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Dr. Alisha DSouza
Review Editor
JoVE

MS: JoVE55525

Title: Turbidimetry on human washed platelets: the effect of the Pannexin1 inhibitor Brilliant Blue FCF on collagen-induced aggregation.

Dear Dr. DSouza,

Thank you for your recent letter regarding the above-mentioned manuscript. We thank the editors and reviewers for their constructive comments and suggestions.

We thoroughly revised the manuscript to address the points raised by the editors and reviewers. In short, more details on the protocol steps are now given, figures have been modified following the suggestions of the reviewers and the discussion has been extended.

For your information, all the changes specified under Editorial comments have been applied to the references, keywords, figures and figure legends, table of essential supplies, reagents and equipment, protocol steps as well as to the discussion have been performed. Of note, the signed Article and Video License Agreement has been sent by Email to Teena Mehta on October 6th, 2016. We have now uploaded it again in the *JoVE* website.

We are looking forward to your response regarding the acceptability of our revised manuscript for publication in the *JoVE*.

Yours Sincerely,

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Reviewer #1:

We thank the reviewer for his/her constructive suggestions that helped us to improve our manuscript. We respond to each of his/her remarks in detail below. *Comments of the reviewer are cited in italics.*

Major concerns:

*Protocol step 1.2.1 requires 80g NaCl, which results in a concentration of 2.7 molar, not millimolar, NaCl in stock solution 1. Also, by my calculations, 0.58g NaH2PO4*H2O (138g/mol) is 8.4mM, not 8.6mM. Additionally, given the complexity of the stock buffers being mixed to create final buffers for use, it would be helpful to list the final concentrations of each component of the actual buffers, not only the stocks to be further diluted. As multiple variants of Tyrode's Buffer are searchable online, it is important to clarify what final ionic content your protocol seeks. E.g. at the end of 1.3, "Final concentrations are (in mM) NaCl 136.5, KCl 2.7, NaHCO3 11.9, NaH2PO4 0.4..."*

We thank the reviewer for this thoughtful comment. All the concentrations have been recalculated and the correct values are now given in the text. As suggested, the final concentrations of each component of the solutions and buffers are now given in protocol steps 1.2.1, 1.3 and 1.4.

- *Apyrase is added to "avoid [allowing] washed platelets to become resistant against the action of ADP," per the introduction. However, apyrase is added at step 3.10 in the protocol and appears to remain in the suspension of platelets throughout the experiment. As you are describing a response that you claim is based on an extracellular ATP/ADP-driven signaling pathway, the presence of "a relatively high concentration of" a potent ATP/ADP-hydrolyzing enzyme throughout the experiment is a major methodological concern*

Apyrase is added to the platelet suspension in order to reduce the risk of potential activation of platelets by extracellular ATP/ADP, which will cause desensitization of purinergic receptors. In this study, we investigate aggregation responses to collagen, which critically depends on a fast activation of P2X1 receptors by ATP released from activated platelets. To further clarify this issue, we have now added a phrase in the note following protocol step 3.10.

- *The overall conclusion exceeds the scope of the paper for two reasons:*
1) No mention of Panx1 is made in this paper, beyond citations of previous work discussing Brilliant Blue and a role for Panx1 in platelet aggregation. To claim that Brilliant Blue has the claimed effects on platelets via action on Panx1, additional experiments would be needed. You currently offer as comparison the activation of a different pathway via arachidonic acid, showing that BB effects are specific to the collagen pathway, but don't show that the BB effects are due to inhibition of Panx1 in particular. Use of other pharmacologic inhibitors of Panx1 (e.g. Trovafloxacin, Probenecid, Carbenoxolone, 10Panx1) or genetic knockout of Panx1 to confirm the effects of BB as acting via Panx1 would solidify this claim.

Our manuscript has been written to join the overall goal of *JoVE*, *i.e.* providing the readers with written and visual detailed protocols of scientific experiments. As such the experiments involving Brilliant Blue were intended as a working example and justified by and asked for

by *JoVE* on the basis of our earlier publication in *Thrombosis and Haemostasis*. We agree with the reviewer that investigating the effect of Brilliant Blue FCF on collagen responses of platelets extracted from Panx1$^{-/-}$ mice would confirm that the inhibitory action of the dye on platelet aggregation is due to its action on Panx1 channels. Given the short time given by *JoVE* for this revision (2 weeks), these experiments can unfortunately not be performed due to lack of availability of Panx1$^{-/-}$ mice. Moreover, the protocol to prepare washed platelets from mice differs considerably from the protocol for human washed platelets, and is beyond the scope of this manuscript.

2) BB is suggested here as a non-toxic potential inhibitor of platelet function, but earlier in the manuscript you note that intake of BB "likely has to largely exceed the normal levels before any side effects on platelet aggregation may be anticipated." Thus you have already stated that BB dye cannot be used as a potential platelet inhibitor at the concentrations known to be non-toxic, and no detail is given of the therapeutic window of BB or the extent of the gap between food-related concentrations and platelet-inhibiting concentrations of the dye. To quote the age-old toxicology adage, "the dose makes the poison." It is inappropriate to claim that BB dye may be expected simultaneously to be non-toxic while used at levels presumably far higher than for existing uses, unless evidence exists to support this claim.
Unless data or citations exist to back up this conclusion, it should be revised to reflect more accurately the limited extent of the data contained in this paper.

The reviewer is right that it is inappropriate to claim that BB dye may be expected simultaneously to be non-toxic while used at levels presumably far higher than for existing uses in daily food practice. Our intentions with the word “toxic” were however different, i.e. we were not referring to toxicity for the human race but rather to side-effects in the experiments that could mask an anticipated experimental outcome. We have changed the paragraph in the discussion accordingly (p.9; 4th paragraph of the discussion section).

Minor concerns:

- The first paragraph of introduction should include a citation, e.g. to a review of platelet function. It should also be edited to read in complete sentences instead of as an unformatted list in paragraph form.

As suggested by the reviewer, we added two new references in the first paragraph of the introduction (Patel *et al. J Clin Invest.* **115** (12), 3348-3354, Andrews *et al. Thromb Res.* **114** (5-6), 447-453).

In protocol step 1.4, what is the osmolarity of the buffer? This should be calculable. Does an osmolarity adjustment always require the addition of 10% total volume of H2O at this step? If so, this volume should be included in the final volume of the previous sentence (110mL instead of 100mL diH2O). Or, simply instruct to test osmolarity and adjust to 295mOsm/L. How much H2O is acceptable to add for this purpose before significantly changing the ionic concentration of the buffer to an unacceptable extent? Please advise.

We provide the value of the desired osmolarity (295 mOsm/L) in case other investigators would like to measure it. In practice however it always appeared correct to simply add 10% of the total volume H_2O, which we now standardly do.

In protocol step 3.2, clarify what portion of the centrifuged blood to collect, e.g. "PRP will be the top layer of liquid in the tube, above the cell pellet of white and then red blood cells." Additionally, in 3.3, avoiding contamination should reference avoiding the entire cell pellet, including the buffy coat of white blood cells, not only the underlying red blood cell pellet.

Following the suggestion of the reviewer, we have now added the requested information in a note after protocol step 3.2. (page 5).

Protocol step 3.7 should read "TA buffer" per the previously noted abbreviation, not "TA."

The change has been made.

In the discussion, what are "normal levels' of blue food dye? Can you provide a rough estimate of order of magnitude of dietary levels versus that required for platelet aggregation?

We thank the reviewer for raising this interesting point. We give now information published by the European Food Safety Authority on the levels of Brilliant Blue FCF in food and compare them to the concentration used in our experiments. Moreover we state now that "assuming that only a small fraction of the dye will be absorbed in the intestines after oral ingestion and that the dye will finally be diluted in the 5 L blood of an adult person, the daily intake of this Blue food dye likely has to largely exceed the normal levels before any side-effects on platelet aggregation may be anticipated." (p.9; 4th paragraph of the discussion section).

In protocol step 3.8, give the parameters for identification of platelets using an automated cell counter - e.g. min/max size, roundness, etc. It should not be assumed that all counters use the same parameters, or have preset parameters by cell type.

The automatized cell counter used to determine the number of platelets detects the size of blood cells by measuring the changes in direct-current resistance. This information has been added to protocol step 3.8.

In the last sentence of the second-to-last paragraph of your discussion, why would a lower concentration of apyrase be sufficient to avoid desensitization of other PRs in platelets? If you have data or a citation for ATP levels that cause desensitization of different PRs in platelets, please back up this claim. Otherwise, it would not logically follow given the expression of other PRs in platelets, including P2YRs (including P2Y1 and P2Y12, both accepted to have roles in platelet aggregation) with ATP/ADP affinities more sensitive by an order of magnitude or greater, than the P2X1R that is your main concern.

As specified in the manuscript, we use a relatively high concentration of apyrase to avoid the desensitization of P2X1 receptors by spontaneous secretion of ATP in absence of agonists. However, when the studied platelet signaling pathway does not critically require preservation of P2X1 function, a final concentration 0.02 U/mL apyrase may be used. This statement is in accordance with many studies (reviewed in Mahaut-Smith *et al*. *Purinergic Signal.* **7** (3), 341-356) demonstrating that 0.02 U/mL apyrase avoids ADP receptor P2Y1 desensitization with negligible P2X1 responses. This information and reference is now added to the manuscript in a note after protocol step 3.10. (page 5/6)

Reviewer #2:

In the paragraph 1 in the Introduction, at platelet adhesion step, besides collagen, VWF is also involved. This part should be rewrote.

We thank the reviewer for this insightful remark. We now mention of Von Willebrand factor's role in platelet adhesion after injury in the first paragraph of the introduction. (page 2)

Some spelling error in the text," a-nuclear" --"anuclear","such a myocardial infarction"--"such as myocardial infarction","resupension"--"resuspension".Please check the whole manuscript carefully.

A careful check throughout the manuscript has been done to correct spelling errors.

Reviewer #3:

Major concerns:

Figure 1: instead of representative images of the aggregometer herein employed, which may vary depending on the manufacturer, authors could provide imagens of cuvettes containing washed platelets either in solution of after the aggregation process. These imagens easily apply to any device.

We thank the reviewer for this attractive suggestion. Accordingly, we modified Figure 1A into a glass cuvette containing resting platelets or activated platelets that formed aggregates after collagen activation.

Figure 2: Should we expect intermediary aggregation curves by using 250 or 500 µM Brilliant Blue FCF? Representative curves showing the maximum effect (i.e. no aggregation) vs no effect towards collagen-induced aggregation fall to exemplify a dose-response curve. In addition, it would be informative to make clear that other parameters could be extracted from the aggregation curves (slope, area etc.).

Our manuscript has been written to join the overall goal of *JoVE*, *i.e.* providing the readers with written and visual detailed protocols of scientific experiments. As such the experiments involving Brilliant Blue were intended as a working example and justified by and asked for by *JoVE* on the basis of our earlier publication in *Thrombosis and Haemostasis*. We completely agree with the reviewer that other parameters could be extracted from the aggregation curves (slope, area etc.) and have added this valuable information to the manuscript (point 4.8; page 7). Although we also agree that showing aggregation curves after pre-incubation of human washed platelets with intermediate concentrations of Brilliant Blue FCF might be informative, these additional experiments have not been performed given the short time given by *JoVE* for this revision (2 weeks).

Minor concerns:

On page 3, Introduction section, 1st paragraph: Here the authors provide the basic information concerning the role of platelets in the hemostatic system but no references are given.

As suggested by the reviewer, we added two new references in the first paragraph of the introduction (Patel *et al. J Clin Invest.* **115** (12), 3348-3354, Andrews *et al. Thromb Res.* **114** (5-6), 447-453).

On page 9, Discussion section, 2nd paragraph: authors discuss the possible sources of individual variability in the aggregation assays but, again, no references have been provided.

Some references (Fusegawa *et al. Thromb Res.* **93** (6), 271-278, Davis *et al. Med Sci Sports Exerc.* **22** (1), 49-53, Patel *et al. FEBS Lett.* **588** (8), 1372-1378) concerning the individual variability point have now been added to the manuscript.

It would be nice to expand the discussion by including other inhibitors of collagen-mediated platelet aggregation including synthetic compounds and naturally-occurring factors.

This *JoVE* manuscript focusses on the methodology of preparing washed human platelets and performing aggregation experiments. The effect of Brilliant Blue on collagen-induced platelet aggregation is only intended as an illustration. We therefore consider an extensive discussion on other inhibitors of collagen-mediated platelet aggregation beyond the scope of this manuscript.