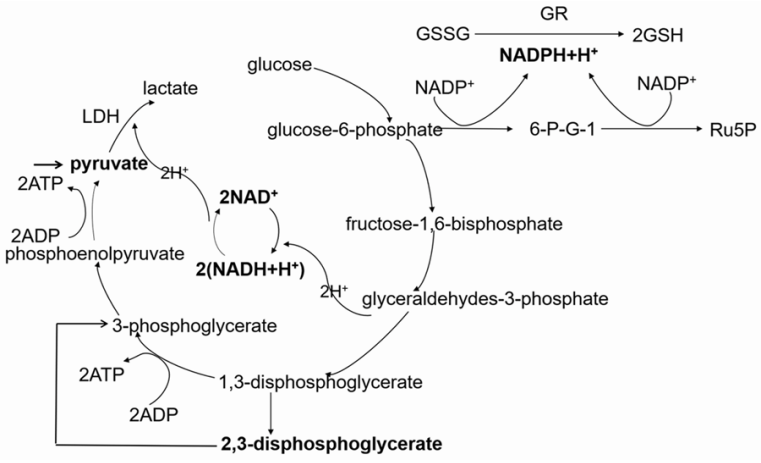

Figure 6. Pyruvate protects glycolytic metabolism in RBCs suffering methemoglobinemia. Pyruvate raised the NAD^+/NADH ratio by enhancing LDH activities. Thus the level of 2,3-DPG increased. It also increased NADPH levels via bolstering hexose monophosphate shunt to increased GSH production. LDH: lactate dehydrogenase; 6-P-G-1: 6-phosphoglucono-δ-lactone; Ru5P: ribulose 5-phosphate; GSSG: glutathione; GSH: reduced glutathione; GR: glutathione reductase.

increase of intracellular pyruvate contents in neutrophils, indicating a relevant transport across the plasmatic cell membrane [30]. Also, Sokolowska et al. suggested the non-enzymatic protective effect of alpha-keto acids (including pyruvate) in the oxidative hemolysis of human RBCs [31]. Therefore, additional parameters were detected to verify the indirect effects within cells in the experiment.

First, we speculated that exogenous pyruvate could enhance redox potentials in RBCs indirectly. To verify it, we detected the level of GSH, an important endogenous antioxidant in RBCs. It was reported that pyruvate could enhance GSH levels in hypoxia, inflammation and diabetes in various organs [32, 33]. Consistent with the literature, our results showed that SP pretreatments at 20 mM and 50 mM significantly increased GSH levels. Further, it suggested that a high concentration of SP was capable of improving redox status in the present lethal methemoglobinemia model due to raising the reducing equivalents, NADPH, to reduce MetHb to hemoglobin, which could result from the effect of pyruvate on pentose phosphate pathway, also called hexose monophosphate shunt (**Figure 6**). Patients with G6PD deficiency are unable to use the NADPH MetHb reductase pathway. Our results suggest that pyruvate could take the place of methylene blue to treat the patients with G6PD deficiency with methemoglobinemia, which needs further verification.

Second, the effect of exogenous SP on RBCs metabolism was explored. RBCs obtain energy mainly through anaerobic glycolysis. Pyruvate is an important metabolic intermediate in glycolysis and reduced into lactate by LDH catalysis. LDH activities were found to be recovered to normal levels by SP pretreatment. Although the NAD^+/NADH ratio and lactate level were not monitored in this experiment, recovery of LDH might be accompanied by the increased NAD^+/NADH ratio. In the LDH reductive reaction, one pyruvate consumes one proton from the cytosolic hydrogen pool to raise the NAD^+/NADH ratio, as described in **Figure 6** [34]. The raised NAD^+/NADH ratio could facilitate glycolytic flux and promote glycolytic ATP synthesis [23]. In response to this reaction, the ATP generation in results showed a great increase with the SP pretreatment, which possibly indicated an overall recovery of metabolic integrity, as summarized in **Figure 6**. Studies documented that Rejuvesol, a rejuvenation solution containing pyruvate, could dramatically restore ATP levels when added into stored RBCs [35, 36]. It was also reported that exogenous pyruvate preserved glycolytic ATP generation in mouse lens, retina, and human spermatozoa [17, 37, 38]. Our results were in agreement with these findings and demonstrated that pretreatment with pyruvate could maintain glycolysis in RBCs subjected to lethal methemoglobinemia. The results also displayed that SP restored the Na^+-K^+ATPase activity at the concentration higher than 20 mM. The Na^+-K^+ATPase activity is dependent on glycolytic ATP levels and is critical for maintaining basic cellular biological function, such as the ion-gradient across cell membranes and intracellular pH in RBCs. Oxidative damage can inhibit enzymatic activities [39, 40]. The improved activity of Na^+-K^+ATPase in our result supported that glycolysis was maintained by pretreatment with pyruvate in RBCs subjected to lethal methemoglobinemia.

Finally, we determined the effect of exogenous SP on RBCs function of oxygen delivery. P_{50} is a parameter to characterize RBCs oxygen affinity during oxygen transport. Several factors influence P_{50}, including the state of Hb, 2,3-DPG, pH and temperature. When Hb is oxidized to metHb, the oxyhemoglobin dissociation curve shifts left and P_{50} decreased. In the present model, RBCs showed significantly increased metHb, left-shifited oxyhemoglobin dissociation curve and decreased P_{50} induced by methemoglobinemia. We also detected levels of 2,3-DPG, the key allosteric effector of hemoglobin, which is produced via the 2,3-DPG pathway. The results show that oxidative stress also decreased 2,3-DPG levels. However, all the changes above were reversed by the pretreatment with SP. Although pyruvate is not involved in the direct synthesis of 2,3-DPG, pyruvate promotes the glycolytic pathway and facilitates the Luebering-Rapoport pathway in RBCs, which are essential for 2,3-DPG production, as shown in **Figure 6**.

We noticed that there were inconsistencies among individual parameters. For example, metHb and MDA reductions and ATP recovery are shown with SP concentrations of 5 mM, while the GSH and 2,3-DPG augmentation were significant at as high as 20-50 mM. The paradox may be induced by various metabolic pathways shared different proportions in glucometabolism. About ~90% of glucose is metabolized via glycolysis pathway, while only ~10% of glucose is used in hexose monophosphate shunt to generate NADPH, which is used primarily to reduce glutathione. Similarly, only ~15% of 1,3-DPG resulted from glycolysis can be used to produce 2,3-DPG. Therefore, there is a highly effective onset concentration of SP (≥ 20 mM) in the 2,3-DPG pathway and hexose monophosphate shunt in the present lethal methemoglobinemia model. Moreover, the lower (10 mM) concentration of SP resulted in a significant increase in the LDH level, while 50 mM of SP induced a decrease of LDH. We speculate that it is due to increased substrate causing a negative feedback.

Limitations

First, the pretreatment concentration of SP (5-50 mM) in the present study was higher than that in other reports. The high SP level was used because the concentration of metHb in the model of methemoglobinemia reached ~70%, which was lethal and would lead to death in clinical settings. Although the concentration of SP in the experiment was extremely high, no adverse effects on RBCs had been proved in a previous pilot study, which showed that SP was superior to methylene blue in prevention of rebound methemoglobinemia (data not show). Second, the experiments were carried out with rat RBCs *in vitro*, which could not fully replace the effect and mechanism *in vivo*. Therefore, further investigations are needed to determine the impact of SP with regular concentrations on methemoglobinemia *in vivo*.

Conclusions

In the present study*, in vitro* exogenous SP protected rat RBCs against H_2O_2-induced lethal methemoglobinemia via decreasing oxidant status and improving glycolic pathways due to the dual actions of SP as an ROS scavenger and a metabolic substrate (**Figure 6**). Therefore, SP may be a prospective drug in protection of RBCs against acute methemoglobinemia. Further in depth investigations are warranted to verify SP protection of RBCs*, in vivo*.

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Disclosure of conflict of interest

None.

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