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Concentration-dependent effect of hypocalcaemia on in vitro clot strength in patients at risk of bleeding: a retrospective cohort study

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SUMMARY

Aim

It is uncertain whether hypocalcaemia is associated with an increased risk of bleeding. This study assessed the dose-related relationship between ionised calcium concentrations and in vitro clot strength measured by maximum amplitude (MA) on the thromboelastograph (TEG).

Methods

A total of 610 patients who were at risk of bleeding or had active bleeding between 2010 and 2014 were considered in this retrospective cohort study. A scatter plot with Pearson correlation coefficient (r) and multiple linear regression was used to assess the dose-related relationship between ionised calcium concentrations and MA on the TEG.

Results

The mean ionised calcium of the patients was 1·10 mmol L⁻¹ (interquartile range: 1·04–1·17) and 235 (38·5%) of them had hypocalcaemia (<1·1 mmol L⁻¹). Hypocalcaemia was more common in patients with significant coexisting coagulopathy. Ionised calcium concentrations (r = 0·285, 95% confidence
interval (CI) 0.211–0.356, \( P = 0.001 \), as well as fibrinogen concentrations, platelet counts, international normalised ratio (INR) and activated Partial Thromboplastin Time (aPTT), had a significant linear correlation with the MA on the TEG. Ionised calcium concentrations and its interaction term with platelet count were both significantly associated with the MA on the TEG (slope of the regression line 1.1 per 0.1 mmol L\(^{-1}\) increment, 95%CI 0.3 to 1.9, \( P = 0.011 \)), after adjusting for fibrinogen concentrations, platelet counts, INR and aPTT.

**Conclusions**

Ionised calcium concentrations had a concentration-dependent association with *in vitro* clot strength after adjusting for other coagulation abnormalities in patients with coexisting coagulopathy. Maintaining a normal ionised calcium concentration, >1 mmol L\(^{-1}\), during critical bleeding is recommended.

**Keywords:** calcium; clotting; coagulopathy; critical bleeding; dose-response relationship; haemorrhage

Calcium has a fundamental role as a cofactor in enzymatic reactions, transmembrane ion flux, muscle contraction, neuronal activity, coagulation cascade, platelet aggregation, regulation of vasomotor tone and cardiac contractility (Kauvar *et al.*, 2006; Lier *et al.*, 2008; Sihler & Napolitano, 2010). Hypocalcaemia may occur in patients with substantial amount of bleeding requiring transfusion of blood products (Dzik & Kirkley, 1988; British Committee for Standards in Haematology *et al.*, 2006). Citrate toxicity has been suggested as the mechanism of hypocalcaemia during massive transfusion (Hayes *et al.*, 1980; Dzik & Kirkley, 1988), however, recent evidence suggests that intravenous colloid solutions and ischaemia reperfusion can also induce hypocalcaemia and hypocalcaemia-induced coagulopathy (Vivien *et al.*, 2005).
Although an *in vitro* study demonstrated that ionised calcium concentrations >0·56 mmol L$^{-1}$ would be adequate for clot formation in blood donated from healthy volunteers (James & Roche, 2004), clinical studies indicate that even mild hypocalcaemia may contribute to a higher risk of bleeding requiring multiple transfusions and mortality in patients with critical bleeding (Lier et al., 2008; Ho & Leonard, 2011; Magnotti et al., 2011). This discrepancy may due to the fact that the effect of mild hypocalcaemia on bleeding may only be apparent in the presence of coexisting coagulopathy.

The commonly used standard tests of haemostasis, such as International Normalised Ratio (INR), activated Partial Thromboplastin Time (aPTT), have had their limitations for many reasons, most being time based and failing to give relevant information about the nature and stability of the platelet-fibrin clot which may be affected by ionised calcium concentrations.

We hypothesised that hypocalcaemia may have a concentration-dependent effect on *in vitro* clot strength in patients with coexisting coagulopathy and conducted a retrospective cohort study to assess whether hypocalcaemia is associated with reduced *in vitro* clot strength after adjusting for coexisting coagulopathy.

**MATERIALS AND METHODS**

After obtaining Royal Perth Hospital Ethics Committee approval, we retrieved the data of patients who had concurrent thromboelastograph (TEG) and ionised calcium concentration measured between January 2010 and March 2014. The TEG test was not a routine coagulation blood test in this study centre and was performed only at the time when patients were considered either at risk of bleeding or actively bleeding. Patients were considered at risk of bleeding if they required transfusion and with abnormal coagulation parameters (INR > 1·5, aPTT > 40 s, fibrinogen <2 g L$^{-1}$ or platelet count <150 \times 10^9 L$^{-1}$). In this study, the patients' platelet count and coagulation blood test results closest to the time when TEG was performed were retrieved from a centralised laboratory database. Patients were excluded if ionised calcium concentrations were not performed within 4 h prior to the TEG testing. All ionised calcium concentrations were measured by an arterial blood gas analyser (ABL800 FLEX,
Radiometer, Copenhagen, Denmark), and ionised calcium concentrations below 1·1 mmol L$^{-1}$ were considered as abnormal and required replacements according to the study centres' massive transfusion protocol. All patients included in this study were critically ill patients who were admitted to the Intensive Care Unit (ICU) of Royal Perth Hospital. This multidisciplinary ICU admitted over 1500 critically ill medical and surgical patients per year, including patients with multiple trauma, burns and cardiothoracic surgery. Because of the retrospective observational nature of this study, informed consent was waived by the ethics committee.

In this study, *in vitro* whole blood (without citrate) clot strength was assessed using the maximal amplitude (MA) (normal range: 54–72 mm) and angle (normal range: 47–74°) of the TEG (Medtel Thromboelastograph, Haemostasis Analyser, Medtel, Lane Cove, New South Wales, Australia). The TEG provides a comprehensive evaluation of the overall coagulation function of the whole blood sample *in vitro* and MA on the TEG reflects the strength of the clot during the clotting process (Gibbs, 2011). According to the recommendation of the manufacturer of the TEG machine, a MA < 54 mm or an angle <47° was considered as having a reduced *in vitro* clot strength. Two channels of TEG, one using kaolin alone as activating agent and the other using kaolin with heparinase, were conducted for all study patients. All whole blood samples were tested within 4 min of sampling at the patient's body temperature. Because some study patients might have received unfractionated heparin subcutaneously for venous thromboembolism prophylaxis, TEG data on the MA of the TEG tracing from the heparinase channel were used in this study for consistency purpose.

**Statistical analysis**

If we assumed coagulation and calcium concentration results were available in 500 patients and 100 of them had hypocalcaemia (<1·1 mmol L$^{-1}$), and a reduced *in vitro* clot strength was also observed in 100 patients, this sample size would have >80% power to detect a relative risk difference of >2·0 in risk of a reduced *in vitro* clot strength between patients with and without hypocalcaemia, if the incidence of hypocalcaemia among those without reduced *in vitro* clot strength was 25%.
The quantitative relationship between ionised calcium concentrations and MA on the TEG was first analysed by a scatter plot, using Pearson correlation coefficient ($r$) to assess the degree of linear relationship between these two variables. The relationship between ionised calcium concentrations and MA was then further analysed after adjusting for platelet counts, INR, aPTT and fibrinogen concentrations in a multiple linear regression. During the modelling process, no variables were removed in the multiple linear regression analysis. Because age itself was unlikely to be related to the *in vitro* clot strength, this factor was not analysed in the multivariate linear regression. Transfusion requirements and blood pH were not recorded in this dataset, and hence, they were also not analysed in the multivariate analysis.

As a sensitivity analysis, trauma diagnosis and four interaction terms between ionised calcium concentrations and platelet count, INR, aPTT or fibrinogen concentration were also entered to the full multivariate model to assess whether they would increase the model's predictive ability in terms of adjusted coefficient of determination ($R^2$). Insignificant interaction terms ($P > 0.05$) were then removed by a backward stepwise procedure to improve the precision of the final results of this sensitivity analysis. A $P$-value $<0.05$ was taken as significant in this study, and all analyses were two-tailed and conducted by spss for Windows (version 22.0, 2014, IBM, Chicago, IL, USA).

**RESULTS**

Of the 780 patients who were tested with a TEG during the study period, 610 patients had a concomitant ionised calcium concentration measured. The mean ionised calcium of the patients was 1.10 mmol L$^{-1}$ (interquartile range: 1.04–1.17) (Fig. 1), and 235 (38.5%) of them had hypocalcaemia (<1.1 mmol L$^{-1}$). The distribution of the MA among those with hypocalcaemia is described in Fig. 2. The patients with hypocalcaemia were more likely to have cardiac surgery and were associated with thrombocytopenia, hypofibrinogenaemia, a prolonged INR or aPTT, and a smaller MA on the TEG compared with patients without hypocalcaemia (Table 1). The proportion of patients with hypocalcaemia was significantly higher among those with reduced *in vitro* clot strength.
compared with those without reduced in vitro clot strength (50·4 vs 35·2%, \(P = 0·002\)). Likewise, moderate hypocalcaemia (<0·9 mmol L\(^{-1}\)) (67 vs 19%, \(P = 0·001\)) and severe hypocalcaemia (<0·6 mmol L\(^{-1}\)) (100 vs 22%, \(P = 0·001\)) were both associated with an increased risk of having reduced in vitro clot strength.

As a continuous predictor, ionised calcium concentrations had a significant correlation with MA on the TEG \([r = 0·285, 95\% \text{ confidence interval (CI)} 0·211–0·356, P = 0·001]\) (Fig. 3), with ionised calcium <1·0 mmol L\(^{-1}\) appeared to be the likely cut-point for MA to be affected by ionised calcium concentrations. Platelet counts \((r = 0·495)\) and fibrinogen concentrations \((r = 0·501)\) were also positively associated with the MA on the TEG, whereas INR \((r = −0·221)\) and aPTT \((r = −0·282)\) were inversely associated with the MA on the TEG. Ionised calcium concentrations remained significantly associated with the MA on the TEG, after adjusting for platelet counts, fibrinogen concentrations, INR and aPTT (Table 2). The slope of the regression was 1·1 per 0·1 mmol L\(^{-1}\) increment in ionised calcium increment and this suggested that for every 0·1 mmol L\(^{-1}\) increment in ionised calcium, there was a 1·1 mm increment in MA on the TEG (95% CI: 0·3–1·9) while holding platelet count, aPTT, fibrinogen and INR constant. INR was, however, no longer significantly associated with the MA on the TEG after adjusting for other covariates.

The full linear regression model explained about 33·3% of the variability in MA \((R^2 = 0·333, \text{ adjusted } R^2 = 0·314)\), and the predicted values of MA from the multivariate linear regression were associated with the observed values of MA on the TEG in a relatively linear fashion (Fig. 4). Among all the predictors assessed, fibrinogen concentrations and platelet counts were more important than ionised calcium concentrations, INR and aPTT in explaining the variability of the final multivariate model.

In the sensitivity analysis, the only significant interaction term was between ionised calcium concentration and platelet count (with a negative regression coefficient), suggesting that changing ionised calcium concentrations would be associated with a larger change in in vitro clot strength in the presence of thrombocytopenia. The adjusted \(R^2\) of the model was further improved by incorporating the interaction term between ionised calcium concentrations and platelet count (0·345 vs 0·326).
Trauma diagnosis was, however, not significantly associated with an increased risk of reduced *in vitro* clot strength (*P* = 0.448), after adjusting for ionised calcium concentration and other coagulation parameters.

**DISCUSSION**

This study showed that hypocalcaemia was common in patients with abnormal coagulation parameters, and ionised calcium concentrations had a concentration-dependent association with *in vitro* clot strength measured by a TEG in patients who were at risk of bleeding or had active bleeding, particularly when there was coexisting thrombocytopenia. These results are clinically relevant and require careful consideration.

First, our results confirmed that hypocalcaemia was common in patients who had critical bleeding and coexisting coagulopathy (Lier *et al.*, 2008; Ho & Leonard, 2011). Hypocalcaemia can be induced by transfusion of blood products such as fresh frozen plasma as well as intravenous fluid (Vivien *et al.*, 2005; Ho & Leonard, 2011; Magnotti *et al.*, 2011), and these therapies can also induce haemodilution and coagulopathy at the same time in patients with critical bleeding. As such, it is possible that any adverse associations between hypocalcaemia and increased transfusion requirements or mortality of patients who have critical bleeding may be purely related to confounding. A previous healthy volunteer study inducing hypocalcaemia in normal blood samples *in vitro* showed that hypocalcaemia did not appear to have any apparent effect on the MA of the TEG tracing provided the ionised calcium concentrations were above 0.56 mmol L⁻¹ (James & Roche, 2004). This study was, however, limited by the use of blood samples without coexisting coagulopathy and, hence, whether mild hypocalcaemia may contribute to an increased risk of bleeding in patients with coexisting hypocalcaemia and abnormal coagulation parameters remains uncertain. Our results have extended the implication of this mechanistic study and showed that even mild hypocalcaemia may have some effect on the strength of the clot formation, as measured by the TEG, in patients with coexisting coagulopathy. Recent observational studies suggested that hypocalcaemia may be associated with a
higher risk of intracerebral haemorrhage after thrombolytic therapy (Guo et al., 2015) and a larger haematoma volume in haemorrhagic stroke compared with those without hypocalcaemia (Inoue et al., 2013). Given previous studies also showed that calcium is an essential cofactor for vitamin K-dependent coagulation factors (factors II, VII, IX, X) and platelet aggregation, and citrate is a very effective regional anticoagulant (Brass et al., 2005; Schilder et al., 2014), our results certainly support the current critical bleeding and transfusion guidelines in recommending that ionised calcium concentrations should be maintained at a normal range in patients with critical bleeding (British Committee for Standards in Haematology et al., 2006; Spahn et al., 2013).

Second, our results confirmed the increasing recognition of the importance of platelets and fibrinogen in the pathogenesis of critical bleeding, haemostasis and thrombosis (Duff et al., 2012; Ho et al., 2012; Hagemo et al., 2014). Our multivariate analysis results showed that fibrinogen concentrations and platelet counts certainly had a larger effect on in vitro clot strength than hypocalcaemia. As such, normalising hypofibrinogenaemia and thrombocytopenia should take a higher priority than correcting hypocalcaemia in the management of critical bleeding. These results were different from our previous work which showed that hypocalcaemia was more important than hypofibrinogenaemia or thrombocytopenia in critical bleeding (Ho & Leonard, 2011). This difference could, at least in part, be explained by the use of fresh frozen plasma in patients with more severe bleeding and as fresh frozen plasma can also cause hypocalcaemia, this could confound the strength of association between mortality and hypocalcaemia. Nevertheless, the sensitivity analysis of this study suggested that correcting hypocalcaemia may have a larger effect on improving in vitro clot strength in the presence of thrombocytopenia. It is thus prudent to correct both hypocalcaemia and thrombocytopenia simultaneously for patients who are actively bleeding. Third, there are also other point-of-care whole blood coagulation tests, including ROTEM or the new TEG6s, which can be used to assist management of critical bleeding. Because most institutions use citrated blood samples for a ROTEM test (or the new TEG6s), any potential adverse effect of hypocalcaemia on in vitro clot strength, as demonstrated in this study, would not be apparent if the analysis is conducted on citrated blood samples. Ionised calcium concentrations should be measured and hypocalcaemia should be
excluded when citrated blood samples are used for point-of-care whole blood clotting testing to guide transfusion management in critical bleeding.

Finally, we would like to acknowledge the limitations of this study. Although this study lends mechanistic support to the significance of hypocalcaemia in critical bleeding, it is by no means definitive. Firstly, some of our patients did not have the ionised calcium concentration and TEG measured simultaneously which could have affected the strength as well as accuracy of the association between MA on the TEG and ionised calcium concentration. Secondly, because MA was primarily used to guide transfusion management, we also did not have data on the fibrinolysis in our patients and hence whether fibrinolysis could modify the association between calcium concentrations and MA on the TEG remains uncertain. Thirdly, whether maintaining a normal calcium concentration in critical bleeding can reduce mortality and other patient-centred outcomes compared with allowing mild hypocalcaemia not corrected remains scientifically unproven. An adequately powered randomised controlled trial targeting at different ionised calcium concentrations (>0.6, >0.9 or >1.0 mmol L\(^{-1}\)) during critical bleeding is needed to confirm the best way to manage hypocalcaemia in patients with critical bleeding. Fourthly, TEG does not assess platelet adhesion function or effect of hypothermia on bleeding tendency (Gibbs, 2011). As such, whether hypocalcaemia would have substantial concentration-dependent interactions with hypothermia and anti-platelet agents remains uncertain. Finally, the size of this study limited the number of variables including higher order interaction terms (e.g. INR × platelet count × ionised calcium concentrations or aPTT × platelet count × ionised calcium concentrations) we could analyse in the multivariate analysis.

In summary, hypocalcaemia was common in patients with coagulopathy and at risk of active bleeding. Ionised calcium concentrations had a relatively weak but significant concentration-dependent effect on in vitro clot strength with and without adjusting for INR, aPTT, platelet count and fibrinogen concentration. A change in ionised calcium concentration was associated with a larger change in in vitro clot strength in the presence of thrombocytopenia. Our results support the current critical bleeding and transfusion guidelines in recommending that ionised calcium concentrations should be maintained at a normal range, >1 mmol L\(^{-1}\), in patients with critical bleeding.
ACKNOWLEDGMENTS

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Both K. M. H. and C. B. Y had made substantial contributions to conception and design, or acquisition of data or analysis and interpretation of data; had been involved in drafting the manuscript or revising it critically for important intellectual content; had given final approval of the version to be published; and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

The authors have no financial and non-financial interest in relation to the subject matter or equipment described in this manuscript.

REFERENCES


Figure 1. Histogram showing the distribution of ionised calcium concentrations of the patients ($n = 610$).

Mean = 1.10 mmol/L, standard deviation = 0.15, interquartile range: 1.04-1.17
Figure 2. Histogram showing the distribution of MA on the thromboelastograph for patients with hypocalcaemia (<1·1 mmol L\(^{-1}\)). The dotted vertical line indicates the lower limit of normal for MA.

Mean MA = 59 mm (standard deviation = 13)
Figure 3. Scatter plot showing the relationship between ionised calcium concentrations and MA on the thromboelastograph. The horizontal-dotted line defines the lower limit of normal MA (54 mm) and the vertical-dotted line defines the ionised calcium = 1·0 mmol L$^{-1}$ which appears to be the likely cut-point for MA to reduce with a reduction in ionised calcium concentrations. The solid line is the loess line for fitting at least 50% of the data points by non-parametric regression.
Figure 4. The relationship between the observed and predicted values of MA on the thromboelastograph by the multilinear regression model.
Table 1. Characteristics of the patients with and without hypocalcaemia (≥1·1 mmol L⁻¹)

<table>
<thead>
<tr>
<th></th>
<th>With hypocalcaemia (n = 235)</th>
<th>Without hypocalcaemia (n = 375)</th>
<th>P-value¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (IQR)</td>
<td>58 (49–68)</td>
<td>55 (42–70)</td>
<td>0·209</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>151 (64)</td>
<td>242 (65)</td>
<td>0·999</td>
</tr>
<tr>
<td>Diagnosis, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trauma</td>
<td>22 (9·4)</td>
<td>76 (20·3)</td>
<td></td>
</tr>
<tr>
<td>Cardiac surgery</td>
<td>69 (29·4)</td>
<td>70 (18·7)</td>
<td></td>
</tr>
<tr>
<td>Non-cardiac surgery</td>
<td>23 (9·8)</td>
<td>31 (8·3)</td>
<td></td>
</tr>
<tr>
<td>Sepsis</td>
<td>44 (18·7)</td>
<td>52 (13·9)</td>
<td></td>
</tr>
<tr>
<td>Non-infective medical causes</td>
<td>77 (32·7)</td>
<td>146 (38·8)</td>
<td></td>
</tr>
<tr>
<td>Platelet count (×10⁹ L⁻¹) (IQR)</td>
<td>162 (88–195)</td>
<td>238 (109–268)</td>
<td>0·001</td>
</tr>
<tr>
<td>Fibrinogen concentration, g L⁻¹ (IQR)</td>
<td>3·3 (2·0–4·1)</td>
<td>4·4 (2·5–6·1)</td>
<td>0·009</td>
</tr>
<tr>
<td>INR (IQR)</td>
<td>1·6 (1·3–1·9)</td>
<td>1·4 (1·2–1·6)</td>
<td>0·001</td>
</tr>
<tr>
<td>aPTT, s (IQR)</td>
<td>49 (35–50)</td>
<td>42 (33–46)</td>
<td>0·001</td>
</tr>
<tr>
<td>MA on TEG, mm (IQR)</td>
<td>59 (53–67)</td>
<td>64 (58–72)</td>
<td>0·001</td>
</tr>
<tr>
<td>Abnormal MA, &lt;54mm, n (%)</td>
<td>67 (29)</td>
<td>66 (18)</td>
<td>0·002</td>
</tr>
<tr>
<td>Angle on TEG, degrees (IQR)</td>
<td>60 (53–72)</td>
<td>62 (56–72)</td>
<td>0·215</td>
</tr>
<tr>
<td>Abnormal angle, &lt;47⁰, n (%)</td>
<td>49 (21)</td>
<td>60 (16)</td>
<td>0·158</td>
</tr>
<tr>
<td>CRP, mg L⁻¹ (IQR)</td>
<td>145 (29–260)</td>
<td>147 (59–200)</td>
<td>0·401</td>
</tr>
</tbody>
</table>

CRP, C-reactive protein; IQR, interquartile range.

¹P-value generated by either χ² or Mann–Whitney test.

²CRP was available only in 235 patients.
Table 2. Association between ionised calcium concentrations and MA on the thromboelastograph after adjusting for other haematological parameters

<table>
<thead>
<tr>
<th>Variables</th>
<th>Slope of the variable (95% confidence interval)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionised calcium concentration (per 0·1 mmol L(^{-1}) increment)</td>
<td>1·1 (0·3 to 1·9)</td>
<td>0·011</td>
</tr>
<tr>
<td>Platelet count (per 10 \times 10^9 L(^{-1}) increment)</td>
<td>0·2 (0·13 to 0·24)</td>
<td>0·001</td>
</tr>
<tr>
<td>Fibrinogen concentration (per g L(^{-1}) increment)</td>
<td>1·7 (1·2 to 2·3)</td>
<td>0·001</td>
</tr>
<tr>
<td>aPTT (per second increment)</td>
<td>−0·1 (−0·05 to −0·14)</td>
<td>0·001</td>
</tr>
<tr>
<td>INR (per unit increment)</td>
<td>0·7 (−1·3 to 2·7)</td>
<td>0·486</td>
</tr>
</tbody>
</table>

Colinearity was not significant between the variables \((r < 0·5)\) and the \(R^2\) of the model was 0·333. The regression standard error of the regression model was 10·2, suggesting that we can expect the model to be accurate in predicting the MA within approximately ±20·4 mm at a 95% confidence level. The maximum Cook's distance was 0·08 confirming that there were no outliers or excessive leverage in the data points used to generate this model. Residual plot suggested that the zero mean, constant variance and independence assumptions for ionised calcium concentrations were met and the QQ plot suggested that normality assumption for the regression analysis was met. The adjusted \(R^2\) of a reduced model by removing one predictor was substantially lower after removing fibrinogen \((R^2 = 0·254)\) or platelet count \((R^2 = 0·272)\), than ionised calcium \((R^2 = 0·318)\), INR \((R^2 = 0·327)\) and aPTT \((R^2 = 0·341)\).