

Neutralisation of the anti-coagulant effects of heparin by histones in blood plasma and purified systems

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Summary

Neutrophil extracellular traps (NETs) composed primarily of DNA and histones are a link between infection, inflammation and coagulation. NETs promote coagulation and approaches to destabilise NETs have been explored to reduce thrombosis and treat sepsis. Heparinoids bind histones and we report quantitative studies in plasma and purified systems to better understand physiological consequences. Unfractionated heparin (UFH) was investigated by activated partial thromboplastin time (APTT) and alongside low-molecular-weight heparins (LMWH) in purified systems with thrombin or factor Xa (FXa) and antithrombin (AT) to measure the sensitivity of UFH or LMWH to histones. A method was developed to assess the effectiveness of DNA and non-anticoagulant heparinoids as anti-histones. Histones effectively neutralised UFH, the IC₅₀ value for neutralisation of 0.2 IU/ml UFH was 1.8 µg/ml histones in APTT and 4.6 µg/ml against 0.6 IU/ml UFH in a purified sys-

tem. Histones also inhibited the activities of LMWHs with thrombin (IC₅₀ 6.1 and 11.0 µg/ml histones, for different LMWHs) or FXa (IC₅₀ 7.8 and 7.0 µg/ml histones). Direct interactions of UFH and LMWH with DNA and histones were explored by surface plasmon resonance, while rheology studies showed complex effects of histones, UFH and LMWH on clot resilience. A conclusion from these studies is that anti-coagulation by UFH and LMWH will be compromised by high affinity binding to circulating histones even in the presence of DNA. A complete understanding of the effects of histones, DNA and heparins on the haemostatic system must include an appreciation of direct effects on fibrin and clot structure.

Keywords

Neutrophil extracellular traps, DNA, histones, heparin, APTT

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Introduction

The inter-relationship between inflammation, coagulation and thrombosis, mediated through the action of neutrophil extracellular traps (NETs), has been the focus of much recent attention (reviewed [1, 2]). The combination of fibrin and NETs forms a system of immune defence, which has been termed immunothrombosis (3). Nevertheless, there is a balance between beneficial effects of NETs and pathological consequences, and there are strong associations between NET components and many disease states (4), including venous thrombosis (5), coronary artery disease (6), stroke (7), cancer (8), infection (9), sepsis (10), transplant rejection and reperfusion injury (11), transfusion related acute lung injury (TRALI) (12), and links with auto-immune disease (13). The principle components of NETs, DNA and histones, are found in *ex*

vivo clots from patients suffering from myocardial infarction and peripheral arterial disease (14, 15) and they are implicated at several points in haemostatic cascades to enhance fibrin formation and retard fibrinolysis. Nucleic acids can activate coagulation (16) and bind fibronectin (17); while histones are known to activate platelets (18), stimulate thrombin generation through a thrombomodulin dependent mechanism (19) and promote the release of von Willebrand Factor (vWF) (5) which binds to histones (20). Histones are toxic to microbial cell pathogens (21), and also cytotoxic to endothelial and epithelial cells (22, 23). Thus, DNA and histones are targets for therapeutic interventions. Heparinoids can bind and disassemble NETs (15, 24) and promote breakdown by DNase (24, 25). In addition, non-anticoagulant heparinoids are being actively developed as potential therapies to bind and neutralise histones that are correlated with poor prognosis in sepsis patients (26, 27).

Hence there is interest in experimental systems that can quantify the neutralising potencies of heparins and heparinoid molecules on histones and explore the consequences of heparin-histone-DNA interactions on haemostasis, which is the focus of the current work. The results are also relevant to our understanding of the effectiveness of heparin therapy for prophylaxis following surgery or treatment for DVT (28, 29) and sepsis (10, 27). Histones may be added to the list of plasma proteins that account for inter-individual sensitivity to heparin or heparin resistance (30).

Materials and methods

Materials

Heparins used were unfractionated heparin (UFH, Heparin Sodium, Wockhardt, Wrexham, UK) and low-molecular-weight heparin (LMWH, WHO, International Standard code 11/176, 342 anti-IIa IU and 1068 anti-FXa IU per ampoule, 9.2 mg/ampoule; and Enoxaparin, NIBSC reagent code 11/174, 275 anti-IIa IU and 1030 anti-FXa IU per ampoule, 9.4 mg/ampoule, NIBSC, S Mimms, UK). Results are reported in the most appropriate units for the technique being used: anti-IIa units in APTT and against

thrombin; anti-FXa units for FXa inhibition studies; and in $\mu\text{g/ml}$ for physical studies. Modified heparins were a partially desulfated version of N-desulfated re-N-acetylated heparin (31), and a fully desulfated variant, N-acetylated de-O-sulfated heparin, characterised by NMR. DNA (calf thymus DNA) and fibrinogen (human, plasminogen-depleted) were from Calbiochem (San Diego, CA, USA). Freeze dried pooled normal platelet-poor plasma was used (NIBSC reagent, code 06/156). Histones (type IIIS) were from Sigma (St Louis, MO, USA). Thrombin was the WHO IS alpha thrombin standard 01/580, NIBSC (32) and antithrombin (AT) was the WHO IS, 06/166, NIBSC. The chromogenic substrates S2288 (H-D-Ile-Pro-Arg-p-nitroanilide) and S2765 (Z-D-Arg-Gly-Arg-p-nitroanilide) were from Chromogenix, Milan Italy. Human factor Xa (FXa) was a gift from Baxter (Vienna, Austria).

Inhibition studies

Activated partial thromboplastin time (APTT) was determined with APTT-SP (Instrumentation Laboratory, Bedford, MA, USA) a synthetic phospholipid reagent with micronised silica activator using a manual Tetra Coagulometer (Diagnostica Grifols, Barcelona, Spain). Heparins and DNA where present were included in

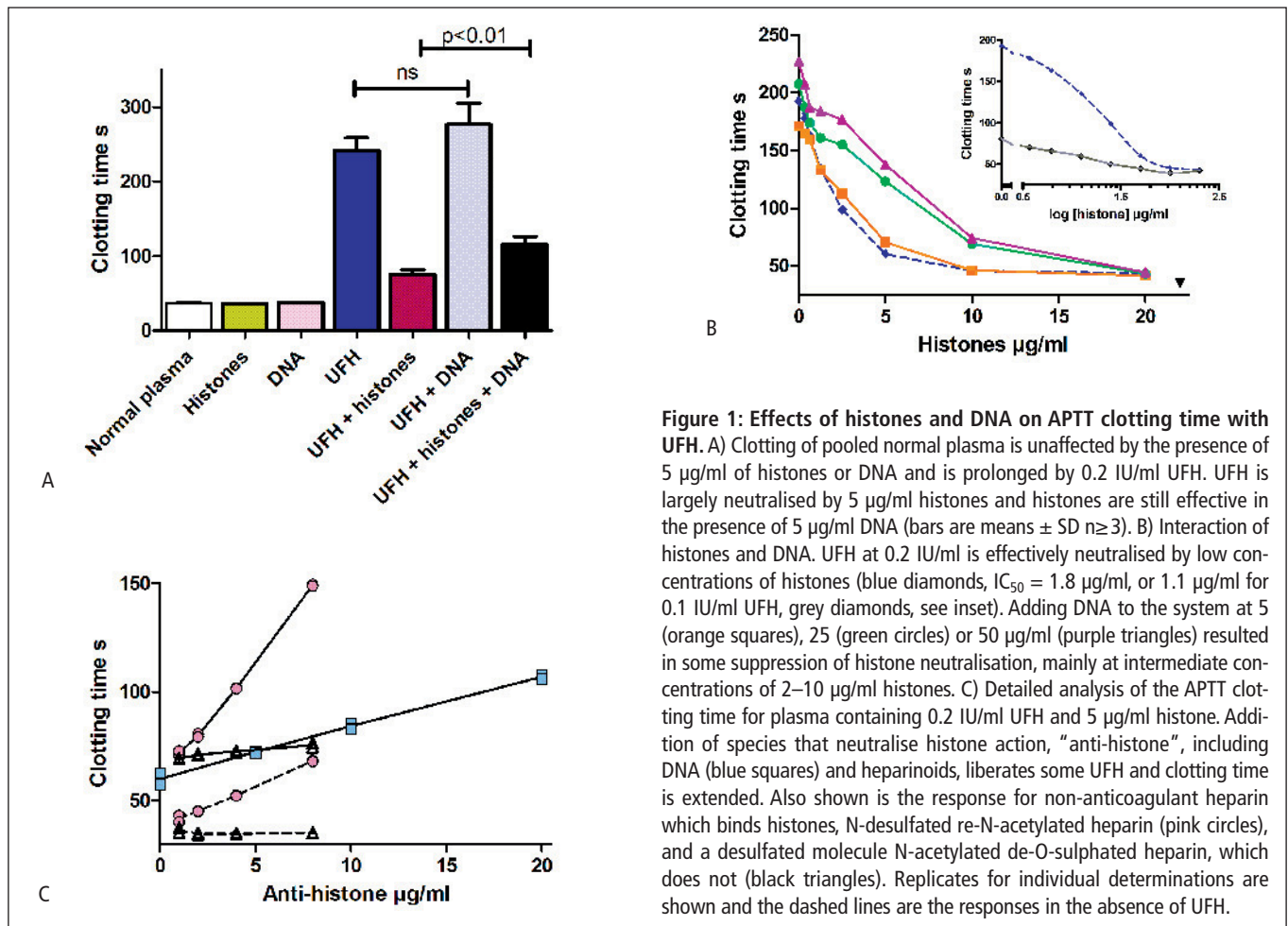


Figure 1: Effects of histones and DNA on APTT clotting time with UFH. A) Clotting of pooled normal plasma is unaffected by the presence of 5 $\mu\text{g/ml}$ of histones or DNA and is prolonged by 0.2 IU/ml UFH. UFH is largely neutralised by 5 $\mu\text{g/ml}$ histones and histones are still effective in the presence of 5 $\mu\text{g/ml}$ DNA (bars are means \pm SD $n \geq 3$). B) Interaction of histones and DNA. UFH at 0.2 IU/ml is effectively neutralised by low concentrations of histones (blue diamonds, $\text{IC}_{50} = 1.8 \mu\text{g/ml}$, or 1.1 $\mu\text{g/ml}$ for 0.1 IU/ml UFH, grey diamonds, see inset). Adding DNA to the system at 5 (orange squares), 25 (green circles) or 50 $\mu\text{g/ml}$ (purple triangles) resulted in some suppression of histone neutralisation, mainly at intermediate concentrations of 2–10 $\mu\text{g/ml}$ histones. C) Detailed analysis of the APTT clotting time for plasma containing 0.2 IU/ml UFH and 5 $\mu\text{g/ml}$ histone. Addition of species that neutralise histone action, “anti-histone”, including DNA (blue squares) and heparinoids, liberates some UFH and clotting time is extended. Also shown is the response for non-anticoagulant heparin which binds histones, N-desulfated re-N-acetylated heparin (pink circles), and a desulfated molecule N-acetylated de-O-sulphated heparin, which does not (black triangles). Replicates for individual determinations are shown and the dashed lines are the responses in the absence of UFH.

0.1 ml reconstituted freeze dried plasma to which 0.1 ml APTT-SP was added. Then after 300 s at 37°C, 0.1 ml of 25 mM CaCl₂ ± histones was added and clotting time recorded.

Thrombin and AT reactions were also monitored in chromogenic solution assays with 10 mM Tris HCl buffer, pH 7.4 containing 0.2 M NaCl and chromogenic substrate S2288 at 37°C using a Thermomax plate reader (Molecular Devices, Sunnyvale, CA, USA). End point readings were taken as a measure of thrombin activity after 5 hours to optimise sensitivity and minimise substrate consumption. The ability of histones to neutralise heparin is shown as a plot of heparin activity versus log of histone concentration in µg/ml. Heparin activity was calculated as $1 - [(Abs_{AT+heparin+x \mu g/ml \text{ histones}} - Abs_{AT+heparin}) / (Abs_{no AT no heparin} - Abs_{AT+heparin})]$. FXa binding to AT was studied with 0.6 mM S2765 by monitoring absorbance over time assuming irreversible FXa-AT complex formation under the conditions used. Inhibitory kinetics of FXa and AT were monitored in the absence of LMWHs and with either type of LMWH at 0.3 IU/ml (anti-FXa units) in the presence of histones at 0, 0.63, 1.25, 2.5, 5, 10, 20, 40 µg/ml. Values for initial rate and observed FXa-AT reaction rate (k_{obs}) were fitted to these data using the equation $A_{405} = A_0 + (v_0 / k_{obs}) * (1 - \exp(-k_{obs} * t))$ where k_{obs} is the observed reaction rate between FXa and AT. Plots of k_{obs} versus histone concentration for each heparin, were fitted to a four-parameter binding equation (GraphPad Software, San Diego, CA, USA) to derive IC₅₀ values for neutralisation of heparin by histones.

Viscoelasticity studies

The viscoelastic properties of fibrin clots were studied in a cone-and-plate type HAAKE RheoStress 1 oscillation rheometer (ThermoScientific, Karlsruhe, Germany), as previously described (15). Briefly, a 500 µl mixture of fibrinogen at 2.4 mg/ml in 10 mM Hepes buffer pH 7.4 containing 0.15 M NaCl was prepared with the additives: histones, DNA, UFH and LMWH; alone, or in various combinations; and at various concentrations, as specified in Results. Clotting was initiated by adding 410 µl of the fibrinogen mixture to 25 µl of 166 nM thrombin, and 410 µl of the clotting mixture was quickly transferred to the rheometer plate thermostatted at 37°C. Oscillatory strain (γ) of 0.015 at 1 Hz frequency was imposed on the composite fibrin clot 2 minutes (min) after the initiation of clotting, and storage modulus (G') and loss modulus (G'') were determined with HAAKE RheoWin data management software v.3.5.00.012 (ThermoScientific) for 15 min. At the end of the 15 min clotting phase G' and G'' plateaued and the flow curves for the same clot samples were taken by applying increasing shear stress (τ) from 0.01 up to 1000 Pa stepwise in 250 steps within 300 seconds (s), and measuring the resulting strain values. Flow curves are presented as calculated clot viscosity vs applied stress, where the collapse of the clot structure is mirrored as a sharp decline of its viscosity, and critical shear stress (τ_0) for the gel/fluid transition is determined from an extrapolation of this decline to a theoretically zero viscosity. All measurements were performed in 3–5 replicas for each combination of additives in the fibrin clots. Because the normal or any other tested standard theoretical distribution of

the evaluated numeric data could not be confirmed, a non-parametric statistical test was used to compare the results from different samples. The Kolmogorov-Smirnov test was chosen because of its robust power to compare distributions of two data sets independently of their shape and used with the Statistics Toolbox v. 10.0 of Matlab R2015a (The MathWorks Inc., Natick, MA, USA).

Binding and structural studies

Direct interaction of heparins with histones and disruption of DNA-histone complexes were studied by surface plasmon resonance (SPR), as described in the accompanying Suppl. Material (available online at www.thrombosis-online.com). Fibre diameter values of fibrin clots containing UFH or LMWH (where present) were evaluated by scanning electron microscopy (SEM) and small-angle X-ray scattering (SAXS) as previously described in (15). Porosity of the fibrin network was assessed from the liquid permeability of the clots under constant hydrostatic pressure as described in (33). Full details of these experimental procedures are given in the Suppl. Material (available online at www.thrombosis-online.com).

Results

Neutralisation of UFH by histones in plasma

APTT is a routine plasma-based test system, widely used clinically to monitor heparin levels in patients (28), and may be employed to study neutralisation of heparin by histones. ► Figure 1A summarises the effects of histones and DNA on heparin in a series of APTT determinations. Clotting time was unaffected by low concentrations of histones or DNA (5 µg/ml) but, as expected, clotting was delayed in the presence of therapeutic levels of 0.2 IU/ml UFH. However, prolongation by UFH was almost normalised by 5 µg/ml histones but DNA (5 µg/ml) was only partially effective at reversing the neutralising effect of histones (compare 5th and 7th columns of ► Figure 1A). The behaviour of DNA as an anti-histone was explored in detail in this system and results are shown in ► Figure 1B. At 0.2 IU/ml heparin, 50% normalisation of clotting time (the IC₅₀) occurred at 1.8 µg/ml histones (see inset ► Figure 1B). When 5, 25 and 50 µg/ml DNA was added to the plasma in this system, the potency of histones was abrogated, but 20 µg/ml histones neutralised 0.2 IU/ml heparin at all DNA concentrations investigated. Furthermore, using the APTT system, it was possible to develop a method to measure the potency of anti-histone agents with little or no anticoagulant activity, as shown in ► Figure 1C, with N-desulfated re-N-acetylated heparin, a low anticoagulant activity heparin variant. Also shown is a fully desulfated variant, N-acetylated de-O-sulfated heparin, with no anticoagulant activity or anti-histone capacity. In this system the balance between 0.2 IU/ml UFH and 5 µg/ml histones is modulated by further addition of anti-histone heparinoids or DNA causing an extension of clotting time which can be explained by the liberation of UFH. The data shown indicate that the partially desulfated N-desulfated re-N-acetylated heparin had a strong anti-histone activity (and low intrinsic anticoagulant activity, shown by the

dashed line response), whereas the N-acetylated de-O-sulphated heparin did not displace histones (or possess any intrinsic anticoagulant activity), probably due to low sulfation or negative charge. DNA had an intermediate anti-histone effect (blue squares) and low intrinsic anticoagulant activity (not shown).

Effects of histones on UFH and LMWH in purified systems

In clinical practice, LMWH is more commonly used than UFH for prophylaxis of thrombosis (28) and detailed investigations of the effects of histones on the activity of UFH and two forms of LMWH were pursued in purified systems. ►Figure 2 shows results for thrombin cleavage of chromogenic substrate in the presence of AT and LMWH, which catalyses thrombin-AT formation. Further addition of histones to the system reduces the activity of UFH or LMWH, as shown. The fitted curves for the data presented in ►Figure 2 indicate an IC_{50} of 4.8 $\mu\text{g/ml}$ histones with 0.6 IU/ml UFH, comparable to results in the plasma-based APTT system ($IC_{50} = 1.8 \mu\text{g/ml}$ with 0.2 IU/ml UFH). In the thrombin-chromogenic substrate system, IC_{50} values for 0.6 IU/ml (anti IIa units) LMWH IS and Enoxaparin were 6.1 and 11.0 $\mu\text{g/ml}$ histones, respectively (for more details see ►Figure 2 legend). Thus, LMWH activity was severely compromised in the presence of low concentrations of histones, to a similar extent as UFH as assessed using chromogenic assay methods or APTT.

LMWH activity is more appropriately measured in FXa-AT assay systems and results showing the effect of 0–40 $\mu\text{g/ml}$ histones are presented in ►Figure 3. In this approach, slow binding kinetics curves were monitored by following the cleavage of chro-

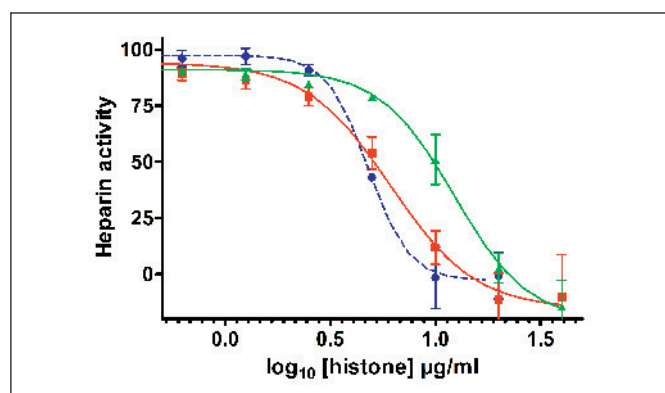


Figure 2: Titration of heparin stimulation of thrombin-AT binding with histones. Mixtures of 0.02 nM thrombin and 50 nM AT and 0.6 IU/ml UFH or LMWH (anti-IIa units) were incubated in the presence of increasing concentrations of histones, 0, 0.63, 1.25, 2.5, 5.0, 10, 20, 40 $\mu\text{g/ml}$ and S2288 chromogenic substrate for 5 hours at 37 °C. Absorbance at 405 nm was read as a measure of thrombin activity, which was converted into % heparin activity. IC_{50} (\pm SE) values for the neutralising effect of histones on heparin activity were calculated by fitting a four-parameter binding isotherm to determine the concentrations of histones required for 50% inhibition: 4.8 (\pm 1.0) $\mu\text{g/ml}$ (against UFH blue circles), 6.1 (\pm 1.1) $\mu\text{g/ml}$ (against Enoxaparin, green triangles) and 11.0 (\pm 1.1) $\mu\text{g/ml}$ (against LMWH IS, red squares). Data points are means \pm SD error bars, $n=3$.

mogenic substrate by FXa as AT binds to form an inactive complex. Inhibition of FXa is very slow without LMWH present and the apparent rate constant for the formation of the FXa-AT complex, k_{obs} , provides a measure of stimulation of the reaction by LMWH. Hence, the potency of histones in neutralising LMWH can be followed by monitoring k_{obs} for this reaction. The curves shown in ►Figure 3A illustrate poor inhibition of FXa by AT in the absence of LMWH (dashed line), as expected, but rapid inhibition in the presence of LMWH (grey line). Increasing concentrations of histones effectively neutralised added LMWH as indicated by the intermediate curves. k_{obs} values fitted to these curves were used to generate binding isotherms for histone neutralisation of heparins as shown in ►Figure 3B for UFH, and the two LMWHs. The results were similar to those in ►Figure 2 for thrombin inhibition. Once again a simple dose response was seen for histones over the range 0–40 $\mu\text{g/ml}$, and the IC_{50} values were 8 $\mu\text{g/ml}$ histones or lower, comparable to those seen with thrombin and AT (see figure legends for details). Thus the sensitivity of LMWH to histones when FXa-AT complex formation kinetics were studied was similar to UFH or LMWH in thrombin-AT systems.

To directly investigate the interaction between heparins, DNA and immobilised histones, SPR was used, which provided evidence of high affinity binding (see Suppl. Figure 1, available online at www.thrombosis-online.com). Because of the heterogeneous size distribution of heparin preparations, it is not possible to calculate molar concentrations and hence real K_d values. However, for comparative purposes in this system, analysis of binding curves of stable response unit (RU) values versus $\mu\text{g/ml}$ heparin injected over immobilised histones gave apparent equilibrium dissociation constants of 0.4, 0.8 and 0.5 $\mu\text{g/ml}$ for UFH, LMWH IS and Enoxaparin, respectively. Furthermore, these heparins were effective at displacing DNA from the immobilised histones. In competition with 50 $\mu\text{g/ml}$ DNA, IC_{50} values of 0.5, 2.5 and 3.1 $\mu\text{g/ml}$ were observed for UFH, LMWH IS and Enoxaparin, respectively, in displacing DNA (see Suppl. Figure 1, available online at www.thrombosis-online.com). Expressed as anti-FXa units, these IC_{50} values are in the range 0.1–0.2 IU/ml similar to concentrations used in ►Figures 1–3, above, supporting the conclusion that clinically relevant concentrations of UFH and LMWH will be bound by histones even in the presence of significant concentrations of DNA.

Effects of heparins, histones and DNA on fibrin structure and clot stability

We and others have previously investigated the direct effects of DNA, histones and UFH on fibrin structure and clot properties (15), but less is known about interactions with LMWH and heparinoids. Direct effects of DNA, histones and heparins on fibrin fibre structure and clot properties were further investigated by SEM, clot permeability and SAXS. These results are summarised in ►Table 1 and Suppl. Figure 2 (available online at www.thrombosis-online.com). It can be seen that there are similar trends for both UFH and LMWH to directly affect fibrin and clot structure. The thinner fibres found by SEM, and the decreased clot porosity

in the fluid permeability measurements suggest a denser fibrin mesh, which is also supported by the SAXS data that show that in the periodically ordered fibrin structure the most prevalent cell unit size is decreased in the presence of heparins, especially LMWH. The SAXS method may require the modifiers at a higher concentration than our functional studies, since it is looking at fibrin structure at the level of practically every single fibrin monomer to get periodical scattering signals. Fibrin fibre size or branching density, on the other hand, might be influenced by the same modifier at a much lower molar ratio to fibrin monomers. LMWH is if anything more effective than UFH, though the situation is complicated by the heterogeneity of the heparin species and how best to report concentrations. Nevertheless, clinical concentrations of heparins do appear to have effects on fibrin and clot structure that are independent of their activity in regulating thrombin activity.

Direct effects of heparins, histones and DNA were also addressed by viscoelasticity studies to explore the potential modulatory effects of histones and DNA, alone or in combination with either UFH or LMWH, on clot resilience to shear stress (τ). The flow curves (► Figure 4) express the changes in clot structure as a result of increasing shear stress τ in terms of viscosity η (higher η values indicating a structure more resistant to mechanical deformation). The critical shear stress τ_0 where η returns to zero is an indication of where clot structure has disintegrated and detailed values are presented in ► Figure 4. The effects of DNA and/or histones on clot resilience are shown in ► Figure 4A where it can be seen that the addition of 25 or 50 $\mu\text{g/ml}$ DNA resulted in a weaker structure which broke down at 193 or 186 Pa rather than 235 Pa. By contrast, the addition of histones, at only 5 or 10 $\mu\text{g/ml}$, resulted in a stronger clot that broke down at shear stress of >500 Pa, and this effect was counteracted by DNA at increasing concentrations. UFH at therapeutic concentrations had a small, but statistically non-significant, destabilising effect. UFH alone did not significantly reduce the clot-strengthening effects of 10 $\mu\text{g/ml}$ histones (► Figure 4B), whereas UFH in combination with DNA was more effective than DNA alone in counteracting histone effects. Histone/DNA-clots were stronger than DNA-clots (► Figure 4A); UFH/histone/DNA clots were equivalent in shear resistance to UFH/DNA clots (► Figure 4B), and were definitely softer than histone/DNA-clots. The combined neutralising effects of DNA and UFH were overcome by an increase in the histone concentration from 10 to 25 $\mu\text{g/ml}$. Modulation of clot strength by positively charged histones that strengthen the clot and negatively charged DNA or UFH that weaken it, was not explained by simple charge rules as indicated by data with LMWH, which is also negatively charged like UFH, but with shorter chains. ► Figure 4C shows a similar set of curves to those in 4B, but with LMWH (Enoxaparin here, and LMWH IS behaved identically, not shown) in place of UFH. In this case, addition of LMWH alone made the clot more resistant to breakdown (red line) and even enhanced the stabilising effect of 10 $\mu\text{g/ml}$ histones (blue line). DNA at 25 $\mu\text{g/ml}$ could counteract the LMWH-effect (compare red and light blue lines) and partially neutralised the combined effects of LMWH plus histones (dark blue vs green lines).

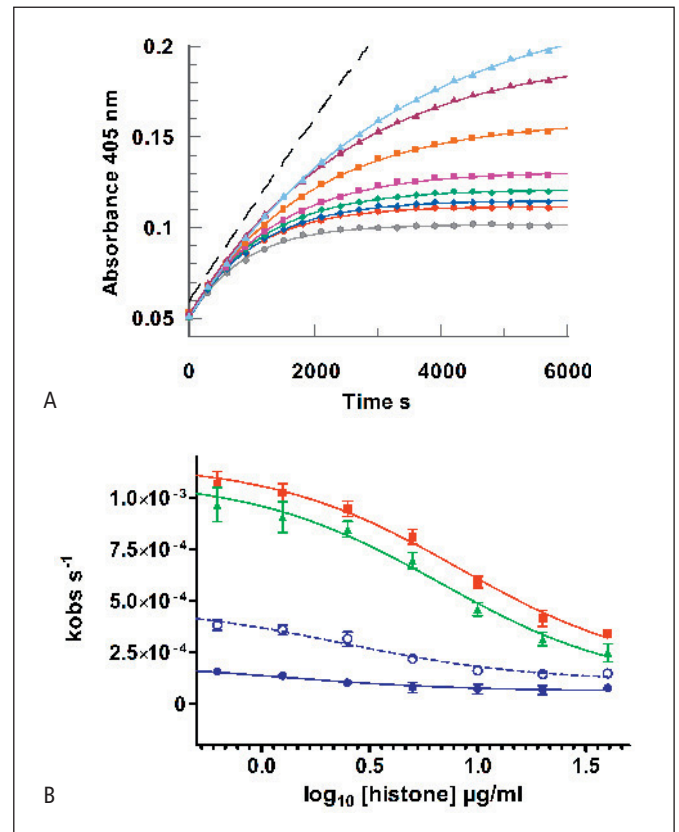


Figure 3: FXa –AT inhibition in the presence of heparin and histones. A) Series of time courses for the slow binding inhibition of 0.2 nM FXa by 25 nM AT in the presence of 0.3 IU/ml LMWH IS, where absorbance was monitored over time, following cleavage of 0.6 mM S2765, at 37 °C. Curves show the presence of histones at 0, 0.63, 1.25, 2.5, 5.0, 10, 20, 40 $\mu\text{g/ml}$ (grey, red, blue, green, pink, orange, maroon, pale blue, respectively; only every tenth data point is shown for clarity). The dashed line shows FXa activity in the presence of AT but without heparin. Values for initial rate and observed FXa-AT reaction rate (k_{obs}) were fitted to these data (solid lines) using the equation $A_{405} = A_0 + (v_0 / k_{\text{obs}}) * (1 - \exp(-k_{\text{obs}} * t))$ where k_{obs} is the observed reaction rate between FXa and AT. B) Summarised data and fitted plots (solid lines) of k_{obs} versus histone concentration for each heparin, from which IC_{50} (\pm SE) values for neutralisation of heparin by histones were determined: 7.8 (\pm 1.3) $\mu\text{g/ml}$ (with LMWH IS, red squares), 7.0 (\pm 1.4) $\mu\text{g/ml}$ (with Enoxaparin, green triangles), in both cases LMWH was 0.3 IU/ml (anti-FXa units). With UFH, IC_{50} values were 1.2 (\pm 1.4) $\mu\text{g/ml}$ histones (solid blue circles with 25 nM AT and 0.3 IU/ml UFH) or 2.4 (\pm 1.4) $\mu\text{g/ml}$ histones (open blue circles with 50 nM AT and 0.6 IU/ml UFH). All data points are means \pm SD error bars, $n=3$.

Discussion

Neutralisation of heparin by histones

Heparin is known to disrupt NETs (24), presumably by binding to histones in competition with DNA, but another consequence of this interaction is that circulating histones can interfere with heparin therapy. The purpose of the current work was to improve our quantitative understanding of the interaction of heparins, histones and DNA in purified and plasma-based systems. Histones, being

highly positively charged, behave like other biological molecules such as protamine sulfate, platelet factor 4, polybrene and LDL in their ability to bind to negatively charged heparins, as shown many years ago (34). This early report identified the possibility that histones could neutralise heparins in circulation but considered the interaction was not biologically significant because histones were confined to the nucleus of the cell. More recent studies on NETs and the occurrence of circulating nucleosomes identified in many disease states (see *Introduction*) suggest there is a need to investigate neutralisation of therapeutic heparins by histones as a contributing factor to heparin resistance and the well-known variability of individual responses to heparin treatment (30). Direct binding of UFH, LMWH and DNA to immobilised histones was demonstrated using SPR (see Suppl. Figure 1, available online at www.thrombosis-online.com) and the concentration range of analytes used corresponded to those in other purified and plasma systems shown in ► Figures 1–3. Thus SPR is a complementary technique for direct quantitative study of heparin-histone binding alongside functional plasma-based methods such as APTT (shown in ► Figure 1A–C) and in purified systems (► Figures 2 and 3). A striking feature of the data presented in the present report was the relatively low concentrations of histones, below 10 µg/ml, needed to reduce the activity of therapeutic concentrations of heparin by 50% (IC₅₀). While in earlier studies histone levels up to 40 µg/ml (e.g. [15, 26, 35]) or in animal models >70 µg/ml (10) were reported, an IC₅₀ of 4.8 µg/ml histones was observed by our monitoring thrombin-AT with 0.6 IU/ml UFH (► Figure 2); and an IC₅₀ of 7.0 µg/ml for histones with FXa-AT in the presence of 0.3 IU/ml

Enoxaparin (► Figure 3). These are heparin concentrations within ranges used for prophylaxis or acute treatment (28, 30), and similar concentration ranges were also investigated in the plasma-based APTT system. Even in the presence of DNA UFH potency was much reduced by 5–20 µg/ml histones (► Figure 1B), suggesting that circulating histones could compromise heparin activity even in the presence of significant amounts of DNA.

Disruption of NETS by heparins

In some clinical situations, the interaction between histones and heparins can be used for therapeutic benefit. For example, recent studies on the treatment of sepsis and disseminated intravascular coagulation highlight heparinoids with low intrinsic anticoagulant activity as anti-histone therapies (26) that “mop up” circulating histones and destabilise NETs in clots (15, 24). Esmon has proposed that histones in circulation are always found associated with DNA (1) so some caution is needed when interpreting experimental results from systems where NET components are investigated individually. However, we and others have previously published evidence that histones and DNA have different distributions in thrombi isolated from patients (14, 15) and in purified systems containing activated neutrophils (33). The inhibition studies described in the current work employed a preparation of mixed histones and DNA and this model may not exactly replicate the mixture of histones available in and around NETs and within fibrin clots. The behaviour of histone subtypes, and the level of citrullination, which reduces the positive charge on histones (36, 37) has not been investigated in the current study but is an important area for future work. It is also becoming clear that alternative mechanisms to classical suicidal NETosis exist, and DNA and histones may be released from a variety of cell types or mitochondria by several distinct mechanisms (38). These observations suggest a range of histone species and DNA-histone interactions *in vivo* and simple model systems will not capture the full complexity. Nevertheless, taken together our observations suggest heparins can bind histones with high affinity and will be effective in destabilising NETs in an environment rich in DNA.

Fibrin and clot structure

Direct effects of mixtures of heparins, histones and DNA on clot structure and strength were also explored in the current work as summarised in ► Figure 4. Generally speaking, histones were found to strengthen clot structure, DNA alone weakened clots, and the effects of their combinations were concentration-dependent (► Figure 4A), in agreement with our earlier findings (15). The clot strengthening effects of histones at 10 µg/ml were only partially neutralised by therapeutic concentration of UFH; however, UFH plus DNA was definitely more effective in counteracting histones than DNA alone. Again, the clot softening effects of 25 µg/ml DNA could prevail depending on histone/DNA ratios. Strikingly, LMWH, which is also negatively charged but has shorter chain lengths than UFH, made clots more resilient to shear stress and also potentiated the stabilising action of histones. The

Table 1: Summary of effects of UFH and LMWH on fibrin structure as measured by SEM, permeability analysis and SAXS.

Technique ^a	Concentration of heparin µg/ml ^b	Parameter
SEM		Median Fibre diameter (bottom-top quartiles) nm
	0	108.7 (93.8–124.8)
	0.94 UFH	96.3 (81.7–112.3)
	1.72 LMWH IS	85.9 (73.4–100.5)
Permeability analysis		Relative permeability constant ± standard error of mean
	0	1 ± 0.06
	1.18 UFH	0.9 ± 0.04
	2.76 LMWH IS	0.51 ± 0.07
SAXS		Cluster cell periodicity (Peak 1) nm
	0	22.1
	25 UFH	19.0
	25 LMWH IS	16.4

^aFull details of methods and results can be found in the Suppl. Material to this paper (available online at www.thrombosis-online.com). ^bConcentrations are given in µg/ml for these studies, corresponding to 0.2 IU/ml and 0.25 IU/ml anti-IIa units for SEM and permeability analysis, respectively.

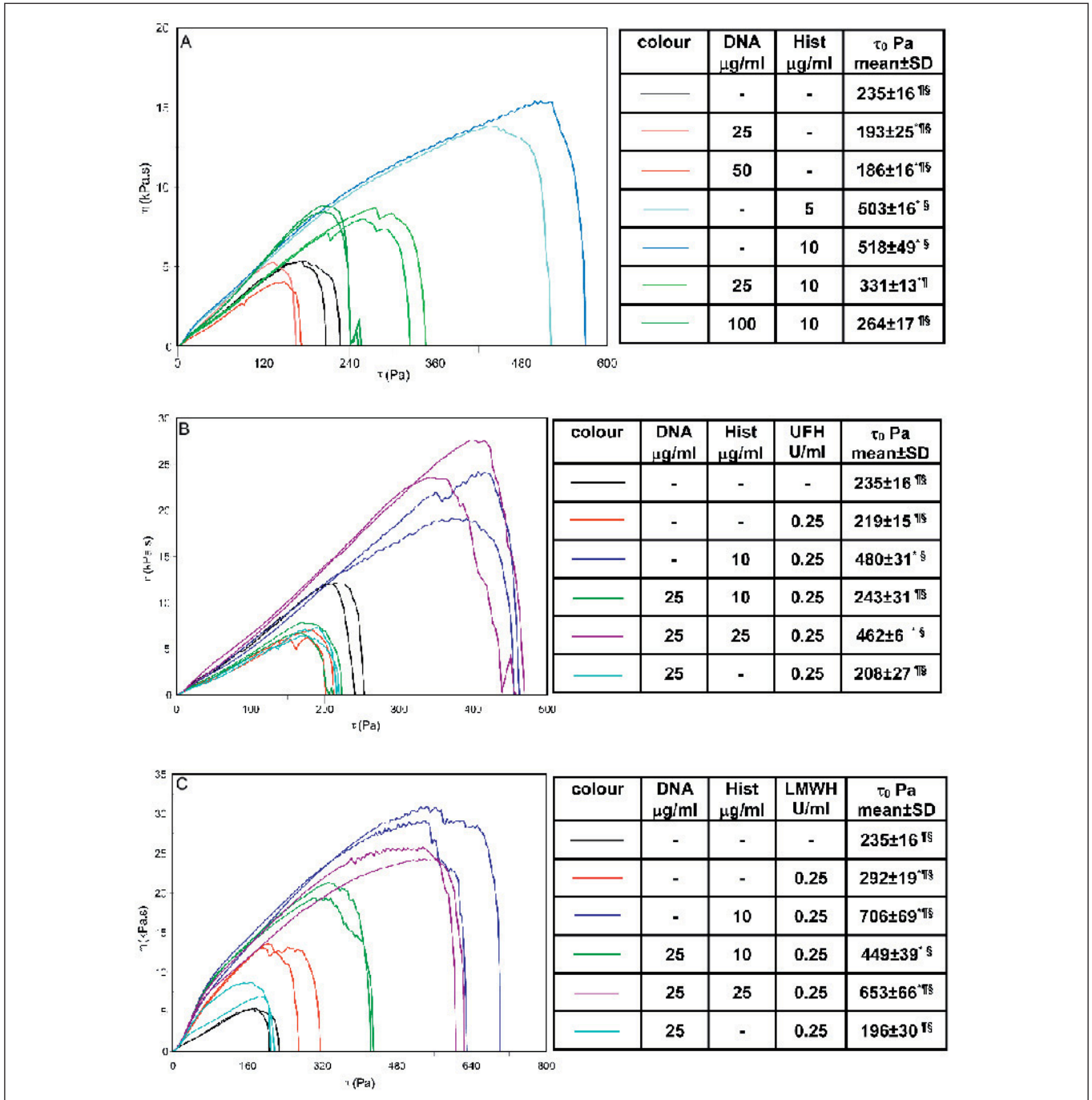


Figure 4: Effects of DNA, histones and UFH or LMWH on fibrin clot strength. Representative flow curves depict the viscosity η of composite fibrin clots as they are probed by applying increasing shear stress τ . Critical shear stress τ_0 values (at which viscosity falls to a theoretically zero value) are presented as mean \pm standard deviation values ($n=3-6$). Symbols indicate significant differences at $p<0.05$ according to Kolmogorov-Smirnov test in comparison to pure fibrin (*), fibrin with 10 $\mu\text{g/ml}$ histones (¶), or fibrin with 10 $\mu\text{g/ml}$ histones plus 25 $\mu\text{g/ml}$ DNA (§). A) DNA alone at 25 or 100 $\mu\text{g/ml}$ when clotted with fibrin resulted in a clot that is less resistant to shear

stress. Histones strengthened the clot significantly, even at 5 or 10 $\mu\text{g/ml}$, whereas DNA neutralised the effects of histones in this system at 25, or 100 $\mu\text{g/ml}$. B) Effects of 0.25 IU/ml UFH on clot viscosity. UFH alone demonstrated some effects similar to DNA in panel A, but histone at 10 $\mu\text{g/ml}$ could counter the presence of UFH. DNA cooperated with UFH to neutralise histones. C) Effects of LMWH on fibrin viscosity. LMWH (Enoxaparin, anti IIa units) alone made the clot more resistant to breakdown and enhanced the stabilising effect of 10 $\mu\text{g/ml}$ histones, whereas DNA at 25 $\mu\text{g/ml}$ counteracted LMWH.

disparate effects of UFH and LMWH on clot resilience suggest different mechanisms of action. It has long been known that ternary complexes of UFH-thrombin-fibrin monomer can form to modulate the activity of thrombin, affecting the cleavage of fibrinogen (39) which may modify the structure of fibrin. In addition, in plasma, where AT is present, UFH promotes thrombin inhibition and lower thrombin activity will produce clots with thicker fibres, more open structure and increased susceptibility to fibrinolysis (e.g. [40]). However, UFH may have more direct effects that lead to clots with more open structure with increased porosity (41). Fibrin formation in the presence of LMWH has been observed to produce a more rigid fibrin structure with thin fibres (42), in line with our findings; but others found minor effects of LMWH on plasma clot structure or sensitivity to lysis (43). More recent studies identified biphasic effects of both LMWH and UFH on clot structure in a purified system and a mechanism was proposed of incorporation of LMWH into fibrin fibres to account for changes in clot structure (44). Interestingly, polyphosphate (polyP), another highly anionic polymer like heparin was able to incorporate into fibrin if added to fibrinogen before clotting and produce thick fibres, with higher turbidity that were resistant to fibrinolysis (45), but no rheology data were provided. It may be significant that polyP chain length was important such that >100 phosphate residues were required to affect turbidity (and hence structure), but this effect could be blocked by very short chain polyP (46). In our, antithrombin-free fibrin clot model, the rheology data suggest a direct impact of heparins, DNA and histones on fibrin structure that was not simply related to charge. Thus, LMWH and LMWH plus histones demonstrated a prothrombotic potential through stabilisation of clot structure, not seen with UFH, by a route independent of antithrombin action. These observations warrant further studies with other clinically important LMWH products,

and polyP, and investigations using more physiological environments.

Results from the current study provide a rationale for understanding sources of heparin resistance in situations where circulating histones may be present. Methods are presented for studying non-anticoagulant heparinoid molecules that may be effective at neutralising histones as potential therapeutics in treating sepsis, for example. These methods will provide a more complete understanding of charge and structure and interactions with histones and DNA complexes, and effects on fibrin that may influence their success in treating patients.

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Conflicts of Interest

None declared.

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What is known about this topic?

- NETs formed primarily of histones and DNA are associated with thrombosis and infection and act as a defence mechanism but also have pathophysiological consequences.
- NETs can be destabilised by heparin.
- Histones generated during NET formation are positively charged and bind to a range of other molecules and cells.

What does this paper add?

- Histones can bind both UFH and LMWH with high affinity, relevant to circulating histone concentrations expected during some disease states.
- Circulating histones are likely to interfere with heparin therapy.
- Combinations of histones, heparins and DNA act on fibrin to directly influence clot stability.
- Clot stabilisation by histones is reduced by UFH but enhanced by LMWH so the outcomes are not explained by simple charge interactions.

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