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CRYOPRESERVATION OF L929 CELL CULTURE IN PROTECTIVE SOLUTIONS CONTAINING HYALURONIC ACID

Recently, cryobiological studies have focused on the prospects of using hyaluronic acid (HA) as a component of protective media during cryopreservation of various cell types. HA is a polysaccharide of natural origin and an integral component of the extracellular matrix, which determines its high biocompatibility and potential protective properties for cells under stressful conditions, in particular during freezing. The paper presents the results of cryopreservation of L929 cells using protective solutions containing 0.5% HA of various molecular weights as well as 5% of the classical endocellular cryoprotectant DMSO. Experimental protocols for the freezing of cells differed in the cryoprotective solution composition, the method of adding its components to cells, and the duration of cell exposure. The effectiveness of the cryopreservation protocols used was assessed by the viability of L929 cells and their adhesive properties. The results obtained showed that HA, regardless of its molecular weight, did not affect the penetration of DMSO through the membranes of L929 cells. The use of a cryoprotective solution containing only low-molecular-weight HA ensured cell survival at (72 ± 4.2) %, which did not differ from the values for the standard protocol. For high-molecular-weight HA, this index decreased to (42 ± 4.8) %. Regardless of the cryopreservation protocol, L929 cells retained the ability to attach to an adhesive surface. However, further growth and proliferation of cells largely depended on the composition of the cryoprotective solution and the conditions of administration of its components. Thus, it was shown that both low-molecular-weight HA and high-molecular-weight HA exhibited pronounced cryoprotective properties and can be used either as components of protective media in combination with DMSO, or as an independent impermeable cryoprotectant.

Key words: *cryopreservation, cryoprotectants, L929 cell culture, dimethyl sulfoxide, hyaluronic acid, cell adhesion.*

One of the key issues related to low-temperature storage of cell suspensions is optimizing the composition of cryoprotective solutions. Traditionally, they include permeable low-molecular cryoprotectants such as dimethyl sulfoxide (DMSO), glycerol, propylene glycol, etc. These substances prevent intracellular crystallization, but can have a cytotoxic effect that depends on their concentration. As a rule, to stabilize cell membranes, traditional cryoprotective solutions contain serum or albumins, which can cause additional contamination [6, 7]. To eliminate these risks, high-molecular impermeable impurities (sucrose, trehalose, polyampholytes, polyvinyl alcohol, dextran, *etc*.) are added to the composition of cryoprotective solutions [8—10]. They allow the reduced concentration of permeable cryoprotectants, ensure effective cell dehydration and extracellular protection, affecting the course of ice crystallization and recrystallization.

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Recently, a number of publications have appeared on the use of hyaluronic acid (HA) of various molecular weights as a cryoprotectant, which is a natural glycosaminoglycan and an integral component of the extracellular matrix [5, 11, 15]. HA molecules are involved in such biological processes as cell signaling, regulation of cell adhesion and proliferation, and regeneration [4, 5, 14]. The listed properties determine the low cytotoxicity of HA, and the high ability of its molecules to hydrate allows us to consider this substance as a promising extracellular cryoprotectant [3]. There are some reports on the effectiveness of HA in protective solutions for cryopreservation of various cell types [5, 11, 12] and on its stabilizing effect for plasma membranes [15]. In future, such properties of HA will contribute to the creation of serum-free media for cryopreservation of cell suspensions [10]. However, it should be noted that the protective mechanism of HA is not fully understood. In addition, the question of determining the concentration of HA in cryoprotective solutions and its dependence on molecular weight remains unresolved.

The research aim was to study the effect of hyaluronic acid solutions of different molecular weights on the preservation of L929 cell cultures during cryopreservation under different conditions of adding hyaluronic acid and dimethyl sulfoxide to the protective solution.

MATERIALS AND METHODS

The object of the study was the L929 cell culture, which was stored at Low-temperature Bank of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine (Kharkiv). The samples for the study were previously thawed in a water bath at 37 °C, washed from the cryopreservation medium and cultured in DMEM/F12 (BIOWEST, France) with the addition of antibiotics (200 U/ml benzylpenicillin, 200 μg/ml streptomycin and 5 μg/ml amphotericin B) and 10% fetal calf serum (FCS) (BIOWEST) in plastic culture bottles (Bioswesstec, Switzerland) under conditions of 37 °C with a CO_2 content in the atmosphere of 5%. The cell line was maintained for three passages. For experiments, the cell monolayer was detached from the surface of the flask by sequential treatment for 2 min with Versene solution (Vetline, Ukraine) and 0.25% trypsin solution (BIOWEST) for 10 min. Then, the cells were washed from the enzymatic solution by 3-min centrifugation in DMEM/F12 with the addition of 10% FCS at 800 g and used in further experiments. DMSO (Sigma-Aldrich, USA) was added to the protective solutions as a permeable cryoprotectant. In addition, the experimental solutions contained 0.5% HA (Bang&Bonsomer, Finland): low molecular weight (LMWH) (10—100 kDa) or high molecular weight (HMWH) (> 2000 kDa).

L929 cells were cryopreserved according to standard protocol 1 : exposure of cells in a solution of 10% DMSO with the addition of 10% FCS and subsequent freezing [1]. The study comprised experimental protocols (2—10), which differed from the standard freezing regimen in the composition of the cryoprotective solution, duration of incubation and the way of adding its components to the cells: 2—10-min exposure in a solution of 0.5% LMHA, subsequent freezing; 3—10-min exposure in a solution of 0.5% HMGA, subsequent freezing; 4—10-minute exposure in a solution of 5% DMSO, subsequent freezing; 5—10-min exposure in a solution of 0.5% LMHA and 5% DMSO, subsequent freezing; 6—10-min exposure in a solution of 0.5% HMHA and 5% DMSO, subsequent freezing; 7—5-min exposure in a solution of 5% DMSO, addition of 0.5% solution of LMHA, 5-min exposure, subsequent freezing; 8—5-min exposure in a solution of 5% DMSO, addition of 0.5% solution of HMHA, 5-min exposure, subsequent freezing; 9—5-min exposure in a solution of 0.5% LMHA, addition of 5% solution of DMSO, 5-min exposure, subsequent freezing; 10—5-min exposure in a 0.5% HMHA solution, addition of 5% DMSO solution, 5-minexposure, subsequent freezing.

The use of protocols 5—10, which investigated the effect of simultaneous or alternate addition of solution components to cells, was due to the need to clarify the question of the effect of HA molecules of various molecular weights on the permeability of L929 cell membranes for DMSO as an endocellular cryoprotectant.

After incubation in experimental solutions at room temperature, the cells (concentration 10^6 cells/ml) were placed in cryoampoules (Nunc, USA) with a capacity of 1.8 ml and frozen at a rate of 1 degree/min to –80 °C with subsequent immersion in liquid nitrogen. Cryoampoules were stored in liquid nitrogen for 3 days.

The samples were thawed in a water bath at 37 °C until the solid phase disappeared, washed from

the cryoprotectant solution by centrifugation in DMEM/F12 with the addition of 10% FCS and transferred to 24-well culture plates for further cultivation under conditions of 37 °C, 5% CO_2 and 100% humidity. The seeding concentration of cells per well was 10^4 cells/ml, the number of replicates for each sample was at least three.

The survival of cells in suspension immediately after thawing was assessed by the standard staining with 0.4% trypan blue solution, which was added to the cell suspension in a 1 : 1 ratio.

Cell development in culture was observed for 5 days, after which the samples were fixed in ethanol solutions and stained with hematoxylin solution. Monolayer confluency was assessed in percent according to the method of E.M. Plaksina et al. [13]. The monolayer area was determined using the "AxioVision Rel. 4.8" software(Carl Zeiss, Germany). The relative monolayer area was calculated by the formula:

$$S = \Sigma Si/Sn \times 100\%,$$

where Si is the area of the region with stained cells; Sn is the plate well area.

Quantitative experimental data were presented in the form of M ± m, where M is the mean value, m is the standard deviation. Statistical significance was assessed using one-way analysis of variance, with differences considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Fig. 1 shows the data on the survival of L929 cells by trypan blue staining immediately after thawing. The survival of cells cryopreserved according to standard protocol 1 was (68 ± 3.1)%. Reducing the concentration of DMSO in the cryoprotective solution to 5% (protocol 4) almost threefold reduced the survival rate (22 ± 2.9)%. Adding HA to the cryoprotective solution containing 5% DMSO significantly increased the survival rate of cells regardless of its molecular weight and the way of the protective agent introduction (protocols 5—10). It can be assumed that the presence of HA molecules, which is a natural polymer, regardless of its molecular weight does not affect the permeability of DMSO molecules through the membranes of L929 cells. This assumption is consistent with the data of S. Garantziotis *et al.* [2], where the authors showed that HA, due to its molecular structure and ability to bind a significant amount

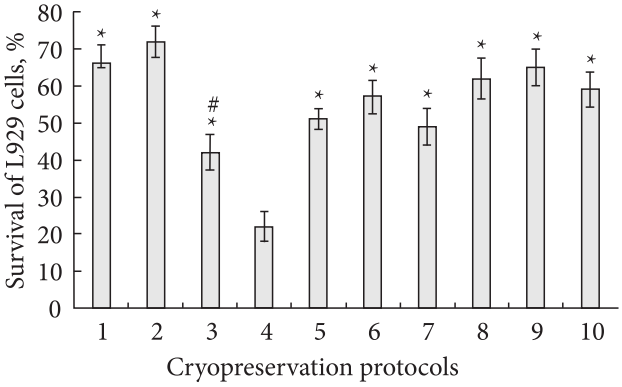

Fig. 1. Survival of L929 cells after freeze-thawing by trypan blue staining according to cryopreservation protocols. * — significant differences compared to the indices of protocol 4, $p < 0.05$; # — significant differences compared to the indices of protocol 1, $p < 0.05$

of free water, forms a viscoelastic shell around the cell, which allows low-molecular substances such as electrolytes and nutrients to diffuse.

Regarding the results of cryopreservation of L929 cells in a protective solution containing only LMHA (protocol 2), the cell survival (72 ± 4.2) % did not differ from that using the standard protocol 1, while for solutions containing HMHA (protocol 3) it was significantly lower (42 ± 4.8) %. The findings are consistent with the literature data and indicate a possible stabilizing effect of HA on cell membranes. This fact has also been proven for other cell types [11, 15]. In addition, a pronounced cryoprotective effect of LMHA was shown in murine fibroblasts [15] and encapsulated stem cells [5].

The survival of L929 cells after cryopreservation according to protocols 5 and 6 (simultaneous addition of HA and DMSO) did not differ significantly, but was lower after using the standard protocol and almost twice as high compared to protocol 4.

The alternate addition of DMSO and NMHC (protocols 7 and 9) affected cell survival. No significant differences in cell survival were found under the conditions of alternate addition of DMSO and NMHC (protocols 8 and 10). The results of observation of the development of L929 cells in culture for 5 days showed that using all experimental protocols, the cells demonstrated the ability to attach to the adhesive surface. However, the further growth of the cell culture depended on the composition of the cryoprotective solution and the conditions of adding its components. On the 5th day of cultivation, the confluency of the monolayer in samples cryopreserved according to

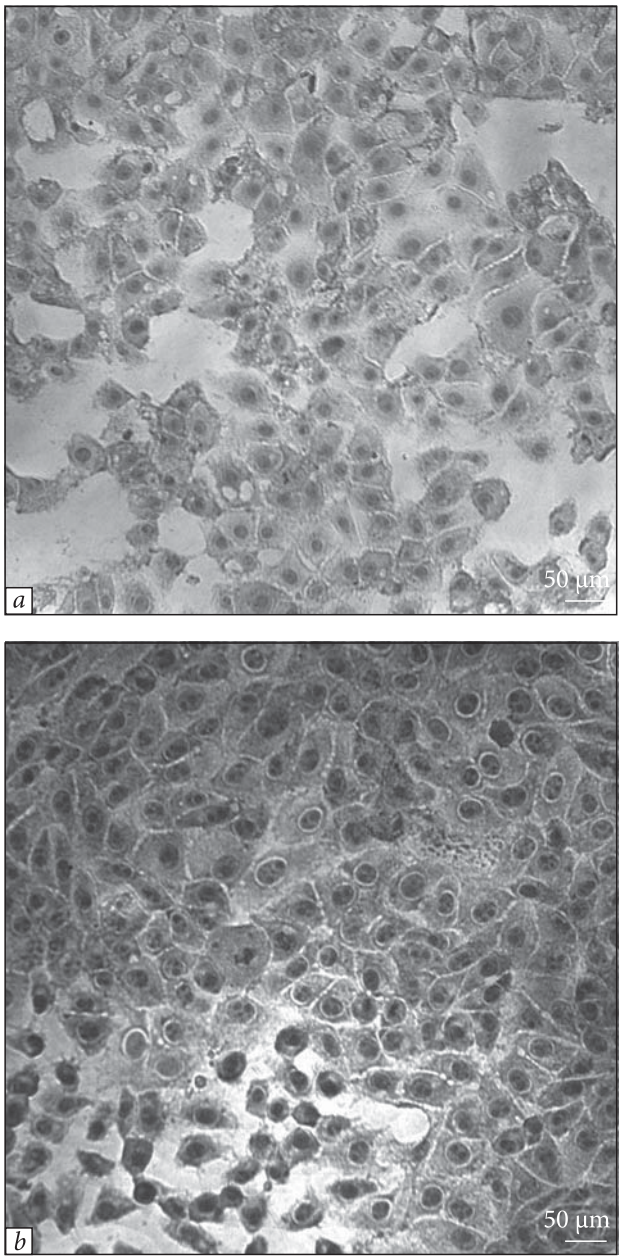

Fig. 2. L929 cell culture on day 5 of cultivation after cryopreservation according to protocol 2 (0.5% solution of LMHA) (*a*) and protocol 3 (0.5% solution of HMHA) (*b*)

protocols 2 (Fig. 2, *a*) and 3 (Fig. 2, *b*) was 58 and 51%, respectively. These indicators differed slightly from those for protocol 1 (65%). The lowest monolayer confluency values (14%) were observed in samples cryopreserved according to protocol 4. The obtained data confirm the results described above regarding the low efficiency of 5% DMSO as an independent protective solution during cryopreservation of L929 cells.

Despite the widespread use of L929 cell lines in experimental studies, there are few literature sources devoted to the study of the influence of cryopreservation conditions on their preservation (justification of the choice of the cryoprotectant solution composition, concentrations of its components and the conditions of addition, temperature-time parameters of cell exposure). The most detailed study devoted to the influence of various cryoprotectants and their concentrations on the viability of L929 cells during cryopreservation was reported by K. Matsumura et al. [9]. However, the main attention in this research has been paid to the study of the protective properties of poly-L-lysine derivatives in the composition of cryoprotectant solutions.

Thus, further studies should be aimed at finding effective concentrations of HA taking into account its molecular weight in order to further reduce the concentration of DMSO and prevent its toxic effect on cells during cryopreservation.

CONCLUSIONS

1. In protective solutions for cryopreservation of L929 cells, HA can be used as a component of the cryoprotective solution both in combination with DMSO and as an independent impermeable cryoprotectant.

2. The effectiveness of using HA in the composition of the cryoprotective solution depends on its molecular weight. LMHA ensured the preservation of L929 cells at the level of (72 ± 4.2) % and did not differ from the indices of the standard protocol. The use of HMHA reduced the preservation of cells to (42 ± 4.8)

3. The adhesive properties of L929 cells did not depend on the composition of the cryoprotective solution and the conditions of the introduction of its components, but affected their further growth and proliferation for all experimental cryopreservation protocols.

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КРІОКОНСЕРВУВАННЯ КУЛЬТУРИ КЛІТИН L929
У ЗАХИСНИХ РОЗЧИНАХ ІЗ ВМІСТОМ ГІАЛУРОНОВОЇ КИСЛОТИ

Останнім часом у кріобіологічних дослідженнях приділяється увага перспективності використання гіалуронової кислоти (ГК) як компонента захисних середовищ під час кріоконсервування різного типу клітин. ГК є полісахаридом природного походження і невід'ємною складовою позаклітинного матриксу, що обумовлює її високу біосумісність та потенційні захисні властивості щодо клітин у стресових умовах, зокрема під час заморожування. У роботі наведено результати кріоконсервування клітин L929 з використанням захисних розчинів з вмістом 0,5 % ГК різної молекулярної маси та 5 % класичного ендоцелюлярного кріопротектора ДМСО. Експериментальні протоколи заморожування клітин відрізнялися складом кріозахисного розчину, способом додавання його складових до клітин та тривалістю експозиції клітин. Ефективність застосованих протоколів кріоконсервування оцінювали за показниками життєздатності клітин L929 та їх адгезивних властивостей. Отримані результати показали, що ГК незалежно від її молекулярної маси не впливає на проникнення ДМСО крізь мембрани клітин L929. Використання кріозахисного розчину, який містив лише низькомолекулярну ГК, забезпечувало збереженість клітин на рівні (72 ± 4,2) %, що не відрізнялося від показників для стандартного протоколу. Для високомолекулярної ГК цей показник знижувався до (42 ± 4,8) %. Незалежно від протоколу кріоконсервування клітини L929 зберігали здатність до прикріплення до адгезивної поверхні. Проте подальший ріст та проліферація клітин значною мірою залежали від складу кріозахисного розчину та умов введення його компонентів. Таким чином, показано, що як низькомолекулярна ГК, так і високомолекулярна ГК проявляють виражені кріозахисні властивості і можуть використовуватися як складові захисних середовищ у поєднанні з ДМСО, або як самостійний непроникний кріопротектор.

***Ключові слова**:* кріоконсервування, кріопротектори, культура клітин L929, диметилсульфоксид, гіалуронова кислота, адгезія клітин.