Analysis of Anticoagulant Effect of Unfractionated Heparin by Using Thrombin Generation Assay *in Vitro*

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Abstract

Unfractionated heparin (UFH) is widely used in the treatment and prophylaxis of thrombosis. The anticoagulant effect of UFH is monitored according to activated partial thromboplastin time (APTT). However, there are reports of cases in which hemorrhagic complications develop despite APTT being maintained within the therapeutic range. This indicates there are problems with the monitoring method using APTT during UFH treatment. To assess the actual anticoagulant effect in UFH therapy using APTT as a monitoring method, we investigated changes in APTT and thrombin generation (TG) potential *in vitro* with the addition of UFH to samples from 10 healthy adults. There were large individual differences in the degree of APTT prolongation with the addition of UFH to samples from healthy adults. Furthermore, individual differences in the degree of TG change exceeded those in APTT in 7 subjects with comparable APTT changes caused by UFH (coefficients of variation: 2–5% for APTT, 10–50% for TG potential). Thus, with UFH treatment, there are large individual differences in the degree of TG inhibition even when APTT is within the appropriate range for coagulation control. These findings provide invaluable information for resolving the problem of hemorrhagic complications during UFH treatment based on the current APTT method.

Key words

unfractionated heparin, anticoagulant effect, thrombin generation,
activated partial thromboplastin time, individual difference

Introduction

Unfractionated heparin (UFH) is widely used in the treatment and prophylaxis of thrombosis. Doses are adjusted to ensure the prolongation of activated partial thromboplastin time (APTT) is within 1.5–2.5 times the normal value when UFH is used for treatment¹⁾. However, it has been reported that there are large individual differences in the APTT-prolongation effect of UFH, and how precisely APTT reflects the

actual anticoagulation effect of UFH *in vivo* is unknown^{2–4)}. Furthermore, the effect of UFH on APTT measurement varies markedly depending on the reagents and/or measurement equipment used^{5–9)}. Thus, there are problems with using APTT as a monitoring method in UFH treatment. The incidence of hemorrhagic complications when UFH treatment is performed with APTT monitoring is reportedly 2–5%¹⁰⁾¹¹⁾, and there are cases in which hemorrhagic complications occur despite APTT being maintained

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within the therapeutic range. In these cases, the actual anticoagulant effect of UFH may be excessive, despite appropriate APTT prolongation.

Thrombin is well known to play an important role in coagulation mechanisms. Measuring the amount of thrombin generated more precisely reflects overall hemostasis than coagulation times, such as prothrombin time (PT) and APTT. The thrombin generation (TG) test was recently proposed to be an informative method for studying the anticoagulant effect of antithrombotic drugs¹²⁾¹³⁾.

Therefore, in order to assess the anticoagulant effect of UFH therapy using APTT as a monitoring method, this study investigated changes in APTT and TG potential in response to the addition of UFH using the TG test according to the calibrated automated thrombogram method.

Methods

Blood Samples

Ten healthy adults (6 men and 4 women) aged 20–40 years with normal PT and APTT served as the study subjects. Whole blood was drawn into citrated tubes. Platelet-poor plasma (PPP) was prepared by 2 centrifugations at 3000 rpm for 15 min at 15°C; it was subsequently aliquotted and stored at –80°C until use.

This study was approved by the Ethics Committee at St. Marianna University School of Medicine (approval #1210), and informed consent for blood collection was directly obtained from all subjects.

Heparin

The UFH preparation used was the Heparin Na Lock (1000 U/10 mL) Syringe® (Otsuka Pharmaceutical, Tokyo, Japan). Heparin was diluted with saline, 20 μ L heparin solution was added to 980 μ L plasma, and the final concentration was adjusted to 0.1, 0.2, 0.3, 0.4, and 0.5 U/mL. In addition, 20 μ L saline was added to 980 μ L plasma to create 0 U/mL sample.

Determination of APTT

UFH concentration (0, 0.2, 0.3, 0.4, and 0.5 U/mL) was added to PPP obtained from 10 healthy adults, and changes in coagulation time were evaluated by the APTT method. APTT was measured using a CA-7000® analyzer (Sysmex, Kobe, Japan), and APTT-SLA® (Sysmex Corporation) was used as an APTT reagent.

TG Assay

UFH (0, 0.1, 0.2, and 0.3 U/mL) was added to PPP obtained from 7 healthy adults in whom APTT changes as a result of UFH addition were comparable, and changes in TG potential were evaluated. TG potential was measured using Fluoroskan Ascent® (Finggal Link, Tokyo, Japan) and analyzed using the Thrombinoscope® Program (Finggal Link). PPP Reagent (5 pM tissue factor and 4 μ M phospholipid) and Flu-Ca kit (16.6 mM CaCl₂) were used as reagents (Finggal link). Prepared plasma samples were incubated at 37°C for 5 min until immediately before measurement. TG potential was evaluated using the following 5 parameters: lag time, thrombin peak (Peak), endogenous thrombin potential (ETP), time to peak (ttPeak), and start tail.

Statistical Method

For analyzing correlations of APTT with TG parameters when UFH was added to samples, Spearman's rank correlation test was used for statistical analysis. P-values <0.05 were considered to be statistically significant.

Results

APTT

The concentration at which APTT was prolonged at least 1.5-fold was 0.2 U/mL in 7 of the 10 subjects and 0.3 U/mL in the other 3. Individual differences in APTT changes became significant with increasing UFH concentration; APTT was prolonged at slightly more than 2-fold with the addition of 0.5 U/mL UFH in the subject with the smallest APTT change, while APTT was prolonged approximately 6-fold in another subject with the same concentration of UFH (**Figure 1**). The antithrombin (AT) level of the subject with the smallest APTT change was within the normal range (data not shown).

TG test

TG potential was evaluated in 7 subjects in whom APTT prolongation as a result of UFH was comparable. APTT was prolonged 1.5–2.5-fold with the addition of 0.2 or 0.3 U/mL UFH (**Figure 2**).

The thrombin generation curve (thrombogram) shifted to the right with the addition of UFH, the peak decreased, and the amount of thrombin generated was inhibited in a dose-dependent manner (**Figure 3**). Regarding the TG parameters, ETP and Peak decreased, while lag time, ttPeak, and start tail were prolonged with the addition of UFH (**Figure 4**).

Figure 1. APTT prolongation with in vitro addition of UFH in samples from 10 healthy adults. The UFH concentration at which APTT prolongation became at least 1.5-fold was 0.2 U/mL in 7 of the 10 subjects and 0.3 U/mL in the other 3 subjects.

Figure 2. APTT prolongation with the addition of UFH to samples from 7 healthy adults in whom clotting times were equally prolonged by UFH. APTT prolongation became at least 1.5-fold in all subjects with the addition of UFH 0.2 U/mL, and prolongations with the addition of 0.3 U/mL were within the range of 2– to 2.5-fold in all subjects.

These results indicate TG potential is inhibited by the addition of UFH in a dose-dependent manner.

In 7 subjects with comparable UFH sensitivity measured by the APTT method, APTT was negatively correlated with ETP ($r=-0.86$, $p<0.01$) and Peak ($r=-0.77$, $p<0.01$) and positively correlated with lag time ($r=0.85$, $p<0.01$), ttPeak ($r=0.94$, $p<0.01$), and start tail ($r=0.90$, $p<0.01$); however, large prediction intervals were detected in correlation

with APTT and any TG parameters (**Figure 5**). When the change ratios (calculated as after UFH addition / before UFH addition) in APTT and TG parameters were compared using coefficient of variations (CVs), the CV of APTT was very small at 2–5%, while those of ETP and Peak were 10–46% and 39–50%, respectively; meanwhile, the CVs of lag time, ttPeak, and start tail, which are the TG parameters reflecting time, were relatively low (**Table 1**).

Figure 3. Dose-dependent inhibition of UFH (0, 0.1, 0.2, and 0.3 U/mL) on TG test in a healthy adult.

Each thrombin generation curve (thrombogram) was shown in the presence of UFH 0U/ml ($\square$), UFH 0.1U/ml ($\triangle$), UFH 0.2U/ml ($\times$), and UFH 0.3U/ml ($\circ$), respectively.

Figure 4. The alteration of each TG parameters by addition of UFH in 7 healthy adults with comparable APTT changes when several UFH concentrations (0 to 0.3 U/mL) were added in vitro.

ETP (A) and Peak (B) decreased, while lag time (C), ttPeak (D), and start tail (E) were prolonged with the addition of UFH.

Figure 5. Correlations of APTT with TG parameters in 7 healthy adults with comparable APTT changes when several UFH concentrations (0 to 0.3 U/mL) were added in vitro.

Spearman's rank correlation test was used for statistical analysis. The solid line represents regression line, dot lines represent 95% confidence interval, and dashed lines represent 95% prediction interval. APTT was negatively correlated with ETP (A) and Peak (B), while it was positively correlated with lag time (C), ttPeak (D), and start tail (E). However, large prediction intervals were detected in correlation with APTT and any TG parameters.

Table 1. Change Ratio in TG Parameters with the Addition of UFH to Samples from 7 Healthy Adults with Comparable APTT Changes

Parameters	UFH (U/ml)					
	0.1		0.2		0.3	
	Change ratio (Mean $\pm$ SD)	CV (%)	Change ratio (Mean $\pm$ SD)	CV (%)	Change ratio (Mean $\pm$ SD)	CV (%)
APTT	1.26 $\pm$ 0.03	2.2	1.66 $\pm$ 0.07	4	2.20 $\pm$ 0.12	5.3
ETP	0.63 $\pm$ 0.06	9.7	0.31 $\pm$ 0.11	35	0.14 $\pm$ 0.06	46
Peak	0.26 $\pm$ 0.10	39	0.07 $\pm$ 0.03	46	0.03 $\pm$ 0.01	50
Lag time	1.47 $\pm$ 0.13	8.6	2.55 $\pm$ 0.42	17	5.01 $\pm$ 1.63	33
ttPeak	2.13 $\pm$ 0.41	19	4.46 $\pm$ 0.73	16	6.27 $\pm$ 0.95	15
Start tail	1.74 $\pm$ 0.30	17	2.61 $\pm$ 0.30	12	2.82 $\pm$ 0.31	11

CV: Coefficient of variance

Discussion

Although low molecular weight heparin and factor Xa inhibitors have recently been developed for the treatment and prophylaxis of thrombosis, UFH is still the most widely used anticoagulant agent. UFH deactivates coagulation factors II, IX, X, XI, and XII via AT. Its anticoagulant effect is known to differ among individuals because of the multiple underlying anticoagulant mechanisms¹⁴⁾. The present investigation demonstrates that there are large individual differences in the degree of APTT prolongation with the addition of UFH *in vitro*. Furthermore, the anticoagulant effect of UFH is known to be less effective in subjects with low AT levels (<60%) than in subjects with normal AT levels¹⁵⁾. In the present study, the AT level of the subject with the smallest APTT prolongation as a result of UFH addition was within the normal range.

In subjects with comparable APTT changes in response to UFH, each TG parameter obtained in the presence of UFH was strongly correlated with APTT values, demonstrating TG inhibition in a UFH concentration-dependent manner. However, individual differences in the change ratios of each TG parameter were larger than those with APTT, especially for ETP and Peak. These results suggest the degree of TG inhibition differs among individuals, even if APTT prolongation is equivalent. This is considered to be because strong inhibition of TG potential as a result of UFH may tend to be associated with hemorrhage; conversely mild TG inhibition may tend to be related to an insufficient anticoagulant effect. Individual differences in TG inhibition as a result of UFH addition may be the reason for the occurrence of hemorrhagic complications despite APTT being maintained within the therapeutic range during UFH treatment.

Conclusion

The degree of TG inhibition is associated with large individual differences, even when APTT is maintained within the appropriate range during UFH treatment. These findings provide invaluable information for resolving the problem of hemorrhagic complications during UFH treatment based on the current APTT method. This issue awaits clarification in further investigations using clinical samples.

Disclosure of Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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