

SECTION 2. CLINICAL RESEARCH: NEURODEGENERATIVE AND CARDIOVASCULAR DISEASE

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THROMBOELASTOGRAPHIC STUDY OF FIBRIN CLOT AND MOLECULAR BASIS OF MAXIMUM CLOT FIRMNESS

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Maximum clot firmness (MCF) is the main parameter of thromboelastography (TEG) reflecting the stability of a clot. In this work, we looked for markers that can influence the enhancement of MCF and detected molecular markers and blood clotting parameters that can be involved in such mechanisms. Blood samples of pregnant women with placental disorders were collected in the Kyiv Perinatal Center. TEG was performed on whole blood in EXTEM and INTEM tests. APTT, INR, fibrinogen concentration and platelet aggregation were measured using traditional laboratory approaches. D-dimer was detected in sandwich ELISA using monoclonal antibodies III-3B and II-4D. The relative cross-linking activity of factor XIIIa was measured by the direct quantification of the cross-linked γ -chain of fibrin using Western-Blotting with monoclonal antibody II-4D. D-dimer and fibrinogen concentrations, clotting time in the APTT test, INR and rate of platelet aggregation did not correlate with the MCF. However, we found positive correlations of MCF with factor XIIIa activity: 0.51 and 0.87 for EXTEM and INTEM, respectively. These data indicate that for normal and slightly increased fibrinogen concentrations, fibrin clot firmness will depend mostly on the activity of factor XIIIa. Thus the direct determination of factor XIIIa activity in blood plasma of patients can be relevant for predicting the risk of intravascular coagulation. Evaluation of the content and activity of individual clotting factors or other components of the coagulation system can be useful additions to the TEG diagnostics and should not be neglected.

Key words: thromboelastography, factor XIIIa, fibrinogen, maximum clot firmness, thrombosis.

Introduction

Thromboelastography (TEG) is a widely used and useful approach that allows determination of overall hemostasis potential and clot properties in the whole blood [1]. Its results mainly provide general information about the risk of bleeding [2, 3].

The use of TEG for the prediction of intravascular thrombus formation is under discussion [4]. TEG was shown to be informative for the detection of disseminated intravascular coagulation in patients with sepsis [5]. It has also been applied for the characterization of blood coagulation potential in patients with

chronic kidney disease [6]. Currently, its application is widely discussed for patients with severe consequences of COVID-19 [7, 8].

TEG provides a series of coagulation parameters for the curve of thrombus formation and destruction *in vitro*, and each of these parameters is associated with molecular markers of blood coagulation and fibrinolysis that can be evaluated by other methods [9].

In this work, we focused on the possible use of TEG for the prediction of intravascular thrombus formation. We assumed that maximum clot firmness (MCF) is the main TEG parameter reflecting the stability of clot, thus indicating the danger of thrombosis [10, 11]. We examined the correlation of MCF with the concentration of fibrinogen, D-dimer, platelet aggregation, APTT, INR and factor XIIIa activity in order to select those molecular markers that most affect fibrin clot firmness.

Materials and Methods

Ethical statement. This clinical trial was approved by experts of the Ethics Commission of the Shupyk National Medical Academy of Postgraduate Education (protocol № 14, 07.12.2020) and the Ethics Commission of the Kyiv Perinatal Center (protocol No 3, 05.05.2020) and complies with current legislation of Ukraine, modern ethical standards and principles of scientific clinical trials.

Reagents. ADP, acrylamide, bis-acrylamide and thrombin (50 NIH/ml) were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Molecular weight markers were from ThermoFisher (Waltham, Massachusetts, USA). APTT-reagent, thromboplastin, protein C activator and control donor blood plasma were from Siemens (Munich, Germany). β -mercaptoethanol was purchased from Bio-Rad Laboratories (Hercules, California, USA). All other reagents were of chemical grade and provided by local suppliers.

Monoclonal antibodies. Mouse IgG monoclonal antibodies II-4D and III-3B both specific to the different parts of NH₂-terminal portion of the γ -chain in the fibrin D-domain were obtained at the Palladin Institute of Biochemistry of NAS of Ukraine using a hybridoma technique [12].

Thrombin-like enzyme. Thrombin-like enzyme was purified from the venom of *Agkistrodon halys halys* using Blue-Sepharose (St Louis, Missouri, Sigma-Aldrich, USA) at the Palladin Institute of biochemistry of NAS of Ukraine [13].

Blood samples. Somatically healthy women ($n = 42$) of 18-42 years with single spontaneous pregnancies with placental disorders (fetal growth retardation and/or impaired blood flow in the arteries of the umbilical cord in the second or third trimesters) were enrolled in the study. These placental dysfunction patients did not receive specific antithrombotic treatment. Blood samples of pregnant women were collected at the Kyiv Perinatal Center and analyzed immediately. Venous blood sampling for testing was collected from a peripheral vein using vacuum systems into sterile plastic 4 ml tubes, containing 3.8% sodium citrate solution (Eximlab, Kyiv, Ukraine). All women gave oral and written informed consent to be included in the study. This prospective cohort study was approved by the Ethics Commission of the Shupyk National Medical Academy of Postgraduate Education and the Ethics Commission of the Kyiv Perinatal Center (# 3 from 05/05/2020).

Platelet rich plasma (PRP). For the aggregometry study, PRP was obtained from whole blood by centrifuging at 160 g for 30 min at 25°C.

Platelet-poor plasma (PPP). PRP was centrifuged at 300 g for 15 min at 25°C. PPP was collected above the platelet pellet and frozen at -35°C. PPP was thawed prior to the measurements at 37°C during a period of at least 30 min.

TEG. TEG was performed on whole blood using Rotem Delta (Tem Innovations GmbH, Munich, Germany). EXTEM and INTEM modes that reflect the formation of fibrin clots initiated by the extrinsic and intrinsic pathways, respectively were measured.

Fibrinogen concentration. Fibrinogen concentration in blood plasma was determined by the modified spectrophotometric method. Blood plasma (0.2 ml) and phosphate-buffered saline (PBS, 1.7 ml) were mixed in a glass tube. Coagulation was initiated by the addition of 0.1 ml of thrombin-like enzyme from the venom of *Agkistrodon halys halys* (1 NIH/ml) that prevented fibrin cross-linking. The

Abbreviations: PRP – platelet rich plasma; PPP – platelet poor plasma; APTT – activated partial thromboplastin time; INR – international normalized ratio; TEG – thromboelastography; MCF – maximum clot firmness; PT – prothrombin time.

mixture was incubated for 30 min at 37°C. The fibrin clot was removed and dissolved in 5 ml of 1.5% acetic acid. The concentration of protein was measured using a POP spectrophotometer (Optizen, Daejeon, Korea) at 280 nm ($\epsilon = 1,5$) [14].

D-dimer concentration measurements in sandwich ELISA. DD-specific monoclonal antibody III-3B was used as the catch-agent. 100 μ l of analyzed blood plasma was dissolved 1:10 in PBS with 5% of milk and 0.1% of tween-20. Another DD-specific monoclonal antibody (II-4D) was used as the tag-agent. Concentration of D-dimer was measured using the calibration curve obtained for purified D-dimer [15]. Normal parameters of D-dimer content in the developed test were calculated as 80 ng/ml.

Activated partial thromboplastin time (APTT). APTT was measured according to the following procedure: 0.1 ml of blood plasma was mixed with an equal volume of APTT-reagent and incubated for 3 min at 37°C. Then the coagulation was initiated by adding 0.1 ml of a 0.025 M solution of CaCl_2 . The clotting time was monitored using the Coagulometer Solar (Solar, Minsk, Belarus).

Prothrombin time (PT). PT was measured as follows: clotting was initiated by mixing 0.1 ml of blood plasma with 0.1 ml of 0.025 M CaCl_2 and 0.1 ml of thromboplastin reagent (Siemens, Munich, Germany). The clotting time was monitored. Thromboplastin acts through the tissue factor pathway of coagulation and activates only carboxylated and uncleaved forms of prothrombin. Results were presented as international normalized ratio (INR) that was calculated by the formula: $\text{INR} = (\text{CTp}/\text{CTd})^{\text{ISI}}$; where CTp – patient's blood plasma clotting time, CTd – donor's blood plasma clotting time, ISI – International Sensitivity Index of thromboplastin.

Platelet aggregation. Platelet aggregation was measured based on changes in the turbidity of human PRP [16]. In a typical experiment, 250 μ l of PRP was incubated with 25 μ l of 0.025 M CaCl_2 and 25 μ l of 12.5 μ M ADP at 37°C. Aggregation was monitored for 10 min using the aggregometer Solar 2110 (Solar, Belarus).

SDS-PAGE/Western-Blotting. The polypeptide chains of fibrin formed in blood plasma were separated using SDS-PAGE in 6% gels according to Laemmli [17] in the presence of 0.2% β -mercaptoethanol. The separated bands were transferred to a nitrocellulose membrane to specify the γ -chain by immunoprobings. The membrane was blocked with 5% milk in PBS for one hour, incubated

with a mouse monoclonal antibody II-4D (specific to the NH_2 -terminal portion of the γ -chain in fibrin D-domain) for an additional hour and then developed with a HRP-labelled goat anti-mouse secondary antibody. The bands were visualized using 0.001 M 4-chloro-1-naphthol solution in 0.5 M Tris pH 7.5 and 0.03% H_2O_2 . Concentrations of the cross-linked γ -chains were calculated using densitometry of scanned electrophoregrams with TotalLab TL100 software (Nonlinear Dynamics, Newcastle-Upon-Tyne, UK) [18].

Statistical analysis. Statistical analysis was performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). All assays were performed in a series of three replicates. The data were fitted with standard errors using Statistica 7 (StatSoft Power Solutions, Round Rock, Texas, USA). Pearson's correlation coefficient was calculated using the Pearson Correlation Coefficient Calculator provided by Social Science Statistics (<https://www.socscistatistics.com/>).

Results

Measuring MCF in patients' blood. Routine coagulation tests do not provide any information on the kinetics of clot formation, clot strength or interactions between the coagulation components. Application of TEG allowed us to select the group of patients with an increased value of MCF. This parameter was estimated in EXTEM and in INTEM systems when blood coagulation in the test was initiated by the thromboplastin or APTT-reagent, respectively. MCF in EXTEM and INTEM tests is dependent on platelet concentration and function as well as platelet-fibrin interactions. Concentrating on only the fibrin properties, we expected not much difference between these two modes. However, the results indicated that 38% and 20% of patients had increased MCF (> 72 mm) in the INTEM and EXTEM, respectively, indicating an increase in total coagulation potential in pregnant women (Fig. 1).

Interestingly, we did not find many patients that had a decreased value of MCF (< 50 mm) in either mode. However, this very parameter had to be important for predicting an increased risk of intravascular coagulation. Thus, we studied the correlation of other blood coagulation parameters with MCF using Pearson's correlation coefficient (Table).

Correlation of MCF with basic coagulation markers. Analysis of blood coagulation parameters in patients with placental disorders showed they

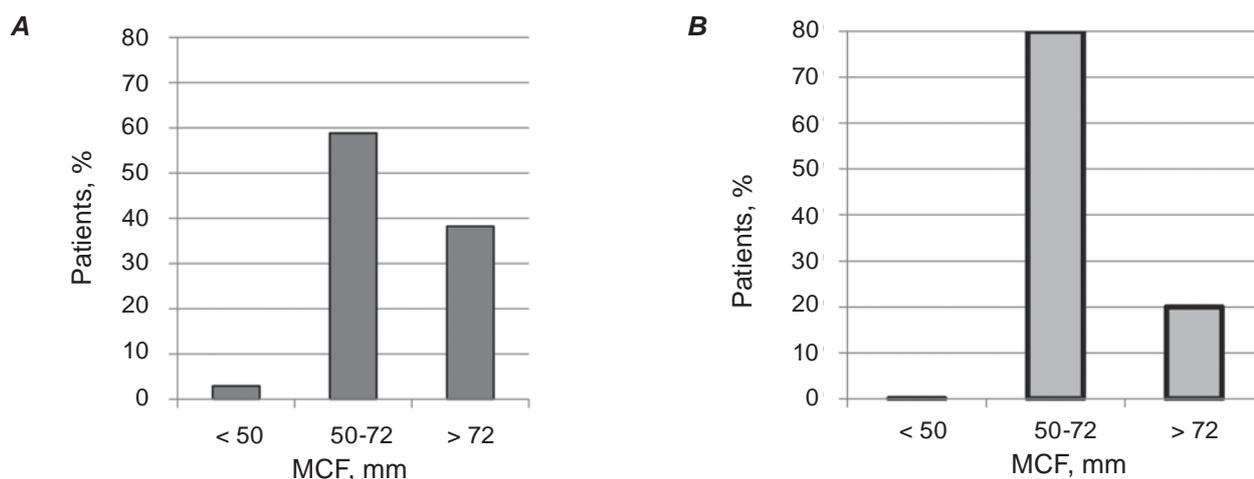


Fig. 1. Percentage of patients with placental disorders according to the maximum clot firmness (MCF) in (A) INTEM and (B) EXTEM tests. Total number of patients = 42. Less than 50 mm – patients with decreased MCF; 50-72 mm – patients with normal MCF; greater than 72 mm – patients with increased MCF

Basic parameters of the blood coagulation system of patients with placental disorders and their correlation with maximum clot firmness (MCF)

Parameters	Control values	Measured values	Correlation with MCF	
			EXTEM	INTEM
Fibrinogen, mg/ml	2.53 ± 1.0	4.7 ± 1.0 ^a	0.46 ^b	0.22
D-dimer, ng/ml	90 ± 30	85 ± 47	0.20	0.20
APTT, s	28 ± 2	30.3 ± 3.9	-0.16	0.13
INR	1.0 ± 0.1	1.1 ± 0.1	-0.03	-0.46 ^b
Platelet aggregation, %	32 ± 15	48 ± 14	0.06	-0.18
Platelet count, x1000/mkl	250 ± 50	228 ± 57	- 0.1	0.06

APTT – activated partial thromboplastin time; INR – international normalized ratio. ^aSignificant according to Mann-Whitney test at $P \leq 0.05$. ^bData indicate the weak or moderate correlation according to Pearson's test.

generally had a higher fibrinogen level compared to the healthy nonpregnant donors, in accord with a previous report [19]. All other studied parameters including platelet aggregation induced by ADP, as well as APTT and INR were not changed (Table). Also, we did not find an increase in D-dimer concentration, as only 30% of patients had accumulation of the D-dimer above 100 ng/ml.

In addition, none of the studied parameters were correlated with MCF. We detected weak or moderate correlation only between MCF in the EXTEM mode and the fibrinogen concentration ($r = 0.46$) and between MCF in the INTEM mode and the INR ($r = -0.46$). These findings emphasize once again that the division of the external and internal blood clotting pathways is only a conditional distribution. Since all these links are quite intercon-

nected and work almost simultaneously, they cannot be considered separately from each other.

Factor XIIIa cross-linking activity. Factor XIIIa cross-linking activity was evaluated according to the method that was developed by Gryshchuk and co-authors [20]. This method is based on the direct evaluation of cross-linked polypeptide chains of polymerized fibrin using SDS-PAGE. To adapt it to the study in blood plasma we added the immunodetection of cross-linked chains in Western-Blotting.

Commercially available methods are based on measuring the ammonium released during fibrin stabilization using an ammonium-sensitive electrode or alternatively using the NAD(P)H-dependent glutamate dehydrogenase indicator reaction [21, 22]. The main advantage of our immunodetection method is the direct measurement of the amount of cross-

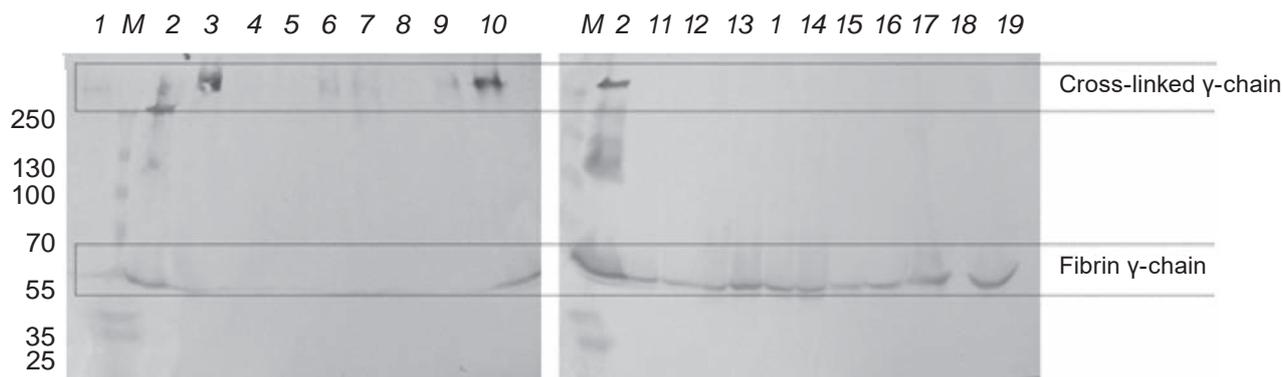


Fig. 2. Western-Blotting of blood plasma samples of patients with placental disorders obtained after the factor XIIIa-induced cross-linking of fibrin. Monoclonal antibody II-4D was used to detect – γ -chain monomers (denoted “Fibrin γ -chain”) and γ -chain oligomers (denoted “Cross-linked γ - γ ”). Lanes 2-10 – blood plasma of patients with high level of MCF (> 72 mm). Lanes 1, 11-19 – blood plasma of patients with normal level of MCF (50-72 mm). M – molecular weight markers. Samples 1 and 2 were presented on both blots to avoid possible differences due to protein transfer or staining

linked molecules of fibrin, i.e., the object of interest of our studies.

To 0.05 ml of PPP we added an equal volume of Tris HCl buffer pH 7.4, with 0.13 M NaCl and 10^{-3} M CaCl_2 and initiated polymerization by thrombin at the final concentration of 1.5 NIH/ml.

Fibrin γ -chain-specific monoclonal antibody II-4D was used for detection of cross-linked γ -chain oligomers. Densitometry of protein bands detected with monoclonal antibody allowed us to estimate the relative cross-linking activity of factor XIIIa in each sample of blood plasma.

The Western-Blotting analysis of blood plasma samples from patients with normal or high levels of MCF is presented in Fig. 2. As it is seen in Fig. 2, the amount of cross-linked γ -chain oligomers was substantially bigger in the blood plasma samples of patients with high level of MCF (> 72 mm) in comparison to those with normal level (50-72 mm). Such a difference indicated the differentiation in the activity of cross-linking factor XIIIa. We applied the Pearson’s correlation analysis to quantify this difference.

Pearson’s correlation coefficients were determined for the relative cross-linking activity of factor XIIIa and MCF in INTEM or EXTEM. Strong ($r = 0.87$) and moderate ($r = 0.51$) positive correlations were found for INTEM and EXTEM, respectively.

Discussion

In this study, we examined the blood coagulation system parameters in patients with placental disorders, focusing mainly on thrombotic compli-

cations that can be extremely dangerous for mother and fetus [23]. Activation of intravascular coagulation is currently considered as one of the reasons for placental disorders with placental-associated complications such as fetal growth retardation, premature birth, preeclampsia, fetal growth retardation and premature detachment of the normally located placenta [24, 25].

In our analysis of the blood coagulation system of patients with placental disorders, we focused on those factors that could influence blood clot firmness as reflected in changes of the MCF parameter. The most important factors should be fibrinogen and platelets. As the core of a clot, fibrinogen can obviously influence mechanical properties of a clot [26]. Furthermore, platelets are important for the filling of a clot ‘body’ and make it stiffer due to retraction [27]. However, only the fibrinogen level weakly correlated with the MCF. The platelet aggregation level showed no correlation with the MCF.

The correlation of MCF with the APTT and INR was predictable as far as these two tests can be assumed as another way to study intrinsic and extrinsic pathways of the coagulation cascade [28]. The only difference is that in these tests, the coagulometer monitors the time of clot formation in blood plasma and the TEG monitors the maximum rigidity of the blood clot. However, again we did not find any correlations. We found the same situation with the D-dimer, which is assumed to be the most important molecular marker for indicating intravascular thrombosis [29].

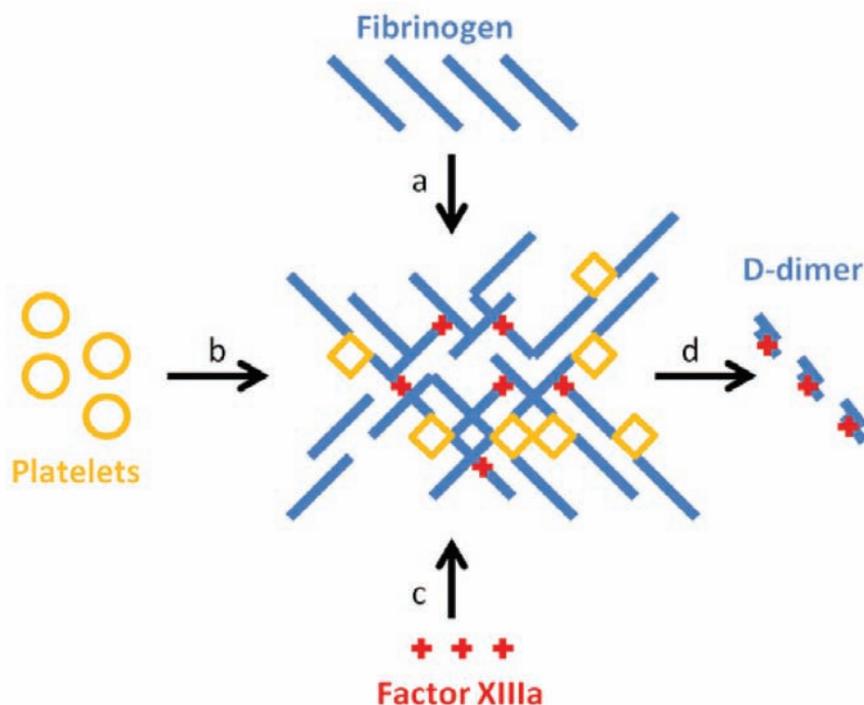


Fig. 3. Principal scheme of fibrin clot composition indicating factors that provide clot firmness. **a.** Thrombin converts fibrinogen to fibrin that is then able to polymerize and form the core of the thrombus; **b.** Platelets are activated and bind to fibrinogen and fibrin, forming the 'body' of the thrombus; **c.** Factor XIIIa cross-links fibrin, thereby covalently bonding the fibrin molecules to each other; **d.** Polymerized cross-linked fibrin is later degraded by plasmin resulting in the formation of D-dimers

Our results indicated strong positive correlations of MCF with factor XIIIa activity: 0.51 and 0.87 for EXTEM and INTEM, respectively. Thus, our findings indicated a noteworthy contribution of factor XIIIa cross-linking to the MCF (Fig. 3). Neither the platelet aggregation level nor the total fibrinogen concentration influenced this parameter. Also, the accumulation of D-dimer that appears as a result of plasmin hydrolysis of covalently cross-linked fibrin does not allow the prediction of the mechanical properties of a clot.

Summarizing the obtained data, we can predict that in some range of fibrinogen concentrations covering normal and slightly increased values, the MCF of fibrin will depend only on the activity of factor XIIIa. Being proved *in vitro* in model systems this conclusion would open a new direction in the search for ways to regulate thrombus formation and digestion. Also, our findings indicate the potential relevance of determining factor XIIIa activity in blood plasma of patients when intravascular coagulation is expected.

The study of parameters of clot firmness by TEG and factor XIIIa cross-linking activity in patients with high risk for placental disorders during pregnancy is a promising strategy for predicting perinatal complications and making timely decisions about anticoagulant therapy. Our findings that suggest the impact of factor XIIIa cross-linking activity on fibrin clot firmness correspond to data indicating the importance of this parameter as a part of TEG studies [30].

Conclusion. Factor XIIIa cross-linking activity had the most influence on the MCF. Platelet aggregation and fibrinogen concentration had minor effects on this parameter. Also MCF did not correlate with molecular markers of intravascular thrombus formation (D-dimer) and with basic clotting tests (APTT, PT). Factor XIIIa cross-linking activity should be evaluated for the accurate analysis of mechanical properties of fibrin clots during pathologies accompanied by intravascular thrombosis.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ТРОМБОЕЛАСТОГРАФІЧНЕ ДОСЛІДЖЕННЯ ФІБРИНОВОГО ЗГУСТКА ТА МОЛЕКУЛЯРНІ ОСНОВИ МАКСИМАЛЬНОЇ ЩІЛЬНОСТІ ЗГУСТКА

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Максимальна щільність згустка (MCF) основний параметр тромбоеластографії (TEG), який відображає стабільність згустка. Проведено пошук маркерів, які можуть впливати на збільшення MCF та визначено молекулярні маркери та параметри зсідання крові, задіяні у таких механізмах. Зразки крові вагітних жінок із плацентарною дисфункцією було відібрано в Перинатальному центрі міста Києва. TEG проведено на цільній крові в EXTEM та INTEM тестах. АЧТЧ, МНВ, концентрацію фібриногену та агрегацію тромбоцитів вимірювали за допомогою традиційних підходів лабораторної

діагностики. D-димер визначали в сандвіч-імуноензимному аналізі з використанням моноклональних антитіл III-3В та II-4D. Відносну активність прошивки фактором XIIIa вимірювали шляхом прямого кількісного визначення прошитого γ -ланцюга фібрину методом Вестерн-блот із використанням моноклональних антитіл II-4D. Концентрації D-димеру та фібриногену, час зсідання за тестом АЧТЧ, МНВ та ступінь агрегації тромбоцитів не корелювали з MCF. Однак, ми виявили позитивну кореляцію MCF із активністю фактора XIIIa: 0,51 та 0,87 для EXTEM та INTEM відповідно. Одержані дані свідчать про те, що за нормальної чи дещо підвищеної концентрації фібриногену щільність фібринового згустка буде в основному залежати від активності фактора XIIIa. Таким чином, безпосереднє визначення активності фактора XIIIa в плазмі крові пацієнтів може бути важливим для прогнозування ризику внутрішньосудинного зсідання. Оцінка вмісту та активності окремих факторів зсідання крові або інших компонентів системи зсідання може бути корисним доповненням до MCF діагностики, чим не слід нехтувати.

Ключові слова: тромбоеластографія, фактор XIIIa, фібриноген, максимальна щільність згустка, тромбоз.

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