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CRYOPRESERVATION OF MESENCHYMAL STEM CELLS WITHIN MACROPOROUS MATRICES AFTER SUCROSE PRETREATMENT

Mesenchymal stromal/stem cells (MSCs) attract the attention of scientists and specialists in various fields of medicine due to their high immunomodulatory and regenerative potential, ability to multilineage differentiation. Effective storage technologies are essential for the implementation of MSCs into medical and laboratory practice. The article investigates the effect of pretreatment with sucrose on the viability, metabolic activity and differentiation potential of MSCs after cryopreservation in three-dimensional (3D) macroporous matrices. The results of the study showed that pretreatment with sucrose increased the efficiency of cell cryopreservation in collagen matrices by slow cooling in the presence of 10% DMSO and serum. The viability and metabolic activity of cells after cryopreservation in 3D matrices was significantly higher when treated with sucrose. It was also found that cells after cryopreservation retained the ability to proliferate and multilineage differentiation. The findings suggest that using sucrose for cell pretreatment is a promising approach to reduce cryodamage during their cryopreservation in 3D matrices and opens up new opportunities for increasing the efficiency of storage of tissue-engineered constructs.

Key words: *mesenchymal stem cells, pretreatment, sucrose, cryopreservation, DMSO, viability, metabolic activity, induced differentiation, tissue-engineered constructs.*

Cultivation of mesenchymal stromal/stem cells (MSCs) in three-dimensional (3D) matrices enables to study the biological properties of cells in conditions that are as close to natural as possible. A number of different methods have been developed to create cell carriers: salting out, lyophilization, cryogelation, electrospinning, and 3D printing. The options for choosing the polymer that will make up the matrix are even more diverse: polylactic and polyglycolic acids, alginate, chitosan, collagen, gelatin, and their composites [2]. In this work, we used the collagen-based polymer cryogels. The choice of collagen carrier was due, first of all, to the high biocompatibility, nontoxicity, and low immunogenicity of the material [8, 12]. Skin biopsies were chosen as the source of MSCs. These skin-derived MSCs have found wide application in tissue engineering and regenerative medicine [19].

Cryopreservation and the establishment of low-temperature banks allow not only to store samples for a long time and use them almost immediately when needed, but also ensure the availability of certified and standardized biological material. In contrast to the effective and simple methods proposed for cell suspension freezing, the development of methods for cryopreservation of cells in 3D structures remains one of the most pressing problems in cryobiology. It is known that the most

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pronounced damaging effect on adherent cells is exerted by the action of osmotic and mechanical effects caused by the formation of ice crystals [4, 20].

Currently, methodological approaches have been developed for cryopreservation of cells of different origins, which differ in the composition of the cryoprotectant solution, the rate and stages of freezing. However, the method of slow two-stage freezing under the protection of the permeable cryoprotectant DMSO and fetal bovine serum (FBS) is considered as the "gold" standard for MSC cryopreservation [10]. In this regard, this method was chosen as the initial option for cryopreservation of MSC suspensions and cells cultured in collagen matrices. We have previously showed [17] that the inclusion of saccharides (lactose, trehalose, sucrose, mannitol or raffinose) in the culture medium 24 h before the cryopreservation procedure, which did not contain a cryoprotectant, significantly increased the viability of human skin-derived MSCs, *i. e.*, we demonstrated the effectiveness of saccharide pretreatment to enhance the resistance of MSCs to low temperatures. The highest and almost identical cell viability rates were obtained when sucrose, trehalose, and raffinose were applied. Therefore, sucrose was used in this study as a more accessible and common saccharide.

The aim of this work was to investigate the effect of sucrose pretreatment on the survival rates of cells in 3D constructs after cryopreservation using the two-stage slow freezing method.

MATERIALS AND METHODS

The human MSC cultures obtained by the method of Rogulska *et al.* [18] from skin biopsies were used in the experiments. Cells were isolated in compliance with biomedical ethics and stored in the cryobank of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine (IPC&C of the NAS of Ukraine). Tissue sampling procedures were performed after obtaining the written informed consent from donors in accordance with the recommendations of the Declaration of Helsinki of the World Medical Association for biomedical research and the requirements of the Bioethics Committee of the IPC&C of the NAS of Ukraine.

Monolayer culture of mesenchymal stromal/stem cells. Cells were cultured at 37 °C, 95% humidity, and 5% CO_2 in expansion medium consisting of α-MEM (Biowest, France) supplemented with 2 mM L-glutamine, 50 μg/ml streptomycin, and 50 U/ml penicillin, and additionally containing 10% FBS (Biowest). After the cell cultures reached a 70% confluency, they were trypsinized using standard methods and further plated at a 1 : 3 ratio. Cells from passages 4—7 were used for experiments.

Three-dimensional cultivation of mesenchymal stromal/stem cells in collagen carriers. For 3D cultivation, polymeric macroporous collagen-based carriers were used, manufactured by the method described by us earlier [18]. To populate the carriers with cells in the form of disks with a diameter of 4 mm and a thickness of 2 mm, the perfusion method was used [15]. The resulting constructs, which contained 200—300 thousand cells, were transferred to the wells of plates (SPL, Korea) and cultured under standard conditions in α-MEM medium with 10% FBS. Complete replacement of the culture medium was performed every 3—4 days.

Cryopreservation of mesenchymal stromal/stem cells in suspension and in collagen matrices. For cryopreservation, DMSO cryoprotectant was used at a final concentration of 10%. Cryoprotective medium consisted of α-MEM medium with 10% FBS, and a doubled concentration of DMSO, was slowly added dropwise to an equal volume of culture medium with cell suspension or matrices. Cells were exposed to a cryoprotective medium at 4 °C for 15 min. Cells were frozen in 1 ml cryovials (SPL) using a two-stage slow freezing regimen. Cryopreserved samples were stored in liquid nitrogen for at least 2 weeks before thawing and analysis.

The pretreatment was carried out by culturing the cells for 24 hours with sucrose at a final concentration of 100 mM. In this case, in addition to 10% DMSO and FBS, 200 mM sucrose was added to the cryoprotectant solution.

Warming was performed in a water bath at 37 °C. The cryoprotectant medium was removed by diluting the cryopreservation medium ten-fold with Hanks' solution (Biowest, France).

Analysis of viability and metabolic activity of mesenchymal stromal/stem cells. Cell viability at different stages of the study was determined using combined ethidium bromide (EB) and fluorescein diacetate (FDA) staining [8]. The incorporation of fluorescent dyes into the cells was assessed using an inverted confocal laser scanning microscope "Zeiss LSM 510 META" (Carl Zeiss, Germany) and LSM 510 v. 4.2 software (Carl Zeiss). Confocal images were acquired along the Z axis at 15 μm intervals at

an excitation wavelength of 488 nm for FDA and 543 nm for EB. Viability indices were determined as the percentage of fluorescein-positive (green) cells out of the total number of stained cells, counted for each sample in a series of images acquired from the surface to 150–210 μm depth of the carrier.

To assess the metabolic activity of cells in two- and three-dimensional cultivation conditions, the redox indicator AlamarBlue (AB, Serotec, USA) was used [14]. The content of the reduced form of AB was determined fluorimetrically after 3 hours of cultivation in the expansion medium containing 10% AB on a Tecan GENios spectrofluorimeter (Tecan Inc., Austria) at excitation wavelengths of 550 nm and emission wavelengths of 590 nm. The background was the medium with an indicator without cells. Data processing was performed using the XFluor v.4.50 software (Tecan Inc.). The results were presented in relative fluorescence units (RFU), which were calculated as the ratio between the fluorescence level of the experimental and blank samples (without cells).

Assessment of the ability of mesenchymal stromal/stem cells to undergo directed osteogenic and adipogenic differentiation. To determine the ability of stromal cells to undergo directed osteogenic differentiation under culture conditions, they were transferred to α-MEM medium containing 10% FBS, L-glutamine, antibiotics, and osteogenesis inducers: 100 nM dexamethasone, 10 mM β-glycerophosphate, 0.2 mM L-ascorbic acid 2-phosphate (all inducers manufactured by Sigma-Aldrich, USA) [5].

To assess the ability of stromal cells to undergo directed adipogenic differentiation, they were transferred to α-MEM medium containing 10% FBS, 0.2 mM L-glutamine, and antibiotics, as well as adipogenesis inducers in the amount of 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 10 μg/ml insulin, and 100 μM indomethacin (all inducers manufactured by Sigma-Aldrich, USA) [21].

Cells were cultured in induction media for 21 days, after which the cultures were fixed for 30 min at 4 °C in 10% formalin prepared in phosphate buffer (pH 7.2). The ability to osteogenic differentiation was assessed by alkaline phosphatase expression using the Fast Blue RR Salt, Naphtol AS-MX Phosphate Alkaline Solution kit No. 85 (Sigma-Aldrich) according to the manufacturer's instructions.

Lipid accumulation was indicated by staining the cultures with Oil Red O [6].

The experimental results were statistically processed. The normality of the distribution of the obtained results was assessed using the Shapiro-Wilk test. Depending on the nature of the data distribution, the Student's t test with Bonferroni correction or one-way analysis of variance ANOVA and the Mann-Whitney test were used to compare the samples.

RESULTS AND DISCUSSION

Cryopreservation of MSCs in the form of suspensions after thawing allowed obtaining more than (89 ± 4)% viable cells. Unlike cell suspension, cryopreservation of MSCs in matrices significantly reduced the percentage of viable cells. The indices obtained by calculating the quantitative ratio of FDA-positive (live) cells and EB-positive (dead) cells were (48 ± 4)%. Matrices with cells frozen without the use of cryoprotectants served as negative controls. After this freezing option, only single viable MSCs remained on the surface of the carrier pores. Characteristic micrographs of cells cryopreserved in collagen matrices are shown in Fig. 1.

The metabolic activity of cells was assessed one day after warming and recultivation under the standard conditions (37 °C, 5% CO_2). The fluorescence level of the reduced form of AB in cells cryopreserved in suspension was 15% lower compared to the level before cryopreservation (Fig. 2). The metabolic activity of MSCs after cryopreservation in the collagen carrier decreased by 60% compared to the control group (before cryopreservation).

Thus, in contrast to successful cryopreservation of MSC suspension, freezing of three-dimensional bioconstructs results in the preservation of less than half of viable cells after thawing.

Based on the need to modify the cryopreservation method using 10% DMSO and FBS, we attempted to improve cell preservation by modifying the cryopreservation method.

In our previous series of experiments [17], a new approach was developed to increase the resistance of MSC suspensions to cryoinjury, which involved the combined use of saccharides as a supplement to the cell culture medium (pretreatment), and to cryoprotective solution as well. Among the studied saccharides, sucrose showed the highest activity. The proposed method of modifying the cryopreservation procedure using sucrose was selected for further studies in 3D structures.

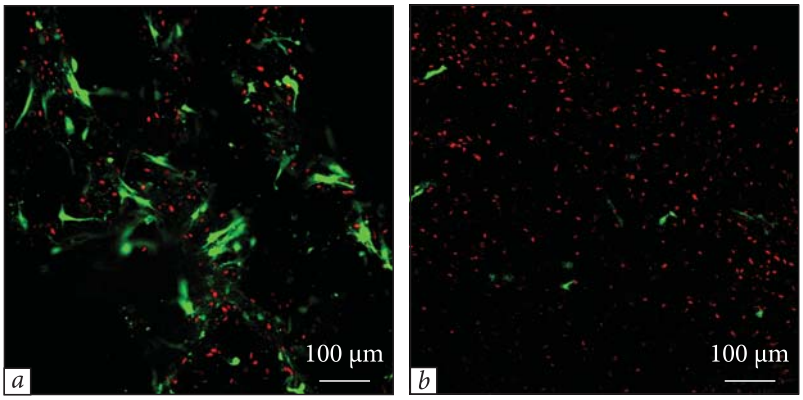

Fig. 1. MSCs within collagen matrices cryopreserved under protection of 10% DMSO (*a*) and without using cryoprotectant (*b*). FDA/EB staining, confocal laser microscopy

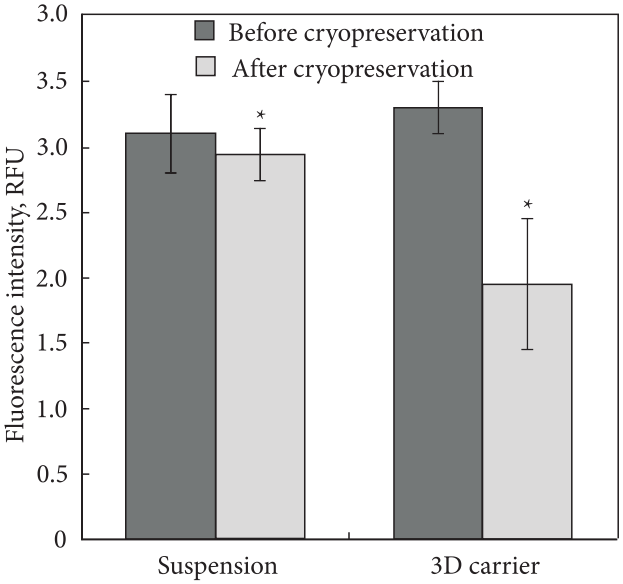

Fig. 2. Metabolic activity of MSCs after cryopreservation in three-dimensional collagen-based carriers and in suspension; * — differences are statistically significant ($p < 0.05$) compared to non-cryopreserved samples

The combined addition of sucrose 24 h before cryopreservation to the MSC culture medium and to the cryoprotective solution significantly affected the viability and properties of the cells. Fig. 3 shows that sucrose pretreatment and its inclusion in cryoprotective solution allows to increase the viability rates to (64 ± 6)% (Fig. 3, *a*). It should be noted that after cryopreservation, no differences in viability were found between the cells located on the periphery and in the deeper layers of collagen carriers (within the range of 150–210 μm depth). During further cell cultivation within collagen matrices, they were able to proliferate, and the dead cells were gradually eliminated. To day 5 after warming and subsequent recultivation, almost the entire volume of the carrier was evenly filled with cells, which indicated an active recovery (Fig. 3, *b*).

The metabolic activity of cells within collagen matrices cryopreserved using sucrose decreased by (40 ± 5)% compared to the pre-cryopreservation value, which was 18% higher than the results obtained with no pretreatment used. Thus, the study shows the possibility of increasing the resistance of adherent cells to cryodamage factors by culturing in the presence of sucrose.

It should be noted that MSCs cryopreserved using sucrose pretreatment retained the ability for multilineage differentiation in the osteogenic and adipogenic directions: during cultivation in the presence of adipogenesis inducers, the cells accumulated neutral lipids (Fig. 4, *a*), and in the presence of osteogenic inducers, they expressed alkaline phosphatase (Fig. 4, *b*).

Traditional monolayer 2D culture does not allow preserving the properties of MSCs during long-term propagation, while 3D culture, which partially reproduces the regulatory mechanisms operating in natural systems, creates better conditions for MSC growth [11]. In this regard, new approaches to cryopreservation of cells in matrices are needed.

These findings suggest the cells within macroporous 3D bioconstructs to be very sensitive to cryopreservation factors. Conditions that ensure the preservation of almost all the cells in suspension after cryopreservation (slow two-stage cooling in the presence of 10% DMSO and serum) lead to the death of half of the cells. The low efficiency of cryopreservation of 3D bioconstructs is conditioned by more pronounced osmotic and mechanical effects caused by ice crystal formation as compared to cells in suspension. The quite large linear dimensions of the bioconstruct create obstacles to the uniform distribution of cryoprotectants in different areas of the matrix. This causes the appearance of local areas with a rather high concentration of toxic cryoprotectant, as well as the areas

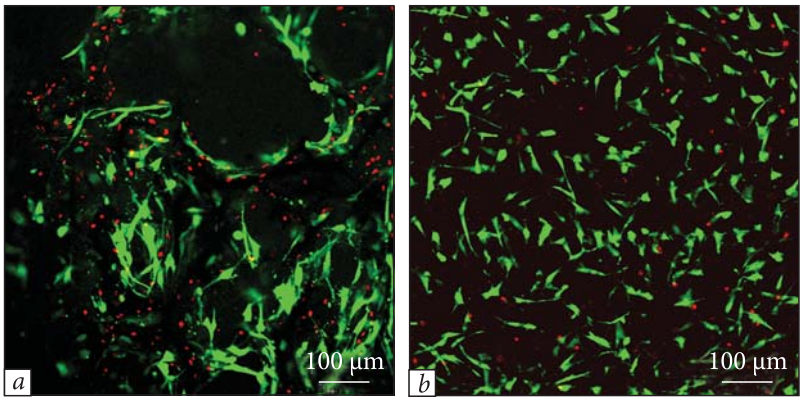

Fig. 3. MSCs within collagen matrices cryopreserved with sucrose and 10% DMSO, immediately after warming (*a*) and 5 days after culture (*b*). FDA/EB staining, confocal laser microscopy

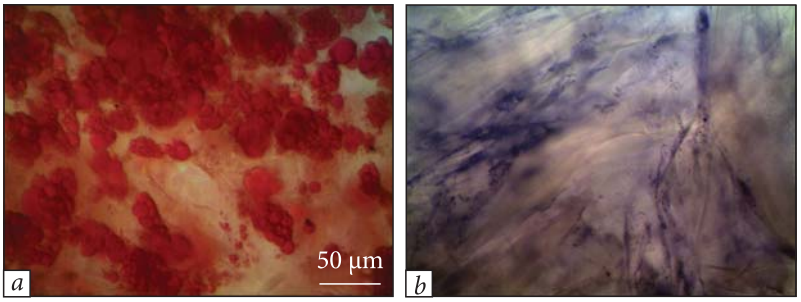

Fig. 4. The ability of MSCs to undergo adipogenic (*a*) and osteogenic (*b*) differentiation after cryopreservation with sucrose pretreatment use. *a* — accumulation of neutral lipids; *b* — expression of alkaline phosphatase

where, due to slow diffusion, there is almost no cryoprotectant solution.

Obviously, during cryopreservation of 3D constructs, the cell viability is influenced by a number of factors, including the sample volume, cooling and thawing rates, physical properties of the scaffold, *etc*. This issue has been the subject of several studies. In particular, the approach of regulating the degree of cell spreading on the matrix surface had a positive effect [7]. Other researchers have shown that cryopreservation in a minimal volume of cryoprotectant, the so-called "air" cryopreservation, also enhances the viability of MSCs [11]. We applied the procedure for pretreatment of MSCs within macroporous matrices with sucrose during cultivation, which significantly improved the efficiency of their cryopreservation. In addition, we supplemented the cryoprotective medium with sucrose, which acted as an extracellular cryoprotectant, increased the osmolarity of the medium, reduced the formation of ice crystals inside the cell and stabilized the plasma membrane during freezing. The positive effect of sucrose application was demonstrated in the study of cryopreservation of MSCs within allogeneic bioscaffolds [9]. The results of this work are consistent with the findings of X. Xu *et al*. [20], which also showed that surface-attached MSCs were more vulnerable to low temperatures than cells in suspension upon slow cooling. A cooling rate of 10 °C/min resulted in a more significant decrease in intracellular pH, actin filament deformation, and mitochondrial aggregation than cooling at slower rates (1—1.5 °C/min).

A significant difference between cells in a scaffold and in suspension is their attachment to the pore surface. One possible explanation for higher cryosensitivity is that the flattened cells have a limited ability to reorganize their cytoskeleton in response to changes in osmotic pressure and ice crystal formation. Unlike cells in suspension, which can freely change the shape, the flattened cells are subjected to additional mechanical stresses, which increases the risk of membrane damage and cell death. It is known that the physical properties of the matrix significantly affect the level of cell damage. The findings on the effect of relaxation/stretching of cells on pore surface showed that modulated compression/decompression of cells promoted higher cell attachment and survival [1]. That is, when using non-rigid collagen macroporous matrices with stretching/compression patterns, in response to the applied physical forces that arise during cryopreservation, we can speak of a certain degree of adaptation of both the cytoskeleton and the membrane dependent on it. It can be assumed that the development and use of matrices with lower stiffness compared to those selected in our work will allow to obtain higher indices of cell viability after cryopreservation.

The positive effect of sucrose pretreatment on cell survival during cryopreservation, as observed in our work, may be due to several mechanisms. First, during sufficiently long cell culture (24 h), sucrose can be slowly transported across the plasma membrane in a concentration-dependent manner, which may be due to passive diffusion or an active clathrin-dependent pathway, as shown for trehalose [3]. V. Mutsenko *et al.* [13] found out that increasing the intracellular concentration of sucrose by permeabilization of MSCs by electroporation allowed to obtain high cell viability (more than 80%) after cryopreservation without DMSO and other cryoprotectants. Secondly, the presence of osmotically active sucrose in the culture medium leads to partial dehydration of cells and, accordingly, to a change in the area and density of contact with the surface of the carrier. In this regard, it is appropriate to refer to the findings of Y. Petrenko *et al.* [16], which showed that MSCs that were moderately spread out on surface of scaffold pores were less sensitive to cryopreservation effects than those with longer attachment times and larger contact areas with the carrier. Interestingly, the positive effect of sucrose pretreatment is only realized when sucrose is present in both the culture and cryopreservation media. In the absence of sucrose in the cryopreservation medium, the effect of the treatment is insignificant. This fact, as well as the significantly lower viability of cells not treated with sucrose after cryopreservation under sucrose protection [17], may be due to a decrease in the intracellular concentration of sucrose when it is absent in the external solution.

These findings demonstrate that sucrose pretreatment promotes the cell survival during cryopreservation not only in suspension, but also in the composition of macroporous matrices, where they are attached to the surface. However, despite the significant improvement in MSC preservation, the proposed cryopreservation method requires additional improvement. Further research can be aimed at studying the mechanisms of enhancing the MSC resistance within 3D bioconstructs, the protective effect of cryoprotectants, as well as the development of new types of matrices, optimization of the modes of saturation with cryoprotectant solution, freeze-thawing of MSCs within 3D bioconstructs.

CONCLUSIONS

Cryopreservation of MSCs in 3D matrices using programmed slow freezing under the protection of 10% DMSO allows preserving viability and metabolic activity at the level of (48 ± 4) and (40 ± 5)%, respectively.

The use of sucrose at the stage of pretreatment and during subsequent cryopreservation under the protection of 10% DMSO ensures an increase in cell survival rate within macroporous collagen carriers by 18%. During subsequent 3D cultivation, MSCs cryopreserved in carriers using sucrose pretreatment have a typical fibroblast-like shape, retain the ability for multilineage differentiation, actively proliferate and populate the volume of the collagen matrix.

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КРІОКОНСЕРВУВАННЯ МЕЗЕНХІМАЛЬНИХ СТОВБУРОВИХ КЛІТИН
У СКЛАДІ МАКРОПОРИСТИХ МАТРИЦЬ ПІСЛЯ ПЕРЕДОБРОБКИ САХАРОЗОЮ

Мезенхімальні стромальні/стовбурові клітини (МСК) привертають увагу науковців та спеціалістів різних галузей медицини завдяки високому імуномодуляторному та регенеративному потенціалу, здатності до мультилінійного диференціювання. Важливою умовою впровадження МСК у медичну та лабораторну практику є розробка ефективних технологій їхнього зберігання. Досліджено вплив попередньої обробки (передобробки) сахарозою на життєздатність, метаболічну активність та диференціювальний потенціал МСК після кріоконсервування у складі тривимірних (3D) макропористих матриць. Показано, що передобробка сахарозою підвищує ефективність кріоконсервування клітин у складі колагенових матриць шляхом повільного охолодження в присутності 10% ДМСО та сироватки. Життєздатність і метаболічна активність клітин після кріоконсервування у 3D матрицях була суттєво вище за умови передобробки сахарозою. Встановлено, що клітини після кріоконсервування зберігали здатність до проліферації та мультилінійного диференціювання. Доведено, що використання сахарози для передобробки клітин є перспективним підходом, який забезпечує зменшення кріопошкоджень під час кріоконсервування клітин у складі 3D матриць і відкриває нові можливості для підвищення ефективності зберігання тканинно-інженерних конструкцій.

***Ключові слова*:** мезенхімальні стовбурові клітини, попередня обробка, сахароза, кріоконсервування, диметилсульфоксид, життєздатність, метаболічна активність, індуковане диференціювання, тканинно-інженерні конструкції.