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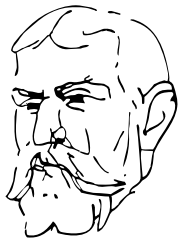

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ORIGINAL ARTICLE

THE ROLE OF ENTEROVIRUSES IN THE DEVELOPMENT OF ISCHEMIC STROKE AND ITS OUTCOMES

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ABSTRACT

The aim: To study the role of enteroviruses (EV) in the development of ischemic stroke and its outcome.

Materials and methods: The main group (MG) included 72 patients with acute cerebrovascular disorders were examined using the National Institutes of Health Stroke Scale and Barthel Index. The comparison group (CG) included 35 patients without cerebrovascular disease. Viruses were isolated from patients' sera and identified in neutralization test. EV genomes were detected in polymerase chain reaction (PCR). Serological diagnosis was performed by enzyme-linked immunosorbent assay.

Results: EV genomes were more frequently detected in the patients' sera in MG than in CG (23.6 ± 5.9% and 2.9 ± 2.8%, $p < 0.05$). The greater level of neurological deficits was in patients with positive PCR test results comparatively with patients with negative PCR test results (11.76 ± 0.31 and 10.97 ± 0, 27, $p = 0.040$). The regression of neurological deficit during the treatment was a worse in patients with positive PCR test results and presence of specific IgG compared with patients with positive PCR test results and absence of specific IgG (11.2 ± 2.6% and 19.6 ± 2.4%, $p = 0.031$).

Conclusions: The trigger role of EV in the development of IS is established. PCR is recommended for diagnosis of EV in patients with IS.

KEY WORDS: ischemic stroke, neurological deficit, enteroviruses

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INTRODUCTION

Acute cerebrovascular accident (ACM) is the second leading cause of disability and death in the world [1]. Nearly 50% of patients have disability after ishemic stroke (IS) [2]. A second IS develops in 25-30% of cases in a year after the first IS, and only 10% of patients return to work. IS is the cause of permanent disability in 53% of patients [3, 4].

The most common etiology of ischemic stroke in adults is atherosclerosis (AS). There are a number of hypotheses about the emergence, development and progression of AS that complement each other (lipid-infiltrative, inflammatory with free radicals, lower nitric oxide, peroxide, «response to damage» to vascular walls, chronic endothelial damage and dysfunction, autoimmune and bacterial infections, etc.) [2, 4-6]. The cause of IS remains unclear in about 40% of cases. This necessitates research on risk factors (RF) of AS and its cardiovascular and cerebrovascular complications [7]. Infectious can initiate the inflammatory process with subsequent damage of vascular endothelium, which leads to endothelial dysfunction as an early sign of AS. It is associated with severity of IS and determines the development of complications [2, 7, 8].

Inflammatory changes in AS can be initiated by human herpesviruses (HSV-1, HSV-2, EBV, CMV), human T-cell lymphoma virus type 1, Chlamydia pneumoniae, Helicobacter pylori, because these pathogens were found in affected blood vessels and atherosclerotic plaques [5, 10, 11]. The simultaneous persistence of several infectious agents in patients with severe immunoreactivity on the background of genetic predisposition in the presence of other RF can lead to AS. It can be confirmed by the detection of Ig G simultaneously to several infectious agents in the serum of patients with AS [4]. Therefore, the study of the role of infectious agents in the development of IS is important.

THE AIM

Study of the role of enteroviruses in the development of ischemic stroke and its consequences.

MATERIALS AND METHODS

A total of 107 patients who were hospitalized in the Neurological department and the Department of Cerebrovascular Pathology of the Oleksandrivska Clinical Hospital in Kyiv from 2009 to 2015 were enrolled in the study. The study was approved by the Bioethic Commission of the O.O. Bogomolets National Medical University. All patients gave voluntary written consent to participate in the study.

MG consisted of 72 patients with ACVD who were examined using the NIHSS stroke severity scale (the National Institutes of Health Stroke Scale) and Barthel Index (IB). Verification of the diagnosis was performed using Doppler ultrasound (DU), computed tomography (CT) or magnetic resonance imaging (MRI).

Inclusion criteria were the following: age of the patient over 18 years, confirmed diagnosis of ACVD (ischemic stroke or transient ischemic attack (TIA)), and absence of exclusion criteria. Exclusion criteria were: length of hospital stay less than 2 weeks, inability to collect serum at intervals of two weeks.

The control group (35 people) consisted of patients who were treated at the hospital in case of neurological diseases not related to the vascular pathology (13 patients with peripheral nerve neuropathy and polyneuropathy, 8 – with radiculopathy and ganglionitis, 9 – with hereditary diseases of the nervous system).

The age of the examined patients of MG ranged from 31 to 86 years and the average age was 62.6 ± 12.0 years. The sample included 41 women (56.9%) and 31 men (43.1%). CG included 35 patients aged from 16 to 86 years, the average age was 56.3 ± 19.3 years. The sample included 19 women (63.3%) and 11 men (36.7%).

Clinical and neurological parameters were studied using modern neurological scales: the dynamics of neurological disorders was determined by the NIHSS stroke severity assessment scale; functional stroke recovery was assessed using BI. The pathogenetic subtype of stroke was determined using TOAST criteria.

All patients underwent ultrasound and transcranial Doppler (TCD) examination of the brachiocephalic arteries using a Medison ACCUVIX V10 with a 2-4 MHz phased array sensor to determine the severity of the stenosis, the thickness of the intima-media complex (IMT) of the common carotid artery (CCA), internal carotid arteries, middle cerebral, anterior cerebral arteries (respectively ICA, MCA, ACA), subclavian, extracranial parts of vertebral, basilar and posterior cerebral arteries (respectively SCA, VA, BA, PCA). Examination of the main vessels of the head in the extracranial region was performed using an ultrasound Doppler "Multigon 500M" (MultigonIndustriesInc, USA). The analysis of cerebral hemodynamics parameters was performed with the determination of average indicators of the maximum and mean velocity in ICA and VA. The generally accepted method provided blood flow determination in CCA, ICA, ACA, VA, BA, PCA by qualitative (audiovisual) and quantitative characteristics.

Magnetic resonance imaging (MRI) of the brain was performed on a device "FLEXATAR" (Toshiba) with a field strength of 1.5 T.

Isolation of viruses from blood sera and their identification was performed by micromethod on cell cultures (RD, HEp-2 and HeLa) with 100% cytopathic effect, followed by identification in the virus neutralization test. The genomes of EV were determined in reverse transcriptase-polymerase chain reaction (RT-PCR) using the AmpliSens test system [12]. Serological testing was performed in the paired sera of patients of MG and CG by enzyme-linked immunosorbent assay (ELISA) using the test system for the determination of Ig G and Ig M to EV "Ig G Teskit / Ig A Teskit / Ig M Teskit", manufactured by Sekisui Virotech GmbH, Germany. The results were recorded using the ELISA HumaReader (Human GmbH, Germany). All calculations were performed using the statistical data analysis package IBM SPPS Statistics 22.

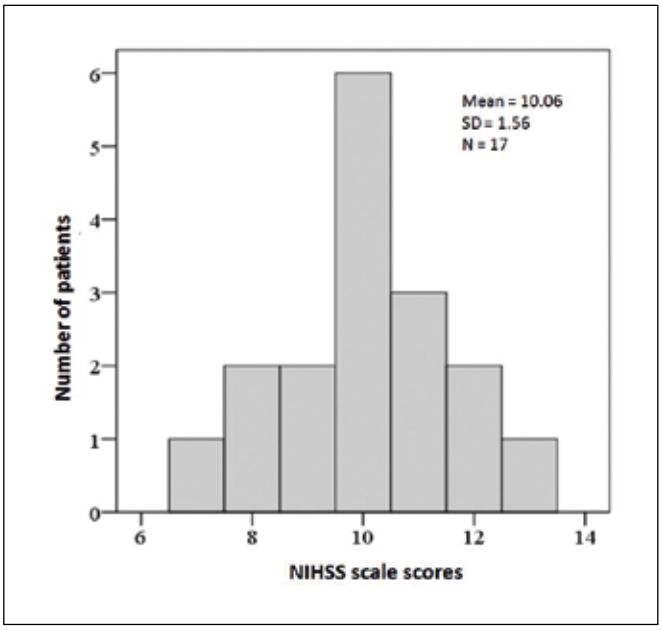

Fig.1. Distribution of patients in the main group by NIHSS score

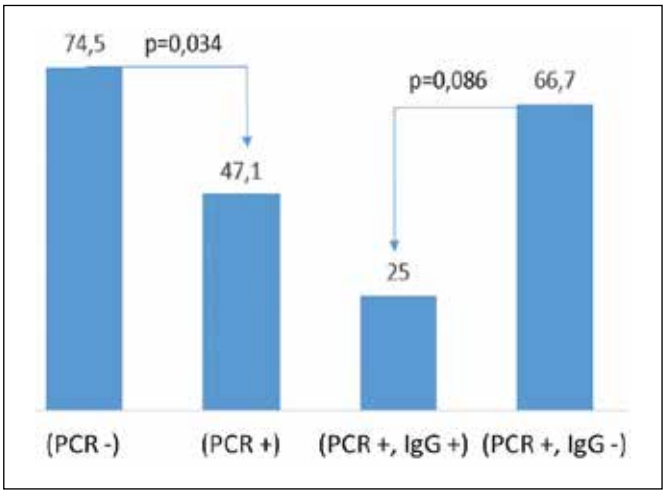

Fig. 2. Frequency of minimal functional dependence of ≥ 75 points according to the BI three months after stroke

RESULTS

It was proved that EV genomes were detected in 17 of 72 sera of patients with MG (23.6 ± 5.9%) and in the serum of one patient of 35 patients in CG (2.9 ± 2.8%). EV were isolated on cell cultures from 11 sera of 17 PCR-positive sera of patients in MG. There were identified 8 isolated strains as Coxsackie B viruses (serotypes 2, 3, 4) and ECHO viruses (serotypes 6, 9, 27 (two strains), 29), however other 3 isolated strains were not identified. Virus was not isolated from one PCR-positive serum of a patient in GP. Detection of EV genomes in the sera of examined patients indicates the presence of enterovirus infection [13].

The paired sera of patients were studied by ELISA to detect specific IgM and IgG to EV. IgM to EV were not detected in any serum of patients in MG and CG. IgG to EV were detected in both sera of 17 patients in MG (23.6 ± 5.1%). IgG to EV were detected in 8 of 17 PCR-positive

Table I. Indicators of neurological deficit scores according to the NIHSS scale for different values of the PCR test results and IgG

NIHSS, points	PCR-test		p	PCR + IgG +	PCR + IgG -	p
	+	-				
Admission	11,8±0,3	10,9±0,3	0,040	12,3±0,3*	11,3±0,5	0,153
Discharge	10,1±1,6	7,07±2,1	0,001	10,9±0,4*	9,3±0,5*	0,046
Dynamics of points, %	15,6±8,2	34,6±1,6	0,001	11,2±2,6*	19,6±2,4*	0,031

Note. * - significant difference (p <0.05) compared to patients with negative PCR test results.

Table II. Characteristics of risk factors in patients of the main and comparison groups

Evaluation Criteria	MG (n=72)	CG (n=35)	P
	Absolute value (%) M±m	Absolute value (%) M±m	
Age of patients, years	63,0±13,0	56,4±19,3	p> 0,05
Male, %	31 (43,1%)	13 (37,1%)	p> 0,05
Female, %	41 (56,3%)	22 (62,9%)	p> 0,05
Smoking, %	16 (22,2%)	5 (14,3%)	p> 0,05
Alcohol abuse, %	11 (15,3%)	1 (2,9%)	p> 0,05
Hypertension, %	63 (87,5%)	24 (68,6%)	p> 0,05
Overweight, %	11 (15,3%)	1 (2,9%)	p> 0,05
Blood cholesterol (mmol/l)	5,3±1,0	4,74±0,8	p> 0,05

patients in MG, and only in 2 patients in CG who were PCR-negative.

It was found that the genomes of EV were more common detected in patients aged 51 to 55 years old (58.3 ± 14.8%). The lowest incidence of EV genomes was observed in patients under 60 years old (9.7 ± 5.3%). Detection of EV in patients in MG was significantly higher than in CG (p<0.05).

The frequency of detection of structural and morphological changes in cerebral vessels in patients with ACVD was high. In all patients of MG relative to CG significant changes in the inner vascular wall of the carotid arteries during ultrasound examination – an increase IMT: in CG – 0.62 ± 0.09 mm (p <0.001), in patients of MG – 1.03 ± 0.07 mm (p <0.001) were detected. The high risk of IMT increase may be caused by an association with a viral infection. Of the 72 patients of MG, cerebral stenosis was found in 64: 24 patients had minor ICA stenosis > 25%, which was accompanied by an asymmetry of blood flow velocity, 30 had minor VA stenosis <50%, and 10 had moderate ICA stenosis > 50%. A cerebral hemodynamic parameters analysis revealed that in patients with ACVD, the average linear and mean blood flow velocities in ICA relative to CG are much lower. According to CT or MRI data, the MG patients had confirmed changes typical for stoke foci; the TIA patient over 50 had occasional foci of encephalomyelitis. At the time of hospitalization, the mean value of neurological deficit according to the NIHSS scale was 10.06 ± 0.38 points (7 to 13 points), Fig. 1.

According to the mean NIHSS scores at the time of admission to the hospital in the subgroup of patients with positive PCR test results, the severity of neurological deficit was higher and differed significantly compared with patients who had negative PCR test results (11.76 ± 0.31 vs. 10.97 ± 0.27 points, p = 0.040, respectively).

PCR-positive patients who had IgG to EV in serum did not differ in severity of neurological manifestations from PCR-positive patients who did not have IgG to EV in serum (12.25 ± 0.31 points and 11.33 ± 0.51 points, respectively, p = 0,153).

During treatment in patients with a positive PCR test result and the presence of IgG a slower regression of neurological deficit was observed and at the time of discharge from the hospital and was on average 11.2 ± 2.6% compared to patients with the positive PCR test results and no IgG presence – 19.6 ± 2.4%, p = 0.031. Slow recovery of neurological functions led to significantly higher values of the neurological scores at the time of discharge: 10.88 ± 0.39 points compared with 9.33 ± 0.53 in the case of seronegative results for IgG, p = 0.046, table I.

The presence of enterovirus infection signs in patients was reflected in a functional dependence for three months after stroke, as assessed according to BI. In particular, in patients that have the enterovirus's genome the recovery to the level of minimal functional dependence of ≥ 75 points on the BI scale occurred in 8 (47.1%) patients; in the case of RNA virus genome absence, 41 (74.5%) patients, p = 0.034.

The combination of the positive enteroviruses genome RNA and IgG was associated with poorer functional recovery of <75 points on the BI in 6 (75.0%) patients compared with the 3 PCR-negative patients (33.3%), but the data did not reach statistical significance, p = 0.086, fig. 2.

Clinical and laboratory patient data of MG and CG was analyzed to assess the impact of enterovirus infection on

the course of the disease and to determine the role of enteroviruses in ischemic stroke occurrence (Table 2). We did not find a significant difference between the data on the proportion of smokers in the MG and the CG. The portion of heavy alcohol users in the MG was higher than in the CG ($p > 0.05$). At the same time, 63 (87.5%) of 72 patients in the MG were diagnosed with hypertension (blood pressure higher than 140/90 mm Hg), while in the CG only 24 (68.6%) of 35 patients were diagnosed with hypertension. Also 11 (15.3%) of 72 patients of the MG were overweight, while in the CG only 1 (2.9%) of 35 patients was overweight. A higher level of cholesterol in blood was found in the MG patients – 5.3 ± 1.0 mmol / l compared with the CG patients (4.74 ± 0.84 mmol / l), but these differences in indicators were not statistically significant ($p > 0,05$).

DISCUSSION

The results of experimental studies confirm the initiation of atherogenesis by existing viral infection. At the heart of the initial stage of AS are foci of endothelial cell damage, which occur due to changes in the vascular wall under the influence of hypertension, viral infection, accompanied by increased endothelial permeability [10, 11]. Under conditions of hypertension, the increase in vascular pressure narrows, the autoregulation of vascular tone changes in the direction of promoting further vasodilation with a decrease in muscle tone of unstriated muscles. This mechanism changes the state of the vascular wall endothelium, plasma cells impregnate the latter, which leads to the development of perivascular edema, which leads to diapedetic hemorrhage, which causes edema and dystrophic changes in nerve cells [2, 7].

Numerous studies suggest that latent infections may be predictors of IS. The accumulation of infectious agents that can act as triggers of the inflammatory process is obvious. In most cases, the development of CVD is influenced by the association of viral infection and other RF [7, 9, 11, 14]. Recent studies confirm the similarity of the process of atherogenesis with the typical inflammatory response.

Enterovirus infection is laboratory confirmed by the isolation of EV or detection of EV RNA in sterile clinical material. Molecular methods based on nucleic acid in vitro amplification is the gold standard for diagnosing EV infections [15]. Reverse transcriptase PCR (RT-PCR) targeting the 5'noncoding regions (5'NCR) is recommended for diagnosis of EV because of their sensitivity, specificity and short turnaround time [13]. Sequencing of part of the VP1 capsid protein gene is used for EV type identification [15]. Virus isolation including neutralization assays is a classical method, but should not be used in primary routine diagnostics due to their known insensitivity and slowness. The European Non-Polio Enterovirus Network (ENPEN) (the network collaboration of clinical and molecular virologists, clinicians, epidemiologists and public health experts functions) was created to develop and share knowledge on diagnostic techniques for EV and parechoviruses detection and characterization, disease presentations and prognosis, virus evolution and pathogenesis [15].

According to our study laboratory markers of EV infection (isolated EV or EV genome) were not detected in 9 of 72 patients in MG (12.5 ± 3.9%), but specific Ig G to EV were detected in paired sera of these patients in ELISA. These results indicate that such patients have suffered from enterovirus infections in the past, resulting in antibodies to EV that remain in diagnostic titers.

Thus, we determined the presence of laboratory markers of EV infection in 26 patients of 72 patients (36.1%) in MG: laboratory markers of acute EV infection were detected in 6 patients (EV were detected by PCR and / or virological method and specific Ig M were detected in ELISA); laboratory markers of chronic persistent EV infection in the acute stage were detected in 8 patients (EV were detectted by PCR and / or virological method, and specific Ig G or both Ig M and Ig G were detected in ELISA); laboratory markers of EV infection were detected in 9 patients (no EV were detected in serum by PCR and / or virological method, but specific Ig G in ELISA were detected); laboratory markers of persistent EV infection were detected in 3 patients (PCR revealed EV RNA, but no specific Ig M and Ig G were detected). No laboratory markers of EV infection were detected in 46 patients in CG.

We found that 33 patients (45.8%) in MG had acute respiratory disease 1-14 days before hospitalization for IS. This explains the detection of laboratory signs of EV infection in these patients on the first day of hospitalization and confirms the possible role of EV as a trigger in the development of IS.

When testing the patients with atherothrombotic (AT) ischemic stroke subtype for the presence of EV, a poorer functional recovery was observed in patients with the positive PCR test results and the presence of IgG to viruses associated with the functional recovery; <75 points by BI, $p = 0.086$.

We studied the effect of viral infection on the condition of cerebral vessels, the course and consequences of IS. The functional recovery in patients with confirmed EV persistence was significantly worse one year after IS, than in patients without one. Recovery of neurological function one year after ischemic stroke was significantly worse in patients in MG than in patients in CG. This was reflected in a lower mean score of IB in patients in MG. These data support the hypothesis regarding the triggering effect of EV infection on the development of structural changes in atherosclerotic plaques.

According to the literature, the study of pathogenetic mechanisms of the origin and development of AS, which confirms the participation of viruses in its development, opens new perspectives for the use of complex anti-viral therapy in the complex therapy of ACD / IS.

This study demonstrates the potential of using PCR to detect EV in patients with IS as a more sensitive, rapid and specific method than virological and allows to detect EV that are not isolated in cell culture. This will adequately adjust the therapy and improve the course of the disease and prognosis [16, 17]. Nesting RT-PCR, which involves

amplification of the genomic region encoding the viral structural protein VP1, has been developed for direct identification of EV in clinical specimens. It allows to detect EV group B, including relatively rare viruses ECHO and ECHO 15, ECHO, as well as enteroviruses A71 (EV-A71), that can be used for direct identification of EV in clinical material with low viral load [18]. Further research will focus on the etiopathogenetic role of viral associations in the onset and development of IS.

CONCLUSIONS

EV genomes were isolated from serum of patients with IS in 23.6 ± 5.9%, that is significantly higher than in patients in CG: 2.9 ± 2.8% ($p < 0.05$). EV were isolated on cell cultures in 11 cases of 17 PCR-positive sera of patients from the main group and identified as Coxsackie B viruses (serotypes 2, 3, 4), ECHO viruses (serotypes 6, 9, 27 (two strains), 29), but in three samples the isolated viruses were not identified.

Thus, studies have found an association between the presence of EV RNA genome and the severity of developed IS, as well as a positive correlation between the effectiveness of neurological recovery and the presence of EV in the blood, especially in presence of IgG in serum. The presence of viral infection predicted a negative regression of neurological deficit for next three years after IS. The functional recovery was significantly worse, which was reflected in a lower average score of IB and a lower percentage of patients with minimal limitation of function.

The use of PCR to detect enteroviruses in patients with IS in the future will complement the arsenal of diagnostic methods. Expanding knowledge about the pathogenetic processes of IS and confirming the possible role of EV in the development of atherosclerotic changes opens new perspectives for improving existing and new diagnostic methods, as well complex therapy and prevention.

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Conflict of interest:

The Authors declare no conflict of interest.

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