BLOOD RESEARCH
VOLUME 53 · NUMBER 1
March 2018
ORIGINAL ARTICLE

Global hemostatic assay of different target procoagulant activities of factor VIII and factor IX

Ki-Young Yoo[1], Soo-Young Jung[1], Sung-Ho Hwang[2], Su-Min Lee[2], Jong-Ho Park[2], Hyun-Ja Nam[2]

[1]*Korea Hemophilia Foundation, Seoul,* [2]*Mogam Institute for Biomedical Research, Yongin, Korea*

p-ISSN 2287-979X / e-ISSN 2288-0011
https://doi.org/10.5045/br.2018.53.1.41
Blood Res 2018;53:41-8.

Received on June 26, 2017
Revised on October 11, 2017
Accepted on October 29, 2017

*This study was supported by a grant from the Korea Hemophilia Foundation.

Correspondence to
Ki-Young Yoo, M.D., Ph.D.
Korea Hemophilia Foundation,
70 Saimdang-ro, Seocho-gu, Seoul 06641, Korea
E-mail: gowho@hanmail.net

Background
Korean National Health Insurance reimburses factor VIII (FVIII) and factor IX (FIX) clotting factor concentrate (CFC) infusions to discrepant activity levels, allowing elevation of FVIII activity to 60 IU/dL and FIX to 40 IU/dL. We aimed to assess hemostatic response to these target levels using global hemostatic assays.

Methods
We enrolled 34 normal healthy men, 34 patients with hemophilia A, and 36 with hemophilia B, with residual factor activity of 3 IU/dL or less and without inhibitors. Patients with hemophilia A and B received injected CFCs according to reimbursement guidelines. Fifteen minutes after injection, we assessed hemostatic response with global hemostatic assays: thrombin generation assay (TGA), thromboelastography (TEG), and clot waveform analysis (CWA).

Results
Normal healthy men and patients with hemophilia A and B were 36.7, 37.2, and 35.1 years old, respectively. FVIII and recombinant FIX concentrate doses were 28.8 IU/kg and 43.6 IU/kg. Post-infusion FVIII activity rose from 0.5 IU/dL to 69.4 IU/dL, while FIX activity rose from 1.4 IU/dL to 46.8 IU/dL. Post-infusion peak thrombin concentrations in hemophilia A and B were 116.6 nM/L and 76.4 nM/L ($P<0.001$). Post-infusion endogenous thrombin potential (ETP) in hemophilia A and B was 1349.8 nM/min and 915.6 nM ($P<0.001$). TEG index of hemophilia A and B was 0.11 and −0.51 ($P=0.006$).

Conclusion
Current reimbursed doses for FIX concentrates are insufficient to achieve hemostatic responses comparable to those after reimbursed doses for FVIII concentrates in terms of peak thrombin concentration, ETP, and TEG index.

Key Words Hemophilia A, Hemophilia B, Thrombin

INTRODUCTION

Hemophilia is the most common hereditary bleeding disorder caused by a deficiency of coagulation factors. Patients with hemophilia A and hemophilia B lack factor VIII (FVIII) and factor IX (FIX), respectively. According to plasma procoagulant activity, hemophilia is classified as severe (<1 IU/dL), moderate (1–5 IU/dL), or mild (>5 IU/dL) [1]. Patients with hemophilia can bleed into joints and/or muscles after minimal trauma or even spontaneously. In the event of bleeding, the deficient factor should be replaced with clotting factor concentrate (CFC) to a sufficient activity level as soon as possible [2]. Bleeding occurs most commonly into joints, and prompt infusion of CFC is crucial to effectively stop bleeding. However, the recommended factor activity levels to treat hemarthrosis in hemophilia A and hemophilia B differ among various reports. According to some studies [2, 3], the desired activity levels of FVIII and FIX are equal, while in others the desired activity level of FIX is lower than that of FVIII [4, 5].

More than 95% of Korean patients with hemophilia B have been exposed to recombinant factor IX concentrates (rFIX) since 2003. According to the reimbursement guidelines of the Korean National Health Insurance (NHI) system, the rFIX dose for which reimbursement is provided to treat

mild to moderate bleeding episodes was limited until 2014 to an increase in FIX activity to 30 IU/dL. The dose of rFIX to stop moderate bleeds was then escalated to increase the FIX activity level to 40 IU/dL. The expanded coverage, however, is still lower than the dose recommended by the World Federation of Hemophilia (WFH) guidelines or the reimbursed dose to treat moderate bleeds experienced by patients with hemophilia A, which is established at a level to reach 60 IU/dL of FVIII activity.

This discrepancy in coverage may be explained by some reports that have suggested different phenotypes for hemophilia A and B. Patients with hemophilia B bleed 35% less frequently [6], as well as less severely [7], than patients with hemophilia A. In severe hemophilia A, the median age at first hemarthrosis is 1.9 years, as opposed to 2.4 years in severe hemophilia B [8]. Hemophilic arthropathy is less common in patients with hemophilia B than in patients with hemophilia A [9]. The relatively milder phenotype of patients with hemophilia B may be explained by less common severe gene defects and more detectable FIX:Ag [10]. Moreover, intra-articular FVIII activity level is less than 1% of the factor level found in normal pooled plasma, while intra-articular FIX activity level is about 10% [11]. These findings seem to support the current reimbursement guidelines of the Korean NHI recommending lower doses of FIX CFC per infusion than of FVIII.

On the other hand, other recent reports have indicated that the phenotypes of hemophilia A and B are similar [12] or that the differences are not statistically meaningful [8]. Considering the basic pathophysiology, the symptoms of hemophilia are principally related to the lack of generation of thrombin, due to the inability to form the tenase and prothrombinase complexes during the amplification phase in the coagulation process. Although FVIII and FIX have different mechanisms of action in inducing coagulation, the outcome of replacing deficient factors in hemophilia A and B should, in both cases, be thrombin generation. Hence, the WFH guidelines for the management of hemophilia have recommended the same target pro-coagulant activities to manage hemarthrosis and muscle bleeding, regardless of the type of hemophilia [2]. However, in cases of life-threatening bleeding or other forms of major bleeding such as iliopsoas muscle bleeding, the WFH guidelines recommend smaller amounts of FIX concentrate. Although the reason for this recommendation is not stated in the guidelines, it can be presumed that high FIX activity levels may result in undesirable thrombosis. Individuals with high FIX activity (>129 IU/dL) are exposed to a more than two-fold increased risk of deep vein thrombosis [13].

The aim of the present study was to compare hemostasis induced by the doses of factor VIII and factor IX for which reimbursement is provided by the Korean NHI for hemarthrosis and muscle hematoma, which are common moderate bleeds seen in clinical practice. To evaluate hemostatic efficacy more objectively, the investigators adopted laboratory assessments rather than clinical observations or patient ratings regarding hemostatic efficacy. Global hemostasis assays such as thrombin generation assay (TGA), thromboelastography (TEG), and clot waveform analysis (CWA) were used.

MATERIALS AND METHODS

Study population

Subjects were recruited at the Korea Hemophilia Foundation from October 2016 to March 2017. Inclusion criteria were age of 15 years and older, and residual clotting factor activity of less than 3 IU/dL, based on a cohort study finding that the first joint bleeding that represents a more frequent bleeding phenotype occurs significantly earlier in patients who have less than 3 IU/dL residual clotting factor activity [14]. Although two kinds of recombinant FIX concentrates are available in Korea, only nonacog gamma was used in order to minimize any potential impact on the results of activated FIX content and potency variation [15]. Regarding hemophilia A, only users of full-length FVIII were included in the study. We also enrolled normal healthy males as a reference group. Patients with hemophilia with inhibitors or in a bleeding state at the start of this study were excluded. To compare clinical phenotypes, we reviewed the number of annual joint bleeds in participants with hemophilia A and B for the past 12 months at the time of enrollment. This study was approved by the institutional review board of Korea Green Cross Laboratories and informed consent was obtained from all subjects.

Laboratory assessments

We tested for the presence of inhibitors with the Bethesda assay prior to the first injection of CFCs. One Bethesda unit (BU) is defined as the amount of an inhibitor that will neutralize 50% of 1 unit of FVIII:C in normal plasma after 120 minutes incubation at 37°C. The cutoff value of inhibitor positivity was 0.6 BU/mL. We measured baseline aPTT and performed one-stage factor assays for each patient. To assess hemostatic effect, we conducted global hemostatic assays (TGA, TEG, and CWA), and also assessed in-vivo recovery (IVR) at 15 minutes after injections of CFCs. As controls, one-stage clotting assays of FVIII and of FIX, as well as TGA, TEG, and CWA were assessed in normal healthy males. aPTT was measured with a CS-2500 automated coagulation analyzer (Sysmex Corporation, Kobe, Japan) using Dade Actin FS (Siemens Healthcare Diagnostics, Marburg, Germany) reagent. The reagents used for one-stage clotting assays were coagulation factor VIII and IX deficient plasma (Siemens Healthcare Diagnostics, Marburg, Germany), and the coagulometer was a CS-2500 automated coagulation analyzer. TGA was measured with a Techno Thrombin TGA kit (Technoclone GmbH, Vienna, Austria), and the fluorescence reader was FLx800 (BioTek Instruments, Inc., Winooski, VT, USA). TEG was assessed with ROTEM (TEM International, Munich, Germany) using citrated whole blood that had been fixed at room temperature for 30 minutes after adding calcium. TEG index (normal range, -2–+2) was calculated with the following equation: (-0.1227) CT+

(-0.092) CFT+(0.1655) MCF -7.7922 [16]. The reagent for CWA was Dade Actin FS and the equipment was a CS-2500 automated coagulation analyzer. MIN2 (maximum acceleration) of CWA was derived from the 2nd derivative of aPTT. After taking 9 mL of whole blood for baseline tests, we infused FVIII concentrates to reach 60 IU/dL of FVIII:C in patients with hemophilia A, while for patients with hemophilia B, we infused FIX concentrates to reach 40 IU/dL of FIX:C, according to the reimbursement guidelines of the Korean NHI. At 15 minutes after injection of FVIII and FIX concentrates, we obtained the same volume of whole blood from each patient through different veins. To produce platelet-poor plasma (PPP), samples preserved in an evacuated, 3.2% sodium citrate tube were centrifuged at a 3000 rpm for 10 minutes and stored frozen at -70°C. PPP was thawed in a 37°C water bath for 5 minutes and subjected to TGA and CWA.

Assessment of hemostatic responses

As the end product in the coagulation process is thrombin generation, comparable levels of thrombin generated by FVIII and FIX concentrate replacement may be a good indicator of comparable hemostatic responses, in spite of the difference in mechanism of actions of FVIII and FIX in the clotting process. Thrombin generation can be analyzed by the thrombin generation curve (thrombogram). Four parameters characterize a specific thrombogram; the lag time (min), the peak thrombin concentration (nM/L), the maximal velocity of thrombin generation (nM/min), and the endogenous thrombin potential (ETP). The primary endpoint in the present study was the peak thrombin concentration, which reflects "thrombin bursting" and discriminates clinical severity [17]. ETP, TEG index, and MIN2 were secondary endpoints.

Statistical analysis

Based on our pilot study of 19 patients with severe hemophilia (12 patients with hemophilia A and 7 patients with hemophilia B), the study population size was determined as 34 per group with a 5% significance level and 80% power. Parameters that displayed normal distribution were analyzed with one-way analysis of variance (ANOVA) or the two-sample t-test. Nonparametric data were analyzed with the Mann-Whitney or Kruskal-Wallis test, with Bonferroni correction as the post-hoc test. All mean values of each parameter falling within the range of normal distribution were included in the analysis. Values outside the normal distribution range were substituted with median values.

RESULTS

Study population

A total of 104 males (34 normal healthy males, 35 patients with hemophilia A, 38 patients with hemophilia B) were enrolled in the study. The mean FVIII:C and FIX:C of normal healthy males were 129.5 IU/dL and 105.8 IU/dL, respectively. The median baseline FVIII:C of patients with hemophilia A and FIX:C of patients with hemophilia B were 0.8 IU/dL and 1.5 IU/dL, respectively ($P<0.001$). One patient with hemophilia A and two with hemophilia B who had greater than 3 IU/dL of their baseline factor activity were excluded (Table 1). The mean ages of normal healthy males, patients with hemophilia A, and patients with hemophilia B were 36.7 years (range, 21–54 yr), 37.2 years (range, 19–57 yr), and 35.1 years (range, 17–56 yr, $P=0.686$), respectively. No significant difference in annual joint bleeds was observed between patients with hemophilia A and B ($P=0.925$). All 36 patients with hemophilia B were tested with nonacog gamma, 23 patients with hemophilia A used full-length, 3rd generation recombinant FVIII (rFVIII), and 11 patients used highly purified, plasma-derived FVIII (pdFVIII). The mean doses of FVIII and FIX were 28.8 IU/kg and 43.6 IU/kg, respectively, which are reimbursable by the Korean NHI.

Hemostatic response

Normal healthy males showed mean FVIII:C and FIX:C of 129.5 IU/dL (95% confidence interval [CI], 118.6–140.4 IU/dL) and 105.8 IU/dL (95% CI, 99.8–111.8 IU/dL), respectively. Mean peak FVIII:C and FIX:C measured at 15 minutes after responsible factor concentrate injections were 69.4 IU/dL (95% CI, 65.1–74.2 IU/dL) and 46.8 IU/dL (95%

Table 1. Patient characteristics.

	Healthy male (N=34)	Hemophilia A (N=34)	Hemophilia B (N=36)	$P^{a)}$
Age (yr)	36.7±10.0	37.2±11.8	35.1±10.6	0.686
Body weight (kg)	NT	70.9±1.6	71.0±2.2	0.983
Joint bleeds (annual)	NA	21±23.9	20.2±22.5	0.925
Baseline factor activity (IU/dL)				
FVIII:C	129.5±31.4	0.8±0.6	NA	<0.001
FIX:C	105.8±17.2	NA	1.5±0.5	
Clotting factor concentrate				
Source	NA	rFVIII N=23, pdFVIII N=11	Nonacog gamma N=36	NA
Dosage	NA	28.8±1.1	43.6±0.8	<0.001

[a)] *P*-value is calculated between hemophilia A and hemophilia B.
Abbreviations: NA, not applicable; rFVIII, recombinant factor VIII; pdFVIII, plasma-derived factor VIII.

CI, 43.5–50.1 IU/dL, $P<0.001$) in patients with hemophilia A and B, respectively. The mean IVRs of FVIII:C and FIX:C were 2.4 IU/dL/IU/kg (95% CI, 2.2–2.6 IU/dL/IU/kg) and 1.0 IU/dL/IU/kg (95% CI, 0.9–1.1 IU/dL/IU/kg), respectively. Higher IVR was observed among users of recombinant FVIII compared to users of pdFVIII (Fig. 1). This result can be explained by the body weight of rFVIII users, which is generally correlated with IVR. rFVIII users were heavier than users of pdFVIII (72.0 kg vs. 63.0 kg, respectively).

The mean peak thrombin concentrations of normal

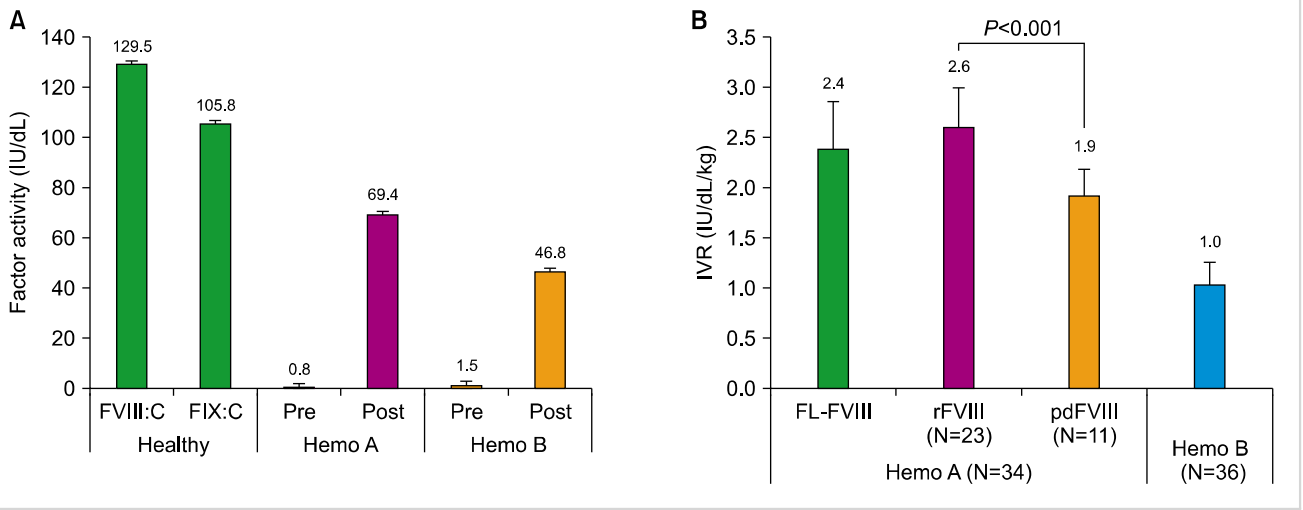

Fig. 1. One-stage clotting factor assay. Factor activity in healthy males, pre- and post-infusion of factor concentrates in hemophilia A and B patients **(A)**. In-vivo recovery of hemophilia A and B patients **(B)**.
Abbreviations: FL-FVIII, full-length factor VIII; IVR, in-vivo recovery; pdFVIII, plasma-derived factor VIII; rFVIII, recombinant factor VIII.

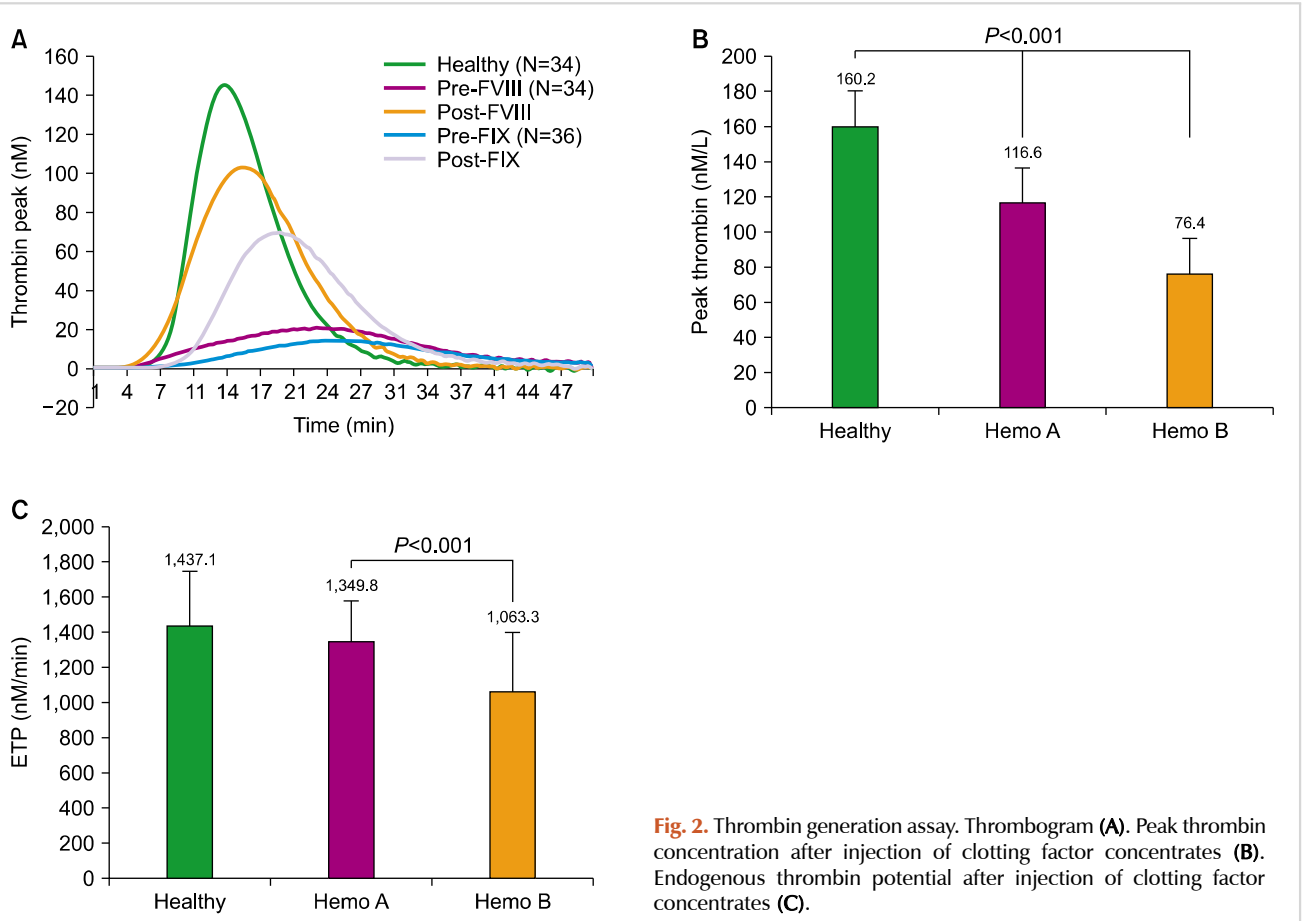

Fig. 2. Thrombin generation assay. Thrombogram **(A)**. Peak thrombin concentration after injection of clotting factor concentrates **(B)**. Endogenous thrombin potential after injection of clotting factor concentrates **(C)**.

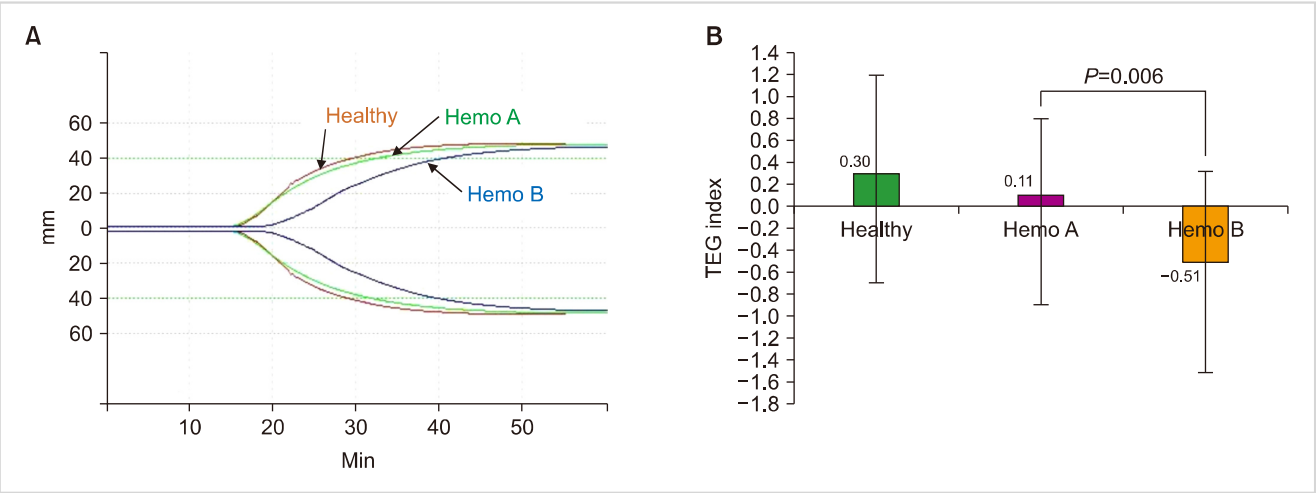

Fig. 3. Thromboelastography. Thromboelastograph after injection of clotting factor Concentrates **(A)**. TEG index after injection of clotting factor concentrates **(B)**.
Abbreviation: TEG, thromboelastography.

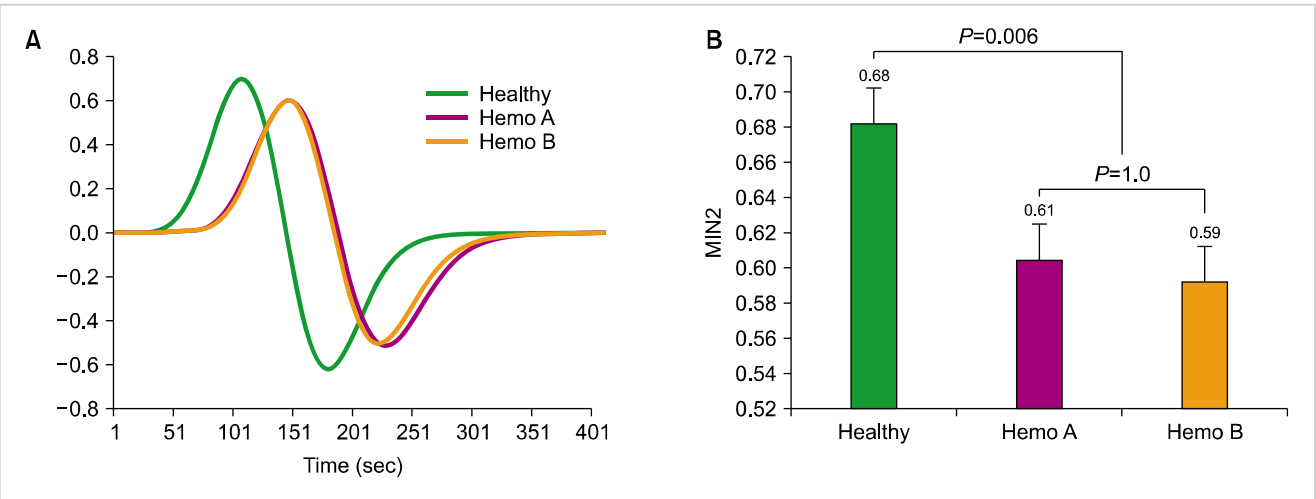

Fig. 4. Clot waveform analysis. Second derivatives of aPTT after injection of clotting factor concentrates **(A)**. MIN2 values after injection of clotting factor concentrates **(B)**.

Table 2. Summary of hemostatic response.

	Healthy male (N=34)	Hemophilia A (N=34)	Hemophilia B (N=36)	$P^{a)}$
Factor activity (t_{max}, IU/dL)				<0.001
FVIII:C	129.5±31.4	69.4±13.5	NA	
FIX:C	105.8±17.2	NA	46.8±9.8	
TGA				
Peak thrombin (nM/L, CV %)	160.2±43.1 (26.9)	116.6±27.9 (24.0)	76.4±33.5 (43.9)	<0.001
ETP (nM/min, CV %)	1437.1±308 (21.5)	1349.8±227 (16.9)	915.6±485 (31.5)	<0.001
TEG				
TEG index	0.29±0.89	0.11±0.69	−0.51±0.83	0.006
MCF (mm)	48.7±5.1	48.3±3.9	47.0±4.6	0.280
CWA				
MIN2	0.68±0.14	0.61±0.10	0.59±0.12	1.0

[a)] *P*-value is calculated between hemophilia A and hemophilia B.
Abbreviations: CWA, clot wave form analysis; ETP, endogenous thrombin potential; MCF, maximum clot firmness; NA, not applicable; TEG, thromboelastography; TGA, thrombin generation assay.

healthy males, patients with hemophilia A, and patients with hemophilia B were 160.2 nM/L (95% CI, 144.4–175.2 nM/L), 116.6 nM/L (95% CI, 107.3–126.6 nM/L), and 76.4 nM/L (95% CI, 65.0–87.6 nM/L), respectively (Fig. 2A). A significant difference in mean peak thrombin concentration was observed among groups with Bonferroni post-hoc correction (Fig. 2B). As for ETP, patients with hemophilia A showed higher ETP than those with hemophilia B (Fig. 2C).

As shown in Fig. 3A, clotting time and clot formation time were longer in patients with hemophilia B. This finding indicates that the onset and rate of clot formation after injection of FIX concentrate are delayed and slow. Even though the TEG index was within the normal range in subjects with both hemophilia A and B, the difference in TEG index results between individuals with hemophilia A and hemophilia B was statistically significant (Fig. 3B).

The MIN2 of CWA in patients with hemophilia B was insignificantly lower than that in those with hemophilia A (Fig. 4).

Table 2 summarizes the hemostatic response assessed by the global hemostatic assays. Peak thrombin concentration, ETP, and TEG index results from subjects with hemophilia A were closer to the reference values than the results obtained from the individuals with hemophilia B.

DISCUSSION

Our study hypothesis was that patients with hemophilia B receiving a dose targeted to achieve 40 IU/dL of FIX activity cannot expect a hemostatic response comparable to that of patients with hemophilia A receiving a dose targeted to achieve 60 IU/dL of FVIII activity. Post-infusion thrombin generation in study subjects with hemophilia A and B showed significant differences in terms of peak thrombin concentration and ETP. Statistically meaningful differences were also observed between the two subject groups in terms of TEG index. These differences imply a delay in both onset (r) and rate (k) of clot formation in individuals with hemophilia B.

Only adult subjects were recruited in this study, to minimize the impact on pharmacokinetic (PK) results from PK variation among different age groups, especially in terms of IVR and clearance. B-domain deleted FVIII concentrates were excluded from investigational drugs to minimize the potential impact on results of product variation. Similarly, of the two rFIX concentrates available in Korea, only nonacog gamma was used in this study. Nonacog gamma is known to have less preactivated FIX, which is considered "impure content" influencing safety and efficacy, and is also known to have actual potency closer to its nominal potency [15].

Three global hemostatic assays, TEG, TGA, and CWA were used to assess the hemostatic response. These assays may be used to evaluate hemostatic response based on objective and measurable parameters, although limitations still exist in that no sole global hemostatic assay can comprehensively and accurately reflect in-vivo hemostatic response [18].

Hemker *et al.* [19] reported that the intra-individual and inter-individual coefficients of variation (CVs) of ETP with PPP sample were 5% and 15%. In the present study, the inter-individual CV of ETP of normal healthy males was 21.5%. In addition, the inter-individual CV of post-infusion ETP in patients with hemophilia A and hemophilia B were 16.9% and 31.5%, respectively. As post-infusion TGA was performed once, intra-individual variation could not be investigated. In terms of peak thrombin and ETP, subjects with hemophilia B had wider inter-individual variation than those with hemophilia A, which means that individuals with hemophilia B have a more heterogeneous hemostatic response.

To our knowledge, this is the first report comparing thrombin generation induced by FVIII and FIX concentrates using global hemostatic assays. As FVIII is a cofactor and FIX is an enzyme, the two clotting factors have different mechanisms of action in the coagulation pathway; hence some investigators may question the validity of a direct comparison of efficacy between FVIII and FIX concentrates. While several studies have compared clinical outcomes of FVIII and FIX deficiencies, the difference in phenotypes of the two diseases remains an open debate. Some publications emphasize the clinical significance of extravascular FIX pooling [20]. As yet, the mechanism of action of extravascular FIX remains to be elucidated, while the factor is known to bind reversibly to type IV collagen, a component of the endothelial basement membrane. We assume that extravascular FIX may be released from collagen and some free FIX can be subsequently activated by adjacent tissue factor-VIIa complex in the initiation phase of the coagulation pathway [21]. However, the amount of activated FIX developed by this mechanism is small because tissue factor pathway inhibitor (TFPI) rapidly inhibits the action of the tissue factor-VIIa complex. Other free extravascular FIX may diffuse and become activated on the surface of activated platelets. Although it is unclear how long collagen-bound FIX can survive, extravascular FIX could generate blood clots in mice even 7 days after intravenous infusion [22]. Extravascular FIX may play a role in prophylaxis to some extent, or in initial thrombin generation to activate platelets or other clotting factors at bleeding sites. To measure the entire generation of thrombin during the coagulation pathway, not only plasma thrombin but also extravascular thrombin should be measured. However, extravascular thrombin cannot be measured yet. The question may be raised whether plasma thrombin measurement can reflect the hemostatic efficacy of CFC. The answer requires an understanding of the physiology of hemostasis. Shearing forces that are caused by laminar blood flow are crucial for adhesion and aggregation of platelets. This means that platelet adhesion and activation take place along the vessel wall [23]. The majority (96%) of thrombin is produced on the activated platelet surface during the propagation phase of the coagulation reaction to generate thrombin bursting in the acute bleeding state [24, 25] and can easily diffuse into plasma to interact with

other clotting substrates [26]. Therefore, maintaining optimal plasma FIX activity has been standard clinical practice.

Patients with hemophilia A and B with FVIII or FIX activity levels of 3 IU/dL or less were enrolled in the present study. In the late 1950s, Biggs and MacFarlane defined patients with severe hemophilia as those with coagulation factor levels of 1 IU/dL or less [27]. However, recent studies have recruited as severe patients those with levels less than 2 IU/dL. Den Uijl *et al.* [14] published an article demonstrating that a clotting factor level of 3 IU/dL is a significant threshold leading to frequent annual joint bleeds. In this study, subjects with hemophilia A and B had mean baseline FVIII:C of 1.5 IU/dL and FIX:C of 0.8 IU/dL, respectively, but their clinical symptoms in terms of annual joint bleeds were not significantly different. The subjects in this study were phenotypically homogeneous.

Considering these findings, we concluded that the current differing dose levels approved for reimbursement by the Korean NHI for patients with hemophilia with similar clinical phenotypes lead to unequal hemostatic responses as measured by global hemostatic assays.

In summary, hemophilia A and B have several different characteristics, some of which—e.g., the mechanism of extravascular FIX—should be further elucidated. However, achieving the target plasma activity to induce adequate plasma thrombin bursting is still standard clinical practice, especially to stop acute bleeds. Taking into consideration the basic physiology of hemostasis, larger amounts of activated FIX and the majority of thrombin are expected to be generated on the surface of activated platelets along the damaged vessel wall. From this perspective, it is worthwhile to measure plasma thrombin generation to evaluate hemostatic efficacy at the different target activities of FVIII and FIX. Global hemostatic assays suggest that FIX dosing targeted to 40 IU/dL of FIX activity, as currently reimbursed by the Korean NHI, is not likely to provide a similar hemostatic response to that expected from the currently reimbursed FVIII dosing.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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