Platelet-based Detection of Nitric Oxide in Blood by Measuring VASP Phosphorylation
--Manuscript Draft--

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Bethesda, August 22, 2018

Dear Dr. Bajaj,

Please find enclosed corrected manuscript (JoVE58647) "Platelet-detection of nitric oxide in blood: measurement of VASP phosphorylation after nitrite reduction by red blood cells" after implementing editorial comments. The "accepted changes file" contains the highlighted protocol that we believe should be a basis of the video part.

We thank the editorial office for valuable help with this manuscript and hope that it is now in form suitable for publication.

Respectfully,

Barbora Piknova, PhD.

1 **TITLE:**
2 Platelet-based Detection of Nitric Oxide in Blood by Measuring VASP Phosphorylation
3

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18 **KEYWORDS:**
19 Platelets, red blood cells, nitrite, nitric oxide, blood clotting, deoxyhemoglobin, VASP protein
20

21 **SUMMARY:**
22 Here, we present a protocol to address the potential use of platelets as a highly sensitive nitric
23 oxide sensor in blood. It describes initial platelet preparation and the use of nitrite and
24 deoxygenated red blood cells as nitric oxide generators.
25
26 **ABSTRACT:**
27 Platelets are the blood components responsible for proper blood clotting. Their function is highly
28 regulated by various pathways. One of the most potent vasoactive agents, nitric oxide (NO), can
29 also act as a powerful inhibitor of platelet aggregation. Direct NO detection in blood is very
30 challenging due to its high reactivity with cell-free hemoglobin that limits NO half-life to the
31 millisecond range. Currently, NO changes after interventions are only estimated based on
32 measured changes of nitrite and nitrate (members of the nitrate-nitrite-NO metabolic pathway).
33 However precise, these measurements are rather difficult to interpret *vis a vis* actual NO changes,
34 due to the naturally high baseline nitrite and nitrate levels that are several orders of magnitude
35 higher than expected changes of NO itself. Therefore, the development of direct and simple
36 methods that would allow one to detect NO directly is long overdue. This protocol addresses a
37 potential use of platelets as a highly sensitive NO sensor in blood. It describes initial platelet rich
38 plasma (PRP) and washed platelet preparations and the use of nitrite and deoxygenated red
39 blood cells as NO generators. Phosphorylation of VASP at serine 239 (P-VASP^{Ser239}) is used to
40 detect the presence of NO. The fact that VASP protein is highly expressed in platelets and that it
41 is rapidly phosphorylated when NO is present leads to a unique opportunity to use this pathway
42 to directly detect NO presence in blood.

43
44 **INTRODUCTION:**

45   Platelets are small disc-shaped cell fragments derived from megakaryocytes that are crucial for
46   blood clotting. The clotting cascade is initiated by various bioactive molecules (such as collagen
47   or ADP), released after the injury of vascular wall. The blood clotting process can be modified,
48   among various effectors by nitric oxide (NO). NO, naturally produced by mammalian cells, is one
49   of the most versatile physiological signals. It acts as a potent vasodilator, neurotransmitter and
50   immune modulator, to name a few of its many functions. In the bloodstream, NO also helps to
51   regulate the extent of blood clotting by inhibiting platelet aggregation. One of the most likely
52   sources of NO in the bloodstream is nitrite, an inorganic ion that has been shown to serve as a
53   precursor of NO. Reacting with red blood cells (RBCs), nitrite is reduced to NO and deoxyHb is
54   oxidized to methemoglobin (metHb)[1]. NO released from RBCs is vasoactive and causes
55   vasorelaxation[2]. This nitrite reduction pathway is an alternate NO generation pathway, acting
56   together with and complementing the classical NO generation path by endothelial nitric oxide
57   synthase at hypoxic conditions.

58

59   Platelets themselves are not able to reduce nitrite into NO but are very sensitive to its presence.
60   In intact platelets, NO in the nanomolar range increases cGMP (EC_{50} = 10 nM) and
61   phosphorylation of VASP (EC_{50} = 0.5 nM)[3]. Therefore, platelets may serve as an excellent sensor
62   of nitrite reduction by RBCs and NO release into blood. There are several methods that can
63   directly measure the extent of platelet activation – such as aggregometry and
64   thromboelastography (TEG)[4,5]. However, these methods require expensive specialized
65   instrumentation and rather large amounts of material. It is also possible to monitor events
66   downstream, after NO is released from RBCs, using the changes in platelet surface protein
67   expression - such as P-selectin[6]. NO is also known to increase the amount of cGMP in the
68   platelets[7]. Previously, we used cGMP to monitor NO release into blood after nitrite reduction by
69   deoxygenated RBC[8]. This proved to be a very sensitive method; however, cGMP is a short-lived
70   molecule and its detection involves extensive labor. Another possibility, described in the
71   presented protocol, uses phosphorylation of the vasodilator-stimulated phospho (VASP)-protein
72   to detect the presence of NO in blood. VASP is a substrate of protein kinase G activation, which
73   is phosphorylated upon the interaction with NO through the sGC/cGMP pathway[9]. Detectable
74   VASP phosphorylation occurs at very low NO concentrations, which could make platelets a very
75   sensitive detector of NO presence in blood. VASP is highly expressed in platelets, but not in other
76   blood cells, which allows to follow selectively the events involving platelets[10].

77

78   The main goal of this protocol is to describe the method in detail for the detection of NO release
79   in whole blood using its interaction with platelets by monitoring VASP phosphorylation[11,12]. The
80   described method allows early detection of low NO concentrations – theoretically in the
81   nanomolar range which makes the present protocol more sensitive than cGMP determination,
82   due to the use of standard Western blot techniques achievable in most laboratory settings.

83

84   **PROTOCOL:**

85

86   Note: Blood samples were obtained from NIH blood bank (IRB approved protocol: 99-CC-0168).

87

88   **1.   Blood Sample Preparation**

89

90 Note: To avoid platelet activation, draw blood slowly and mix gently with citrate by inverting the
91 tube several times.

92

93 1.1.   Platelet-rich plasma (PRP) preparation

94

95 1.1.1. Draw 30-50 mL of blood using a 20G or larger diameter needle and add into a tube
96 containing sodium citrate (3.8%) in a 1:9 ratio or acid citrate dextrose (ACD) (85 mM trisodium
97 citrate, 66.6 mM citric acid, 111 mM glucose, pH 4.5) in a 1:6 ratio.

98

99 1.1.2. Aliquot 5 mL of freshly collected whole blood into empty 15-mL conical tubes. Centrifuge
100 whole blood at 120 × g for 10 min at room temperature.

101

102 1.1.3. Carefully collect the supernatant containing the platelets (platelet-rich plasma, PRP) from
103 the upper portion by a plastic transfer pipette.

104

105 Note: PRP from Step 1.1.3 is ready for use in experiments or for further processing to prepare
106 washed platelets. The soft pellet obtained after centrifugation (Step 1.1.2) contains red blood
107 cells and white cells and can be further purified to obtain washed red blood cells (RBC) – see Step
108 1.3. The experiment needs to be finished within 2 h after drawing blood. When collecting PRP
109 (supernatant), avoid the collection of buffy coat-containing leukocytes accumulated on the
110 PRP/RBC interface.

111

112 1.2.   Washed platelet preparation (optional)

113

114 1.2.1. Centrifuge collected PRP at 400 × g for 10 min at room temperature. Discard the
115 supernatant and keep the platelet pellets.

116

117 1.2.2. Gently wash the pellets by adding 5 mL of CGS buffer (120 mM NaCl, 12.9 mM trisodium
118 citrate and 30 mM glucose, pH 6.5) and centrifuge at 400 × g for 10 min at room temperature.

119

120 1.2.3. Resuspend the platelet pellets with 3 mL of modified Tyrode buffer (134 mM NaCl, 0.34
121 mM NaH_2PO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM
122 glucose pH 7.4).

123

124 1.2.4. Dilute the platelets (1:100 - as example, 10 µL of platelets in 990 µL of Rees-Ecker solution)
125 with Rees Ecker solution and count by hemocytometer.

126

127 1.2.5. Adjust the density of platelets to 3×10^8 cells/mL by adding Tyrode buffer.

128

129 1.2.6. Incubate the platelet suspension at 37 °C for 1 h before starting the experiment.

130

131 Note: Washed platelets are stable for 5-8 h at 37 °C.

132

133 1.3.   Preparation of washed red blood cells (RBC)

134

135 1.3.1. After the collection, centrifuge the soft pellet obtained in Step 1.1.3 at 2500 × g for 10
136 min at room temperature.

137

138 1.3.2. Discard the supernatant and wash the RBC pellet with 5 mL of PBS by centrifugation at
139 2500 × g for 10 min at room temperature. Repeat this step for 3 times.

140

141 1.3.3. Discard the PBS and use washed RBCs for further experiments.

142

143 **2.   Deoxygenation**

144

145 2.1.   Add 1 mL of the mixture of PRP (prepared in Step 1.1.4. or 1.2.4.) and RBCs (prepared in
146 Step 1.3.) at the desired hematocrit into a polypropylene bottle closed with a rubber stopper. For
147 example, at 20% hematocrit, add 200 µL of RBCs to 800 µL of PRP.

148

149 Note: Suggested hematocrits are from 0% up to 40%. Do not use a glass bottle or flask as platelets
150 adhere to glass surface.

151

152 2.2.   Insert the needle connected to helium gas tank into the closed bottle and make the gas
153 outlet by inserting a 26G needle (**Figure 1**).

154

155 2.3.   Slowly swirl the bottle at room temperature.

156

157 Note: Do not allow He gas to bubble through blood preparation. Slow gas flow is preferable for
158 this procedure, and excessively fast He gas flow leads to increased hemolysis. The most efficient
159 gas flow needs to be determined by trial, as it highly depends on the geometry of the
160 experimental setup.

161

162 2.4.   Periodically follow the deoxygenation process by measuring the partial oxygen pressure
163 (pO_2) using a CO-oximeter.

164

165 Note: In our hands, 10 min of deoxygenation decreases pO_2 to 25 mmHg which is required for
166 nitrite reduction by RBCs. Continuing deoxygenation did further reduce pO_2 to 10 mmHg;
167 however, it led to increased hemolysis and increases in cell-free hemoglobin (**Figure 2**).

168

169 **3.   Red Blood Cell Nitrite Reduction**

170

171 3.1.   Add 1 mL of PBS (pH 7.4) into 0.0345 g of $NaNO_2$ to make 500 mM stock solution of nitrite.
172 Dilute 500 mM $NaNO_2$ to 250 µM $NaNO_2$ (25x) by serial dilution in PBS (pH 7.4).

173

174 3.2.   Using a microsyringe, inject nitrite (40 µL) through the septum into the deoxygenated
175 sample of PRP and RBCs to achieve final concentrations of 10 µM in total 1 mL of sample.

176

177   Note: In this experiment, the final concentrations of nitrite used is 10 µM.

178

179   3.3.   Incubate the sample at 37 °C for 10 min or longer.

180

181   Note: Adjust the exact duration of RBC incubation with nitrite needed for maximal nitrite
182   reduction for different type of experiments.

183

184   3.4.   Remove the stopper and pipette 1 mL of the sample into a microcentrifuge tube.

185

186   3.5.   Centrifuge at 200 × g for 3 min at room temperature.

187

188   3.6.   Pipette 300 µL of the platelet suspension from the upper portion of the supernatant and
189   mix with ACD (pH 6.5) in a 1:9 ratio. Discard the RBC pellet.

190

191   3.7.   Centrifuge the platelet suspension at 500 × g for 4 min at room temperature.

192

193   3.8.   Add 80 µL of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, 0.5% NP-40, pH 7.4)
194   containing protease inhibitor cocktail III (1:500) to the platelet pellets.

195

196   **4.   Western Blotting of VASP**

197

198   4.1.   Load 15 µg of the protein from each sample to 2 separate 10% SDS-PAGE gels.

199

200   Note: Harsh stripping buffer, which contains β-mercaptoethanol, cannot remove P-VASP^{Ser239} and
201   VASP antibodies from the membrane; therefore, P-VASP ^{Ser239} and VASP should be run separately.

202

203   4.2.   Run the gels at 120 V for 1.30 h and transfer to the nitrocellulose membrane.

204

205   4.3.   Block non-specific binding with 5% non-fat dry milk.

206

207   4.4.   Incubate the membrane with P-VASP^{Ser239} (1:500), VASP (1:1000) and GAPDH (1:1000) for
208   overnight at 4 °C.

209

210   4.5.   Incubate horseradish peroxidase (HRP) secondary antibody with the membrane for 45
211   min at room temperature.

212

213   4.6.   Expose the membrane with an imager machine and quantify the band density using
214   ImageJ.

215

216   **REPRESENTATIVE RESULTS:**
217   Venous blood samples have pO_2 values between 50-80 mmHg. Deoxygenation by helium rapidly
218   decreases pO_2 to 25 mmHg within 10 min. Increased deoxygenation time slightly further
219   decreases pO_2. However, increased time of deoxygenation also leads to significantly increased
220   levels of cell-free hemoglobin (determined by CO-Oximeter, visually seen on **Figure 2** as

221 increasingly red coloration of plasma) (**Figures 2A-B**). Increased hemolysis is not associated with
222 stirring because stirring without deoxygenation does not induce hemolysis (**Figure 2C**).
223
224 We found that the presence of RBCs in lysed samples decreases P-VASP^{Ser239} and VASP detected
225 by Western blotting (**Figure 3**). Therefore, we first separate platelets and RBCs before running
226 Western blots to detect P-VASP^{Ser239} in platelets.
227
228 To show that there is enough time to separate RBCs out of platelets without affecting VASP
229 phosphorylation, we used a short-lived NO donor. PROLI NONOate (NO donor with half-life of 1.8
230 s at 37 °C pH 7.4), rapidly increased P-VASP^{Ser239} in platelets (within 10 s of incubation). P-
231 VASP^{Ser239} induced by PROLI NONOate is detected for 10 min after the incubation with PRP
232 (**Figure 4**).
233
234 Nitrite increases P-VASP^{Ser239} in platelets in the presence of deoxygenated RBCs (pO$_2$ 25 mmHg).
235 Nitrite reductase activity of deoxygenated RBCs depends on hematocrit and the maximum effect
236 is observed at 20% hematocrit (**Figure 5**).
237
238 **FIGURE AND TABLE LEGENDS:**
239 **Figure 1. Deoxygenation chamber.** Mixture of PRP and RBCs is added into a plastic bottle closed
240 with a rubber septum. The closed bottle is connected to helium (He) line using a needle. Oxygen
241 is flushed out by a small syringe needle (26G) serving as a gas outlet.
242
243 **Figure 2. Partial oxygen pressure (pO$_2$) and cell-free hemoglobin after deoxygenation.** (**A**) PRP
244 and RBCs samples were deoxygenated for 3, 5, 10, 15, 30 and 50 min. pO$_2$ of samples were
245 measured by blood gas analyzer (n = 3). Error bars are SEM. Representative pictures of
246 supernatant of PRP+RBCs samples stirred with (**B**) or without helium (**C**).
247
248 **Figure 3. Representative VASP Western blot bands in samples of PRP/RBCs prepared at various
249 hematocrit.** RBCs were added into PRP to 1, 5, 10, 15, 20, 40% hematocrit. Mixtures of PRP +
250 RBCs were centrifuged at 500 × g for 5 min and lysed with lysis buffer.
251
252 **Figure 4. Representative Western blot bands of P-VASP^{Ser239} and VASP in PRP after exposure to
253 NO donor PROLI NONOate**. PROLI NONOate (10 μM) was added into PRP and incubated at 37 °C
254 for 10 and 30 s and 1, 2, 5, 10, 20 and 40 min.
255
256 **Figure 5. VASP^{Ser239} phosphorylation depends on pO$_2$ and hematocrit levels.** (**A**) PRP + RBCs (20%
257 hematocrit) were deoxygenated to two different pO$_2$ levels, 40 and 25 mmHg. P-VASP^{Ser239} and
258 VASP were measured after the incubation of deoxygenated samples with 10 μM nitrite at 37 °C
259 for 10 min. (**B**) Nitrite (10 μM) was added into deoxygenated PRP + RBCs at 0, 10, 20 and 40%
260 hematocrit (pO$_2$ = 25 mmHg). Fold increased (P-VASP^{Ser239}) was calculated relative to control
261 (PRP+RBCs without nitrite) for each hematocrit value. Error bars are SEM.
262
263 **DISCUSSION:**
264 Since platelets are easily activated, gentle handling of platelet-containing samples is required.

265 Fast pipetting and vigorous shaking should be avoided. Platelet inhibitors such as prostacyclin
266 (PGI$_2$) can be used to prevent platelet activation; however, this may affect some signaling
267 pathways inside the platelets. For the preparation of platelet pellets, we add ACD to the platelet
268 suspensions and use low speed centrifugation.
269
270 Freshly prepared platelets in PRP have a limited life span, up to 2 h. To achieve high
271 reproducibility, all experiments should be done only with fresh platelets and within 2 h after
272 blood collection. The presented data were obtained with platelets in PRP, which is considered
273 more physiological than washed platelets. Although the life span and purity increase for washed
274 platelets, repeated centrifugation during preparation of washed platelets can lead to their
275 spontaneous activation and this may result in inconsistent quality of platelet preparations. PRP
276 is preferred over washed platelets since the procedure is faster and further handling could
277 activate platelets. However, in specific cases, when the interference of plasma blood clotting
278 factors could interfere with experimental setup, washing platelets represents solution to avoid
279 these problems.
280
281 Oxygen contamination in deoxygenated samples is the most important critical step for this assay.
282 RBCs can be pooled and deoxygenated before being added to the PRP. However, transferring
283 deoxygenated RBCs increases the risk of oxygen contamination. In addition, deoxygenation of
284 concentrated RBCs requires more time than deoxygenation of the full preparation at the desired
285 hematocrit, and longer exposure to He gas leads to increased levels of cell-free hemoglobin.
286 Although cell-free hemoglobin can be washed out of deoxygenated RBCs, the phosphate buffer
287 saline or other buffers used for washing must be carefully deoxygenated to prevent oxygen
288 contamination during the centrifugation. To shorten the preparation process and avoid
289 unnecessary oxygen contamination, each PRP+RBCs sample was prepared at the desired
290 hematocrit first and then deoxygenated before the addition of nitrite.
291
292 To quantify P-VASP[Ser239] by Western blot, RBCs must be separated out of the platelets. VASP is
293 expressed in RBCs[13], but the levels are nonsignificant when compared to hemoglobin and much
294 lower than in platelets. Since total protein concentration is used for loading controls in the
295 Western blots, the presence of RBCs in lysate samples interferes with P-VASP and VASP
296 measurement by Western blotting. Due to a slow rate of dephosphorylation by phosphatase
297 enzymes in platelets[14], P-VASP is sustained for 10 min (**Figure 4**) and therefore it is possible to
298 separate RBCs by centrifugation before collecting platelet cell lysates.
299
300 The EC$_{50}$ for VASP phosphorylation is almost an order of magnitude lower than the EC$_{50}$ for the
301 peak cGMP increase, 0.5 nM and 9 nM, respectively[3], which makes VASP phosphorylation one of
302 the most sensitive methods to detect the presence of low amounts of NO. However, the VASP
303 phosphorylation curve in response to NO is bell-shaped; a phosphorylation decrease is observed
304 after peaking at 3-30 nM of NO[3]. This nonlinear response to increasing NO concentrations makes
305 VASP unsuitable for rigorous NO quantification.
306
307 Since platelets in PRP have a short life span, the assay must be done in fresh platelets within 2 h
308 after drawing blood. The inconsistency of pO$_2$ levels after the deoxygenation can be found since

309 the deoxygenation of samples is dependent on the flow rate of helium gas and rate of swirling
310 the bottle. Therefore, the time for deoxygenation needs to be determined by trial. For the
311 measurement of P-VASP by Western blots, the antibody choice is critical, and one needs to use a
312 direct NO donor as a positive control to check the specificity. In addition to NO, P-VASP[Ser239] in
313 platelets can be phosphorylated by the inhibition of phosphodiesterease enzymes and the
314 activation of protein kinase A pathway.
315
316 The measurement of P-VASP[Ser239] has some advantages over cGMP assay. cGMP in platelets has
317 a very short half-life (less than 10 s) and the measurement of cGMP requires the addition of
318 phosphodiesterase inhibitor[3]. Also, the cGMP measurement uses a rather expensive ELISA
319 technique. VASP can be simply measured by an in-house Western blot protocol and therefore is
320 more readily available.
321
322 Increased P-VASP[Ser239] in platelets was successfully used as a sensor for NO generation by inhaled
323 nitrite *in vivo*[6]. Therefore, we showed that it is possible to use P-VASP[Ser239] in platelets as a marker
324 of NO generation *in vitro* and *in vivo* in the blood and possibly in various other situations. The
325 unusually low EC_{50} makes this method an excellent candidate for detecting the presence of very
326 low NO concentrations in blood. Therefore, this protocol provides a possibility to study NO
327 generation in blood at various physiological stimuli, such as during blood coagulation cascade,
328 increased sheer stress or increased inflammation.
329

339 **REFERENCES:**
340 1. Huang, K.T. *et al*. The reaction between nitrite and deoxyhemoglobin. Reassessment of
341 reaction kinetics and stoichiometry. *Journal of Biological Chemistry* **280** (35), 31126-31131
342 (2005).
343 2. Cosby, K. *et al*. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human
344 circulation. *Nature Medicine* **9** (12), 1498-1505 (2003).
345 3. Mo, E., Amin, H., Bianco, I.H., Garthwaite, J. Kinetics of a cellular nitric
346 oxide/cGMP/phosphodiesterase-5 pathway. *Journal of Biological Chemistry* **279** (5), 26149-
347 26158 (2004).
348 4. Park, J.W., Piknova, B., Nghiem, K., Lozier, J.N., Schechter, A.N. Inhibitory effect of nitrite on
349 coagulation processes demonstrated by thromboelastography. *Nitric oxide* **40**, 45-51 (2014).
350 5. Wajih, N. *et al*. The role of red blood cell S-nitrosation in nitrite bioactivation and its modulation
351 by leucine and glycose. *Redox Biology* **8**, 415-421 (2016).

352 6. Akrawinthawong, K. *et al*. A flow cytometric analysis of the inhibition of platelet reactivity due
353 to nitrite reduction by deoxygenated erythrocytes. *PLoS One*. **9** (3), e92435 (2014).

354 7. Friebe, A., Koesling, D. Regulation of nitric oxide-sensitive guanylyl cyclase. *Circulation
355 Research* **93** (2), 96-105 (2003).

356 8. Srihirun, S. *et al*. Platelet inhibition by nitrite is dependent on erythrocytes and deoxygenation.
357 *PLoS One*. **7** (1), e30380 (2012).

358 9. Smolenski, A. *et al*. Analysis and regulation of vasodilator-stimulated phosphoprotein serine
359 239 phosphorylation in vitro and in intact cells using a phosphospecific monoclonal antibody.
360 *Journal of Biological Chemistry*. **273** (32), 20029–20035 (1998).

361 10. Burkhart, J.M. *et al.* The first comprehensive and quantitative analysis of human platelet
362 protein composition allows the comparative analysis of structural and functional pathways.
363 *Blood*. **120** (15), e73–82.

364 11. Parakaw, T. *et al*. Platelet inhibition and increased phosphorylated vasodilator-stimulated
365 phosphoprotein following sodium nitrite inhalation. *Nitric oxide*. **66**, 10-16 (2017).

366 12. Srihirun, S., Piknova, B., Sibmooh, N., Schechter, A.N. Phosphorylated vasodilator-stimulated
367 phosphoprotein (P-VASPSer239) in platelets is increased by nitrite and partially deoxygenated
368 erythrocytes. *PLoS One*. **13** (3), e0193747 (2018).

369 13. Cortese-Krott, M.M. *et al*. Identification of a soluble guanylate cyclase in RBCs: preserved
370 activity in patients with coronary artery disease. *Redox Biology*. **14**, 328-337 (2018).

371 14. Abel, K., Mieskes, G., Walter, U. Dephosphorylation of the focal adhesion protein VASP in
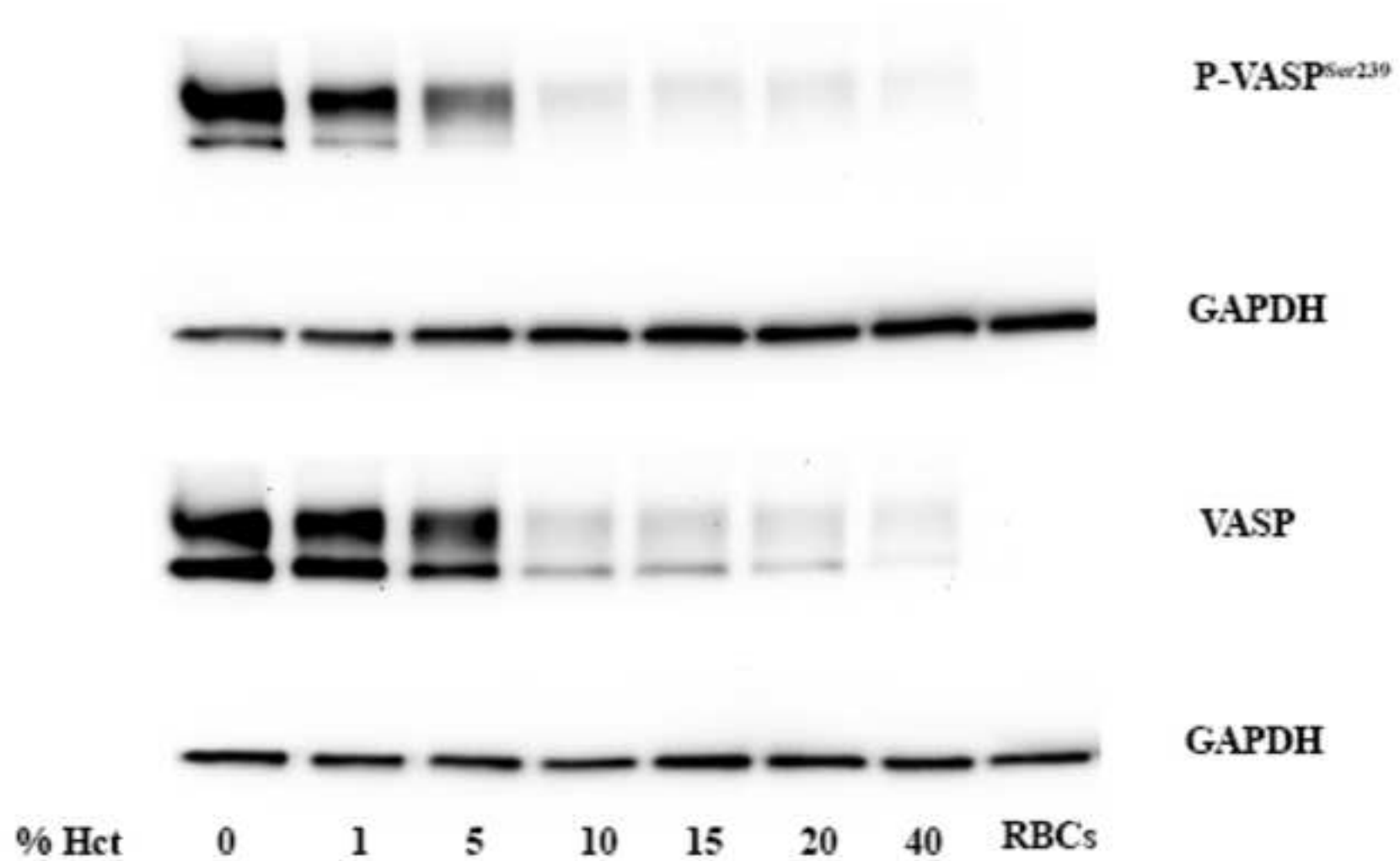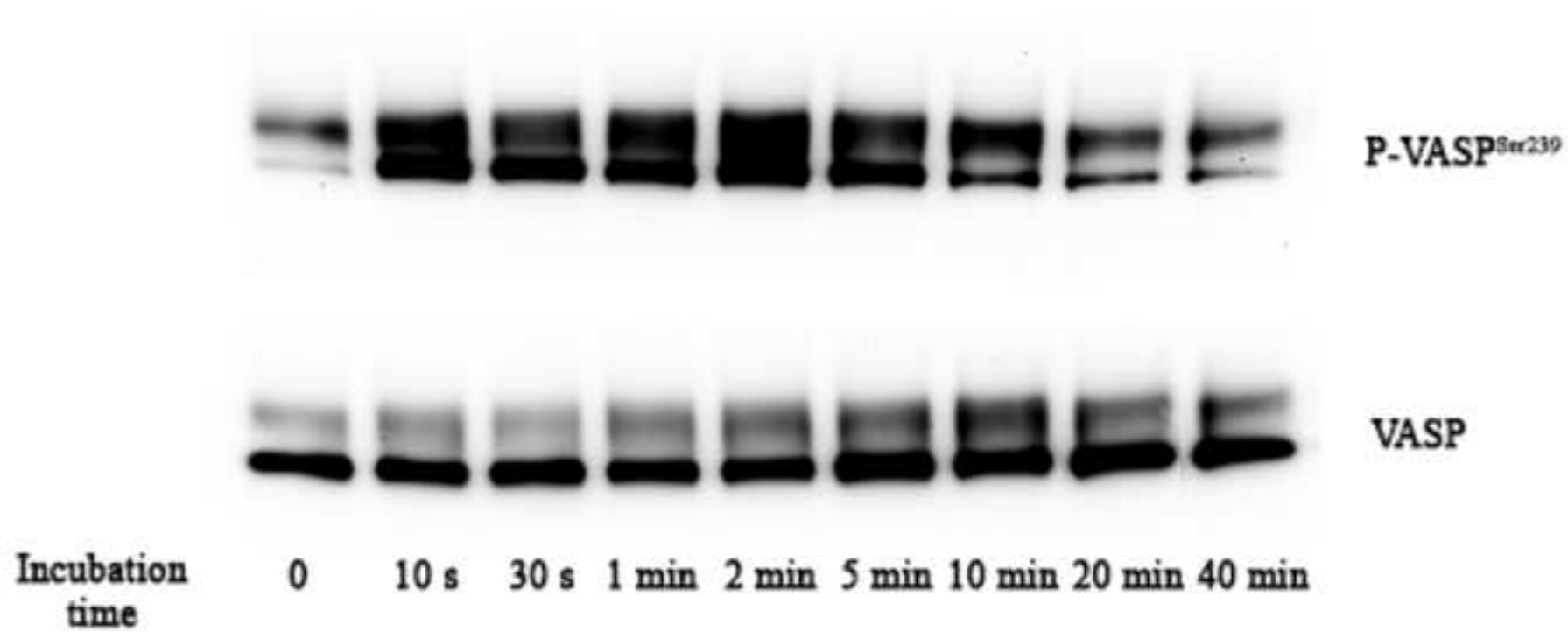372 vitro and in intact human platelets. *FEBS letter*. **370** (3), 184-188 (1995).

373

Figure1
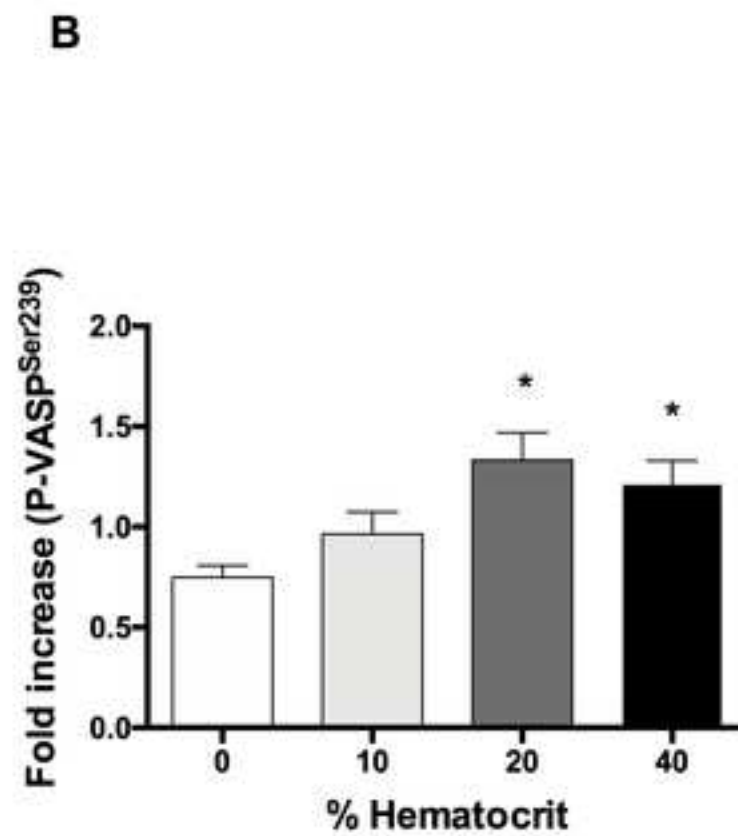
A

B Stirring with helium

C Stirring without helium

| Time (min) | 3 | 5 | 10 | 15 | 30 | 50 |

Figure3 Click here to access/download;Figure;Figure 3.tif ⬇

Figure4 Click here to access/download;Figure;Figure 4.tif ☰

Incubation time: 0   10 s   30 s   1 min   2 min   5 min   10 min   20 min   40 min

P-VASP^{Ser239}

VASP

Figure5   Click here to access/download;Figure;Figure 5.tif ±
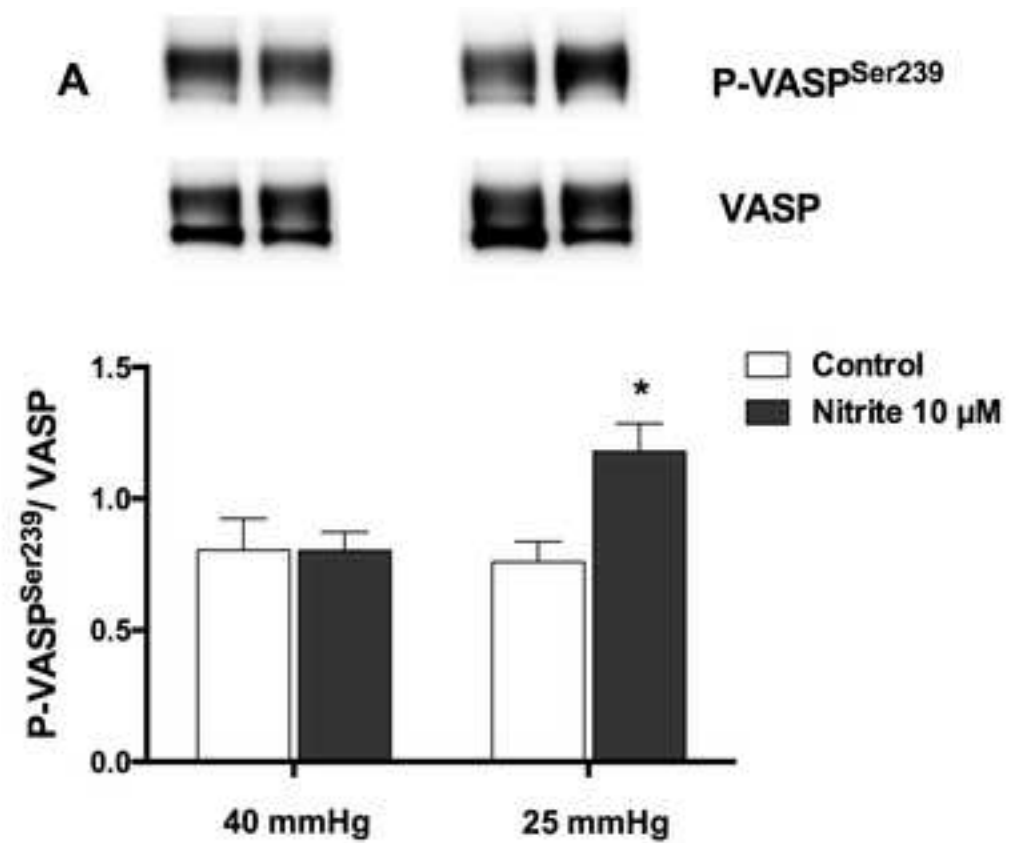
Name of Reagent/Equipment

Tri-sodium citrate

Citric acid

Glucose

NaCl; sodium chloride

$NaH_2PO_{4;}$ sodium phosphate monobasic, monohydrate

KCl; potassium chloride

$NaHCO_3$; sodium bicarbonate

HEPES; N-[2-Hydroxyethyl]piperazine-N'-[-ethanesulfonic acid]

$MgCl_2$ (1 M); magnesium chloride

$CaCl_2$; calcium chloride

Nalgene Narrow-mouth HDPE Economy bottles

Red septum stopper NO.29

$NaNO_2^-$; sodium nitrite

TRIZMA Base; Tris[hydroxymethyl]aminomethane

NP-40; 4-Nonylphenyl-polyethylene glycol

Protease inhibitor cocktail set III

Phospho-VASP (Ser239) antibody

VASP antibody

GAPDH (14C10) Rabbit mAb

2-mercaptoethanol

Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L)

Clarity Western ECL Substrate

CO-oximeter (ABL 90 flex)

Company	Catalog number	Comment/Description
Supply by NIH blood bank		
Supply by NIH blood bank		
Sigma	G7528-250G	
Sigma	S-7653 1kg	
Mallinckrodt Chemical	7892-04	
Mallinckrodt Chemical	6858	
Mallinckrodt Chemical	7412-12	
Sigma	H3375-500g	
Quality Biology	351-033-721	
Sigma	C5080-500G	
Nalgene	2089-0001	
Fisherbrand	FB57877	
Sigma	S2252-500G	
Sigma	T8524-250G	
Sigma	74385-1L	
Calbiochem	539134	
Cell signaling technology	3114	
Cell signaling technology	3112	
Cell signaling technology	2118	
Sigma	M-6250-10ml	
Jackson Immuno Research Laboratories	111-035-003	
BIO-RAD	1705060-200ml	
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Title of Article: *Platelet-detection of nitric oxide in blood: measurement of VASP phosphorylation after nitrite reduction by red blood cell*

Author(s): *Srihirun, Sirada; Schechter, Alan N; Piknova, Barbora*

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1 **TITLE:**
2 **Platelet-based Detection of Nitric Oxide in Blood Using Measurement of VASP**
3 **Phosphorylation.**
4

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19 **KEYWORDS:**
20 Platelets, red blood cells, nitrite, nitric oxide, blood clotting, deoxyhemoglobin, VASP protein
21

22 **SUMMARY:**
23 Here we present a protocol to address the potential use of platelets as a highly sensitive nitric
24 oxide sensor in blood. It describes initial platelets preparation and use of nitrite and
25 deoxygenated red blood cells as nitric oxide generators. Special attention is given to most
26 common pitfalls to avoid.
27

28 **ABSTRACT:**
29 Platelets are blood components responsible for proper blood clotting. Their function is highly
30 regulated by various pathways. One of the most potent vasoactive agents, nitric oxide (NO), can
31 also act as a powerful inhibitor of platelet aggregation. Direct NO detection in blood is very
32 challenging, due to its high reactivity with cell-free hemoglobin that limits NO half-life to the
33 millisecond range. Currently, NO changes after interventions are only estimated based on
34 measured changes of nitrite and nitrate (members of the nitrate-nitrite-NO metabolic pathway).
35 However precise, these measurements are rather difficult to interpret *vis a vis* actual NO changes,
36 due to the naturally high baseline nitrite and nitrate levels that are several orders of magnitude
37 higher than expected changes of NO itself. Therefore, development of direct and simple methods
38 that would allow one to detect NO directly is long overdue.
39

40 This protocol addresses a potential use of platelets as a highly sensitive NO sensor in blood. It
41 describes initial platelet rich plasma (PRP) and washed platelet preparations and the use of nitrite
42 and deoxygenated red blood cells as NO generators. Phosphorylation of VASP at serine 239 (P-
43 VASP[Ser239]) is used to detect the presence of NO. The fact that VASP protein is highly expressed
44 in platelets and that it is rapidly phosphorylated when NO is present, leads to the unique

45   opportunity to use this pathway to directly detect NO presence in blood.
46
47   **INTRODUCTION:**
48
49   Platelets, small disc-shaped cell fragments derived from megakaryocytes, are crucial for blood
50   clotting. The clotting cascade is initiated by various bioactive molecules (such as collagen or ADP),
51   released after the injury of vascular wall. The blood clotting process can be modified, among
52   various effectors by nitric oxide (NO). NO, naturally produced by mammalian cells, is one of the
53   most versatile physiological signals. It acts as a potent vasodilator, neurotransmitter and immune
54   modulator, to name a few of its many functions. In the bloodstream, NO also helps to regulate
55   the extent of blood clotting by inhibiting platelet aggregation. One of the most likely sources of
56   NO in the bloodstream is nitrite, an inorganic ion that has been shown to serve as a precursor of
57   NO. Reacting with red blood cells (RBCs), nitrite is reduced to NO and deoxyHb is oxidized to
58   methemoglobin (metHb)[1]. NO released from RBCs is vasoactive and causes vasorelaxation[2]. This
59   nitrite reduction pathway is an alternate NO generation pathway, acting together with and
60   complementing the classical NO generation path by endothelial nitric oxide synthase at hypoxic
61   conditions.
62
63   Platelets themselves are not able to reduce nitrite into NO but are very sensitive to its presence.
64   In intact platelets, NO in the nanomolar range increases cGMP (EC_{50} = 10 nM) and
65   phosphorylation of VASP (EC_{50} = 0.5 nM)[3]. Therefore, platelets may serve as an excellent sensor
66   of nitrite reduction by RBCs and NO release into blood. There are several methods able to
67   measure directly the extent of platelet activation – such as aggregometry and
68   thromboelastography (TEG)[4,5]. However, these methods require expensive specialized
69   instrumentation and rather large amounts of material. It is also possible to monitor events
70   downstream, after NO is released from RBCs, using changes in platelet surface protein expression
71   - such as P-selectin[6]. NO is also known to increase the amount of cGMP in the platelets[7].
72   Previously, we used cGMP to monitor NO release into blood after nitrite reduction by
73   deoxygenated RBC[8]. This proved to be a very sensitive method, however cGMP is a short-lived
74   molecule and its detection involves extensive labor. Another possibility, described in the
75   presented protocol, uses phosphorylation of Vasodilator-stimulated phospho (VASP)-protein to
76   detect presence of NO in blood. VASP is a substrate of protein kinase G activation, which is
77   phosphorylated upon interaction with NO through the sGC/cGMP pathway[9]. Detectable VASP
78   phosphorylation occurs at very low NO concentrations, which could make platelets a very
79   sensitive detector of NO presence in blood. VASP is highly expressed in platelets, but not in other
80   blood cells, which allows to follow selectively events involving platelets[10].
81
82   Main goal of this protocol is to describe in details method for detection of NO release in whole
83   blood using its interaction with platelets via monitoring VASP phosphorylation[11,12]. Described
84   method allows early detection of low NO concentrations – in theory in nanomolar range which
85   makes the present protocol more sensitive than cGMP determination and, due to the use of
86   standard Western blot technique achievable in most laboratory settings.
87
88   **PROTOCOL:**

89

Blood samples were obtained from NIH blood bank (IRB approved protocol: 99-CC-0168).

1.   Blood sample preparation.

Note: To avoid platelet activation, draw blood slowly and mix gently with citrate by inverting the tube several times.

1.1. Platelet-rich plasma (PRP) preparation.

1.1.1. Draw 30-50 ml of blood using 20G or larger diameter needle and add into a tube containing sodium citrate (3.8%) in a 1:9 ratio or acid citrate dextrose (ACD) (85 mM trisodium citrate, 66.6 mM citric acid, 111 mM glucose, pH 4.5) in a 1:6 ratio.

1.1.2. Aliquot 5 mL of freshly collected whole blood into empty 15-mL conical tubes. Centrifuge whole blood at 120 × g for 10 minutes at room temperature.

1.1.3. Carefully collect supernatant, containing platelets (platelet-rich plasma, PRP) from the upper portion by a plastic transfer pipette.

Note: PRP from step 1.1.3 is ready for use in experiments or for further processing to prepare washed platelets. The soft pellet obtained after centrifugation (step 1.1.2) contains red blood cells and white cells and can be further purified to obtain washed red blood cells (RBC) – see section 1.3. The experiment needs to be finished within 2 hours after blood drawing. When collecting PRP (supernatant), avoid collection of buffy coat-containing leukocytes accumulated on the PRP/RBC interface.

1.2. Washed platelet preparation - optional.

1.2.1. Centrifuge collected PRP at 400 × g for 10 minutes at room temperature. Discard supernatant and keep platelets pellet.

1.2.1. Gently wash the pellets by adding 5 mL of CGS buffer (120 mM NaCl, 12.9 mM trisodium citrate and 30 mM glucose, pH 6.5) and centrifuge at 400 × g for 10 minutes at room temperature.

1.2.3. Resuspend platelet pellets with 3 mL of modified Tyrode buffer (134 mM NaCl, 0.34 mM NaH$_2$PO$_4$, 2.9 mM KCl, 12 mM NaHCO$_3$, 20 mM HEPES, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM glucose pH 7.4).

1.2.4. Dilute platelets (1:100 - as example, 10 µL of platelets in 990 µL Rees-Ecker solution) of Rees Ecker solution and count by hemocytometer.

1.2.5. Adjust number of platelets to 3×10^8 cells/mL by add Tyrode buffer.

Commented [A8]: Made this as step 1.1.1 please check

Commented [A9R8]: It is step 1.1.1.

Commented [A10]: This already has sodium citrate added to it?

Commented [A11R10]: Yes, blood comes from step 1.1.1. where it is collected on citrate

Commented [A12]: This is not a step. Please either use imperative tense or convert to a note.

Commented [A13R12]: converted

Commented [A14]: We cannot have paragraphs of text and notes following each other. Please convert some to sentences to action steps in imperative tense and move some to the discussion.

Commented [A15R14]: modified

Commented [A16]: Wash how? By centrifuging? Condition for centrifugation? Please write every detail.

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Commented [A20R18]: No centrifugation at this step.

Commented [A21]: Volume?

Commented [A22R21]: We added explanation what 1:100 dilution means, but this is a common knowledge, so we think is redundant

133

134 1.2.6. Incubate platelet suspension at 37 °C for 1 hour before the start experiment.

135

136 **Note:** Washed platelets are stable for 5-8 hours at 37 °C.

137

138 **1.3. Preparation of washed red blood cells (RBC):**

139

140 1.3.1. After the collection. centrifuge the soft pellet obtained in step 1.1.3 at 2500 × g for 10
141 minutes at room temperature.

| Commented [A23]: Where was the soft pellet stored? |
| Commented [A24R23]: Soft pellet is immediately centrifuged after finish step 1.1.3., wording changed |

142

143 1.3.2. Discard the supernatant and wash the RBC pellet with 5 mL of PBS by centrifugation at
144 2500 × g for 10 minutes at room temperature. Do this for 3 times.

145

146 1.3.3. Discard PBS and use washed RBCs for further experiments.

147

148 **2. Deoxygenation**

149

150 2.1. Add 1 mL of the mixture of PRP (prepared in step 1.1.4. or 1.2.4.) and RBCs (prepared in step
151 1.3.) at desired hematocrit into a polypropylene bottle closed with a rubber stopper. For
152 example, at 20% hematocrit, add 200 μL of RBCs in 800 μL of PRP.

| Commented [A25]: What is desired hematocrit? Please be specific in terms of volumes and concentration with respect to your experiment. |
| Commented [A26R25]: Desired hematocrit depends on the type of experiment performed. |

153

154 **Note:** Suggested hematocrits are from 0 up to 40%. Do not use glass bottle or flask - platelets
155 adhere to glass surface.

156

157 2.2. Insert needle connected to helium gas tank into closed bottle and make gas outlet by
158 inserting a small syringe needle (26G) (**Figure 1**).

159

160 2.3. Slowly swirl the bottle at room temperature.

161

162 **Note:** Do not allow He gas to bubble through blood preparation! Slow gas flow is preferable for
163 this procedure, excessively fast He gas flow leads to increased hemolysis. The most efficient gas
164 flow needs to be determined by trial, as it highly depends on the geometry of the experimental
165 setup.

166

167 2.4. Periodically follow deoxygenation process measuring partial oxygen pressure (pO_2) using CO-
168 oximeter.

169

170 **Note:** In our hands, 10 min deoxygenation decreases pO_2 to 25 mmHg which is required for nitrite
171 reduction by RBCs. Continuing deoxygenation did further reduce pO_2 to 10 mmHg however, it led
172 to increased hemolysis and increases in cell-free hemoglobin (**Figure 2**).

173

174 **3. Red blood cells nitrite reduction.**

175

176 3.1. Add 1 mL of PBS pH 7.4 into 0.0345 g of $NaNO_2^-$ to make 500 mM stock solution of nitrite.

177   3.2. Dilute 500 mM $NaNO_2^-$ to 250 μM $NaNO_2^-$ (25x) by serial dilution in PBS pH 7.4.
178
179   3.2. Using microsyringe, inject nitrite (40 μL) through septum into deoxygenated sample of PRP
180   and RBCs to achieve final concentrations of 10 μM in total 1 mL of sample.
181
182   **Note:** In this experiment, final concentrations of nitrite used is 10 μM.
183
184   3.3. Incubate the sample at 37 °C for 10 min or longer.
185
186   **Note:** Exact duration of RBC incubation with nitrite needed for maximal nitrite reduction might
187   need adjustment for different type of experiments.
188
189   3.4. Remove the stopper and pipette 1 mL of sample to an Eppendorf tube.3.5. Centrifuge at 200
190   × g for 3 min at room temperature.
191
192   3.6. Pipette 300 μL of platelet suspension from the upper portion of supernatant and mix with
193   ACD (pH 6.5) in a 1:9 ratio. Discard the RBC pellet.
194
195   3.7. Centrifuge the platelet suspension at 500 × g for 4 minutes at room temperature.
196
197   3.8. Add 80 μL of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris, 0.5% NP-40, pH 7.4) containing
198   protease inhibitor cocktail III (1:500) to platelet pellets.
199
200   **4. Western blotting of VASP**
201
202   4.1. Load 15 μg protein of each sample in 2 separate 10% SDS-PAGE gels.
203
204   **Note:** Harsh stripping buffer, which contains β-mercaptoethanol cannot remove P-VASP[Ser239] and
205   VASP antibodies from membrane, therefore P-VASP [Ser239] and VASP should be run separately.
206
207   4.2. Run gels at 120 V for 1.30 hours and transfer to nitrocellulose membrane.
208
209   4.3. Block non-specific binding with 5% non-fat dry milk.
210
211   4.4. Incubate membrane with P-VASP[Ser239] (1:500), VASP (1:1000) and GAPDH (1:1000) for
212   overnight at 4°C.
213
214   4.5. Incubate horseradish peroxidase (HRP) secondary antibody with membrane for 45 minutes
215   at room temperature.
216
217   4.6. Expose membrane with imager machine and quantified band density using Image J software
218   or similar.
219
220   **REPRESENTATIVE RESULTS:**

221
222 Venous blood samples have pO_2 values between 50-80 mmHg. Deoxygenation by helium rapidly
223 decreases pO_2 to 25 mmHg within 10 minutes. Increased deoxygenation time slightly further
224 decreases pO_2. However, increased time of deoxygenation also leads to significantly increased
225 levels of cell-free hemoglobin (determined by CO-Oximeter, visually seen on Figure 2 as
226 increasingly red coloration of plasma) (Figure 2A-B). Increased hemolysis is not associated with
227 stirring because stirring without deoxygenation does not induce hemolysis (Figure 2C).
228
229 We found that the presence of RBCs in lysed samples decreases P-VASP^{Ser239} and VASP detected
230 by Western blotting (**Figure 3**). Therefore, we first separate platelets and RBCs before running
231 Western blots to detect P-VASP^{Ser239} in platelets.
232
233 To show that there is enough time to separate RBCs out of platelets without affecting VASP
234 phosphorylation, we used a short-lived NO donor. PROLI NONOate (NO donor with half-life of 1.8
235 seconds at 37 °C pH 7.4), rapidly increased P-VASP^{Ser239} in platelets (within 10 seconds of
236 incubation). P-VASP^{Ser239} induced by PROLI NONOate is detected for 10 minutes after incubation
237 with PRP (**Figure 4**).
238
239 Nitrite increases P-VASP^{Ser239} in platelets in the presence of deoxygenated RBCs (pO_2 25 mmHg).
240 Nitrite reductase activity of deoxygenated RBCs depends on hematocrit and the maximum effect
241 is observed at 20% hematocrit (**Figure 5**).
242
243 **FIGURE AND TABLE LEGENDS:**
244
245 **Figure 1. Deoxygenation chamber.** Mixture of PRP and RBCs is added into a plastic bottle closed
246 with a rubber septum. A closed bottle is connected to helium (He) line using a needle and He line.
247 Oxygen is flushed out by a small syringe needle (26G) serving as a gas outlet.
248
249 **Figure 2. Partial oxygen pressure (pO_2) and cell-free hemoglobin after deoxygenation.** (A) PRP
250 and RBCs samples were deoxygenated for 3, 5, 10, 15, 30 and 50 minutes. pO_2 of samples were
251 measured by blood gas analyzer (n=3). Error bars are SEM. Representative pictures of
252 supernatant of PRP+RBCs samples stirred with (B) or without helium (C).
253
254 **Figure 3. Representative VASP Western blot bands in samples of PRP/RBCs prepared at various
255 hematocrit.** RBCs were added into PRP to 1, 5, 10, 15, 20, 40% hematocrit. Mixtures of PRP +
256 RBCs were centrifugation at 500 × g for 5 minutes and lysed with lysis buffer.
257
258 **Figure 4. Representative Western blot bands of P-VASP^{Ser239} and VASP in PRP after exposure to
259 NO donor PROLI NONOate**. PROLI NONOate (10 μM) was added into PRP and incubated at 37 °C
260 for 10 and 30 seconds (s) and 1, 2, 5, 10, 20 and 40 minutes (m).
261
262 **Figure 5. VASP^{Ser239} phosphorylation depends on pO_2 and hematocrit levels.** (A) PRP + RBCs (20%
263 hematocrit) were deoxygenated to two different pO_2 levels, 40 and 25 mmHg. P-VASP^{Ser239} and
264 VASP were measured after incubation of deoxygenated samples with 10 μM nitrite at 37 °C for

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265   10 minutes. (B) Nitrite (10 μM) was added into deoxygenated PRP + RBCs at 0, 10, 20 and 40%
266   hematocrit (pO_2 = 25 mmHg). Fold increased (P-VASP^{Ser239}) was calculated relative to control
267   (PRP+RBCs without nitrite) for each hematocrit value. Error bars are SEM.

268
269   **DISCUSSION:**

270
271   **Critical steps.**
272   Since platelets are easily activated, gentle handling of platelet-containing samples is required.
273   Fast pipetting and vigorous shaking should be avoided. Platelet inhibitors such as prostacyclin
274   (PGI$_2$) can be used to prevent platelet activation; however, this may affect some signaling
275   pathways inside platelets. For preparation of platelet pellets, we add ACD to platelet suspensions
276   and use low speed centrifugation.

277
278   Fresh prepared platelets in PRP have only limited life span, up to 2 hours. To achieve high
279   reproducibility, all experiments should be done only with fresh platelets and within 2 hours after
280   blood collection. The presented data were obtained with platelets in PRP, which is considered
281   more physiological than washed platelets. Although life span and purity increase for washed
282   platelets, repeated centrifugation during preparation of washed platelets can lead to their
283   spontaneous activation and this may result in inconsistent quality of platelet preparations. PRP
284   is prefer over washed platelets since the procedure is faster and further handling could activate
285   platelets. However, in specific cases, when the interference of plasma blood clotting factors could
286   interfere with experimental setup, washing platelets represents solution to avoid these
287   problems.

> **Commented [A31]:** This was moved to the discussion section from protocol.

288
289   Oxygen contamination in deoxygenated samples is the most important critical step for this assay.
290   RBCs can be pooled and deoxygenated before adding into PRP. However, transferring
291   deoxygenated RBCs increases risk of oxygen contamination. In addition, deoxygenation of
292   concentrated RBCs requires more time than deoxygenation of the full preparation at desired
293   hematocrit, and longer exposure to He gas leads to increased levels of cell-free hemoglobin.
294   Although cell-free hemoglobin can be washed out of deoxygenated RBCs, phosphate buffer saline
295   or other buffers used for washing must be carefully deoxygenated to prevent oxygen
296   contamination during centrifugation. To shorten the preparation process and avoid unnecessary
297   oxygen contamination, each PRP+RBCs sample was prepared at desired hematocrit first and then
298   deoxygenated before addition of nitrite.

299
300   To quantify P-VASP^{Ser239} by Western blot, RBCs must be separated out of platelets. VASP is
301   expressed in RBCs[13], but levels are nonsignificant when compared to hemoglobin and much lower
302   than in platelets. Since total protein concentration is used for loading controls in the Western
303   blots, the presence of RBCs in lysate samples interferes with P-VASP and VASP measurement by
304   Western blotting. Due to a slow rate of dephosphorylation by phosphatase enzymes in
305   platelets[14], P-VASP is sustained for10 minutes (Figure 4) and therefore it is possible to separate
306   RBCs by centrifugation before collecting platelet cell lysates.

307
308   EC$_{50}$ for VASP phosphorylation is almost an order of magnitude lower than EC$_{50}$ for the peak

309   cGMP increase, 0.5 nM and 9 nM, respectively[3], which makes VASP phosphorylation one of the
310   most sensitive methods to detect presence of low amounts of NO. However, VASP
311   phosphorylation curve in response to NO is bell-shaped, phosphorylation decrease is observed
312   after peaking at 3-30 nM of NO[3]. This nonlinear response to increasing NO concentrations makes
313   VASP unsuitable for rigorous NO quantification.
314
315   **Limitations of the technique**
316   Since platelets in PRP have short life span, the assay must be done in fresh platelets within
317   2 hours after blood drawing. The inconsistence of pO_2 levels after deoxygenation can be found
318   since deoxygenation of samples is dependent on flow rate of helium gas and rate of swirling the
319   bottle. Therefore, the time for deoxygenation need to be determined by trial. For the
320   measurement of P-VASP by Western blots, antibody choice is critical and need to use direct NO
321   donor as a positive control to check specificity. In addition to NO, P-VASP[Ser239] in platelets can be
322   phosphorylated by the inhibition of phosphodiesterease enzymes and the activation of protein
323   kinase A pathway.

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325   **Significance of the method with respect to alternative method**
326   Measurement of P-VASP[Ser239] has some advantages over cGMP assay. cGMP in platelets has very
327   short half-life (less than 10s) and the measurement of cGMP requires the addition of
328   phosphodiesterase inhibitor[3]. Also, cGMP measurement uses rather expensive ELISA technique.
329   VASP can be simply measured by an in-house Western blot protocol and therefore is more readily
330   available.
331
332   **Future directions**
333   Increased P-VASP[Ser239] in platelets was successfully used as a sensor for NO generation by inhaled
334   nitrite *in vivo*[6]. Therefore, we showed that it is possible to use P-VASP[Ser239] in platelets as a marker
335   of NO generation *in vitro* and *in vivo* in the blood and possibly in various other situations. The
336   unusually low EC_{50} makes this method an excellent candidate for detecting presence of very low
337   NO concentrations in blood. Therefore, this protocol provides a possibility to study NO
338   generation in blood at various physiological stimuli, such as during blood coagulation cascade,
339   increased sheer stress or increased inflammation.
340

344
345   **DISCLOSURES:**
346
347   Dr. Alan Schechter is listed as a co-inventor on several patents issued to the National Institutes
348   of Health for the use of nitrite salts for the treatment of cardiovascular diseases. He receives
349   royalties based on NIH licensing of these patents for clinical development but no other
350   compensation.
351
352   **REFERENCES:**

353
354 [1] Huang, KT. *et al*. The reaction between nitrite and deoxyhemoglobin. Reassessment of
355 reaction kinetics and stoichiometry. *Journal of Biological Chemistry* **280** (35), 31126-31131
356 (2005).
357 [2] Cosby, K. *et al*. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human
358 circulation. *Nature Medicine* **9** (12), 1498-1505 (2003).
359 [3] Mo, E. Amin, H. Bianco, IH. Garthwaite, J. Kinetics of a cellular nitric
360 oxide/cGMP/phosphodiesterase-5 pathway. *Journal of Biological Chemistry* **279** (5), 26149-
361 26158 (2004).
362 [4] Park JW, Piknova B, Nghiem K, Lozier JN Schechter AN. Inhibitory effect of nitrite on
363 coagulation processes demonstrated by thromboelastography. *Nitric oxide* **40**, 45-51 (2014).
364 [5] Wajih N, et. al. The role of red blood cell S-nitrosation in nitrite bioactivation and its
365 modulation by leucine and glycose. *Redox Biology* **8**, 415-421 (2016).
366 [6] Akrawinthawong, K. Park, JW. Piknova, B. Sibmooh, N. Fucharoen, S. Schechter, AN. A flow
367 cytometric analysis of the inhibition of platelet reactivity due to nitrite reduction by
368 deoxygenated erythrocytes. *PLoS One*. **9** (3), e92435 (2014).
369 [7] Friebe A, Koesling D. Regulation of nitric oxide-sensitive guanylyl cyclase. *Circulation Research*
370 **93** (2), 96-105 (2003).
371 [8] Srihirun, S. *et. al*. Platelet inhibition by nitrite is dependent on erythrocytes and
372 deoxygenation. *PLoS One*. **7** (1), e30380 (2012).
373 [9] Smolenski, A. *et al*. Analysis and regulation of vasodilator-stimulated phosphoprotein serine
374 239 phosphorylation in vitro and in intact cells using a phosphospecific monoclonal antibody. *J*
375 *Biol Chem*. **273**(32), 20029–20035 (1998).
376 [10] Burkhart, JM. *et al.* The first comprehensive and quantitative analysis of human platelet
377 protein composition allows the comparative analysis of structural and functional pathways.
378 *Blood*. **120** (15), e73–82.
379 [11] Parakaw, T. *et. al.* Platelet inhibition and increased phosphorylated vasodilator-stimulated
380 phosphoprotein following sodium nitrite inhalation. *Nitric oxide*. **66**, 10-16 (2017).
381 [12] Srihirun, S. Piknova, B. Sibmooh, N. Schechter, AN. Phosphorylated vasodilator-stimulated
382 phosphoprotein (P-VASPSer239) in platelets is increased by nitrite and partially deoxygenated
383 erythrocytes. *PLoS One*. **13**(3), e0193747 (2018).
384 [13] Cortese-Krott, MM. *et. al.* Identification of a soluble guanylate cyclase in RBCs: preserved
385 activity in patients with coronary artery disease. *Redox Biology*. **14**, 328-337 (2018).
386 [14] Abel, K. Mieskes, G. Walter, U. Dephosphorylation of the focal adhesion protein VASP in vitro
387 and in intact human platelets. *FEBS letter*. **370**(3), 184-188 (1995).
388