

September 16, 2016

Dr. Jin-Xin Kong  
Science Editor  
World Journal of Critical Care Medicine

RE: Revision of ESPS manuscript NO: 29038; Title: Impact of High Dose Vitamin C on Platelet Function

Dear Dr. Kong:

On behalf of all the authors please accept our sincere thanks for your prompt review of our manuscript entitled: *Impact of High Dose Vitamin C on Platelet Function*. The reviewers have classified it as Grade B (very good) and concluded that it has a High priority for publication. Our work was further commended for making "additive knowledge to clinical transfusion". The reviewers also praised that our new findings were well supported by the quantitative data presented together with clarity of the manuscript. In response to reviewers' suggestions and inquiries, we have made a few changes to the manuscript and also added a few edits to improve the readability of the manuscript.

#### Reviewer 1 Comments:

- **Comment #1:** Whether this so called higher dose really indicates a "high dose" in human body?

**Response #1:** We employed 2 doses of VitC in this study: Lo VitC, 0.3mmol/L (final concentration) and Hi VitC, 3mmol/L (final concentration). These levels were designed to correlate with the plasma levels obtained in our recent clinical trial of intravenous ascorbic acid in sepsis (Reference #16, Fowler et al) and others (Reference #15 and 17 – 19). The plasma levels of VitC achieved in the clinical trial, with repetitive dosing (200mg/kg/day) over 4 days, was 50 times higher than normal plasma levels (40-70µmol/L). In the current in vitro study, a single dose of 3mmol/L VitC added to the platelet concentrates, increased the intracellular level of VitC 13-fold within 48 hours. The intracellular level remained elevated throughout the remainder of the study. Therefore, defining an *in vitro* concentration of 3mmol/L VitC as a relatively high dose is warranted.

- **Comment #2:** Many kinds of platelet function tests have been mention to be representative of platelet function, however, none of them can be the main one especially translate into clinical subjects. Please identify your chosen parameters to be their best performance for clinical translational endpoints.

**Response #2:** We agree with the reviewer that none of these tests, in isolation, can be used to translate findings into clinical subjects. However, the array of tests performed were used to investigate various aspects of human conditions in which the function of platelets comes into question. For example, TEG addresses functions of platelets under sluggish flow conditions that is often observed following wounds and trauma. Indeed, TEG technology has been a major aid in decision-making for surgeons following trauma and cardiac surgery and for triaging patients to receive blood components to aid with clotting, reverse heparin action and screen for blood thinner usage in unconscious patients. Platelet aggregation assays, on the other hand, give insights into the initial platelet response to stimuli like Collagen and ADP under higher shear conditions. Flow cytometry assays enable one to screen for platelets granule pool defects and provide more sensitive technology to detect fine changes that might otherwise go unnoticed. Hence all these platelet function tests have relevance to clinical translational endpoints and the ultimate effect is the aggregate of these various assays.

#### Reviewer 2 Comments:

- **Comment #1:** The authors mentioned in Method section and used 1500 x g to prepare platelet rich plasma however, this speed will sedate platelets. How long the centrifugation is performed is also not clear. Furthermore, 5000 x g will activate platelets which may influence the following studies. In addition, in Sample Processing, the author further mentioned they use 2000 x g to get PPP. It's

completely confusing. These should be clarified and all preparations should follow a mature and standard protocol.

**Response #1:** We thank the reviewer for allowing us to clarify this. We used a 2 step process to isolate platelets for analysis in this study.

**STEP 1: Platelet Concentrate Preparation:** This step describes the initial preparation of platelet concentrates from freshly donated blood. Single donor platelet concentrates preparation was performed by Virginia Blood Services (Richmond, VA), a commercial facility that processes freshly donated blood and prepares platelet concentrate bags for therapeutic or prophylactic transfusion purposes. This facility follows the AABB guidelines described in the AABB Technical Manual for platelet manufacture. In brief, these guidelines state that “First spin: Separates whole blood unit into platelet rich plasma (PRP), buffy coat (WBC’s) and red blood cells (RBCs) by centrifugation at 2000 x g for 3 min (soft spin). Second spin: The first manufacture of the platelet-rich plasma is followed by the second spin at 5,000 x g for 5 min (hard spin) obtaining the platelet concentrate. During the second spin, platelets are pelleted from the PRP to yield platelet concentrate and platelet poor plasma.” This procedure was used by Virginia Blood Services for preparation of platelet concentrates in transfusion bags. We supplemented these bags with one of three additives: normal saline (control); 0.3mmol/L VitC (Lo VitC); or 3mmol/L VitC (Hi VitC). Prior to supplementation, an initial baseline sample was collected at the Virginia Blood Services facility and transported to our laboratories for further processing (STEP 2 below). Bags containing the platelet concentrates remained at Virginia Blood Services until they passed standard screening tests (2 days). These bags were then transported to the Virginia Commonwealth University Transfusion Medicine Center and sampled again on days 2, 5, and 8. This portion is described in the “Platelet Concentrate Preparation” section of the Methods.

**STEP 2: Sample Processing:** This step describes the methodology for preparation of Adjusted PRP and PPP. This step was performed in our laboratory using microcentrifuge tubes. The initial baseline samples as well as samples removed using sterile technique from the bags on days 2, 5 and 8 were processed in our laboratory in tubes. An initial platelet count was obtained and a portion of the sample was used to obtain platelet poor plasma (PPP) by centrifugation at 2,000 x g for 10 minutes. The resultant PPP was then used to adjust the sample platelet concentration to 230-270 x10<sup>3</sup>/μl (Adj. PRP). This portion is described in the “Sample Processing” section of the Methods.

- **Comment #2:** In page 7, last line, and page 8, the first line, for ascorbate detection, platelets were washed with cold PBS, which will activate platelet and induce platelet secrete or shed their component, results in inaccurate ascorbate concentration.

**Response #2:** We thank the reviewer for correcting our error. We used PBS at room temperature (22°C) and not cold PBS as initially mentioned. We have made this change in the revised document.

- **Comment #3:** In platelet secretion assay, it is not clear if the divalent cations were added or not.

**Response #3:** In accordance with manufacturer’s recommendation for running platelet aggregation/secretion assays, no divalent cations were added.

- **Comment #4:** In Flow Cytometry section, what is normal saline? It should be just sterilized saline.

**Response #4:** The normal saline used in these studies was sterile saline (commercially available 0.9% NaCl in water). We have revised the manuscript to show this change.

- **Comment #5:** In page 7, Platelet concentrate preparation, how much residual plasma was used?

**Response #5:** Platelet concentrates were prepared by Virginia Blood Services (Richmond, VA) following a standard platelet-rich plasma (PRP) method using a two-step centrifugation process. After the second spin, platelets were suspended in fifty ml of residual plasma.

- **Comment #6:** It is also not clear if the platelet concentration from each individual was adjusted to similar before they were treated with Lo/Hi VitC.

**Response #6:** Platelet concentrates were prepared by Virginia Blood Services (Richmond, VA). Upon completion of processing, the investigators added Saline or Lo/Hi VitC to the platelet concentrates. The units were held at the Virginia Blood Services facility for 24 hours for screening, then transported to the Virginia Commonwealth University Transfusion Medicine Center for sampling and analysis. While we were unable to perform an initial platelet count before supplementation with Lo/Hi VitC at Virginia Blood Services, analysis of all samples was performed after adjusting each platelet concentration to 230-270  $\times 10^3/\mu\text{l}$  (Adj. PRP)

- **Comment #7:** One interest finding in Figure 5 is that the response of stored platelets to collagen is gradually decreased, but was abolished to ADP at the second day. The authors should discuss this phenomenon and explain why.

**Response #7:** We thank the reviewer for pointing out this phenomenon, which was identified as early as 1979 in blood bank research (Moroff G, *et al.* Aggregation response of human platelets stored at 22C as platelet-rich plasma. Transfusion 1979; 19:704-18). It is thought that this reduced ADP aggregation response results from poor platelet calcium mobilization that occurs during storage. Our data are also consistent with other data by Reddoch, *et al* (Shock 2014;4:54-61). Unfortunately we did not measure calcium mobilization in this study.

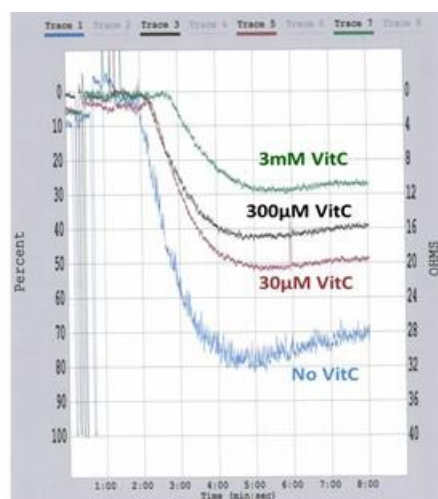
### Reviewer 3 Comments:

- **Comment #1:** I do not observe any significant differences in the intraplatelet level of Vit C between the different days and it appears that the highest levels are already reached after 2 days. Thus, the effects on time might not be specific for Vit C. Please, discuss this point.

**Response #1:** We thank the reviewer for correctly pointing out that there are no significant differences in the intra-platelet level of VitC between the different days and it appears that the highest levels are already reached after 2 days. *In vivo*, intracellular platelet ascorbate levels are dependent on the plasma VitC concentration and are usually 20-40 fold higher. In the current *ex vivo* study, intracellular platelet VitC levels rose rapidly and were 3-15 fold higher depending on the external VitC concentration. The intracellular platelet VitC levels plateaued after 48 hours and remained constant indicating dose-dependent saturation. It is possible that these unanticipated effects arise due to the *ex vivo* storage conditions of the platelets. Platelets in storage bags are out of their normal environment, densely crowded, and have limited access to nutrients. Accumulation of products of metabolism in the storage bags and other factors associated with platelets storage beyond 5 days could also impact the system buffering capacity leading to the drop in pH observed in our study. This change in pH combined with the closed nature of the *ex vivo* system could also account for the observed effects. We have added this to the DISCUSSION section to the revised manuscript.

- **Comment #2:** What happens with platelet function after a short time exposure of VitC, ie: 30-60 min? Since platelets in-vitro shut off many of their activation pathways after 4-6 hrs it is very important to understand the effect of these high Vit C concentrations on platelet functional responses at shorter times after Vit C treatment.

**Response #2:** We thank the reviewer for pointing out this important aspect. While the primary goal of the study was to assess the impact of long-term exposure (5 – 8 days) of platelets to VitC, we also measured the short term (30 minutes) effects of VitC on platelet aggregation. As seen in the figure, we found that in normal volunteers, pre-treatment of platelets with increasing



doses of VitC for 30 minutes incrementally attenuated platelet aggregation following activation with lipopolysaccharide (LPS) /collagen. Since this has already been shown by other researchers (Cowan DH, Graham RC Jr, Shook P, Griffin R, The influence of ascorbic acid on platelet structure and function, Thromb Diath Haemorrh. 1975 Sep 30; 34(1):50-62; Cordova C, Musca A, Violi F, Perrone A, Alessandri C, Influence of ascorbic acid on platelet aggregation in vitro and in vivo, Atherosclerosis. 1982 Jan; 41(1):15-9) and was not the primary focus of our study, we did not include this information in the manuscript.

Thank you for your consideration of this work. If there are any questions I can answer, please feel free to contact me by email or by phone. With warmest regards, I am,

Sincerely,

Ramesh Natarajan, PhD  
Professor of Medicine  
Division of Pulmonary Disease and Critical Care Medicine  
Department of Internal Medicine  
VCU School of Medicine