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Zinc promotes clot stability by accelerating clot formation and modifying fibrin structure

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Summary

Zinc released from activated platelets binds fibrin(ogen) and attenuates fibrinolysis. Although zinc also affects clot formation, the mechanism and consequences are poorly understood. To address these gaps, the effect of zinc on clot formation and structure was examined in the absence or presence of factor (F) XIII. Zinc accelerated a) plasma clotting by 1.4-fold, b) fibrinogen clotting by 3.5– and 2.3-fold in the absence or presence of FXIII, respectively, c) fragment X clotting by 1.3-fold, and d) polymerisation of fibrin monomers generated with thrombin or batroxobin by 2.5– and 1.8-fold, respectively. Whereas absorbance increased up to 3.3-fold when fibrinogen was clotted in the presence of zinc, absorbance of fragment X clots was unaffected by zinc, consistent with reports that zinc binds to the αC-domain of fibrin(ogen). Scanning electron microscopic analysis revealed a two-fold increase in fibre diameter in the presence of zinc and in permeability studies, zinc increased clot porosity by 30-fold with or without FXIII. Whereas FXIII increased clot stiffness from 128 ± 19 Pa to 415 ± 27 Pa in rheological analyses, zinc reduced clot stiffness by 10– and 8.5-fold in the absence and presence of FXIII, respectively. Clots formed in the presence of zinc were more stable and resisted rupture with or without FXIII. Therefore, zinc accelerates clotting and reduces fibrin clot stiffness in a FXIII-independent manner, suggesting that zinc may work in concert with FXIII to modulate clot strength and stability.

Keywords

Zinc, fibrinogen, fibrin, FXIII, clot structure

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Introduction

Fibrin (Fn), the main scaffold of blood clots, forms when thrombin cleaves fibrinopeptides (FP) A and B from the Aα and Bβ chains of fibrinogen (Fg) to expose knobs on the respective chains. The resultant Fn monomers polymerise when the knobs bind to pre-existing complementary holes of other Fn monomers to form two-stranded protofibrils. The protofibrils assemble laterally into long fibres that comprise the Fn clot (1). Thrombin also converts FXIII to activated FXIII (FXIIIa), which covalently cross-links the protofibrils, thereby increasing clot stiffness by two- to five-fold to stabilise the clot by compacting the fibres (2).

Clot stability, the ability to resist mechanical stress, is a critical property of Fn that is determined in part by the nature of the fibres that form upon polymerisation (3). Clot stiffness is represented by elastic and inelastic properties of Fn, and its development, which occurs much later than gelation, may reflect fibre re-arrangement (4). Stiffer clots containing thinner fibres are less stable and more susceptible to rupture and embolisation than softer clots, which can undergo reversible deformation (3). In contrast, clots composed of thick fibres are more readily degraded by the fibrinolytic system (5). Therefore, the physical properties of Fn clots are critical determinants of their role in haemostasis.

Fn fibres formed in plasma are larger than those formed in purified systems (6), consistent with the concept that the structural and physical properties of a clot are a consequence of the conditions under which the clots form (1, 3). In particular, the thrombin concentration and ionic conditions influence clot structure (7). Calcium (Ca^{2+}) facilitates Fn monomer polymerisation, thereby increasing fibre thickness (7–9), whereas chlorine opposes lateral

aggregation and promotes thin fibre formation (10). These observations suggest that the ionic milieu influences Fn monomer polymerisation. Zinc (Zn^{2+}) is another cation that influences clot formation (8, 11–15). Zn^{2+} circulates in plasma at a concentration of ~ 10–20 μM; however, ~ 80 % is weakly bound to albumin, leaving ~ 0.1–2 μM in a free, unbound state (16–19). However, Zn^{2+} is abundant at sites of vascular injury because Zn^{2+} stores in platelets are released upon platelet activation, and platelets accumulate in thrombi in numbers 50– to 100-fold higher than those found in the circulating blood (17, 20). Therefore, the Zn^{2+} concentrations in clots are likely to be higher than those in circulating plasma. These high levels enable Zn^{2+} to modulate the contact activation pathway at sites of vascular injury (21).

Previous studies have demonstrated that Zn^{2+} binds Fg and Fn with a K_d of 9–13 μM, and impairs proteolysis by plasmin and trypsin (17, 22). Furthermore, a Zn^{2+} binding site has been identified on the αC-domain of fibrin(ogen) (22). Although Fn fibres formed in the presence of Zn^{2+} have been shown to be thicker than those formed in its absence (14), mechanistic information about how Zn^{2+} affects clot structure, the mechanical properties of Fn, and clot stability is lacking. To address these gaps in knowledge, we quantified the effect of Zn^{2+} on a) Fg clot times in plasma and purified systems, b) Fn fibre diameter, and c) clot porosity, stiffness, and stability. Fragment X, a high-molecular-weight plasmin degradation product of Fg that lacks the αC-domain, was used in place of Fg to examine the contribution of the αC-domain.

Materials and methods

Reagents

Human α-thrombin and Fg containing FXIII (FIB 1; herein termed Fg^{XIII}) were purchased from Enzyme Research Laboratories (South Bend, IN, USA). A portion of the Fg^{XIII} was depleted of FXIII (herein termed Fg) by affinity chromatography using an immobilised antibody against FXIII (23). Both Fg and Fg^{XIII} were subjected to precipitation with 19 % ammonium sulfate to ensure the highest integrity of the α-chains (24). SDS-PAGE analysis of thrombin-treated Fg or Fg^{XIII} under reducing conditions revealed absence and presence of cross-linked γ-γ dimers and α-polymers, respectively (data not shown). Plasmin was purchased from Haematologic Technologies Inc. (Essex Junction, VT, USA). Aprotinin was purchased from Cedarlane (Burlington, ON, Canada). Fragment X was prepared by digesting 4 ml of a solution containing 60 μM Fg with 100 nM plasmin for 15 minutes (min) at 37°C. Plasmin activity was inhibited by adding 500 KIU/ml aprotinin, and the digested mixture was then subjected to high-performance liquid chromatography to isolate fragment X as described (25). Thrombin was dialyzed against 20 mM Tris, pH 7.4, 150 mM NaCl (TBS) to remove citrate. Batroxobin, a thrombin-like enzyme from the venom of *B. atrox moojeni* that only releases FPA, was purchased from Pentapharm (Basel, Switzerland). Experiments were performed in a Zn^{2+} buffering system containing 10 mM Tricine, 150 mM NaCl, pH 7.4, and 0.01 % Tween 20 (TcBS). The desired free Zn^{2+} concentration in TcBS was obtained by adding ~100-fold higher concentration of $ZnCl_2$, as described (17).

Clot formation in plasma

Blood collected from 7 healthy volunteers into 5-ml tubes each containing 7.2 mg of EDTA was subjected to centrifugation at 2100 × g for 10 min at 23°C. The resultant platelet-poor plasma was harvested, pooled, and dialysed versus TBS at 4°C to remove Zn^{2+} and EDTA. Plasma Zn^{2+} concentrations were determined by atomic absorption spectrometry as described (17). For clotting experiments, 50 μl of dialysed plasma pre-warmed to 37°C was placed into wells of a 96-well plate, and 50 μl of a solution containing 0.5 nM thrombin, 15 mM $CaCl_2$, and 0–10 μM free $ZnCl_2$ in TcBS was added. Absorbance was monitored at 350 nm at 3 second (s) intervals up to 25 min at 37°C using a Spectromax plate reader (Molecular Devices, Sunnyvale, CA, USA). The clot time, defined as the time to achieve half maximal absorbance, was obtained using instrument software.

Clot time in purified systems

Thrombin (0.5, 2, 5, or 10 nM) and 2 mM $CaCl_2$ were separately placed into wells of a 96-well plate, and clotting was initiated by adding aliquots of 3 μM Fg in TcBS containing 0–5 μM free $ZnCl_2$. In a separate experiment, 0.5 nM thrombin and 2 mM $CaCl_2$ were separately placed into wells of a 96-well plate, and clotting was initiated by adding 3 μM Fg or fragment X in TcBS with 0–5 μM free $ZnCl_2$. Absorbance was monitored at 405 nm at 2 s intervals up to 33 min at 37°C using a plate reader, and clotting time was determined as described above.

Preparation and polymerisation of Fn monomers

After placing 3 ml of a 76.3 μM Fg solution in 12,000–14,000 molecular weight cut off dialysis tubing (VWR International), thrombin or batroxobin was added to 5 nM or 5 units/ml, respectively. The tubing was placed in a beaker containing 1 l of TBS and dialysed for 3 hours (h) at 37°C. The buffer was changed to 20 mM acetic acid and dialysis was continued overnight at 4°C with one buffer change. The contents of the tubing were then removed and subjected to centrifugation at 5000 × g for 4 min at 23 °C, after which the supernatant was collected and stored in aliquots at –80 °C. After adding 10-μl aliquots of soluble Fn (final concentration 2 μM) to wells of a 96-well plate, polymerisation was initiated by addition of 190 μl of TcBS containing 2 mM $CaCl_2$ and 0–6 μM free $ZnCl_2$ and absorbance was monitored at 405 nm at 2 s intervals up to 33 min at 37°C using a plate reader. Clot times were determined as described above.

Scanning electron microscopy (SEM)

Clots were formed in the absence or presence of Zn^{2+} and/or FXIII on 0.025 μm circular filter paper membranes (EMD Millipore) placed in wells of a 24-well plate. Thus, using a pipette, 50-μl ali-

quots of a solution containing 3 μM Fg or Fg^{XIII}, 10 nM thrombin, and 2 mM $CaCl_2$ in the absence or presence of 5 μM free $ZnCl_2$ in TcBS were placed on each membrane. Three clots were formed under each of the four conditions. The wells were sealed, and the plate was incubated at 100% humidity for 30 min at 37°C. Membranes were fixed overnight in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, rinsed twice with 0.1 M phosphate buffer and stained for 1 h with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Samples were dehydrated using a series of graded ethanol washes (50%, 70%, 70%, 95%, 95%, 100%, 100% and 100%), and dried in a critical point dryer. The membranes were mounted onto SEM stubs, sputter-coated with gold, and viewed at 30,000× magnification using a Tescan Vega II LSU scanning electron microscope (Tescan, PA, USA) operating at 10 kV. Images were acquired with the VEGA/TESCAN software. Each clot was imaged at five different sites, and the fibre diameter in each image was quantified using Photoshop version 6 Extended (Adobe Systems, San Jose, CA, USA). Contrast and brightness of the images were adjusted and changed from grey scale to black and white. An 11 × 11 inch grid was placed over each image to divide it into multiple cells and the thickness of fibres in each cell of alternate rows was measured manually in Photoshop using the ruler tool set to 2 μm. The numbers of fibres quantified per image ranged from 72–207. After determining the fibre diameters in the five images, the mean fibre diameter for each clot was calculated.

Clot porosity

Samples containing 0.5 ml of 9 μM Fg or Fg^{XIII}, 2 mM $CaCl_2$ and 0 or 5 μM free $ZnCl_2$ were clotted in 3-ml syringes with 5 nM thrombin and incubated for 1 h at 23°C. Syringes were mounted vertically and 2.5 ml TcBS was then placed on top of each clot. The flow through was collected into tubes positioned below the syringes for 6 and 16 min with and without Zn^{2+}, respectively. Darcy's constant K_s, a measure of clot porosity was then calculated using the following equation (26).

$$Ks = \frac{Q \cdot L \cdot \eta}{t \cdot A \cdot \delta P}$$

where Q is the volume of TcBS collected (ml), L is the clot length (1.6 cm), η is the viscosity of flow through (10^{-2} poise), t is the time of collection (s), A is the cross-sectional area of the syringe (0.232 cm^2), and δP is the atmospheric pressure (1,012,950 dyne/cm^2).

Clot strength

The viscoelastic properties of clots were determined using a Discovery Hybrid Rheometer-3 and TRIOS software (TA Instruments, New Castle, DE, USA). Clots were formed by addition of 5 nM thrombin to a solution containing 9 μM Fg or Fg^{XIII} and 0–5 μM free $ZnCl_2$ in TcBS. Samples were gently mixed with a pipette for 3 s before adding 1230-μl aliquots between the Peltier plate and the nickel-coated cone (4° cone angle and 40 mm diameter). Samples were surrounded with mineral oil to prevent dehydration and three sequential studies were then performed. Time sweep experiments were conducted under continuous oscillations at 1 rad/s with an imposed shear strain of 0.5% to monitor network polymerisation. After the storage modulus (G) reached a plateau, a frequency sweep routine was applied under a shear strain of 0.5% with the angular frequency increasing from 0.1–10 rad/s. For both time and frequency sweep experiments, the storage modulus (G), loss modulus (G) and loss tangent (δ) were also calculated. The final peak hold experiment measured the stress-strain relationship by imposing steadily increasing strain on the Fn network.

Statistical analysis

Data are presented as mean ± standard error (SE) unless otherwise indicated. Significance of differences was determined using the Mann-Whitney rank sum test or one-way analysis of variance followed by Tukey's test for multiple pair-wise comparisons (SigmaPlot version 11.0). A nested three-way (condition, clot, image) analysis of variance was performed to determine the dominant sources of variation in Fn fibre diameter. In particular, this analysis addressed the issue of whether there was additional variation in fibre diameter from image to image within a clot, and from clot to clot, relative to the inherent between fibre variation within an image. As a result of this analysis, subsequent comparisons of mean fibre diameter among the four conditions (i.e. with or without Zn^{2+} and/or FXIII) were based on the inter-clot variation in mean fibre diameter. In all cases, p-values less than 0.05 were considered statistically significant.

Results

Effect of Zn^{2+} on clot times in plasma

Zn^{2+}-depleted plasma was clotted with thrombin in the presence of $CaCl_2$ and concentrations of free Zn^{2+} up to 10 μM. In the presence of 5 and 10 μM free Zn^{2+}, clot times were significantly ($p<0.01$ and $p<0.001$) shortened by 1.2– and 1.4–fold, respectively (►Figure 1) Therefore, Zn^{2+} accelerates thrombin-mediated clotting in plasma.

Effect of Zn^{2+} on Fn clot formation

Fg was clotted with 0.5–10 nM thrombin in the presence of 0–5 μM free Zn^{2+}. Zn^{2+} at 5 μM produced an approximate two-fold, concentration-dependent and statistically significant ($p<0.001$) shortening of the clot time (►Figure 2A); an effect similar to that observed on the plasma clot time. The Zn^{2+}-dependence was independent of thrombin concentration because the EC_{50} values for Zn^{2+} ranged from 1.5–1.9 μM ($p=0.8$) with thrombin concentrations ranging from 0.5–10 nM. At 5 μM Zn^{2+}, there was a significant ($p<0.001$) concentration-dependent reduction in relative clot time as the thrombin concentration increased from 0.5 nM to 10 nM; similar to the reduction of clot time with increasing thrombin concentration in the absence of Zn^{2+}. These results confirm previous reports that Zn^{2+} accelerates Fg clotting (8, 11–15).

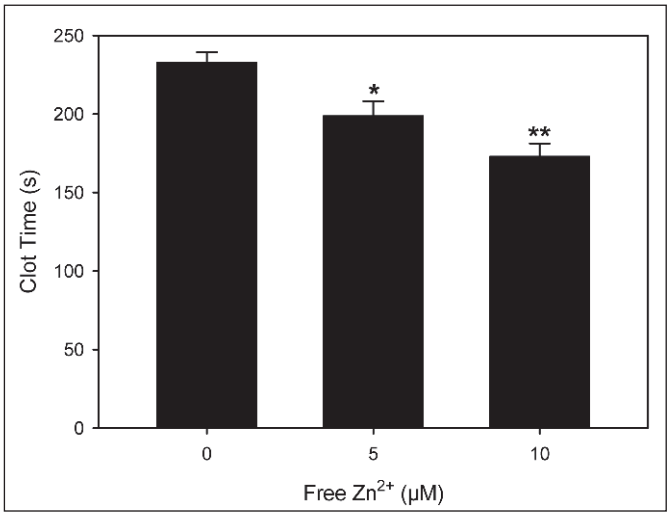

Figure 1: Effect of Zn^{2+} on clotting. Aliquots of dialysed plasma were clotted with 0.5 nM thrombin in the presence of 15 mM $CaCl_2$ and 0–10 μM free Zn^{2+}. Absorbance was monitored at 350 nm and clot times were determined. Bars represent means of three determinations each performed in duplicate, while the lines above the bars indicate SE. Based on parametric one-way analysis of variance, Zn^{2+} significantly ($p<0.001$) accelerates clot time in a concentration-dependent fashion, and Tukey's test for multiple pair-wise comparisons indicates that clot times at 5 μM (*$p<0.01$) and 10 μM Zn^{2+} (**$p<0.001$) are significantly shorter than that with no Zn^{2+}.

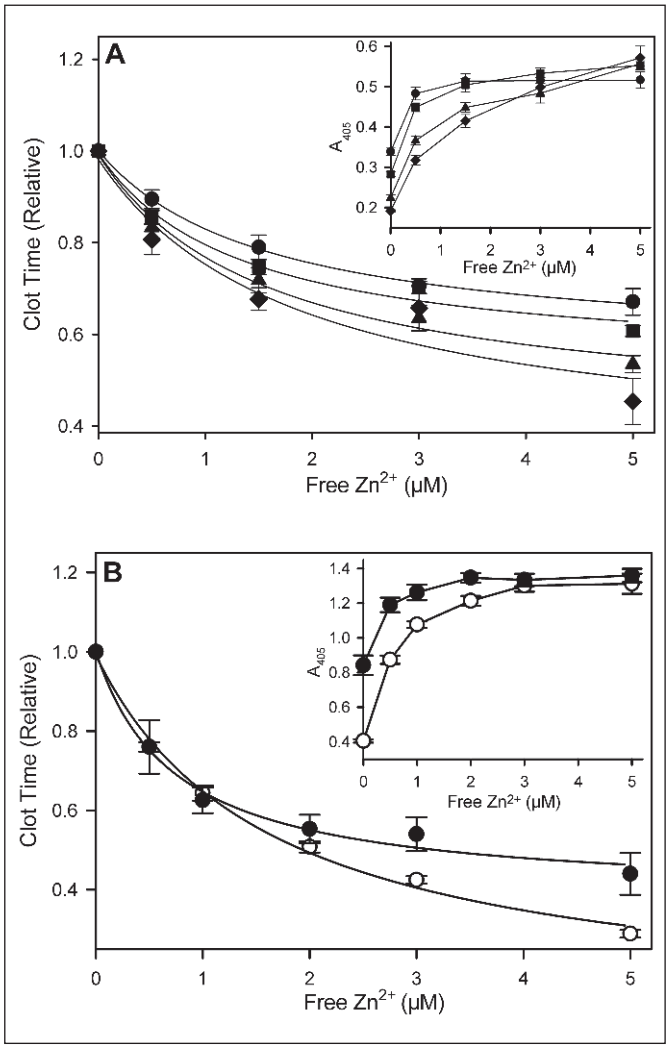

Figure 2: Effect of Zn^{2+} and FXIII on clot formation. A) Samples containing 3 μM Fg, 2 mM $CaCl_2$, and 0–5 μM free Zn^{2+} were clotted with 0.5 (circles), 2 (squares), 5 (triangles), or 10 (diamonds) nM thrombin. Clot times were determined by monitoring absorbance at 405 nm and values measured in the presence of Zn^{2+} were normalised relative to that measured in its absence. Clot times in the absence of Zn^{2+} were 441.9 ± 25, 122.1 ± 8, 47.3 ± 1.7, and 21.3 ± 1.1 s with 0.5, 2, 5, 10 nM thrombin respectively. Symbols represent the mean of three experiments each done in duplicate, while the bars reflect the SE. The data were fitted using nonlinear regression analysis of a rectangular hyperbola (line). Final absorbance values were determined from the experiments in (A) and mean values were plotted along with SE (inset). Arbitrary lines were used to connect the points. B) Samples containing 0–5 μM free Zn^{2+}, 2 mM $CaCl_2$, and 9 μM Fg with (closed) or without (open) FXIII, were clotted with 5 nM thrombin and clot times were determined as outlined above. Initial clot times in the absence of Zn^{2+} were 185.2 ± 16.1 and 144.5 ± 18 s without and with FXIII, respectively. Symbols (circles) represent the mean of three determinations each done in duplicate, while the bars reflect SE. The data were fitted by nonlinear regression analysis of a rectangular hyperbola (line). Final absorbance values from the experiments in (B) and mean values were plotted (inset).

In the presence of Zn^{2+}, absorbance increased in a concentration-dependent manner. The increase ranged from 1.7- to three-fold with thrombin concentrations of 0.5 and 10 nM, respectively (►Figure 2A, inset); a difference that was statistically significant ($p<0.001$). As expected, in the absence of Zn^{2+}, absorbance decreased with increasing concentrations of thrombin ($p<0.001$), confirming that thrombin concentration affects clot formation (7). By contrast, in the presence of 5 μM Zn^{2+}, the thrombin dose-response was negated because the changes in absorbance values were small and not statistically significant ($p=0.3$). Therefore, Zn^{2+} appears to negate the effect of thrombin concentration on absorbance. Together, these data suggest that Zn^{2+} is an independent modulator of clot structure.

Next, we examined the effect of Zn^{2+} in the presence of FXIII because cross-linking is an important determinant of Fn stability. The effect of Zn^{2+} was maintained in the presence of FXIII because clot times of Fg and Fg^{XIII} were significantly ($p<0.001$) accelerated by 3.5– and 2.3-fold, respectively (►Figure 2B), and absorbance was significantly ($p<0.001$) increased by 3.3- and 1.7-fold, respectively (►Figure 2B). Although Zn^{2+} accelerated Fg^{XIII} clotting to a lesser extent than Fg clotting, the EC_{50} values remained between 1 and 2 μM. Therefore, Zn^{2+} accelerates clotting in the absence and presence of FXIII.

In order to bypass the enzymatic steps involving thrombin, we examined the effect of Zn^{2+} on polymerisation of preformed Fn monomers prepared with thrombin or batroxobin. In a concentration-dependent fashion, Zn^{2+} significantly ($p<0.001$) accelerated polymerisation of Fn monomers prepared with thrombin or

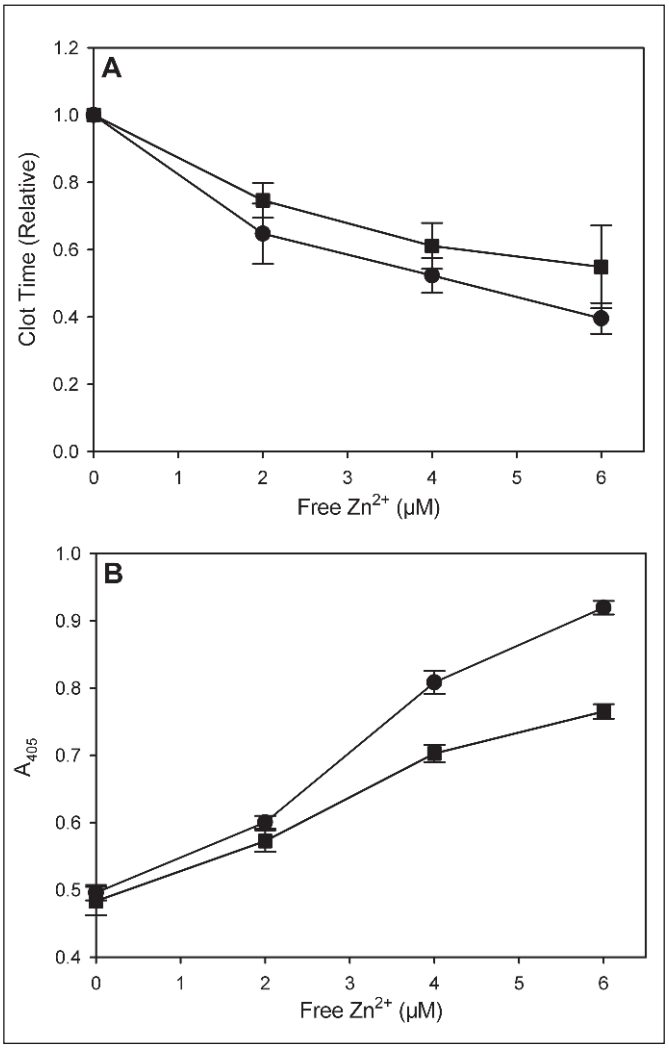

Figure 3: Effect of Zn^{2+} on polymerisation of fibrin monomers. A) Samples containing 2 mM $CaCl_2$, 0–6 μM free Zn^{2+}, were mixed with 2 μM fibrin monomers generated with thrombin (circles) or batroxobin (squares). Clot times were determined by monitoring absorbance at 405 nm, and values measured in the presence of Zn^{2+} were normalised relative to that determined in its absence. Initial clot times in the absence of Zn^{2+} were 26.1 ± 4.2 and 27.3 ± 2.9 s for Fn monomers generated with thrombin or batroxobin, respectively. Symbols represent the mean of four determinations, while the bars reflect SE. Arbitrary lines were used to connect the points. B) Final absorbance values were determined from the experiments in (A) and mean values and SE are plotted.

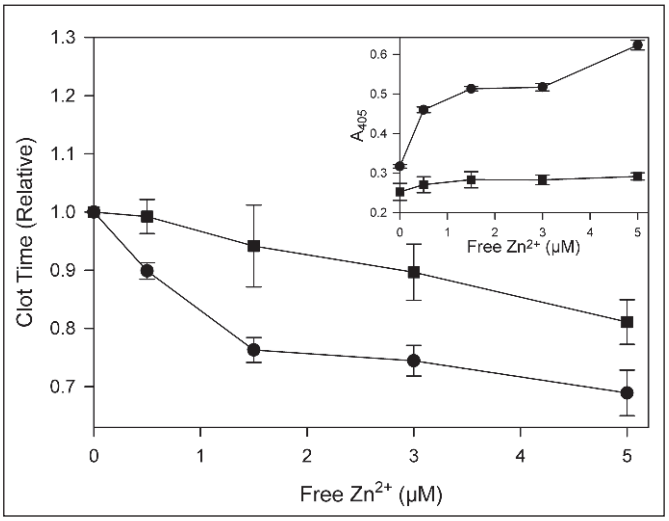

Figure 4: Comparison of the effect of Zn^{2+} on clotting of fibrinogen and fragment X. Samples containing 3 μM Fg (circles) or fragment X (squares) and 2 mM $CaCl_2$ were clotted with 0.5 nM thrombin in the presence of 0–5 μM free Zn^{2+}. Clot times were determined by monitoring absorbance at 405 nm, and values measured in the presence of Zn^{2+} were normalised relative to that determined in its absence. Initial clot times in the absence of Zn^{2+} were 476.2 ± 24.9 and 422.7 ± 67.9 s for Fg and fragment X, respectively. Final absorbance values were determined as above and plotted (inset). Symbols represent the mean of three determinations each performed in duplicate, while the bars reflect SE. Arbitrary lines were used to connect the points.

batroxobin by 2.5- and 1.8-fold, respectively (►Figure 3A). Furthermore, in the presence of Zn^{2+}, clot absorbance significantly ($p<0.001$) increased by approximately two- and 1.6-fold with thrombin- and batroxobin-generated monomers, respectively (►Figure 3B), indicating that Zn^{2+} modulates Fn formation regardless of whether FPB is removed. Therefore, Zn^{2+} appears to influence Fn polymerisation rather than fibrinopeptide release.

To determine the role of the αC-domain in the Zn^{2+} effect on clot formation, fragment X was used in place of Fg (27). At 5 μM, Zn^{2+} significantly accelerated the clotting of Fg and fragment X by 1.5- ($p<0.001$), and 1.3-fold ($p=0.04$), respectively (►Figure 4), suggesting that the effect of Zn^{2+} on clot time is attenuated with fragment X. Although Zn^{2+} increased the absorbance of Fg clots by two-fold ($p<0.001$), the absorbance of fragment X clots was increased by only 1.2-fold ($p=0.6$) (►Figure 4, inset). Therefore, modulation of clotting and clot structure by Zn^{2+} appears to depend, at least in part, on the presence of the αC-domain of Fg.

Effect of Zn^{2+} on clot structure

The effect of Zn^{2+} on three-dimensional clot structure was examined using SEM. Clots prepared by clotting Fg or Fg^{XIII} with thrombin in the absence or presence of 5 μM free Zn^{2+} were imaged at 30,000× magnification (►Figure 5). Nested three-way analysis of variance indicated significant additional variation in Fn fibre diameter between images taken from the same clot ($p<0.0001$) and in fibre diameters between clots ($p<0.0001$), suggesting that the most appropriate statistical approach would be to use "clot" as the unit of analysis by firstly averaging all Fn fibre diameter measurements for a clot (giving three data points per condition). Comparisons between conditions were then based on the average of the three clot means per group and the pooled inter-clot SD of mean diameter. There was no significant interaction between the effects of Zn^{2+} and those of FXIII ($p=0.18$) so that the results in all of the clots formed with Zn^{2+} or FXIII could be compared with those in all of the clots formed without Zn^{2+} or FXIII.

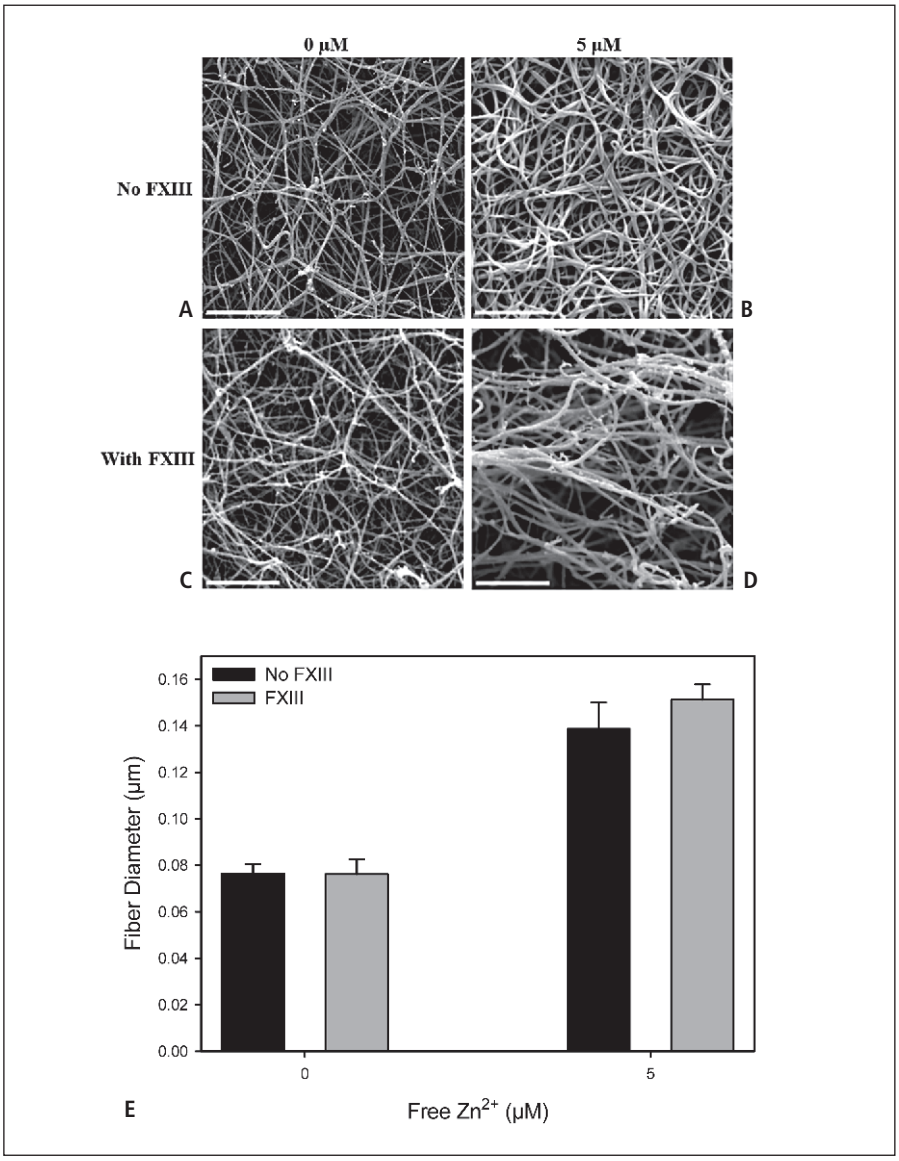

Figure 5: SEM of Fn clots formed in the absence or presence of Zn^{2+} and FXIII. Aliquots of 3 µM Fg containing (A, B) or lacking (C, D) FXIII were clotted with 10 nM thrombin in the presence of 2 mM $CaCl_2$ and in the absence (A, C) or presence (B, D) of 5 µM free Zn^{2+}. Clots were incubated in 100 % humidity for 30 min at 37°C prior to SEM analysis at a magnification of 30,000x. In all images the bar represents 2 µm. E) Fibre diameters were quantified from the micrographs. The bars represent the mean values for three clots, imaged five times per clot, and lines above the bars reflect the inter-clot SD. The effect of Zn^{2+} on Fn fibre diameter was significant ($p < 0.001$), whereas neither the effect of FXIII nor the interaction of Zn^{2+} and FXIII was significant (p=0.20 and p=0.18, respectively).

Zn^{2+} had a strong effect on Fn fibre diameter ($p < 0.001$), increasing it by two-fold (mean increase 0.069 µm, 95 % confidence interval [CI] 0.057 to 0.080). In contrast, FXIII had a non-significant effect on fibre diameter (p=0.20, mean increase 0.006 µm, 95 % CI -0.056 to 0.043); results that diverge from those of a previous study, which reported thinner fibres in the presence of FXIII (28). Therefore, based on our findings, the Zn^{2+}-dependent increase in Fn fibre diameter is independent of FXIII. Because Zn^{2+} increases fibre diameter and changes overall clot structure, studies were done to examine the effect of Zn^{2+} on the porosity of Fn clots.

Effect of Zn^{2+} on clot porosity

Clots formed with Fg yielded $K_s \pm$ SD values of $5.5 \pm 1.5 \times 10^{-12}$ and $1.9 \pm 0.5 \times 10^{-11}$ cm^2 in the absence or presence of Zn^{2+}, respectively, whereas those formed with Fg^{XIII} had $K_s \pm$ SD values of $5.1 \pm 1.1 \times 10^{-12}$ and $2.4 \pm 0.2 \times 10^{-11}$ cm^2, in the absence or presence of Zn^{2+}, respectively. Thus Zn^{2+} evoked a ~30-fold increase in clot porosity (p=0.03), and there was no significant effect of FXIII on porosity (p=0.7).

Effect of Zn^{2+} on clot rheology

Clots were formed by incubating 9 µM Fg or Fg^{XIII} with thrombin in the absence or presence of Zn^{2+} between the Peltier plate and cone geometry of the rheometer for viscoelasticity analysis. G , G , and δ were continuously monitored in the time sweep phase to assess overall stiffness and rigidity as the clots formed. The storage modulus reached a plateau of 127.6 ± 18.6 Pa at about 2,000 s in the absence of FXIII and Zn^{2+} (►Figure 6A, ►Table 1). In the presence of FXIII, the storage modulus increased more slowly to 414.8 ± 26.5 Pa; a value 3.3-fold higher ($p < 0.001$) than that deter-

Table 1: The influence of Zn^{2+} and FXIII on the mechanical properties of clots. Clots were formed by incubating 9 μM Fg or FgXIII with 5 nM thrombin, 2 mM $CaCl_2$, and 0–5 μM free Zn^{2+}. The mean storage and loss modulus, loss tangent ratio and SD were determined at 3605 s from three separate experiments. Statistical significance was determined as $p<0.05$ (*) or $p<0.001$ (**).

Free Zn^{2+} (μM)	No FXIII			With FXIII		
	G′ (Pa)	G″ (Pa)	δ (Pa)	G′ (Pa)	G″ (Pa)	δ (Pa)
0	127.6 ± 18.6	6.4 ± 0.7	0.05 ± 0.003	414.8 ± 26.5	12.2 ± 2.4	0.03 ± 0.006
0.5	90.6 ± 12.5*	7 ± 0.8	0.08 ± 0.005**	207.9 ± 5.2**	15.2 ± 3.4	0.07 ± 0.02*
1	63.8 ± 13.4**	5.7 ± 1.2	0.09 ± 0.007**	151 ± 2.3**	15.4 ± 1	0.1 ± 0.007**
2	39.7 ± 6.4**	3.9 ± 0.7*	0.1 ± 0.004**	104.4 ± 2.4**	11 ± 1.5	0.1 ± 0.01**
3	23.9 ± 5.6**	2.3 ± 0.4**	0.1 ± 0.006**	81.8 ± 1.4**	8.9 ± 1	0.1 ± 0.01**
5	12.8 ± 0.8**	1.3 ± 0.1**	0.1 ± 0.004**	48.5 ± 2.6**	4.7 ± 0.2*	0.1 ± 0.01**

mined without FXIII (►Figure 6B, ►Table 1). Thus, as shown previously, FXIII enhances the elastic properties of Fn, rendering the clots more rigid (2).

In the presence of increasing concentrations of Zn^{2+}, the time during which the storage modulus increased was accelerated, corresponding to faster clotting as observed by absorbance. However, the final storage modulus showed a progressive decrease with increasing Zn^{2+} concentrations. This was observed in both the absence and presence of FXIII, resulting in a significant ($p<0.001$) 10– and 8.5-fold reduction in the storage modulus for Fg and Fg^{XIII} clots, respectively, at 5 μM free Zn^{2+} (►Figure 6C, ►Table 1). The amount of energy dissipated from the clot during deformation, known as the loss modulus, decreased significantly ($p<0.001$) in a concentration-dependent manner, up to 6.5- and 2.6-fold with Fg and Fg^{XIII} clots, respectively, at 5 μM Zn^{2+} (►Figure 6D, ►Table 1). The storage and loss moduli values were used to calculate the loss tangent, which is the ratio of energy dissipated to energy stored in a cyclic deformation. Zn^{2+} increased δ significantly ($p<0.001$) by two- and 3.5-fold with Fg and Fg^{XIII} clots, respectively (►Table 1). Because the overall effects of Zn^{2+} on the mech-

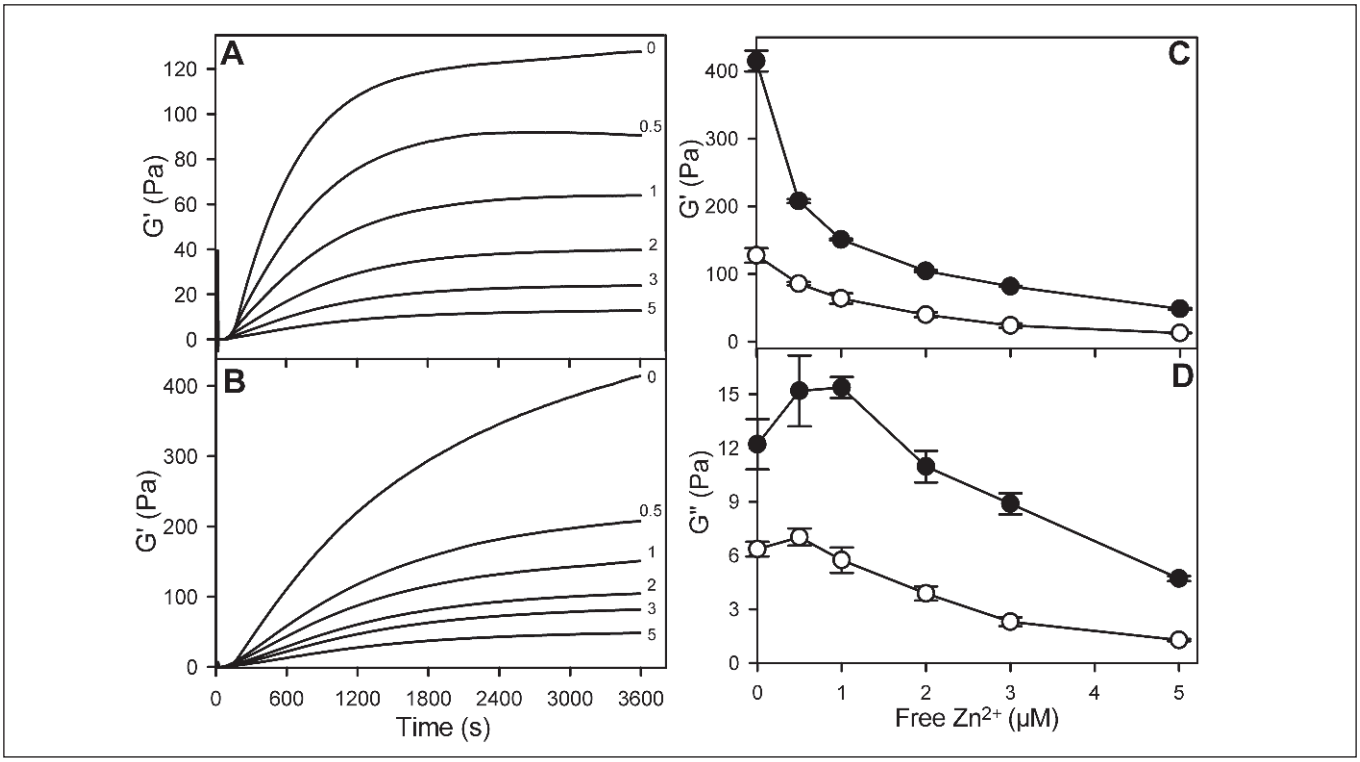

Figure 6: Effect of Zn^{2+} on the rheological behaviour of clots formed in the absence or presence of FXIII. Clots were formed in a rheometer by incubating 9 μM Fg with 5 nM thrombin, 2 mM $CaCl_2$, and 0–5 μM free Zn^{2+} in the absence (A) or presence of FXIII (B). G′ and G″ values at 1 h were averaged from the three sets of data and plotted (circles) without (open) and with (closed) FXIII.

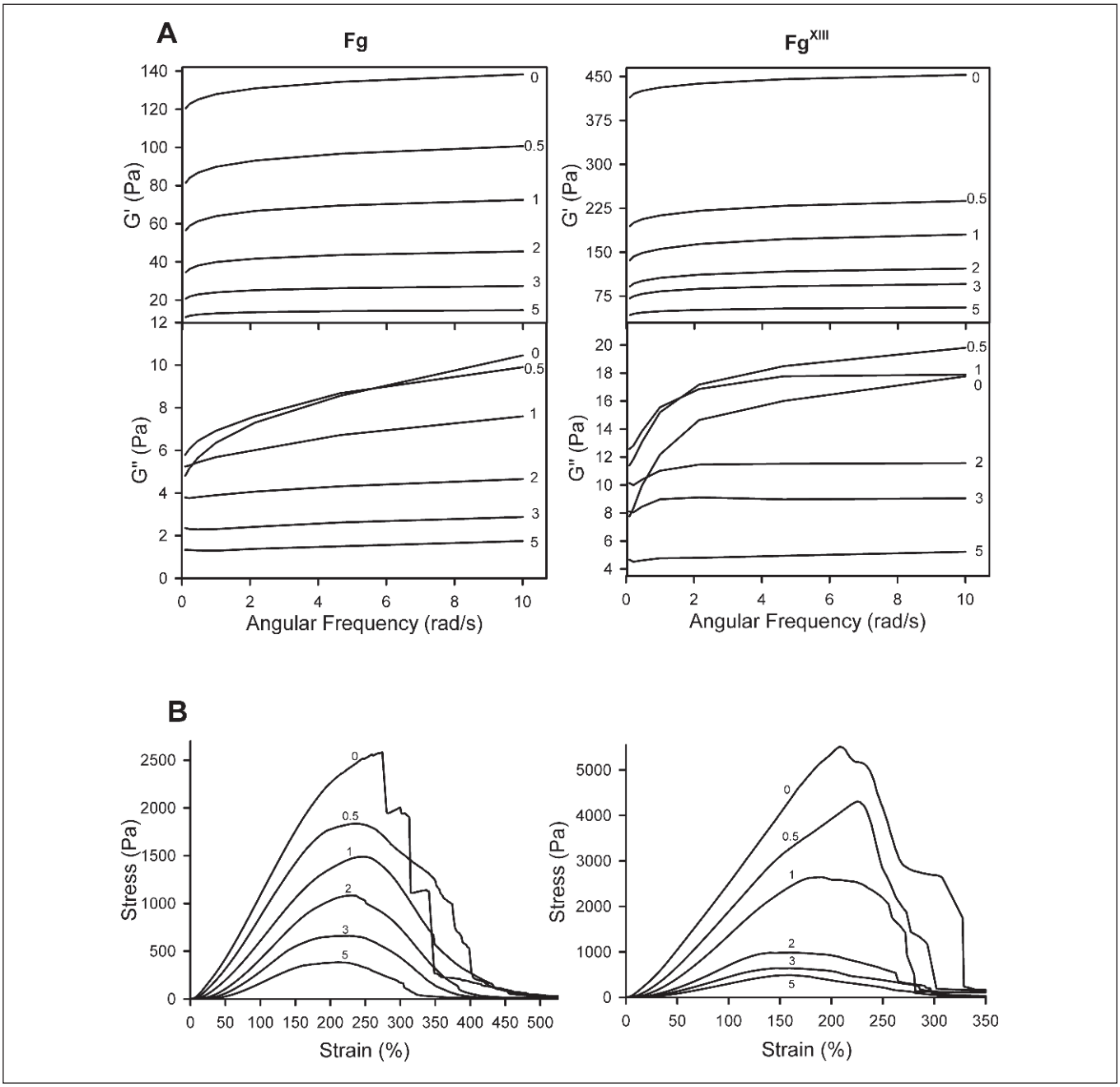

Figure 7: Effect of Zn^{2+} and FXIII on clot stability and rupture. A) Clots from Figure 6 were perturbed with increasing forces to determine changes in G′ or G″. B) After perturbation, the clots were compacted by increasing strain until the fibres broke causing the clots to rupture. Each plot represents the means of the three sets of data.

anical properties of clots were similar in the absence and presence of FXIII, these data confirm the results of the absorbance studies and suggest that the capacity of Zn^{2+} to modulate clot structure is independent of FXIII.

Once the clots were formed, a sweep of frequency oscillations of up to 10 rad/s was applied to measure clot stability (4). Despite differing storage modulus values, no changes in the storage modulus with respect to frequency were observed with clots prepared in the absence or presence of Zn^{2+} or FXIII (►Figure 7A, ►Table 2). This finding suggests that clots generated in the presence of Zn^{2+} remain stable upon perturbation despite the differences in clot structure.

By shearing Fn clots, the strain or deformation of the elastic network can be quantified. Thus, as torque is applied, the fibres become compacted as they are pulled apart, as demonstrated in the ascending phase of ►Figure 7B. As the network is stretched further, individual fibres begin to break until the clot is no longer able to support any additional stress (peak phase) and it then col-

Table 2: The effect of Zn^{2+} on clot stiffness under perturbation. Clots were formed under the same conditions as in Table 1. After formation, clots were perturbed with an initial frequency of 0.1 rad/s, and increased to maximal perturbation at 10 rad/s. The storage modulus and SD were determined for three individual experiments with varying Zn^{2+} concentrations.

Free Zn^{2+} (μM)	No FXIII		With FXIII	
	G′ (Pa; 0.1 rad/s)	G′ (Pa; 10 rad/s)	G′ (Pa; 0.1 rad/s)	G′ (Pa; 10 rad/s)
0	120.5 ± 19.1	138.3 ± 21.9	414.3 ± 31.1	452.8 ± 35.9
0.5	81.4 ± 12.3	100.6 ± 14.6	194.8 ± 8.3	237.7 ± 3
1	56.5 ± 12.1	72.5 ± 15	136.3 ± 3.3	180.3 ± 2
2	34.5 ± 5.4	45.5 ± 7.4	91.8 ± 1.3	122.2 ± 4.1
3	20.8 ± 5	27.4 ± 6.2	71.4 ± 2	95.7 ± 2.5
5	11.1 ± 0.6	14.7 ± 0.6	42.8 ± 3	55.9 ± 2.6

lapses (descending phase). In the ascending phase, more strain was required to reach a given level of stress in clots formed in the presence of Zn^{2+} compared with those formed in its absence, consistent with thicker fibre formation (►Figure 7B). This is in agreement with the SEM and absorbance data, which indicate that Zn^{2+} increases fibre diameter because larger fibres of the same density and structure will require more force to strain than smaller ones. However, peak stress values decreased with increasing concentrations of Zn^{2+} (►Figure 7B). In the descending phase, clots deformed in the presence of Zn^{2+}, but did not fully rupture, as evidenced by broadening of the descending phase. This suggests that Zn^{2+} renders clots more fluid-like, and less susceptible to rupture. The same Zn^{2+} phenomenon was observed in the presence of FXIII (►Figure 7B), even though the initial stiffness of fibres formed in the presence of FXIII was greater than that of fibres formed in its absence.

Discussion

Previous studies suggest that Zn^{2+} and other divalent ions accelerate clot formation by enhancing Fn assembly, resulting in increased fibre thickness (8, 11–15). However, no mechanism for this effect has been elucidated. In the current study, we confirm that Zn^{2+} accelerates Fn clot formation and increases fibre diameter both in the absence and presence of FXIII. Clots formed in the presence of Zn^{2+} are less stiff than those formed in its absence, are resistant to perturbation by mechanical forces and are more porous. Therefore, although FXIII promotes clot stiffness by compacting the Fn fibres, Zn^{2+} counters the effect of FXIII, such that cross-linked fibres remain in a more fluid-like state, possibly rendering clots more tolerant of deformation and less susceptible to rupture.

Cross-linking by FXIII is a critical determinant of the viscoelastic properties of Fn, and renders clots mechanically stronger by compacting the fibres (29). This concept is supported by the bleeding diathesis and impaired wound healing exhibited by patients with congenital FXIII deficiency (30). Cross-linking of the α- and γ-chains of Fn increases clot stiffness and provides resistance to fibrinolysis (28, 31). However, stiff clots are more prone to rupture and embolisation. Therefore, a balance between clot stiffness and elasticity is critical. Consequently, substances such as Zn^{2+} that modify the effect of cross-linking will impact on this balance.

Clot structure can be altered in several ways, including by changing the thrombin, Fg, and ion concentrations and by altering flow dynamics (7). Therefore, local conditions can influence the size of the Fn fibres that are formed, and will impact on whether the fibres are densely or loosely distributed. Dense Fn clots composed of thin fibres are more rigid and brittle compared with porous soft clots containing loosely packed, thick fibres. Thus, with the same applied stress, stiff clots with a higher storage modulus will undergo less deformation and rupture more than soft clots (3). These mechanical properties are particularly important for clots that form in the high shear environment of the arterial system. Zn^{2+} will be abundant in platelet-rich arterial thrombi because of its release from activated platelets (17).

We show that Zn^{2+} serves as a natural clot stabilizer because it offsets the rigidity induced by FXIII-mediated cross-linking by delaying the onset of strain stiffening of the clots. This is an important advantage because the balance between the elastic deformation and rupture of Fn networks determines in part whether clots remain intact and occlude major vessels or undergo fragmentation and subsequent embolisation (4).

Zn^{2+} participates in numerous haemostatic mechanisms, yet it is unclear how it modifies Fn structure. Because it affects clotting in the absence of thrombin, Zn^{2+} must exert its effect on Fn. Zn^{2+} binds to Fg at numerous sites; one in particular is in the αC-domain (22). Thus, histidine residues 544 and 545 constitute a Zn^{2+}-dependent heparin-binding site that attenuates heparin-dependent thrombin inhibition by antithrombin (22). By examining

What is known about this topic?

- Zn^{2+} is released from activated platelets, and is abundant at sites of vascular injury.
- Zn^{2+} binds fibrinogen with high affinity.
- Zn^{2+} accelerates coagulation yet delays fibrinolysis.

What does this paper add?

- Zn^{2+} increases fibrin fiber thickness at least in part by binding to the αC-domain of fibrinogen.
- Zn^{2+} increases pore size and porosity of fibrin clots.
- Zn^{2+} offsets the increase in clot stiffness induced by factor XIII thereby rendering cross-linked clots more elastic.

Abbreviations

δ, loss tangent; F, factor; Fg, fibrinogen; FgXIII, Fg with FXIII; Fn, fibrin; FPA, fibrinopeptide A; FPB, fibrinopeptide B; G′, storage modulus; G″, loss modulus; K_d, binding affinity; K_s, Darcy's constant; SD, standard deviation; SE, standard error; SEM, scanning electron microscopy; TBS, Tris-buffered saline; TcBS, Tricine-buffered saline.

clot formation with fragment X, we show that this Zn^{2+}-binding site is a likely candidate for altering fibre thickness. The Zn^{2+} effect on clot absorbance is abrogated with removal of the αC-domain, which is known to enhance lateral aggregation of protofibrils, thereby generating thick fibres with large pores (27). Consistent with this concept, histidine residues on Fg have been implicated in the regulation of Fn monomer polymerisation (12). Although Fg binds Ca^{2+} and numerous other divalent cations, the Zn^{2+} binding site in the αC-domain may be particularly important because Zn^{2+} affects clot formation more than other positively charged ions (8, 11). Therefore, by binding to Fg, Zn^{2+} may have important structural and functional roles in Fn formation and stabilisation.

The current study demonstrates that Zn^{2+} modifies clot structure, and softens the stiffening that results from FXIII-mediated cross-linking. This modification in structure and pore size may facilitate the passage of plasma components into the interstices of the clot, thereby promoting clot maturation and subsequent fibrinolysis. Any enhancement of fibrinolysis via this mechanism will be offset, at least in part, by the fact that, independent of clot structure, Zn^{2+} slows fibrinolysis by attenuating plasminogen activation and fibrin degradation by plasmin (17). Therefore, Zn^{2+} has competing effects on clot formation and degradation via distinct mechanisms. These results provide additional evidence that Zn^{2+} serves as a dynamic modulator of numerous reactions in haemostasis, and contributes to the localized, rather than systemic, response to perturbation. They also suggest that ionic conditions should be considered when evaluating the effects of FXIIIa on clot properties.

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Conflicts of interest
None declared.

References

1. Weisel JW, Litvinov RI. Mechanisms of fibrin polymerisation and clinical implications. Blood 2013; 121: 1712–1719.
2. Kurniawan NA, Grimbergen J, Koopman J, et al. Factor XIII stiffens fibrin clots by causing fibre compaction. J Thromb Haemost 2014; 12: 1687–1696.
3. Weisel JW. Structure of fibrin: impact on clot stability. J Thromb Haemost 2007; 5 (Suppl 1): 116–124.
4. Weisel JW. The mechanical properties of fibrin for basic scientists and clinicians. Biophys Chem 2004; 112: 267–276.
5. Gabriel DA, Muga K, Boothroyd EM. The effect of fibrin structure on fibrinolysis. J Biol Chem 1992; 267: 24259–24263.
6. Carr ME. Fibrin formed in plasma is composed of fibres more massive than those formed from purified fibrinogen. Thromb Haemost 1988; 59: 535–539.
7. Ryan EA, Mockros LF, Weisel JW, et al. Structural origins of fibrin clot rheology. Biophys J 1999; 77: 2813–2826.
8. Kanaide H, Uranishi T, Nakamura M. Effects of divalent cations on the conversion of fibrinogen to fibrin and fibrin polymerisation. Am J Hematol 1982; 13: 229–237.
9. Marx G. Elasticity of fibrin and protofibrin gels is differentially modulated by calcium and zinc. Thromb Haemost 1988; 59: 500–503.
10. Di Stasio E, Nagaswami C, Weisel JW, et al. Cl– regulates the structure of the fibrin clot. Biophys J 1998; 75: 1973–1979.
11. Carr ME, Powers PL. Differential effects of divalent cations on fibrin structure. Blood Coagul Fibrinolysis 1991; 2: 741–747.
12. Fatah K, Hessel B. Effect of zinc ions on fibrin network structure. Blood Coagul Fibrinolysis 1998; 9: 629–635.
13. Marx G, Eldor A. The procoagulant effect of zinc on fibrin clot formation. Am J Hematol 1985; 19: 151–159.
14. Marx G, Hopmeier P, Gurfel D. Zinc alters fibrin ultrastructure. Thromb Haemost 1987; 57: 73–76.
15. Suzuki K, Hashimoto S. The influences of divalent metal ions on fibrin monomer polymerisation. Biochim Biophys Acta 1976; 439: 310–315.
16. Gorodetsky R, Mou X, Blankenfeld A, et al. Platelet multielemental composition, lability, and subcellular localisation. Am J Hematol 1993; 42: 278–283.
17. Henderson SJ, Stafford AR, Leslie BA, et al. Zinc delays clot lysis by attenuating plasminogen activation and plasmin-mediated fibrin degradation. Thromb Haemost 2015; 113: 1278–1288.
18. Lu J, Stewart AJ, Sadler PJ, et al. Albumin as a zinc carrier: properties of its high-affinity zinc-binding site. Biochem Soc Trans 2008; 36: 1317–1321.
19. Whitehouse RC, Prasad AS, Rabbani PI, et al. Zinc in plasma, neutrophils, lymphocytes, and erythrocytes as determined by flameless atomic absorption spectrophotometry. Clin Chem 1982; 28: 475–480.
20. Marx G, Korner G, Mou X, et al. Packaging zinc, fibrinogen, and factor XIII in platelet alpha-granules. J Cell Physiol 1993; 156: 437–442.
21. Vu TT, Fredenburgh JC, Weitz JI. Zinc: an important cofactor in haemostasis and thrombosis. Thromb Haemost 2013; 109: 421–430.
22. Fredenburgh JC, Leslie BA, Stafford AR, et al. Zn2+ mediates high affinity binding of heparin to the alphaC domain of fibrinogen. J Biol Chem 2013; 288: 29394–29402.
23. Fredenburgh JC, Stafford AR, Leslie BA, et al. Bivalent binding to gammaA/gamma'-fibrin engages both exosites of thrombin and protects it from inhibition by the antithrombin-heparin complex. J Biol Chem 2008; 283: 2470–2477.
24. Walker JB, Nesheim ME. The molecular weights, mass distribution, chain composition, and structure of soluble fibrin degradation products released from a fibrin clot perfused with plasmin. J Biol Chem 1999; 274: 5201–5212.
25. Schaefer AV, Leslie BA, Rischke JA, et al. Incorporation of fragment X into fibrin clots renders them more susceptible to lysis by plasmin. Biochemistry 2006; 45: 4257–4265.
26. Ariens RA, Philippou H, Nagaswami C, et al. The factor XIII V34L polymorphism accelerates thrombin activation of factor XIII and affects cross-linked fibrin structure. Blood 2000; 96: 988–995.
27. Collet JP, Moen JL, Veklich YI, et al. The alphaC domains of fibrinogen affect the structure of the fibrin clot, its physical properties, and its susceptibility to fibrinolysis. Blood 2005; 106: 3824–3830.
28. Hethershaw EL, Cilia La Corte AL, Duval C, et al. The effect of blood coagulation factor XIII on fibrin clot structure and fibrinolysis. J Thromb Haemost 2014; 12: 197–205.
29. Schroeder V, Kohler HP. New developments in the area of factor XIII. J Thromb Haemost 2013; 11: 234–244.
30. Levy JH, Greenberg C. Biology of Factor XIII and clinical manifestations of Factor XIII deficiency. Transfusion 2013; 53: 1120–1131.
31. Bagoly Z, Koncz Z, Harsfalvi J, et al. Factor XIII, clot structure, thrombosis. Thromb Res 2012; 129: 382–387.

