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Assessing NET Formation Using Combined Neutrophil Activation in Diabetic Patients with Osteoarthropathy: A Methodological Approach

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INTRODUCTION & AIM

The formation of neutrophil extracellular traps (NETs) is a mechanism of innate immunity. Dysregulation of this process contributes to tissue damage in various pathologies. However, data on the role of netosis in the pathogenesis of diabetic osteoarthropathy (DOA) remain extremely limited.

This study aimed to evaluate the informativeness of a modified method for assessing NET formation using combined neutrophil activation in patients with Type 2 diabetes mellitus and diabetic foot syndrome (DFS) and DOA.

METHOD

The authors modified a patented technique [1] for evaluating NETs induced by a nonspecific antigenic stimulator – a microbial culture containing *L. reuteri, L. acidophilus, L. rhamnosus,* and *B. longum* – applied to neutrophils isolated from peripheral venous blood. Thrombin solution («Renam») was used as an additional NETosis activator. Microslides were analyzed using combined microscopy techniques: transmitted light for neutrophil visualization and fluorescence emission (excitation 450–480 nm, emission ≥515 nm) for detecting extracellular and intracellular DNA stained with propidium iodide (PI).

Ex vivo neutrophil leukocytes isolated from healthy volunteers, DFS patients, and DOA patients were stimulated with a probiotic or thrombin for 30 minutes at 37°C. Post-activation, we visualized and quantified intact neutrophils, activated neutrophils, early netosis cells, cloud-shaped NETs, and filamentous NETs (fig. 1).

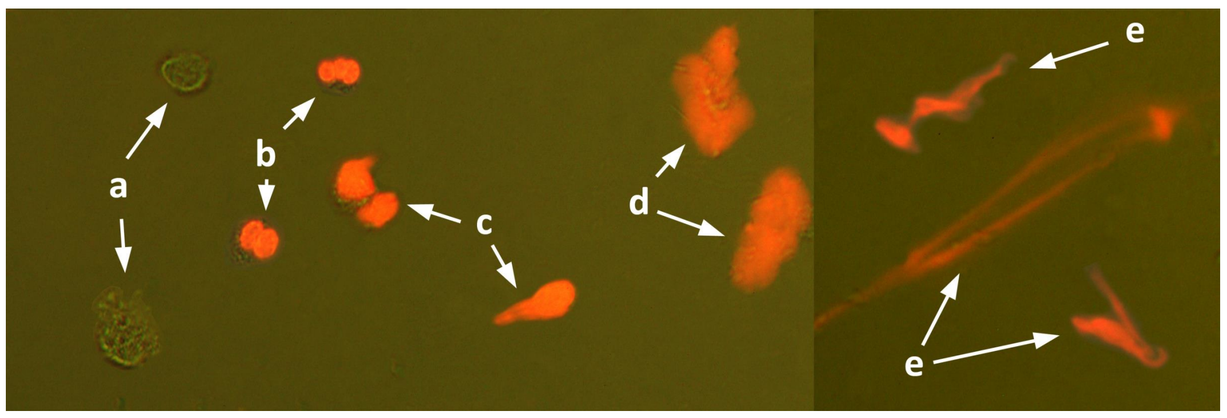

Figure 1. Intact neutrophils (a); activated neutrophils (b), early netosis cell (c); Neutrophil extracellular traps: cloud-shaped NETs (d), filamentous NETs (e). Fluorescence microscopy, x600.

RESULTS & DISCUSSION

Comparative analysis revealed optimal parameters: PI concentration ≥0.1 mg/mL, probiotic preparation at 2.5×10^9 bacteria/mL, thrombin activity at 3 IU/mL, and a leukocyte suspension/activator ratio of 1:10. Interassay coefficients of variation in 10 experimental series with thrombin were 13.8% in a healthy donor and 12.5% in a DOA patient. Neutrophils from DFS patients exhibited enhanced NET formation upon stimulation with both probiotic and thrombin compared to healthy donors (*p*=0.002 and *p*<0.001, respectively) (fig. 2, 3). Notably, neutrophils from DOA patients (Charcot foot) demonstrated more pronounced filamentous NET formation than those from diabetic foot patients without osteoarthropathy (*p*=0.011) (fig. 3, 4).

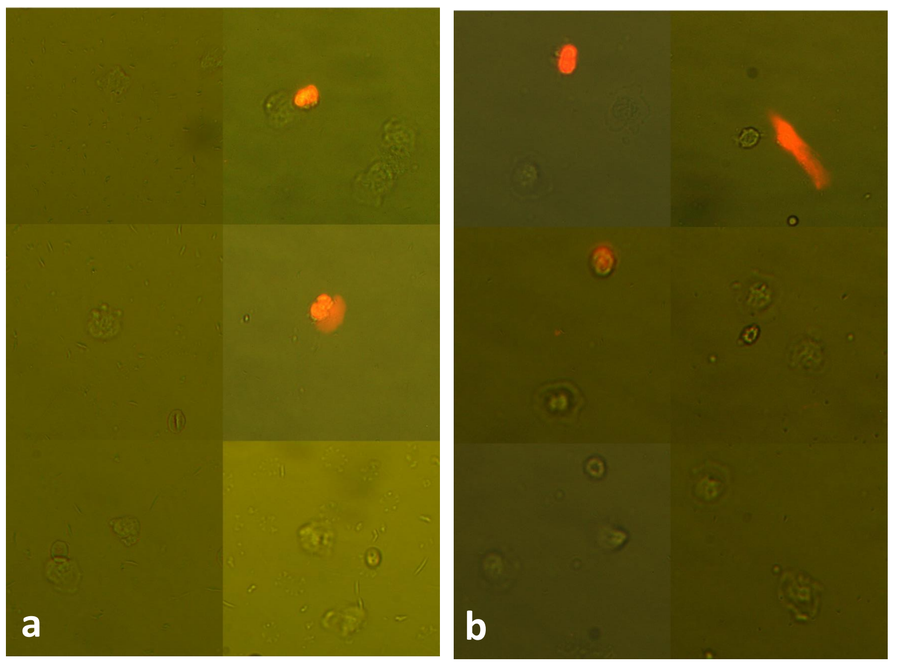

Figure 2. Example of fields of view after exposure of neutrophils from a healthy volunteer to a stimulant: a – activation of neutrophils by probiotic; b – activation of neutrophils by thrombin. Fluorescence microscopy, x600.

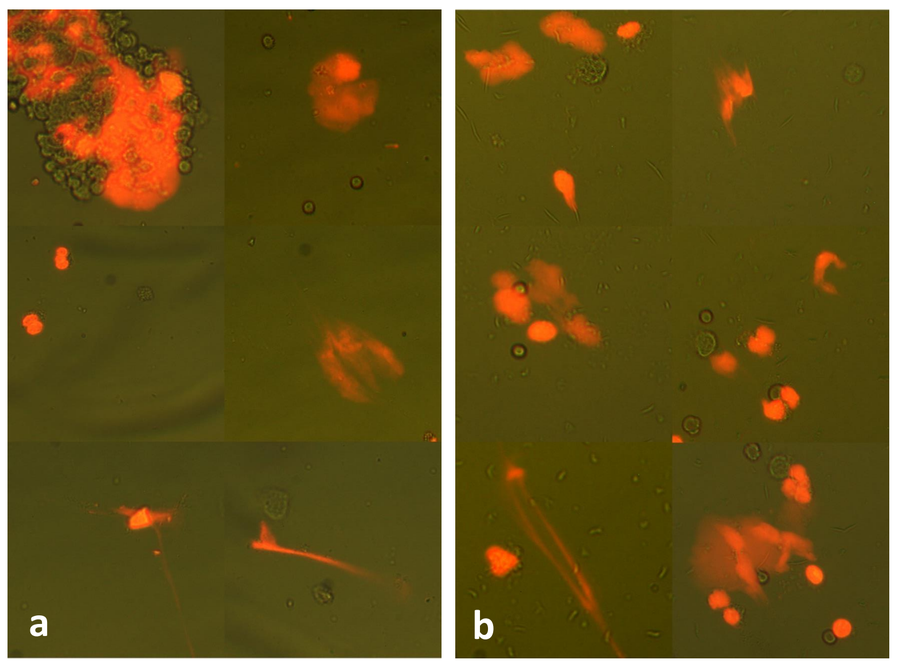

Figure 3. Example of fields of view after exposure of neutrophils from a patient with DFS to a stimulant : a – activation of neutrophils by probiotic; b – activation of neutrophils by thrombin. Fluorescence microscopy, x600.

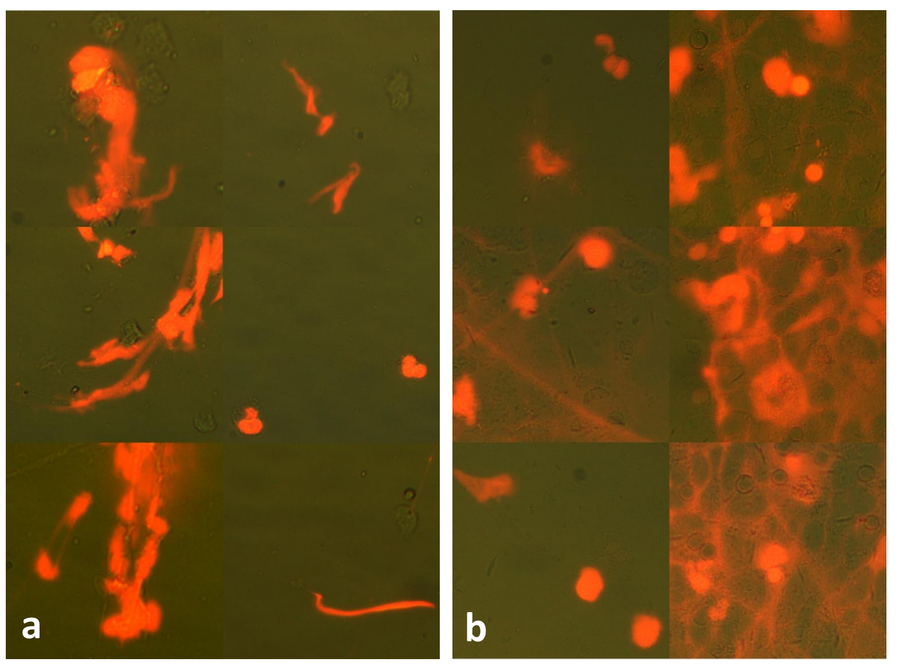

Figure 4. Example of fields of view after exposure of neutrophils from a patient with DOA to a stimulant : a – activation of neutrophils by probiotic; b – activation of neutrophils by thrombin. Fluorescence microscopy, x600.

CONCLUSION

The developed method allows for assessing NET formation in response to nonspecific antigenic stimulation and characterizing potential risks of immunothrombosis progression in DOA.

FUTURE WORK / REFERENCES

1. Novikov D. G., Zolotov A. N., Kirichenko N. A., Mordyk A. V. Method for detection of neutrophil extracellular traps in supravital stained blood preparation. Patent for invention № 2768152. Publ. 23.03.2022, Bull. № 9. (In Russ.)

FUNDING

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