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COMPARATIVE METHODS FOR PROCESSING PLATELET LYSATE

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Abstract

Introduction: Platelets play an important role in numerous physiologic processes through the release of bioactive proteins and growth factors contained in the α granules. These proteins can be collected after physical and chemical processes stimulate their release from platelets. After filtration, this solution contains concentrated growth factors and is then referred to as platelet lysate (PL). Among the several methods used to produce PL, no method has been established as superior in producing the highest concentration of growth factors. This study evaluated six methods of producing PL including $CaCl_2$, freeze-thaw methods at −80˚ C and at −40˚ C, ozone exposure, USb, and USp, along with an "un-activated" Platelet-rich plasma (PRP) sample served as a control. Our goal was to evaluate each sample to determine which method produced the highest concentration of growth factors.

Methods: PRP was produced from whole blood and subsequently divided into seven samples. One sample served as a control while the other six were used to produce PL by different methods, including ultrasonic probe homogenization, ultrasonic bath homogenization, freeze-thaw at −80˚ C, freeze-thaw at −40˚ C, addition of calcium chloride, and ozone administration.

Outcome measures: The concentration of six growth factors were measured using digital enzyme-linked immunosorbent assay from the produced samples.

Results: PL produced by ultrasonic bath produced the highest concentrations of BDNF, EGF HB-EGF, PDGF-BB, and Vascular Endothelial Growth Factor (VEGF). PL produced by ultrasonic probe produced the highest concentration of IL-1 RA. The concentration of growth factors produced by the ultrasonic bath and probe methods did not differ significantly. The growth factors found in the freeze-thaw methods did not statistically differ from control. Ozone was the least effective at releasing measurable growth factors from PRP. The calcium chloride method resulted in a clotted sample which inhibited growth factor analysis.

Conclusion: The results of this study support the effectiveness of ultrasound homogenization in releasing growth factors from PRP over other current activation methods.

Keywords: *platelet lysate, platelet releaseate, platelet-rich plasma, alpha granules, growth factors, PRP*

BACKGROUND

Platelets contribute to numerous physiologic processes including clot formation, wound healing, neurogenesis, angiogenesis, and growth, proliferation and differentiation of various types of cells.[1] These processes occur through the induced release of platelet α granules that contain bioactive molecules, including platelet-specific proteins, growth factors (GFs), coagulation factors, adhesion molecules, cytokines, angiogenic factors, proteoglycans,

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and cytokines/chemokines.[2] There are hundreds of recognized platelet GFs that contribute to anabolic and paracrine effects briefly described in Table 1.[3] Standard preparations of platelet-rich plasma (PRP) can be used to focally deliver GFs to pathologic tissue. This physiologic response is highly dependent on several factors including local tissue environment, activation status of PRP, and the degree to which platelets can degranulate and release GFs contained in their alpha granules. For these, and other reasons, clinicians began to look at applying concentrated GFs as a potential substitute and/or replacement for PRP in various clinical conditions. A concentrated solution of platelet proteins and GFs can be accomplished by exogenous processing of the platelets to induce release of their alpha granules, then separating this solution from the platelets themselves. the resulting solution is called platelet lysate (PL) or platelet releasate (Figure 1). Platelet lysate can then be used for research and clinical applications including the induction and promotion of stalled or slowed physiologic processes. There is a growing volume of preclinical and clinical studies that describe the successful use of both PRP and PL in wound healing[3-7,8] cell differentiation,[9,10] tissue regeneration,[11] improved surgical outcomes,[12] and treatments for specific pathologies, such as intervertebral disc herniations,[13] osteoarthritis[14,15] ligament damage[16] and tendinopathies.[3,4,12,17,18] PL has been shown to carry similar beneficial healing and regenerative effects as PRP in several studies.[1,7,9,19-21] Furthermore, the in vitro release and growth factor concentration of PL has been demonstrated to provide a faster, more robust response to treatment compared to the in vivo release from un-activated PRP.[11,19]

Several established methods of PL processing have been described, but no study has compared them to determine which results in the most substantial and efficient release of proteins and GFs.[4-6,11,22-24] The different processing techniques include methods involving platelet rupture (lysis) and platelet degranulation.

We examined six methods of producing PL to understand which produces the highest concentration of GFs. We evaluated methods of platelet degranulation included calcium chloride ($CaCl_2$) and exposure to ozone gas, along with several methods of platelet lysate including two freeze-thaw methods (at −80˚C and at −40˚C), ultrasonic probe homogenization, and ultrasonic bath homogenization.[5,6,22, 25-29]

METHODS

Study Approval

Institutional Review Board (IRB) approval (*IRB Registration Numbers:* IRB00008463, IRB00003657, IRB00004920, IRB00001035, IRB00006075, IntegReview IRB, Austin, TX, USA) and informed consent was obtained for each participant in this study.

Table 1. Physiologic effects of selected growth factors found in plasma and PRP. Also shown are average growth factor concentrations produced by our laboratory compared to baseline levels. (pg/ml = picogram per milliliter, Bone-Derived Growth Factor (BDNF), Interleukin-1 Receptor Antagonist (IL-1 RA), Epidermal Growth Factor (EGF), Heparin-Binding EGF-like Growth Factor (HB-EGF), Platelet-Derived Growth Factor BB (PDGF-BB), and Vascular Endothelial Growth Factor (VEGF))

Growth Factor	Physiologic Effects	Average Concentration in PRP (pg/mL)
BDNF	Promotes survival, growth and differentiation of neurons	4960
EGF	Growth, proliferation and differentiation of numerous cell types	41
HB-EGF	Role in wound healing, angiogenesis and neurogenesis	15
IL-1 RA	Selectively inhibits inflammatory effects of IL-1	72
PDGF-BB	Angiogenesis and proliferation of mesenchymal cells	4123
VEGF	Angiogenesis	140

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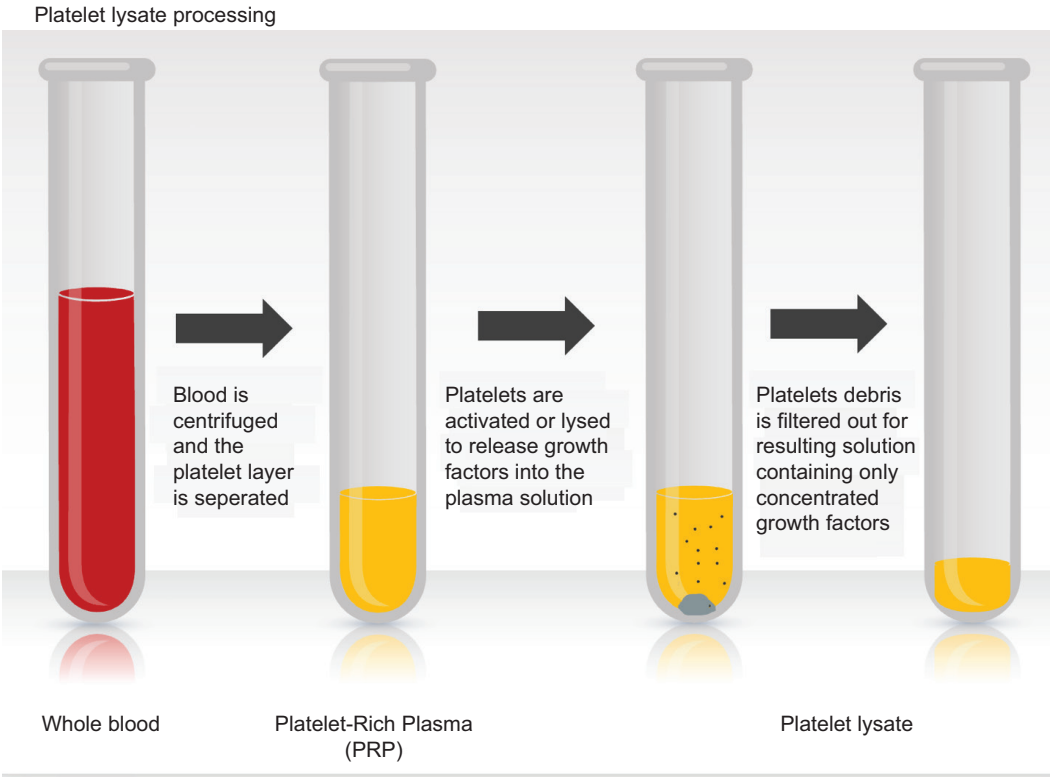

Figure 1. Processing of whole blood to produce platelet lysate.

Procedure

Eight healthy volunteers (two males, six females, aged 21–60) were enrolled in the study. 150 mL of whole, venous blood was collected from each subject and this was drawn into syringes containing a total of 30 mL anticoagulant citrate dextrose (ACD formula A 100mL contains 2.45 g dextrose and 2.2 g sodium citrate and 730 mg citric acid) (Fenwal, IL, USA) for anticoagulation. Each 180 mL sample produced 21 mL of PRP by double-centrifugation method. This was then split into seven 3mL samples. One sample of PRP was left unprocessed and used as a control. The second sample ($CaCl_2$) was mixed with 0.3 mL of $CaCl_2$, a ratio of 1:10, for 5 minutes. The third sample (ozone) was placed into a syringe with an equal volume of ozone gas and mixed for 30 seconds. The ozone was obtained through an ozone generator (Model HTU-500G2, Longevity Resources, AZ, USA) at a concentration of 20mcg/mL. The fourth sample (USp) tube was placed in an ice bath. Then, a sterilized ultrasonic probe (threaded ultrasonic probe tip, tapered microtip; 1/8") (Cole-Parmer, Cole-Parmer, IL, USA) was inserted directly into the solution and the probe cycled between sonication and rest for several cycles totaling 2 ⅓ minutes. The ultrasound processor was a (Hanchen, Luyi, China) run at 95%. The fifth sample (USb) was placed into a closed test tube and submerged in an ice bath within the ultrasonic bath (Kendal Commercial Grade 300 Watts Ultrasonic Cleaner EF-3000D, Shanghai Shining Image Co., Ltd. of Shanghai, Shanghai, China) and sonicated for 30 minutes. The sixth sample (cold freeze-thaw or cFT) was placed into a closed test tube and immersed into an insulated receptacle with 1L of acetone and dry ice. This was maintained until the sample achieved a temperature of –80 °C. The sample was then placed into an incubator at +21 °C to thaw. This freeze-thaw cycle was repeated one time. The seventh sample (warm freeze-thaw or wFT)

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was placed into a closed test tube and stored in a freezer at −40 °C until frozen, then thawed in the incubator at +21 °C. This freeze-thaw cycle was repeated one time. At the end of each procedure, all samples were centrifuged and filtered to remove cellular debris. The supernatant was collected in a closed test tube. The samples were then frozen at −40 °C until thawed for analysis.

Growth Factor Quantification

Growth factor concentrations were measured by ultrasensitive digital enzyme-linked immunosorbent assay using the Human CustomMAP, including the HCANCER2, HMPC18, and HMPCORE2 panels (Myriad RBM, Texas, USA). This is a proven platform that provides reliable biomarker analysis. The Human CustomMAP panel was used to measure the concentration of six markers including Bone-Derived Growth Factor (BDNF), Interleukin-1 Receptor Antagonist (IL-1 RA), Epidermal Growth Factor (EGF), Heparin-Binding EGF-like Growth Factor (HB-EGF), Platelet-Derived Growth Factor BB (PDGF-BB), and Vascular Endothelial Growth Factor (VEGF).

Statistical Analysis

All statistical analyses were performed using SPSS (Version 22.0, IBM Corp., Armonk, NY: USA). A one-way between-groups analysis of variance was conducted to assess the impact of each of the PL methods on levels of each marker compared to each other and to the control. Post-hoc comparisons were done using Tukey HSD (BDNF, HB-EGF, and PDGF-BB) and Games-Howell (IL-1 RA, EGF, and VEGF) methods. A p-value of $< .05$ was considered statistically significant for this project.

RESULTS

Marker Concentrations

The average marker concentrations produced by each PL method and their statistical differences are summarized in Table 2. We obtained data for all methods except the $CaCl_2$ method (see Discussion). Regardless of method, BDNF recorded the highest concentrations with a mean of 37,620 pg/mL. PDGF-BB was the marker with the next greatest concentration release with a mean of 28,477 pg/mL. The other markers were released in lower concentrations with means as follows: EGF (788 pg/mL), VEGF (424 pg/mL), IL-1 RA (273 pg/mL), and HB-EGF (145 pg/mL). Except for the $CaCl_2$ method, all other activation methods produced higher concentrations of GFs compared to control. The USb method released the highest BDNF, EGF, HB-EGF, PDGF-BB, and VEGF concentrations. The cFT method produced the highest concentrations of IL-1 RA (concentration). With the exception of IL-1 RA, both USb and USp methods performed better than the rest of the methods; however, there were no significant differences between the two US methods. Compared to the control, the USp method produced all markers at significantly higher levels, while the USb method released five markers with significant differences (BDNF, EGF, HB-EGF, PDGF-BB, VEGF). Both US methods significantly outperformed

Table 2. Average Marker Concentrations

	BDNF (pg/ml)	EGF (pg/ml)	HB-EGF (pg/ml)	IL-1 RA (pg/ml)	PDGF-BB (pg/ml)	VEGF (pg/ml)
Control	4960	41	15	72	4123	140
Ozone	17138	357	84	78	10013	245
USp	54500^{abcd}	1324^{abcd}	188^{a}	447^{abd}	43913^{abcd}	572^{abd}
USb	69000^{abcd}	1390^{ad}	234^{abd}	259	46813^{abcd}	713^{ad}
cFT	22625	600	123	471^{abd}	20625	310
wFT	24838	269	98	107	21024	279

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the wFT method for all markers. Compared to cFT, USp released three markers at significantly higher concentrations (BDNF, EGF, PDGF-BB), while the USb method was only significantly higher for two markers (BDNF, PDGF-BB). Though the FT and ozone methods produced all markers at higher concentrations than control, only the IL-1 RA released by cFT was significantly higher. cFT produced EGF, HB-EGF, IL-1 RA, and VEGF at higher concentrations than wFT, but only IL-1 RA was significantly different. As stated earlier, the cFT method released the highest concentration of IL-1 RA, however the remaining markers were released at marginal levels. The wFT did not release any marker at higher levels than the other methods. Finally, though higher than control, ozone released the lowest concentrations for all markers. While the FT methods generally did not differ significantly from ozone, both the US methods consistently produced greater levels.

Laboratory Considerations

The ozone method was the quickest method of production, closely followed by the USp and $CaCl_2$ methods, respectively. FT and USb methods were similar in production time and had the longest protocols. Overall, each of our protocols were completed within two hours and produced with a relatively low risk of contamination, demonstrating the ease of producing PL. In addition, as ozone and ultrasound applications are routinely used for sanitization, the contamination risk is dramatically lowered using these methods.

DISCUSSION

This study shows that US methods of producing PL resulted in the highest concentrations of GFs from PRP. Our results support available literature describing successful activation of PRP using US.[25,26]

Ultrasonic homogenization is a common laboratory procedure used to lyse cells, but still in the early stages of translation to use with PRP. Here, sound waves produced at frequencies of >20 kHz rapidly compress and decompress dissolved gases causing violent collisions that rupture the cell membranes causing release of intracellular proteins.[26] Although very efficient at lysing cells, ultrasonic homogenization can transfer heat to the sample, possibly denaturing the proteins.[26] Because of this phenomenon, submersion of the sample into an ice bath is often used to decrease this heat transfer.[26] Limited evidence has shown that ultrasonic techniques are effective in producing PL.[25,26] Two methods of ultrasonic homogenization have been studied: Ultrasonic Bath (USb) and Ultrasonic Probe (USp). USb methods rely on the use of closed containers in which the PRP sample is placed in a water bath within the machine. Ultrasonic waves are then transmitted through the bath.[26] Bernardi, et al. have described the successful use of USb in producing factor-concentrated PL for stimulating mesenchymal stromal cell differentiation.[26] The USp method relies on a probe directly inserted into the PRP solution to directly transfer ultrasonic waves. This has the potential to provide a more efficient transfer of energy to the PRP, but also increases the risk of contamination and greater heat transfer. Fortunato, et al. demonstrated the successful use of an USp to create PL, which contained high factor concentrations and stimulated endothelial colony forming cells to form and expand microvascular networks.[25] Fortunato, et al. described a method of activating PRP using USp stimulation, in conjunction with thrombin incubation, to successfully concentrate factors and stimulate cell proliferation in vitro.[25] We used a similar USp method but removed the thrombin incubation to allow for the effect of USp alone. Our USp protocol produced significantly higher concentrations of all factors than control and other methods. These results expand on previous findings by showing that USp, without the combination of thrombin, can produce high levels of factors.[25]

Bernardi, et al. demonstrated the successful stimulation of cell growth and differentiation using activated PRP produced via USb.[26] Our USb protocol was similar. However, our PRP was left in a sealed test tube, rather than freeze bags to eliminate the additional steps of transferring PRP from different containers. As with our USp protocol, our USb protocol successfully released factors, further supporting the current literature. In comparing the two

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methods, we found that the USb method produced all factors, except IL-1 RA, at higher concentrations than the USp method. However, none of these differences were statistically significant. While this could suggest USb methods as more proficient activators of PRP, further investigation will be needed to confirm any differences.

Freeze-thaw cycles are commonly utilized methods for creating effective PL. These also have considerable variability in methodology including the number and length of time of cycles, and the range of temperatures used.[9,11,14,19,22] During each freeze cycle, water crystals form and lyse platelets' cellular membranes, releasing their intracellular products.[22] (check on[22,32]). In general, we noted lower concentrations of GFs for each of the FT methods. While both FT protocols released all factors at higher concentrations than control, this was less robust than the ultrasound methods. Furthermore, we noted relatively comparable results with our two FT methods. In addition, both US protocols outperformed the FT methods, suggesting US is a more effective means of releasing factors. These results support Bernardi, et al.'s findings that USb releases higher levels of factors than FT methods.[26] In the literature, much data on FT methods is used to produce activated PRP. However, few studies have compared FT to US methods.[25,26] FT methods are one of the most common methods for lysing platelets, though the literature is still inconclusive on the efficiency.[5,6,11,22,28,29] Multiple studies have successfully demonstrated the ability of FT methods to produce higher concentrations of GFs compared to un-activated PRP, though some have failed to show this difference.[5,6,9,14,19,22,28] The reduced efficacy of the FT methods demonstrated in our study further adds to the inconclusiveness of available literature. One possible explanation is the variability of FT protocols (e.g., varying in number of cycles, temperatures used, and length of time for each cycle) complicating the ability to compare results from individual studies. Each of our FT protocols involved two cycles of freeze (−40 °C or −80 °C) and thaw (+21 °C) and were completed in under two hours.

Ozone, a radical oxygen species, has limited description of its effect on PRP or mechanism of action in the literature.[27,33,34] Valacchi, et al. showed that ozone successfully activated the release of PDGF and TGF-beta over PRP.[34] However, Anitua, et al. presented data showing that ozone treatment reduced the concentration of several factors and did not improve cell proliferation when compared to $CaCl_2$ methods.[27] It is theorized that the imbalance of reactive oxygen species vs. antioxidants and the structural changes to proteins from the resultant oxygenation are some of the factors explaining the poor stimulation of PRP by ozone.[27] One theory suggests that hydrogen peroxide generated from the breakdown of ozone can activate the platelets to release their contents.[33] Another theory postulates that ozone can contribute to PRP coagulation in a mechanism similar to $CaCl_2$.[34] However, there is scarce and inconclusive evidence available confirming ozone's effects on PRP.[27]

Our findings show that ozone activation resulted in significantly less GFs compared to all methods, thus suggesting insufficient activation of PRP. The ozone method resulted in the lowest concentrations of factors released by any of the methods in our study, except for the $CaCl_2$ where we could not collect results. Both US and FT methods produced higher concentrations of GFs than ozone, with both of the US methods showing more substantial differences. While there is limited research on the effects of ozone on PRP, our results, in addition to available literature, further question the use of ozone as an effective means of producing activated PRP.

The addition of $CaCl_2$ to PRP is a well-studied method of activation. The $CaCl_2$ stimulates clot formation, promotes platelet degranulation, and releases their intra-corpuscular products into the plasma.[30] The sample can then be centrifuged to remove cellular products and the clot, leaving GFs and other cytokines concentrated in the plasma. Protocols contain variability in the concentration of $CaCl_2$ used, the time allowed for clot formation, and the concomitant administration of other activators, such as thrombin.[7,20,21,31] A potential limitation of the $CaCl_2$ method is the loss of GFs during centrifugation due to entrapment of the proteins within the cellular matrix formed during the clot.[28,30]

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A significant setback to this study was the inability to collect data for our $CaCl_2$ method. We could not derive reliable data on the $CaCl_2$ methods due to technical problems. Though the exact cause is unknown, we know that the introduction of $CaCl_2$ into plasma triggers the clotting cascade, resulting in a gelatinous clot formation in the test tubes and a potential incompatibility with the equipment used by the laboratory.[7] This is an unfortunate loss as $CaCl_2$ methods are one of the most common PL production methods.[11,23,24,35,36] It should be mentioned that current data is yet to determine a significant difference between FT vs. $CaCl_2$ methods, suggesting both are equivocal in their ability to release factors from PRP.[5,6,22,28,29]

Another limitation to our study is the small sample size. This study was designed as an introductory investigation into comparing current and novel methods for activating PRP and future studies with more samples will be required to confirm our findings. Another limitation is using only six factors for analysis when over 400 factors are released from PRP samples.[14] Furthermore, no analysis was performed on the many other variables that could be important in the formulation and standardization of PL. Protocol variables, such as platelet concentration and volume, white and red blood cell concentration, and the various demographic variables (age, gender, sex, etc.), have not been fully investigated.[24,37,38] Lastly, while our study included both common methods and novel methods, it should be mentioned that there are other approaches for activating PRP such as collagen or thrombin, sometimes combined with $CaCl_2$.[24] Also, the use of detergents such as trin-butyl phosphate, Triton X-40, and Triton X-100 have been utilized and studied.[6,28]

PRP and PL are still under investigation and large reviews of available data are inconclusive. One of the major setbacks facing the field is the lack of standardization for producing and reporting PRP products.[24,36] There are numerous methods described for producing and activating PRP, which result in using[24,39] Furthermore, there is variability among researchers in the definition and quality control of PRP end-products.[24] This lack of standardization has resulted in a volume of difficult-to-compare and interpret evidence.[11,18,23,36,40] Another setback is the lack of large, high-quality study designs (randomized, controlled, double-blinded), as most of the current evidence comes from investigations that meet lower standards of evidence.[18,41] While these setbacks hinder the ability to review available data, this has not deterred the rapid application of PRP products in clinical settings.[24,42]

This study helps fill the gap in the literature surrounding activation methods of PRP and confirms previous findings showing the advantages of US approaches. The results of this study suggest that future investigations into methods of activating PRP should include US techniques. Whereas the literature generally supports FT methods ability to activate PRP, our study demonstrated marginal results using these protocols. As this study is an introductory investigation, further work is required to establish efficacy and ideal protocols for US methods.

CONCLUSIONS

This study's results support ultrasound homogenization's effectiveness in releasing GFs from PRP over other current activation methods.

AUTHOR CONTRIBUTIONS

Conceptualization, H.T., B.R. and D.H.; methodology, H.T, B.R. and D.H.; validation, H.T, B.R. and D.H; formal analysis, H.T, B.R. and D.H; investigation, H.T.; data curation, H.T. and D.H..; writing—original draft preparation, H.T.; writing-review and editing, B.R. and D.H.; visualization, B.R.; supervision, D.H.; funding acquisition, D.H.. All authors have read and agreed to the published version of the manuscript.

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INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted according to the guidelines of 260 the Declaration of Helsinki, and

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approved by the Institutional Review Board (or Ethics Committee) 261 of Integra Review (protocol code IRB00008463) 06/01/2016.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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