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Cryopreservation of human Wharton's jelly multipotent mesenchymal stromal cells with reduced concentration of dimethyl sulfoxide

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ABSTRACT

The urgent problem of long-term storage of multipotent mesenchymal stromal cells (MMSCs) is to improve the protocol of their cryopreservation for further application maintaining the therapeutic properties and minimizing the risks of adverse effects on the health of the recipient. As a standard cryoprotectant, a mixture of 90 % fetal bovine serum (FBS) and 10 % dimethyl sulfoxide (DMSO) is used, which, however, can cause a variety of adverse reactions. Therefore, it is important to study the possibility of reducing the concentration of potentially dangerous DMSO by adding other components to the mixture for cell cryopreservation.

PURPOSE. *To determine the efficiency of cryopreservation of human Wharton's jelly MMSCs using cryoprotectants of different composition by studying the proliferative activity, phenotype and features of cell morphology in culture in vitro.*

MATERIALS AND METHODS. *The cryoprotective effect of various combinations of DMSO, ethylene glycol, sucrose and trehalose was studied. The efficacy was assessed by cell viability, their adhesive properties, expansion rate and monolayer formation, as well as the expression of main MMSCs markers.*

RESULTS. *It is shown that the most effective combination is 4 % DMSO with 6 % trehalose which provides the highest level of preservation of cell viability, as well as their adhesive and proliferative properties during thawing. Other combinations of the cryoprotectant components showed a much slower cell division, in some cases, the monolayer was not formed at all. For all investigated variants, the main surface markers of MMSCs were preserved.*

CONCLUSIONS. *The obtained results indicate the possibility of reducing the concentration of DMSO to 4 % in the freezing medium for MMSCs cryopreservation while maintaining their viability, proliferative activity and common surface markers.*

KEY WORDS: *multipotent mesenchymal stromal cells; Wharton's jelly; cell cryopreservation; DMSO; trehalose; sucrose, ethylene glycol*

In recent years, the progress in the treatment of inflammatory-degenerative lesions of the central nervous system (CNS) is associated with the development of cell technology [1]. The most promising ones for use in clinics are multipotent mesenchymal stromal cells (MMSCs), in particular, stromal cells of Wharton's jelly [2, 3]. Due to the fact that the umbilical cord is developed in early pregnancy, these cells have a much greater potential for differentiation and therapeutic potential [4], compared with MMSCs from other sources, because they retain common embryonic stem cells markers [5]. Therefore, the cells from this source are able to effectively impact on the foci of inflammation in the CNS and have pronounced immunomodulatory properties. In addition, this type of MMSCs can be expanded in large number in a short time using simple cell culture techniques [6-9]. It is also important that their isolation has no moral and ethical restrictions.

Given the prospects for clinical application of MMSCs, there is a need to develop a method of long-term storage of MMSCs by cryopreservation, considering the freezing protocol, the type of cryoprotectant and the storage time. The results of clinical use depend on the quality of the cellular product, so effect of cryopreservation should be estimated for minimize its negative effects.

Dimethyl sulfoxide (DMSO) is used as a standard cryoprotectant in the most common freezing medium [2, 10]. The optimal concentration of this reagent is required for successful freezing of different cell populations. It is necessary to take into account the effect of DMSO on the cell membrane when it is added to the cell suspension before freezing. The modelling of cell membrane has shown that the effect of DMSO on the lipid bilayer depends on the concentration of DMSO in solution. When DMSO molecules penetrate into the phospholipid bilayer of the cell membrane, at a concentration of DMSO from 2.5 to 7.5 %, there is a decrease in the bilayer thickness. At concentrations of DMSO from 10 % and more, in addition to reducing the bilayer thickness, the formation of hydrophilic pores and defects in the cell membrane is observed. The DMSO concentration higher than 20 % leads to the complete destruction of the lipid bilayer [11]. For the cryopreservation of cells, the final concentration of DMSO 10 % is the most common. It was found that decrease the concentration of DMSO below 5 % leads to significant losses of cells, and a concentration higher than 10 % does not increase the efficiency of its preservation, but is dangerous for the patient [12].

When DMSO is used as a cryoprotectant in experimental and clinical studies, certain side effects are possible. Thus, among the side effects observed in about 50 % of animals and humans after the introduction of MMSCs cryopreserved with DMSO, there are the following: nausea, vomiting, diarrhea, abdominal pain, headaches, hypotonia or hypertension. No such side effects were observed when using non-frozen cells. Much less often, but more serious conditions caused by disorders of the cardiovascular and respiratory systems have been documented: arrhythmias, respiratory depression, fatal heart failure, seizures, reversible leukoencephalopathy and stroke [13]. In addition, DMSO is not certified for parenteral or oral use in the treatment of patients. For example, today FDA allows only bladder instillation of DMSO in the treatment of interstitial cystitis. The accumulation of such information has led to the search for alternative substances that can protect the cell from damage by ultra-low temperatures, and be safe when administered into body [14].

The analysis of the literature showed that during cryopreservation of cells, a part of DMSO can be replaced by disaccharides [15-17]. However, these substances belong to the impermeable cryoprotectants, and without the permeable agent do not prevent formation of ice crystals completely [15, 16]. Therefore, the concentration of DMSO in the cryoprotective medium can be reduced and disaccharides can be added to the solution (e.g., trehalose). In some studies, it has been shown that multicomponent cryoprotectants are safer [16].

In this regard, the aim of this study was to establish the efficacy of cryopreservation of Wharton's jelly MMSCs using cryoprotectants of different composition by studying the proliferative activity, phenotype and morphology of cells in culture *in vitro*.

MATERIALS AND METHODS

CELL CULTURE

The culture of human Wharton's jelly MMSCs was obtained by the method of explants. The umbilical cord was obtained during normal childbirth from a healthy mother with informed consent. The umbilical cord was kept at room temperature for 30 minutes in DMEM medium (*Biowest*, France) with 10-fold concentration of antibiotics streptomycin and penicillin (*Biowest*, France) at a concentration of 1 mg/mL and 1000 U/mL, respectively, and then washed with saline and minced into pieces up to 0.5 mm. The resulting pieces were placed in DMEM/F12 medium (*Biowest*, France) supplemented with 10 % fetal bovine serum (FBS) (*Biowest,* France), seeded to 25 cm^2 cell culture flasks (Bioswisstec, Switzerland) and cultured in an incubator with 5 % CO_2 at 37 °C until the clones' formation. The culture medium was replaced with fresh ones every 3 days. Upon reaching 70-80 % confluency, a zero passage of the umbilical cord MMSCs was obtained.

The MMSCs culture was passaged according to standard procedures using a solution of 0.25 % trypsin and EDTA 0.02 % (*Biowest*, France) and cultured for 2 passages as described previously. At the 1st passage, the cells were characterized by minimal surface markers typical for MMSCs: CD73, CD90, CD105 (expression over 95 %) and CD34 and CD45 (expression less than 2 %) [18]; and the ability to differentiate into three cell types – adipo-, osteo- and chondrocytes.

CRYOPRESERVATION AND THAWING OF CELLS

For cryopreservation, media with different content of DMSO (*Helicon*, Russia) and combinations of fetal bovine serum (*BioWest*, France), trehalose (*BioFroxx*, Germany), ethylene glycol (*LaboChem*, Germany), sucrose (*BioFroxx*, Germany) were used (**Table 1**).

The MMSCs of the 2nd passage were detached from the culture flask according to standard methods and counted using a hemocytometer. The cells were divided into aliquots for $3 \cdot 10^6$, centrifuged in DMEM/F12 medium at 800 xg for 10 minutes. The cell pellet was resuspend in 3 mL cooled to 8-10 °C experimental freezing media of each variant, aliquoted for $1 \cdot 10^6$ cells/mL in cryotubes and transferred to a programmed freezer Kryo 500 (*Planer*, UK). The cooling rate was 1 °C/min. After reaching the temperature of -80 °C, the tubes were transferred to a cryostorage with liquid nitrogen, where they were stored for 20 days.

After 20 days of storage, the experimental samples were thawed at 38 °C for 1 minute. The cell suspension from cryovials were transferred into 40 ml of serum-free DMEM medium, gently mixed and centrifuged for 10 min at 800 xg. All experiments were performed on the same culture. For each variant of the medium, 3 frozen MMSCs samples were studied. The equipment for cell cryopreservation and thawing procedures was kindly provided by Hemafund LLC (*Kyiv*, Ukraine).

THE COUNT OF CELL NUMBER

To estimate the total number and proportion of viable and dead cells after thawing, 20 µL of cell suspension was stained with Trypan Blue dye (*AppliChem*, Germany) and counted in a Goryaev hemocytometer.

THE ANALYSIS OF PROLIFERATION

To assess the proliferative activity of thawed cells from each group, $1 \cdot 10^5$ viable cells centrifuged in phosphate buffer saline (*Biowest*, France) and seeded into 25 cm^2 culture flask. Complete growth medium consisted of DMEM medium (*Biowest*, France) supplemented with 10 % FBS (*Biowest*, France) and antibiotics penicillin 100 U/mL and streptomycin 100 µg/mL (*Biowest*, France). After 2 days, the cells were detached using trypsin-EDTA solution, their number was counted in a hemocytometer.

The population doubling time after 72 hours was calculated by the formula according to Roth V. [19]:

$$DoublingTime = \frac{duration * log(2)}{log(FinalConcentration) - log(InitalConcentration)}$$

During cultivation, the observation of the cells was performed using an inverted microscope DM IL (*Leica*, Germany). Photomicrographs of the cultures were taken using a Power Shot A640 camera (*Canon*, Japan).

GROUP	THE COMPOSITION OF THE FREEZING MEDIA
1	10 % DMSO, 90 % FBS (standard medium, control)
2	35 % trehalose, 65 % FBS
3	25 % ethylene glycol, 10 % sucrose, 65 % FBS
4	4 % DMSO, 6 % trehalose, 90 % FBS
5	15 % ethylene glycol, 3 % DMSO, 10 % sucrose, 12 % trehalose, 60 % FBS

Table 1. The composition of experimental media for cryopreservation

THE DETERMINATION OF THE SURFACE MARKERS EXPRESSION

The analysis of the expression of surface markers CD105, CD90, CD73, CD34 and CD45 as minimal criteria for determining mesenchymal stromal cells according to the recommendations of the International Society of Cell Therapy [20] was performed by flow cytometry in State Institute of Genetic Medicine NAMS of Ukraine according to standard protocols. Monoclonal antibodies at a working concentration of 1 μg/mL were added to 2-5•10^5 cells in 100 μL of CellWash buffer (*BD Bioscience*, USA) and incubated for 30 min at 4 ^{0}C protected from light. Then the cells were washed in 1 mL of CellWash buffer by centrifugation at 400 xg for 5 min, resuspended in 300 μL of CellWash buffer and filtered through a cell filter with a pore diameter of 70 μm (*BD Falcon*, USA) immediately before analysis. To determine the viability, 5 μL of 7-aminoactinomycin D (*BD Bioscience*, USA) was added to 2•10^5 cells in 300 μL of CellWash buffer and incubated for 5 min. The samples were analyzed on a BD FACSAria cell sorter (*BD Bioscience*, USA) using BD FACSDiva 6.2.1 software, recording at least 20,000 cells for each sample. Fluorochrome-conjugated mouse anti-human monoclonal antibodies for CD105 PerCP-Cy5.5 (Cat. No. 560819), anti-CD90 FITC (Cat. No. 555595), anti-CD73 APC (Cat. No. 560847), anti-CD34 APC (Cat. No. 345804), anti-CD45 FITC (Cat. No. 345808) (all – *BD Bioscience*, USA) were used for immunophenotyping.

THE DETERMINATION OF MMSCs ABILITY TO DIRECTED DIFFERENTIATION

Since directed differentiation is one of minimal criteria for defining multipotent mesenchymal stromal cells, the ability to adipo-, chondro- and osteogenesis of Wharton's jelly cells in the native state and after their freezing-thawing were estimated [20]. Common methods were used for cell differentiation [21, 22]. During induced adipogenesis, cells were placed in a differentiation medium containing DMEM culture medium, supplemented with 10 % FBS, 50 μM/L indomethacin, 10 μM insulin, 1 μM 3-isobutyl-1-methylxanthine (all – *Sigma-Aldrich*, USA), and cultured for two weeks. The cells were fixed in 4 % paraformaldehyde (PFA) solution and the induction of adipogenesis was confirmed by staining cells with Oil Red O according to standard methods [23]. For osteogenic differentiation we used DMEM medium with low glucose supplemented with 10 % FBS, 0.1 μM dexamethasone, 200 μM L-ascorbate-2-phosphate and 10 mM β-glycerol phosphate (all – *Sigma-Aldrich*, USA). Cells were cultured for 21 days, fixed in 4 % PFA and stained with Alizarin Red S [24]. The induction of chondrogenesis was carried out according to the standard procedure by adding to the DMEM culture medium with high glucose of 100 nM dexamethasone, 50 μg/mL ascorbic acid phosphate, basic fibroblast growth factor (bFGF), 100 μg/mL sodium pyruvate, 1.25 mg/mL bovine serum albumin, and 1 % Insulin-Transferrin-Selenium solution. The transforming growth factor beta (TGF-β) was added to the medium at a concentration 10 ng/mL. The cultivation was performed in micromass culture and the medium was replaced every 3 days [22]. After 3 weeks, the cultures were fixed in 4 % PFA, stained with alcyan blue [25] and analyzed under a microscope.

STATISTICAL ANALYSIS OF RESULTS

The Mann-Whitney U test was used to compare differences between the values of the indicators in the groups using the MS Excel software (Microsoft, USA). Differences were considered significant at $p < 0.05$. Data are presented as Mean ± SD.

RESULTS AND DISCUSSION

Given the medium which consisted of 10 % of DMSO and 90 % of FBS as control, we investigated various combinations of cryoprotectants which had to provide the following conditions:

- the highest percentage of cell viability after thawing;
- the minimum content of DMSO to prevent adverse reactions when using cells for transplantation in humans;
- the ability of thawed cells to proliferate at the same rate as cryopreserved in the control medium;
- the expression of the typical surface markers at the control level.

As a result of the research, it was shown that the number of cells after thawing in all variants was almost the same and was 0,925-0,940•10^6 cells compared with 1•10^6 before cryopreservation. The reason for the decrease in the number of cells, most likely, is the loss due to washing from DMSO.

The data on the number of viable cells after freezing/thawing are shown in Table 2. These values were obtained by staining the suspension with Trypan Blue and almost correspond with the data obtained by flow cytometry. They indicate that of the studied cryopreservation media, type 1 and 4, consisting of 10 % DMSO + 90 % FBS and 4 % DMSO + 6 % trehalose + 90 % FBS, respectively, are the best composition to the preservation of cell viability. On the other hand, the lowest content of viable cells was observed during the cryopreservation in medium type 2 ($p \leq 0.05$), which consisted of 25 % ethylene glycol + 10 % sucrose + 65 % FBS (**Table 2**).

According to our data, different media for cryopreservation did not change the expression level of the typical surface markers CD73, CD90 and CD105 (**Fig. 1**). In all options, more than 90 % of the cells expressed these stromal markers, while less than 1 % of the cells expressed the hematopoietic markers CD34 and CD45 (**Table 3**).

The data on the preservation of MMSCs markers after thawing correspond with the results of other researchers [26]. Such stability of markers at changes of other characteristics can testify to a possibility of their

TYPE	THE COMPOSITION OF THE CRYOPRESERVATION MEDIUM	THE PERCENTAGE OF VIABLE CELLS, N = 3
1	10 % DMSO + 90 % FBS	95.9 ± 0.1
2	35 % trehalose + 65 % FBS	31.3 ± 0.07*
3	25 % ethylene glycol + 10 % sucrose + 65 % FBS	71.07 ± 0.03*
4	4 % DMSO + 6 % trehalose + 90 % FBS	94.3 ± 0.2
5	15 % ethylene glycol + 3 % DMSO + 10 % sucrose + 12 % trehalose + 60 % FBS	85.4 ± 0.1

Table 2. The percentage of viable cells in thawed samples after cryopreservation medium of different composition
*Note: * – $p \leq 0.05$ compared to type 1.*

GROUP	CD90	CD73	CD105	CD34	CD45
Before cryopreservation	94.9	97.7	97.0	6.0	0.2
1	97.2	98.5	98.8	0.4	0.1
2	99.9	100	96.3	0.2	0.1
3	99.8	100	99.2	0.4	0.1
4	99.9	100	100	0.1	0.0
5	99.9	100	98.9	0.3	0.1

Table 3. The expression of minimal surface markers of MMSCs after freezing in cryoprotective medium of different composition, %.
Notes: 1 - 10 % DMSO + 90 % FBS; 2 - 35 % trehalose + 65 % FBS; 3 - 25 % ethylene glycol + 10 % sucrose + 65 % FBS; 4 - 4 % DMSO + 6 % trehalose + 90 % FBS; 5 - 15 % ethylene glycol + 3 % DMSO + 10 % sucrose + 12 % trehalose + 60 % FBS.

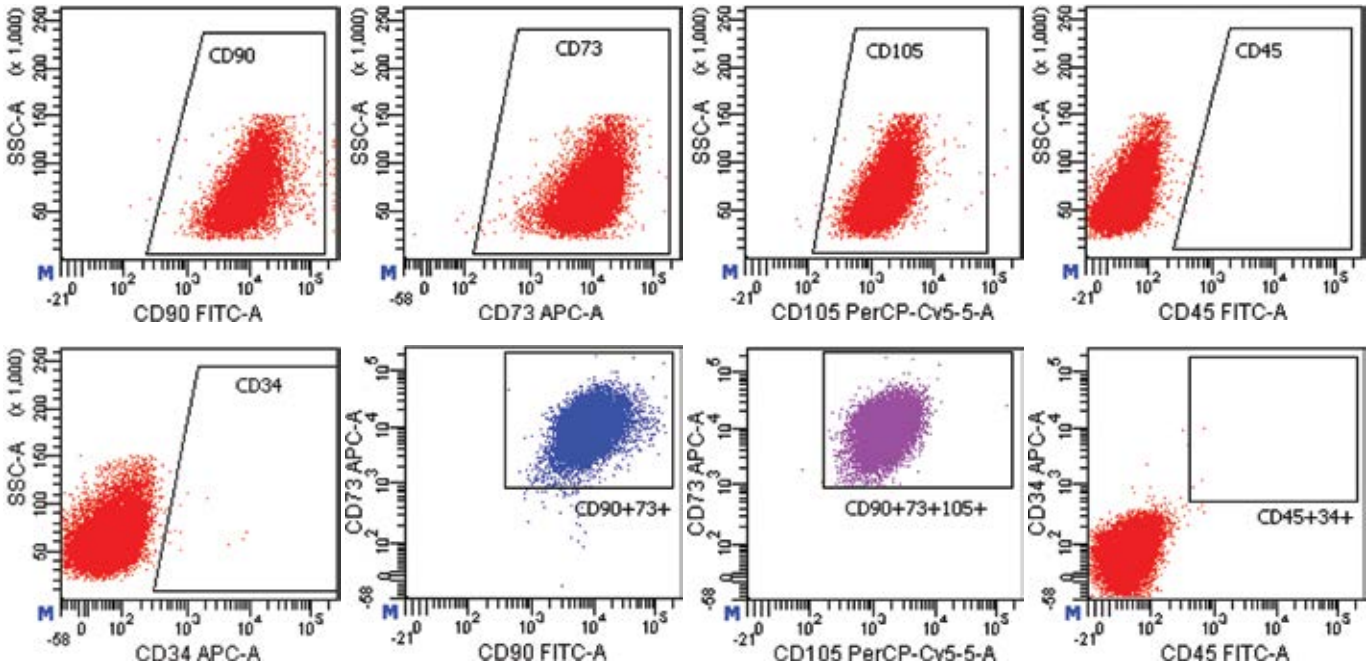

Fig. 1. Dot-lot histograms of CD90, CD73, CD105, CD34, and CD45 expression in human Wharton's jelly MMSCs culture after freezing/thawing in cryopreservation medium type 4.

use only for the rough characteristic of population of cells, first of all, for confirmation of their belonging to the type of mesenchymal stromal cells.

The defining of cells to the type of multipotent mesenchymal stromal cells is also confirmed by their ability for directed differentiation into the osteogenic, chondrogenic and adipogenic lineages after freezing and thawing as well as for native MMSCs without cryopreservation. It is shown that this property of MMSCs does not change after cryopreservation, which is confirmed by the presence of lipid granules stained by Oil Red O during differentiation into adipocytes. Osteogenic differentiation revealed alizarin staining of calcium deposits in a bright orange-red color, while undifferentiated MMSCs were pale pink. The presence of characteristic bright blue clusters of proteoglycans, stained with alcyan blue around the cells which differentiated into chondrocytes, was also demonstrated. Our data on thawed cells are confirmed by the results of other authors [27, 28], which indicate the preservation of the ability of frozen/thawed cells to typical differentiation into three cell types – adipo-, osteo- and chondrocytes.

The obtained results are interesting because they show that the concentration of DMSO has no effect on this characteristic, as in the case of surface markers expression. Our results correspond with the review of Bahsoun S. et al. [29], which summarizes the data of many authors who performed freezing of MMSCs using both commercial ready-to-use cryopreservation media, including low concentrations of DMSO, and cryoprotectants freshly prepared immediately before the use. At the same time, it should be noted that in some experiments presented in this review, there was an increase and decrease in some quantitative indicators of MMSCs differentiation (e.g., alkaline phosphatase activity) after the cryopreservation, although, in general, qualitative characteristics preserved.

Thus, none of the cryoprotectants used in our experiments changed either the expression of surface markers or the ability of cryopreserved cells to differentiate. In this regard, the question what characteristics change during cryopreservation arises. Therefore, the next stage of research was to study the proliferation of MMSCs after cryopreservation using different cryoprotectants.

The growth rate of cells after thawing in different variants of cryopreservation media differed significantly. After 1 day, in type 1 and 4, a significant number of adhered cells were observed (**Fig. 3-A** and **5-A**). It was found that when seeding $1 \cdot 10^5$ cells, their number increased to $3.8\text{-}3.9 \cdot 10^5$ after 2 days, which corresponds to the usual growth rate of Wharton's jelly MMSCs (**Fig. 2-B** and **5-B**). After 3 days, almost 80 % of the monolayer confluence was observed, which indicated to the intensive cell proliferation. In group 2, a minimal number of viable cells was observed, after 1 day there were single adherent cells, and after 2 days there was cell degeneration (**Fig. 3-A, B**). Group 3 and 5 occupied an intermediate position. Some of the cells adhered, but after 2 days the number of cells was only $2 \cdot 10^5$ (**Fig. 4** and **6-A, B**). Upon further observation, by the 5th day, the growth rate slowed down, and the monolayer did not form (**Fig. 7**).

The explanation for this phenomenon was found when determining the MMSCs population doubling time after thawing. The results are shown in table 4, which shows that the shortest doubling time was observed in groups 1 and 4 (25-26 hours). After 48 hours, in these groups the monolayer confluency was observed. On the contrary, for groups 2 and 3, a much longer population doubling time was calculated – 83 and 63 hours, respectively, which was reflected in the slow increase in cell number. The lack of cell contact led to their degeneration in group 2 or the impossibility of monolayer formation in groups 3 and 5. Remarkably, the population doubling time determined for group 5 was significantly shorter (33 hours) than for group 3, but MMSCs proliferation in these variants was almost the same. This may be due to the changes in cell metabolism during cryopreservation in this cryopreservation medium, given that type 5 contains the maximum number of components compared to other groups. However, this assumption is only speculative and requires special studies on the effect of individual components on maintaining the metabolism of MMSCs unchanged.

Our results differ from those described in the review [29]. Bahsoun S. et al. analyzed the data of many authors on this issue and identified the variable and unchangeable characteristics after cryopreservation. The first ones include proliferation, morphology, differentiation and immunophenotype. If the immunophenotype and differentiation did not change in our experiments, then in contrast to the stable proliferation described by other authors, we showed that when using different cryoprotectants, cell proliferation can change. This may be due to the fact that in our work we studied the umbilical cord-derived MMSCs, while other authors cultured bone marrow-derived stromal cells. Although some of the studies described in the review show a slight decrease in cell proliferation after

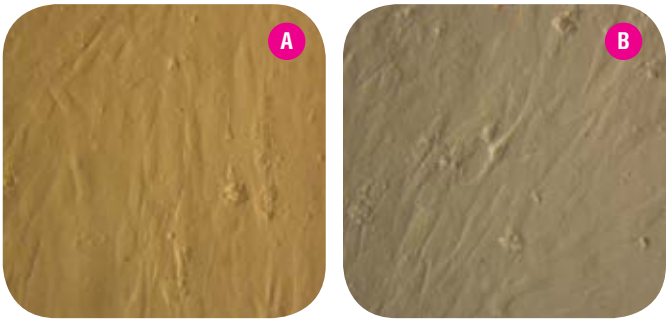

Fig. 2. Micrographs of MMSCs cultures after freezing in cryoprotective medium type 1 (10 % DMSO + 90 % FBS); phase-contrast microscopy; x100. **A** – 1 day after thawing, **B** – 2 days after thawing.

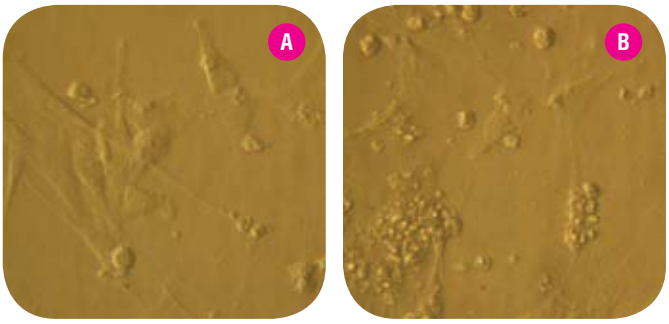

Fig. 3. Micrographs of MMSCs cultures after freezing in cryoprotective medium type 2 (35 % trehalose + 65 % FBS); phase-contrast microscopy; x100. **A** – 1 day after thawing, **B** - 2 days after thawing.

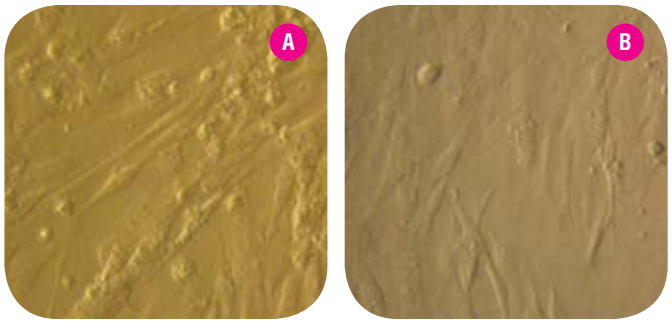

Fig. 4. Micrographs of MMSCs cultures after freezing in cryoprotective medium type 3 (25 % ethylene glycol + 10 % sucrose + 65 % FBS); live unstained drug, phase-contrast microscopy; x 100. **A** – 1 day after thawing, **B** – 2 days after thawing.

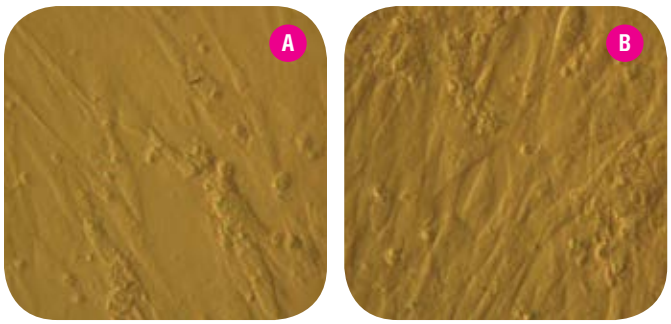

Fig. 5. Micrographs of MMSCs cultures after freezing in cryoprotective medium type 4 (4 % DMSO + 6 % trehalose + 90 % FBS); phase-contrast microscopy; x100. **A** – 1 day after thawing, **B** – 2 days after thawing.

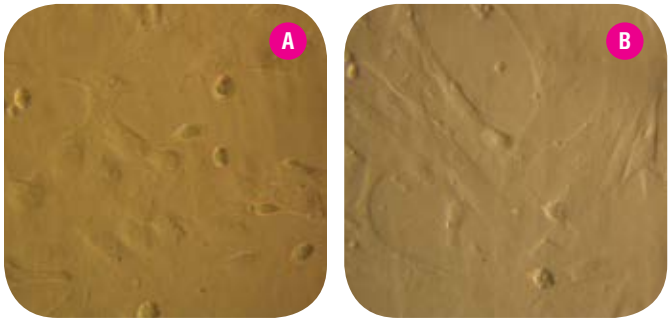

Fig. 6. Micrographs of MMSCs cultures after freezing in cryoprotective medium type 5 (15 % ethylene glycol + 3 % DMSO + 10 % sucrose + 12 % trehalose + 60 % FBS); phase-contrast microscopy; x100. A - 1 day after thawing, B - 2 days after thawing.

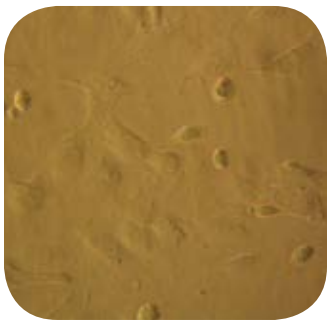

Fig. 7. Micrographs of MMSCs cultures after freezing in cryoprotective medium type 3 (25 % ethylene glycol + 10 % sucrose + 65 % FBS) 5 days after thawing; phase-contrast microscopy; x100.

freezing/thawing, this is especially true for MMSCs of later passages. According to our results, freezing of MMSCs after the 2nd passage can reduce the concentration of DMSO from 10 % to 4 % with the addition of 6 % trehalose. When using this composition of the cryoprotective medium we observed maximum preservation of proliferative activity of umbilical cord-derived MMSCs after thawing.

Variable characteristics that change during cryopreservation include viability, adhesion, immunomodulation, and metabolism [29]. Our results on maintaining cell viability confirm this. In terms of metabolic characteristics and immunomodulatory properties, this is especially important given the clinical application of MMSCs in the treatment of autoimmune diseases. Regarding these characteristics, there are differences in the analyzed studies – from complete preservation to a significant decrease

GROUP	THE COMPOSITION OF THE CRYOPROTECTIVE MEDIUM	POPULATION DOUBLING TIME, HOURS
1	10 % DMSO + 90 % FBS	25.78 ± 0.1
2	35 % trehalose + 65 % FBS	83.72 ± 0.12
3	25 % ethylene glycol + 10 % sucrose + 65 % FBS	63.30 ± 0.1
4	4 % DMSO + 6 % trehalose + 90 % FBS	26.18 ± 0.07
5	15 % ethylene glycol + 3 % DMSO + 10 % sucrose + 12 % trehalose + 60 % FBS	33.68 ± 0.09

Table 4. The MMSCs population doubling time when using cryoprotective media of different composition.
*Note: * – $p \leq 0.05$ compared to group 1.*

in metabolic activity. Therefore, to maintain these properties of MMSCs at the required level, it is necessary to develop the optimal conditions for their cryopreservation.

Our results allowed us to conclude that it is possible to significantly reduce the concentration of DMSO in the freezing medium to 4 % instead of 10 % due to the addition of at least 6 % of trehalose. This composition of the cryopreservation medium is the most suitable for use, due to the fact that DMSO is a permeable cryoprotectant that prevents the formation of water crystals inside the cell protecting its organelles. In this case, trehalose is an impermeable cryoprotectant that covers and protects the cell from the outside. This combination has significantly reduced the concentration of DMSO, which will definitely help prevent its possible negative side effects.

In the future, our research will focus on replacing xenogeneic fetal bovine serum, which is used in experiments, with an autologous or allogeneic human serum.

CONCLUSION

1. ***The human Wharton's jelly MMSCs, cryopreserved in cryoprotective media of different composition, after thawing differ in viability, adherence, expansion and monolayer formation.***
2. ***According to morphological studies and analysis of growth dynamics of cryopreserved human umbilical cord-derived MMSCs, among the studied cryoprotective media, the most effective one for maintaining cell viability, proliferative activity and confluent monolayer formation is the combination of 4 % DMSO + 6 % trehalose + 90 % FBS.***

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