Effects of Activated Neutrophils on Blood Clot Contraction

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INTRODUCTION

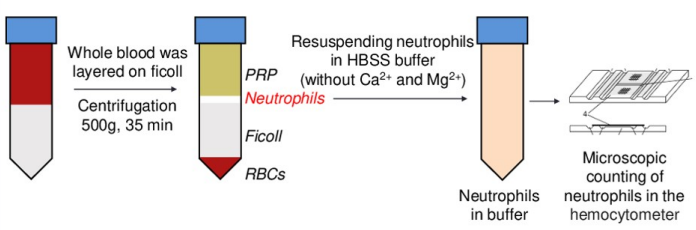
Neutrophils are a crucial component of the immune system. Platelet-driven contraction or retraction of blood clots is an important pathophysiological mechanism that could be modulated by neutrophils accumulated in inflammatory thrombi. Activation of neutrophils is accompanied by NETosis, that is, the release of neutrophil extracellular traps (NETs) comprising uncondensed chromatin, i.e. a network of DNA with histones and other proteins. Intravascular NET formation is known to enhance blood clotting and promote platelet activation, but the effects of NETs on blood clot contraction are unknown.

AIM

To assess the effects of activated neutrophils on the rate and extent of platelet-driven contraction of blood clots.

METHODS

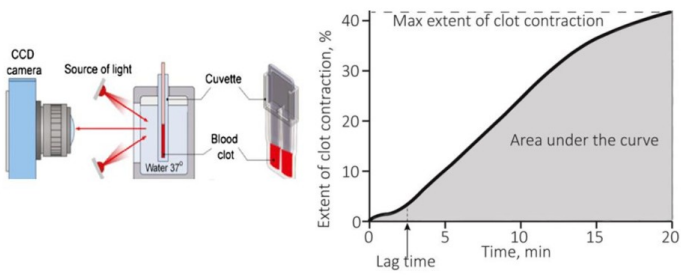
1) *Isolation of human neutrophils*



2) *Activation of neutrophils by PMA and their characterization*

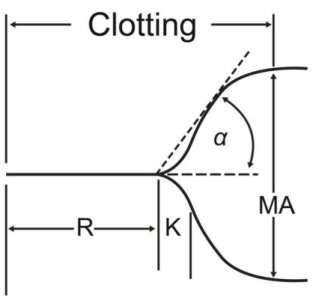
Isolated human neutrophils were activated with 100 nM PMA (Phorbol 12-myristate 13-acetate) 3.5 hours at 37°C and added to normal human blood or platelet-rich plasma (PRP) followed by thrombin-induced clotting and clot contraction tracked optically as reduction of clot size. Using light (including standard histological H&E staining) and confocal fluorescent microscopy, neutrophils were stained for NET-specific CitH3 histones and DNA that highlighted neutrophilic nuclei and NETs within isolated PMA-activated neutrophils, supernatant, and fibrin clots. High-resolution scanning electron microscopy was also employed to visualize the presence of NETs with activated neutrophils and within PRP clots.

3) *Clot contraction*



4) *Thromboelastography*

R - reaction time
K - coagulation time
MA - maximum amplitude
α - alpha angle
G is the storage modulus calculated from the maximal amplitude (MA) using the formula:



*G (dyn/cm²)=(5000*MA/(100-MA));*
1 dyn/cm²=0.1 Pa.

RESULTS

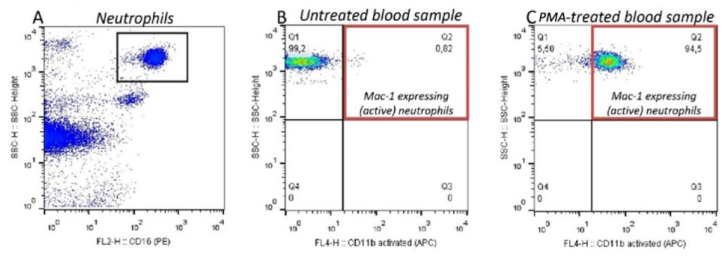


Figure 1. Gating of CD16+ neutrophils (**A**). No active Mac-1 (CD11b) expressed by neutrophils (Q2) in a control untreated blood sample (**B**) and a large fraction of neutrophils expressing active Mac-1 (Q2) within the gate of CD16+ neutrophils in PMA-treated blood sample (**C**). **Treatment of neutrophils with PMA leads to the expression of activated Mac-1.**

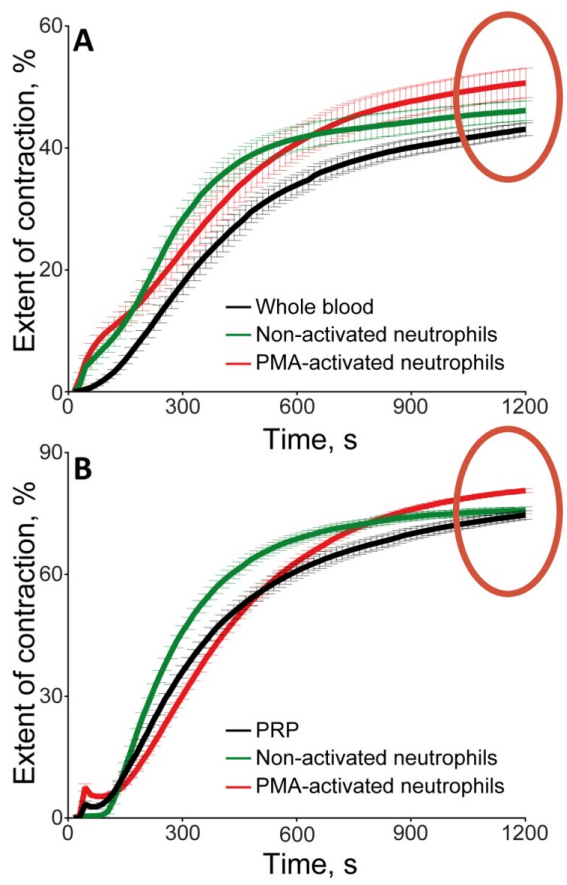


Figure 3. Averaged kinetic curves of clot contraction in whole blood (**A**) and PRP (**B**) in the absence (control) and presence of non-activated or PMA-activated neutrophils. **Activated neutrophils increase the extent of clot contraction without visible effect on the rate of contraction.**

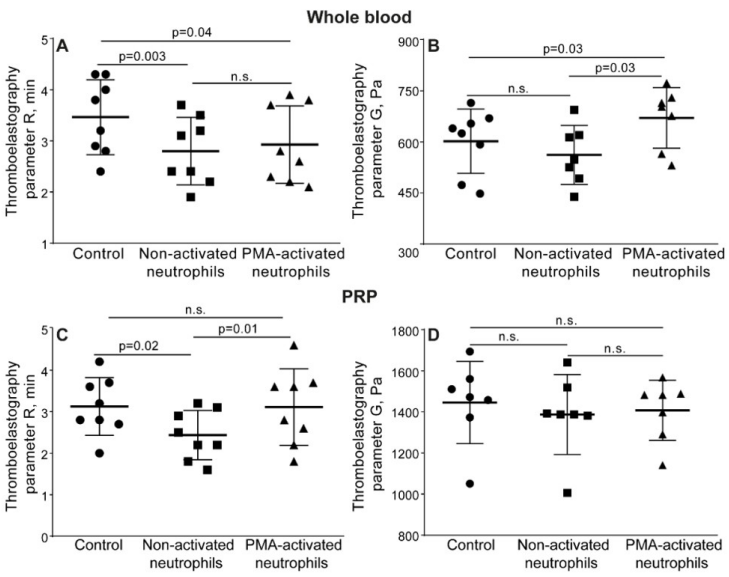


Figure 5. Effects of non-activated and PMA-activated neutrophils on the parameters of thromboelastography (R, G) in whole blood (**A** and **B**) and PRP (**C** and **D**). *R* is the clotting time. *G* is a storage modulus. Results are presented as a mean ± SD. RM one-way ANOVA test. n.s. - not significant. **The hypothesis about the softening effect of NETs on fibrin as a mechanism for promoting clot contraction is not supported by this data.**

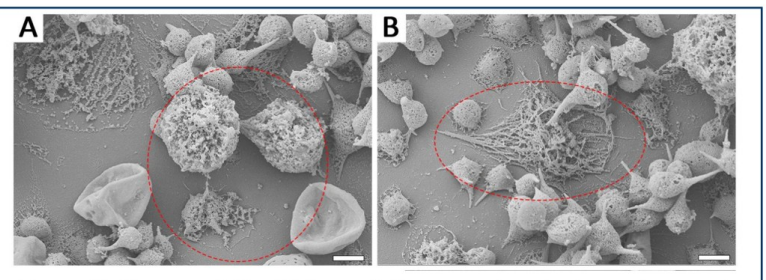


Figure 2. Representative high-resolution scanning electron micrographs of NETs (*dashed ovals*) released by isolated PMA-activated neutrophils at the initial (**A**) and final (**B**) stages of NETosis. Magnification bars = 2 µm. **Activation of isolated neutrophils with PMA is accompanied by NETosis.**

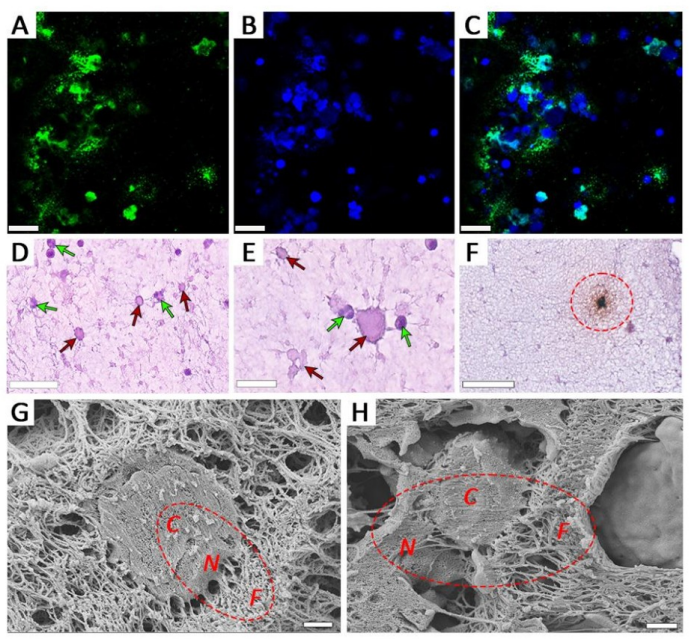


Figure 4. **(A-C)** Fluorescence microscopy of a PRP clot formed in the presence of PMA-activated neutrophils and stained for *CitH3 (*A), *DNA (B), and overlay* (C). Magnification bars = 20 µm. **(D, E)** Non-activated (*green arrows*) and activated (*red arrows*) neutrophils within a PRP clot formed in the presence of PMA-activated neutrophils. H&E stain. **(F)** A single PMA-activated neutrophil is stained for CitH3 (*dashed circle*) to visualize the nucleus and pericellular NETs. Magnification bars = 50 µm (D, F) and 25 µm (E). **(G, H)** Scanning electron micrographs showing the incorporation of PMA-activated neutrophils and NETs (*dashed ovals*) in the PRP clot. *C* - cell, *N* - NETs, *F* - fibrin. Magnification bars = 1 µm. **Clotting of blood plasma in the presence of PMA-activated neutrophils results in the embedding of NETs into a fibrin clot.**

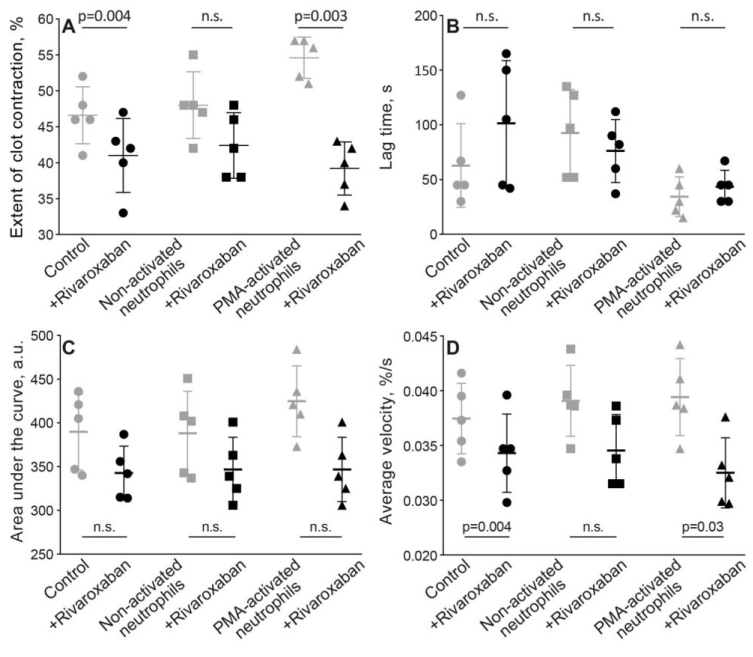


Figure 6. Effects of rivaroxaban on clot contraction in whole blood in the absence or presence of PMA-activated neutrophils. The parameters of clot contraction: final extent of contraction **(A)**, lag time **(B),** area under the curve **(C)** and average velocity **(D)**. Results are presented as a mean ± SD (n=5). Student's paired *t*-test. n.s. - not significant. **Inhibition of factor Xa by rivaroxaban abrogates the stimulating effect of activated neutrophils on clot contrcation, suggesting that the effect is due to generation of the endogenous thrombin.**

CONCLUSIONS

Activated neutrophils promote blood clot contraction and this effect is associated with formation of extracellular traps embedded into the fibrin network. NETs stimulated clot contraction by enhancing the production of endogenous thrombin rather than affecting the elastic properties of blood clots.

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CONTACT INFORMATION

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