Title
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Journal
Journal of Trauma and Acute Care Surgery, 89(5)

ISSN
2163-0755

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Publication Date
2020-11-01

DOI
10.1097/ta.0000000000002820

Peer reviewed

HHS Public Access

Author manuscript

J Trauma Acute Care Surg. Author manuscript; available in PMC 2021 November 01.

Published in final edited form as:

J Trauma Acute Care Surg. 2020 November ; 89(5): 871–879. doi:10.1097/TA.0000000000002820.

Dynamic Effects of Calcium on *In Vivo* and *Ex Vivo* Platelet Behavior After Trauma

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Abstract

Background: Mobilization of intra and extracellular calcium is required for platelet activation, aggregation, and degranulation. However, the importance of alterations in the calcium-platelet axis after injury is unknown. We hypothesized that in injured patients, *in vivo* initial calcium concentrations (pre-transfusion) predict *ex vivo* platelet activation and aggregation, viscoelastic clot strength, and transfusion of blood products. We additionally hypothesized that increasing calcium concentrations *ex vivo* increase the expression of platelet activation surface receptors and platelet aggregation responses to agonist stimulation in healthy donor blood.

Methods: Blood samples were collected from 538 trauma patients on arrival to the emergency department. Standard assays (including calcium), platelet aggregometry (*PA*) and thromboelastometry (*ROTEM*) were performed. In *PA*, platelet activation (pre-stimulation impedance, Ω) and aggregation responses to agonist stimulation (AUC) with adenosine diphosphate (ADP), thrombin receptor-activating peptide (TRAP), arachidonic acid (AA), and collagen (COL) were measured. Multivariable regression tested the associations of calcium with *PA*, *ROTEM*, and transfusions. To further examine the calcium-platelet axis, calcium was titrated in healthy blood. *PA* and ROTEM were performed, and expression of platelet glycoprotein IIb/IIIa and P-selectin were measured by flow cytometry.

Results: The patients were moderately injured with normal calcium and platelet counts. Higher calcium on arrival (pre-transfusion) was independently associated with increased platelet activation (pre-stimulation, Ω; $p<0.001$), aggregation (ADP-stimulated, AUC; $p=0.002$; TRAP-

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Author Contribution Statement:

Study design, literature search, data analysis, data interpretation and writing were performed by Z.M and L.Z.K. Recalcification experiments on healthy donor blood were performed by Z.M. and A.T.F. Data collection was performed by Z.M., A.T.F., M.P., and B.N.G. Data interpretation and critical revision were performed by R.A.C. and M.J.C.

Disclosure outside the scope of this work: none.

Meeting presentation: Presented at the 50th Annual Meeting of the Western Trauma Association held February 23rd to February 28th, 2020 in Sun Valley, Idaho.

Conflicts of Interest:

Disclosure outside the scope of this work: none.

stimulated, AUC; p=0.038) and clot-strength (ROTEM max clot firmness; p <0.001), and inversely associated with 24h transfusions of blood, plasma, and platelets (all p<0.005). Up-titrating calcium in healthy blood increased platelet activation (pre-stimulation, Ω; p<0.001), aggregation (ADP, AA, COL-stimulated AUCs; p<0.050), and expression of P-selectin (p=0.003).

Conclusions: Initial calcium concentrations (pre-transfusion) are independently associated with platelet activation, aggregation, clot-strength, and transfusions after injury. These changes may be mediated by calcium driven expression of surface receptors necessary for platelet activation and aggregation. However, the therapeutic benefit of early, empiric calcium repletion in trauma patients remains undefined.

Keywords

Platelets; platelet activation; platelet aggregation; platelet function tests; blood coagulation disorders; calcium; hypocalcemia; trauma

Background

Hemorrhage remains a leading cause of preventable death after trauma (1). Accordingly, a focus on elucidating drivers of impaired hemostasis that are characteristic of trauma-induced coagulopathy (TIC) continues to be paramount for improving mortality after injury (2, 3). The role of cellular mediators in TIC has become increasingly recognized, including alterations in platelet activation and aggregation (2–6). Recent advances have identified potential mechanistic drivers of these alterations including changes in platelet structure, platelet signaling, and platelet-immune regulation (7–14).

Importantly, calcium is a universal secondary messenger whose mobilization from the extracellular space into platelets is essential for proper platelet activation, aggregation, cytoskeletal rearrangements, and degranulation (15, 16). Furthermore, hypocalcemia is predictive of blood product transfusions and mortality in hemorrhaging trauma patients undergoing massive transfusion, exacerbated with calcium chelation by citrated blood products (17–19). Additionally, evidence suggests that calcium concentrations may mediate viscoelastic clot strength through effects on platelets in hemorrhaging, critically ill patients who have a preexisting coagulopathy (20). However, the calcium-platelet axis after injury is poorly characterized, and given that hypocalcemia can be rapidly identified at point-of-care and is easily treated, significant equipoise exists to define the relationships of calcium with platelet behavior during traumatic hemorrhage.

In this study, we sought to delineate the dynamic effects of calcium concentrations on platelet activation, aggregation, and platelet dependent viscoelastic clot strength to better understand the calcium-platelet axis after injury. We hypothesized that in injured patients, *in vivo* initial calcium concentrations (pre-transfusion) predict *ex vivo* platelet activation and aggregation, viscoelastic clot strength, and transfusion of blood products. We additionally hypothesized that increasing calcium concentrations *ex vivo* increase the expression of platelet activation surface receptors and platelet aggregation responses to agonist stimulation in healthy donor blood.

Methods

Patient Enrollment, Inclusion and Exclusion Criteria, and Sample Preparation:

Whole blood samples from 1,651 trauma patients were collected in standard laboratory vacuum-sealed tubes containing 3.2% (0.109mol/L) sodium citrate on arrival to the emergency department for coagulation assays as part of a longitudinal prospective study evaluating abnormalities of coagulation and inflammation after injury (2010–2018) (6, 21–23). All highest level trauma activations were enrolled under an initial waiver of consent approved by the Committee on Human Research at the University of California, San Francisco. After excluding patients who were pregnant, in-custody, transferred, under the age of 18, on anticoagulant or antiplatelet medications, had burns greater than 20% body surface area, and those who did not have platelet aggregometry (*PA)* performed, 538 patients remained in the study. Comprehensive demographics, injury characteristics, and clinical outcomes were collected prospectively on all patients.

Laboratory Assays of Clinical Samples:

Platelet Aggregometry (PA): *PA* was performed using the Multiplate® multiple electrode aggregometer (Verum Diagnostica GmbH; Munich, Germany) (24). Citrated whole blood (300uL) was diluted in warmed normal saline containing 3mM $CaCl_2$ for recalcification and incubated for 3 minutes at 37°C with continuous stirring in a Multiplate® test cell. Platelet aggregation was induced by agonist stimulation with adenosine diphosphate (ADP, final concentration 6.5μM; via P2 receptors), thrombin receptor activating peptide-6 (TRAP, final concentration 32μM; via PAR receptors), arachidonic acid (AA, final concentration 0.5mM; via the cyclooxygenase pathway), and collagen (COL, final concentration 3.2μg/mL; via GpIa/IIa and GpVI receptors) as per manufacturer. Platelet adhesion to the electrodes was detected as increasing electrical impedance, measured by duplicate sets of sensor wires in each test cell. Platelet activation was measured as the baseline impedance (Ω) of the whole blood prior to agonist stimulation. Platelet aggregation responses to agonist stimulation were measured as area under the aggregation curve (AUC) over a 6-minute measurement period.

Rotational Thromboelastometry (ROTEM): Viscoelastic testing was performed using the ROTEM® delta machine (Pentapharm GmbH; Munich, Germany) with EXTEM and FIBTEM reagents (star-TEM recalcifier; ex-TEM tissue factor extrinsic pathway activator; fib-TEM cytochalasin D platelet inhibitor + ex-TEM reagent). Citrated whole blood was warmed to 37°C in the TEM cup, prepared according to manufacturer specifications, and the test initiated within 20 seconds of mixing 300uL of whole blood with 20μL of 0.2M $CaCl_2$ for recalcification, and respective activator. Clotting time (CT) in seconds (sec), clot formation time (CFT) (sec), alpha angle (α) (degrees), clot firmness after 10 (A10) and 20 minutes (A20) (mm), maximum clot firmness (MCF) (mm), and maximum lysis (ML%) were recorded.

Standard coagulation assays: The Zuckerberg San Francisco General Hospital (ZSFG) clinical laboratory measured hematocrit, platelet count, pH, and base excess on whole blood samples, and total calcium concentration, international normalized ratio (INR), and partial

thromboplastin time (pTT) on plasma samples. Ionized calcium was measured on whole blood using the calcium electrode of a point of care blood gas analyzer (GEM® 4000 Analyzer, Instrumentation Laboratory).

Ex Vivo Calcium Titration Assays:

To test effects of calcium concentrations on expression of platelet activation surface receptors, platelet aggregation responses to agonist stimulation, and platelet dependent measures of viscoelastic clot strength, whole blood from healthy volunteers (n=4) was drawn in standard laboratory vacuum-sealed tubes containing 3.2% (0.109mol/L) sodium citrate.

Calcium titration: The previously described recalcification process for *PA* was performed with $CaCl_2$ was dissolved in saline, but adjusted such that the final molarity of $CaCl_2$ added to blood samples from each donor was 0mM, 1.5mM, 3.5mM, 4.25mM, or 5mM (Figure 1). These titrations corresponded to unmeasurable (0mM-with no added $CaCl_2$, all endogenous ionized calcium is chelated by citrate), sub-physiologic (<0.50mM), low-physiologic (0.81mM), physiologic (1.25mM), and high-physiologic (1.61mM) concentrations of ionized calcium, confirmed by measurement in the ZSFG clinical laboratory (Gem ® 4000 Analyzer). *PA* was performed as described above using the recalcified healthy donor blood.

Rotational Thromboelastometry (ROTEM): Recalcification was performed as was done for *PA* described above, with final sample ionized calcium concentrations of 0mM, 0.51mM, 0.71mM, 0.97mM, 1.71mM, and 3.00mM (3.00mM corresponding to the ROTEM standard recalcification amount using star-TEM recalcifier reagent). Because the commercial fib-TEM reagent contains premixed recalcifier with cytochalasin D (a platelet cytoskeletal inhibitor), in order to titrate calcium concentrations in the fib-TEM channel, 5μg of cytochalasin D (1mg/ml in DMSO) was used rather than the commercial reagent, and this demonstrated equivalent platelet inhibition to the fib-TEM reagent.

Flow Cytometry: Expression of the platelet activation surface receptors Glycoprotein IIb/IIIa and P-selectin was measured by flow cytometry. Citrated whole blood was recalcified according to the same calcium titration protocol used for *PA* described above, generating final ionized calcium concentrations of 0mM, <0.50mM, 0.81mM, 1.25mM, and 1.61mM (Figure 1). Recalcification and staining was commenced no more than one hour after draw. After five minutes of incubation, the blood was diluted (1:25) with modified HEPES-buffered Tyrode's Solution (137mM NaCl, 2.7mM KCl, 2mM $MgCl_2$, 0.42mM NaH_2PO_4, 5mM glucose, 10mM HEPES, pH 7.4) for antibody staining. Allophycocyanin (APC) -conjugated anti-CD41 (clone HIP-8) was used to distinguish platelets, phycoerythrin (PE) -conjugated anti-CD62p (clone AK4) to distinguish platelets expressing surface P-selectin, and fluorescein isothiocyanate (FITC) -conjugated anti-CD41/CD61 (clone PAC-1) to distinguish platelets with Glycoprotein IIb/IIIa (Integrin αIIbβ3) complex. Staining with fluorophore- and antibody isotype- matched controls was performed to determine positive staining. All antibodies and immunoglobulin (Ig) isotype controls were purchased from Biolegend (San Diego, CA). Stained samples were treated with FACS Lysing Solution at a ratio of 2:1 (Becton Dickinson, San Jose, CA) for 15 minutes at room temperature prior to

storage at 4°C for no more than 24 hours. Data were collected with a BD LSRII cytometer (Becton Dickinson, San Jose, CA) and analyzed using FlowJo (FlowJo, LLC, Ashland, OR).

Statistical Analyses:

Secondary analysis of prospectively collected data was performed. Data are presented as mean (± standard deviation), median (interquartile range [IQR]), or percentage; univariate comparisons were made using Student's *t* test or using Analysis of Variance (ANOVA) for normally distributed data, Kruskal-Wallis testing for nonparametric data, and Fisher's exact test for proportions. Multivariate linear regressions were performed to test the independent associations of calcium concentrations with *PA* and *ROTEM* measures. To account for non-normal distributions of variables consisting of count data, multivariable Poisson regressions with robust standard errors were performed to test the independent associations of calcium concentrations with units of blood products transfused. Of note, no pre-hospital blood products are administered during transport to our institution given the short travel times and small geographic catchment area for our patient population. Pearson's correlation was used to define the relationship between plasma and ionized calcium. Univariate linear regressions were performed to test the associations of ionized calcium concentrations with *PA*, ROTEM, and expression of platelet activation surface receptors in healthy donor whole blood. Given the inter-person variability in the baseline expression of platelet surface receptors, these values were normalized to generate relative changes in expression of surface receptors prior to univariate linear regression analysis. An [alpha] <0.05 was considered significant. Bonferroni corrections were applied for univariate multiple comparisons. All analysis was performed using Stata version 15 (StataCorp, College Station, TX). Power calculations were performed for our *ex vivo* recalcification experiments based on the effect sizes and standard deviations observed in our clinical data set. The estimates demonstrated that a sample size of n=4 with 5 concentrations of calcium tested per subject would yield sufficient observations to detect significant differences in our outcomes of interest with 80% power and alpha set at 0.05.

Results:

Laboratory Assays of Clinical Samples:

The 538 patients were predominately young, male, and moderately injured (median ISS 10; IQR 1–24). While admission platelet counts (median $263\times10^3/\mu L$; IQR $218–310\times10^3/\mu L$) and mean calcium concentrations (8.8 mg/dL SD +/−0.63) were normal, 28% received blood transfusions and 2% died within the first 24 hours (Table 1). Although the cohort was only moderately injured, 42% of patients presented with evidence of impaired platelet aggregation responses to agonist stimulation, and 18% demonstrated coagulopathy with an INR≥1.3 on arrival to the emergency department (Table 1 and Table S1).

Patients with the lowest quartile of calcium (7.2–8.4mg/dL) on presentation had significantly lower platelet activation (pre-stimulation impedance Ω), platelet aggregation responses to ADP stimulation (AUC), and platelet dependent viscoelastic clot strength (EXTEM A10, A20, MCF, all $p<0.0083$ corrected for multiple comparisons; Table 2). However, they also

had higher burdens of injury and shock, and worse outcomes including higher rates of transfusion, and longer ICU and hospital stays (all $p<0.001$; Table S2).

On multivariate regression controlling for confounding demographics (age and gender), injury and shock burden (ISS, base excess), platelet count, and pre-hospital crystalloid volume, increasing initial calcium concentrations (pre-transfusion) were independently and strongly associated with increased levels of platelet activation (pre-stimulation Ω, $p<0.001$), platelet aggregation responses to ADP and TRAP stimulation (AUC, $p=0.002$ and $p=0.038$ respectively; Figure 2 panels A and B and Table S3), faster clot initiation (EXTEM CFT, $p<0.005$), and stronger platelet dependent early (EXTEM A10 and A20) and late (EXTEM MCF) viscoelastic clot strength (all $p<0.001$; Table 3).

Finally, with respect to clinical outcomes, there was an expected inverse, independent relationship between initial calcium concentration (pre-transfusion) and 24 hour transfusions of blood products—for each 1mg/dL increase in calcium there was an associated decrease of 0.55 units of blood, 0.54 units of plasma, and 0.78 units of platelets transfused at 24 hours ($p=0.005$, $p=0.003$, and $p=0.001$ respectively; Table 3). This association was the strongest for the most severely injured patients (ISS≥25, n=95)--for each 1 mg/dL increase in calcium there was an associated decrease of 0.71 units of blood, 0.48 units of plasma, and 0.88 units of platelets transfused at 24 hours ($p=0.011$, $p=0.040$, and $p=0.006$ respectively).

While total plasma calcium concentrations were measured on all participants on arrival to the emergency department, ionized calcium was not routinely captured on arrival for all trauma patients, and therefore was only available for 88 of the 538 patients (primarily from the years 2014–2015; Table S4). For the 88 patients with both ionized and total plasma calcium measurements, there was a linear correlation between the two variables (Pearson's correlation coefficient=0.63; Figure S1), and there was no difference in the mean albumin concentrations between those with and without ionized calcium measurements (3.54 and 3.66 g/dL respectively, $p=0.26$). A sub-analysis of these 88 patients confirmed significant independent associations of ionized calcium with platelet activation (pre-stimulation impedance Ω, $p=0.002$), and important trends for platelet aggregation in response to agonist stimulation with ADP, AA, and COL (Table S5). Lastly, paralleling our results for the entire cohort, there were important trends between ionized calcium and 24 hour blood product transfusions on multivariable analysis (Table S5).

Ex vivo Calcium Titration Assays:

Corroborating our findings in trauma patients, increasing *ex vivo* calcium concentrations in healthy donor whole blood titrated from unmeasurable (~0mM) to high-physiologic (1.61 mM) concentrations were significantly associated with increased levels of platelet activation (pre-stimulation impedance Ω), and platelet aggregation responses to ADP, AA, and COL stimulation (AUC, all $p<0.05$; Figure 2 panels C and D). Paralleling this, there was a stepwise increase in the normalized expression of the platelet activation surface receptor P-selectin with increasing calcium concentrations, while for the expression of Glycoprotein IIb/IIIa, non-significant trends were observed (Table 4). Lastly, there were non-significant trends towards shortened time to viscoelastic clot formation and increased measures of early

viscoelastic clot strength (A10 and A20) (though not for overall clot strength [MCF]) with *ex vivo* recalcification of healthy donor whole blood (Table S6).

Discussion:

In this study, we found that initial calcium concentrations in injured patients, prior to any transfusion or major resuscitation, were independently and significantly associated with *ex vivo* platelet activation (pre-stimulation), platelet aggregation responses to agonist stimulation with ADP, platelet dependent viscoelastic clot formation, and transfusions. These relationships are remarkable, given the assays were performed on whole blood samples drawn in citrate containing tubes, which chelates calcium resulting in undetectable ionized calcium concentrations prior to the *ex vivo* assays. This suggests that higher *in vivo* calcium concentrations confer priming to the platelets that provides functional benefits to activation and aggregation behaviors in the setting of injury, which is durable enough to be detected with aggregometry and viscoelastic testing despite being performed on calcium chelated whole blood. Clinically, these findings are manifested by a moderate, but relevant decrease in blood product transfusions for patients with higher pre-transfusion calcium concentrations. For example, our multivariate model predicts that patients in the highest quartile of plasma calcium concentrations compared to the lowest have a decrease in transfusions by 1.1 units of blood, 1.1 units of plasma, and 1.5 units of platelets at 24 hours. Given the independent relationship between initial calcium concentrations and transfusions were strongest for the most severely injured patients, this may have valuable clinical implications for patients at the highest risk of blood loss, transfusions, and associated poor outcomes.

We hypothesize this biology occurs via the well-known mechanisms of calcium influx inducing downstream platelet signaling, including increased expression and translocation of surface receptors involved in platelet activation and aggregation (15). Supporting this, in our injured patient aggregometry results, there was a strong relationship between calcium concentrations and levels of platelet activation as well as ADP and thrombin stimulated aggregation. Furthermore, in our *ex vivo* healthy donor blood results, we found statistically significant calcium dependent increases in the surface expression of P-selectin, and a trend for the expression of activated Glycoprotein IIb/IIIa in association with up titration of calcium across physiologic ranges. P-selectin and Glycoprotein IIb/IIIa both have critical roles in platelet adhesion and aggregation, but require stimulatory signaling to become functional, through either translocation to the platelet membrane surface (as in the case of P-Selectin) or by conformational activation (for Glycoprotein IIb/IIIa). Our findings suggest that differential expression of these platelet surface receptors mediated by *in vivo* calcium could explain how platelets retain differential activation and aggregation potential *ex vivo* despite being collected in citrated environments.

It is also notable that platelet dependent measures of viscoelastic clot strength in ROTEM (EXTEM A10, A20, and MCF) were significantly associated with calcium concentrations, but that this association was not present for FIBTEM measures, in which the platelet contribution to clot strength is specifically blocked by cytochalasin D in order to measure fibrin’s contribution. This supports that the strong associations between viscoelastic

measures of clot strength and calcium are primarily platelet-dependent. To our knowledge, only one other study has evaluated the relationship between viscoelastic measures and calcium concentrations in hospitalized patients, and found that in hemorrhaging, critically-ill patients, calcium concentrations were significantly associated with clot strength, but only in the setting of pre-existing coagulopathy (20).

Multiple authors have demonstrated relationships between hypocalcemia and poor outcomes in trauma patients (17–19, 25). In our study, we found that there was a statistically significant and independent inverse relationship between the initial calcium concentrations (pre-transfusion) and the total number of transfused units of blood products in the first 24 hours after injury, but there was no independent association of initial calcium concentrations with mortality. Profound hypocalcemia that develops in trauma patients is thought to be secondary to both dilutional effects of resuscitation and due to chelation by citrate in blood products, which, although usually rapidly metabolized by the liver in a healthy individual, may be subject to impaired clearance in the setting of hemorrhagic shock (18). While these associations with hypocalcemia are well established, and corroborated by our study, it remains unknown whether empiric calcium administration in trauma patients could reduce morbidities both for patients with minor and moderate injury burdens as well as for severely injured patients with traumatic hemorrhage.

It is interesting to note that although we observed linear increases in platelet activation and aggregation across the physiologic range of calcium in our aggregometry results, in contrast there appeared to be a calcium concentration threshold effect in our ROTEM experiments in healthy donor blood of ~0.51mM, above which most samples showed normal clotting behavior. One other study in healthy volunteers similarly reported a threshold effect of calcium concentration of 0.56mM for normal *ex vivo* clot formation and strength in thromboelastography (26). While these results from healthy volunteers do not recapitulate the many complex changes in coagulation that occur in trauma patients, they underscore the physiologic importance of preventing hypocalcemia, which could be accomplished by empiric early calcium administration to hemorrhaging patients for whom significant transfusions are anticipated. Furthermore, if there is indeed a priming effect of calcium on platelet behavior in the setting of injury that persists despite calcium chelation, inducing supraphysiologic calcium concentrations prior to transfusion may provide further benefit in the setting of calcium chelation with the large amounts of citrate delivered in massive transfusion. However, given the potential risks of calcium administration, including untoward effects on the cardiovascular system (27) and cellular metabolism (28), the overall risk to benefit profile of empiric, supraphysiologic calcium administration warrants further investigation prior to implementation. It should be noted however that we identified significant effects on both platelet-dependent hemostasis and transfusion outcomes even with calcium concentrations in the high physiologic range. This is important because there may even be hemostatic benefits to calcium repletion to high-normal ranges, mitigating risks that may exist with repletion to supraphysiologic concentrations.

Although we have demonstrated strong associations between initial calcium concentrations and multiple measures of platelet dependent hemostasis, there are important limitations to these results. Given the prospective but observational nature of this study, it is possible that

unknown, unaccounted confounders may be driving these observations. Furthermore, we recognize that our calcium measurement, total plasma calcium, may not necessarily reflect ionized calcium which was only available on a subset of our cohort. However, samples were drawn on arrival to the emergency department, prior to significant crystalloid resuscitation or any blood product transfusions. Furthermore, our sub-analysis of the 88 patients with ionized calcium results demonstrated a linear correlation of ionized calcium with plasma calcium, and no significant difference in albumin concentrations between patients with and without ionized calcium measurements. Although our ionized calcium results are underpowered based on the effect sizes observed for our analysis with plasma calcium, we still demonstrated significant associations between ionized calcium and platelet activation, clot strength, and plasma transfusions (and trends for both platelet aggregation in response to agonist stimulation as well as blood product transfusions), paralleling our study's overall findings. Lastly, further investigations are needed to confirm our mechanistic hypothesis that with increasing calcium concentrations, circulating platelets undergo a priming of expression of platelet activation surface receptors, which enhances their ability to activate and aggregate regardless of subsequent calcium chelation. This in turn, may increase their contributions to hemostasis and improve bleeding outcomes after injury.

In summary, initial calcium concentrations (pre-transfusion) were significantly associated with platelet activation and aggregation, platelet dependent viscoelastic clot strength, and 24 hour blood product transfusions in a cohort of adult trauma patients. These biologic associations were confirmed with *ex vivo* titration of calcium in healthy donor blood in platelet aggregometry, viscoelastic testing, and flow cytometry studies. Our findings support that calcium is intimately involved in platelet-dependent hemostasis after injury, and normalization of calcium during active hemorrhage deserves close attention. Furthermore, future studies of inducing early supraphysiologic calcium concentrations to both prime platelet function and to mitigate hypocalcemia induced alterations in platelet dependent hemostasis should be an active area of resuscitation research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

Alan Wu, PhD; Erin Ross BS; John Parks BA.

Funding Sources:

Dr. Kornblith is supported by NIH 1K23GM130892-01, Dr. Cohen is supported by NIH UM1HL120877 and DoD W911QY-15-C-0044, Dr. Callcut is supported by NIH K01ES026834 and DoD W911QY-15-C-0044, Dr. Matthay is supported by The National Center for Advancing Translational Sciences of the NIH 5TL1TR001871-04.

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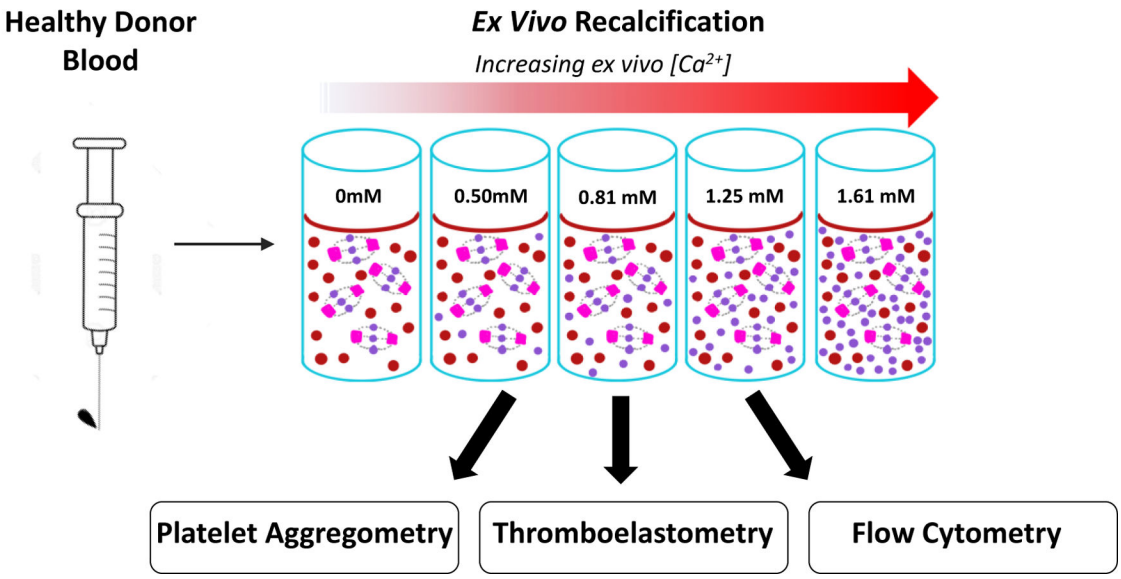

Figure 1: Methodology for Ex Vivo Recalcification of Citrated Blood and Assessment of Calcium Dependent Effects on Platelet Behavior.

Healthy donor blood was collected (n=4) in citrated tubes ([ionized calcium]=0mM due to chelation by citrate]), calcium was added to overcome citrate chelation and generate a range of calcium concentrations from 0 to supraphysiologic, and platelet aggregometry, rotational thromboelastometry, and flow cytometry were performed.

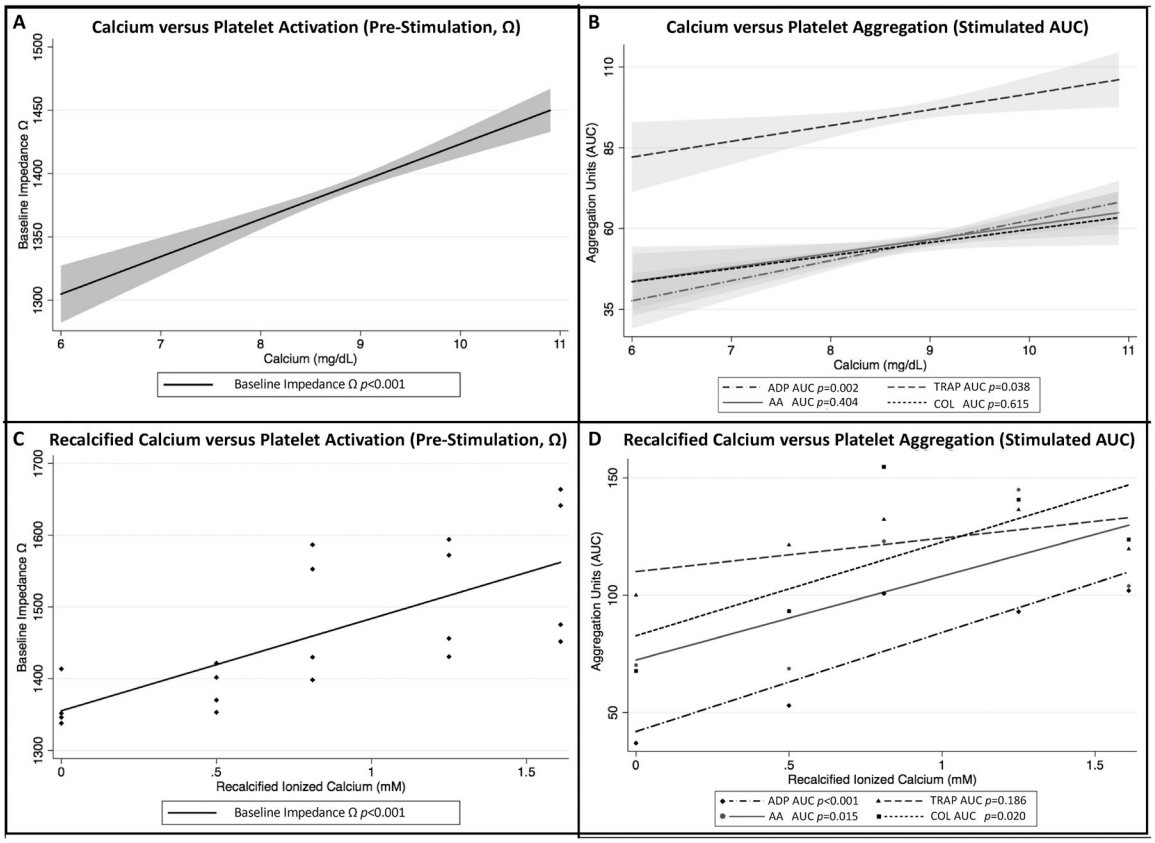

Figure 2: Effect of Calcium Concentrations on Platelet Aggregation.

Panels A and B) Linear regression between admission plasma calcium concentration with platelet activation (pre-stimulation impedance, Ω) (panel A) and platelet aggregation (stimulated Area Under the Curve-AUC) (panel B) in response to all agonists adenosine diphosphate (ADP), thrombin receptor activating peptide (TRAP), arachidonic acid (AA), and collagen (COL). All *p* values listed for multivariate regression controlling for age, gender, injury severity, base excess, platelet count, and pre-hospital crystalloid volume. Shaded regions represent 95% confidence intervals. Panels C and D) Linear regression of recalcified ionized calcium concentration with platelet activation (pre-stimulation impedance, Ω) (panel C) and platelet aggregation (stimulated AUC) (panel D). Recalcified *ex vivo* ionized calcium concentration tested in panels C and D: 0mM, 0.50mM, 0.81mM, 1.25mM, and 1.61mM.

TABLE 1.

Patient Demographics and Outcomes

Variable	
Age (years)	36 (26–50)
Male	86%
BMI (kg/m2)	27 +/− 5
Blunt mechanism	53%
Injury severity score	10 (1–24)
Pre-hospital crystalloid volume (mL)	0 (0–200)
Admit calcium (Total Plasma mg/dL)	8.8 +/−0.63
Admit temperature (C°)	36.5 +/− 0.7
Admit pH	7.33 +/− 0.11
Admit base excess (mmol/L)	2.8 +/− (5.7)
Admit INR ≥1.3	18%
Admit INR	1.1 (1.0–1.2)
Admit PTT (sec)	27.6 (25.5–30.3)
Admit platelets (× 10^3/μL)	263 (218–310)
Admit functional fibrinogen level (mg/dL)	425 +/− 98
Clot strength on admit (ROTEM EXTEM MCF, mm)	64 (60–67)
Overall low platelet response * (%)	42%
Transfused blood at 24 hours	28%
Transfused plasma at 24 hours	19%
Transfused platelets at 24 hours	9%
Total hospital days	5 (2–12)
Total ICU days (to 28 days)	2 (0–4)
Ventilator free days (to 28 days)	28 (24–28)
Mortality at 24 hours	2%
Mortality at discharge	10%

Patient demographics for the 538 patients. Data are mean +/− SD, median (inter-quartile range [IQR]), or percentage. Data for skewed variables reported as median with IQR. Ventilator free days are counted for the first 28 days of hospitalization. Patients who expired received 0 ventilator free days. ROTEM-rotational thromboelastometry, EXTEM- extrinsic pathway channel, MCF- maximum clot firmness.

* Patients categorized as having overall low platelet response if they had aggregation (AUC) values below the lower limit of the manufacturers reference range in response to stimulation to any of the four agonists (adenosine diphosphate, collagen, thrombin receptor activating peptide, or arachidonic acid).

TABLE 2.

Platelet Aggregometry and Rotational Thromboelastometry (ROTEM) Results by Calcium Quartiles on Arrival to the Emergency Department

Variable	Calcium Quartile 1 7.2–8.4mg/dL	Calcium Quartile 2 8.4–8.8mg/dL	Calcium Quartile 3 8.8–9.2mg/dL	Calcium Quartile 4 9.2–10.2mg/dL	P value
Platelet Aggregometry					
ADP AUC (U)	49	50	58	60	0.002*
COL AUC (U)	47	49	54	54	0.134
TRAP AUC (U)	93	92	99	101	0.180
AA AUC (U)	53	57	61	59	0.017
Pre-Stimulation Impedance (Ω)	1367	1381	1392	1415	<0.001*
ROTEM					
EXTEM					
CFT (sec)	90	85	86	84	0.024
Alpha (°)	72	73	73	74	0.020
A10 (mm)	54	56	57	57	<0.001*
A20 (mm)	60	62	63	63	<0.001*
MCF (mm)	61	64	64	65	<0.001*
ML (%)	13	13	14	13	0.721
FIBTEM					
CFT (sec)	207	306	264	658	0.982
Alpha (°)	70	72	71	72	0.263
A10 (mm)	13	14	14	14	0.066
A20 (mm)	14	16	16	15	0.163
MCF (mm)	15	16	16	15	0.348
ML (%)	7	5	6	9	0.085

Data are presented as median for skewed distributions and mean for normal distributions, categorized by calcium quartile. *p* values based on Kruskal Wallis tests for skewed distributions, or ANOVA tests (for normally distributed continuous variables) and chi squared tests (for normally distributed categorical variables). *p* values are marked with * to denote statistical significance (set at <0.0083 adjusting for multiple comparisons). Ω- impedance; AUC- area under the curve; U- aggregation units; ADP- adenosine diphosphate; COL- collagen; TRAP- thrombin receptor activating peptide; AA- arachidonic acid; EX- extrinsic channel, FIB- fibrinolysis channel. CFT- clot formation time (sec), alpha-alpha angle (degrees), A10 and A20- clot firmness after 10 and 20 minutes (mm), MCF-maximum clot firmness (mm), ML-maximum lysis (%). Sec-seconds; ° – degrees; mm- millimeters.

TABLE 3.

Multivariable Analysis of Calcium Concentrations with Rotational Thromboelastometry (ROTEM) and Transfusion

ROTEM*	**Coefficient**	**P-value**	**95% Confidence Interval**
EXTEM			
CFT (sec)	−6.86	0.005	−11.59– −2.13
Alpha (°)	0.95	0.018	0.17–1.73
A10 (mm)	1.78	<0.001	0.84–2.72
A20 (mm)	1.69	<0.001	0.84–2.54
MCF (mm)	1.53	<0.001	0.71–2.35
ML (%)	−1.85	0.131	−4.25–0.55
FIBTEM			
CFT (sec)	60.27	0.580	−168.5–289.0
Alpha (°)	0.66	0.444	−1.03–2.35
A10 (mm)	0.63	0.131	−0.19–1.45
A20 (mm)	0.74	0.096	−0.13–1.61
MCF (mm)	0.64	0.151	−0.24–1.52
ML (%)	−1.53	0.390	−5.05–1.98
Transfusions**			
Blood units in 24 hours	−0.55	0.005	−0.93– −0.16
Plasma units in 24 hours	−0.54	0.003	−0.91– −0.18
Platelet units in 24 hours	−0.78	0.001	−1.26– −0.31

* Multivariable regression analysis results of calcium concentration with viscoelastic testing results controlling for age, injury severity score, platelet count, base excess, gender, and pre-hospital crystalloid volume. EX=-xtrinsic channel, FIB- fibrinolysis channel. CFT- clot formation time (sec), alpha-alpha angle (degrees), A10 and A20- clot firmness after 10 and 20 minutes (mm), MCF-maximum clot firmness (mm). FFP- Fresh Frozen Plasma; RBC- Red Blood Cells

** Multivariable Poisson regression analysis results of admission calcium with 24 hour transfusion requirements controlling for age, injury severity score, base excess, and pre-hospital crystalloid volume and additionally controlling for hemoglobin for RBC transfusions regression, INR for FFP transfusions regression, and platelet count for platelet transfusions regression.

TABLE 4.

Effect of *Ex Vivo* Calcium Concentrations on Expression of Platelet Activation Surface Receptors by Flow Cytometry

Surface Receptor & Agonist Condition	Change in Relative Expression (%) per mM Calcium	P value	95% Confidence Interval
GP2b3a			
Untreated	16.2	0.187	−8.5–40.9
ADP	8.4	0.108	−2.0–18.8
TRAP	0.5	0.750	−2.4–3.3
Convulxin	−0.8	0.048	−1.6–0.0
Aggregate (all 4 conditions)	6.1	0.100	−1.3–13.4
P Selectin			
Untreated	12.6	0.122	−3.6–28.9
ADP	20.1	0.092	−3.5–43.7
TRAP	6.6	0.045	0.2–13.1
Convulxin	0.2	0.700	−0.9–1.3
Aggregate (all 4 conditions)	9.1	0.003	3.4–14.8

Linear regression results of recalcified ionized calcium concentration with the percent change in relative expression of platelet surface receptors of activation, Glycoprotein IIb/IIIa (GpIIb/IIIa) and P-Selectin in healthy donor blood. Percent expression was normalized across samples to account for baseline differences in expression across healthy volunteers. Agonists used: Untreated-none, ADP- adenosine diphosphate, TRAP-thrombin receptor activating peptide, and convulxin- collagen analogue. Aggregate- composite result for the average fold increase per 1mM increase in calcium concentration across all four agonist conditions. Recalcified *ex vivo* ionized calcium concentrations tested to generate results: 0mM, 0.50mM, 0.81mM, 1.25mM, and 1.61mM. N=4.