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CRYOPRESERVED MESENCHYMAL STEM CELLS IMPACT ON THE SPINAL CORD TISSUE IN RATS WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

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Mesenchymal stem cells from human umbilical cord (1×10^6) are able to correct neurodegenerative changes in the spinal cord of rats with chronic experimental allergic encephalomyelitis under the suboccipital administration. Cells cryopreserved in solution with 3 % dimethyl sulfoxide, 15 % ethylene glycol, 10 % sucrose, 12 % trehalose, 60 % fetal calf serum, slow the build-up of pathological changes at day 35; cryopreserved in solution with 4 % dimethyl sulfoxide, 6 % trehalose, 90 % fetal calf serum, – at days 35–60; cryopreserved in solution with 10 % dimethyl sulfoxide, 90 % fetal calf serum, – have no effect. Selection of optimal cryoprotectants to save the biological properties of these cells and working out the modes and ways of their administration remains relevant.

Key words: mesenchymal stem cells, human umbilical cord, suboccipital administration, spinal cord, pathologically altered neurons, demyelination, cryoprotectants.

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ВПЛИВ КРІОКОНСЕРВОВАНИХ МЕЗЕНХІМАЛЬНИХ СТОВБУРОВИХ КЛІТИН НА ТКАНИНУ СПИННОГО МОЗКУ ЩУРІВ З ЕКСПЕРИМЕНТАЛЬНИМ АЛЕРГІЧНИМ ЕНЦЕФАЛОМІЄЛІТОМ

Мезенхімальні стовбурові клітини пуповини людини (1×10^6) здатні коригувати нейродегенеративні зміни у спинному мозку щурів з хронічним експериментальним алергічним енцефаломієлітом за субокципітального введення. Клітини, кріоконсервовані у розчині з 3 % диметилсульфоксиду, 15 % етиленгліколю, 10 % сахарози, 12 % трегалози, 60 % фетальної телячої сироватки, сповільнюють зростання патологічних змін на 35-у добу; кріоконсервовані у розчині з 4 % диметилсульфоксиду, 6 % трегалози, 90 % фетальної телячої сироватки, – на 35–60-у добу; кріоконсервовані у розчині з 10 % диметилсульфоксиду, 90 % фетальної телячої сироватки – не виявляють впливу. Актуальним є вибір оптимальних кріопротекторів для збереження біологічних властивостей цих клітин та відпрацювання режимів і способів їх введення.

Ключові слова: мезенхімальні стовбурові клітини, пуповина людини, субокципітальне введення, спинний мозок, патологічно змінені нейрони, демієлінізація, кріопротектори.

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Cell therapy using mesenchymal stem cells (MSCs) is considered a promising direction for the treatment of multiple sclerosis (MS) – a chronic immune-mediated demyelinating disease of the central nervous system (CNS), which socio-economic significance has acquired a global scale. A combination of inflammatory and autoimmune mechanisms is involved in the pathogenesis of the demyelinating process in MS [5]. The cascade of immunopathological reactions leads to myelin decay and baring of axial cylinders of axons during the acute inflammatory process and the formation of sclerosis foci in areas of defect in the myelin sheath of axons due to proliferation of astrocytes and histiocytes in the chronic course of the disease [3]. Since the treatment of MS should be aimed at regeneration and correction of immunopathological processes, MSCs are most widely used in cell therapy due to their properties [1, 7]. According to the US National Institutes of Health resource *www.ClinicalTrials.gov*, there are currently 29 registered clinical trials in the world on the safety and efficacy of MSCs applying in MS [11]. The results of preclinical experimental studies and clinical trials show a positive therapeutic effect of transplanted MSCs without the additional use of immunosuppressants [6, 7, 12, 14]. MSCs are heterogeneous population of multipotent cells with strong regenerative potential, the ability to differentiate and transdifferentiate into cells of the nervous system (astrocytes, oligodendrocytes, neurons) [10, 15], weak immunogenicity due to low expression of MHC-I, -II antigens [15], immunosuppressive properties mediated by the production of a number of cytokines and growth factors (Il-6,-10, TGF-β, VEGF, HGF, LIF etc.) [1, 7].

MSCs can be obtained from various body tissues [10, 15]. One of the MSC sources is the human umbilical cord (namely, Wharton's jelly), which has great advantages in terms of moral and ethical aspects and relative ease of obtaining, but requires finding the best options for long-term storage (cryopreservation) and the type of cryoprotectant [2]. At the same time, MSCs cryopreserved according to different protocols need to determine safety and efficacy in preclinical studies in experimental models. Experimental allergic encephalomyelitis (EAE) with a chronic remitting course is considered to be a generally accepted model of MS [4].

The purpose of the work was to study the effects of suboccipital administration of mesenchymal stem cells from human umbilical cord cryopreserved in preservatives of different composition on the spinal cord tissue of animals with experimental allergic encephalomyelitis.

Materials and methods. Studies have been conducted in outbred female rats (n=32, age 3 months, weight (230±20) g) breeding vivarium of the State Institution "Romodanov Neurosurgery Institute, National Academy of Medical Sciences of Ukraine" in compliance with the principles of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes" (Strasbourg, 1986) and the Law of Ukraine No. 3447-IV "On protection of animals from cruel treatment" (2006). The animals were anethesized with intraperitoneal administration of a mixture of xylazine (15 mg/kg, "Sedazin", "Biovet", Poland) and ketamine (70 mg/kg, "Calypsol", "Gedeon Richter Ltd", Hungary). EAE of moderate severity was simulated by a single subcutaneous injection into the pads of the hind limbs of rats of 0.2 ml of a suspension of homogenized in saline spinal cord (SC) of adult rats (50 mg per 100 g weight), emulsified (1:1) with complete Freund's adjuvant ("Sigma", USA).

On the 18th day from the induction of EAE (the peak of clinical manifestations) rats were suboccipitally injected with a suspension of MSCs from human umbilical cord (hUC-MSCs, 1×10^6 cells in 0.1 ml of saline per animal) cryopreserved in solutions of different composition: 1) cryo-1: 3 % dimethyl sulfoxide (DMSO), 15 % ethylene glycol, 10 % sucrose, 12 % trehalose, 60 % fetal calf serum (FCS); 2) cryo-2: 4 % DMSO, 6 % trehalose, and 90 % FCS; 3) cryo-3, standart: 10 % DMSO, 90 % FCS. After thawing, the relative number of living cells was: hUC-MSCs (cryo-1) – 85.4±0.1 %; hUC-MSCs (cryo-2) – 94.3±0.2 %; hUC-MSCs (cryo-3) – 95.9±0.1 %; more than 90 % of cells were positive for CD -73, -90, -105 (specific surface markers of MSCs).

Experimental groups of animals were formed accordingly: group 1 – EAE (n=7); group 2 – EAE+hUC-MSCs (cryo-1) (n=7); group 3 – EAE+hUC-MSCs (cryo-2) (n=6); group 4 – EAE+hUC-MSCs (cryo-3) (n=8); control (intact animals, n=4).

On day 35 and 60 after the induction of EAE (on day 17 and 42 after the hUC-MSCs administration) pathomorphological examination was carried out. SC fragments (lumbar region) were fixed in 10 % formalin solution (pH 6.9, "Merck", Germany), poured into paraffin and serial sections were prepared (5–7 μm, microtome "Microm HM430" (Germany), stained with hematoxylin-eosin, hematoxylin-picrofuxin and thionine ("Janssen Chimica"). The obtained histological specimens (n=6 for each animal) were examined on a light microscope "NIKON Eclipse E200" (Japan) with a video recording system (x400, x800) to assess the morphological features of nervous tissue (gray matter (GM), white matter (WM), nerve roots (NR)) according to the following criteria: the presence of pericellular and perivascular edema; manifestations of dystrophic and necrobiotic changes in the nucleus and cytoplasm of cells; signs of demyelination of nerve fibers in WM). Morphometric analysis of digital images of histological specimens was performed in 20 randomly selected fields of vision at the same magnification (x800) to estimate the quantitative changes of neurons (N) and gliocytes (G). According to morphological criteria, the unchanged N (typical, normal structure) and the pathologically changed N (PCN) were tentatively distinguished: dystrophic (hyperchromic and vacuolated) and dead (the shadow cells).

Data were processed using the software package "Statistica 8.0" ("StatSoft, Inc.", 2007). The rank dispersion Kruskal-Wallis H test for multiple and Mann-Whitney U-test for pairwise comparison of independent groups were used. The normality of data distribution was determined by Shapiro-Wilk test. Differences at $p<0.05$ were considered statistically significant. Data are presented as M (25 %; 75 %), where M is the median; (25 %; 75 %) – the quartile interval between the 25th and the 75th percentiles.

Results of the study and their discussion. *Group 1 (EAE).* On day 35 after EAE induction, significant changes were observed in the histological slides of the rats' SC lumbar region in comparison with the overall histological structure and architecture of SC in the intact animals (control, fig. 1a, b, c). In the SC GM the vast majority of N are deformed, hyperchromically colored, with a deformed nucleus (in 50 % the nucleolus is not differentiated), the cytoplasm with signs of tigrolysis of the Nissl substance, the processes are shortened (fig. 1d). The PCN content significantly exceeds the control indicators ($p=2.3\cdot10^{-}$

^{10}; table 1). These changes are exacerbated on the 60th day: the SC GM loosens, the number of large motor N decreases, in some of them – a pronounced tigrolysis of the Nissl substance, homogenized content of the nucleus and cytoplasm, lysed nucleoli (fig. 1e). Most small N have deformed bodies, hyperchromic color, deformed homogenized nuclei with lysis of nucleoli, thickened shortened processes. The normal structure is preserved in 19–31 % N, the PCN fraction is 48–53 %, shadow cells – 21–28 %.

On day 35 after EAE induction, SC WM is loosened and sponged with cavities of various sizes (fig. 1f). While maintaining the overall structure in some areas there are clear signs of axons demyelination. Axial cylinders are swollen, axons are deformed, some of them – in a state of lysis. G are mainly hyperchromically colored, deformed, containing shrunken nuclei with homogenized content. On the 60th day after the induction of EAE, these changes intensify (fig. 1g). The amount of G, which is mainly deformed, hyperchromic, with a homogenized nucleus content in the areas with more pronounced demyelination decreasing. Perivascular edema and small focal hemorrhages are noted.

On the 35th day of the experiment, NR retain the structure with significant loosening (fig. 1h), there is swelling and deformation of axons, some of them are lysed. On the 60th day, these phenomena intensify, there are areas of almost complete demyelination (fig. 1i).

Group 2 (EAE+injection of hUC-MSCs (cryo-1). On day 35 of the experiment, (the 17th day after the administration of hUC-MSCs) the general architecture of SC is preserved against the background of moderate looseness of the nervous tissue. In SC GM less than half of N retain a cellular structure close to normal (fig. 1j). The PCN content exceeds indices of intact animals, but is significantly less than that of rats with EAE (respectively $p=6.0\cdot10^{-5}$, $p=0.03$; table 1). On the 60th day of the experiment, the proportion of PCN increases (fig. 1k), exceeding the corresponding rate of animals in this group on the 35th day ($p=0.002$; table 1) and approaching the rate of rats with EAE.

In SC WM of rats in group 2, on the 35th day there is a significant swelling of nerve fibers with the formation of rounded cavities around the deformed swollen axons and their almost total demyelination in common areas (fig. 1l). G are in reduced quantities, deformed, with hyperchromic color and homogenized nucleus content. On the 60th day of the experiment, these signs of demyelination increase (fig. 1m), there are small hemorrhages.

In SC NR of rats of group 2 signs of demyelination (uneven edema of axial cylinders, deformation and partial lysis of axons) intensify from the 35th to the 60th day (fig. 1n, o).

*Group 3 (EAE+injection of hUC-MSCs (cryo-2)***.** On day 35 of the experiment in the SC GM, the number of N that retains a morphology close to normal is 33-40 %. Most N has pathological changes of varying severity (fig. 1p). On the 60th day, the structural characteristics of the SC GM in this group are almost no different from those on the 35th day (fig. 1q). The PCN proportion in both studied terms exceed the corresponding rate of intact animals ($p=1.0\cdot10^{-6}$, $p=2.0\cdot10^{-4}$), but is significantly lower than the rate of rats with EAE at 35th day, and, especially significantly, at 60th day ($p=0.02$; table 1).

The SC WM of animals in the group 3 on the 35th day has individual differences from insignificant to moderately pronounced signs of demyelination of nerve fibers (fig. 1r), while on the 60th day – spongy, fibrous with clear signs of demyelination. Axial cylinders are swollen, axons are deformed, a significant proportion of them is lysed. G are in small quantities, mainly have a hyperchromic color, deformed with a homogenized content of the nucleus (fig. 1s). Signs of demyelination are also observed in NR: the tissue is fibrous, axons are unevenly swollen and deformed, a significant proportion of them – with varying degrees of lysis (fig. 1t, u).

Group 4 (EAE+injection of hUC-MSCs) (cryo-3). SC GM on the 35th day is loosened to varying degrees. A smaller proportion of N (27–37 %) retains a normal cell structure, contains a rounded, well-contoured nucleus with a nucleolus and a gentle pattern of chromatin. More N is at different stages of necrobiosis (fig. 1v). On the 60th day, the SC GM is largely spongy, contains rounded cavities of different sizes, the predominant amount of N is pathologically altered (fig. 1w). The PCN proportion exceeds the corresponding rate of intact animals on the 35th and 60th day ($p=8.5\cdot10^{-10}$, $p=4.4\cdot10^{-7}$) and does not differ from the rate of rats with EAE (table 1).

SC WM in rats of group 4 on the 35th and 60th day is loosened and spongy, with rounded cavities of different diameters due to manifestations of demyelination of nerve fibers and partial lysis of axons, but the overall structure is preserved (fig. 1x, y). Axons are swollen, deformed, in some areas with signs of lysis. G have a hyperchromic color, shrunken bodies with a homogenized content of the nucleus. There are small focal hemorrhages.

The NR general structure on the SC sections at the 35th and 60th day of the experiment is relatively preserved; there are signs of demyelination in the form of swollen axial cylinders with deformed axons and the formation of separate cavities in place of lysed ones (fig. 1z1, z2).

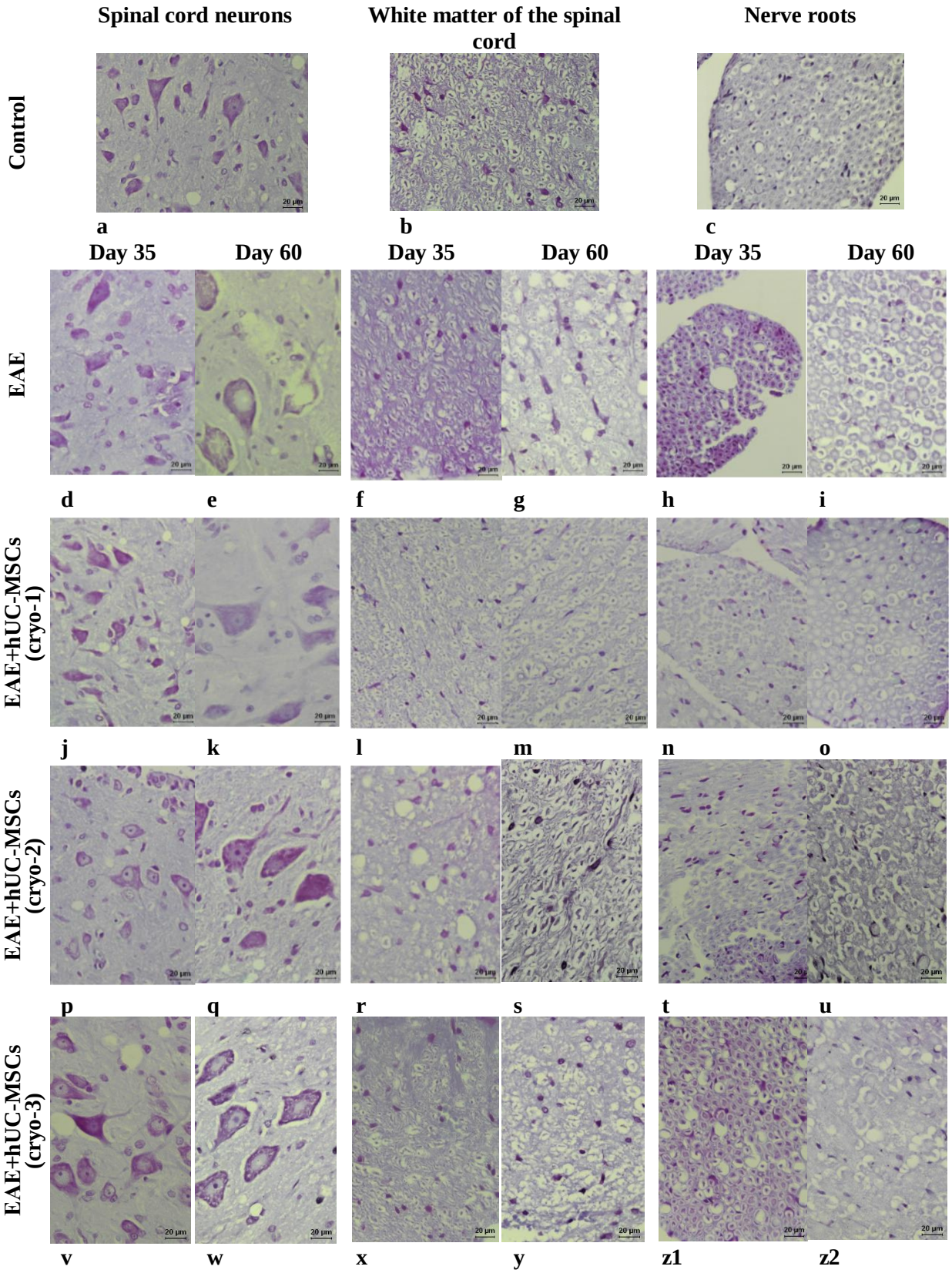

Fig.1. Morphological changes in spinal cord tissue of rats with EAE after suboccipital injection of mesenchymal stem cells from human umbilical cord cryopreserved in solutions of different composition. Thionine staining.

Our study showed that hUC-MSCs administered suboccipitally can affect the neurodegenerative and demyelinating processes in SC of rats with EAE, but the extent of this effect depends on the characteristics of cells after cryopreservation in solutions of different composition. Administration of hUC-MSCs (cryo-1) to rats at the peak of clinical manifestations of EAE (18th day after induction of the disease) slows down the process of increasing pathological changes in the SC in 1 month after EAE induction (17

days after cell administration), however, after 2 months of the experiment, the pathological changes intensify to the level characteristic of rats with EAE without additional effects, which indicates the short duration of the hUC-MSCs (cryo-1) effect and justifies their repeated administration to prolong the effect when using this variant of the solution for cryopreservation. The administration of hUC-MSCs (cryo-2) restrains the pathological process in the SC for 2-month period, certifying the preservation of the hUC-MSCs (cryo-2) influence for 42 days after administration and arguing in favor of the applied variant of the composition of cryoprotectants. No differences were found in the studied parameters in the SC tissue in rats with EAE during 2 months of the experiment, which could be used to record the probable effect of the administered hUC-MSCs (cryo-3), cryopreserved in standard solution (10 % DMSO, 90 % FCS). Therefore, the studied cryopreserved hUC-MSCs can be approximately arranged in a linear series according to the degree of growth of the level and the effect duration: hUC-MSCs (cryo-3) → hUC-MSCs (cryo-1) → hUC-MSCs (cryo-2). The obtained results are correlated with the hUC-MSCs (cryo-2) characteristics, demonstrated *in vitro*, – better adhesive properties and ability to expand and form a monolayer, compared with hUC-MSCs (cryo-1) and (cryo-3) and correspond to the known data on greater safety of multicomponent cryoprotectants [8, 9].

Table 1

Morphofunctional state of motor neurons of the gray matter in the spinal cord lumbar region of rats with EAE under the influence of suboccipital injection of mesenchymal stem cells from human umbilical cord cryopreserved in solutions of different composition

No.	Experimental conditions (groups of animals)	Number of pathologically changed neurons (M (25 %; 75 %))		p (Mann –Whitney U-test)
		Day 35	Day 60	
1	control (intact animals, n=7)	30 (20; 40) $*_1*_2*_3*_4$	30 (20; 40) $*_5*_6*_7*_8$	$*_1$ – p 1, 2=$2.3\cdot10^{-10}$ $*_2$ – p 1, 3=$6.0\cdot10^{-5}$ $*_3$ – p 1, 4=$1.0\cdot10^{-6}$ $*_4$– p 1, 5=$8.5\cdot10^{-10}$ $*_5$ – p 1, 2=$1.0\cdot10^{-10}$ $*_6$– p 1, 3=$2.9\cdot10^{-9}$ $*_7$ – p 1, 4=$2.0\cdot10^{-4}$ $*_8$ – p 1, 5=$4.4\cdot10^{-7}$ $\#_1$ – p 2, 3=0.03 $\#_2$ – p 2, 4=0.02 $\&_1$ – p 3, 3=0.002
2	group 1 (EAE, n=7)	70 (60; 80) $*_1\#_1$	70 (70; 80) $*_5\#_2$	
3	group 2 (EAE+hUC-MSCs (cryo-1), n=7)	60 (50; 70) $*_2\#_1\ \&_1$	70 (60; 80) $*_6\ \&_1$	
4	group 3 (EAE+hUC-MSCs (cryo-2), n=6)	60 (60; 70) $*_3$	60 (60; 70) $*_7\#_2$	
5	group 4 (EAE+hUC-MSCs (cryo-3), n=8)	70 (60; 80) $*_4$	70 (60; 80) $*_8$	

Note: M is the median; (25 %; 75 %) – interquartile range; differences are statistically significant: * – in comparison with control; # – between group values; & – between the values of one group on days 35 and day 60.

Obviously, better preservation of the hUC-MSCs properties during cryopreservation in solution (cryo-2) is provided by a successful combination of reduced concentration of DMSO (4 %, compared to the standard solution (cryo-3) – 10 %) with a content of 6 % disaccharide trehalose. DMSO penetrates through the cell membrane, so it provides maximum maintenance of their properties and viability after thawing; while trehalose is considered a safe substitute for DMSO, but is an impermeable substance, so to ensure complete cryoprotection the presence of a permeable substance is required, i.e. DMSO [9, 13].

Therefore, to obtain a therapeutic effect on SC of rats with EAE, during hUC-MSCs cryopreservation it is advisable to give preference to cryoprotective solution (cryo-2), which confirms the validity of the position to reduce the concentration of DMSO in cryoprotective solution and addition of disaccharides to its composition [9, 13]. However, in our study of SC tissue of experimental animals with EAE after suboccipital administration of hUC-MSCs, cryopreserved in solutions (cryo-1) and (cryo-2), only a slowing of the neurodegenerative and demyelinating process is marked that unfolds in rats with EAE; morphological signs of improvement of the SC condition in the lumbar region (where the most common pathological signs in EAE) or remyelination were not detected. In this regard, further search for the optimal components of cryoprotectants for hUC-MSCs and the development of modes and methods of their administration remain relevant.

Conclusion

In rats with a reproduced model of EAE of moderate severity with a peak of clinical manifestations on the 18th day after EAE induction and chronic remitting course, signs of neurodegenerative and demyelinating process in SC tissue increase in the dynamics of observation from the 35th to the 60th day. hUC-MSCs (cryo-1), cryopreserved in solution with a content of 3 % DMSO, 15 % ethylene glycol, 10 %

sucrose, 12 % trehalose, 60 % FCS, after suboccipital administration to rats with EAE at the peak of clinical manifestations show a short-term effect, slowing the growth of pathological changes in the SC on the 35th day of the experiment. hUC-MSCs (cryo-2), cryopreserved in solution with a content of 4 % DMSO, 6 % trehalose, 90 % FCS, after suboccipital administration to rats with EAE at the peak of clinical manifestations show a longer effect, inhibiting the progression of pathological changes in the SC on the 35-60th day of the experiment. hUC-MSCs (cryo-3), cryopreserved in standart solution (10 % DMSO, 90 % FCS), after suboccipital administration to rats with EAE at the peak of clinical manifestations do not affect the structural changes of the SC tissue of experimental animals.

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