



The Understanding of the Importance of Oxidant/antioxidant Status in Hemophilia Patients with Arthropathy

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Received: 7 July 2025 / Accepted: 16 October 2025 / Published online: 6 November 2025
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Abstract

This study aimed to investigate the roles of pro-inflammatory cytokine [interleukin-1 β (IL-1 β)], receptor activator of nuclear factor-kappa B ligand (RANKL), prooxidant [reactive oxygen species (ROS), thiobarbituric acid reactive substances (TBARS), advanced oxidation protein products (AOPP), advanced glycation end-products (AGEs)], and antioxidant markers [ferric reducing antioxidant power (FRAP), total-thiol content (t-SH)] in the pathogenesis of arthropathy in patients with hemophilia (PwH) with/without arthropathy, and to identify potential therapeutic targets for preventing arthropathy and improving quality of life. The study included 18 PwH without arthropathy [31.0 years; (13–65)] and 39 PwH with arthropathy [10 years; (3–36)]. Among PwH with arthropathy, 6% were inhibitor positive, and 26.3% had involvement of more than two joints. Serum ROS and AGE were measured by fluorometric method, while TBARS, AOPP, FRAP, and t-SH were assessed via spectrophotometrically. IL-1 β and RANKL concentrations were determined using ELISA. AGE and RANKL concentrations were significantly higher in PwH with arthropathy compared to those without ($p=0.002$ and $p=0.010$, respectively), and differences remained significant after age adjustment. FRAP and t-SH levels were initially elevated in PwH with arthropathy ($p=0.030$ and $p=0.007$, respectively), but differences lost significance after age adjustment. No significant differences were observed in ROS, TBARS, AOPP, or IL-1 β levels between groups. Elevated AGE and RANKL levels in PwH with arthropathy suggest their potential involvement in joint damage and highlight the importance of maintaining oxidant/antioxidant balance. Therefore, monitoring these biomarkers may provide supportive information for early detection and inform future research on preventive strategies to reduce joint complications and preserve long-term quality of life in hemophilia.

Keywords Hemophilia A · Hemophilic arthropathy · Pro-oxidant/Antioxidant balance · Reactive oxygen species

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Introduction

Hemophilia is an X-linked congenital bleeding disorder caused by a deficiency of coagulation factor VIII (FVIII) in hemophilia A or factor IX (FIX) in hemophilia B. The major complication of severe hemophilia is spontaneous or trauma-induced bleeding, which predominantly affects large synovial joints such as the elbows, knees, and ankles [1].

Several studies have conducted that bleeding into the intra-articular cavity results in a triple pathology consisting of synovial inflammation, cartilage damage and angiogenesis [2]. For normal cellular functions, tight regulation of the iron homeostasis is crucial. During the early stages of joint damage, exposure of chondrocytes to blood and iron directly contributes to osteochondral injury. Following an acute hemarthrosis, the intra-articular blood is removed from the joint cavity by the synovial cells, along with macrophages and other inflammatory cells [3]. The presence of hemosiderin deposits has been remarked as a critical finding for early phase of hemophilia [4]. In repetitive or continuing bleeding, the accumulation of iron as the hemosiderin deposits are responsible for the destructive effect by inducing proinflammatory cytokines, and stimulates synovial inflammation and angiogenesis [5, 6]. Angiogenic factors including vascular endothelial growth factor (VEGF), endothelial growth factor (EGF) which are secreted in response to bleeding may also enhance neovascularization even in joints not yet affected by arthropathy [7].

In hemophilia patients, the destruction of joint tissue because of recurrent bleeding is the main complication of the disease, causing movement restriction and significant morbidity. Iron-catalyzed oxygen metabolites may contribute the persistent cartilage damage by stimulating the peroxidation of membrane lipids, mitochondrial dysfunction, cellular protein and nucleic acid damages, and ultimately ferroptosis [6–8]. Reactive oxygen species (ROS) contain unstable metabolites of molecular oxygen (O_2) such as superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$) and hydrogen peroxide (H_2O_2), mostly produced in the mitochondria. These species are involved in oxidative stress responses and the regulation of antioxidant enzymes. Advanced oxidation protein products (AOPP), formed under oxidative stress conditions, further stimulate increase ROS generation via cytosolic superoxide generation [9, 10], and act through the receptor of advanced oxidation end-products (RAGE) in endothelial cells and induce endothelial dysfunction like advanced glycation end products (AGEs). In the presence of iron, H_2O_2 may be converted into highly reactive and toxic $HO^{\cdot}$, promoting in the apoptosis of chondrocytes [9, 10]. AOPPs are also recognized as the stimulators of vascular inflammation, and increases in AOPPs have been shown in both experimental and human atherosclerotic lesions

associated with endothelial dysfunction and monocyte activation [9, 11]. AGEs are irreversible, non-enzymatically formed protein crosslinks with a long half-life. Studies have showed that accumulation of AGEs increases with aging, hyperglycemia, chronic inflammation, and oxidative stress. In hemophilia, iron deposition in synovial tissues fosters oxidative stress and promotes the production of interleukin (IL)-1 β , IL-6, tumor necrosis factor-alpha (TNF- α), and interferon-gamma (IFN- γ), contributing to synovial hypertrophy and cartilage degeneration in patients with hemophilic arthropathy [12]. These oxidative mediators have also been demonstrated to activate gene transcription pathways, notably nuclear factor kappa B (NF- κ B), and enhance the expression of receptor activator of NF- κ B ligand (RANKL) [13, 14]. RANKL, a transmembrane ligand expressed by lymphocytes and synovial cells, promotes osteoclastogenesis and bone resorption by acting synergistically with cytokines such as TNF- α , IL-1, and IL-17 [15]. Increased proinflammatory cytokines might be the reason of high expression of RANKL in the patients with hemophilia (PwH) [16]. Additionally, proinflammatory cytokines have been demonstrated to induce hydrogen peroxide production by chondrocytes, exacerbating oxidative damage in hemophilic arthropathy [17].

The aim of this study is to reveal the roles of proinflammatory cytokine IL-1 β and RANK-L, prooxidant (ROS, TBARS, AOPP, and AGEs), and antioxidant factors (FRAP and t-SH) in the pathogenesis of arthropathy in hemophilic patients with and without arthropathy in order to prevent arthropathy development and maintain the quality of life of PwH.

Materials and methods

Study Group

The study group comprised 57 male patients with severe Hemophilia A who were diagnosed and regularly followed at Hematology Department, Oncology Institute, Istanbul University. The participants were stratified into two groups based on the presence of arthropathy; group 1 ($n=18$): PwH without arthropathy [median age: 10 years (range: 3–36)], and group 2 ($n=39$): PwH with arthropathy [median age: 31 (range: 13–65)]. The diagnosis of hemophilic arthropathy was established through comprehensive physical examination and radiologic evaluation, including X-radiography and ultrasonography. For the investigation of early soft tissue changes, ultrasonographic examination was particularly utilized [18]. Demographic and clinical data including body mass index (BMI), inhibitor status, and smoking habits were recorded. Individuals with acute infection, chronic hepatitis,

renal, cardiac or autoimmune/rheumatological diseases were excluded from the study to avoid confounding factors.

Approximately 75% of the PwH received treatment with recombinant FVIII (rFVIII). Additionally, therapeutic agents including fitusiran, emicizumab (a bispecific monoclonal antibody), activated Prothrombin Complex Concentrate (aPCC), marstacimab, and concizumab were used in patients with inhibitor development.

Blood Sampling

Venous blood samples were collected from PwH into serum separator tubes (SST, Becton Dickinson, Plymouth, UK) at the Department of Hematology, Oncology Institute, Istanbul University between January and August 2024. Samples were transferred to the Department of Medical Biochemistry within 15 min, followed by centrifugation at 2000×g for 15 min. The serum samples were aliquoted and stored at −80 °C until biochemical analyses were performed.

Determination of Oxidative Stress Markers

The generation of ROS and AGE levels in serum samples were quantified using fluorometric methods. The fluorescence intensity of the final product was measured using a fluorometric microplate reader (Thermo Electron Corporation Fluoroskan Ascent, FL) and expressed in relative fluorescent units (RFU) [19, 20]. The measurement of TBARS, AOPP, FRAP, and t-SH concentrations were determined using spectrophotometric methods according to established protocols [21–24].

Table 1 The clinical and demographic characteristics of the patients with hemophilia (PwH)

	PwH without arthropathy (n=18)	PwH with arthropathy (n=39)	p value*
Age (years)	10.0	31.0	0.001
(median; min-max)	(3–36)	(13–65)	
BMI (kg/m ²)	22.5	24.8	0.066
(median; min-max)	(12.8–34.8)	(14.5–37.2)	
Age (<18 years/>18 years) (%)	14 (77.8)/4 (22.2)	8 (20.5)/31 (79.5)	0.001
Inhibitor positive (%)	11%	16%	NS
Inhibitor titer (BU)	4.5	6.1	NS
Walking disorder (yes/no)	2/16	25/39	NS
Smoking (yes/no) (%)	17 (94.4)/1 (5.6)	25 (64.1)/14 (35.9)	0.016

*Pearson's χ^2 test was used for categorical variables and Independent Samples t test or Mann Whitney U test were used for continuous variables.

Determination of IL-1 β and RANKL Levels

Serum IL-1 β and RANKL concentrations were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA) using commercial kits (#E-EL-H0149 and #E-EL-H5313, Elabscience, Houston, Texas, USA) according to the manufacturer's instructions. The presence and titer of anti-FVIII inhibitors were determined using the Bethesda assay [25].

Statistical Analysis

All statistical analyses were carried out using Jamovi 2.5, SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA), and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). The normality of distribution for continuous variables was assessed using the Shapiro–Wilk test, supplemented by examination of skewness and kurtosis. In the presence of non-normal distributions, appropriate transformations were applied: square-root for IL-1 β , and log₁₀ for ROS, MDA, and AOPP. Descriptive statistics are expressed as mean $\pm$ standard error (SEM) for normally distributed variables, and as median (minimum–maximum) for non-normally distributed ones. Group comparisons were made using the Independent Samples *t* test or Mann–Whitney *U* test, with chi-squared tests for categorical data. Since the groups were not age-matched, analysis of covariance (ANCOVA) was used to adjust for potential confounding effects of age and, when relevant, smoking status. In ANCOVA models, effect sizes are reported as partial eta squared (η^2p). According to Cohen's (1988) criteria, η^2p values of 0.01, 0.06, and 0.14 indicate small, medium, and large effects, respectively. Bonferroni-adjusted post hoc comparisons were applied when appropriate. Correlations were analyzed using Pearson's or Spearman's tests, with partial correlations controlling for covariates. Bonferroni corrections were applied where appropriate. A two-tailed *p*-value < 0.05 was considered statistically significant.

Results

The clinical and demographic characteristics of PwH were shown in Table 1. The median age was 10 years (range, 3–36) in PwH without arthropathy and 31 years (range, 13–65) in those with arthropathy (*p* = 0.001). Overall, 38.6% of patients were younger than 18 years. 39 of 57 (61.4%) PwH had hemophilic arthropathy, and more than two joints affected in 12 patients (31.6%).

Inhibitors were detected in 16% of PwH with arthropathy, and 10 of 38 patients (26.3%) had involvement of more than two joints. The inhibitor rate was 11% among the PwH without arthropathy. BMI of both groups was similar

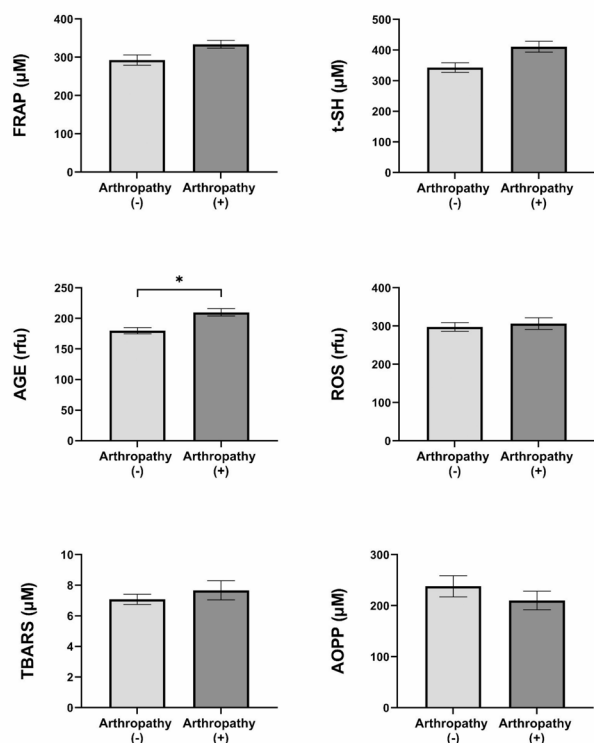


Fig. 1 The serum levels of pro-oxidant and anti-oxidant parameters in individuals with and without hemophilic arthropathy (Mean±SEM)

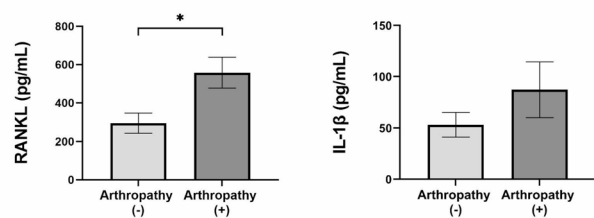


Fig. 2 The serum levels of pro-inflammatory cytokines in individuals with and without hemophilic arthropathy (Mean±SEM)

(22.5 vs. 24.8 kg/m²). The percentage of smoking habit was higher in the PwH with arthropathy group than that of PwH without arthropathy ($p=0.016$) (Table 1).

Analysis of oxidative and antioxidative parameters revealed significantly higher FRAP and t-SH concentrations in PwH with arthropathy compared to those without [329.2 μM (240.8–533.4) vs. 292.6 μM (188.9–422.1), $p=0.023$ for FRAP; 413.6 μM (250.4–522.9) vs. 353.5 μM (198.8–456.6), $p=0.007$ for t-SH]. AGE levels were also significantly higher in the PwH with arthropathy compared to the PwH without arthropathy [208.8 RFU (104.2–287.2) vs. 183.1 (146.5–221.8), $p=0.003$]. The concentrations of ROS, TBARS, and AOPP remained unchanged between both PwH groups ($p=0.825$, $p=0.714$, $p=0.364$, respectively) (Fig. 1). The RANK-L concentrations of the arthropathy group were significantly higher compared to the

non-arthropathy group [502.5 pg/mL (32.8–1050) vs. 201.2 (73.9–898.8); $p=0.019$], whereas no significant difference in serum IL-1β levels were obtained between both groups [55.8 pg/mL (5.41–262.5) vs. 38.2 (0.54–158.1); $p=0.330$] (Fig. 2).

When we adjusted according to the age and smoking habit, differences in the concentrations of FRAP and t-SH between both groups of the hemophilic patients lost statistical significance ($p=0.097$, and 0.136, respectively). In contrast, the significance in AGE and RANK-L of both groups remained unchanged after adjusting for age. When age was added as a covariate for IL-1β, the group effect approached a significance ($p=0.093$).

Correlation analyses adjusted for age demonstrated significant associations between BMI and FRAP ($r=0.47$, $p<0.001$), and between IL-1β and AOPP levels ($r=0.43$, $p=0.029$). However, when both age and smoking were included in the model, only the correlation between BMI and FRAP persisted ($r=0.47$, $p<0.001$).

Discussion

In this study, the levels of oxidative/antioxidant stress markers and proinflammatory cytokines which are key mediators of the pathway in bone remodeling, were investigated in PwH with and without arthropathy. In addition, potential risk factors for arthropathy development including the presence of inhibitor, treatment strategy, BMI, and smoking habits were compared between PwH with and without arthropathy. According to the results of this study, the arthropathy rate was 67.8% in all hemophilia patients and positive inhibitors were detected in 16% of PwH with arthropathy. These findings were consistent with previous reports, which documented inhibitor rates ranging from 9.7% to 24.5% in severe hemophilia [26, 27]. The rate of joint involvement was also similar with the other studies [24]. Although obesity has been linked to increased susceptibility to hemarthrosis due to excess mechanical loading [28], the BMI of both groups were compared between the groups, but no significant changes were observed [28]. Nevertheless, prior studies have suggested that a BMI of less than 18 kg/m² may be associated with a lower incidence of arthropathy seen in patients with hemophilia [29].

Our results demonstrated significantly elevated AGE levels in PwH with arthropathy compared to those without, whereas TBARS and ROS concentrations did not differ. These results remained unchanged after adjusting for age. Interestingly, higher t-SH and FRAP levels were also observed in the arthropathy group, though these differences lost significance after adjusting for age. AGEs are known as an accelerator of oxidative stress and inflammation, and

their accumulation has been linked to ferroptosis, a form of cell death driven by iron-dependent lipid peroxidation [30]. Beyond their role in ferroptosis, AGEs also disrupt collagen synthesis by enhancing the degradation and glycation. The glycation of collagen causes changes in the architecture, mass, and electrical charge of collagen fibers, making the collagen less soluble and less flexible, and associated with decreased bone strength [31]. Normally, NOX4 and nuclear factor E2-related factor 2 (Nrf2) work together to maintain redox homeostasis, limiting AGE and ROS mediated damage. NOX4 can produce hydrogen peroxide, while Nrf2-regulated antioxidants and glutathione can prevent excessive ROS accumulation by neutralizing ROS and oxidant metabolites [32]. Beside the effectiveness of compensatory mechanism, the slow formation and long half-life of the AGE molecules compared to the ROS and AOPP may also explain unchanged concentrations of ROS, AOPP, TBARS between both hemophilic groups [33].

Importantly, excessive AGE accumulation has been implicated in several chronic diseases, including cartilage degeneration, through enhancement of ROS production and impairment of DNA repair mechanisms [32, 34]. Additionally, prior studies have demonstrated the deleterious impact of ROS and lipid peroxidation products such as TBARS on synovial tissues in hemophilic arthropathy [35, 36]. Moreover, iron deposition within synoviocytes has been shown to promote hydroxyl radical generation, chondrocyte apoptosis, and reduced proteoglycan synthesis [35, 37].

Antioxidant defense mechanisms are known to play a role in many chronic bone diseases, such as rheumatoid arthritis, osteoporosis and hematologic diseases [38]. However, until today, there are limited studies to show the importance of antioxidant defense mechanisms on bone/cartilage destruction in hemophilia patients. Nevertheless, some studies have demonstrated the association between decreased plasma antioxidant levels, antioxidant enzymes such as glutathione reductase (GR), superoxide dismutase (SOD), and osteoporosis [39]. There are studies to note the antioxidants including vitamin C, glutathione, and N-acetylcysteine (NAC) might have beneficiary effect on bone in individuals with osteoporotic patients [40]. Depending on our results, increased FRAP, and t-SH levels in the hemophilic arthropathy group, may be indicative of increased antioxidant responses to maintain the oxidant/antioxidant balance against high concentrations of AGE in PwH with arthropathy. Nevertheless, other studies reported altered thiol-disulfide homeostasis without a consistent increase in antioxidant capacity among hemophilia patients [38, 41].

Oxidative stress and pro-inflammatory cytokine development are interrelated and interdependent mechanisms. The hemosiderin and ferritin also cause the secretion of inflammatory cytokines (TNF- α , IL-6, IL-1- β) from type

A synoviocytes, resulting in inflammation, and synovial hyperplasia [42]. All these factors act on T cells, fibroblasts, and osteoclasts, ultimately leading to the degradation of articular cartilage and subchondral bone tissue. It has been reported that the destructive effect of proinflammatory cytokines on synovium and cartilage occurs through IL-1 β and TNF- α and is strengthened by IL-6 [43]. IL-1 β has degenerative effect directly on the joints by inducing some enzymes, and reduction the synthesis of collagen type II. At the same time, it damages the structure of cartilage by increasing ROS and oxidative stress generating the production of HO $\cdot$ radicals [44, 45]. Elevated synovial IL-1 β levels have been demonstrated in hemophilic patients compared with non-hemophilic controls [3]. Although we did not detect significant intergroup differences in serum IL-1 β , the group effect approached significance after adjusting for age. TNF- α has also destructive effect on the synovium by inhibiting both proteoglycan and type II collagen synthesis, as well as stimulating metalloproteinase activity. While some studies reported decreased levels of IL-1 β , IFN- γ , and/or TNF- α in hemophilia patients, some studies have revealed an increase in IL-1 β concentration in synovial fluid following injury [13, 46]. In an experimental study, intra-articular injection of whole blood was shown to cause long-term inhibition of cartilage matrix proteoglycan synthesis with only 4 days of blood exposure [37]. Supporting the findings of this study, a transient increase in inflammatory macrophages was demonstrated, accompanied by an increase in inflammatory cytokine (IL-1 β) levels during acute bleeding, and an increase in angiogenic and bone remodeling factors was demonstrated following recurrent bleeding [47].

We also observed significantly higher RANK-L concentrations in PwH with arthropathy, consistent with the results of Anagnostis et al. [48]. Given the importance of the RANK/RANKL/osteoprotegerin (OPG) axis in bone turnover, elevated RANK-L may contribute to cartilage destruction and synovial changes in hemophilic arthropathy [17]. Oxidative stress has been shown to upregulate RANK-L while downregulating OPG via MAPK signaling pathways [49]. Therefore, increased osteoclastic activation is not completely compensated with bone formation by OPG in the synovial tissue of the hemophiliacs [50]. Decreased expression of OPG with increased expression of RANK and RANKL in the synovium was reported in hemophilic arthropathy patients [3]. All these findings may indicate the bone destruction of hemophilic patients are complex and multifactorial and should be taken into consideration in the selection for the prevention and treatment options of arthropathy.

This study has several limitations, including the relatively small sample size and the lack of age-matched study groups. Furthermore, the absence of detailed data on intra-articular

bleeding frequency or annual bleeding rates limits differentiation between acute and chronic arthropathy. These factors may also account for the absence of significant differences in AOPP and TBARS between groups.

According to our results, preserving the oxidant/antioxidant balance is crucial in preventing arthropathy in patients with hemophilia, as well as in protecting synovial tissue and cartilage during the acute phase following bleeding. Antioxidant-based strategies, such as sirtuins or combined glutathione and vitamin C therapy, administered locally or via extracellular vesicles (PEVs), may represent promising approaches to mitigate joint damage and reduce long-term complications.

Our findings underscore the potential value of timely monitoring of biomarkers such as prooxidant markers and RANKL for identifying hemarthrosis sequelae during the asymptomatic, early, and potentially reversible phase of disease. While further studies are warranted to confirm these observations, targeted antioxidant strategies may hold promise in preventing progressive joint damage and preserving long-term quality of life in patients with hemophilia.

Funding This work received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Data Availability All data generated or analyzed during this study is included in this published article.

Declarations

Conflict of interest The authors have declared that there is no conflict of interest.

Ethical Statement All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent The study was approved by the independent ethics committee/institutional review board at Istanbul University Istanbul Faculty of Medicine, Clinical Research Ethical Committee. Ethical Committee convened on 25.07.2024 and received the protocol number 2746053.

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