Premorbid Hemostasis in Women with a History of Pregnancy Loss

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Abstract

Background Congenital and acquired hemostatic disorders are among the pathogenic factors of pregnancy loss. Studying mechanistic relations between impaired hemostasis and fetal losses is important for the prognosis and prophylaxis of obstetric complications.

Objective This article aims to establish latent hemostatic disorders in nonpregnant women as an important premorbid risk factor of pregnancy loss.

Methods and Results Hemostasis was characterized using two relatively new in vitro assays, namely thrombodynamics (spatial clot growth) and kinetics of blood clot contraction, which together reflect the hemostatic or thrombotic potential. In addition, platelet functionality was assessed using flow cytometry. Our study included 50 women with a history of pregnancy loss and 30 parous women without previous obstetric complications. In patients with pregnancy loss, hypercoagulability was observed along with significant impairment of blood clot contraction associated with chronic platelet activation and dysfunction. Both hypercoagulability and defective clot contraction were significantly more pronounced in patients with a history of three or more miscarriages compared with patients with a history of one or two miscarriages. In addition, a significant inhibition of clot contraction was found in patients with miscarriage occurring after 10 weeks of gestation compared with those who lost a fetus earlier in pregnancy.

Conclusion These results indicate that chronic hypercoagulability and impaired clot contraction constitute a premorbid status in patients with pregnancy loss. The data confirm a significant pathogenic role of hemostatic disorders in pregnancy loss and suggest the predictive value of thrombodynamics and blood clot contraction assays in evaluating the risk of pregnancy loss.

Keywords
- blood clotting
- contraction of blood clots
- recurrent pregnancy loss
- miscarriage

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Introduction

Pregnancy loss (PL) is one of the most common and important medical and social problems. PL is a broad term that includes fetal death in any trimester of pregnancy, including miscarriage ($<$ 20 weeks of gestation) and stillbirths and premature births ($\geq$ 20 weeks of gestation).[1] Recurrent pregnancy loss (RPL) has historically been defined as three consecutive PLs prior to 20 weeks from the last menstrual period. There is data that suggests that risk of miscarriage in women with two versus three or more miscarriages without a history of live birth is 30% versus 33%, respectively.[2] In response to this narrow difference in risk, some contemporary professional organizations, such as the American Society for Reproductive Medicine, expanded the definition of RPL to include women who have two or more failed clinical pregnancies prior to 20 weeks.[3]

One of the pathogenic factors of PL is congenital and acquired hemostatic disorders. The physiological activation of the blood coagulation system starting from the beginning of pregnancy is beneficial because it promotes the implantation of a blastocyst and prevents peri-implantation hemorrhage during the endovascular invasion of the trophoblast. On the other hand, excessive deposition of fibrin at the site of implantation and microthrombosis of spiral arteries and arterioles disrupt the implantation of a fertilized egg and can lead either to abortion or to the placental insufficiency underlying various obstetric complications.[4] It has been shown that hereditary thrombophilias, including factor V Leiden, a mutation of the prothrombin gene as well as deficiencies of protein C, protein S, and antithrombin III (ATIII), can all cause PL in both early and late pregnancy.[5,6] These and other data show that PL is associated with a shift of the hemostatic balance toward a prothrombotic state or hypercoagulability, which cause a predisposition to intravascular fibrin formation. Therefore, during preconception counseling it is important to identify women with pro- or prethrombotic conditions and to start prevention of miscarriage with prophylactic anticoagulants as soon as possible. Despite the time-sensitive diagnosis of pro- and prethrombotic conditions, traditional laboratory hemostatic assays do not always reveal a latent thrombogenic potential and cannot help to assess the risk of obstetric complications.[6] A new integral hemostatic test, called *thrombodynamics*, has been recently introduced. This test is sensitive to both hypo- and hypercoagulability, capturing subtle hemostatic disorders including underlying (pro)thrombotic states.[7,8] The test is based on local activation of the coagulation of blood plasma from one side by a surface-attached tissue factor, mimicking procoagulant subendothelium in a damaged vessel wall. The activation of clotting is followed by subsequent optical tracking of the spatial growth of a fibrin clot.

After a blood clot forms in vitro or in vivo, it undergoes volumetric shrinkage called clot contraction or retraction. Clot contraction is driven by activated platelets; hence, this process can be used as a test to evaluate platelet count and functionality. In addition to platelets, blood clot contraction is modulated by pathological changes in the cellular and molecular composition of blood.[9] Therefore, the study of clot contraction may provide important additional information on the overall hemostatic/thrombotic potential. Clinical studies of the blood of patients with (pro)thrombotic conditions, such as ischemic stroke, venous thrombosis, and systemic lupus erythematosus, have shown that clot contraction is significantly decreased due to platelet dysfunction as a result of their chronic hyperactivation followed by energetic exhaustion and refractoriness to biochemical stimulation.[10–13] Presumably, similar processes may take place in the blood of women with chronic hypercoagulability, which can predispose them to PL. Studying clot contraction of blood from these patients may provide additional information about the role of hemostatic and thrombotic disorders in the pathogenesis of PL.

The aim of this work was to study the hemostatic status in women with a history of PL, using a combination of two relatively new laboratory tests, namely the thrombodynamics and kinetics of contraction of blood clots. The results of these tests in correlation with other laboratory finding and clinical characteristics suggest the important role of premorbid hemostasis disorders in the pathogenesis of PL.

Materials and Methods

Patients, Healthy Subjects, and Exclusion Criteria

The patient group consisted of 50 women with a history of PL. The obstetric history included a combination of two or more miscarriages before 10 weeks of gestation, one or more miscarriages at 10 to 22 weeks of gestation, and one or more stillbirths or premature births at 22 to 34 weeks of gestation. The clinical characteristics of the group studied are presented in ►**Table 1**. This patient group represents a

Table 1 Clinical characteristics of patients with a history of PL ($n = 50$)

Characteristics	Patients with the following abnormalities
Obstetrical risks from patient history	
Early miscarriage (before 10 wk)	40 patients (80%)
Late miscarriage (10–22 wk)	14 patients (28%)
Premature birth or stillbirth (22–34 wk)	17 patients (34%)
Hereditary thrombophilia	
Deficiency of antithrombin III	1 (2%)
Deficiency of protein S $<$ 60%	11 (22%)
Deficiency of protein C $<$ 70%	1 (2%)
Mutation of factor II G20210A	2 (4%)
Acquired prothrombotic features	
Hyperhomocysteinemia $>$ 12 μmol/L	3 (8%)
Hyperfibrinogenemia $>$ 4 g/L	1 (2%)
Plasminogen activator inhibitor-1 $>$ 7 U/mL	8 (16%)

Abbreviation: PL, pregnancy loss.

wide range of PL in terms of gestational age and frequency, including some women with a history of RPL per the classical and updated definitions mentioned in the "Introduction."[1–3] The control group consisted of 30 parous women without previous complications of pregnancy and without a personal and/or family history of thrombosis. The patients and the control individuals were comparable in age (30 ± 1 and 31 ± 1 years, respectively). The study was conducted at the stage of pregnancy planning. Patients and healthy subjects were excluded from the study if for any reason anticoagulation, thrombolytic, or antiplatelet medications were taken at least 2 weeks before the examination. At the time of examination neither patients with a history of PL nor healthy subjects received any hormonal therapy. Also, patients were excluded from the study if they had antiphospholipid syndrome or any comorbidities that could potentially affect the hemostatic tests. All women gave informed consent to participate in the study.

Blood Collection and Processing

Blood was obtained according to the requirements of the Ethical Committee of the Kazan Federal University (Protocol No. 3 as of 03/23/17). Blood was collected into vacutainers containing 3.8% trisodium citrate 9:1 by volume and analyzed within 4 hours. Sample I of whole blood was used for the clot contraction assay. Sample II was centrifuged ($2{,}000 \times g$, 10 minutes) to obtain platelet-poor plasma used for blood coagulation tests. For the thrombodynamics test, platelet-free plasma was used, which was obtained by additional centrifugation of the platelet-poor plasma for 5 minutes at $10{,}000 \times g$ at room temperature. Sample III of whole blood was used for platelet isolation. Blood sample IV was stabilized with K_3-ethylenediaminetetraacetic acid (EDTA) and used for hematological tests. A portion of the sample stabilized with K_3-EDTA was used for genotyping. Comparative results of routine laboratory tests in the patients and control subjects are summarized in ►**Table 2** and ►**Supplementary Table S1** (available in the online version).

Kinetics of Spatially Directed Clot Formation in Blood Plasma (Thrombodynamics)

The thrombodynamics assay is based on the optical registration of the spatial growth of a fibrin clot in blood plasma after contacting a surface with immobilized tissue factor that triggers coagulation in the adjacent plasma layer. The process of formation and propagation of a fibrin clot is tracked and recorded optically by a charge-coupled device camera built in an automated Thrombodynamics Analyzer System (Hema-Core Ltd., Russia) supplied along with a diagnostic kit. To perform the test, a sample of citrated platelet-free plasma was mixed with 200 µg/mL of corn trypsin inhibitor to block the contact phase and 20 mM calcium acetate (final concentrations). Then the recalcified plasma sample (120 µL) was transferred to a measuring cuvette and a plastic activator coated with tissue factor (enclosed in the kit) was immersed. Fibrin growth beginning from one side of the cuvette was registered every 6 seconds for 30 minutes. The images obtained were automatically processed by a program that calculated the following parameters: (1) lag time – the time required to start the formation of fibrin from the moment of contact of the plasma with the activating surface; (2) the initial growth rate of the clot – the average growth rate of the clot calculated in the range of 2 to 6 minutes after the onset of clot growth; (3) the stationary growth rate of the clot – the average growth rate of the clot calculated in the range of 15 to

Table 2 Laboratory parameters in women with a history of PL compared with the control group

Parameters (in parentheses – reference values)	Control group ($n = 30$)	Women with PL ($n = 50$)
Hemostatic parameters		
aPTT (24–38) s	34 ± 1	31 ± 1[a]
Fibrinogen (1.8–3.5) g/L	2.6 ± 0.1	2.7 ± 0.1
Prothrombin time (10.7–12.9) s	12.6 ± 0.2	14.2 ± 1.9
Thrombin time (14–21) s	18.6 ± 0.2	18.0 ± 0.2
Antithrombin III (79.4–112)%	99 ± 1	96 ± 1[a]
Plasminogen (75–150)%	91 ± 2	94 ± 2
Soluble fibrin (3-4) mg/100 mL	4.1 ± 0.2	5.6 ± 1.7
Protein C (70–140)%	85 ± 4	92 ± 2
Protein S (60–130)%	77 ± 3	70 ± 3
D-dimer (0–550) µg/L	281 ± 32	448 ± 165
Factor XIIa-dependent fibrinolysis (5–12) min	6.0 ± 0.3	6.0 ± 0.2
Prothrombin index (88–116)%	90 ± 2	91 ± 2
Hematologic parameters		
Platelet count (180–320) $\times 10^9$/L	228 ± 9	264 ± 11[a]
Red blood cells (3.7–4.7) $\times 10^{12}$/L	4.40 ± 0.06	4.40 ± 0.05
Leukocytes (4–9) $\times 10^9$/L	5.7 ± 0.2	5.8 ± 0.2
Eosinophils (0–5)%	2.3 ± 0.3	2.4 ± 0.3
Monocytes (2–9)%	8.0 ± 0.3	8.1 ± 0.3
Lymphocytes (19–37)%	36 ± 1	35 ± 1
Basophils (0–1)%	1.00 ± 0.08	0.81 ± 0.05[a]
Neutrophils (47–72)%	52 ± 1	53 ± 1
Hemoglobin (115–140) g/L	127 ± 2	120 ± 4
ESR (2–20) mm/h	8 ± 1	9 ± 1
Hematocrit (35–45)%	39 ± 1	35 ± 2
Mean corpuscular volume (MCV) (80–100) fL	87 ± 1	85 ± 1
Red blood cell distribution width (RDW) (11.5–14)%	12.6 ± 0.3	13.2 ± 0.3
Mean platelet volume (MPV) (7.4–10.4) fL	8.3 ± 0.3	9.2 ± 0.2[a]
Platelet distribution width (PDW) (10–18)%	14.0 ± 0.6	17.5 ± 0.8[b]
Thrombocrit (0.15–0.4)%	0.19 ± 0.01	0.26 ± 0.01[b]

Abbreviations: aPTT, activated partial thromboplastin time; ESR, erythrocyte sedimentation rate; PL, pregnancy loss.
[a] $p < 0.05$.
[b] $p < 0.001$.

25 minutes after the onset of clot growth; (4) the size of the fibrin clot after 30 minutes following contact of plasma with the activator insert; and (5) clot density – an optical indicator equal to the intensity of light scattering by the fibrin clot, proportional to the spatial density of the fibrin network (►**Supplementary Fig. S1A**, available in the online version).

Continuous Tracking of Contracting Blood Clots

The kinetics of contraction of blood clots was recorded in vitro using the same Thrombodynamics Analyzer System. Citrated blood samples from patients and controls were activated with 1 U/mL human α-thrombin (Sigma-Aldrich) and 2 mM $CaCl_2$ (final concentrations). The activated blood samples (80 µL) were quickly transferred to a 12 × 7 × 1 mm transparent plastic cuvette that was precoated with a thin layer of 4% vol/vol Triton X-100 in 0.15 M NaCl to prevent the clot from sticking to the chamber without affecting the clot structure and platelet functionality. The transparent cuvette was placed into the 37°C temperature-controlled chamber of the Thrombodynamics Analyzer instrument. The cuvette had two compartments and the experiments were performed simultaneously in duplicate. Images of the clots were taken every 15 seconds for 20 minutes to track the changes in the relative clot size based on the light scattering. The images collected were analyzed computationally to extract the following parameters of clot contraction: (1) extent of contraction calculated as $[(S_0–S_t)/S_0] \times 100$, where S_0 is the initial clot size and S_t is the final clot size at the end point $t = 20$ minutes; (2) lag time – time from the addition of thrombin until the clot reaches 95% of its initial size; (3) average contraction velocity; and (4) area under the kinetic curve, roughly corresponding to the amount of mechanical work of clot compression done by the contracting platelets (►**Supplementary Fig. S1B**, available in the online version).

Platelet Isolation

Citrated venous blood from PL patients or healthy subjects was spun at 200 × *g* for 10 minutes to obtain platelet-rich plasma (PRP). Isolated platelets were collected in the void volume after gel filtration of the PRP on Sepharose 2B equilibrated with Tyrode's buffer (4 mM HEPES, 135 mM NaCl, 2.7 mM KCl, 2.4 mM $MgCl_2$, 5.6 m MD-glucose, 3.3 mM NaH_2PO_4, 0.35 mg/mL bovine serum albumin, pH 7.4). Cell viability was 97% based on the maintenance of the mitochondrial membrane potential (ΔΨm) determined by flow cytometry using a ΔΨm-sensitive fluorescent dye MitoTracker Deep Red FM (Invitrogen). Platelet count was performed in a hemocytometer. Platelets were used within 3 hours of blood collection.

Flow Cytometry of Resting and Activated Platelets

Platelet functionality was analyzed in 27 PL patients and 15 healthy individuals in parallel by expression of P-selectin (CD62p) and active integrin αIIbβ3 (determined by its fibrinogen-binding capacity) before and after activation with a thrombin receptor-activation peptide 6 (TRAP-6, the protease-activated receptor 1 [PAR1]-specific hexapeptide Ser-Phe-Leu-Leu-Arg-Asn; Bachem Americas Inc.). TRAP-6 was added to isolated platelets at 50 µM and incubated for 3 minutes at room temperature. Then platelets (200,000 in 50 µL) were incubated for 10 minutes with anti-human-CD62p phycoerythrin-labeled murine antibodies (BD Biosciences) (0.045 µg/mL) or Alexa Fluor 488-labeled human fibrinogen (ThermoFisher Scientific) (5 µg/mL). After incubation with the labeled ligands, the platelets were analyzed using a FacsCalibur flow cytometer equipped with BD Cell-Quest software (►**Supplementary Fig. S2**, available in the online version). Platelets were gated based on their size and granularity and 5,000 platelets were counted in each sample. FlowJo X software was used for data analysis.

Coagulation, Hematological Tests, and Genotyping

Hemostasis was assessed using an automated Sysmex CA-1500 coagulometer (Sysmex, Canada) based on the following parameters: activated partial thromboplastin time (aPTT), prothrombin time, thrombin time (TT), fibrinogen concentration (Clauss method), ATIII activity, plasminogen level, and proteins C and S levels. D-dimer was measured using an immunochemiluminescent analyzer Immulite 2000 (Siemens Healthcare Diagnostics Inc., United States). Soluble fibrin monomer complexes in plasma were determined using the orthophenanthroline test (reagents of the company "Technology-Standard," Russia). Cell count was performed using a hematology analyzer (Siemens Inc., Japan). Erythrocyte count, mean erythrocyte volume, hematocrit, hemoglobin levels, mean erythrocyte hemoglobin levels, color index, total leukocyte counts, monocytes, neutrophils, lymphocytes, eosinophils, basophils, platelets, and average platelet volume were analyzed.

All women in the main and control groups were genotyped for the presence of the following prothrombotic gene polymorphisms: FV:1691G/A; FII:20210G/A; FXIII:Val34Leu (163G/T); FGB:455G/A; ITGA 2:807C/T; ITGB 3:1565 T/C; and PAI-1:4G/5G (►**Supplementary Table S1**, available in the online version). A real-time polymerase chain reaction was used with the equipment and reagents from DNA Technologies (Russian Federation).

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 7 software package (GraphPad Software, San Diego, California, United States). After assessing normality with the Shapiro–Wilk and D'Agostino–Pearson criteria, two-tailed Student's *t*-test was used to confirm the statistical significance of the observed differences. Correlation analysis was performed using Pearson's correlation coefficient. The level of significance was 95% ($p < 0.05$). Data are presented as mean ± standard error of the mean.

Results

Hypercoagulability in Patients with a History of PL Revealed by the Thrombodynamics Assay

In the thrombodynamics assay, patients with a history of PL, when compared with the control group, had significantly higher average initial and stationary growth rates of the clot

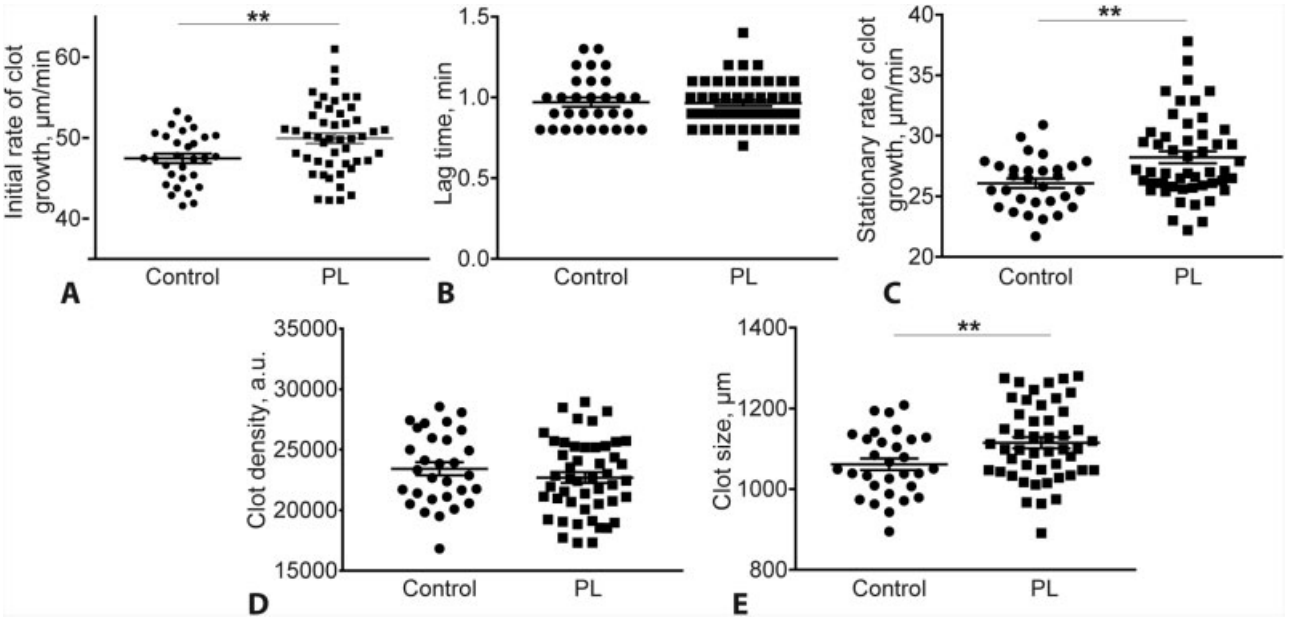

Fig. 1 Parameters of thrombodynamics in women with a history of pregnancy loss (PL) compared with those of the control group. The initial growth rate of the clot (**A**), the lag time (**B**), the stationary growth rate of the clot (**C**), the clot size (**D**), and the clot density (**E**). $^{**}p < 0.01$.

as well as a larger clot size (►**Fig. 1**, and the numbers are shown in ►**Table 3**). These differences indicate a shift toward hypercoagulability in the patients with a history of PL that was observed in the absence of pregnancy. Significant shortening of aPTT in the patients with PL compared with controls (►**Table 2**) confirmed hypercoagulability revealed by the thrombodynamics assay. When the parameters of thrombodynamics were analyzed with respect to hemostasis-related genetic polymorphisms, a significant hypercoagulability documented by the abovementioned parameters of thrombodynamics was found in patients with a SERPINE 1 (plasminogen activator inhibitor-1 [PAI-1]) gene polymorphism 675:5G > 4G. In particular, in patients with 4G/4G homozygous and 5G/4G heterozygous polymorphisms with a hereditary predisposition to thrombosis, the initial and stationary clot growth rates and the clot size were moderately but significantly increased and the lag time was shortened, in contrast with the patients with a "neutral" genotype 5G/5G (►**Supplementary Table S2**, available in the online version). In addition, a significant difference was found for the FXIII:103 G/T polymorphism in the initial rate of clot growth and clot size between G/G homozygotes and T/T homozygotes (►**Supplementary Table S3**, available in the online version). For the polymorphism FBG:455 G/A, a significant difference was revealed in the initial and stationary rate of clot growth as well as in clot size between G/G homozygotes and G/A heterozygotes (►**Supplementary Table S4**, available in the online version). The differences in thrombodynamics and contraction parameters between patient subgroups with genetic polymorphisms FII:20210 G/A, FV:1691 G/A, FVII:10976 G/A, and ITGB3:1565 T/C were statistically insignificant (data not shown).

Table 3 Parameters of thrombodynamics and blood clot contraction in women with a history of PL compared with the control group

Parameters	Control group ($n = 30$)	Women with PL ($n = 50$)
Parameters of thrombodynamics		
Stationary rate of clot growth, μm/min	26.0 ± 0.4	28.2 ± 0.5^a
Lag time, min	1 ± 0.03	1 ± 0.02
Initial rate of clot growth, μm/min	47.4 ± 0.6	49.9 ± 0.6^a
Clot size, μm	1061 ± 14	$1{,}119 \pm 16^a$
Clot density, a.u.	$23{,}410 \pm 546$	$22{,}692 \pm 447$
Parameters of blood clot contraction		
Extent of clot contraction, %	43 ± 1	35 ± 1^b
Lag time, s	196 ± 14	253 ± 17^c
Area under the curve, a.u.	310 ± 11	249 ± 13^a
Average velocity, %/s	0.034 ± 0.001	0.028 ± 0.001^b

Abbreviation: PL, pregnancy loss.
[a] $p < 0.01$.
[b] $p < 0.001$.
[c] $p < 0.05$.

Kinetics of Contraction of Blood Clots in Patients with PL

Despite the standard clot initiation and platelet activation conditions, clots formed from the blood of patients with a history of PL contracted significantly slower and to a lesser extent than clots formed from the blood of healthy subjects (►**Fig. 2** and ►**Table 3**). Specifically, the average degree of contraction, rate of contraction, and the area under the kinetic curve all were significantly reduced, while the lag

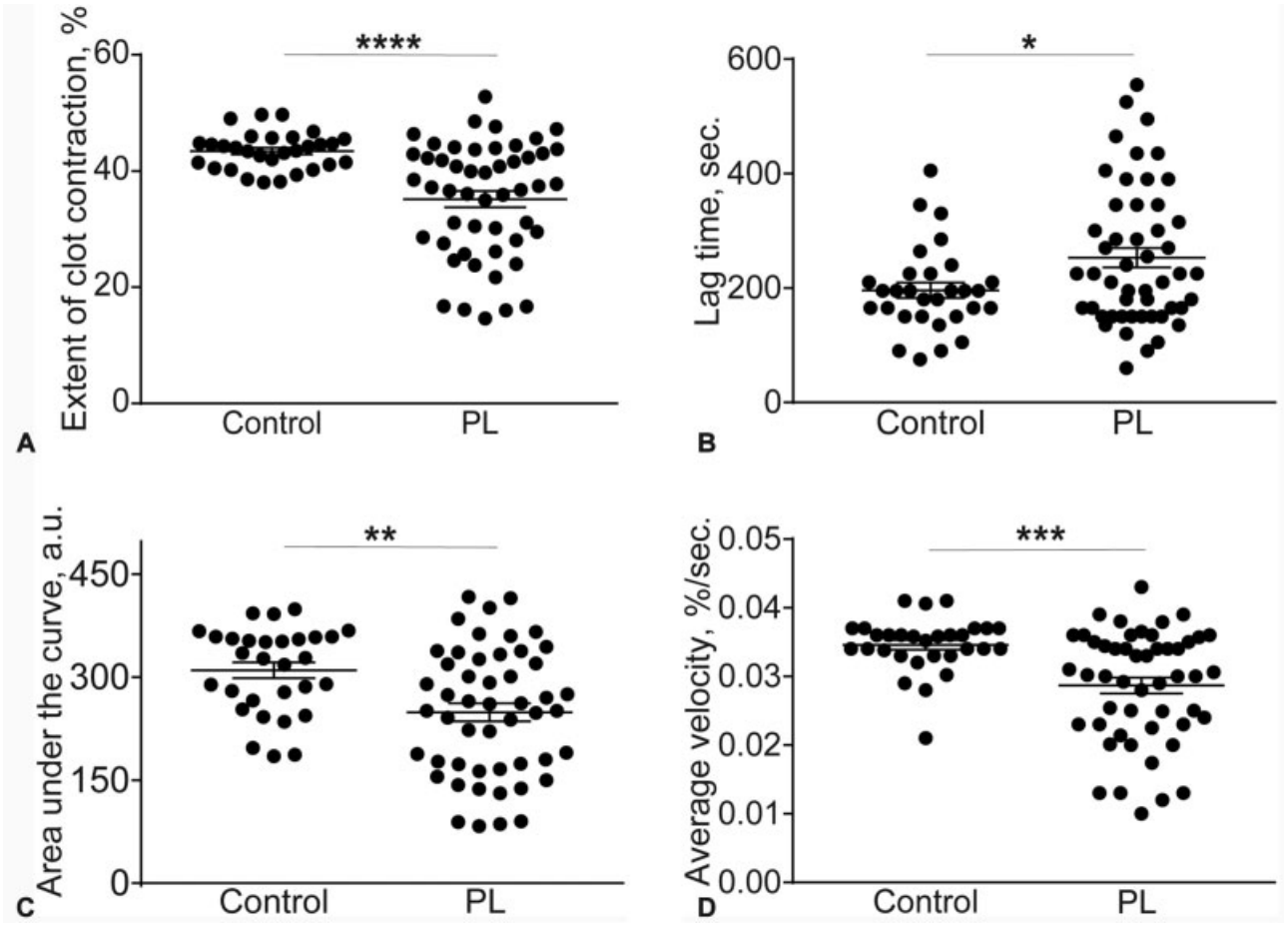

Fig. 2 Parameters of blood clot contraction in women with a history of pregnancy loss (PL) compared with those of the control group. The extent of clot contraction (**A**), the lag time (**B**), the area under the curve (**C**), and the average velocity (**D**). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

period of contraction was prolonged, altogether indicating substantial impairment of clot contraction.

It has been previously shown that clot contraction occurs in three phases: initiation of contraction (phase 1), linear contraction (phase 2), and mechanical stabilization (phase 3).[8] Regression analysis conducted on the averaged kinetic curves and their first derivatives (►**Fig. 3A**) revealed that in PL patients the rate constants of phases 1 and 2 were significantly reduced compared with healthy subjects, indicating impairment of the mechanisms of contraction initiation and compaction of the clots (►**Fig. 3B, C**). However, there were no significant differences in the rates of phase 3 between controls and patients, suggesting that there were no defects in the stabilization of contracted clots in PL patients (►**Fig. 3D**).

Relationship of the Clot Contraction Parameters to Laboratory Tests

To reveal possible mechanisms underlying the observed hemostatic variations in patients with PL history, the parameters of clot contraction were analyzed with respect to other hemostatic and hematological laboratory tests characterizing the blood composition obtained in the same patients with PL and the control group (►**Table 2**).

Correlation analysis of indicators of blood clot contraction and other blood tests revealed reasonable significant correlations (►**Supplementary Table S5**, available in the online version). Inverse correlation of TT with the area under the contraction kinetic curve was found as well as a direct correlation between D-dimer and the extent and rate of contraction. A moderate positive correlation was found between the level of PAI-1 and -2 parameters, namely the extent of clot contraction and the average velocity. Direct correlations between the platelet count and the degree and rate of contraction as well as with the area under the kinetic curve were predictable, because clot contraction is driven by activated platelets. In addition, there was a moderate positive correlation of some contraction parameters with white blood cells, namely neutrophils and basophils. These correlations between the contraction parameters and blood tests suggest that the kinetics of clot contraction are sensitive to changes in blood composition beyond variations of the number and functional state of platelets.

Analysis of Platelet Function in PL Patients

In addition to the results of correlation analysis, the laboratory tests presented in ►**Table 2** provide evidence for changes in platelets in patients with PL. Particularly, an increased

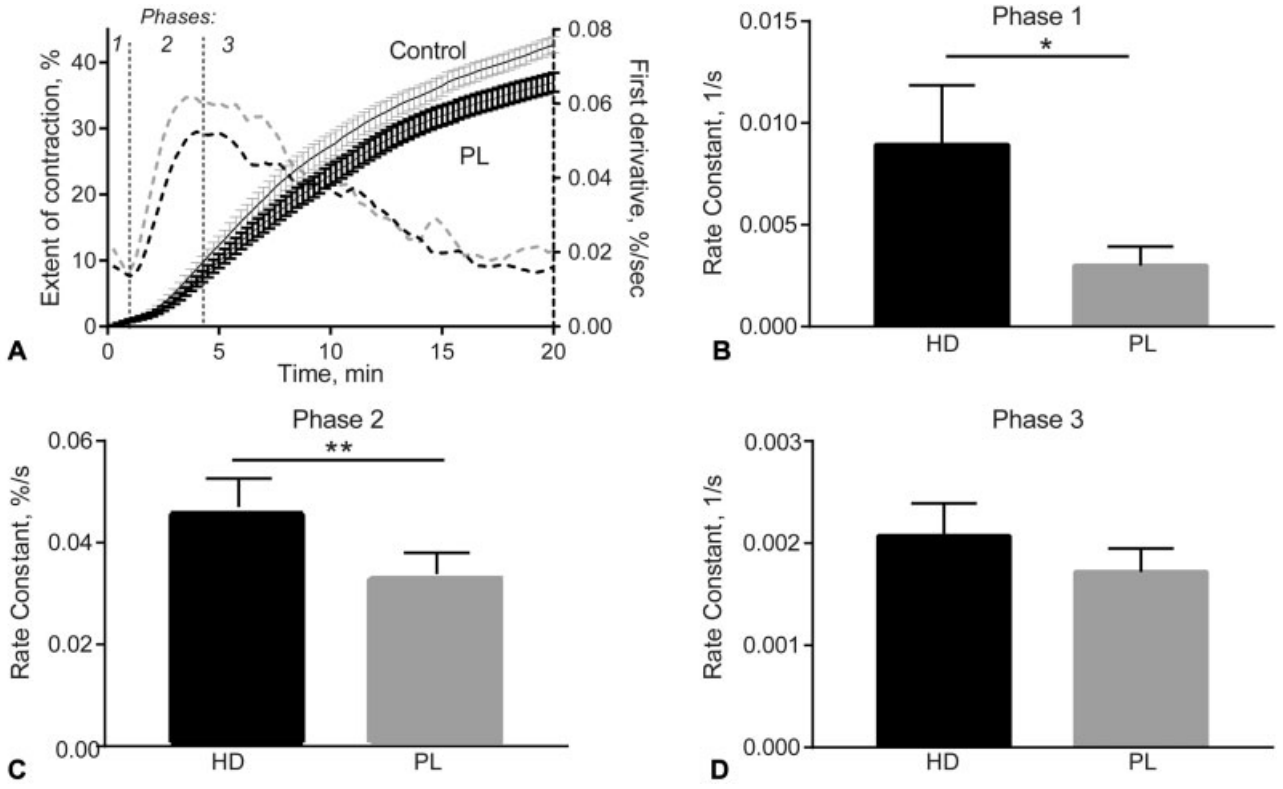

Fig. 3 Contraction of blood clots in patients with pregnancy loss (PL) and control group. (**A**) Averaged kinetic curves of clot contraction in the blood of patients with PL ($n = 50$) and control healthy subjects ($n = 30$). The kinetics curves are segregated into the three phases (dashed vertical lines) determined by assessing the local maxima and minima of the instantaneous first derivative for PL (black dashed curve) and control (gray dashed curve). (**B**–**D**) Rate constants of phase 1 (**B**), phase 2 (**C**), and phase 3 (**D**).

platelet count, elevated mean platelet volume (MPV), platelet distribution width (PDW), and thrombocrit suggest that platelets undergo quantitative and qualitative alterations.[14,15] Because platelets are critical for the contractile force generated during clot contraction, we studied directly the functionality of platelets in PL patients using flow cytometry-based evaluation of the baseline activity of quiescent platelets and their responsiveness to chemical activation. We used TRAP to mimic the effect of thrombin on PAR1 receptors. Platelet reactivity was assessed by surface expression of P-selectin and by the ability to bind fibrinogen as a measure of the integrin αIIbβ3 activation.

In unstimulated platelets isolated from PL patients and healthy subjects, there was a significant difference in the background platelet activation assessed by the levels of P-selectin expression and αIIbβ3 activation (►**Table 4**). However, unlike in resting untreated platelets, in response to TRAP-induced stimulation, platelets from the blood of PL patients had a significantly lower expression of P-selectin compared with TRAP-activated normal platelets (►**Table 4**).

Table 4 Functional characterization[a] of platelets isolated from the blood of women with a history of PL and healthy subjects before and after stimulation with TRAP

	P-selectin expression			Fibrinogen-binding capacity		
	Untreated platelets	TRAP-activated (3-min activation)	TRAP-activated (10-min activation)	Untreated platelets	TRAP-activated (3-min activation)	TRAP-activated (10-min activation)
Healthy subjects ($n = 15$)	1.6 ± 0.2	64.4 ± 3.0	92.7 ± 2.0	0.9 ± 0.2	78.6 ± 3.2	89.2 ± 2.8
Women with PL ($n = 27$)	3.6 ± 0.2[b]	51.3 ± 4.3[b]	77.8 ± 3.3[b]	1.6 ± 0.4[b]	65.3 ± 4.2[b]	75.0 ± 3.5[b]

Abbreviations: PL, pregnancy loss; SEM, standard error of the mean; TRAP, thrombin receptor-activating peptide.
[a]The numbers (mean ± SEM) represent relative flow cytometry counts (%) for the fractions of platelets bearing fluorescently labeled antibodies to P-selectin or fibrinogen.
[b]$p < 0.05$ between controls and patients.

TRAP-induced stimulation also resulted in a reduced fibrinogen-binding capacity compared with the TRAP-activated normal platelets. Collectively, these results indicate that in PL patients, platelets are spontaneously activated to a certain extent but have a remarkably reduced responsiveness to a thrombin-like stimulus. It is noteworthy that the ratio of activated versus quiescent cells with both fluorescent markers was approximately two- to threefold higher in healthy subjects compared with PL patients. The combination of this finding with the reduced exposure of P-selectin and fibrinogen-binding activity in PL patients, suggest that their platelets have a substantially decreased overall activation potential. Accordingly, thrombin-induced contraction in the group of PL patients studied for platelet functionality was significantly impaired with an average extent of $37 \pm 1\%$ versus $43 \pm 1\%$ for controls ($p < 0.01$).

Relation of Thrombodynamics and Clot Contraction to Clinical Characteristics of PL Patients

►**Table 5** shows how changes in thrombodynamics and contraction of blood clots in patients with PL depend on the number of miscarriages. The most pronounced hypercoagulability was found in patients with three or more PLs (fitting the classical definition of RPL), as revealed by dramatic changes in the stationary growth rate reaching pathological values over the normal range as well as by a larger clot size. Patients who lost one or two pregnancies, some of whom fit the newer definition of RPL, had significantly fewer pronounced signs of hypercoagulability assessed by thrombodynamics. The same regularity was revealed for the changes in clot contraction, which was also significantly more suppressed in patients with three or more PLs compared with those who lost one or two pregnancies. Thus, there was a direct association between the number of miscarriages and the severity of hemostatic disorders with increased thrombogenic potential, suggesting an important pathogenic role of chronic hypercoagulability in predisposition to PL.

Another important clinical observation was that alterations in thrombodynamics (initial rate of clot growth and clot size) and contraction of blood clots (extent and rate of contraction as well as lag time) were significantly more pronounced in patients who lost pregnancies after 10 weeks of gestation compared with patients with miscarriages at earlier gestations (►**Table 6**). There was no significant association revealed between the parameters of thrombodynamics and the time of PL (►**Table 6**).

Discussion

In this study, hemostasis in women with a history of PL was studied using two relatively new assays that have never been applied to obstetrics, one based on directional clot growth (thrombodynamics) and the other on contraction of blood clots. These tests revealed a pathogenic role of chronically impaired hemostasis in PL as a premorbid condition. Moreover, in correlation with other laboratory assays and clinical characteristics of PL, these hemostatic tests were shown to

Table 5 Comparison of thrombodynamics and blood clot contraction in women with a history of PL segregated by the number of fetal losses

Parameters	1 or 2 fetal losses ($n = 37$)	More than 2 fetal losses ($n = 13$)
Thrombodynamics parameters		
Initial rate of clot growth, μm/min	49.1 ± 0.7	51.3 ± 1.2
Lag time, min	0.9 ± 0.02	0.9 ± 0.04
Stationary rate of clot growth, μm/min	27.3 ± 0.5	30.5 ± 0.9[a]
Clot size, μm	$1{,}090 \pm 18$	$1{,}174 \pm 25$[b]
Clot density, a.u.	$22{,}335 \pm 499$	$23{,}718 \pm 842$
Blood clot contraction parameters		
Extent of clot contraction, %	38 ± 1	33 ± 3[b]
Lag time, s	258 ± 21	219 ± 26
Area under the curve, a.u.	288 ± 10	246 ± 27[b]
Average velocity, %/s	0.031 ± 0.001	0.027 ± 0.002[b]

Abbreviation: PL, pregnancy loss.
[a] $p < 0.01$.
[b] $p < 0.05$.

Table 6 Comparison of thrombodynamics and blood clot contraction in women with a history of PL depending on the gestational age at which miscarriage occurred

Parameters	Early miscarriage (before 10 wk) ($n = 22$)	Late miscarriage (10–34 wk) ($n = 28$)
Thrombodynamics parameters		
Initial rate of clot growth, μm/min	48 ± 1	51 ± 1[a]
Lag time, min	0.90 ± 0.03	0.9 ± 0.02
Stationary rate of clot growth, μm/min	27.4 ± 0.6	28.7 ± 0.7
Clot size, μm	$1{,}073 \pm 18$	$1{,}138 \pm 22$[a]
Clot density, a.u.	$22{,}968 \pm 650$	$22{,}385 \pm 595$
Blood clot contraction parameters		
Extent of clot contraction,%	39 ± 1	33 ± 2[a]
Lag time, sec	203 ± 16	282 ± 26[b]
Area under the curve, a.u.	263 ± 20	246 ± 17
Average velocity, %/sec	0.031 ± 0.001	0.029 ± 0.001

Abbreviation: PL, pregnancy loss.
[a] $p < 0.05$.
[b] $p < 0.01$.

have a potential diagnostic and prognostic value at the pregnancy planning stage to evaluate the risk of PL.

There is reason to think that repeated fetal loss is a consequence of pathological hypercoagulability during pregnancy, which leads to microthrombosis of the chorionic vessels or placenta and ultimately to miscarriage, premature birth, or stillbirth.[16] It is known that early miscarriages prevail over late spontaneous abortions, which may be due to the fact that placental and uterine vessels formed by this time may be partially or completely occluded as a result of microthrombosis without compensatory blood flow through collateral bypass circulation.[17]

Clinical studies of thrombodynamics and contraction of blood clots in the case of PL have not been conducted previously. The results of this study show that in patients with PL there is a propensity to chronic hypercoagulability associated with a significant reduction in the ability of blood clots to contract compared with healthy women. The hypercoagulability and reduced contraction revealed in PL patients are consistent with the increased levels of fibrinogen and D-dimer, as well as increased formation of soluble fibrin monomer complexes in the blood described in previous studies.[18,19] These characteristics found in patients with a history of PL could be applicable to a narrower population of patients with RPL.

Reduced contraction of blood clots suggests platelet dysfunction in patients with PL caused by their continuous activation in the circulation due to hypercoagulability and thrombin generation, as was shown in other prothrombotic states.[10,20] This assumption is confirmed by the studies of platelet functionality that have led to two conclusions: (1) platelets are continuously activated in the blood of PL patients and (2) platelets are dysfunctional because their response to an activating stimulus is partially impaired (►**Table 4**). These findings are consistent with the data showing a high level of background or "spontaneous" platelet activation in patients with two or more miscarriages in their histories.[21,22] Notably, platelet dysfunction that manifests as a decrease in platelet contractility and impaired clot shrinkage in patients with PL develops notwithstanding a significant increase of platelet counts in the blood compared with the control group (►**Tables 2** and **3**). The relative thrombocytosis may be a compensatory mechanism for metabolic exhaustion and functional impairment of platelets resulting from their chronic activation and reduced lifespan.

In patients with a history of more than two miscarriages, changes of contraction and thrombodynamics were more pronounced than in patients with one or two miscarriages (►**Table 5**). This is consistent with the existing evidence that hemostatic disorders are revealed more often in patients with multiple miscarriages compared with patients with one or two miscarriages.[17]

The time period of pregnancy also matters for the likelihood of miscarriages and associated hemostatic disorders. According to our data, the degree of clot contraction is reduced significantly more in patients with late rather than early miscarriages. This association is consistent with the notion that the late PL (>10 weeks of gestation) is largely due to hemostatic abnormalities (especially thrombophilia), while early fetal losses (<10 weeks of gestation) are usually associated with other pathogenetic factors (genetic, endocrine, and gynecological), not related to the prothrombotic status.[22,23] Remarkably, the reduced basophil count in women with a history of PL (►**Table 2**) may reflect chronic inflammation, such as endometritis, associated with implantation failure and a nondeveloping pregnancy.[24] This finding is consistent with the observation that prothrombotic conditions are associated with reduced basophil counts in blood.[25]

Given the importance of hereditary thrombophilia in the pathogenesis of PL, the clinical data were correlated with eight variants of genetic polymorphism of prothrombotic genes. We revealed a significant predominance of impaired thrombodynamics in the women with PL that were 4G/4G homozygotes and 5G/4G heterozygotes for PAI-1:675 (►**Supplementary Table S2**, available in the online version), which is consistent with an earlier publication showing that 4G homozygosity constituted an independent risk factor for adverse pregnancy complications and that PAI-1 activity was an independent risk factor for PL.[26,27] A growing number of studies suggested that the PAI-1 – 675G/A (4G/5G) polymorphism, when combined with other prothrombotic factors, contributes to the pathogenesis of PL. This cooperative interaction, in turn, facilitates impaired fibrinolysis, thereby promoting PL.[28,29] Since the blood protein PAI-1 is an inhibitor of fibrinolysis, its increased expression in 5G/4G hetero- and 4G/4G homozygous women may contribute to the deposition of fibrin in the vessels of the placenta and exacerbate implantation disorders in women with PL.[30,31]

The results obtained in this study in combination with data in the literature suggest that congenital and acquired hemostatic disorders play a role in the pathogenesis of PL. Irrespective of their primary cause(s), chronic systemic hypercoagulability presumably has two major consequences: (1) it promotes microthrombosis and (2) it leads to continuous chronic platelet activation followed by platelet exhaustion and dysfunction, including impairment of the contractile function, an important mechanism of mechanical remodeling of blood clots and thrombi. A combination of these disorders can impair blood flow in the microvasculature, including the placental circulation, which may result in placental insufficiency and abortion. In addition to the importance of prothrombotic disorders revealed as a potential pathogenic mechanism of PL, our results also suggest that the thrombodynamics and clot contraction assays can be used as laboratory criteria to assess the risk of miscarriage and promptly diagnose obstetric complications before and during pregnancy. These newer tests can complement routine laboratory tests, such as a decrease in aPPT and ATIII as well as an increase in platelets and platelet indices (for example, MPV, PDW, and thrombocrit), which comprise signs of hypercoagulablility, a risk factor for PL.

Conclusion

In women with a history of PL compared with healthy subjects, a significant increase in the clot growth rate and clot size was

found using the thrombodynamics assay. These changes were combined with a significant suppression of blood clot contraction, which together indicate chronic hypercoagulability and predisposition to thrombosis. In patients with three or more fetal losses, the hypercoagulability and impairment of clot contraction were significantly more pronounced compared with patients who have lost one or two fetuses. Also, the revealed hemostatic abnormalities were significantly more prominent in patients with a history of late rather than early miscarriages. The results support the important role of hemostasis disorders in the pathogenesis of miscarriage. In addition, the data obtained suggest a possibility of using thrombodynamics and contraction of blood clots as diagnostic and prognostic tests to determine the risk of PL and possibly RPL, creating the opportunity for timely prophylactic treatment. Future studies are needed to establish the mechanistic and causal relationship between premorbid hypercoagulability and PL.

What is known about this topic?

- Pregnancy loss is a big medical and social problem with unclear etiology and pathogenesis.
- Pregnancy loss may be related to congenital and acquired hemostatic disorders.

What does this paper add?

- Pregnancy loss is associated with chronic hypercoagulability and exhaustive platelet dysfunction, resulting in impaired contraction of blood clots.
- The latent hemostatic disorders constitute a premorbid status in patients with pregnancy loss and directly correlate with the number of miscarriages that occurred at a later time of gestation.
- Thrombodynamics and blood clot contraction assays have the predictive value in evaluating the risk of pregnancy loss, especially recurrent pregnancy loss.

Authors' Contributions

R.I.L., S.I.S., F.I.A., and J.W.W designed the research; A.D.P. and N.G.E. performed experiments; A.D.P., S.I.S., Y.S.B., F.I.A., J.W.W., and R.I.L. analyzed the data; A.D.P., J.W.W., and R.I.L. wrote the paper.

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Conflict of Interest

None declared.

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