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INCREASED THROMBIN GENERATION AS A POTENTIAL MARKER FOR ADVERSE PREGNANCY OUTCOMES

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Aim. *To estimate the effectiveness of the thrombin generation assay (TGA) in identifying high thrombogenic risk in pregnant women.*
Material and methods. *The study comprised 30 healthy non-pregnant women (control group) and 32 women in the third trimester of pregnancy. Of the latter group, 22 women with complicated pregnancy comprised the study group while the remaining ten women made up the comparison group. After delivery, the patients in the study group were divided into subgroups with a favorable (n = 15) and adverse (n = 7) pregnancy outcomes. The state of the hemostatic system was examined using standard coagulation tests and TGA.*
Results. *Thrombin generation assessed as endogenous thrombin potential (ETP), was statistically significantly higher in pregnant than in non-pregnant women (2300 ± 400 vs. 1700 ± 400, respectively, p <0.005). The patients with adverse pregnancy outcomes had statistically significantly higher ETP compared with women with favorable outcomes (2700 ± 600 vs. 2300 ± 300, respectively, p <0.005).*
Conclusion. *TGA can be used to predict adverse pregnancy outcomes. Elevated ETP is associated with adverse pregnancy outcomes.*

Keywords*: complications of pregnancy, thrombin generation assay*

Authors' contributions. Gribkova I.V., Koroleva N.S., Davydovskaya M.V., Murashko A.V.: developing of research design, obtaining data for analysis, rewieving publications on the topic of the article, statistical analysis of the obtained data, article writing.

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The risk of thrombosis in women during pregnancy poses a significant challenge for today's health care services. The changes in the coagulation system during gestation are considered to be adaptive mechanisms and are aimed to reduce obstetrical bleeding in childbirth. Normal pregnancy is associated with the excessive generation of maternal fibrinogen and other coagulation factors as well as with decreased anticoagulant properties, and suppression of fibrinolysis [1, 2]. These changes in the hemostatic system act as a physiological adaptive mechanism, but may be the cause of both maternal and fetal complications [2-4]. These complications may include venous insufficiency, preeclampsia, intrauterine growth restriction, preterm labor, and placental abruption, late and early recurrent miscarriage, and fetal demise. S.A. Mastrolia et al. [2] in their review showed the relationship between thrombosis and complications of pregnancy. Risks are higher in women with acquired or hereditary thrombophilia. V.O. Bitsadze et al. [1] reported the presence of an etiopathogenetic link between thrombophilia and obstetrical complications and the high effectiveness of anticoagulant prophylaxis, provided its early initiation.

However, at present, the decision-making regarding anticoagulant therapy for pregnant women is based only on risk factors, that is, a family history of thrombosis, the presence of a particular thrombophilia (factor V mutation, Leiden mutation, prothrombin mutations, antithrombin deficiency, antiphospholipid antibodies, etc.). These indications for anticoagulant therapy may not always be appropriate, because the existing laboratory techniques are not sensitive enough to detect hypercoagulable state. Accordingly, using these indications for anticoagulant therapy can lead to both an unjustified use of anticoagulants during pregnancy, and vice versa, leaving patients without anticoagulant therapy when it needed, due to an underestimation of the risk. As a rule, the state of the hemostasis system is studied using standard coagulation tests (activated partial thromboplastin time, thrombin time, Quick time, etc.). However, these blood coagulation tests have insufficient predictive value and are difficult to use to assess the risk of thrombosis. Specific molecular markers reflecting the clotting process (D-dimers, fibrinopeptide, thrombin-antithrombin complex) can detect deviations from the reference range, but do not allow for the assessment of the imbalance in the coagulation system as a whole, since changes in the individual components of the coagulation system can be leveled by compensatory mechanisms. A possible solution is the use of tests for global evaluation of the hemostatic system, which can show the state of the hemostatic system as a whole [5, 6]. The thrombin generation assay (TGA) is one of the most investigated and internationally adopted global

hemostasis assays. Unfortunately, in Russia, this test is still rarely used.

Several studies [7-9] have reported using TGA for the examination of women during pregnancy, including for monitoring the effectiveness of anticoagulant therapy [9].

This study aimed to estimate the effectiveness of the TGA in identifying high thrombogenic risk in pregnant women who need anticoagulant therapy to improve pregnancy outcomes.

Material and methods

The study was conducted by national and local laws and regulations for the conduct of human and animal research. All participants signed an informed consent form before entering the study as per the Helsinki Declaration. The study was approved by a local ethics committee.

The study comprised 30 healthy, non-pregnant women (control group) and 32 pregnant women. All pregnant women were divided into a study group (n=22) and a comparison group (n=10). The study group included patients with complicated pregnancies, the presence of thrombogenicity factors and comorbidities (venous insufficiency, placental insufficiency, thrombophilia, and preeclampsia) at the time of enrollment. Exclusion criteria were blood diseases (Von Willebrand disease, thrombocytopenic purpura). The comparison group included ten pregnant women with a physiological pregnancy. All women enrolled in the study were comparable in age (mean age 35.4 ± 5.5 years), gestational age (26-33 weeks gestation) and parity. In the study group, 17 pregnant women received low-molecular-weight heparin (LMWH) in the prophylactic dosing to correct the hemostatic system. The remaining women did not receive any anticoagulant therapy.

The hemostatic system was evaluated using standard coagulation tests (Quick time, fibrinogen concentration, platelet aggregation using ADP and collagen as inducers) and TGA. Blood sampling was performed at the hospital admission. Standard coagulation tests were performed immediately after drawing the blood.

Venous blood was taken from the ulnar vein into VACUETTE test tubes containing a 9 NC coagulation sodium citrate 3.2%. The blood was centrifuged at 1500 g for 15 min at room temperature to produce platelet-poor plasma. Before carrying out laboratory tests, the plasma was stored at -40°C. Before TGA, the plasma was thawed in a water bath at 37°C for 1 hour. Thrombin generation was measured according to the method developed by H.C. Hemker et al. [10]. The detailed procedure is described in the work of I.V. Gribkova et al. [11].

This assay measures the total amount of thrombin formed in the sample in response to standard recalcification and activation. The concentration of thrombin was measured using a specific fluorescent substrate Z-Gly-Gly-Arg-AMC× HCl (Bachem, Switzerland), which triggers the formation of a fluorescent product. 80 µl of plasma was dispensed into standard 96 round-bottom well plates, to which 20 µl of a 2.5 mM fluorescent substrate was added, followed by 20 µl of the activator solution. A mixture of thromboplastin (final concentration in the well 4 pM) and phospholipid vesicles (final concentration in the well 4 µM) was used as an activator. Fluorescence intensity was measured and recorded in real time at 37°C continuously for 60 min using an Appliskan fluorimetric reader (Thermo Fisher Scientific, Finland) at 355 nm (excitation) and 460 nm (emission). All plasma samples were tested in duplicate, and the duplicate measurements were averaged. The error of duplicate measurements did not exceed 2-5%. Statistical analysis was performed using the standard graphics program Origin 6.0 (Microcal Software Inc., MA, USA). The area under the curve reflecting the thrombin concentration as a function of time was calculated based on the total amount of thrombin formed in the sample in 50 minutes (endogenous thrombin potential, ETP). Results are expressed as the peak concentration of thrombin (A_{max}), the time to reach this concentration (t_{max}), and lag time (t_{lag}) - the time to reach a thrombin concentration of 10 nM.

Results

Allocation to subgroups after delivery

After delivery, the study participants were allocated to a subgroup of women with favorable (n = 15) and adverse (n = 7) pregnancy outcomes. Adverse outcomes included premature delivery due to the fetal critical state (n = 6) and partial placental abruption (n = 1). Patients with thrombophilia (n = 7), venous insufficiency (n = 7), placental insufficiency (n = 1) were included in the subgroup with favorable outcomes. Several patients with thrombophilia (n = 2), placental insufficiency (n = 4), and preeclampsia (n = 4) were included in the subgroup with adverse outcomes. Six of seven women in the subgroup with adverse pregnancy outcomes required operative delivery (85%). The critical fetal condition was the indication for preterm delivery.

All women in the subgroup with adverse pregnancy outcomes had an adequate blood loss consistent with the method of delivery. Four newborns from this subgroup were referred to the neonatal intensive care unit (57%). The minimum and maximum newborn birth weight in this subgroup was 1148 g and 3110 g (partial placental abruption), respectively.

In the subgroup with favorable outcomes, 5 (33%) women had had a full term vaginal delivery, and 10 (67%) underwent operative delivery at full term. All newborns of this subgroup were born in a satisfactory condition with first- and fifth-minute Apgar scores of at least 8. The blood loss during operative delivery was 600 ± 100 ml and150 ± 50 ml during vaginal delivery. In this subgroup of women with favorable pregnancy outcomes, newborn birth weight exceeded 2500 g, the minimum and maximum newborn birth weight was 2740 g and 4000 g, respectively.

Results of coagulation assays

Comparison of ETP in healthy, non-pregnant (control group) (n = 30) and healthy pregnant women in the 3rd trimester (n = 10) showed a significantly higher ETP in pregnant women (Figure 1) (paired Student's t-test, ANOVA, $p < 0.05$). Mean TGA parameters in healthy, non-pregnant and pregnant women in the 3rd trimester

are shown in Table. 1. As can be seen from the table, during normal pregnancy all TGA parameters change in the direction of hypercoagulability: t_{max} and t_{lag} are shortened, A_{mach} and ETP increase. In this regard, all further comparisons were made between the study group and the comparison group, which included pregnant women in the 3rd trimester.

Patients with adverse pregnancy outcomes had higher ETP. Examples of experimental TGA curves for patients with favorable and adverse pregnancy outcomes compared to the curve for patients in the comparison group are shown in Fig. 2. As can be seen from the figure, the ROC curve representing an adverse pregnancy outcome is significantly different from the normal curve.

Mean TGA parameters of the patients with favorable and adverse pregnancy outcomes are shown in Table. 1.

As can be seen from the table, the women in the subgroup with adverse pregnancy outcomes had higher ETP compared with both the subgroup with a favorable outcome and the comparison group. These differences were statistically significant (paired Student's t-test, ANOVA, $p < 0.05$) (Figure 3).

Coagulation parameters (Quick prothrombin, fibrinogen, platelet aggregation (inducers: ADP and collagen)) for each group are given in Table. 2. As it can be seen, all parameters in all three groups are within the reference range. Quick prothrombin tended to increase, but no statistically significant difference between the groups was found for any parameter (paired Student's t-test, ANOVA, $p > 0.05$).

Discussion

In recent years, there has been an increasing amount of literature on the use of TGA for examining women during pregnancy [7, 9, 12-18]. Most research is focused on the change in TGA parameters during normal pregnancy [7, 12, 14-17]. Consistent with previous studies, our findings showed a statistically significant ETP increase in the pregnant women in the third trimester compared to the non-pregnant group (Fig. 1). In the present work, in addition to the group of healthy women, the comparison groups, women with complicated pregnancy were examined, 7 of whom had adverse pregnancy outcomes. To identify possible causes of adverse pregnancy outcomes, parameters of the standard coagulation tests and TGA were analyzed. All women had normal standard coagulation tests.

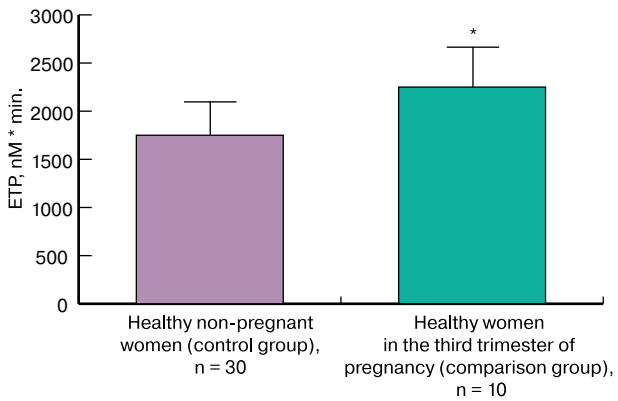
However, the women with adverse pregnancy outcome had statistically significantly higher ETP compared with women with favorable outcomes and women in the comparison group (Fig. 3).

M. Efthymiou et al. [18] reported similar results concluding that the TGA can reflect the current hypercoagulability, while the INR shows adequate anticoagulant therapy. The association of increased thrombin generation with adverse pregnancy outcomes was also reported by the authors of the study [19], showing an increase in the level of thrombin-antithrombin complexes in women in the 2nd trimester who subsequently had a preterm birth.

Therefore, our findings of increased ETP suggest that women from the subgroup with adverse outcomes did not receive sufficient anticoagulant therapy to compensate for hypercoagulability, which can lead to complications. Differences in outcomes in women of both subgroups of the study group who received similar anticoagulant therapy can be explained by an inadequate dose of anticoagulants for a given patient or by the different patient response to the drug, or by the presence of an additional pathological mechanism. These results suggest the feasibility of investigating the hemostatic system in pregnant women to assess hypercoagulability with greater accuracy and choose possible ways of correction, which may include administration of anticoagulant therapy or a change in the dose of already prescribed drugs to avoid thrombotic complications of pregnancy.

In studies [9, 18, 20], TGA was successfully used to evaluate the effectiveness of anticoagulant therapy and the appropriateness of anticoagulant dosing. In

Fig. 1. Mean ETP in healthy non-pregnant and pregnant women in the 3rd trimester



* Differences are statistically significant (Student's paired t-test, ANOVA, $p < 0.05$).

Fig. 2. Examples of experimental curves for patients with favorable and adverse pregnancy outcomes

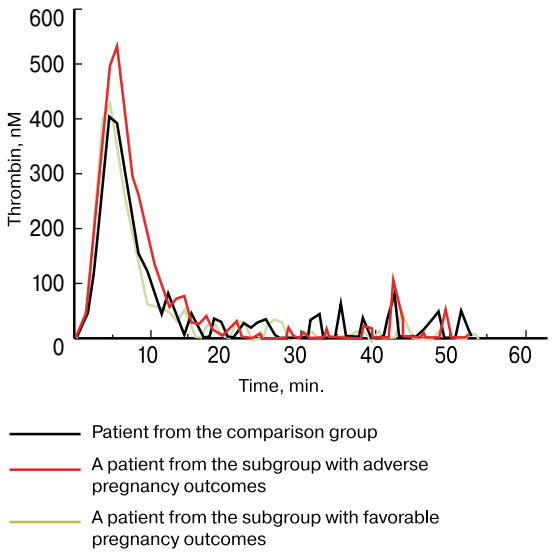


Table 1. TGA results (mean ±SD)

Study groups	ETP	tmax	Amax	tlag
Control group (*n*=30)	1700±400	6±2	280±60	0.6±0.5
Comparison group (*n*=10)	2300±400	5±1	450±80	0.5±0.2
Favorable outcome (*n*=15)	2300±300	5±1	400±70	0.6±0.4
Adverse outcome (*n*=7)	2700±600	5±1	460±130	0.4±0.5

Table 2. Anticoagulant therapy and coagulation parameters in the study groups (mean ±SD) (median and range)

Coagulation parameters	Comparison group (n=10)	Favorable outcome (n=15)	Adverse outcome (n=7)
Anticoagulant therapy	No therapy (n=10, 100%)	LMWH (n=12, 80%) No therapy (n=3, 20%)	LMWH (n=5, 71%) No therapy (n=2, 29%)
Quick prothrombin, % (reference range: 78–142)	107,9±19,6 106 (87–133)	109,4±18,7 106 (75–140)	113,1±27,8 108 (86–168)
Fibrinogen, g/l (reference range: up to 6,5)	5,54±0,99 5,19 (4,58–7,05)	5,13±0,78 5,25 (3,68–6,23)	5,26±0,90 5,20 (4–6,46)
Platelet aggregation (ADP), % (reference range: 50–75)	55,0±8,7 58 (40–64)	64,0±7,1 63,5 (51–79)	60,1±4,3 60 (53–66)
Platelet aggregation (collagen), % (reference range: 50–75)	60,6±6,0 59 (52–68)	66,2±6,5 66 (53–78)	62,9±4,8 62 (56–70)

There were no statistically significant differences between the groups (paired Student's t-test, ANOVA, p> 0.05).

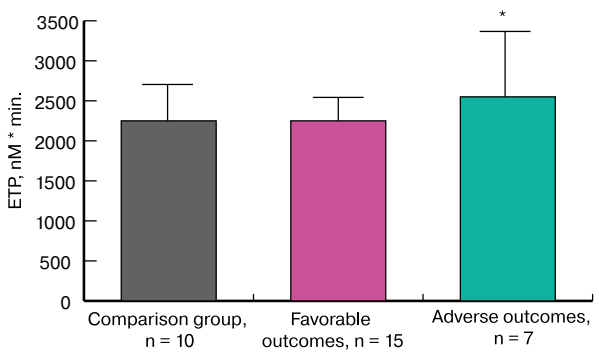
our work, women with favorable pregnancy outcomes and those in the comparison group had similar ETP. Therefore, it might be concluded that normal ETP in patients receiving anticoagulants may predict a favorable pregnancy outcome. However, S.A. Bennett et al. [21] reported that the parameters of TGA in the group of women with unexplained recurrent pregnancy loss (patients with thrombogenic factors were excluded from this group) do not differ from those in the comparison group. This suggests that there are other, non-thrombotic causes of adverse pregnancy outcomes that are not reflected in the TGA parameters.

Using this test, we can only cut off the complications arising from excessive activation of the blood clotting process.

Conclusion

Thus, the results of this study have shown that TGA can be applied to predict adverse pregnancy outcomes.

Fig. 3. Mean endogenous thrombin potential (ETP) by groups



* Differences are statistically significant (Student's paired t-test, ANOVA, p <0.05).

Comparing ETP of the patient with the mean ETP of the comparison group consisting of pregnant women of the same gestational age allows for an accurate estimate of the need for anticoagulant therapy and/or dose adjustment for the already prescribed therapy. However, it should be noted that in this pilot study, only seven patients had adverse pregnancy outcomes, which is not enough for definite conclusions. Therefore, based on the study findings, we can only talk about trends, and further research is needed in this direction.

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