Effects and Mechanisms of Vitamin C Post-Conditioning on Platelet Activation after Hypoxia/Reoxygenation

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Keywords

Vitamin C · Reactive oxygen species · Platelets · Apoptosis · Platelet aggregation

Abstract

Background: Platelet activation occurs upon ischemia/reperfusion and is related to the generation of reactive oxygen species (ROS) during this process. Vitamin C (VC) is a powerful antioxidant. VC scavenges ROS, reduces platelet activation, and attenuates reperfusion injury. However, the effects of VC on platelets undergoing hypoxia/reoxygenation (H/R) remain unclear. **Objectives:** Herein, we evaluated the effects of VC on platelets in vitro following H/R and the related mechanisms. **Method:** Fresh platelets were collected from 67 volunteers at the Blood Center of Hebei Province. Platelets were diluted with saline to a concentration of $2.00 \times 10^{11}/L$. Aggregation and the curve slope were evaluated within 4 h with a whole-blood impedance analyzer. To determine the optimal experimental time, platelets were treated with hypoxia or reoxygenation for different times, and impedance aggregometry was carried out by measuring changes in electrical impedance induced by arachidonic acid (0.5 mM) and adenosine diphosphate (10 μM), thereby establishing the H/R model. Three antioxidants (VC, melatonin, and probucol) were used to treat platelets after H/R, and impedance aggregometry was used to determine their effects on platelet aggregation. The influence of VC on apoptosis-related indicators was detected. ROS and the mito-

chondrial membrane potential were observed by inverted fluorescence microscopy and flow cytometry, respectively. Related protein levels were detected by Western blotting. **Results:** ROS scavengers inhibited platelet activation and aggregation in a concentration-dependent manner. VC post-conditioning scavenged ROS, downregulated cytochrome C, Bax, and caspase-9 proteins, and upregulated Bcl-2 protein. These effects collectively blocked platelet apoptosis and inhibited platelet aggregation. **Conclusions:** VC inhibited platelet aggregation by blocking apoptosis. Thus, VC may have applications in the treatment of platelet-related diseases.

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Introduction

In recent years, major advancements in medical treatments have improved blood flow to ischemic tissues and organs via reperfusion; however, secondary injury is caused by ischemic and anoxic/hypoxic tissue reperfusion (i.e., ischemia/reperfusion injury), and has become an important clinical issue. Many studies have shown that platelet activation plays a critical role in this process.

Platelets are cells derived from mature megakaryocytes in the bone marrow. These cells have no nucleus,

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but have mitochondria and a plasma membrane. They also exhibit an irregular shape and function, primarily to maintain vascular integrity and prevent bleeding after vascular injury. In-depth studies of the physiological functions of platelets have revealed their key roles in human health and disease [1]. A study by Xu et al. [2] showed that activated platelets directly promoted the occurrence of reperfusion injury. Additionally, oxidative stress occurs during the reperfusion process of ischemic tissue, promoting the production of reactive oxygen species (ROS), triggering platelet activation, and causing secondary damage. Notably, the degree of platelet activation reflects the degree of myocardial ischemia. Ziegler et al. [3] ligated the left anterior descending arteries of the heart in mice for 0, 20, or 60 min to cause different degrees of myocardial ischemia, and then injected a new type of radioactive tracer combined with the activated GP IIb/IIIa molecule. Subsequent positron emission tomography of activated platelets to detect the extent of myocardial ischemia showed that this method was highly sensitive, particularly for patients who had undergone cardiac ischemia but had no significant changes in electrocardiograms or biomarkers.

Vitamin C (VC) is a natural water-soluble antioxidant with weak acidity and is involved in various antioxidant mechanisms in the human body. Notably, VC can inhibit platelet activation during ischemia/reperfusion [4], although the specific mechanisms are still unclear. Accordingly, in this study, we aimed to establish a hypoxia/reoxygenation (H/R) platelet model *in vitro*, observe the effects of VC post-treatment on platelet activity, and explore the specific mechanisms involved in VC-mediated changes in platelet activity to provide more theoretical evidence for its clinical use.

Materials and Methods

Materials

In this study, 67 platelet samples from healthy donors were obtained from Hebei Province Blood Center (Shijiazhuang, China). The platelet concentrations ranged from $9.60 \times 10^{11}/L$ to $1.28 \times 10^{12}/L$. Written informed consent was obtained from all donors, and the experimental procedures were approved by the Ethics Committee of the Second Hospital of Hebei Medical University (2017-R226). Samples included in this study were from blood donors who were strictly in accordance with the national blood donors' health examination standards. The blood platelet concentration was determined using a whole-blood cell analyzer (XE-Z100, Symex, Kobe, Japan). Platelets were then diluted to $2.00 \times 10^{11}/L$ with saline. The experiment was completed within 4 h after the collection of platelets.

Chemicals/Reagents

Arachidonic acid (AA) and adenosine diphosphate (ADP) were supplied by Chrono-Log (Havertown, PA, USA). VC was procured from Sigma-Aldrich (St. Louis, MO, USA). Melatonin

was from Biovision (Milpitas, San Francisco, CA, USA). Probucol, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and JC-1 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Antibodies targeting cytochrome C, Bax, Bcl-2, caspase-9, and β -actin were provided by Affinity Biosciences (Cincinnati, OH, USA). Dimethyl sulfoxide (DMSO) was purchased from Bosheng Chemical Reagents (Shijiazhuang, China). RIPA lysis buffer and phenylmethylsulfonyl fluoride (PMSF) storage solution were from Beijing Zhuang League International Biological Gene Technology (Beijing, China). All other reagents were of analytical grade.

Establishment of the H/R Platelet Model *in vitro*

Impedance aggregometry was carried out by measuring changes in electrical impedance using a Chrono-Log Aggregometer (model 590). One milliliter of diluted platelet protoplasm was added to the five incubators and placed in a hypoxic CO₂ incubator (N₂ concentration: 94%, O₂ concentration: 1%, CO₂ concentration: 5%). Samples were removed after 0, 5, 10, 20, and 30 min. We found that 20 min was the optimal time for hypoxia. After hypoxia for 20 min, reoxygenation was carried out for 0, 1, 3, 5, or 10 min, and platelet aggregation was detected. The activation rate of platelet aggregation was calculated according to the following formula: platelet aggregation activation rate (%) = (platelet aggregation rate of experimental group [Ω]/control group of platelet aggregation rate [Ω] - 1) $\times$ 100. We found that 5 min was the optimal time for reoxygenation.

Treatment of Platelets Subjected to H/R with Antioxidants

For the preparation of VC, 1.7612 g of VC powder was dissolved in normal saline to 100 mL, yielding a concentration of 100 mM (pH 2.37). This sample was stored at 4°C and diluted to 10, 100, or 1,000 μ M for use.

Melatonin (0.023228 g) and probucol (0.05168 g) powders were dissolved separately in DMSO to 10 mL, yielding concentrations of 10 mM. The solutions were stored at -20°C in the dark and each diluted to 0.01, 1, or 100 μ M for use in experiments.

One milliliter of diluted platelets was added to the five incubators and placed in the hypoxic CO₂ incubator as before for 20 min. Next, the diluted platelets were incubated with various concentrations of VC, melatonin, or probucol for 5 min, after which AA (0.5 mM) or ADP (10 μ M) was added. To evaluate the antiplatelet effects of these drugs, the percentage inhibition of platelet aggregation by various agents was determined.

Detection of Platelet Apoptosis-Related Indexes following VC Treatment

Determination of Endogenously Generated ROS

Endogenous ROS production in platelets was determined using DCFH-DA, an ROS-sensitive fluorescent probe. Untreated control and treated platelets were incubated with 10 μ M of DCFH-DA for 30 min at 37°C. An inverted fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) was used to image samples at an excitation wavelength of 530 nm.

Determination of Changes in Mitochondrial Membrane Potential ($\Delta\psi$ m)

A FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) was used to analyze $\Delta\psi$ m after the addition of 3 μ L of JC-1 (final concentration 3 μ g/mL) to platelets. The platelets were then incubated at 37°C for 20 min in the dark. Green fluorescence, indicating combination with the JC-1 monomer, was used to evaluate the percentages of mitochondria-depolarized platelets.

Fig. 1. Effects of hypoxia and reoxygenation for various times on platelet aggregation. **a, b** Compared with control platelets under atmospheric conditions, platelets exposed to hypoxia showed increased aggregation and curve slope, which reached a maximum after 20 min of hypoxia. **c, d** Compared with the 0 min group,

platelet aggregation and the curve slope in other groups increased and the activation rate reached a maximum in the 5 min group, but no statistical difference was observed ($p > 0.05$). Data are presented as the mean $\pm$ SD. $\blacktriangle$ $p < 0.05$ versus the 5 min group; $\&$ $p < 0.05$ versus the 10 min group; $\#$ $p < 0.05$ versus the 20 min group.

Determination of Apoptosis-Related Proteins in Platelets

For the preparation of lysates, platelets were placed at a low temperature in a high-speed centrifuge (8,000 g, 4 °C for 10 min). The supernatant was then removed, leaving only a white precipitate, and 50 μ L of phosphate-buffered saline was added to an equal amount of platelet lysate (RIPA lysate: PMSF storage solution = 100:1). The solution was then mixed and incubated on ice for 30 min for complete cell lysis. Lysates were centrifuged at 12,000 g for 10 min, and supernatants were collected as total platelet protein samples.

For Western blot analyses, proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% gels and transferred to polyvinylidene difluoride membranes. The membranes were blotted with specific antibodies against cytochrome C (1:1,000), Bax (1:1,000), Bcl-2 (1:1,000), and caspase-9 (1:1,000; all from Abcam, Cambridge, MA, USA) at 4 °C overnight and then incubated with goat anti-rabbit secondary antibodies (1:5,000) for 2 h. The protein bands were detected using a Chemiluminescence Plus Western Blot Analysis Kit (Bio-Rad, Hercules, CA, USA). Relative quantification was carried out by measuring the band density with Quantity One V4.6.2 software (Bio-Rad). Bands of interest were normalized to the expression of β -actin. The experiments were replicated at least three times.

Data Analysis

All data are expressed as means $\pm$ standard deviation. Statistical analysis was performed with SPSS 22.0 software (IBM, Chicago, IL, USA). Statistical significance among groups was determined by one-way analysis of variance followed by Tukey's test for comparison of means. Differences with p values < 0.05 were considered significant.

Results

Effects of Hypoxia or Reoxygenation Time on Platelet Aggregation

Compared with control platelets maintained under atmospheric conditions, platelets exposed to H/R showed an increased platelet aggregation and curve slope, with maximum responses after 20 min of hypoxia. Significant differences were observed between the 5 and 20 min groups ($p < 0.05$); however, there were no significant differences among the 5, 10, and 30 min groups ($p > 0.05$; Fig. 1a, b).

In our analysis of reoxygenation time, platelet aggregation and curve slopes increased in all groups compared with the 0 min control group, and the activation rate reached a maximum in the 5 min group; however, there were no significant differences observed among all groups ($p > 0.05$; Fig. 1c, d).

Effect of Antioxidant Post-Treatment on Platelets Subjected to H/R

Compared with the H/R group, platelet aggregation in cells treated with 1,000 μ M of VC was decreased significantly ($p < 0.05$). In contrast, no significant differences

Fig. 2. Effects of different concentrations of VC on platelet aggregation and inhibition rates after hypoxia/reperfusion. **a** Compared with the hypoxia/reperfusion group, platelet aggregation in the VC3 group decreased significantly ($p < 0.05$), while no statistical difference was found for the VC1, VC2, and HCl groups ($p > 0.05$). **b** Compared with the hypoxia/reperfusion group, the platelet in-

hibition rate in the VC3 group increased significantly ($p < 0.05$), while no statistical difference was found for the VC1 and VC2 groups ($p > 0.05$). Data are presented as the mean $\pm$ SD. $\blacktriangle$ $p < 0.05$ versus VC1; $\&$ $p < 0.05$ versus VC2; $\#$ $p < 0.05$ versus VC3. VC1, vitamin C 10 μM ; VC2, vitamin C 100 μM ; VC3, vitamin C 1,000 μM .

were observed in the 10 and 100 μM and HCl groups ($p > 0.05$). Treatment with 1,000 μM of VC showed the highest inhibition rate ($p < 0.05$; Fig. 2).

Notably, platelet aggregation in cells treated with 100 μM melatonin was decreased significantly ($p < 0.05$) compared with the H/R group. There were no significant differences in the 0.01 and 1 μM groups ($p > 0.05$). Moreover, the 100 μM group showed the highest inhibition rate ($p < 0.05$), and 0.01 μM melatonin activated, but did not inhibit, platelet aggregation (Fig. 3).

Finally, we analyzed the effects of probucol on platelet aggregation. Compared with the H/R group, platelet aggregation in the probucol group (100 μM) decreased significantly ($p < 0.05$), whereas no significant differences were found in the 1 and 10 μM groups ($p > 0.05$). Again, the 100 μM group showed the highest inhibition

rate compared with the 1 and 10 μM groups ($p < 0.05$; Fig. 4).

Effects of VC on Platelet Apoptosis-Related Indicators

Compared with the control group, ROS levels were significantly increased in the H/R, VC, and HCl groups. Moreover, ROS levels in the VC group decreased, whereas those in the HCl group showed no changes compared with ROS levels in the H/R group. Thus, these findings suggested that VC acted as a powerful ROS scavenger (Fig. 5a).

Next, we evaluated the $\Delta\psi\text{m}$. Compared with the control group, $\Delta\psi\text{m}$ values in the H/R, VC, and HCl groups increased significantly ($p < 0.05$). Additionally, $\Delta\psi\text{m}$ values in the VC group decreased compared with those in the H/R group ($p < 0.05$), and there were no

Fig. 3. Effects of different concentrations of melatonin on platelet aggregation and inhibition rates after hypoxia/reperfusion. **a** Compared with the hypoxia/reperfusion group, platelet aggregation in the M3 group decreased significantly ($p < 0.05$), while no statistical difference was found for the M1 and M2 groups ($p > 0.05$). **b** Compared to the M1 and M2 groups, the M3 group showed

the greatest inhibition rate ($p < 0.05$). Data are presented as the mean $\pm$ SD. $\blacktriangle$ $p < 0.05$ versus M1; $\&$ $p < 0.05$ versus M2. H/R1, 10 μ L 0.9% sodium chloride injection solution; H/R2, 10 μ L DMSO; M1, melatonin 0.01 μ M; M2, melatonin 1 μ M; M3, melatonin 100 μ M.

significant differences ($p > 0.05$) between the HCl and H/R groups, suggesting that the ability to alleviate mitochondrial injury was related to ROS scavenging, not acidity (Fig. 5b).

Finally, we analyzed changes in the expression of apoptosis-related proteins, including Bax, Bcl-2, cytochrome C, and caspase-9. Our results showed that the levels of cytochrome C, Bax, and caspase-9 increased in the experimental groups compared with those in the control group, suggesting that cytoplasmic apoptotic events related to the mitochondrial pathway were stimulated in platelets. Notably, in the VC group, the expression levels of cytochrome C, Bax, and caspase-9 decreased, whereas the expression level of Bcl-2 increased significantly compared with the H/R group. These results demonstrated that VC suppressed activation of the

hypoxia-induced intrinsic apoptosis pathway and hindered AA-induced platelet aggregation and adhesion (Fig. 5c, d).

Discussion

Ischemia/reperfusion injury involves many pathophysiological processes, including platelet activation [5]. The process of organ/tissue ischemia/reperfusion can cause the production and release of active oxygen in nucleated cells [6]. Moreover, platelets can produce ROS after various physiological or pathological stimuli, and the formation of thrombi is accompanied by the release of ROS [7, 8]. ROS are involved in platelet formation, activation, or enzyme digestion. In recent years, studies have

Fig. 4. Effects of different concentrations of probucol on platelet aggregation and inhibition rates after hypoxia/reperfusion. **a** Compared to the hypoxia/reperfusion group, platelet aggregation in the P3 group decreased significantly ($p < 0.05$), while no statistical difference was found for the P1 and P2 groups ($p > 0.05$).

b Compared to the P1 and P2 groups, the P3 group showed the greatest inhibition rate ($p < 0.05$). Data are presented as the mean $\pm$ SD. $\blacktriangle$ $p < 0.05$ versus P1; $\&$ $p < 0.05$ versus P2. H/R1, 10 μ L 0.9% sodium chloride injection solution; H/R2, 10 μ L DMSO; P1, probucol 1 μ M; P2, probucol 10 μ M; P3, probucol 100 μ M.

shown that ROS in platelets are derived from the NADH/NADPH oxidase system [9, 10] and the metabolism of AA; the former is the main source of ROS and plays a major role in determining the levels of ROS in platelets.

Platelet apoptosis is a major pathway for platelet death [11]. Similar to other cells, platelet homeostasis is the result of a balance between platelet production and destruction. Although platelets are nucleus-free blood cells, they contain many apoptotic and anti-apoptotic proteins, and ribosomes from megakaryocytes permit protein synthesis in platelets [12]. Many factors can induce apoptosis in platelets, including high blood flow shear force [13], thrombin [14], collagen [7], the calcium ion carrier A23187, and platelet storage conditions [15]. Additionally, ROS play very important roles in thrombocytopenia and collagen-induced apoptosis [16]. Application of an-

tioxidants can inhibit apoptosis [17], and thrombin and collagen induce platelet activation and aggregation, suggesting that there is a correlation between platelet apoptosis and activation [18].

Previous studies have shown that apoptotic platelets produce particles that subsequently promote platelet activation and accelerate platelet aggregation [19, 20]. To eliminate the interference of a variety of confounding factors in the body, we simulated ischemia/reperfusion in an isolated platelet H/R model [21, 22]. We then selected three antioxidants (VC, melatonin, and probucol) to observe the effects of antioxidants on platelet aggregation and to detect apoptosis-related indexes in platelets in order to explore the specific mechanisms through which VC affects H/R in platelets. Our results showed that the three antioxidants inhibited platelet activation to varying

Fig. 5. Effects of VC on platelet apoptosis-related indicators. **a** ROS generation in the different groups, as observed by inverted fluorescence microscopy (×10). Compared with the control group, ROS levels significantly increased in the H/R, VC, and HCl groups. Compared with the H/R group, ROS levels in the VC group significantly decreased ($p < 0.05$). **b** Mitochondrial membrane potential in the different groups, as determined by flow cytometry. Mitochondrial membrane potential ($\Delta\psi_m$) values in the VC group decreased compared with those in the H/R group ($p < 0.05$). **c** In

the VC group, the expression level of cytochrome C decreased significantly compared with the H/R group ($p < 0.05$). **d** The expression levels of Bax and caspase-9 decreased in the VC group, whereas the expression of Bcl-2 increased significantly compared with the H/R group ($p < 0.05$). H/R, hypoxia/reperfusion; VC, vitamin C 1,000 μM ; HCl, 10 μL hydrochloric acid (pH = 2.37); C1, platelets at room temperature; C2, platelets at 37 °C in order to exclude the influence of temperature.

degrees in concentration-dependent manners. Additionally, ROS produced by H/R increased, and the activation degree increased, while antioxidants removed ROS and inhibited the activation and further aggregation of blood platelets.

VC, also known as L-ascorbic acid, is a natural water-soluble, weakly acidic vitamin with strong antioxidant ability [23]. Recent studies have reported the antiplatelet effects of VC [24]. In this study, we confirmed that high concentrations of VC strongly inhibited platelet aggregation, whereas the HCl group did not show obvious inhibitory effects. Additionally, inverted fluorescence microscopy showed that ROS levels were significantly

lower in the VC group than in the H/R group, indicating that acidity was not related to platelet aggregation effects.

Melatonin is an indole neuroendocrine hormone secreted by the pineal body. Many basic and clinical studies have confirmed that melatonin has extensive effects on biological rhythm and on the reproductive, immune, digestive, central nervous, and cardiovascular systems [25]. Moreover, melatonin has antioxidant and antitumor effects and is widely used for immunomodulation, antiaging, and antitumor treatments [26]. Importantly, in this study, we found that low concentrations of melatonin activated platelet aggregation, whereas higher concentra-

tions inhibited platelet aggregation, similar to the results of previous studies [27].

Probucol was used in the 1970s in the USA and was believed to reduce serum cholesterol. Recent studies have shown that probucol also has antiatherosclerosis effects, modulates oxidative stress, and protects the blood vessel endothelium [28]. During exposure to hypoxia, platelets produce large amounts of ROS, and active oxygen can induce aggregation by participating in platelet-related signal transduction pathways [29]. Moreover, antioxidants can inhibit platelet aggregation by removing the produced ROS. In this study, we showed that probucol inhibited platelet activation and aggregation in a concentration-dependent manner. Thus, our results showed that all three antioxidants inhibited the aggregation of platelets exposed to H/R, indicating that activation and aggregation of platelets exposed to H/R were closely related to oxidative stress.

VC is not widely used in the clinical setting during ischemia/reperfusion therapy. In this study, we confirmed that VC post-conditioning inhibited platelet activation during H/R. The decreased ROS and platelet apoptosis index in the VC group supported the relationship between inhibition of platelet aggregation and anti-apoptotic effects. Moreover, the results of the HCl group (at the same pH) were not significantly different from those of the H/R group, indicating that the inhibitory effects on platelet aggregation were not related to the weak acidity of the treatment.

As described above, platelet apoptosis is a type of programmed cell death induced by various physiological and pathological stimuli. Apoptosis induced in platelets occurs mainly through signaling pathways in the mitochondria; these signaling pathways affect the function of the mitochondria, decrease the $\Delta\psi_m$, and activate apoptotic proteins, resulting in activation of caspase-3 and induction of apoptosis [30]. During this process, mitochondria provide energy for cell survival and act as the center of apoptosis regulation. Proapoptotic Bax and antiapoptotic Bcl-2 play important roles in the regulation of platelet apoptosis, and the relative ratio of these proteins determines whether cells undergo programmed cell death. In this study, we found that H/R significantly increased the $\Delta\psi_m$ and cytochrome C, caspase-9, and Bax expression, and significantly decreased Bcl-2 expression, indicating that platelet apoptosis played an important role in platelet activation. When VC was added before reoxygenation, the $\Delta\psi_m$ and cytochrome C, caspase-9, and Bax expression decreased, whereas Bcl-2 expression increased. Thus, these results showed that VC inhibited platelet aggregation by blocking apoptosis. Moreover, these effects were related to ROS scavenging ability, not acidity.

In clinical trials, Basili et al. [31] found that VC treatment inhibited platelet activation to some extent, consis-

tent with the results of the present study. This suggests that VC could be used as an antiplatelet drug. Indeed, we propose that VC could be applied to reduce the extent of tissue and organ ischemia/reperfusion injury. However, further studies are needed to assess these effects, particularly in patients with various comorbidities.

This study had some limitations. First, the use of an isolated platelet H/R model cannot accurately assess the effects of leukocytes, macrophages, and various cytokines in the body, and the drug may interact with other drugs in the body. Therefore, the effects of VC on platelet aggregation are expected to be more complex in vivo. In addition, platelet collection and experimental procedures may lead to partial platelet activation, affecting the accuracy of the results.

Conclusion

We found that ROS scavengers concentration-dependently inhibited platelet activation and aggregation. Moreover, VC post-conditioning scavenged ROS and caused downregulation of cytochrome C, Bax, and caspase-9 proteins and upregulation of the Bcl-2 protein. VC treatment also reduced platelet apoptosis and inhibited platelet aggregation.

Statement of Ethics

Written informed consent was obtained from all blood donors, and the experimental procedures were approved by the Ethics Committee of the Second Hospital of Hebei Medical University (2017-R226).

Disclosure Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by the National Natural Science Foundation of China (No. 81402510) and Hebei Province Natural Science Foundation (No. H2015206139).

Author Contributions

W.C. was involved in study design and manuscript editing; D.L. and D.P. performed the majority of the laboratory work and contributed to writing the manuscript; H.H. and G.G. were responsible for the analyses of data.

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