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The imbalance of the haemostasis cascade in people suffered ischemic stroke.

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ABSTRACT

Multiple anomalies of hemostasis parameters in patients with stroke are a well known fact. In addition ischemic stroke causes a lot long-term disorders of the almost every links of hemostasis. Thus it is shown that even a year after an post ischemic stroke deviations still persist in the organism. Including deviations at the fibrinolytic level which is the main marker of recovery. Thus patients with AIS like with CIS had abnormal concentration of PAI-1, vWF and t-PA in acute phase as well as one year past acute phase. Also the increased reactivity of platelets obtained from healthy donors after their incubation with IgG fractions from blood plasma of patients with ischemic stroke in both the acute phase and post stroke period was shown. The results confirm the differentiation in qualitative composition of tested subtypes of ischemic stroke IgG fractions. Also significantly different effect of IgG from AIS patients compared to IgG from heathy donors on the tested process during the acute stroke as well as one year past was clearly expressed.
Keywords: Ischemic stroke, platelets aggregation, t-PA, PAI-1, vWF.

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INTRODUCTION

Based on the high prevalence, morbidity and mortality of ischemic stroke this disease is one of the most pressing health problem nowadays (1). Each person has a different recovery time and need for long-term care. Problems with moving, thinking, functional deficit, depression, and cognitive disorders often improve in the first weeks or months after a stroke. But some people will keep improving years after a stroke (2). It is known that the leading mechanism of stroke realization correlated with haemostasic profile (3). In particular coagulation activation, deficiency of natural anticoagulant and disorders in fibrinolytic status were observed during the stroke (4). It was proved that lot of protein markers exist in the bloodstream during the acute stroke as well as months/years past disease (5-8, 16). The complete return to normal life after stroke was not observed and repetition of the illness happen. In this frame it take sense to characterize the chosen parameters of haemostasis system in acute phase as well as one year past disease.

MATERIALS AND METHODS

Blood plasma samples were taken from 35 healthy donors and 123 patients: 62 (50,4%) patients with atherothrombotic ischemic stroke (AIS) and 61 (49,6%) patients with cardioembolic ischemic stroke (CIS) during the first 6 hours of the disease onset and after one year past stroke in 86 patients: 45 (52,3%) patients with AIS and 41 (47,7%) patients with CIS (37 (30,1%) died during the year). Patients were hospitalized in neurological department of the Hospital №4 (Kyiv, Ukraine). The diagnosis of ischemic stroke was confirmed through computed tomography or magnetic resonance imaging. At the first day of admission to hospital, all patients received aspirin 325 mg orally, then 100 mg aspirin daily. From the second day of hospital stay, patients received low molecular weight heparin in prophylactic dose. All donors and patients or their relatives had been warned about the conduct of clinical research and provided written agreement about participation. Current experiment was approved by the ethics committee from ESC “Institute of Biology”, Kyiv, Ukraine.

Fasting blood samples were collected from the cubital vein of all patients on the 1st and the 7th day of hospitalization as well as from the absolutely same group of patients one year past acute phase of disease. Thus we obtained three blood samples from the each patient: 1st, 7th and one year past acute phase. Blood was collected into 3.8% sodium citrate (9:1). The sample was immediately centrifuged at 1,000g for 10 min at the room temperature, and aliquot of platelet-rich plasma (PRP) was taken for platelet aggregation measurement. Blood plasma was separated by further centrifugation at 2,000g for 15 min at the room temperature and stored at −20° C until assays were performed.

Platelet aggregation was analyzed after stimulation with ADP (final concentration 2.5 mM) using photo-optical aggregometer AT-02 (Medtech, Russia) within 3 hours of sampling. Aggregation analysis was performed according to the manufacturer instructions. (18). Each aggregation measurement was repeated three times.

The plasma levels of plasminogen activator inhibitor-1 (PAI-1), tissue plasminogen activator (t-PA), and von Willebrand factor (vWF) were measured by standard enzyme linked immunosorbent assay (ELISA) techniques (15, 19). The ELISA plates were coated overnight at 4°C with blood plasma samples previously diluted 10-fold with 50 mM Tris-HCl containing 130 mM NaCl, pH 7.4. After being washed, plates were blocked with 5% nonfat dry milk in 50 mM Tris-HCl containing 130 mM NaCl, pH 7.4 for 1 h at 37ºC and washed again. Then plates were incubated for 1 h at 37ºC with specific primary antibodies against the t-PA, PAI and vWF (Millipore, Germany). Plates were washed and incubated for 1 h at 37ºC with corresponding secondary antibodies (Sigma, USA) conjugated to horseradish peroxidase. After washing, substrate (*o*-phenylenediamine and hydrogen peroxide) was added. Plates were read at 492 nm by a microplate spectrophotometer (QuantTM, BioTek Instruments, Inc). The healthy donor's (control) samples concentration was set as 100%, and changes in concentration are given as percentage of controls.

IgG was separated by affinity chromatography on protein A Sepharose (13). 1 ml of blood plasma was applied on the column of protein A Sepharose (total volume of the column was 5 ml). Nonspecifically bound proteins were washed by 50 mM Tris-HCl containing 130 mM NaCl, pH 7.4. Elution was performed by 0.1 M glycine-HCl, pH 2.2. The purity of separated IgG fractions was controlled by 7,5% PAGE (13, 16) using the following protein standards: myosin (200 kDa), b-galactosidase (116 kDa), phosphorylase b (97 kDa), albumin (66.2 kDa) and ovalbumin (45 kDa). Gels were stained with 0.125% solution of Coomassie Brilliant Blue G-250

in 25% isopropanol and 10% acetic acid. The concentration of the separated IgG was measured by spectrophotometer (Bio-Rad, USA).

RESULTS

A total of 123 patients were included to study after signing informed-consent form. Clinical characteristics of patients are presented in table 1. There were no significant differences between patients with AIS and CIS in gender, age, hypertension and ischemic heart disease and stroke severity. In patients with AIS expected significant difference was a higher percentage of patients with hyperlipidemia – 51,6% patients with AIS and 32,8% patients with CIS (p<0,001). In patients with CIS were registered a significantly higher neurological deficit (11,5±0,6) compared patients with AIS (9,1±0,6 , p=0,048).

IgG fractions were obtained from blood plasma of healthy donors, patients with AIS and patients with CIS by affinity chromatography on protein A Sepharose (11, 12). The purity of separated IgG was controlled by PAGE. The presence of clean IgG in elute was determined. The presence of light chain 25 kDa and heavy chain 55 kDa of IgG was proved under the conditions of disulfide bonds recovery by b-ME (Figure 1).

The IgG concentration determined by spectrophotometric method for healthy donors was equal 7,6 ± 0,5 mg/ ml. The concentrations of IgG obtained from the blood plasma of stroke patients varied during the experimental period. In particular one year past acute phase as well as on the 1st day of hospitalization concentration of IgG fractions both stroke subtypes were close to the healthy donor's value. Besides on the 7th day of hospitalization concentration of one year past AIS IgG fraction elevated to 9,3 ± 0,3 and on the 14th day to 10,7 ± 0.9 mg/ml (Figure 2). Statistically significant changes in the CIS IgG fractions concentration were not observed during tested period.

The investigation of ADP-dependent aggregation of the platelet obtained from group of patients with ischemic stroke has shown significant difference comparing to healthy donors platelets aggregation. The maximum aggregation value for the healthy donors was equal 66,2 ± 3,5 %. Platelets from AIS patients shoved fluctuation of maximum aggregation during tested period and was equal in acute phase 83,4 ± 5,1 %, on the 7th day of hospitalization 82,6 ± 4,2 % and one year past acute phase 55,8 ± 3,6 %. Platelets from CIS patients were characterized by significantly increased platelet's response to ADP only at the 7th day of hospitalization and was equal 87,7 ± 3,5 %. (Table 2).

Table 1: Clinical characteristics of ischemic stroke patients

	AIS (n=62)	CIS (n=61)	p
Age, years (M±SD)	**72,1 ± 10,7**	**74,3 ± 9,8**	**0,652**
Gender, male, n (%)	**31(50)**	**29 (47,5)**	**0,714**
Hypertension, n (%)	**43 (69,3)**	**41 (67,2)**	**0,502**
Hyperlipidaemia, n (%)	**32 (51,6)**	**20 (32,8)**	**<0,001**
Ischemic heart disease, n (%)	**19 (30,6)**	**18 (29,5)**	**0,353**
Baseline NIHSS (M±SE)	**9,1±0,6**	**11,5±0,6**	**0,048**

Table 2: ADP-dependent platelet aggregation of the healthy donors and patients with atherothrombotic and cardioembolic ischemic stroke

Group Healthy donors, n=35	**Maximum aggregation, %** 66,2 ± 3,5		
	1th day of hospitalization (acute phase)	7th day of hospitalization	1 year past acute phase
Atherothrombotic ischemic stroke, n=66	83,4 ± 5,1 *	82,6 ± 4,2 *	55,8 ± 3,6 *#
Cardioembolic ischemic stroke,n=56	74,3 ± 4,1	87,7 ± 3,5 *#	60,6 ± 2,4

* statistically significant changes in comparison with healthy donors
\# statistically significant changes in comparison with acute phase of stroke

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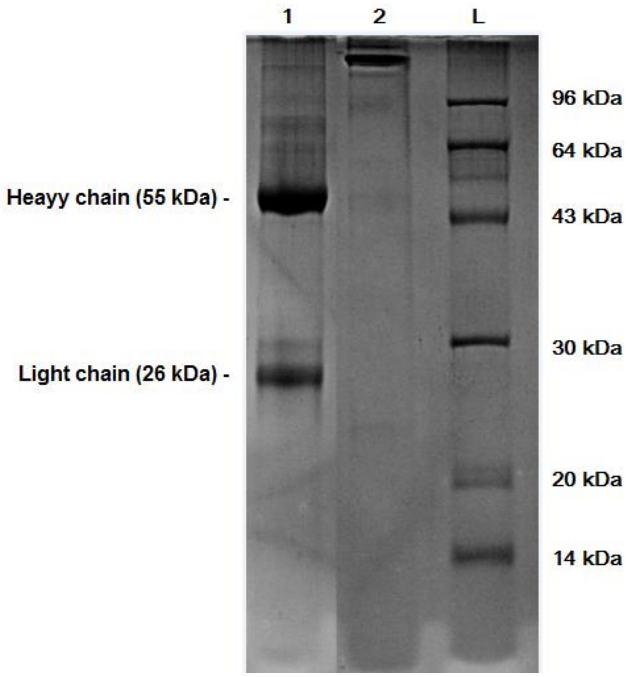
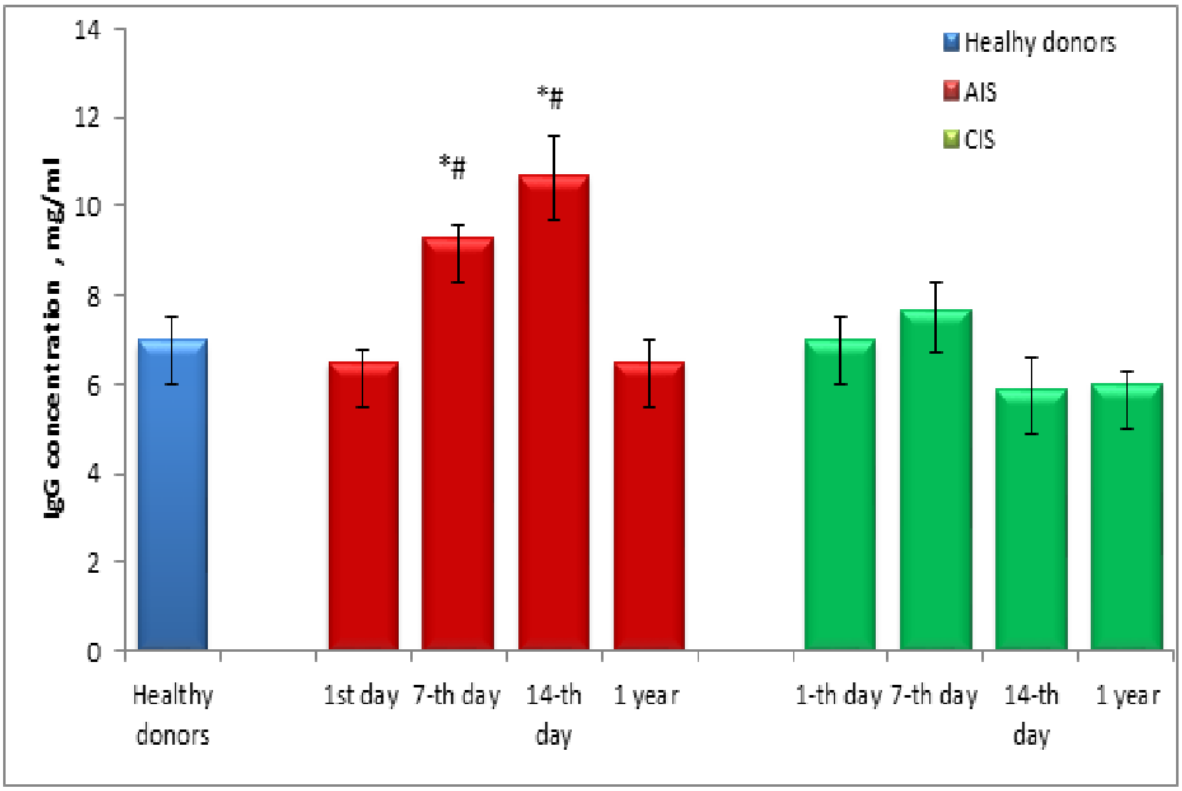


Figure 1. Electrophoregrame of IgG separated by affinity chromatography on a column of protein A Sepharose.

1 – Antibodies in the presence of b-mercaptoethanol (heavy and light chains);
2 - Native antibodies;
L – molecular weight ladders.

Figure 2. Concentrations of IgG on the each testing points



* statistically significant changes in comparison with healthy donors
# statistically significant changes in comparison acute phase

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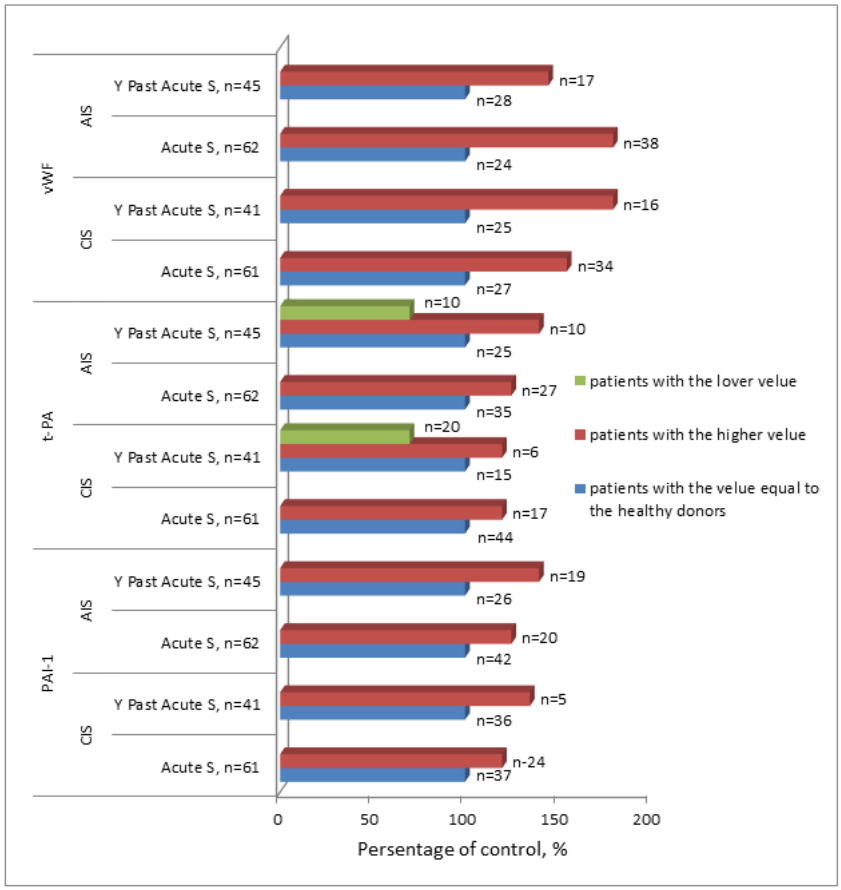
Figure 3. Concentration of the t-PA, PAI-1 and vWF in the blood plasma of the patients with the stroke



Pathological conditions observed during the acute phase of both subtype of ischemic stroke accompanied with abnormal concentrations of tissue plasminogen activator (t-PA), plasminogen activator inhibitor type 1 (PAI-1) and von Willebrand factor (vWF) that. Moreover the full recovery these parameters to the health level have not been noticed for AIS as well as for CIS one year past acute phase (Figure 3). Some amount of patients with the value close to the norm existed in each tested groups of patients. Number of such patients becomes more one year past acute phase.

DISCUSSIONS

It was shoved that AIS like CIS accompanied with changes in haemostasis system. For some numbers of the patient haemostasis disorders had saved one year past acute phase. The important fact is that CIS accompanied with low intensive chagges in haemostasis parameters level is comparison with AIS. Mostly haemostasis parameters were not equal to healthy donors in acute phase of disease but become close to the norm past one year of acute phase for patients with CIS. Opposite situation belong to AIS which provoke 'shaking' of some parameters level. ADP-dependent platelet aggregation of the patients with AIS was veered in range from the 25 % higher (1st day of hospitalization) to the 16 % lower (1 year past acute phase) comparing to the healthy donors. As explanation can be used idea about depletion of the ADP-dependent platelet aggregation potential as an adaptive mechanism.

Characterization of key components of fibrinolysis: tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1); and von Willebrand factor (vWF) as part of coagulation process provided basic pictures of the haemostasis profile of stroke patients. Patients with AIS like with CIS have abnormal concentration all parameters in acute phase as well as one year past acute phase. In each tested group included some number of the patients with similar to healthy donors tested parameters. Percentage of this patients become bigger by the time. But each group include anther patients with index saved abnormal level. This phenomenon can be explained by the effect of medical therapy and individual characteristics of

haemostatic profile of the organism on the one hand. On the other some mechanism of molecules saved in the organism after stroke persisted in the blood and provoked repetition variety.

Our data suggest that AIS and CIS accompanied with generation of the different qualitative composition of autoantibody that are able to significant selectively influence of haemostasis factors in acute phase as well as past acute phase of disease.

CONCLUSIONS

The aberrations in the haemostasis system of the patients with atherotrombotic and cardioembolic ischemic stroke during the acute phase as well as one year past disease were proved. The concentration of the t-PA, PAI-1 and vWF were significantly different in comparison with healthy donors. Unfortunately not all patients was characterized by normal value of the t-PA, PAI-1 and vWF concentration in the blood plasma one year past disease. The ADP-dependent platelet aggregation after the incubation of healthy donor's platelets with IgG fractions separated from blood plasma of stroke patients was significantly higher comparing to control aggregation which did not include IgG. The fact is maximum platelets aggregation after incubation of platelets with the healthy donor's IgG fraction was equal to the value of control aggregation. One year past acute phase as well as acute phase of ischemic stroke stable disorders of the tested parameters was saved.

FOOTNOTES

The abbreviations used are: IgG, immunoglobulin class G; AIS, atherothrombotic ischemic stroke; CIS, cardioembolic ischemic stroke; b-ME, β-mercaptoethanol; ADP, adenosine diphosphate; t-PA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; vWF, von Willebrand factor.

REFERENCES

[1] Hoyert D, Land Xu J. National vital statistics reports: from the centers for disease control and prevention. National center for health statistics, National vital statistics system, 2012; 61.6, 1-52

[2] Panteleyenko L, Sokolova L. Determinants of quality of life one year after ischemic stroke. *Ukrainian Bulletin*, 2010 18, 64-98

[3] Woodward M, Lowe G, Campbell D. Associations of inflammatory and hemostatic variables with the risk of recurrent stroke. *Stroke*, 2005; 36, 2143–2147

[4] Vovk T, Savchuk O, Ostapchenko L. Characteristic of autoimmune antobodies to components of hemostasis, which are producing in blood flow during systemic lupus erythematosus. *Physics of life*, 2010; 18.3, 59-63

[5] Burlova-Vasylieva M, Raksha N, Torgalo E, Savchuk O. The appearance of molecules of prothrombin origin in blood upon development of atherothrombotic and cardioembolic ischemic stroke. *Bulletin of Taras Shevchenko National University of Kyiv*, 2014; 3.68, 57-60

[6] Burlova-Vasylieva M, Savchuk O. Assessment of t-PA and PAI-1 levels in patients with atherothrombotic stroke and atrial fibrillation stroke. *Materials of conference IX Jakub K. Parnac: Proteins from Birth to Death. Israel,* 2013; 91

[7] Burlova-Vasylieva M, Kravchenko N, Savchuk O. Polymorphisms in FII, FV and PAI-1 genes in Ukrainian patients with atherothrombotic and cardioembolic ischemic stroke. *European Journal of Human Genetics,* 2014; 411

[8] Burlova-Vasylieva M, Savchuk O, Kravchenko N. ADP-induced platelet aggregation and plasma vWF level in Ukrainian patients with atherothrombotic and cardioembolic ischemic stroke. *Materials of conference 21st International Student Congress of BioMedical Sciences,* 2014; 222

[9] Shuaib A, Hussain M. The past future of neuroprotection in cerebral ischemic stroke. *Eur. Neurol*, 2008; 59, 41-43

[10] Vovk T, Savchuk O, Ostapchenko L. Characteristic of autoimmune antobodies to components of hemostasis, which are producing in blood flow during systemic lupus erythematosus. *Physics of life*, 2010 18.3, 59-63

[11] Katrii T, Vovk T, Kravchenko N, Savchuk O, Ostapchenko L. (2015) Influence of IgG separated from blood plasma of patients with ischemic stroke on the process platelet's proteins secretion. *International Journal of Chemical and Biomolecular Science*. 278-283

[12] Katrii T, Kravchenko N, Vovk T, Ostapchenko L, Savchuk O. (2016) The influence of immunoglobulin class G from blood plasma of patients with stroke on the activity of some parameters of haemostasis system. *Journal Blood Coagulation & Fibrinolisis*. Doi:10.109/MBC.0000000000000497. (in print)

[13] Zaremba E, Slaba N, Kopchak L. (2004) The main features of immune changes in acute forms of coronary heart disease. *Family Medicine*. 2.8, 52-53

[14] Ben Shimon M, Lenz M, Ikenberg B. (2015) Thrombin regulation of synaptic transmission and plasticity: implications for health and disease. *Front Cell Neuroscience.* 9, 151

[15] 15. Hockfield S, Carlson S, Evans C. (1993) Selected methods for antibody and nucleic acid probes. 4.1, 679

[16] Raksha N, Burlova-Vasylieva M, Torgalo E,Savchuk O. (2014) The appearance of molecules of prothrombin origin in blood upon development of atherothrombotic and cardioembolic ischemic stroke. *Bulletin of Taras Shevchenko National University of Kyiv, series Biology*. 3.68, 57-60

[17] Weber K, Osborn M. (1969) The reliability of molecular weight determinations by dodecyl sulfatepolyacriylamide gel electrophoresis. *J. Biol. Chem*. 244.16, 4406–4412

[18] Halenova T, Nikolaeva I, Nakonechn A [at el] (2016) *Ukr. Biochem. J*. 87.5, 83-92

[19] Kemeny M. (1991), A Practical Guide to ELISA, NY: Pergamon Press

[20] Volkov G., Savchuk A., Karbovskyy V. Method of extraction protein C activator from Agristrodon blomhoffii ussuriensis venom. Patent WO 2008/020740.2008

[21] Volkov G, Platonov T, Savchuk O, Krasruff E, Chernyshenko T, Gornitskaya O. Modern views on the haemostasis system / Naukova Dumka, (2005) Ukr.