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The effects of human umbilical cord multipotent mesenchymal stromal cells on the behavior and oxidative stress in the brain of mice of different ages with a cuprizone-induced model of demyelination

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ABSTRACT

The transplantation of umbilical cord multipotent mesenchymal stromal cells (UC-MMSCs) is a promising strategy in the treatment of multiple sclerosis. The features of cells effect in the recipients of different ages, as well as the ways of its implementation, remain poorly understood.

PURPOSE. *To study the effect of UC-MMSCs transplantation on the behavior, oxidative stress factors and antioxidant protection in the brain of mice of different ages with an experimental model of multiple sclerosis.*

MATERIALS AND METHODS. *Male 129/Sv mice 6-7 months old and 14-16 months old received neurotoxin cuprizone with food for 3 weeks. Since the 10th day of the cuprizone diet, human UC-MMSCs were injected intravenously at a dose of 5•10^5 cells per mice. We evaluated the parameters of behavior in the open field test and rotarod test. The level of malondialdehyde (MDA) and the activity of antioxidant enzymes were studied in the brain.*

RESULTS. *In mice of both age groups with a cuprizone diet, motor, emotional and exploratory activity decreases. The level of MDA in the brain increases and the activity of antioxidant enzymes decreases. The transplantation of UC-MMSCs leads to positive changes in the behavior of mice with a cuprizone diet. In the adults, the motor and emotional activity improved; in the aging ones exploratory activity and muscle tone increased. After the injection of UC-MMSCs in adult mice, the level of MDA in the brain decreases.*

CONCLUSION. *The therapeutic effect of transplanted UC-MMSCs on the behavioral parameters and MDA level in the brain of mice with a cuprizone model of demyelination largely depends on their age and is more pronounced in adult animals.*

KEY WORDS: *human umbilical cord multipotent mesenchymal stromal cells; demyelination; cuprizone; behavior; oxidative stress*

Multiple sclerosis is one of the most common demyelinating diseases of the central nervous system (CNS) [1]. Patients with multiple sclerosis have motor, emotional, vegetative and cognitive disorders due to the progression of demyelination and neurodegeneration in the central nervous system. In the pathogenesis of multiple sclerosis, oxidative stress and neuroinflammation in the brain are of great importance. Currently, this disease is increasingly common in patients older than 45 years. Existing approaches to the pharmacotherapy of multiple sclerosis are not always effective, and the long-term use of medications is accompanied by significant side effects.

A promising strategy in the treatment of multiple sclerosis may be the use of multipotent mesenchymal stromal cells (MMSCs) of different tissue origin [2, 3]. The MMSCs are capable of multi-lineage differentiation, production of trophic and growth factors that affect neurogenesis,

synaptogenesis and survival of neurons and, in addition, have anti-inflammatory, antioxidant and immunomodulatory properties [4-7].

The properties of the human umbilical cord multipotent mesenchymal stromal cells (UC-MMSCs) and the possibility of their application in neurology are being widely studied [8, 9]. These cells are characterized by low immunogenicity, which allows them to be used for allogeneic transplantation. UC-MMSCs proliferate quite well in vitro, are capable of transdifferentiation into cells of ectodermal origin and the synthesis of IL-10 and TGF-β [10-12]. Their immunosuppressive effect is higher in comparison with similar cells from adipose tissue and bone marrow [13].

One of the models of multiple sclerosis in mice is experimental allergic encephalomyelitis. Using this model, the positive effect of UC-MMSCs on the injured spinal cord myelin fibers, motor and emotional activity of adult animals have been shown [14, 15]. At the same time, there is the evidence of the age importance of not only the donor, but also the recipient for the manifestation of the neuroprotective properties of transplanted adipose-derived MMSCs [16, 17]. However, the age-related features of the UC-MMSCs efficacy, as well as possible ways of its implementation at demyelinating diseases of the central nervous system, remain poorly studied.

The **PURPOSE** is to study the effect of UC-MMSCs transplantation on the functional state of the central nervous system, oxidative stress factors and antioxidant protection in brain of mice of different ages with a model of multiple sclerosis.

MATERIALS AND METHODS

Animals. The studies were performed on male 129/Sv mice (genotype H-2^b) aged 6-7 months and 14-16 months from the vivarium of the State Institute of Genetic and Regenerative Medicine of the NAMS of Ukraine. The animals were kept under standard conditions under a 12:12 light regime with free access to food and water *ad libitum*. To obtain biological material, mice were decapitated under ether anaesthesia in the morning. All experimental studies were carried out in accordance with the Law of Ukraine «On the Protection of Animals from Cruelty» and the «European Convention for the Protection of Vertebrate Animals, which are used for experimental and other scientific purposes» (Strasbourg, 1986).

Experimental models. A toxic cuprizone model of demyelination was used in the study [18]. Mice received the neurotoxin cuprizone [bis (cyclohexanone)-oxaldihydrazone] (*Sigma-Aldrich*, Germany) with food daily (0.2 % of mass of the daily feed), for three weeks.

Isolation and cultivation of UC-MMSCs. UC-MMSCs were isolated using explants technique from the umbilical cord of a healthy woman in labour, as described previously [14]. A woman at the age of 26 who gave birth to a boy signed an informed consent to provide material for research. UC-MMSCs of the 2nd passage were used for the transplantation to the experimental animals. An inverted microscope DM IL (*Leica*, Germany) was used to assess the condition of the cell cultures.

The culture obtained from umbilical cord tissue of the 2nd passage was morphologically homogeneous and contained mainly small mitotically active spindle-shaped cells. The latter expressed the surface marker antigens CD105, CD73 and CD90, but did not express CD45 and CD34, and also differentiated into osteoblasts, adipocytes and chondrocytes *in vitro* which meets the minimal criteria for defining MMSCs. Cell immunophenotyping was performed on a BD FACSAria cell sorter (*Becton Dickinson*, USA).

UC-MMSCs of the 2nd passage were injected once into the tail vein on the 10th day of the cuprizone diet at a dose of $5 \cdot 10^5$ cells per 50 µl of vehicle. The control was treated with one injection of 0.9 % normal saline into the tail vein. The choice of the indicated time of UC-MMSCs administration to mice is explained by the development of demyelination and neurodegeneration in the central nervous system from the 8th to 10th day of cuprizone treatment, as well as the possibility of the manifestation of the therapeutic effect of human bone marrow-derived MMSCs after the transplantation while cuprizone treatment [18-20].

Experimental groups. Adult and aging mice were divided into 3 groups: intact animals (the usual diet); animals that received cuprizone and an injection of UC-MMSCs; animals treated with cuprizone and 0.9 % normal saline injection. The studies were performed 21 days after the start of cuprizone treatment. Each experimental group of adult mice consisted of 10 animals, aging ones – 9 mice.

The CNS functions were evaluated by the behavioral parameters in the open field test and rotarod test [21]. In the "open field" test, we examined the horizontal locomotor activity (the number of crossed squares), the exploratory behavior (the number of rearings and head dipping), and the emotional activity (number of fecal boluses and grooming) in mice. Each animal was tested for 3 minutes. The rotarod test enables to assess muscle tone, motor coordination and balance of experimental animals. In the device, the speed of the rotating rod was changed under continuous acceleration from 10 to 20 rpm. Data was presented as the total time (latency) it takes the mouse to fall off the rod at 10 and 20 rpm.

The factors of oxidative stress and antioxidant protection of the brain. The level of malondialdehyde (MDA) in the brain of mice was determined by the colour intensity of the trimethine complex formed between thiobarbituric acid and MDA [22]. The activity of antioxidant enzymes was evaluated in the supernatant of brain homogenates by spectrophotometry using a µQuant spectrophotometer (*Bio-Tek*, USA), as we described earlier [23]. The activity of superoxide dismutase (SOD) was evaluated by the ability to inhibit the autooxidation of adrenaline into adenochrome in U/mg of protein per minute; catalase enzyme activity represents in µmol of H_2O_2 utilized/mg protein per minute; glutathione peroxidase (GPx) enzyme activity represents in nmol of NADPH oxidized/mg protein per minute. The protein concentration in the brain was measured by the Lowry method. All reagents are Riedel-de Haen (*Fluka*, Germany).

Statistical analysis of the results was carried out using Student's t-test. The difference between the indicators was considered statistically significant at $p < 0.05$. Statistica 7.0 (*StatSoft Inc*., USA) was used for statistical analysis of the results.

RESULTS AND DISCUSSION

The effect of UC-MMSCs transplantation on the behavior of mice of different ages with a cuprizone diet. It was found that in adult mice after cuprizone treatment, the number of crossed squares, rearings, boluses, explored holes, grooming and the latency time on the rod decreases (Table 1). A similar trend in changing the studied parameters (with the exception of the number of grooming) is also observed in aging mice treated with cuprizone.

Moreover, in adult mice with a cuprizone diet, compared with intact animals, the number of squares and boluses decreases by 2.9 and 6.7 times, and in aging mice by 2.3 and 5.7 times, respectively. As a result, in experimental mice, the age difference between the parameters is leveled.

The transplantation of UC-MMSCs in adult mice with a cuprizone diet leads to a significant increase in the number of crossed squares and grooming compared to the group without cells; the number of grooming does not differ from intact animals (see **Table 1**). After the injection of UC-MMSC to the aging mice, the number of rearings significantly increases but remains less than in the intact group.

Thus, in adults and aging mice with a cuprizone diet, motor, emotional, exploratory activities as well as muscle tone are reduced. The changes in the studied parameters are more significant in adult mice. UC-MMSCs transplantation in mice of both age groups has a positive effect on some parameters of behavior, namely, in adult animals, on horizontal locomotor and emotional activity, and in aging animals, on exploratory activity and muscle tone.

Factors of oxidative stress and antioxidant protection in the brain of mice of different ages treated with cuprizone and UC-MMSCs. It was found that after cuprizone treatment, the level of MDA increases and the activity of GPx decreases in the brain of adult mice compared to intact

animals (Table 2). In the brain of experimental aging mice, there is an increase in the level of MDA and a decrease in the activity of SOD, catalase, and GPx (see Table 2).

After the injection of UC-MMSCs, the level of MDA in the brain decreases in adult mice with a cuprizone diet to the values of the intact group, while in aging mice it remains unchanged (**Table 2**). The cell therapy does not affect the activity of antioxidant enzymes in the murine brain of both age groups.

Thus, after transplantation of UC-MMSCs in mice of different ages with a cuprizone diet, positive changes in the behavior and MDA level as one of the oxidative stress factors in the brain are observed. A similar effect of transplanted cells depends on the age of the experimental mice and is more pronounced in adults.

Behavioral parameters in mice of different ages with a cuprizone diet after UC-MMSCs transplantation. In our experiment, studies were performed on a toxic cuprizone demyelination model. It was shown that cuprizone damages mature oligodendrocytes in different parts of the central nervous system, resulting in the development of demyelination [18]. Pathological changes in the central nervous system of animals with the cuprizone model of demyelination are similar to those at multiple sclerosis in humans [24]. Other authors and our studies have shown that not only in adults, but also in aging mice, under the influence of cuprizone, demyelination and neurodegeneration develop in different parts of central nervous system such as the cerebral cortex, cerebellum, corpus callosum, hippocampus, and lumbar spinal cord [18, 25]. Moreover, structural impairment of the central nervous system are combined with functional disorders (changes in motor, exploratory and emotional activity) and are more pronounced in adult mice [25]. In this study, we also revealed similar age-related features of behavior changes in mice with a cuprizone diet. The results obtained in this study suggest that structural disorders of the central nervous system in adults and aging mice have certain differences that may affect the response of neural cells to the effects of various biologically active compounds.

UC-MMSCs were injected to adult and aging mice 10 days after the start of cuprizone treatment. Other authors and we found that already at these terms demyelination, apoptosis of oligodendrocytes, changes in the structure of neurons and behavior, which increase after three weeks of cuprizone treatment, are observed in the brain of mice aged 3-6 months [18,19]. In addition, it was shown that the introduction of biologically active compounds during the cuprizone treatment allows us to study their effects on the processes of demyelination, whereas after the finish of the cuprizone treatment, on the spontaneous remyelination [18]. According to our data, in adult and aging mice with a cuprizone diet and UC-MMSCs injection, some behavioral parameters improve. Thus, in adult mice, these are parameters of motor and emotional activity, in aging mice – the parameters of exploratory activity and muscle tone. Further morphological studies of the central nervous system in such mice will partly explain some of age-related differences in changes in behavioral parameters.

Parameters of oxidative stress and antioxidant protection in the brain of different age mice treated with cuprizone and UC-MMSCs. Oxidative stress in the brain of mice with a cuprizone diet plays an important role in the damage to myelin and neurons [18, 25]. One of its factors is MDA, which is formed as a result of peroxidation of polyunsaturated fatty acids and is able to react with nucleic acids, phospholipids and amino acids. We found an increase in the level of MDA in the brain of adult and aging mice treated with cuprizone. In addition, the activity of antioxidant enzymes decreases in the brain of experimental mice of both age groups (especially in aging), which indicates an imbalance between the factors of oxidative stress and antioxidant protection. At the same time, after the transplantation of UC-MMSCs, the level of MDA significantly decreases in the brain of adult mice with a cuprizone diet. Our results are consistent

INDICATOR	EXPERIMENTAL GROUP		
	INTACT	CUPRIZONE + SALINE	CUPRIZONE + UC-MMSCs
Adult mice			
Number of crossings	67.7 ± 4.0	23.6 ± 3.6*	35.8 ± 3.5*#
Number of rearings	1.6 ± 0.4	0.6 ± 0.2*	0.5 ± 0.1*
Number of boluses	2.0 ± 0.1	0.3 ± 0.1*	0.1 ± 0.03*
Number of explored holes	2.1 ± 0.3	0.4 ± 0.1*	0.4 ± 0.1*
Number of grooming	0.3 ± 0.1	0.1 ± 0.01*	0.3 ± 0.04#
Rotarod, sec	120.1 ± 14.2	76.7 ± 15.2*	63.6 ± 20.6*
Aging mice			
Number of crossings	50.9 ± 6.1&	22.5 ± 5.1*	27.7 ± 6.8*
Number of rearings	1.4 ± 0.3	0.2 ± 0.04*	0.7 ± 0.1*#
Number of boluses	1.7 ± 0.1&	0.3 ± 0.1*	0.1 ± 0.04*
Number of explored holes	2.7 ± 0.4	0.8 ± 0.2*	0.5 ± 0.1*
Number of grooming	0.3 ± 0.1	0.3 ± 0.1&	0.4 ± 0.1
Rotarod, sec	86.0 ± 8.5&	65.2 ± 5.7*	90.6 ± 19.6

Table 1. The behavior indicators in mice of experimental groups of different ages, M ± m.
*Notes: * – p < 0.05 compared to the intact group; # – p < 0.05 compared to cuprizone treatment only; & – p < 0.05 compared to adult mice.*

INDICATOR	EXPERIMENTAL GROUP		
	INTACT	CUPRIZONE + SALINE	CUPRIZONE + UC-MMSCs
Adult mice			
Malondialdehyde (nM/mg)	3.5 ± 0.5	6.2 ± 0.8*	3.6 ± 0.2#
Superoxide dismutase (U/mg•min)	13.8 ± 0.9	14.4 ± 0.8	14.8 ± 0.6
Catalase (μM/mg•min)	0.8 ± 0.1	0.9 ± 0.2	0.9 ± 0.2
Glutathione peroxidase (nM/mg•min)	6.9 ± 0.5	5.3 ± 0.3*	4.6 ± 0.3*
Aging mice			
Malondialdehyde (nM/mg)	4.0 ± 0.2	4.8 ± 0.2*	4.9 ± 0.3*&
Superoxide dismutase (U/mg•min)	15.2 ± 0.3	11.9 ± 1.3*	13.1 ± 0.9*
Catalase (μM/mg•min)	2.2 ± 0.3&	1.1 ± 0.1*	1.4 ± 0.2*
Glutathione peroxidase (nM/mg•min)	5.8 ± 0.2&	4.6 ± 0.3*	4.3 ± 0.6*

Table 2. Indicators of oxidative stress and antioxidant protection in the brain of mice, M ± m.
*Notes: * – p < 0.05 compared to the intact group; # – p < 0.05 compared to cuprizone treatment only; & – p < 0.05 compared to adult mice.*

with other authors' data who have found that after the transplantation of bone marrow MMSCs in the brain of animals with the pathology of the central nervous system, the formation of oxidative stress factors such as free radicals and superoxide anion is reduced [6].

The mechanisms of the antioxidant effect of UC-MMSCs are being actively studied. In particular, an increase in the level of glutathione after UC-MMSCs transplantation has been shown, which plays an important role in the detoxification of oxidative stress products [6]. We did not observe any changes in MDA level in aging mice injected with UC-MMSCs. Perhaps this is due to a more significant decrease in the activity of antioxidant enzymes after cuprizone treatment. Age-specific features of the development of oxidative stress in response to the effects of cuprizone are also important [25, 26]. It is possible that the effect of UC-MMSCs in an aging organism can be enhanced by the combined application of biologically active compounds with an antioxidant effect.

There is another way of therapeutic effect of UC-MMSCs in animals with CNS demyelination. This is an anti-inflammatory effect of cells, which is associated with the activation of IL-10 production, decrease in IL-1β production and manifestations of active gliosis in the brain of such animals [14, 27]. It has been shown that the development of a demyelinating pathology of the central nervous system is accompanied by the activation of microglia cells and the imbalance of pro- and anti-inflammatory cytokines [18, 25].

When studying the possible mechanisms of UC-MMSCs actions in animals with CNS pathology, the important issue is their penetration into the brain after intravenous administration. Literature data prove that UC-MMSCs, after suboccipital injection to adult rats with experimental allergic encephalomyelitis, can migrate from the injection site to different parts of the central nervous system (cerebral hemispheres, cerebrospinal fluid, and thoracic spinal cord) and survive for 5 days [28]. However, with intravenous administration of cells, an important condition for their migration into the brain is an increase in the permeability of the blood-brain barrier. It has been shown that already in the early stages of the development of multiple sclerosis, accompanied by neuroinflammation, the blood-brain barrier permeability increases [29]. In adult mice with a cuprizone model of demyelination, such changes in the blood-brain barrier are observed already 24 hours after the start of toxin treatment [30]. However, the literature data on the penetration of transplanted bone marrow and adipose-derived MMSCs into the brain of young mice with a cuprizone diet indicate both their detection in the brain with the development of remyelination and their absence [20, 31]. UC-MMSCs injected into the mouse tail vein can be found also in the vessels of the brain of young animals with the LPS-induced neuroinflammation model [32].

Considering all the above mentioned, we can assume the likelihood of transplanted UC-MMSCs penetrating into the brain of mice with a cuprizone diet, including aging ones, since blood-brain barrier permeability and neuroinflammation in the brain increase. In this case, the differentiation of UC-MMSCs in the neural lineage cells and the synthesis of neurotrophic factors are not excluded [27]. The paracrine effect of UC-MMSCs may also be important in realizing their neuroprotective effect in mice with CNS demyelination [33].

CONCLUSION

1. ***Transplantation of umbilical cord-derived (UC) MMSCs has a positive effect on altered behavior in mice with a cuprizone treatment. The effect of transplanted cells is largely dependent on the age of the animals: in adults, motor and emotional activity increase, in aging ones – exploratory activity and muscle tone increase.***
2. ***In adult cuprizone-treated mice with UC-MMSCs transplantation, the level of MDA in the brain is decreased compared to the group of experimental animals without cell administration.***
3. ***The results of age-related differences in the therapeutic effect of UC-MMSCs on the indicators of the functional state of the central nervous system and oxidative stress with demyelination can be useful in developing approaches to the clinical application of these cells in adult and aging patients.***

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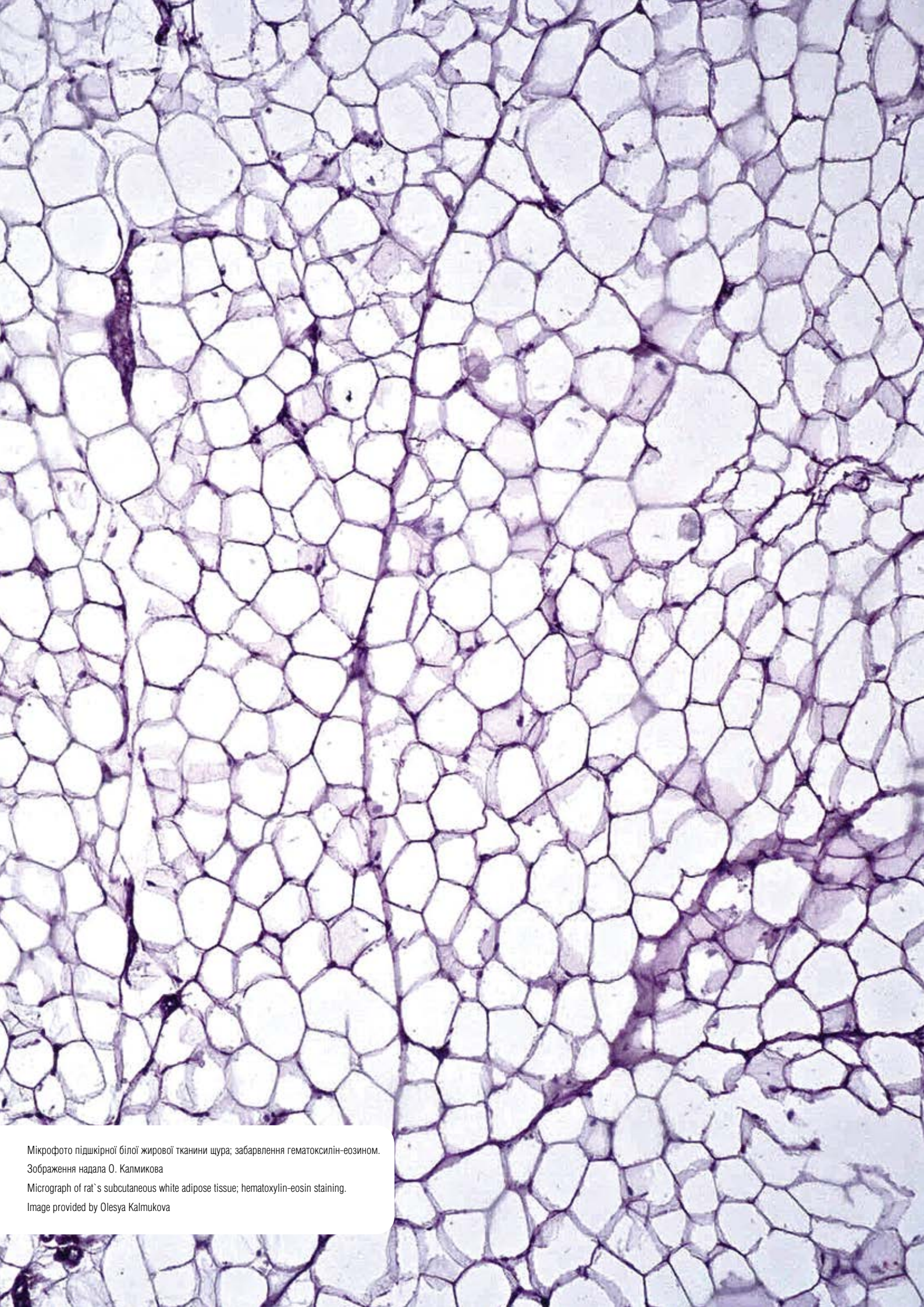

Мікрофото підшкірної білої жирової тканини щура; забарвлення гематоксилін-еозином.
Зображення надала О. Калмикова

Micrograph of rat`s subcutaneous white adipose tissue; hematoxylin-eosin staining.
Image provided by Olesya Kalmukova