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Elucidating the involvement of neutrophil extracellular traps in hemarthrosis pathophysiology

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Abstract:

BACKGROUND: Hemophilia is an inherited bleeding disorder that could cause many complications, one of which is hemarthrosis. Neutrophils are the predominant immune cells that infiltrate joints after hemorrhage. Tissue injury is often accompanied by the production of neutrophil extracellular traps (NETs), which are DNA constructs containing attached granular enzymes.

AIMS OF STUDY: The aim of this study was to identify the presence of neutrophil extracellular traps including the neutrophil elastase (NE) and myeloperoxidase (MPO), in patients with hemophilia A presented with hemarthrosis.

SUBJECTS AND METHODS: During a period of 8 months from November 2022 to June 2023, 50 persons were recruited cross-sectional study was conducted. In the current study, a sample of 25 individuals with hemophilia A presenting with hemarthrosis were included. Additionally, a control group of 25 unrelated, almost healthy persons,matched in terms of age and sex were also included. NE and MPO levels in blood were measured by flow cytometry technique.

RESULTS: The level of MPO and NE in the blood was significantly higher in hemophilia A patients than controls. In the results of hemophilia A patients, the mean and standard deviation of MPO were 3253.36 +_1865.48, while for NE it was 5229.08+_2667.43. These values were found to be statistically significant $P<0.05$ when compared to the control group. In the control group, the mean and standard deviation of MPO were 2285.48+_811.89, and for NE, it was 3816.92+_1890.45.

CONCLUSIONS: Patients with hemarthrosis had a considerably increase level of NETs in their blood than healthy individuals, and these findings indicate a function of NETs in the pathology of hemophilia A with hemarthrosis.

Keywords:

Hemarthrosis, myeloperoxidase, neutrophil elastase, neutrophil extracellular traps

Introduction

Hemophilia is a medical condition in which the affected patient bleeds heavily for an extended period of time after injuries, operations, teeth extractions, or circumcisions. Constant bleeding after minimal trauma or spontaneous bleeding is an indicator of severe hemophilia.[1] It is characterized by a lack of clotting factors FVIII which result from distinct gene variations or mutations.[2] Hemophilia A affects around 1 in 5000 men.[3] Recurrent joint cavity bleeding is one of the complications that cause synovial hyperplasia, and proliferation of fibroblast-like and macrophage-like synoviocytes is responsible for the production of several molecules involved in angiogenesis and vascular remodeling, as well as inflammatory cytokines such as tumor necrosis factor (TNF)-alpha, interleukin-6 (IL-6), and IL-1 beta. Furthermore, these synoviocytes produce vascular endothelial growth factors to facilitate the process of angiogenesis. These increase fibroblast-like synoviocyte (FLS) growth and chondrocyte death by producing reactive oxygen species.[4] Joint cavity

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bleeding may lead to the release of damage-associated molecular patterns from erythrocytes and other tissues, which in turn may lead to sterile inflammation in the joints and the production of neutrophilic proteins, also known as neutrophil extracellular traps (NETs).[5] This is just one of many molecular mechanisms that contribute to the pathogenesis of hemarthrosis. Neutrophil DNA-histone complexes are protein structures that give rise to the formation of extracellular traps (NETs), which are complex net-like structures that are released by activated neutrophils.[6] Brinkmann *et al*. were the first to characterize NET structures in 2004. They did so as a result of the stimulation of neutrophils with phorbol myristate acetate, lipopolysaccharide, and IL-8. The stimulation is an important factor in the generation of NETs by neutrophils. However, in the end, they all lead to the same consequence, which is the release of NETs after chromatin condensation, nuclear breaks, and subsequent disintegration.[7] These include the chromatin-decondensing enzymes active myeloperoxidase (MPO), neutrophil elastase (NE), and protein-arginine deiminase type 4.[8] The study aims to find out how much neutrophil extracellular traps, neutrophil elastase and myeloperoxidase are present in patients with hemophilia A who have hemarthrosis. To get insight into the underlying pathophysiology of hemarthrosis.

Subjects and Methods

Subjects and design study

This case–control study was conducted between November 2022 and June 2023 by researchers at Al-Karama Teaching Hospital in Wasit and Children Welfare Teaching Hospital in Baghdad, as part of the Department of Pathology and Forensic Medicine in the Faculty of Medicine at Mustansiriyah University. Fifty samples were analyzed, and 25 were from people with hemophilia A with hemarthrosis. Patients with hemophilia were first diagnosed by a hematologist based on patient history and physical examination, and then confirmed with a Factor VIII assay. Information was gathered through in-person interviews with patients using a standard questionnaire that inquired about demographics such as age, sex, height, weight, and family history. The other 25 were collected from people of similar age and sex to the patients, apparently healthy subjects.

Methods

Each participant in the research provided their informed written consent after receiving a comprehensive explanation of the study's objectives and any possible health risks. 2 mL of venous blood was drawn from both patients and controls, which was put in an anticoagulant EDTA K3 tube for hematological parameters and flow cytometer analysis, and the sample was shaken gently and then directly used. A complete blood count (CBC) was done using a fully automated hematology auto-analyzer (Cell-Dyn Ruby manufactured in Germany). The BD FACSCanto II flow cytometry apparatus is used in the identification of NE and neutrophil MPO:

- The markers, CD45 and NE (PE), were combined in a falcon tube. Subsequently, 100 µl of a fresh blood sample was added to the tube. The mixture was then incubated for a duration of 15 min in a dark cabinet
- The 100 µl of intra-assurance A was put into the tube, and then 5 minutes of waiting were consumed. Subsequently, a volume of 2 mL of lysing solution was introduced into the tube, followed by an incubation period of 8 min at ambient temperature in dark environment. The samples were subjected to centrifugation at a speed of 2500 cycles per 5 min, after which the supernatants were carefully extracted. A volume of 50 µl of intra-assurance B was introduced into the tube, followed by the addition of the MPO marker
- The mixture was then left undisturbed for a duration of 15 min
- As a wash solution, add 2 ml of normal saline to the sample, mix it in a vortex, centrifuge it for 5 minutes, and discharge the supernatants
- Next, the second cell is washed for a duration of 5 min, serving as the first washing step
- Following two rounds of cell washes, a volume of 1 mL of cell wash was introduced into the tube. The sample was subjected to flow cytometry using the BD FACSCanto II instrument, and the resulting data were presented.

Ethics approval and consent to participates

Written informed consent was provided by all patients or their legal guardians before entry into the study, and the study protocol was approved by each site's independent ethics committee/institutional review board.

Statistical analysis

The information was analyzed using IBM SPSS-29 (IBM Statistical Packages for the Social Sciences, version 29, Chicago, IL, USA). The measures, such as mean and standard deviation (SD), were used to display the data. When comparing three or more independent means, analysis of variance was used to establish statistical significance, whereas the Student's *t*-test was used to establish statistical significance when comparing two separate means. If $P < 0.05$, then it was regarded to be statistically significant.[9]

Results

In this study, the mean age of the study group was 22.0 ± 11.0 years. There was no statistically significant variance between the study groups ($P \geq 0.05$) in age, as shown in Table 1. The mean of duration of illness was 15.0 ± 6.9, as shown in Table 2. None of the hematology measures showed a statistically significant difference from each other $P \geq 0.05$ in (hemoglobin, packed cell

volume, and red blood cell). A statistically significant decrease ($P < 0.05$) was observed in platelet counts throughout the study groups. The mean±SD of platelet count for patients (256.2 ± 58.1/μL) was lower compared to healthy people (342.1 ± 73.2/μL). There was no statistically significant variance in white blood cell count ($P \geq 0.05$) between the study groups, as shown in Table 3. The findings from Tables 4 and 5 indicate a statistically significant increase ($P < 0.05$) in the values of MPO and NE among patients with hemophilia A who have hemarthrosis, in comparison to the levels seen in healthy subjects. The results (mean ± SD) of MPO of patients were 3253.36 ± 1865.48 compared to healthy subjects (2285.48 ± 811.89), and NE results of patients were 5229.08 ± 2667.43 compared with healthy subjects (3816.92 ± 1890.45).

Table 1: Demographic distribution of hemophilia A patients and healthy subjects dependent on the age groups

Age (years)	Hemophilia A, *n* (%)	Healthy subjects, *n* (%)	*P*
<18	11 (44.0)	13 (52.0)	0.571
≥18	14 (56.0)	12 (48.0)	
Mean±SD	19.0±10.0	21.2±13.1	0.515

SD=Standard deviation

Table 2: Distribution of hemophilia A patients according to the duration of illness

Duration of illness (years)	*n* (%)
<10	6 (24.0)
10–19	12 (48.0)
≥20	7 (28.0)
Mean±SD	15.0±6.9

SD=Standard deviation

Table 3: Hematological parameter of hemophilia A patients versus healthy subjects

Parameter	Hemophilia A	Healthy subjects	*P*
Hb (g/dL)	13.17±2.50	13.30±1.40	0.824
PCV (%)	44.77±8.51	45.21±4.75	0.824
RBC (million cells/μL)	5.24±0.93	4.94±0.36	0.142
Platelets (/μl)	256.2±58.1	342.1±73.2	0.0001#
WBC×10^9/L	7.73±2.51	8.08±1.75	0.571
Neutrophil	4.89±2.33	4.37±1.18	0.331

#There is a statistically significant difference seen between two independent means when doing a Student's *t*-test at a significance level of 0.05. PCV=Packed cell volume, RBC=Red blood cell, WBC=White blood cell, Hb=Hemoglobin

Table 4: Comparison between neutrophil myeloperoxidase between hemophilia A patients and healthy subjects

	Parameter flow MPO (mean±SD)	SEM	*P*
Hemophilia A patients	3253.36±1865.48	373.096	0.021#
Healthy subjects	2285.48±811.89	162.378	

#There is a statistically significant difference seen between two independent means when doing a Student's *t*-test at a significance level of 0.05. SEM=Standard error of the mean, SD=Standard deviation, MPO=Myeloperoxidase

Table 5: Comparison between neutrophil elastase between hemophilia A patients and healthy subjects

	NE (mean±SD)	SEM	*P*
Hemophilia A patients	5229.08±2667.43	533.487	0.036#
Healthy subjects	3816.92±1890.45	378.090	

#There is a statistically significant difference seen between two independent means when doing a Student's *t*-test at a significance level of 0.05. SEM=Standard error of the mean, SD=Standard deviation, NE=Neutrophil elastase

Discussion

Identifying the process of inflammation that causes joint damage in hemophilia A patients is important for treating their major complications. This is because neutrophils make up a large portion of human leucocytes, and has a major role in the body's immune system and inflammatory processes. Table 3 shows that there are no differences in the results of the CBC between patients and healthy people, due to patients' commitment to treatment as well as the awareness of patients' families in hospital visits for examination and follow-up of everything related to the condition of their children. Except, there is a significant difference in platelet, $P = 0.0001$, and the reason may be attributed to the presence of infection, as in the study of Yaguchi *et al*., 2004,[10] Fogagnolo *et al*., 2022,[11] and Campo *et al*., 2020.[12] The other cause may be due to patients with hemarthrosis use nonsteroidal anti-inflammatory drugs (NSAIDs) as an analgesic for joint pain, and the role of NSAIDs in inhibiting blood platelets is known, as in the study of Bedene *et al*., 2020,[13] which clarified the relationship between the use of NSAIDs and a decrease in platelets. The results shown in Tables 4 and 5 indicate a statistically significant elevation in the levels of neutrophil MPO and NE in the blood of patients compared to those of individuals in good health. The release of cytokines, including TNF-alpha, IL-6, IL-1 beta, and pro-angiogenic chemicals, into the bloodstream is a consequence of joint hemorrhage, which initiates a persistent inflammatory response. The pro-inflammatory cytokines, such as TNF-alpha and IL-17, have been seen to enhance the production and protein configuration of neutrophil extracellular traps (NETs), leading to their subsequent release.[14] When MPO is activated, granular NE is also released and transported to the nucleus, where it cleaves and alters core histones;[15,16] our study agreed with Oneto *et al*., 2022,[17] van Vulpen *et al*., 2021,[18] and Kaminski *et al*., 2020.[5] Melchiorre *et al*., 2017,[19] confirmed that the pathogenesis of hemarthrosis has several aspects between rheumatoid arthritis and osteoarthritis of both clinical and biological features, such as synovitis, bone resorption, and articular cartilage deterioration. Carmona-Rivera *et al*., 2020,[20] Clarke, 2020,[21] and Song *et al*., 2020,[22] all established that

NETs are critical synovial structures that orchestrate inappropriate immune responses, inflammation, and joint destruction, so when synovial neutrophils interact with FLS, an inflammatory stimulus to a pathogenic immune response is set to occur. Some evidence suggests that synovial NE actually degrades cartilage components rather than only boosting inflammatory pathways in FLS and macrophages.

Conclusions

Patients who developed hemarthrosis had a significantly elevated level of NETs in their blood compared to persons who were considered to be healthy. These findings suggest that NETs have a role in the development of hemarthrosis in individuals with hemophilia A.

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Conflicts of interest
There are no conflicts of interest.

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