Assessment of selected ROTEM parameters, kinetics of fibrinogen polymerization and plasmin amidolytic activity in patients with congenital fibrinogen defects

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Introduction

Congenital fibrinogen disorders (CFD) are very rare fibrinogen deficiencies, which may be either quantitative (afibrinogenemia and hypofibrinogenemia) or functional (dysfibrinogenemia and hypodysfibrinogenemia). In contrast to afibrinogenemia, a high proportion of hypofibrinogenemia or dysfibrinogenemia patients is asymptomatic at the time of diagnosis, which is often made due to different abnormalities in routine tests of hemostasis. However, in rare cases, the diagnosis of congenital dysfibrinogenemia (dysFI) can be challenging, and its confirmation requires specialized tests, including genetic analysis. The natural history of patients with dysFI is also very unpredictable, since the risk of bleeding, thrombotic events or pregnancy-related complications cannot be precisely determined for the majority using standard hemostasis tests.

The last few years has seen growing interest in using thromboelastography, a global hemostasis test which measures the dynamics of the entire clotting and fibrinolysis process, for the assessment of bleeding and thrombotic risk in various clinical settings. Recently, it has been shown that rotation thromboelastometry (ROTEM) may be helpful in indicating the prothrombotic state, which is present at diagnosis in patients with multiple myeloma and essential thrombocythemia. Until now, few attempts have been made to characterize the usefulness of thromboelastography in patients with dysFI or hypodysfibrinogenemia. The aim of the present study was to compare a spectrum of ROTEM parameters in a cohort of patients with CFD, and to determine whether ROTEM can be used to discriminate patients with dysFI and hypoFI. The study also compares the results of fibrin plasma polymerization and clot lysis tests, as well as plasmin amidolytic activity, in different types of CFD.

Patients

The study was performed in eight CFD patients, four women and four men, with a median age of 40 years (range 21-85), as given in Table 1. Informed consent was obtained from all subjects. The diagnosis of afibrinogenemia (patient nb. 4 – table 1), hypoFI (patients nb. 1,2,6,7 – table 1) and dysFI (patients nb. 3,5,8 - table 1) was made based on the assessment of thrombin time (TT), functional (von Clauss method) and antigenic (ELISA method) fibrinogen concentration following the exclusion of acquired causes of fibrinogen defects. The majority of patients (6/8) revealed no bleeding or thrombotic complications in the medical history, with exception of one female subject with afibrinogenemia (nb. 4 - several bleeding events since early childhood) and one male subject with hypoFI (nb. 7 – deep vein thrombosis at the age of 45). The patients enrolled into the study did not receive any drugs strongly influencing hemostasis for at least 14 days prior to taking a blood sample. The control group consisted of 15 healthy volunteers of similar age. Fibrin plasma polymerization and clot lysis tests, as well as plasmin amidolytic activity, were assessed at the Department of General Biochemistry, University of Łódź. Reference plasma (in 12 repetitions) was used as a control for the tests.

Results 1

The concentration of fibrinogen, measured using ELISA and the von Clauss method, together with the results of the hemostasis screening tests are shown in Table 1. All patients demonstrated reduced values of functional fibrinogen, while five of the eight patients showed lower than normal values of antigenic fibrinogen. Patients with dysFI demonstrated higher median values of functional and antigenic fibrinogen, APTT (activated partial thromboplastin time) and TT than patients with hypoFI (Table 2).

Results 2

ROTEM

Parameters reflecting Initiation and Speed at which a Solid Clot Forms (CT, CFT, α- angle)

Median CT and CFT readings were found to be markedly higher while α- angle values were found to be markedly lower in the cohort of patients with CFD than in controls according to EXTEM, INTEM, FIBTEM and APTEM tests (Table 3). Patients with hypoFI showed markedly higher readings of CFT according to EXTEM and lower α- angle values according to EXTEM and APTEM than patients with dysFI (Table 4).

Parameters reflecting Clot Firmness (MCF)

MCF readings were significantly lower in the samples of patients than in controls according to EXTEM, INTEM, FIBTEM or APTEM tests (p< 0.001 in all ROTEM tests) (Table 3). Cases with hypoFI demonstrated markedly lower readings of MCF according to all ROTEM tests than cases with dysFI (Table 4). The most significant differences concerned the MCF EXTEM test (p< 0.001).

Parameters reflecting Clot Lysis

None of the ROTEM tests found any significant differences with regard to ML values between the cohort of patients and healthy volunteers, nor between the patients with hypoFI and those with dysFI (Table 4).

Parameters of fibrin plasma polymerization, clot lysis and plasmin amidolytic activity

All patients demonstrated different disturbances of fibrin polymerization process while patients nb. 3,4 showed no fibrin polymerization at all. The values for maximal velocity of fibrin polymerization (V_{max}), maximal absorbance (A_{max}) and velocity of clot lysis (V_{Lys}) were found to be significantly lower in the group of CFD patients than in reference plasma (Table 5). In contrast, no marked differences were identified between studied groups in reference to Lag time and plasmin amidolytic activity (Table 5). Patients with hypoFI showed higher median readings of Lag time, V_{max} and plasmin amidolytic activity and lower median readings of A_{max} and V_{Lys} than patients with dysFI (Table 6). Figure 1 presents the process of fibrin plasma polymerization and clot lysis graphically in all studied patients and in the reference plasma.

Methods

General

The following tests were performed in all patients and controls: basic hemostasis screening, fibrinogen concentration analysis by von Clauss and ELISA, ROTEM analysis, fibrin plasma polymerization, clot lysis and plasmin amidolytic activity assays.

ELISA for the quantitative determination of fibrinogen concentration in plasma

Abcam's Fibrinogen Human *in vitro* competitive ELISA kit (Cambridge, MA, USA) is designed for the quantitative measurement of fibrinogen levels in plasma. A fibrinogen specific antibody was pre-coated onto 96-well plates and blocked. Standards and test samples were added to the wells, followed by biotinylated fibrinogen, before the wells were washed with wash buffer. Streptavidin-Peroxidase Complex was added and unbound conjugates were washed away with wash buffer. TMB was then used to visualize Streptavidin-Peroxidase enzymatic reaction. TMB was catalyzed by Streptavidin-Peroxidase to produce a blue color product that changed to yellow after the addition of acidic stop solution. The density of yellow coloration was inversely proportional to the amount of fibrinogen captured in the plate.

ROTEM

Citrated samples of blood were collected under standardized conditions and ROTEM (Pentapharm GmbH, Munich, Germany, software version 1.5.3.) measurements were processed within a maximum of two hours. Four routine ROTEM tests (EXTEM, INTEM, FIBTEM and APTEM) were conducted to assess coagulation time (CT), clot formation time (CFT), α – angle, maximum clot firmness (MCF) and maximum lysis (ML) according to the manufacturer's instructions. The details of ROTEM methodology has been provided in our previous publications.

Determination of fibrin plasma polymerization and clot lysis by the turbidimetric method

The kinetics of fibrin plasma polymerization and clot lysis was evaluated by turbidimetry according to Kostka et al. To each microtiter well was added 100 µl of citrated plasma and then 200 µl of activation mixture (0.75 U/ml thrombin, 225 ng/ml rt-PA, 7.5 mM $CaCl_2$ in TBS, pH 7.4) which had been preheated to 37ºC. Immediately after the addition of enzymes, the absorbance changes were monitored every 12 s for 50 minutes (λ=360 nm) at 37ºC in a SPECTROstar Nano (BMG LABTECH) microplate reader. The following parameters were determined from the fibrin polymerization and clot lysis curve: Lag time – time required for the formation and growth of protofibrils from fibrin monomer after the removal of fibrinopeptides; the maximal velocity of the polymerization process (V_{max}) – reflecting the velocity of lateral protofibril association; maximal absorbance (A_{max}) – indicating the fiber thickness and the degree of crosslinking; velocity of clot lysis (V_{Lys}) – the susceptibility of the clot to lysis.

Plasmin amidolytic activity assay by the spectrophotometric method.

Plasma plasmin was assayed by chromogenic substrate (Chromogenix S-2251) after streptokinase activation. Assays were performed at 37ºC in 96-well polystyrene flat-bottom plates. Briefly, 20 µl of test plasma was diluted with 220 µl of buffer (50 mM Tris/HCl, pH 8.2) and preincubated at 37°C for 10 min with 10µl of streptokinase (10 000 U/ml). Following this, 30 µl of chromogenic substrate (3 mM) was added to each reaction well. The rate of liberation of p-nitroaniline from the substrate was determined by kinetic method at 415 nm in a SPECTROstar Nano (BMG LABTECH) microplate reader.

Table 1. Characteristics of study participants
APTT – activated partial thromboplastin time; F- female; M- male; PT – prothrombin time; TT – thrombin time

Patients	Age (years)/sex	Fibrinogen concentration von Clauss method (g/l)	Fibrinogen concentration ELISA method (g/l)	PT (s)	APTT (s)	TT (s)
1	24/M	0.81	1.04	11.4	31.3	33.2
2	64/F	1.21	1.31	14.0	30.5	28.2
3	40/M	1.86	2.96	9.2	46.8	178.5
4	21/F	<0.03	0.04	>120.1	>180.1	>240.1
5	40/F	1.47	3.33	12.1	28.2	39.7
6	85/M	1.34	1.48	10.6	36.0	24.1
7	47/M	1.13	1.38	10.4	27.8	27.9
8	24/F	1.27	2.35	10.6	44.7	23.2
reference values		2-4	2-4	7.0-10.5	26.0-40.0	16.0-21.0

Table 2. Results of fibrinogen concentration and screening hemostasis tests in patients with hypo and dysfibrinogenemia.
PT – prothrombin time; APTT – activated partial thromboplastin time; TT – thrombin time; SD – standard deviation

	Patients with hypofibrinogenemia (n)		Patients with dysfibrinogenemia (n)		Reference values
Fibrinogen concentration (g/l): von Clauss method	mean ± SD	1.13± 0.23	mean ± SD	1.93±0.98	2-4
	median	1.17	median	1.27	
	range	0.81-1.47	range	1.47-305	
Fibrinogen concentration (g/l): ELISA method	mean ± SD	1.3±0.19	mean ± SD	2.88±0.49	2-4
	median	1.345	median	2.96	
	range	1.04-1.48	range	2.35-3.33	
PT (s)	mean ± SD	11.6±1.7	mean ± SD	10.6±1.5	7.0-10.5
	median	11.0	median	10.6	
	range	10.4-14.0	range	9.2-12.1	
APTT (s)	mean ± SD	31.4±3.4	mean ± SD	39.9±10.2	26.0-40.0
	median	30.9	median	34.7	
	range	27.9-36.0	range	28.2-44.7	
TT (s)	mean ± SD	28.4±3.7	mean ± SD	80.5±85.3	16.0-21.0
	median	28.05	median	39.7	
	range	27.9-33.2	range	23.3-178.5	

Table 3. Thromboelastometry data: CT- coagulation time, CFT – clot formation time, α – angle, MCF- maximum clot firmness and ML- maximum lysis in the cohort of patients with hypo and dysfibrinogenemia and in control group
p-values lower than 0.05 are marked in bold.

Test	CT			CFT			α – angle			MCF			ML		
	Patients (n=7)	Controls (n=15)	p	Patients (n=7)	Controls (n=15)	p	Patients (n=7)	Controls (n=15)	p	Patients (n=7)	Controls (n=15)	P	Patients (n=7)	Controls (n=15)	p
EXTEM	72.4±16.7	59.1±7.8	<0.001	129.4±41.9	79.5±19.6	<0.001	65.9±7.3	74±3.9	<0.001	54.9±8.2	63.1±4.5	<0.001	18±4.5	21.9±3.9	0.13
INTEM	197±19.9	172.7±20.6	0.02	110.3±34.9	63.9±13.6	<0.001	69.7±4.7	77.2±2.5	<0.001	52.6±8.9	61±3.9	<0.001	16.7±3.4	18.7±2.8	0.16
FIBTEM	86.6±23.4	52.6±8.1	<0.001	-	-	-	62±7.1	69.9±8.7	<0.001	10.9±5.3	15.7±3.8	<0.001	18.6±15	4.4±5.1	-
APTEM	79.9±26.41	55.9±8.1	0.002	129±53.2	83.5±21.2	<0.001	66.4±7.1	73.4±4.1	<0.001	53.3±8.1	62.2±4.4	<0.001	18.1±3.2	20.6±13.5	0.53

Table 4. Comparison between selected parameters of thromboelastometry: CT- coagulation time, CFT – clot formation time, α – angle, MCF- maximum clot firmness and ML- maximum lysis in the cohort of patients with hypo- and dysfibrinogenemia.
p-values lower than 0.05 are marked in bold letters.

Test	CT			CFT			α – angle			MCF			ML		
	Patients HypoFI (n=4)	Patients DysFI (n=3)	p	Patients HypoFI (n=4)	Patients DysFI (n=3)	p	Patients HypoFI (n=4)	Patients DysFI (n=3)	p	Patients HypoFI (n=4)	Patients DysFI (n=3)	P	Patients HypoFI (n=4)	Patients DysFI (n=3)	p
EXTEM	63±9.9	85±16.6	0.08	159.5±25.3	89.3±9.3	<0.001	60.8±4.9	72.7±3.9	0.01	49±3.8	62.7±4.2	<0.001	19.5±4.1	16±5	0.35
INTEM	198.5±24.5	195±16.5	0.8	128.5±34.6	86±17.4	0.11	67.5±4.8	72.7±2.9	0.16	46.5±4.4	60.7±6.1	0.02	18±2.9	15±3.6	0.28
FIBTEM	88.5±32.3	84±7	0.8	-	-	-	-	62±7.1	-	7.5±2.1	15.3±5.1	0.03	24.3±16.8	11±101	0.28
APTEM	72,5,±28,4	89,7±25,0	0,44	161,5±47,6	72,5,5±28,4	0,47	62,08,1	72,3±2,5	0,04	47,8±5,4	60,7±3,5	0,02	19,5±2,4	16,3±3,8	0,23

Table 5. Results of selected parameters of fibrin plasma polymerization, clot lysis and plasmin amidolytic activity in the cohort of patients (mean ± SD, median from three tests in each patient) and in reference plasma (mean ± SD, median values from 12 tests). P values reflect differences between medians.
p-values lower than 0.05 are marked in bold. Lag time - time required for the formation and growth of protofibrils; V_{max} -maximal velocity of fibrin polymerization process; A_{max} - maximal absorbance; V_{Lys} – velocity of clot lysis; 0-lack of polymerization; ns - non significant; SD - standard deviation; ΔmA/min - delta mili absorbance/minute.

Patients	Lag time (s)	V_{max} [ΔmA/min]	A_{max}	V_{Lys} [ΔmA/min]	Plasmin amidolytic activity [ΔmA/min]
1	432±32	20.0±8.9	0.039±0.005	6.7±5.8	152±14
2	617±27	11.3±3.8	0.018±0.004	5.0±1.0	178±14
3	0	0	0	0	137±9
4	0	0	0	0	120±11
5	410±16	38.3±2.9	0.122±0.009	14.0±1.0	129±5
6	195±8	85.3±4.2	0.166±0.017	21.0±1.7	120±37
7	177±2	81.3±5.0	0.132±0.002	12.3±1.2	138±11
8	150±4	78.3±3.2	0.151±0.004	21.7±1.5	123±17
Patients (8) median, range	186,0; 0-617	29.1; 0-85.3	0.08; 0-0.166	9.5; 0-21.7	129; 120-178
reference values (12) medians; range	178; 169-190 ns	240; 201,0-273.0 0.000248	0.42; 0,35-0.45 0.000248	46,0; 37.0-49.0 0.000248	119; 98-135 ns

Table 6. Comparison between selected parameters of fibrin plasma polymerization, clot lysis and plasmin amidolytic activity in the cohort of patients with hypo and dysfibrinogenemia dysFI – dysfibrinogenemia; hypoFI – hypofibrinogenemia; SD – standard deviation; ΔmA/min – delta mili absorbance/minute.

Lag time (s)		Maximal velocity of polymerization process (V_{max}) [ΔmA/min]		Maximal absorbance (A_{max})		Velocity of clot lysis (V_{Lys}) [ΔmA/min]		Plasmin amidolytic activity [ΔA/min]	
Patients hypoFI (n=4) median range	Patients dysFI (n=3) median range	Patients hypoFI (n=4) median range	Patients dysFI (n=3) median range	Patients hypoFI (n=4) median range	Patients dysFI (n=3) median range	Patients hypoFI (n=4) median range	Patients dysFI (n=3) median range	Patients hypoFI (n=4) median range	Patients dysFI (n=3) median range
313 177-617	150 0-410	50.6 11.3-85.3	38.3 0-78.3	0.086 0.018-0.166	0.122 0-0.15	9.9 5-21	14.0 0-21.7	0.145 0.120-0.178	0.129 0.123-0.137

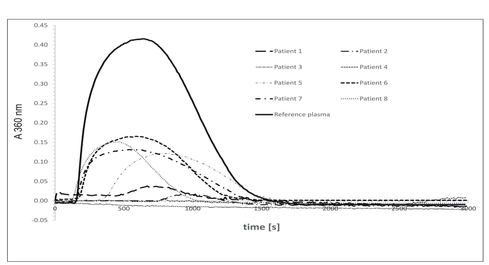

Figure 1. Graph depicting fibrin plasma polymerization and clot lysis in all studied patients and in reference plasma.

Conclusions

In conclusion, our data suggests that both rotation thromboelastometry and fibrin plasma polymerization by turbidimetry have a high sensitivity towards the detection of different congenital fibrinogen disorders. While the assessment of ROTEM MCF may help discriminate patients with hypo or dysfibrinogenemia, its effectiveness has to be confirmed on larger groups of patients.

Poster Presented at:
Rare bleeding disorders
Jacek Trelinski
DOI: 10.3252/pso.au.WFH2016.2016
214--PO-M
WFH2016