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Effect of Storage Conditions on the Integrity of Human Umbilical Cord Mesenchymal Stromal Cell-Derived Microvesicles

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We studied the effect of storage conditions on the safety of microvesicles produced by human multipotent umbilical cord mesenchymal stromal cells into the conditioned medium. It was found that microvesicles can be stored without serious degradation for up to 1 week at 4ºC, but were almost completely destroyed during freezing and thawing cycles irrespective of the storage temperatures (-20ºC, -70ºC, or -196ºC). Similar results were obtained for lyophilized medium conditioned by human multipotent umbilical cord mesenchymal stromal cells. Addition of a cryoprotectant (5-10% DMSO) followed by freezing and/or lyophilization preserved microvesicles at a nearly initial level. These findings indicate that during storage, microvesicles, being membrane structures, behave similar to living cells and require appropriate conditions for prolonged storage.

Key Words: *microvesicles; multipotent mesenchymal stromal cells; umbilical cord; storage; flow cytometry*

In recent years, human umbilical cord-derived multipotent mesenchymal stromal cells (MSC) are considered to be most available and promising cell therapy product for regenerative medicine [9], due to their unique biological properties, in particular, their extremely highest proliferative and secretory activities that distinguish umbilical cord MSC from MSC derived from "adult" sources (bone marrow and adipose tissue) [7]. It is not surprising that umbilical cord MSC have become the object of not only experimental, but also clinical research. The efficacy of umbilical cord MSC has already been convincingly demonstrated for graft-versus-host disease (GVHD), myocardial infarction, chronic heart failure, type 1 and type 2 diabetes mellitus, spinal cord injuries, joint and cartilage damage, liver cirrhosis, cerebral palsy, *etc*. [9,10,11,13,16]. At the same time, most authors associate therapeutic effects of MSC to a greater extent with products of their secretion (cytokines, growth factors, and microparticles) [12,18], rather than with the ability of cells to differentiate and directly replace the affected elements in the injured area, as it was previously believed [14].

The paracrine effects of MSC can be mediated by two types of cell-secreted biological products: soluble (cytokines, chemokines, and growth factors) that have anti-inflammatory, anti-apoptotic and immunomodulatory properties and insoluble that are presented by extracellular vesicles such as exosomes, microvesicles and apoptotic bodies [17,19,21]. These structures were first described in 1983 as products of reticulocyte secretion. Later, it was discovered that extracellular vesicles are produced by other cell types: T and B lymphocytes, dendritic cells, platelets, epithelial and endothelial cells, and other cells, including umbilical cord-derived MSC [8] and play an important role in intercellular interactions, regulation of immune responses, blood coagulation, inflammation, and angiogenesis.

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Extracellular vesicles were found in various biological fluids (blood plasma, breast milk, cerebrospinal, intra-articular, and amniotic fluids, ascites, *etc*.). In contrast to soluble molecules, extracellular vesicles are more resistant to degradation in the bloodstream and apparently can transfer biological information in the body over a longer distance. At least under experimental conditions, MSC-derived microparticles were effective in models of cardiovascular diseases, oxidative stress, ischemia-reperfusion injury, as well as in injuries of the CNS, bone and cartilage, lungs and immune system [11,19,21].

It is believed that the use of MSC-secreted derivatives is associated with lesser risk in clinical applications in comparison with application of therapeutic products containing viable cells. In the most typical case, the culture medium conditioned by tens of millions MSC can represent almost ready to use therapeutic product [12,20]: most soluble factors produced by umbilical cord MSC [14] exhibit strong angiogenic (VEGF, HGF, FGF, IL-6, and MCP-1), anti-apoptotic (VEGF, HGF, FGF, GM-CSF, and IL-6), immunomodulatory (HGF, IL-6), or neuroprotective (BDNF, NGF, and GDNF) effects; some of them can regulate several parts of homeostasis [9].

This is why the products secreted by umbilical cord MSC, including extracellular vesicles, are not less interesting than MSC themselves. Potentially, they can become a universal therapeutic product due to the relative simplicity and low cost of manufacturing, quality control during production, and the absence of potential risks associated with the use of whole cells [6]. Meanwhile, if the optimal storage conditions of MSC-secreted soluble molecules of protein or peptide origin are well known, the data on extracellular vesicles (in particular microvesicles) safety are scanty and sometimes contradictory.

In the previous study [8], we obtained quantitative data on the microvesicles production by cultured umbilical cord MSC and provided their phenotypic characteristics.

The purpose of this work was to assess the integrity of microvesicles in the conditioned medium (CM) of umbilical cord MSC in different storage regimens and to select conditions ensuring maximum protection of the therapeutic potential of this cell-free "cellular product".

MATERIALS AND METHODS

Detailed methods of MSC isolation from human umbilical cord tissue (UC-MSC) and their characterization, as well as the procedure for obtaining UC-MSC CM were described in detail in our previous publications [2-8]. The cells were cultured in DMEM/F-12 medium (Gibco Invitrogen) supplemented with antibiotics and 10% fetal calf serum (HyClone). Passage 2-3 cultures from 3 donors were used in the study. As in the previous study, the culture medium was filtered through 0.1-μ filters before addition to MSC, to reduce the unwanted "background" [8].

Storage conditions. The media conditioned for 48 h were collected, centrifuged for 20 min at 3000 rpm to precipitate cell debris, and divided into aliquots of 2-3 ml. The "standard" storage conditions included incubation at 4-6°C or freezing and storage at -20°C, -70°C, and -196°C (in liquid nitrogen) in cryotubes. To some aliquots of fresh conditioned media, DMSO was added at a concentration of 10%, and the samples were frozen either under the conditions mentioned above or using programmable freezing according to the protocol designed for cryopreservation of umbilical cord blood hematopoietic stem cells [1] followed by storage in liquid nitrogen. The number of microvesicles in the corresponding fresh CM was taken as 100% (control).

Lyophilization of CM was carried out in glass vials (1 ml CM in 10-ml vial) in FreeZone system (Labconco) according to manufacturer's program after freezing the samples at -70ºC in the presence of 2.5-10% DMSO. The storage time for cooled, frozen, or freeze-dried CM was 2-3 weeks.

Identification and assessment of microvesicles integrity. Flow cytometry (FACSCalibur, BD) was used to detect microvesicles and determine their number before and after storage [8]. Prior to staining, the aliquots of frozen CM were thawed in a water bath at 37ºC, and the freeze-dried samples were dissolved in distilled water to the initial volume of 1 ml. Then, 20 μl phycoerythrin-labeled antibodies (CD29, Beckman Coulter) specifically staining more than 95% microvesicles were added to 200 μl CM and incubated overnight at 4ºC. For evaluation of the absolute amount of microvesicles, they were stained in Trucount tubes (BD).

Statistical analysis. Statistical processing of the results was performed using Statistica 7.0 software.

RESULTS

The analysis of native microvesicles coincided with the results obtained earlier: microvesicles were highly CD29-positive and their concentration in the media conditioned by MSC was ~20-30 mln/ml.

Storage for 1 and 2 weeks at 4°C was not associated with a "dramatic" decrease in the concentration of microvesicles compared to the initial media (95.0±2.0 and 64.8±36.0% of intact microvesicles, respectively; Fig. 1). Only by the end of the third week, the concentration of microvesicles decreased by more than 50% of the initial level.

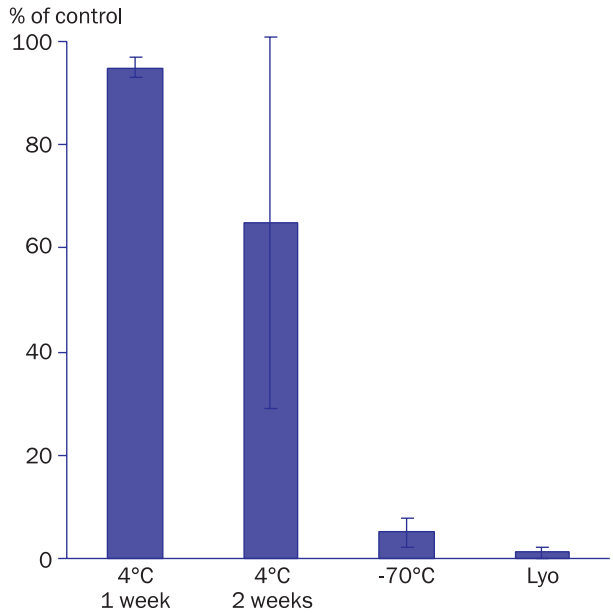

Fig. 1. Changes in the content of $CD29^+$ microvesicles in UC-MSC CM stored at 4ºC (standard cooling), -70ºC (freezing), and freeze-dried (Lyo). The data are presented as *M±SD* for 3 parallel UC-MSC cultures.

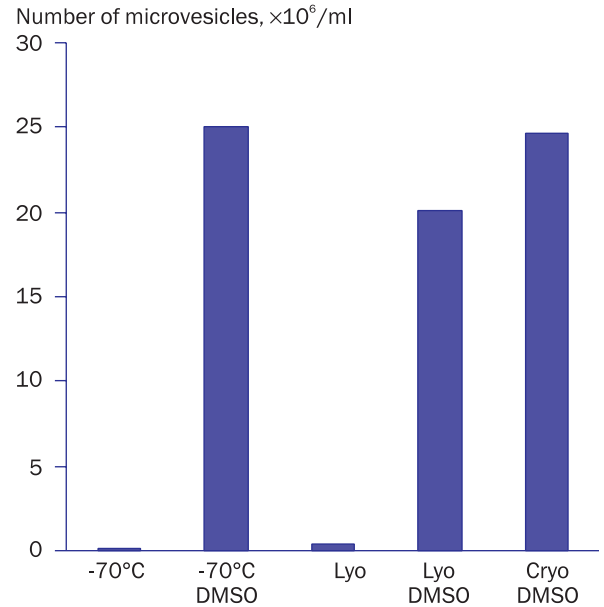

Fig. 2. The effect of 10% DMSO on microvesicle integrity in frozen (-70ºC), cryopreserved (Cryo), and lyophilized (Lyo) UC-MSC CM. Results of one representative experiment are shown.

Quantitative analysis of microvesicles in frozen CM yielded disappointing results: <1-5% preserved microvesicles irrespectively of storage temperatures (Fig. 1; the data for -20ºC and -196ºC are not shown). Repeated freezing/thawing cycles led to almost complete degradation of microvesicles. Similar results (1.1±1.0%) were obtained for CM subjected to freezing at -70ºC followed by lyopholization.

Based on the obtained results, it can be concluded that microvesicles poorly tolerate storage in a frozen state and the damage occurs, most likely, not during storage, but during freezing procedure. Therefore, in further experiments freezing and storage of CM were performed after addition of 10% DMSO. The results were apparent: after "standard" or programmed freezing, the concentration of microvesicles increased to 80-95% of the initial level (Fig. 2). Similar results were obtained in CM samples frozen at -70ºC in the presence of DMSO and then subjected to lyophilization.

DMSO is widely used for freezing and cryogenic storage of mammalian cells, including human MSC and UC-MSC. The optimal DMSO concentration in the freezing medium is ~7.5-10%. Lesser concentrations are inefficient in maintaining cell viability and the higher concentrations are cytotoxic. However, there are no published data on optimal concentrations of the cryoprotectant for storage of microvesicles.

Therefore, in a special series of experiments we selected optimal conditions ensuring effective protection of both soluble bioactive factors and membrane structures present in MSC CM. In this case, CM samples were subjected to freeze-drying after addition of 2.5, 5, 7.5, and 10% DMSO. Initial CM samples or media lyophilized without DMSO were used as the control. The best results were obtained at DMSO concentration of 5% (Figs. 3, 4). It is noteworthy that higher (7.5 and 10%) DMSO concentrations, usually used in cryopreservation of living cells, in the case of microvesicles were less effective, probably due to membrane damage caused by solvent and subsequent destruction.

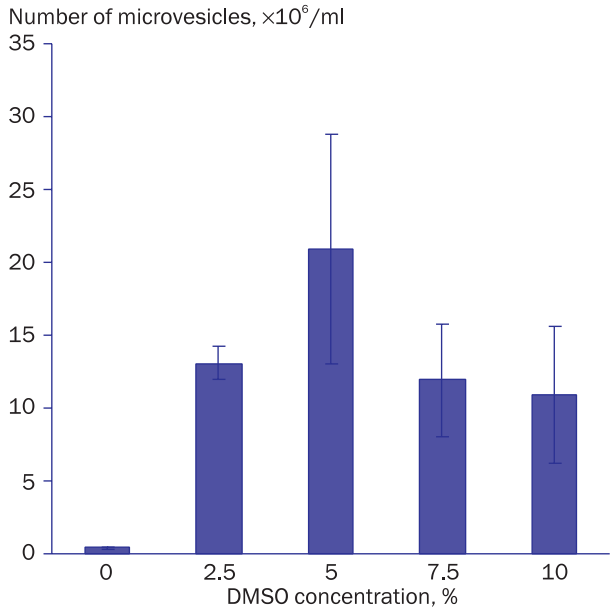

Fig. 3. Effect of DMSO concentration on microvesicle integrity in freeze-dried UC-MSC CM. The data for 3 parallel UC-MSC cultures are presented.

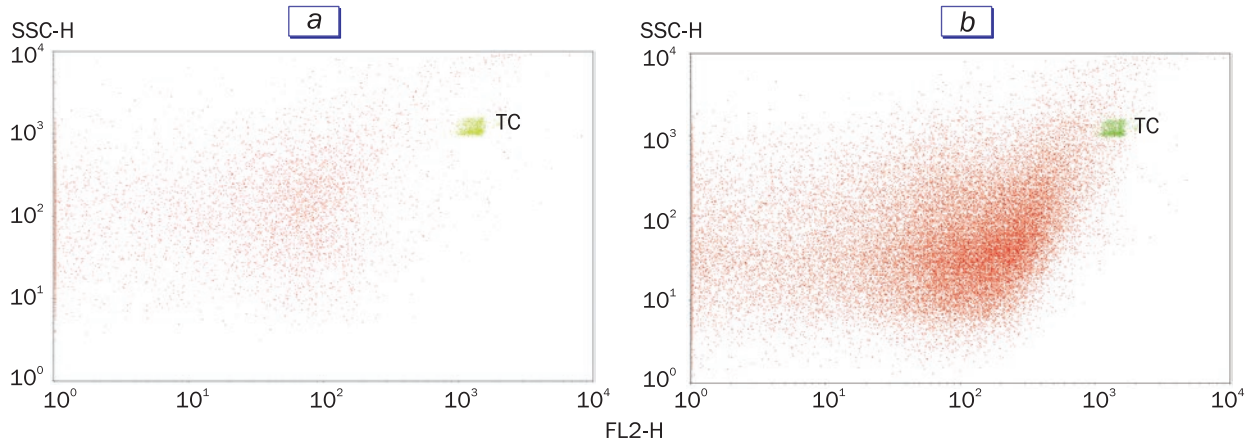

Fig. 4. Flow cytometry results reflect low and high efficiency of microvesicle protection during freeze-drying of UC-MSC CM without (*a*) or with (*b*) 5% DMSO. The number of Trucount beads (TC) is identical (1000±25 microspheres).

Taken together, the data obtained show that microvesicles during freezing and storage "behave" similar to living cells, and their maximum preservation can be achieved under conditions excluding damage to membranes by ice crystals. The best results (>90-95% intact microvesicles from their initial content) can be achieved lower DMSO concentration in comparison with those used for freezing living cells. Subsequent lyophilization increases the effective shelf life of such combined cell-free therapeutic product to several years, which is sufficient for appropriate preclinical and/or clinical studies.

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