

DYNAMICS OF INDICATORS OF THE ENDOTHELIUM MORPHOFUNCTIONAL STATE OF THE BRAIN MICROCIRCULATORY BED VESSELS IN RATS WITH NITRITE-INDUCED ALZHEIMER'S TYPE DEMENTIA ON THE BACKGROUND OF MESENCHYMAL STEM CELL ADMINISTRATION

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MIKROCIRKULATORNOM KAPILARNOM KORITU PACOVA SA DEMENCIJOM ALCHAJMEROVOG TIP A INDUKOVANOG NITRITOM NA TERENU MEZENHIMALNIH MATIČNIH ČELIJA

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ABSTRACT

Objective. The aim of this study was to assess the vascular endothelium morphofunctional state of the brain microcirculatory bed in rats with nitrite-induced Alzheimer's type dementia on the background of stem cells administration.

Methods. 14 days after the experiment's end, the endothelin-1, VEGF-A, eNOS, von Willebrand factor were determined in blood serum by the enzyme immunoassay and photometric methods in rats with a model of nitrite-induced dementia (14 and 28 days of sodium nitrite intraperitoneal introduction) with and without mesenchymal stem cells (MSCs) administration. The brain slices were stained according to the Einarson's method and immunohistochemically by staging the reaction with antibodies to VEGF.

Results. With an increase in the sodium nitrite administration period, the degree of damage of brain capillaries and neurons increased, dystrophy of "surviving" neurons developed and ability to produce VEGF decreased. After 14 days of "regeneration period" in groups without MSCs administration, further stimulation of VEGF production by endotheliocytes, cortex and hippocampus neurons of varying degrees was observed. In groups where stem cells were introduced, the number of capillaries increased, with endothelial hyperplasia in some cases.

Conclusion. In animals with nitrite-induced dementia, dose-dependent damage to the endothelium of the capillary bed is noted. From the first day damage the vascular regeneration can be proved by VEGF expression. The stem cells administration more effectively stimulates capillary regeneration, as evidenced by a noticeable increase of the number of brain capillaries.

Key words: Alzheimer disease; sodium nitrite; mesenchymal stem cells.

SAŽETAK

Cilj. Cilj ove studije bio je da se proceni morfofunkcionalno stanje vaskularnog endotela u mikrocirkulacijskom sloju mozga kod pacova s demencijom Alzheimerovog tipa izazvanom nitritima na terenu administracije matičnih ćelija.

Metode. Četrnaest dana po završetku eksperimenta određivani su endotelin-1, VEGF-A, eNOS, Fon Vilebrandov faktor u krvnom serumu, imunoanalizom enzima i fotometrijskim metodama kod pacova s modelom demencije izazvane nitritima (14 i 28 dana intraperitonealne indukcije natrijum nitrita) s primenom mezenhimalnih matičnih ćelija (MSC) i bez nje. Moždani preseci su obojeni prema Einarsonovoj metodi i imunohistohemijski postavljanjem reakcije antitela na VEGF.

Rezultati. S povećanjem perioda primene natrijum nitrita povećava se stepen oštećenja moždanih kapilara i neurona, razvija distrofija „preživelih“ neurona i smanjuje sposobnost stvaranja VEGF. Nakon 14 dana „perioda regeneracije“ u grupama bez administracije MSC primećuje se dalja stimulacija proizvodnje VEGF endotelocitima, korteksom i hipokampusom neurona različitog stepena. U grupama gde su uvedene matične ćelije broj kapilara se povećava, ponegde s endotelnom hiperplazijom.

Zaključak. Kod životinja s demencijom izazvanom nitritima primećuju se oštećenja endotela kapilarnog korita zavisna od doze. Od prvog dana oštećenja vaskularna regeneracija može se dokazati ekspresijom VEGF. Administracija matičnih ćelija efikasnije stimuliše regeneraciju kapilara, što dokazuje приметно povećanje broja moždanih kapilara.

Ključne reči: Alchajmerova bolest; natrijum nitrit; mezenhimske matične ćelije.

INTRODUCTION

The attention of many researchers has been focused on the study of the interconnection between neuropathy and vasculopathy in the brain. After all, the role of vascular factors is decisive in the mechanisms of neuronal function damage in neurodegenerative diseases. The study of Alzheimer's disease (AD) etiopathogenesis remains an urgent issue, which is associated with the need to search for new, effective means of combating the progression of this pathology. Every year the number of AD patients all over the world is growing steadily. Perhaps this is the result of a combined negative influence of different exogenous factors that can cause genetic mutations in the human body, and subsequently lead to the development of neurodegenerative processes.

Back in 2009, Suzanne M. de la Monte et al. (1) established the fact of an increase of AD cases with excessive accumulation of nitrosamines, which are formed in the body during the interaction of sodium nitrite with proteins after its entering into the body with water, food, etc. Besides their carcinogenic effect, nitrosamines reinforce the oxidative stress, have a mutagenic effect, which plays an important role in the development of neurodegenerative changes. The role of this exogenous factor in the development of Alzheimer's type dementia was proven in our previous studies on the created nitrite-induced model, where after prolonged intraperitoneal administration of aqueous solution of sodium nitrite rats developed cognitive deficit, changed behavioral reactions, and, along with this deposition of congophilic masses in the arteries' walls of the brain and focal - in the white matter of the hemispheres (2).

It has also been previously experimentally stated, but on the scopolamine model of dementia, that animals simultaneously developed insulin resistance and energy deficiency in the brain (3, 4). Other studies also revealed similar changes (mitochondrial dysfunction, ATP deficiency, oxidative stress) in combination with the development of cognitive impairment in animals after the administration of low doses of streptozotocin, which is close to nitrosamine in chemical structure (5).

Amyloidosis of the brain has several hypotheses. Thus, the hypothesis of "amyloid cascade" provides for the excessive formation of β -amyloid by neuronal and glial cells as a result of pathological proteolysis of APP-amyloid precursor protein, which causes the accumulation of amyloid plaques and the development of neurodegenerative processes (6). Some authors believe that a mandatory stage in the accumulation of amyloid and the formation of cognitive impairment is cerebrovascular dysfunction due to vascular damage and the development of chronic ischemia (7). Possibly, neurodegeneration without previous impairment of endothelial function does

not occur in AD patients (8).

The aim of the paper was to study of indicators characterizing the vascular endothelium morphofunctional state of the brain microcirculatory bed in rats with nitrite-induced Alzheimer's type dementia on the background of stem cells administration.

MATERIAL AND METHODS

1. *Animals and groups*

The experiment involved 48 male Wistar Albino Glaxo (WAG) rats weighing 180-250 g, which were divided into 5 groups. Animals of group N-14 (nitrite 14 days, $n = 8$) and group N-28 (nitrite 28 days, $n = 8$) received intraperitoneal injections of an aqueous solution of sodium nitrite (NaNO_2) in a dose of 50 mg/kg for 14 days and 28 days, respectively. Animals of group N-14-SC (nitrite 14 days + stem cells, $n = 8$) and group N-28-SC (nitrite 28 days + stem cells, $n = 8$) received single intravenous injections of mesenchymal stem cells (MSCs) in a dose of 500,000 cells per each rat at the appropriate time after sodium nitrite injections (Figure 1). Animals of the control group (C, $n = 16$) received injections of 0.9% sodium chloride solution at the same time and in the same way. All animals were housed in 41x41x20 cm cages (4 rats in each cage) at a controlled temperature of $20 \pm 2^\circ\text{C}$ and humidity of $60 \pm 10\%$ under standard vivarium conditions. The rats were removed from the experiment 14 days after the last day of injections in order to assess both the degree of damage and the regenerative capabilities of the cerebral vessels in rats. This period is conventionally called by us "the regeneration period" as the regeneration begins after the first day of damage, and in the period from 14 to 28 days the damaging factor was removed and regeneration continued. Blood was collected in sterile EDTA VACUTAINER tubes, the brain was removed for the preparation of histological sections.

All institutional and national guidelines for the care and use of laboratory animals were followed. When working with experimental animals, we were guided by the provisions of the European Convention for the Protection of Vertebrate Animals (Strasbourg, 03/18/1986, revised and supplemented in 2006), Law of Ukraine No. 3447-IV, Art. 26, 31 "Animal Protection Law", "General Ethical Principles of Animals Experimentation", adopted by the Fifth National Congress on Bioethics (Kyiv, 2013). The Commission on Ethics and Bioethics of KhNMU at its 8th meeting on 10/10/2018 approved that this experiment complied with the bioethical requirements of the EU Directive 2010/63/EU on the protection of animals, the Council of Europe Convention for the Protection of Vertebrate Animals (ETS123) and did not violate ethical standards in science and standards for conducting biomedical research.

2. Obtaining stem cells

Primary culture of mesenchymal stem cells was obtained from the bone marrow of the femur. The suspensions were washed with Hank's saline solution, centrifuged at 450 g for 10 min and placed in cultivation flasks with a capacity of 75 cm² at a density of 4x10⁵ cells/cm² in Dulbecco's Modified Eagle's Medium DMEM/F12 (1/1) medium containing 2 mM L-Glutamine, 10% fetal bovine serum (FBS) (SIGMA-ALDRICH, F7524) and 2 µl/ml antibiotic antimycotic solution (SIGMA-ALDRICH, A5955). The medium with non-adherent cells was discarded after 24 hours of cultivation and fresh medium was added to adhered fibroblast-like MSCs. They were cultured at 37°C and 5% CO₂ in air in a CO₂-incubator for 14 days, the medium was changed every 3 days (9). All reagents were purchased from SIGMA-ALDRICH (USA).

3. Endotelin -1(ET-1), vascular endothelial growth factor A (VEGF-A), endothelial NO-synthase (eNOS), von Willebrand factor (vWF) determination in blood serum

Concentrations of ET-1, VEGF-A and eNOS in blood serum were determined by enzyme immunoassay using a standard set of reagents on a semi-automatic enzyme immunoassay analyzer STAT FAX 303+ (Elabscience, Wuhan, Hubei, China, 2019).

Determination of the vWF level was based on the aggregation of formalin-fixed platelets obtained from rats by ristomycin by a photometric method using a Solar PV12521 spectrophotometer (Belarus).

4. Obtaining morphological preparations and immunohistochemical determination of vascular endothelial growth factor (VEGF)

The brain immediately after decapitation of the animals was fixed in 10% neutral formalin, followed by preparation of slices using gallocyanine chromium alum staining according to the Einarson's method to assess the state of DNA and RNA of various cells. For immunohistochemical studies sections with a thickness of 3-4 microns were prepared which were applied to glass slides with high adhesive ability SuperFrost (Thermo Scientific, USA). After dewaxing and standard processing in xylene and ethanol the samples were boiled in a water bath in citrate buffer (pH = 6) at a temperature of 95-98°C. A detection system "Ultra Vision Quanto Detection System HRP DAB Chromogen" (Thermo scientific, USA) was used to detect the antigen-antibody VEGF interaction reaction (10). The study of histological and immunohistochemical preparations of the brain was carried out using Zeiss Axiostar plus binocular microscope with ProgRes C10Plus digital camera (Germany). The expression of VEGF antigen in neurons of the parietal lobe cortex, CA1 zone of the hippocampus, in vascular endotheliocytes of microcirculatory bed (MCB) and in

venous blood plasma was studied. The mean values of the levels of optical density of VEGF-tagged cells cytoplasm (I) and the background (I₀) were determined in the program of the raster graphic editor GIMP (GNU Image Manipulation Program). Optical density (D) of VEGF was calculated by the formula $D = \lg I_0 / I$ (11).

5. Statistical analysis

Normal distribution of the sample was assessed using the Shapiro-Wilk test. Based on its results, non-parametric tests were used to compare the independent groups of variables. The study selected one-way Kruskal-Wallis analysis and Dunn's multiple comparison test to assess the differences between five independent groups. If p-values were below 0.05, the difference was considered to be statistically significant. All numerical data were analyzed using GraphPadPrism 5.0 (GraphPad Software Inc., California, USA) and the Social Science Statistical Package (SSPS).

RESULTS

As described in the previously published articles, 2 weeks after completing a course of 14-day sodium nitrite injections, in addition to the development of motor protective inhibition in rats, and a decrease in exploratory activity and cognitive deficit, the following changes were noted morphologically in the cerebral vessels: amyloid deposition in the walls' arteries; the formation of blood clots, both parietal in large vessels and obstructing in small vessels; infiltration of blood vessels by macrophages and lymphocytes (vasculitis); presence of pronounced perivascular edema and areas of "empty spaces" that follow the contours of capillaries (12, 13).

In this article, the morphofunctional state of the MCB vascular endothelium in animals of the experimental groups was assessed more deeply. In a microscopic survey, in rats of group N-28, the damage to the cerebral vessels is more pronounced than in group N-14 (Figure 2). At the same time, the signs of endothelial recovery and growth of new capillaries were observed in all groups for 14 days of regeneration, and in groups with stem cells – multiple areas of endothelial hyperplasia (Figure 3, 4).

Among the biochemical indicators assessing the state of the endothelium, two indicators characterizing the level of damage and endothelial dysfunction (ET-1 and vWF) were studied, one of the factors reflecting the compensatory capabilities of endothelial cells (eNOS), and the factor indicating the regenerative capabilities of the endothelium (VEGF-A) (Table 1). Thus, the average level in ET-1 in the blood serum of animals of group N-14 was 3.8 times higher than in group C. After stem cells administration at the beginning of the recovery period, the level of ET-1 became significantly lower than in group N-14, but did not reach the level of group C.

In animals of group N-28 the mean value of ET-1 concentration became approximately 7 times higher than in rats of group C. At the same time, the administration of stem cells to rats of group N-28-SC contributed to a significant decrease in ET-1 by 3.5 times compared to group N-28, which, however, was higher than that in group C.

Changes similar in direction were observed in the study of von Willebrand factor level (marker of endothelial dysfunction) in all experimental groups. It should be mentioned that in animals of group N-28-SC, vWF concentration practically reached control values.

It was found that the administration of sodium nitrite to rats for 2 weeks caused a significant increase in the activity of endothelial NO synthase (1.4 times higher than

in group C), and the administration of stem cells into group N-14-SC caused decrease in enzyme activity almost to the level of that in group C.

In group N-28, in contrast to group N-14, the eNOS activity insignificantly exceeded that in group C. Stem cell injections in group N-28-SC caused a statistically insignificant (almost 1.1-times compared to group N-28) decrease in eNOS activity, while at the same time, the level of enzyme activity did not practically differ from the control.

As it was seen from the data obtained, the concentration of VEGF A increased under the influence of prolonged administration of sodium nitrite after 2 weeks of the regeneration period: in group N-14 - 1.4 times, and in group N-28 - reliably 3.8 times compared to the control.

Table 1. Biochemical parameters of endothelial state

Parameters	Control group (C)	Group (1) N-14	Group (2) N-14-SC	Group (3) N-28	Group (4) N-28-SC
ET-1, pg/ml	0.88±0.08 (1, 3) *	3.36±0.39 (4, C) *	1.49±0.11	5.33±1.12 (4, C) *	1.24±0.07 (1, 3) *
vWF, %	84.15±0.92 (1, 3) *	115.9±0.8 (C) *	94.85±1.26 (3) *	132.8±3.5 (2, 4, C) *	85.33±25.81 (3) *
eNOS, pg/ml	95.96±1.63 (1) *	137.6±2.3 (4, C) *	104.3±2 (4) *	100.5±1.89	90.03±1.14 (1, 2) *
VEGF A, pg/ml	39.46±1.17 (2, 3, 4) *	57.38±0.85 (2, 3) *	62.78±0.93 (1, C) *	151.8±1.2 (1, C) *	60.43±0.53 (C) *

Values are mean± confidence interval (CI) for the mean.

* Related groups with significantly different results ($p < 0.05$, Kruskal–Wallis test and Dunn's multiple comparisons test) are listed in each column in parentheses, e.g. (4,C).

Table 2. Optical density of VEGF expression in different cells of brain tissue

Parameters	Control group (C)	Group (1) N-14	Group (2) N-14-SC	Group (3) N-28	Group (4) N-28-SC
In endotheliocytes	0.04±0.006 (1, 3, 4) *	0.059±0.008 (2, C) *	0.026±0.004 (1, 3, 4) *	0.057±0.006 (2, 4, C) *	0.08±0.009 (2, 3, C) *
In hippocampal neurons	0.036±0.005 (2, 3) *	0.04±0.006 (2, 3) *	0.024±0.003 (1, 4, C) *	0.016±0.002 (1, 4, C) *	0.04±0.005 (2, 3) *
In cortical neurons	0.039±0.006 (1, 2) *	0.058±0.004 (3, C) *	0.059±0.004 (3, C) *	0.046±0.007 (1, 2) *	0.052±0.008
In plasma	0.025±0.007 (1,4) *	0.061±0.01 (2, 3, C) *	0.018±0.005 (1, 4) *	0.01±0.003 (1, 4) *	0.08±0.012 (2, 3, C) *

Values are mean± confidence interval (CI) for the mean.

* Related groups with significantly different results ($p < 0.05$, Kruskal–Wallis test and Dunn's multiple comparisons test) are listed in each column in parentheses, e.g. (4,C).

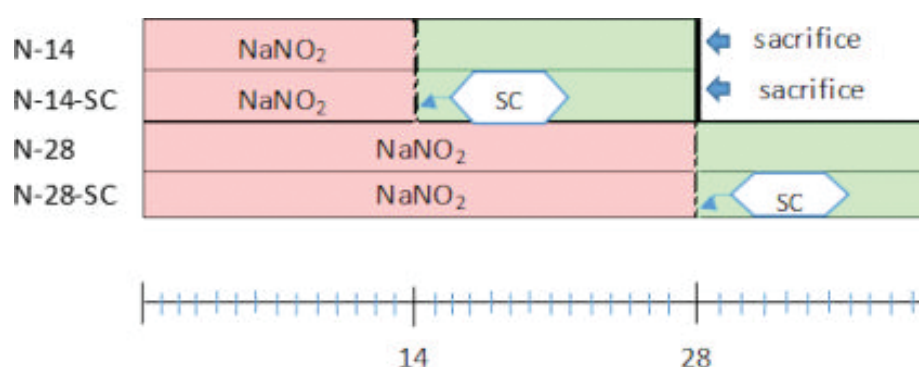


Figure 1. Experimental design. Groups N-14 - nitrite 14 days, N-28 - nitrite 14 days, N-14-SC-nitrite 14 days+stem cells (SC), N-28-SC- nitrite 28 days+stem cells, NaNO₂ – sodium nitrite.

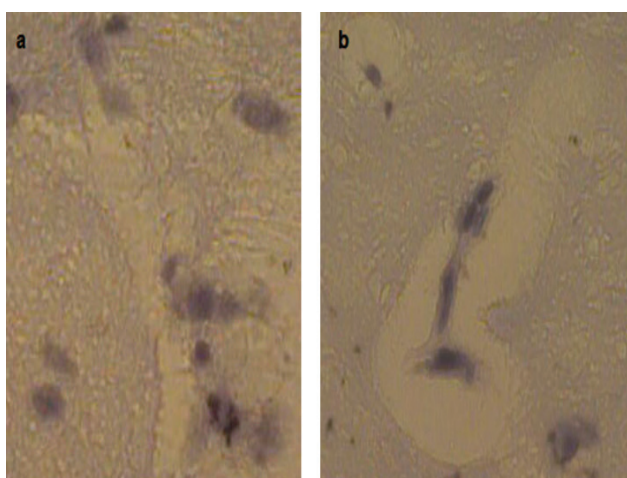


Figure 2. A – presence of “empty space”, repeating the contour of the dead vessel. B – capillary with flat hyperchromic nuclei of endothelial cells and expressed peri-vascular edema. Staining according to the Einarson's method. Group N-28. x400

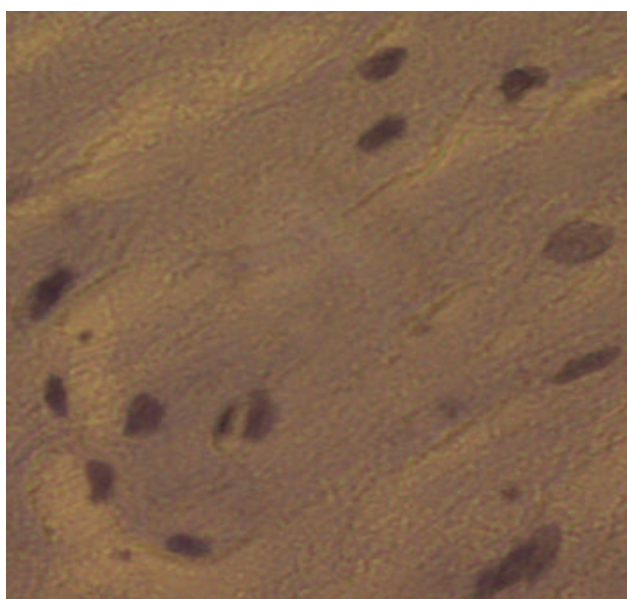


Figure 3. A great number of “young” endothelial cells (cells with large elongated oval nucleus). Staining according to Einarson's method. Group N-14-SC. x400

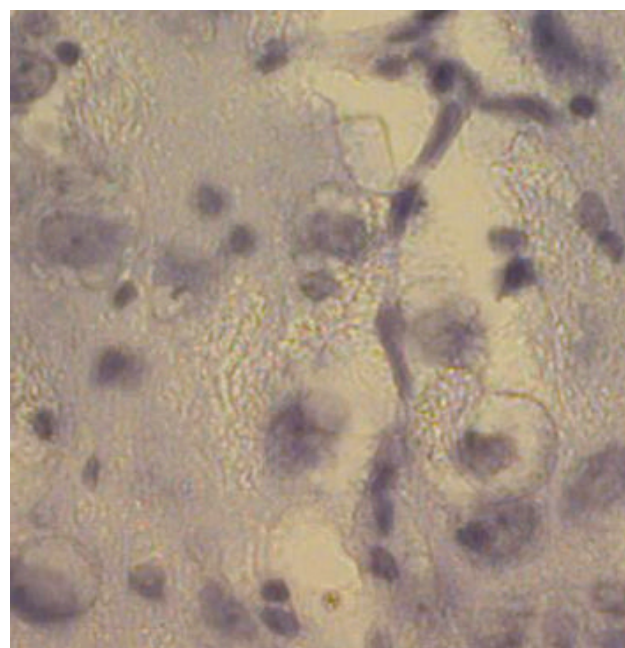


Figure 4. Endothelial hyperplasia. Hippocampal neurons are light, rounded shaped. Staining according to Einarson's method. Group N-28-SC. x400

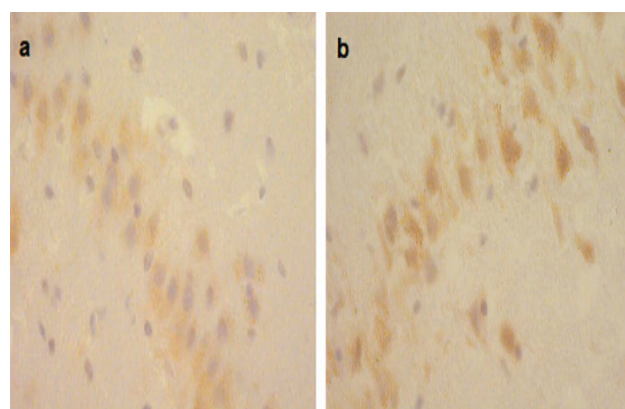


Figure 5. Immune histochemical reaction with antibodies to VEGF. CA1 hippocampal zone. a – diffused weak color of neurons of group N-28. b – intensely tagged neurons of group N-28-SC. Presence of “empty” spaces between neurons in both groups. x400

After the administration of stem cells to rats of group N-14-SC there was a slight increase in VEGF A synthesis, and in group N-28-SC there was a sharp decrease in VEGF A synthesis (2.5 times compared to group N-28). Nevertheless, its concentration remained reliably higher than in the rats of the control group.

Simultaneously with the change of VEGF level in the blood serum, the production of this peptide regulating the proliferation of endothelium change in the cytoplasm of neurons in the cerebral hemispheres, in the neurons of the hippocampus (CA1 field), in the endotheliocytes of capillaries, in the blood plasma of cerebral venules, which is assessed by staging immunohistochemical reaction on VEGF, study of the total number of tagged cells in the field of view of the microscope and the optical density of the cytoplasm of these cells (Table 2). Thus, in group N-14 the number of VEGF-tagged endothelial cells was higher and the optical density of the cytoplasm was 1.5 times higher than in the control group. At the same time, in group N-28 where the endothelial damage was more significant compared to group N-14, the number of tagged endothelial cells in the field of view was smaller, and the average optical density of the cytoplasm of tagged endothelial cells did not differ from that in group N-14. The administration of stem cells promoted a decrease in the number of tagged endothelial cells in group N-14-SC, which contained a small amount of VEGF granules in the cytoplasm, and the optical density of their cytoplasm was reliably reduced. In group N-28-SC changes were of the opposite nature: an increase in the number of capillaries in the brain tissue with intensely tagged endothelial cells and their optical density was reliably higher (1.4 times) compared to group N-28.

In the CA1 field of the hippocampus in all main groups the number of neurons decreased, which was especially pronounced in the groups with 28 days of sodium nitrite administration. In this context the following picture was observed at the end of the regeneration period: in group N-14 – an increase in the number of tagged neurons and a low (as in group C) optical density of the cytoplasm; in group N-14-SC in comparison to group N-14 – a decrease in the number of tagged neurons and a decrease in the optical density of their cytoplasm; in group N-28 hippocampal CA1 field was strongly atrophied and the remaining neurons contained a label where optical density of the cytoplasm was low; in group N-28-SC on the background of a similar atrophization of hippocampus CA1 field the optical density of the cytoplasm of tagged neurons significantly increased compared to group N-28 (2.5 times) (Figure 5).

Optical density of VEGF in the neurons of the cerebral cortex in all studied groups was higher than in the control group. At the same time, visually, in group N-14-SC the greater number of neurons were tagged in comparison to

group N-14. However, the level of VEGF expression in neurons of the cerebral cortex of rats of group N-14 and group N-14-SC practically did not differ from each other. In animals of group N-28, the number of neurons in the cerebral cortex was noticeably reduced, there were “empty” gaps between neurons which indicated that atrophization of the cortex had occurred, the remaining neurons had a less intense label than in group N-14. After the administration of stem cells in group N-28-SC, the VEGF expression did not statistically differ in comparison to group N-28.

Determination of VEGF content in venous blood plasma allows: to assess the level of its synthesis by brain cells and the degree of reception by endothelial cells to stimulate their division. This indicator significantly differs from the one in blood serum (biochemically determined) and reflects the general organism level of synthesis and use of this peptide. Compared to the control group, optical density of tagged plasma in animals of group N-14 increased reliably by 2.4 times, and in group N-28 it decreased by 2.5 times. After the administration of stem cells in group N-14-SC a significant decrease in the content of the label was observed in the plasma as compared to group N-14 and in group N-28-SC the plasma optical density was reliably higher – 8 times, compared to group N-28.

DISCUSSION

Currently, the point of view about significant contribution of cerebrovascular dysfunction to the development of Alzheimer's disease (AD) has been gaining increasing recognition. Thus, in the study by Vercllyte S. et al. (14), a decrease of cerebral circulation in the cortex of the frontal and parietal lobes of the brain of patients with early stages of AD was found. It was revealed that hypoperfusion and ischemic brain damage were accompanied by the activation of enzymes involved in the proteolysis of the APP amyloid precursor protein, thereby increasing the accumulation of beta-amyloid both in the vascular wall (the development of cerebral amyloid angiopathy (CAA) and in the brain tissue) (15). At the same time signs of endothelial mitochondrial dysfunction were observed, which could be primary regarding the appearance of amyloid in the vessels wall (16).

In our work, when simulating dementia of the Alzheimer's type of vascular origin by daily intraperitoneal injection of an aqueous solution of sodium nitrite for 14 days, this substance, circulating in blood, had a damaging effect on the vascular endothelium. Indeed, in group N-14 and, especially, in group N-28 a decrease in the number of capillaries and endothelial cells in them, an increase in the number of pericytes and a decrease in the size of the remaining hyperchromic endothelial cells were

noted during morphological examination of the brain in the MCB vessels (13). These changes occurred despite the fact that the course of sodium nitrite injections had been completed and 14 days had already passed, when only the regeneration of the endothelium was taking place without damaging it.

The study of biochemical parameters characterizing the state of the endothelium in this study demonstrated that the administration of sodium nitrite caused a significant increase of ET-1 and vWF content in blood serum, which confirms the violation of the integrity of the endotheliocyte monolayer and vasoconstriction and thrombosis increase in the vessels. The data obtained are consistent with the results of Savitskiy I. et al. (17), where animals developed endothelial dysfunction on the background of chronic nitrate load (daily intake of 0.03% nitrate solution instead of drinking water, which is converted into nitrite in the body). Moreover, the increase of the dose of sodium nitrite received by the animals (group N-28 compared to group N-14) was accompanied by a significant increase of the values of these two indicators. Taking into account that these biochemical parameters reflected the reaction of the endothelium in the entire bloodstream of the body, a histological examination of the brain carried out in parallel demonstrated that the capillaries of the brain tissue in animals of group N-28 were damaged more than in group N-14.

The eNOS enzyme responds to endothelial damage by performing, to a certain extent, a compensatory role, catalyzing the formation of nitric oxide (NO). Besides vasodilation, NO has an anti-thrombogenic, anti-inflammatory effect and promotes transendothelial migration of progenitor endothelial cells (18). A slight increase of eNOS activity after 2 weeks of the regeneration period in animals of group N-28, in contrast to the significant activity of endothelial NO synthase in animals of group N-14, proved a stronger damage of endothelial cells and, apparently, a decrease in their compensatory capabilities in general.

The histologically observed regeneration of capillaries, the proliferation of endothelial cells in group N-28 developed on the background of a more significant damage of capillaries than in group N-14, as mentioned earlier, therefore show that, indeed, capillaries endothelium of the brain tissue in group N-28 recovered to a lesser extent than in group N-14, i.e. the estimated explanation of eNOS lower activity in group N-28 compared to group N-14 can be considered convincing.

The VEGF content in arterio-venous blood was significantly increased in group N-14 and in group N-28, but in group N-28 it was almost 3 times higher than in group N-14. Obviously, high VEGF index in blood of group N-28 could be formed due to high possibilities of cell regeneration of many parenchyma cells, which

suffered due to disturbed blood supply during endothelial death and capillaries destruction; new cells of such tissues actively synthesized VEGF. Maximum endothelial death is observed only in group N-28 (judging by the histology of the brain), the restoration of which is not completed until the moment of withdrawal from the experiment, and in group N-14, apparently, the degree of endothelial cell division stimulation by this active protein decreased by the end of 14-day regeneration period, i.e. there was a significant, but not yet complete restoration of the endothelial layer in the bloodstream vessels.

There was an increase in the number of tagged corresponding cells in comparison to group C and at the same time an increase in the optical density of the label, i.e. content of this protein in the cytoplasm, were found during immunohistochemical determination of VEGF content (i.e. production) in hippocampus neurons, cerebral cortex and capillary endotheliocytes in group N-14. In group N-28, as mentioned earlier, damage to the capillary endothelium was more pronounced and was accompanied by cerebral cortex and CA1 zone atrophy of the hippocampus, whereas almost all neurons were tagged, but optical density of the cytoplasm was less than in group N-14, which can be due to dystrophy of the “surviving” neurons and their reduced ability to produce VEGF.

The number of tagged cells in the capillary bed of the brain in group N-14 and group N-28 had sharply increased in comparison to group C, and the optical density of the cytoplasm in such endotheliocytes increased equally. To assess the VEGF level in brain blood the optical density of blood plasma in veins was examined. We have never seen publications with such studies. But this indicator can really be regarded as an indicator reflecting VEGF concentration in the venous blood of the brain. The interpretation of the results of this indicator is based on the point of view that VEGF plasma concentration in the brain veins is the result of two divergent processes occurring with VEGF in the brain: VEGF production by different cells and “consumption”, i.e. the reception by endotheliocytes. It turned out that significantly more VEGF was removed from the brain tissue with venous blood in group N-14 than in group N-28, apparently because much more of this protein was consumed in group N-28, as endothelial regeneration was not yet completed. These results do not contradict the data obtained by Li Huang et. al. (19), when the VEGF level was significantly lower in patients with AD than in patients with mild cognitive impairment.

Thus, our experiment has demonstrated that cortex and hippocampus neurons reacted to the damage of the capillary endothelium and stimulated the production of vascular growth factor. In the study of K. Okabe et. al. (20), it was also demonstrated that VEGF secreted by cortex and hippocampus neurons induced the blood

vessels growth, which was important for the blood supply to areas of the brain with dystrophic changes.

When stem cells were used in both groups (N-14-SC, N-28-SC), a sharp decrease of both ET-1 and vWF content in blood serum was observed, which indicates that to the blood vessels endothelium damage in the body decreased to the values of group C due to stem cells administration. Accordingly, eNOS content in these groups decreased as this enzyme was activated in response to endothelial damage. Histological examination of brain tissue directly demonstrated that in these groups there was a sharp increase in the number of capillaries, with areas of endothelial hyperplasia in some places.

Despite this, the VEGF content in total blood remained slightly higher than in group C, while the administration of stem cells did not significantly change the VEGF content in blood during the 14-day course of sodium nitrite administration, and after 28 days of administration it significantly decreased, approximately 2.5 times. Decreased VEGF circulation and improved angiogenesis were obtained in people with heart failure and advanced endothelial dysfunction who received allogeneic (from a compatible donor) mesenchymal stem cells (21). It can be assumed that in the tissues of the body as a whole (on average) the need to stimulate angiogenesis in group N-14-SC after a preliminary short activation (not studied by us) decreased due to the end of the period of active regeneration of the damaged endothelium. And in group N-28-SC, in comparison to group N-28, acceleration of regenerative processes was obvious and in this connection the decrease in VEGF content in total blood serum. An analysis of plasma optical density in the lumen of small veins (immunohistochemical reaction for VEGF) fully confirmed this assumption, as a small amount of this peptide was removed from the brain tissue in the N-14-SC group with venous blood (at the level of group C), and in group N-28-SC its plasma concentration was very high, i.e. it remained unclaimed.

A significant decrease in the cytoplasm optical density of the capillary endothelium and CA1 neurons of the hippocampal field, proved already completed (or ending) endothelial regeneration in the vessels of the brain tissue in group N-14-SC, as only the neurons of the cerebral cortex still produced VEGF at the same level as without the administration of stem cells, but at the same time the cortex showed signs of atrophization. But in group N-28-SC, VEGF synthesis remained increased relative to group N-28 in endotheliocytes and in neurons of the cerebral cortex and in neurons of the hippocampus, i.e. stimulation of the endothelial cells division continued, as their damage by sodium nitrite was more significant.

Thus, using the NaNO₂ model of Alzheimer's type dementia, dose-dependent endothelial damage with a reduction in the capillary network in the brain tissue with

biochemically proved signs of endothelial cell destruction is demonstrated. Stimulation of endothelial cell regeneration studied by the VEGF content both in blood (plasma) and in neurons and endothelial cells cytoplasm, as well as by the histological picture of the brain tissue, is more active in animals that received one-time mesenchymal stem cells. It can be assumed that damage of the capillary wall followed by hypoxia and tissue nutrition disturbance is one of the primary stages in the formation of amyloid in the brain tissue due to the activation of pathological breakdown of APP protein.

CONCLUSION

In experimental animals with a nitrite-induced model (14 daily injections) of Alzheimer's type dementia, biochemical and morphological evidence of significant damage of the vascular endothelium both in the body as a whole and in the brain tissue is revealed after 14 days of the "regeneration" period. Intensive VEGF production is observed with ongoing restoration of the endothelial layer in vessels, which is clearly seen in the vessels of the brain tissue microcirculatory bed. With an increase in the duration of sodium nitrite administration, the degree of endothelial damage increases significantly both in the body as a whole and in the brain tissue with simultaneously more reduced production of VEGF.

Stem cells therapy the next day after the end of sodium nitrite administration promotes the acceleration of the regeneration of the endothelial lining of blood vessels, as evidenced by a noticeable increase in the number of capillaries in the brain tissue. Comparison of the observed changes in the indicators of the endothelial morphofunctional state suggests that the state of endothelial regeneration is closer to completion after a 14-day administration of sodium nitrite compared to 28-day administration.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no conflict of interests.

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ABBREVIATIONS

AD - Alzheimer's disease; C – control; CAA - cerebral amyloid angiopathy; DMEM - Dulbecco's Modified Eagle's Medium; eNOS - endothelial NO-synthase; ET-1-Endotelin -1; EU – European Union; FBS - fetal bovine serum; KhNMU – Kharkiv National Medical University; MSCs - mesenchymal stem cells; N-14 - nitrite 14 days; N-14-SC - nitrite 14 days + stem cells; N-28 - nitrite 28 days; N-28-SC - nitrite 28 days + stem cells; NaNO₂ - sodium nitrite; SC - stem cells; VEGF-A - vascular endothelial growth factor A; vWF - von Willebrand factor; WAG - Wistar Albino Glaxo

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