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Fibrinolytic parameters under ischemic stroke with diabetes mellitus combination

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List of abbreviations:
α_2-AP – α_2-antiplasmin,
BMI – body mass index,
DM – diabetes mellitus,
ECLT – euglobulin clot lysis time,
IS – ischemic stroke,
PAI-1 – plasminogen activator inhibitor 1,
tPA – tissue-type plasminogen activator.

Key words: α2-antiplasmin; type 2 diabetes mellitus; euglobulin clot lysis time; plasminogen activator inhibitor 1; tissue-type plasminogen activator

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Abstract

Background: *Fibrinolysis and thrombosis alterations include important parts of stroke pathophysiology. At the same time fibrinolytic system disorders are a common feature of patients with metabolic syndrome and diabetes. So it may increase the possibility of developing atherosclerotic lesions and occlusive intravascular thrombi. The present study investigated the influence of type 2 diabetes mellitus presence on the indicators of fibrinolytic parameters (plasminogen activator inhibitor 1 (PAI-1), tissue-type plasminogen activator (tPA) content, streptokinase-activated plasminogen and α_2-antiplasmin activities, euglobulin clot lysis time (ECLT) and Hageman-factor-dependent fibrinolysis time) under ischemic stroke (IS).*

Materials and methods: *Participants were 87 subjects with IS, 22 of them had diabetes mellitus. Blood samples besides for aforementioned parameters were analyzed for glucose and glycosylated haemoglobin content.*

Results: *The research established increase of plasma PAI-1 and tPA levels, ECLT, Hageman-factor-dependent fibrinolysis time in IS and IS with diabetes mellitus patient groups in comparison with the control. PAI-1 concentration in plasma was positively correlated with both lysis time tests but tPA content was negative correlated with glucose level and PAI-1 for only IS patients. But there was a high negative correlation between tPA and ECLT as well as Hageman-factor-dependent fibrinolysis time for both investigated IS forms.*

Conclusions: *The results showed important differences in the characteristics of the fibrinolytic mechanism in IS patients compared with healthy population. The major differences were elevated PAI-1 and t-PA contents and prolonged ECLT in IS patients but no significant differences in these parameters were observed between the patients with IS and IS with diabetes.*

INTRODUCTION

There are a lot of evidences of impaired fibrinolysis under metabolic syndrome as well as non-insulin-dependent (type 2) diabetes mellitus (DM) *(1)*. Haemostasis abnormalities are usually associated with tissue-type plasminogen activator (tPA) - mediated conversion of proenzyme plasminogen into active protease plasmin, which, in its turn, degrades fibrin structure of intravascular thrombi. Plasmin cleaves fibrin thus breaking down meshwork of clot and it is extremely short-lived due to inactivation by α_2-antiplasmin (α_2-AP), which is an abundant inhibitor restricting plasmin action *(2)*.

In addition, it has been known for many years, that increased concentration of other fibrinolysis regulation factor, which is a plasminogen

activator inhibitor 1 (PAI-1), is also a concomitance of type 2 diabetes *(3)*. So, evolving evidence of the central role of PAI-1 and tPA in fibrinolysis and thrombosis mediating increasingly supports a theory that they are a significant risk factor for macrovascular complications and cardiovascular diseases, particularly in patients with diabetes.

It should be noted, that abnormal endogenous fibrinolytic activity may also be a risk factor for such abundant cerebrovascular pathology as stroke. Both normal and high levels of PAI-1 have been observed in the acute phase of stroke *(4)*. PAI-1 is a primary physiological inhibitor of endogenous fibrinolysis that acts via tPA inhibition, often leading to fibrin accumulation in basement membranes and interstitial tissues *(5)*.

In view of the above tPA is a blood factor orchestrating the breakdown of blood clots. Therefore the US Food & Drug Administration approved it as the only thrombolytic drug for treatment of patients with acute ischemic stroke (IS).

The other factor with crucial role in reducing plasmin activity and thus inhibiting fibrinolysis is a α_2-antiplasmin. However, earlier studies of genetically deficient in either tPA, plasmin inhibitor, or α_2-AP mice had paradoxically unexpected results *(6)*. The removing profibrinolytic tPA or antifibrinolytic α_2-AP had similar beneficial outcomes in a murine model of stroke. Other authors established higher concentration of plasmin-α_2-AP complex in diabetics *(7)*. In addition, the fibrin network of plasma clots from patients with diabetes contains more plasmin-α_2-AP than the fibrin network of plasma clots from control subjects *(8)*.

Thus, despite the large amount of information about the important role of fibrinolytic system disorders under stroke, as well as diabetes, data characterising changes of mentioned parameters under conditions of IS with diabetes mellitus combination lack. However, the important question about fibrinolytic system peculiarities under combination of pathologies remains unclear, especially in case of standard treatment such complicated deseases *(9)*. So, the aim of the current study was to investigate possible differences in indicators of fibrinolytic parameters (PAI-1, tPA content, streptokinase-activated plasminogen and α_2-antiplasmin activities, euglobulin clot lysis time (ECLT) and Hageman-factor-dependent fibrinolysis time) under ischemic stroke with and without type 2 diabetes mellitus.

MATHERIALS AND METHODS

The experimental group included 87 patients (median age, 74.2 ± 9.0 years) with suspected IS were admitted to Kyiv city hospital #4 and 25 population controls (median age, 70.2 ± 10.3 years).

The diagnosis of IS was confirmed by neurovisualization with computed tomography and magnetic resonance imaging of brain in all 87 patients, 22 (26.4%) of them had type 2 DM, diagnosed according to the World Health Organization guidelines *(10)*.

All the patients underwent of PAI-1, tPA content, streptokinase-activated plasminogen and α_2-antiplasmin activities, ECLT and Hageman-factor-dependent fibrinolysis time, glucose and glycosylated haemoglobin content and body mass index (BMI) measurements.

Patients were excluded if they had a past history of predisposition to hypercoagulability, coma, cancer, recent surgery or other estimated diseases.

This study was approved by the ethics committee of Educational and Scientific Centre Institute of Biology and Medicine of Taras Shevchenko National University of Kyiv (Ukraine) and performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki. Informed consent was obtained from all individual participants included in the study.

All the patients had aspirin therapy in dose of 325 mg during the first 24 h in the hospital. Blood samples for haemostasis investigations were collected from the patients shortly after their admission to the hospital (acute phase of stroke) in tubes containing 1/10 volume of 0.129 M sodium citrate. Venous fasting blood for DM identifying was collected from the cubital vein in tubes with sodium citrate and K_3EDTA from 8 to 9 am.

Glucose content was estimated by enzymatic glucose oxidase method.

PAI-1 and t-PA were measured by enzyme-linked immunosorbent assay.

Plasminogen and α_2-antiplasmin plasma activities were estimated by chromogenic substrate S_{2251} cleavage with next spectrophotometry assay *(11)*. Plasminogen activation to plasmin was catalyzed by streptokinase. The incubation medium for plasminogen activity determination consisted of 0.05 M Tris-HCl buffer (pH 7.4), 25 µl of blood plasma; 3 mM S_{2251} substrate and 5-10 IU/ml of streptokinase. The volume of reaction medium was 250 µl and the temperature was 37 °C. Absorption of released p-nitroaniline was measured in two-wave mode at 405 nm and 492 nm with microplate spectrophotometer Synergy HT (BioTeck, USA).

The incubation medium for α_2-AP activity assay consisted of 0.05 M Tris-HCl buffer (pH 7.4), 25 µl of blood plasma, 3 mM S_{2251} substrate and 0.5 IU/ml of plasmin. Measurement of released p-nitroaniline absorption was performed as described above.

ECLT assay included two steps plasma euglobulin fraction obtainment and fibrinolytic analysis. 200 µl of chilled plasma were placed in a glass centrifuge tube with 1.8 ml of cold distilled H_2O and 150 µl of 0.25% acetic acid. The mixture was gently stirred and incubated for 30 min at 4 °C. The precipitate was consisted of plasma euglobulin reach fraction that contained plasminogen, plas-

minogen activators (primarily tPA) and fibrinogen. The supernatant was discarded after centrifugation at 700 g for 15 min at 4 °C and the precipitate was dissolved in 250 µl of 0.05 M Tris-HCl buffer (pH 7.4).

Total fibrinolytic activity was determined by the time of fibrin clot lysis by euglobulin fractions. Clots were formed by $CaCl_2$ solution adding and polymerization of fibrin from euglobulin factions. 0.1 ml of 25 mM $CaCl_2$ was added to 250 µl of obtained euglobulin fractions dissolved in 0.05 M Tris-HCl buffer (pH 7.4). Samples were incubated at 37 °C and time of complete clot lysis (in hours) was determined *(12)*.

Hageman-factor-dependent fibrinolysis time was detected after kaolin-stimulated coagulation precipitate obtainment *(12)*. 0.5 ml of plasma was mixed with 8 ml of distilled H_2O, 0.2 ml of 1% solution of acetic acid and 0.5 ml of 0.5% kaolin suspension in plastic tubes. The mixture was gently stirred and incubated at 37 °C for 30 min with next centrifugation for 6 min at 700 g. The supernatant was discarded and the residue was dissolved in 0.5 ml of 0.05 M Tris-HCl buffer (pH 7.4). After induced by 25 mM $CaCl_2$ (in equal volume) clot formation time (in minutes) of its complete lysis was determined.

Glycosylated hemoglobin was assayed with HbA1c IMU-LA-TEST (Erba Lachema s.r.o., CzechRepublic).

BMI as an index of obesity extent was calculated as body weight (kg) divided by square of height (m^2).

The results were statistically analysed by Statistica 7.0 (StatSoftInc., USA) and presented as median with quartilies for all numerical variables, minimum value of statistical series (min) and maximum value of statistical series (max). Distribution within groups was assessed using the Shapiro-Wilk test. Because of the fact that test variables were not normally distributed, further analysis by non-parametric tests was performed. The Mann-Whitney U-test was used to compare differences between studied and control groups. Correlations between variables were assessed using Spearman's rank correlation coefficient. All statistical tests were 2-tailed with p-values <0.05 taken as significant.

RESULTS

There were no statistically significant differences noted between the groups of IS patients with or without DM in age and sex. BMI was significantly higher under IS with DM (20.26 vs. 34.01, p=0.02).

The group of patients with IS+DM included individuals, whose mean indicator of blood glucose was 9.47±2.65 mmol/L, while in the group with IS this parameter was 4.93±0.97 mmol/L.

The research has established the statistically significant changes of PAI-1 and tPA levels in both investigated patient groups in comparison with the control (**Figure 1**).

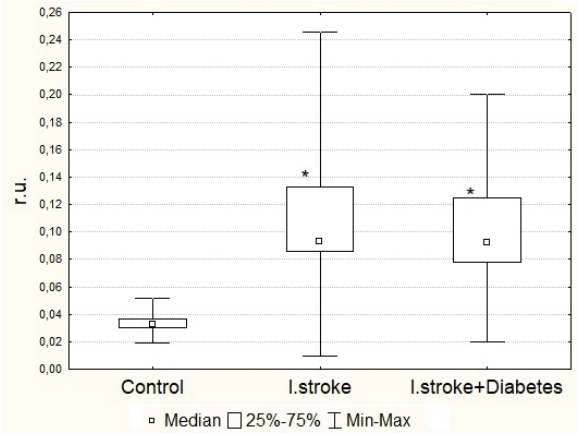

Figure 1. *Plasminogen activator inhibitor-1 plasma content under ischemic stroke and ischemic stroke with diabetes mellitus. (r.u. – relative units)*

** - The result is significant at p<0.05 in comparison with the control*

PAI-1 concentration in plasma of the patients with IS only was significantly higher compared to the control group (0.093 vs. 0.033, p<0.001). This index was increased in the patients with IS complicated by DM relatively the control values as well (0.092 vs. 0.033, p<0.001) but no significant differences (p=0.51) in plasma PAI-1 contents were observed between the patients with IS and IS with diabetes. It should be noted for IS 95% CI 0.101-0.121 and for IS+DM 95% CI 0.085-0.116 were estimated, so more pronounced deviations of PAI-1 content compared with the control were observed under IS without DM.

There was no a significant correlation between plasma PAI-1 and any investigated blood parameters of patients with IS+DM. However, plasma PAI-1 positively correlated with both lysis time tests of patients with IS alone. The established Spearman's rank correlation coefficient for PAI-1 and ECLT was r=0.393; p=0.0004, this parameter for PAI-1 and Hageman-factor-dependent fibrinolysis

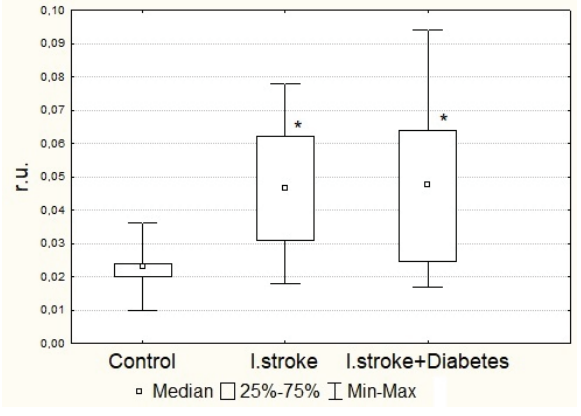

Figure 2. *Tissue-type plasminogen activator plasma content under ischemic stroke and ischemic stroke with diabetes mellitus. (r.u. – relative units)*

** - The result is significant at p<0.05 in comparison with the control*

Table 1. *Spearman rank order correlation of fibrinolytic parameters and glucose content in groups of ischemic stroke and ischemic stroke complicated by diabetes mellitus.*

	Ischemic stroke		Ischemic stroke + Diabetes mellitus	
	Spearman Rank Order Correlation (r)	p	Spearman Rank Order Correlation (r)	p
PAI-1 & tPA	-0.249737	0.028494*	0.133188	0.459962
PAI-1 & ECLT	0.392519	0.000414*	0.079920	0.658407
PAI-1 & Hageman-dependent fibrinolysis time	0.332444	0.003137*	0.255439	0.151363
tPA & ECLT	-0.646481	0.000000*	-0.565201	0.000610*
tPA & Hageman-dependent fibrinolysis time	-0.578119	0.000000*	-0.492712	0.003579*
Hageman-dependent fibrinolysis time & ECLT	0.776947	0.000000*	0.799899	0.000000*
α_2-AP & ECLT	-0.152304	0.186058	-0.348789	0.046663*
α_2-AP & Hageman-dependent fibrinolysis time	-0.084503	0.464966	-0.410659	0.017602*
Glucose & PAI-1	0.246195	0.030900*	-0.180958	0.313554
Glucose & tPA	-0.260160	0.022309*	0.187725	0.295490
Glucose & α_2-AP	0.284286	0.012220*	0.149967	0.404843

* - p<0.05

time was 0.364; p=0.003. We also found not so significant but negative correlation between plasma PAI-1 and tPA level in the patients with IS (r=–0.249; p=0.028).

The tPA plasma content investigation revealed increase of this parameter under the both IS conditions (**Figure 2**). The median of tPA concentrations of IS patient group (n=65) did not differ from IS+DM group (n=22) (0.046 versus 0.048 r.u). But tPA levels were more than two times higher in both IS and IS+DM groups in comparison with the control subjects (n=25; 0.023 r.u).

We have found the negative correlation of plasma tPA content with glucose level and PAI-1 for IS patients (r=–0.26; p=0.022 and r=–0.249; p=0.028, respectively). There was no significant correlation of these parameters in the case of IS with type 2 DM. But it was high negative correlation between tPA and ECLT as well as Hageman-factor-dependent fibrinolysis time for both investigated IS forms. Spearman rank order correlation for tPA and ECLT was –0.65 under IS and -0.56 under IS with DM (p<0.001) (**Table 1**).

As it could be seen from the **Figure 3** and **Figure 4**, the aforementioned parameters were significantly prolonged. So Hageman-factor-dependent fibrinolysis time was more than threefold higher for both diabetic (21 min; 95% CI 20.85–22.73) and non-diabetic (22 min; 95% CI 21.7–22.98) patients with IS compared with

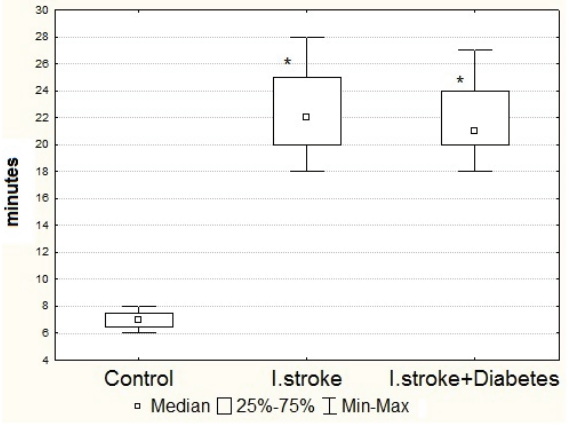

Figure 3. *Hageman-factor-dependent fibrinolysis time under ischemic stroke and ischemic stroke with diabetes mellitus.*
** - The result is significant at p<0.05 in comparison with the control*

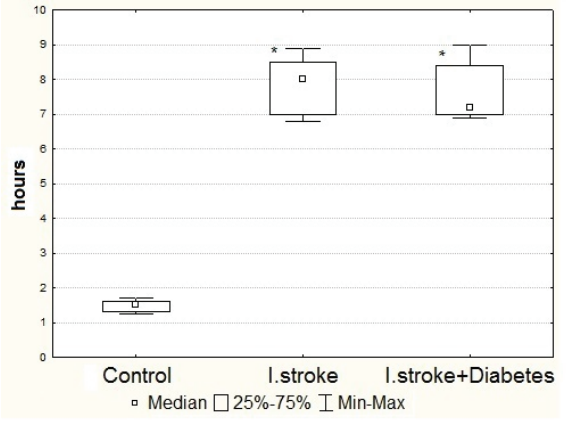

Figure 4. *Euglobulin clot lysis time under ischemic stroke and ischemic stroke with diabetes mellitus.*
** - The result is significant at p<0.05 in comparison with the control*

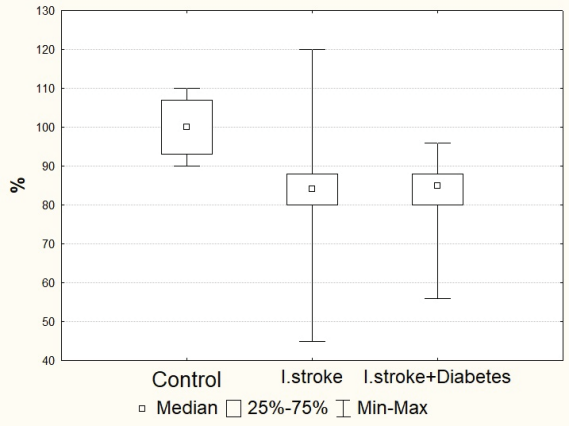

Figure 5. *α_2-Antiplasmin plasma activity under ischemic stroke and ischemic stroke with diabetes mellitus*
** - The result is significant at p<0.05 in comparison with the control*

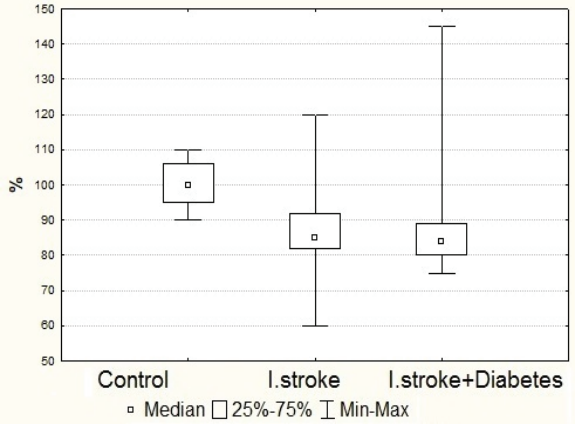

Figure 6. *Plasminogen plasma activity under ischemic stroke and ischemic stroke with diabetes mellitus.*
** - The result is significant at p<0.05 in comparison with the control*

relatively healthy control donors (7 min; 95% CI 6.74–7.87) indexes.

The other fibrinolysis time parameter ECLT had even more pronounced deviations. Its value was 1.5±0.2 h for the control group but there were 7.77±0.7 h for the IS patients and 7.64±0.73 h in the case of IS with diabetes. So statistically significant diversities of Hageman-factor-dependent fibrinolysis time and euglobulin clot lysis time between the control and IS patients ($p<0.01$) were established and no differences between presence of hyperglycemia under IS or its absence were observed.

The Spearman rank order correlation revealed the strong correlation not only for tPA content with fibrinolysis time tests (ECLT and Hageman-factor-dependent fibrinolysis time) but also the high level of positive correlation exactly between ECLT and Hageman-factor-dependent fibrinolysis time ($r=0.79$ for IS+DM; $r=0.78$ for IS $p<0.001$) independently of diabetes presence under stroke. Thus, despite the identical trend of PAI-1 content changes and prolongation of time lysis tests in patients with IS and stroke complicated by DM, in the latter case, there was no correlation between PAI-1 content and ECLT, which was observed under IS alone. However, ischemic stroke with diabetes mellitus was characterized by negative correlation in both time lysis parameters with α_2-antiplasmin activity (**Table 1**). This parameter was lower in both pathological conditions and amounted 80–85% compared with the control (**Figure 5**).

It should be noted that IS patients without hyperglycemia manifested statistically significant correlation of glucose level with PAI-1, tPA content and α_2-AP activity (**Table 1**), that wasn't observed under stroke with DM.

As the quantity of tPA was increased in stroke groups and main substrate of this secreted serine protease is glycoprotein plasminogen (PLG), which is synthesized in liver and circulates in blood, to assay streptokinase stimulated blood circulating PLG activity seems to be relevant. So the general tendency to some decrease of PLG activity under IS similarly like under IS+DM was shown (**Figure 6**). The median of PLG activity of IS patients fluctuated near 87.3% compared with the parameter from relatively healthy donors, this activated fibrinolytic potential amounted 87.2% of the control data in the case of diabetes mellitus stroke. It should be noted that this parameter didn't correlate with any of the investigated criteria neither under the stroke nor under the stroke with glycemic conditions.

Plasmin, which is generated from plasminogen, cleaves fibrin, thus breaking down meshwork of clot and is extremely short-lived due to α_2-antiplasminin activation *(2)*. The α_2-AP activity was shown to be some depleted under both investigated IS conditions compared with the control. The median value was almost similar for stroke and stroke with diabetes. But significantly more pronounced fluctuations of indicators were observed under the stroke only. In addition, α_2-AP activity positively correlated with glucose level under stroke but not under DM, and negatively correlated with both time lysis parameters (Hageman-factor-dependent fibrinolysis time and euglobulin clot lysis time) in the case of hyperglycemia presence under stroke.

DISCUSSION

It was established that the main markers of fibrinolysis had significant changes in the patients with acute ischemic stroke and such disturbances were independent of type 2 diabetes mellitus presence. Thus, plasma quantity of PAI-1 increased almost threefold under acute stroke compared with the control group and there was the earlier study describing the elevated level of PAI-1 under IS *(4)*. PAI-1 indicator of patients under stroke with diabetes was similar and these data coincided with a huge number

of fibrinolysis investigations under metabolic syndrome *(13, 14)*, obesity *(15)* as well diabetes mellitus *(16)*. Also PAI-1 and antithrombin III inhibiting fibrinolysis, consistently have been found to be elevated in diabetic patients and in those with insulin resistance. Additionally, there were both cross-sectional and prospective studies showing that increased tPA antigen and PAI-1 levels have been significantly associated with IS *(17)*. PAI-1, which abnormal high quantity was shown under stroke, is the most efficient inhibitor of tPA in plasma and the majority of circulating tPA is bound with this inhibitor. In the present report, we documented that tPA level was abnormally high in subjects with both conditions of acute IS as well. Our findings coincided with previous studies, showing that high tPA antigen levels can be detected in the same group of individuals, who were examined in either acute or subacute phase after stroke *(18)*. It should be noted that recombinant tissue plasminogen activator treatment has long been the only available therapeutic agent for IS *(2)*. Moreover recent study has found a decreased recanalization rate following recombinant tPA administration in the presence of hyperglycemia *(17)*. E. C. van Overbeek et al. found that higher baseline plasma tPA-activity (but not PAI-1 level) was associated with progression of white matter hyperintensities after 2 years of follow-up in lacunar stroke patients *(19)*.

The interaction between tPA and PAI-1 and their coordinated appearance in plasma are highly complex. Total amount of tPA and PAI-1 antigens in plasma usually shows a strong positive correlation, suggesting that synthesis and/or clearance of tPA and PAI-1 are biologically linked. However, an increased tPA production due to increased PAI-1 levels does not automatically result in increased amount of active tPA. Instead of that, tPA activity reveals a negative correlation with both PAI-1 and tPA antigen. This latter correlation may seem confusing, since increase in total amount of tPA results in tPA activity decrease. In other words, high tPA antigen concentration in stroke patients may indicate inactivation of fibrinolytic system or may be due to a delayed clearance of complexed with inhibitors tPA. High PAI-1 antigen concentrations represent increased fibrinolytic inhibition in patients with cerebral infarction *(4)*.

The results of this investigation revealed significant reduction of fibrinolysis that was concluded from extend euglobulin clot lysis time as well as Hageman-dependent fibrinolysis time. Corresponding changes of ECLT under stroke were also described by *(20)*. The literature data in the case of diabetes mellitus are not so single-valued, so a prolonged ECLT may occure due to diabetes *(21)*. But it was showed ECLT activation under type 1 DM (203.4+/–76.8 min vs. in the control group 276.08+/–84.87 min, $p < 0.05$) and conversely a reduction of euglobulin fibrinolysis under type 2 DM (448+/–117 min) compared with the control ($p<0.05$) *(22)*. Moreover there were authors, who postulated the highest PAI correlation with ECLT *(23)*, which was also established in this investigation but only under IS. The literature data of Hageman-factor-dependent fibrinolysis time were quite rare under the studied pathologic state.

Tissue-type plasminogen activators, dramatically increasing in quantity under stroke, mediate conversion of plasminogen to active protease plasmin. Maybe such excessive changes of tPA level led to depletion of plasminogen activity pool under ischemic stroke, which, in its turn, would have negative consequences for fibrinolysis. Similar results *(24)* showed lower mean of plasminogen activity in total cerebral infarction group and in patients with cardioembolic stroke, compared with the control. Although it is well known that glycation of plasminogen in diabetes directly affects fibrinolysis by decreasing plasmin generation and reducing protein-specific activity, we haven't revealed any difference between stroke patients with or without diabetes mellitus. The same, independent of glycaemia presence, changes were indicated for α_2-antiplasmin activity.

The obtained results showed that there were important differences in the characteristics of the fibrinolytic mechanism in ischemic stroke patients compared with healthy population. The major differences were prolonged euglobulin clot lysis time and elevated PAI-1 and t-PA contents in ischemic stroke patients. In addition, there were not any significant differences in deviations of aforementioned fibrinolytic parameters under ischemic stroke complicated by type 2 diabetes mellitus in comparison with ischemic stroke but it was found changes of Spearman rank order correlation between PAI-1 & tPA, PAI-1 & ECLT, Glucose & PAI-1, Glucose & tPA in case of the diabetes. These findings support the hypothesis that fibrinolysis disturbances are accompanied cerebrovascular events.

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ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

CONFLICT OF INTEREST

Author Olha Kravchenko, author Nataliia Raksha, author Tetiana Halenova, author Tetiana Ishchuk, author Tetiana Vovk, author Mariia Tymoshenko, author Oleksii Savchuk, and author Liudmila Ostapchenko declare that they have no conflict of interest.

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