MECHANISM FOR SHORTENING PT AND APTT IN DOGS AND RATS – EFFECT OF FIBRINOGEN ON PT AND APTT –

Masaaki KURATA, Yukiko SASAYAMA, Naoko YAMASAKI,
Ikue KITAZAWA, Yoshimasa HAMADA and Ikuo HORII

*Worldwide Safety Sciences, Pfizer Global Research and Development,
Nagoya Laboratories, Pfizer Inc.,
5-2 Taketoyo, Aichi 470-2393, Japan*

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ABSTRACT — The possible mechanisms for shortening prothrombin time (PT) and activated partial thromboplastin time (APTT) were investigated using citrated plasma from rats and dogs *in vitro*, especially focusing on increased fibrinogen concentrations. When purified canine fibrinogen was added to citrated canine plasma at final concentrations of 2, 4 and 8 mg/mL, PT and APTT were significantly shortened. The increased concentrations of clottable fibrinogen in the test system were confirmed by markedly shortened thrombin time (TT). In citrated rat plasma, while purified rat fibrinogen had no effect on PT or APTT at final concentrations of 2, 4 and 8 mg/mL, it did shorten TT. These results suggest that an increased concentration of fibrinogen is a possible mechanism to shorten PT and APTT in dogs, but not in rats.

KEY WORDS: Prothrombin time, Activated partial thromboplastin time, Rats, Dogs, Fibrinogen

INTRODUCTION

In preclinical toxicity studies, prothrombin time (PT) and activated partial thromboplastin time (APTT) are routinely measured as the standard parameters of blood coagulation, according to IHCPT* recommendation (Weingand *et al.*, 1996) and guidelines (*e.g.*, issued from MHLW** in Japan) based on ICH***. Examination of prolonged PT and APTT is useful to screen for impairment in extrinsic and intrinsic pathways, respectively.

Although shortened PT and APTT in laboratory animals (*e.g.*, dogs and rats) are frequently observed in general toxicological studies, the significance has not been fully explored or evaluated. In recent studies on human clinical pathology, shortened APTT was reported to be associated with increases in (i) fibrin/fibrinogen degradation products, (ii) thrombin-ATIII complex and (iii) factor VIII (Ten Boekel and Bartels, 2002), and to be possibly useful to predict thrombosis (Reddy *et al.*, 1999; Korte *et al.*, 2000) and myocardial

infarction (Madi *et al.*, 2001). It is thus significant to investigate the mechanism for shortening PT and APTT in laboratory animals.

In this study, we especially focused on the influence of fibrinogen concentrations in shortening PT and APTT. Fibrinogen is the coagulation factor related with hypercoagulation (Zwaginga *et al.*, 1994) and an acute phase protein for inflammatory reaction (Gardner *et al.*, 2000). Furthermore, Acang and Jalil (1993) reported an increased concentration of fibrinogen and shortened PT and APTT in patients with diabetes mellitus.

The present study was therefore undertaken to investigate the effect of increased fibrinogen concentrations on PT and APTT, using citrated plasma from dogs and rats *in vitro*.

* : The International Harmonization for Clinical Pathology Testing

** : The Ministry of Health, Labour and Welfare

***: The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

MATERIALS AND METHODS

Reagents

Fibrinogen from dog plasma (Cat No. F-7128, Sigma, St. Louis, MO) or from rat plasma (Cat No. F-6755, Sigma) was dissolved in saline (0.9% NaCl). Other reagents were as follows; PT reagent (Thrombocheck PT, International Reagent, Kobe, Japan), APTT reagent (Dade Actin, Dade Behring, Marburg, Germany) and thrombin reagent (Thrombin Reagent, Dade Behring, Marburg, Germany).

Animals

Male beagle dogs (Marshall beagle dogs) were purchased from Charles River Japan, and used in the experiment at the age of 12–15 months old. Dogs were housed in individual cages of one animal room under the following conditions: temperature at $21 \pm 2^\circ\text{C}$, relative humidity at $55 \pm 15\%$, 12 air changes per hour and 12-hr illumination (06:00 to 18:00). Dogs had free access to tap water and to a commercial laboratory animal feed (DS-A, Oriental Yeast Inc., Tokyo, Japan).

Ten male rats (Strain: Crj:CD(SD)IGS, SPF; 8-week-old) were purchased from Charles River Japan, and used in the experiments when they were 9 to 11 weeks old. Rats were housed in individual cages of one animal room under the following conditions: temperature at $23 \pm 2^\circ\text{C}$, relative humidity at $55 \pm 15\%$, 12 air changes per hour and 12-hr illumination (06:00 to 18:00). Rats had free access to tap water and to a pelleted commercial laboratory animal feed (CE-2, Clea Japan Inc., Tokyo, Japan).

This study was carried out in accordance with the ethical guidelines for the use of experimental animals from Pfizer Inc.

Blood collection and plasma sample preparation

For dogs, 9 volumes of blood were collected into a syringe containing 1 volume of 3.8% sodium citrate solution, from a cephalic vein without anesthesia, after an approximate 15-hr fast.

Rats were anesthetized by intraperitoneal injection of pentobarbital 35–70 mg/kg after approximately 15 hr fasting. Nine vol. of blood was collected from the abdominal aorta into a syringe containing 1 vol. of 3.8% sodium citrate solution.

Citrated plasma was separated from citrated blood by centrifugation (2000 g, 15 min, 4°C). The intrinsic fibrinogen level (against human fibrinogen standard) was 1.8 ± 0.4 mg/mL (mean $\pm$ SD, $n = 9$) in

canine plasma and 2.2 ± 0.2 mg/mL (mean $\pm$ SD, $n = 10$) in rat plasma.

Prothrombin time, activated partial thromboplastin time and thrombin time

A 20 μL -aliquot of fibrinogen solution (or saline control) was mixed with 180 μL of citrated plasma. Prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) were measured using the PT reagent, the APTT reagent, the thrombin reagent and a semi-automatic coagulation analyzer (KC-10A, Amelung, Lehbrinksweg, Germany).

Thromboelastogram

Samples and reagents were combined as follows: 320 μL of citrated blood plus 40 μL of fibrinogen solution (or saline control) plus 40 μL of 100 mM CaCl_2 in the case of samples from dogs; or 160 μL of citrated blood plus 200 μL of saline plus 20 μL of fibrinogen solution (or saline control) plus 20 μL of 100 mM CaCl_2 in that of samples from rats. The whole process of blood coagulation was monitored by thromboelastography (Clot-Tracer, TE-700, Erma Inc., Tokyo, Japan). The angle (rate) of increasing amplitude and the maximum amplitude for clot elasticity were measured on the thromboelastogram (TEG), according to the description given by Mallett and Cox (1992) and Otto *et al.* (2000) (Fig. 1).

Statistical analysis

Student's unpaired *t*-test (Steel *et al.*, 1997) was applied for statistical analysis. A probability (*P*) less than 0.05 was considered statistically significant.

RESULTS

PT, APTT and TT

When purified canine fibrinogen was added to

Fig. 1. Measurement of the thromboelastogram pattern. Units: angle (degree $^\circ$) and maximum amplitude (mm).

Short PT and APTT.

citrated canine plasma at final concentrations of 2, 4 and 8 mg/mL (as added fibrinogen concentration), PT and APTT were significantly shortened ($p < 0.05$ - 0.001, t -test). The increased concentrations of clottable fibrinogen were confirmed by the markedly shortened TT (upper column in Table 1).

In the case of citrated rat plasma, while purified rat fibrinogen had no effect on PT and APTT at the final concentrations of 2, 4 and 8 mg/mL, it shortened TT (lower column in Table 1).

TEG

Canine fibrinogen at a final concentration of 4 mg/mL induced an increased rate of clot formation and increased the maximum amplitude of TEG (Fig. 2 and Table 2). In rats, TEG also showed a hypercoagulation pattern at a fibrinogen concentration of 1 mg/mL (Fig. 3) and an increase in the maximum amplitude of TEG (Table 2).

DISCUSSION

In the case of canine plasma, when purified canine fibrinogen was added to citrated canine plasma at concentrations of 2 - 8 mg/mL, PT and APTT were shortened. In both PT and APTT reactions, the final step is fibrin-formation from fibrinogen. Thus, an increased concentration of fibrinogen is considered to enhance the rate of fibrin formation in canine plasma, resulting in shortened PT and APTT in this species. As supporting evidence of this, TEG showed that a high concentration of fibrinogen increased the rate of fibrin

formation in canine plasma.

The concentration of fibrinogen in normal

Fig. 2. Canine thromboelastogram pattern showing hypercoagulation induced by adding canine fibrinogen. The final concentration of added fibrinogen was 4 mg/mL. The combination ratio for samples and reagents was as follows: 320 μ L of canine citrated whole blood plus 40 μ L of 40 mg/mL fibrinogen solution plus 40 μ L of 100 mM CaCl_2 . Arrows indicate the addition of reagents.

Table 1. Changes in PT and APTT when adding fibrinogen to citrated rat or canine plasma.

Dog plasma + Dog fibrinogen		Added Fibrinogen Final Conc (mg/mL)					
		0	2	4	8		
PT (sec)	n=10	6.1 $\pm$ 0.3	5.6 $\pm$ 0.2 ***	5.4 $\pm$ 0.3 ***	5.3 $\pm$ 0.3 ***	92%	87%
APTT (sec)	n=10	10.6 $\pm$ 0.5	10.3 $\pm$ 0.4 *	10.1 $\pm$ 0.4 ***	9.8 $\pm$ 0.4 ***	95%	92%
TT (sec)	n=9	15.3 $\pm$ 3.6	11.1 $\pm$ 2.0 **	8.1 $\pm$ 1.0 ***	6.3 $\pm$ 0.9 ***	53%	41%

*, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$, significantly different from 0 mg/mL.

% means the ratio against 0 mg/mL.

Rat plasma + Rat fibrinogen		Added Fibrinogen Final Conc (mg/mL)					
		0	2	4	8		
PT (sec)	n=10	15.9 $\pm$ 2.3	15.8 $\pm$ 2.3	16.2 $\pm$ 2.4	16.9 $\pm$ 2.6	99%	106%
APTT (sec)	n=10	22.1 $\pm$ 2.8	22.0 $\pm$ 2.6	22.3 $\pm$ 2.6	23.6 $\pm$ 2.6	100%	107%
TT (sec)	n=10	11.9 $\pm$ 1.0	8.9 $\pm$ 0.3 ***	7.4 $\pm$ 0.3 ***	5.2 $\pm$ 0.3 ***	75%	44%

***: $p < 0.001$, significantly different from 0 mg/mL.

% means the ratio against 0 mg/mL.

Table 2. Changes in thromboelastogram parameters when adding fibrinogen *in vitro*.

Species	Parameter	n	Added Fibrinogen Final Conc (mg/mL)	
			0 mg/mL	4 mg/mL
Dog	Angle (degree)	4	63.0 ± 5.0	75.3 ± 2.2 **
	MA (mm)	4	56.8 ± 1.7	68.3 ± 2.8 **

Species	Parameter	n ¹⁾	Added Fibrinogen Final Conc (mg/mL)	
			0 mg/mL	1 mg/mL
Rat	Angle (degree)	5	58.8 ± 8.1	68.8 ± 12.6
	MA (mm)	5	40.6 ± 3.7	46.0 ± 2.1 *

*, p<0.05, **p<0.01: Significantly different from 0 mg/mL.

Angle: Angle (rate) of increasing amplitude with clot formation.

MA: Maximum amplitude for clot elasticity.

1): We tested 9 animals. The other 4 samples were out of measurement range, due to marked hypercoagulation at 1 mg/mL.

Fig. 3. Rat thromboelastogram pattern showing hypercoagulation induced by adding rat fibrinogen. The final concentration of added fibrinogen was 1 mg/mL. The combination ratio for samples and reagents was as follows: 160 μ L of rat citrated whole blood plus 200 μ L of saline plus 20 μ L of 20 mg/mL fibrinogen solution plus 20 μ L of 100 mM CaCl_2 . Arrows indicate the addition of reagents.

plasma is approximately 2 mg/mL in dogs and rats. We added fibrinogen at concentration of 2 - 8 mg/mL. Thus, the fibrinogen concentration in this study was from 2 to 5 times that of the normal one. Lewis *et al.* (1989) reported 5 times elevation of plasma fibrinogen concentration in rat models of induced-inflammation. Otto *et al.* (2000) also reported an increased plasma concentration of fibrinogen in canine enteritis (up to 10 mg/mL, *i.e.*, approximately 5 times of normal). The fibrinogen concentrations used in this study were within the range possibly observed in an inflammatory status *in vivo*. These findings strongly suggest that PT and APTT are shortened in plasma from dogs affected by inflammation, due to the increased fibrinogen concentrations.

In the case of rat plasma, purified rat fibrinogen had no effect on PT or APTT at the same concentrations described for canine plasma. This was not at least due to inactivity of the purified rat fibrinogen, since rat fibrinogen shortened TT in rat plasma. TEG also showed hypercoagulation after the addition of rat fibrinogen, confirming the coagulability of the purified rat fibrinogen. The present results suggest a species difference between rats and dogs regarding shortening of PT and APTT by increased concentrations of fibrinogen.

As for rat plasma, fibrinogen might not be a slow rate-limiting factor in PT and APTT. The normal PT and APTT were 2 or 3 times longer in rats than in dogs, but TT was comparable. This suggests the presence of a slow coagulation-cascade step(s) that is not a fibrinogen-fibrin step in rats. Further studies are necessary to clarify the mechanisms for shortening PT and APTT in rats.

In conclusion, the present results suggest that an increased concentration of fibrinogen is associated with shortened PT and APTT in dogs, but not in rats. At present, therefore, the mechanism(s) for shortening PT and APTT in rats remains unknown.

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