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Original article

THE EFFECT OF LYOPHILIZED AND FROZEN UMBILICAL CORD CRYOEXTRACT ON L929 CELL CULTURE

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Background. The human umbilical cord is a promising source of biologically active substances with regenerative properties. However, the potential of lyophilized cryoextract from the umbilical cord for regenerative medicine, which could facilitate storage and transportation, remains unexplored. Therefore, it is important to study the effect of such cryoextracts using a cellular model.
Objective. To evaluate the effect of lyophilized and frozen umbilical cord cryoextracts on the L929 cell line to assess their therapeutic potential.
Methods. This study was conducted on L929 cell cultures. Cryoextracts from the human umbilical cord were obtained through cryoextraction and lyophilized forms at –80 and –20 °C. These extracts were added to Dulbecco's Modified Eagle Medium (DMEM) at three concentrations: 0.1, 0.5, and 1.0 mg/ml. The control groups included cells cultured in DMEM with and without fetal bovine serum. Cell morphology and monolayer confluency were observed. To assess the impact of the cryoextracts, several assays were performed: cell viability (adhesion), migration activity (scratch test), pinocytosis activity (neutral red uptake assay), metabolic activity (MTT assay), and (proliferation) population doubling time.
Results. The addition of umbilical cord cryoextract and its lyophilized form at –80 °C was non-toxic to the cells. The most effective concentration was 0.1 mg/ml, which significantly stimulated cell adhesion and proliferation compared to the culture medium without fetal serum. The lyophilized cryoextract at –20 °C did not enhance cell viability but did increase pinocytosis activity.
Conclusions. These findings suggest that umbilical cord cryoextract and its lyophilized form at –80 °C can be used as growth factors in cell line cultivation. The lyophilized cryoextract shows promise for use in conditions where specialized storage equipment is not available. However, the lyophilized form at –20 °C primarily stimulates pinocytosis activity and inhibits proliferation.
Keywords: cryoextract; cryopreservation; L929 Cell Line; lyophilization; umbilical cord; cell culture; viability; metabolic activity.

Introduction

The human umbilical cord is a crucial physiological structure that connects the fetus to the placenta throughout pregnancy, ensuring vital functions that support fetal development. It comprises three main vessels – two arteries and one vein – encased in Wharton's jelly, a gelatinous substance rich in hyaluronic acid, chondroitin sulfate, and growth factors. This composition protects the vessels from damage and maintains stable blood circulation between the fetus and placenta [1, 2]. Furthermore, the umbilical cord lacks innervation, enhancing its resistance to external influences, while both umbilical cord blood and Wharton's jelly are rich sources of mesenchymal stem cells (MSCs) [3].

Recent medical research has shown growing interest in the umbilical cord as a source of biologically active substances and stem cells for therapeutic applications. A key focus has been the effects of umbilical cord cryoextracts and their components on cells and tissues. Umbilical cord MSCs, in particular, have potent regenerative properties and immunomodulatory effects, positioning them as promising candidates for cell therapy [4, 5]. The use of umbilical cord-derived biomaterials avoids the ethical concerns associated with obtaining cells from embryonic tissues [6].

Umbilical cord derivatives are actively used in regenerative medicine for the treatment of conditions such as diabetes, cardiovascular diseases, nervous system disorders, bone regeneration, and wound healing [7–9]. For instance, MSCs from the umbilical cord have been shown to reduce inflammation and promote tissue regeneration via paracrine signaling [10]. However, the potential of umbilical cord cryoextract remains underexplored.

A key challenge is optimizing the storage and processing of umbilical cord biological material. Cryopreservation and lyophilization are promising

methods in this regard. Lyophilization, a dehydration process, enables long-term storage of biologically active substances without significant loss of their properties. This method is advantageous because lyophilized preparations can be stored without specialized equipment and are easily transported, making them highly applicable in regenerative medicine [11, 12]. Lyophilized umbilical cord products maintain their therapeutic properties and are applied in the treatment of wounds and tissue damage [13].

The L929 cell line is a well-established model for studying cytotoxicity and regenerative processes. It is commonly used to evaluate the effects of biologically active substances on cell growth, differentiation, and apoptosis. Examining the impact of umbilical cord cryoextracts and lyophilized products on L929 cells is crucial for understanding their potential applications in regenerative medicine and therapy [14, 15]. In the future, this knowledge could contribute to developing novel treatments for tissue damage and chronic conditions.

Thus, the the aim of the study was to examine the effects of umbilical cord cryoextract and its lyophilized forms on L929 cells, which will help determine their potential for use in medical research and clinical practice.

Materials and Methods

The study was conducted using L929 cell cultures obtained from the low-temperature biological object bank at the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. The cryopreserved culture, stored in liquid nitrogen at –196 °C, was thawed in a water bath at 37 °C until the ice completely melted, resuspended in Dulbecco's Modified Eagle Medium (DMEM) (Biowest, France), centrifuged at 1500 rpm, and the supernatant was removed. After thawing, the cells were seeded into 25 cm^2 culture flasks (SPL, Republic of Korea) and cultured in a CO_2 incubator (Thermo Fisher Scientific, USA). The medium was changed every 3 days until 100% confluency was achieved, after which the cells were used for experiments. They were cultured in DMEM medium (Biowest, France) supplemented with 10% fetal bovine serum (FBS) (Lonza, Germany) and 1% antibiotic-antimycotic solution (BioWest, France) at 37 °C, 100% humidity, and 5% CO_2 in culture vessels (SPL, Republic of Korea).

The umbilical cord was obtained under conditionally sterile conditions during delivery with informed consent from the maternity. It was rinsed with sterile phosphate-buffered saline (PBS) (Biowest, France) to remove erythrocytes. The umbilical cord cryoextract (UCC) was prepared by minced the washed cord with scalpel, washed again to remove residual erythrocytes, and centrifuged at 1500 rpm for 10 minutes in a laboratory centrifuge (LMSC-P10-01-Elekon, Ukraine). The samples were homogenized and diluted 1:2 with PBS, then stored in a ultra-low temperature freezer DW-86W100J (Haier, China) at –80 °C in 15 ml sterile polypropylene Falcon-type tubes (SPL, Republic of Korea) each containing 5 ml of the suspension. Before use, the suspension was thawed at 37 °C in a water bath WB-4 (Micromed, Ukraine), centrifuged for 10 minutes at 1500 rpm, and the supernatant transferred to sterile tubes.

To ensure the proper preservation of biological objects such as bacteria, vaccines, serums, and medical preparations, lyophilization (freeze-drying) is typically used with pre-cooling to –60 to –80 °C. This temperature ensures complete freezing of water without leaving a liquid phase and allows for efficient "dry" sublimation of ice. However, it complicates the biotechnological process due to the need for complex refrigeration equipment. At the same time, for storing protein-based biostimulants such as FBS for cell culture work, higher temperatures (up to –20 °C) are used, which significantly simplifies storage. The question remains whether cooling to –20 °C can be applied in sublimation technologies for biostimulants.

Two lyophilized UCC samples were studied: one was freeze-drying at –80 °C (-80LUCC) and another one at –20 °C (-20LUCC). For -80LUCC obtaining, UCC was cooled to –80 °C and lyophilized using a freeze-dryer Alpha 1-2 LDplus (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). To obtain -20LUCC, the samples were frozen to –20 °C and lyophilized in the freeze-dryer. The lyophilized material was stored in hermetically sealed tubes at +8 °C for 3 months.

To assess the effect of the studied substances, L929 cells were seeded into 24-well plates (SPL, Republic of Korea) at a concentration of 2×10^5 cells/well and cultured in DMEM medium at 37 °C, 100% humidity, and 5% CO_2. The study included five groups differing in the content of biologically active compounds: group 1 – DMEM, used as a control to assess the impact of the absence of growth factors on cell development and proliferation; group 2 – medium with fetal bovine serum (DMEM + FBS), control with standard growth conditions with FBS added at a conventional con-

centration of 10%; group 3 – medium with added UCC; group 4 – medium with added -80LUCC; group 5 – medium with added -20LUCC. The concentrations of the substances were standardized by protein content, based on literature recommendations (0.1, 0.5, and 1.0 mg/ml of protein in the culture medium) [16].

Cell morphology and monolayer confluency based on adhesive properties were evaluated after 24 hours of cultivation [17]. To assess cell migration and wound healing capacity, the scratch test was used. A scratch was made in the monolayer cell culture at the center of the well using a sterile 200 μL pipette tip, and observations were made daily until the wound area was completely closed [18].

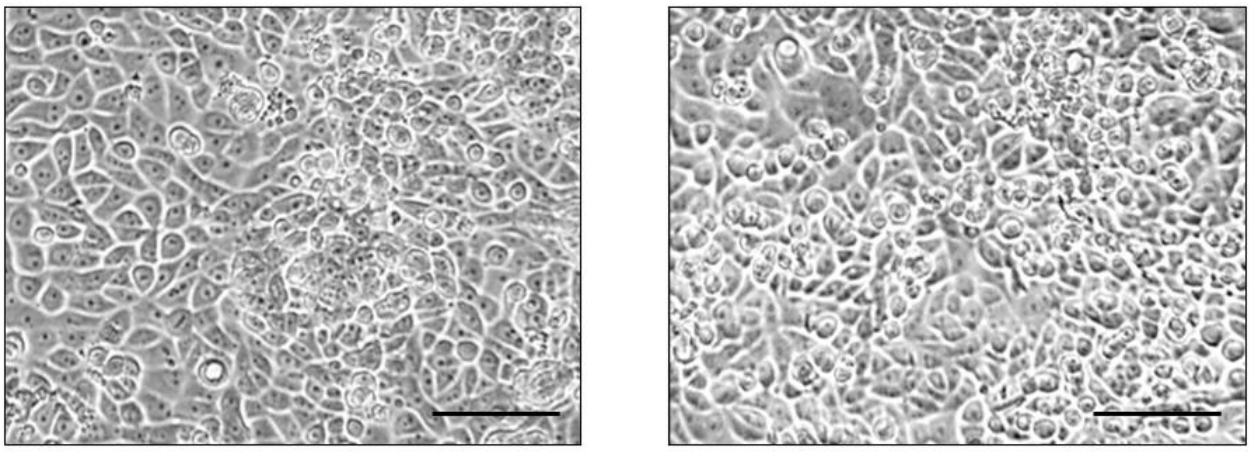
To assess cell viability, the neutral red uptake assay was used [19]. The population doubling time was measured [20, 21], and the MTT test was conducted to determine the functional state of mitochondria [22]. For the MTT assay, cells were seeded into 96-well plates (1×10^4 cells/well), incubated for 24 hours with the tested substances, and then treated with MTT solution (5 mg/ml) for 3 hours. After incubation, dimethyl sulfoxide with sodium dodecyl sulfate was added, and absorbance was measured at 570 nm using a microplate spectrophotometer "SM600" (Utrao, China). For the NR assay, cells were seeded into 96-well plates (1×10^4 cells/well), incubated for 24 hours with the tested substances, and then treated with a solution of 50% ethanol and 3% acetic acid. Absorbance was measured at 540 nm using the same spectrophotometer.

Images from microscopy were processed using ToupView v3.7 (Hangzhou ToupTek Photonics Co., Ltd, Hangzhou, China) and ImageJ v1.48 (NIH, USA). The significance of the differences was determined using the Mann–Whitney U test. Statistical analysis was performed using Past V. 3.15 software (University of Oslo, Norway).

All experiments are consistent with the main provisions of the Law of Ukraine 'On the Protection of Animals Against Cruelty' (No. 3447-IV dated 21.02.2006), 'European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes' (Strasbourg, 1986) and discussed at the meeting of the Bioethics Committee of the Institute for Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine.

Results

Our conducted studies on the effects of umbilical cord cryoextracts revealed the following. L929 cells cultured in DMEM medium (group 1) had a heterogeneous morphology, with the monolayer confluency reaching approximately 100%, However, the density of the cell layer was notably lower compared to the group where FBS was added to the culture medium (group 2). The majority of cells in group 1 displayed irregular, amorphous, or elongated shapes (Fig. 1a), which is likely indicative of stress due to the absence of growth factors in the medium. In contrast, cells in group 2 (DMEM + FBS) had a more uniform morphology, predominantly showing regular, rounded, or polygonal shapes. These cells adhered closely to each other, forming a confluent monolayer without visible gaps (Fig. 1b), a characteristic typical of cultures grown under optimal conditions with adequate growth support.



a b

Figure 1: Microphotographs of the L929 cell monolayer after 24 hours of cultivation (a) in DMEM and (b) in DMEM + FBS. Phase contrast, scale bar 100 μm

The cells in group 3, cultured in the presence of UCC at three different concentrations, exhibited monolayer formation. Notably at a concentration of 0.5 mg/ml, the cells formed a denser monolayer with regular morphology and well-defined nuclei (Fig. 2b). In comparison, at concentrations of 0.1 mg/ml and 1.0 mg/ml, some heterogeneity in cell shape was evident (Figs. 2a, 2c). In groups 4 and 5, which were cultured with -80LUCC and -20LUCC, normal monolayer morphology was maintained, particularly at the lowest concentration of the respective samples (Figs. 3a, 4a).

The study of L929 cell adhesion capacity revealed that in the control group 2, where FBS was added to the culture medium, the number of fully adhered cells was significantly higher compared to all the experimental groups. This result was anticipated, as FBS contains growth and adhesion factors that promote cell attachment in standard cell cultivation protocols. When cultured with UCC at a concentration of 0.1 mg/ml, adhesion and cell spreading increased by 13% compared to cells cultured in DMEM without growth factors. However, the effect of UCC on cell adhesion was less pronounced than that observed with FBS (group 2). Increasing the UCC concentration to 0.5 mg/ml did not further enhance adhesion, while a concentration of 1.0 mg/ml led to a reduction in both adhesion and cell spreading. For cells cultured with -80LUCC, effects similar to those of UCC were observed, with a 19% increase in adhesion and spreading at 0.1 mg/ml, but suppression at 1.0 mg/ml. No significant differences were found between groups 4 (-80LUCC) and 3 (UCC). In contrast,

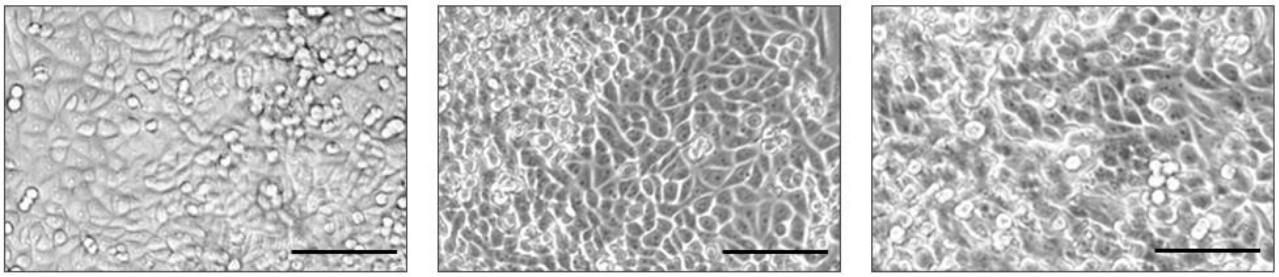


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Figure 2: Microphotographs of the L929 cell monolayer after 24 hours of cultivation in the presence of umbilical cord cryoextract at concentrations of (a) 0.1 mg/ml, (b) 0.5 mg/ml, and (c) 1.0 mg/ml. Phase contrast, scale bar 100 μm

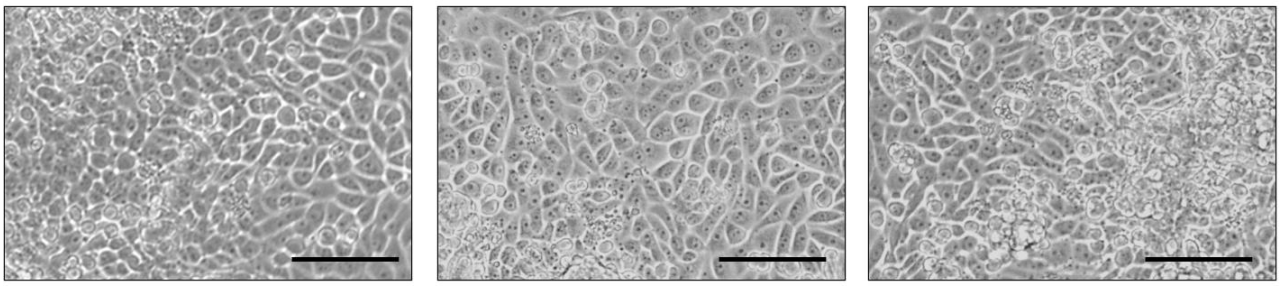

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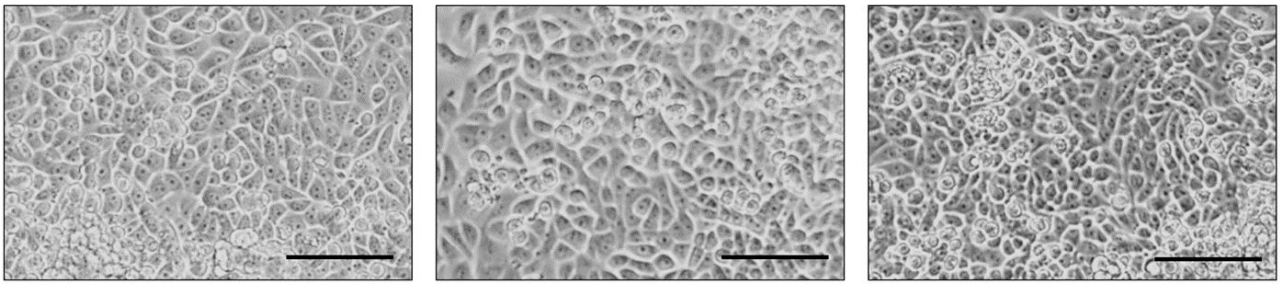
Figure 3: Microphotographs of the L929 cell monolayer after 24 hours of cultivation in the presence of lyophilized umbilical cord cryoextract (–80 °C) at concentrations of (a) 0.1 mg/ml, (b) 0.5 mg/ml, and (c) 1.0 mg/ml. Phase contrast, scale bar 100 μm



a b c

Figure 4: Microphotographs of the L929 cell monolayer after 24 hours of cultivation in the presence of lyophilized umbilical cord cryoextract (–20 °C) at concentrations of (a) 0.1 mg/ml, (b) 0.5 mg/ml, and (c) 1.0 mg/ml. Phase contrast, scale bar 100 μm

cells cultured with -20LUCC at all tested concentrations showed no improvement in adhesion compared to the medium without biologically active substances (Fig. 5).

The analysis of the migration activity test results revealed that the wound closure rate, which indicates the cell migration rate, was approximately 20% higher in group 2 (DMEM + FBS) compared to group 1 (without growth factors). Groups 3 and 5 did not demonstrate a significant effect on L929 cell migration, showing no difference from the data obtained for group 1. However, in group 4, where lyophilized at –80 °C cryoextract (-80LUCC) was added at a concentration of 0.1 mg/ml, the wound closure rate matched that of Group 2 (Fig. 6).

The neutral red uptake assay demonstrated that UCC and -80LUCC had no significant effect on the pinocytotic activity of the cells at any tested concentration. However, the addition of -20LUCC to the culture medium notably stimulated the pinocytotic activity (Fig. 7).

The MTT assay showed no significant difference between groups 1 and 2, indicating the presence or absence of growth factors had no impact on the metabolic activity of the cells. Similar results were observed for groups 3 and 4, where the addition of UCC and -80LUCC to the culture medium at all tested concentrations showed no toxic effect on L929 cells. However, a significant increase in unreduced tetrazolium dye was detected

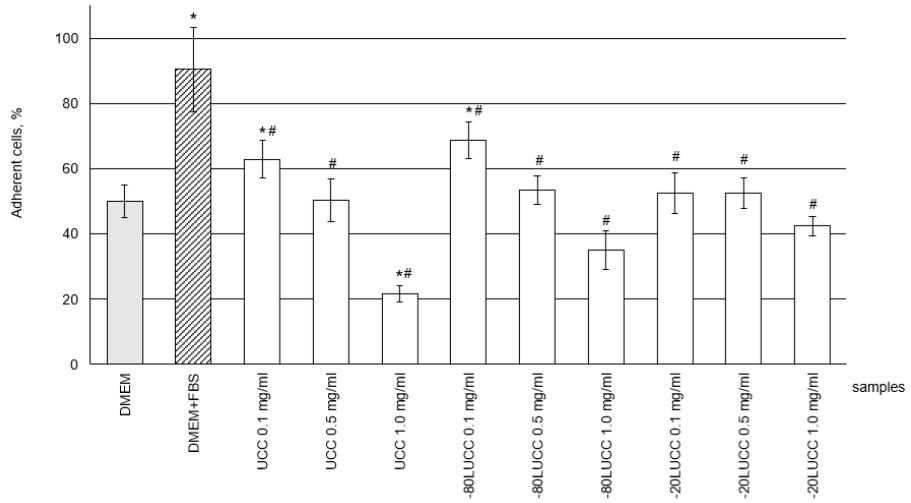


Figure 5: Adhesion rates of L929 cells in the presence of the studied substances, %. * – the indicator is statistically significantly different from the control cultured without the addition of substances, $p < 0.05$; # – the indicator is statistically significantly different from the control cultured with FBS, $p < 0.05$ ($n = 12$)

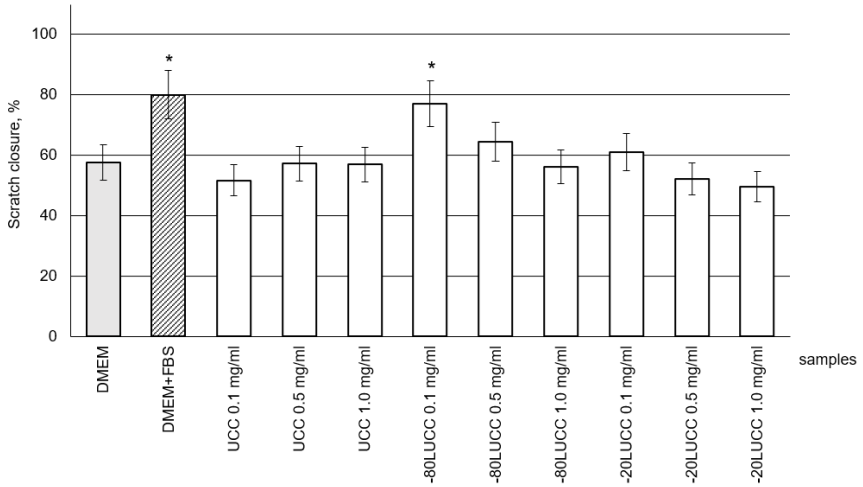


Figure 6: Scratch test results of L929 cells in the presence of the studied substances, %. * – the indicator is statistically significantly different from the control cultured without the addition of substances, $p < 0.05$ ($n = 12$)

in cultures treated with -20LUCC at concentrations of 0.5 and 1.0 mg/ml, suggesting a decrease in the functional activity of the cells (Fig. 8).

The results obtained for population doubling time were as expected for the control groups. In group 2, where cells were cultured with the addition of FBS, the doubling time was twice as fast as in cells cultured in DMEM alone. The tested samples showed fairly heterogeneous results.

The most comparable results to standard cultivation conditions (group 2) were observed in cell cultured with the addition of UCC at concentrations of 0.1 and 0.5 mg/ml, as well as with -80LUCC at 0.1 mg/ml. These concentrations could potentially be used as substitutes for fetal bovine serum. It is important to note that a high concentration of UCC in the culture medium negatively affected the population doubling time, which was also observed when using -80LUCC at a concentration of 1.0 mg/ml. Analysis of the results obtained for group 5, with different concentrations of -20LUCC, L929 cell proliferation was accelerated compared to group 1, but significantly slower than in group 2 (Fig. 9).

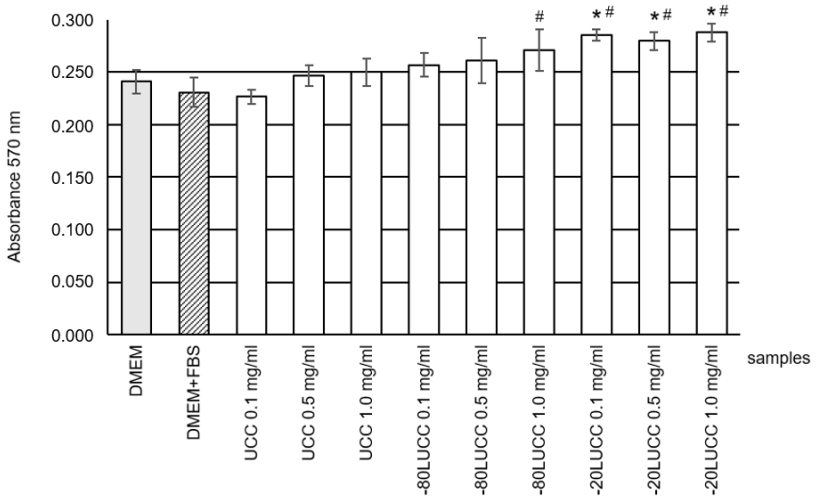


Figure 7: Neutral red uptake assay results for L929 cells in the presence of the studied substances, arb. units. * – the indicator is statistically significantly different from the control cultured without the addition of substances, $p < 0.05$; # – the indicator is statistically significantly different from the control cultured with FBS, $p < 0.05$ ($n = 24$)

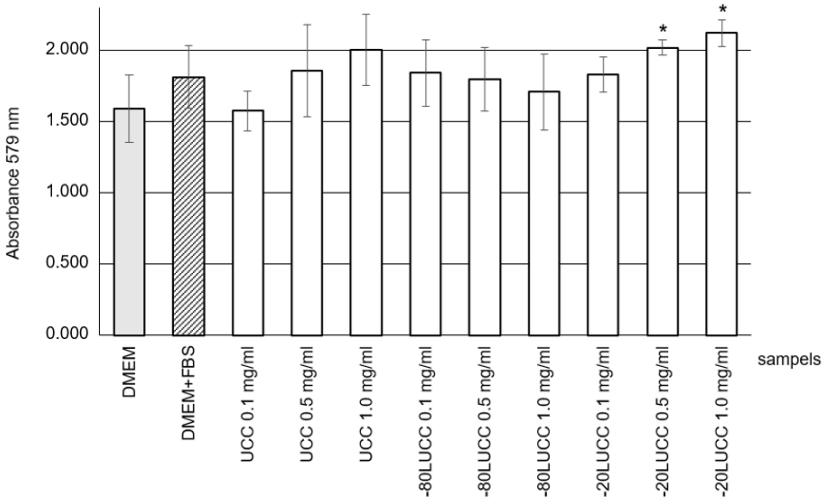


Figure 8: MTT assay results for L929 cells in the presence of the studied substances, arb. units. * – the indicator is statistically significantly different from the control cultured without the addition of substances, $p < 0.05$ ($n = 24$)

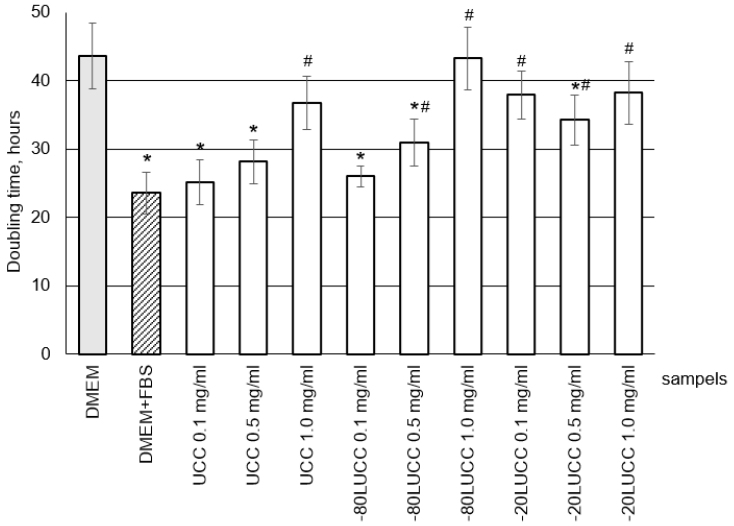


Figure 9: Doubling time of L929 cells in the presence of the studied substances, hours. * – the indicator is statistically significantly different from the control cultured without the addition of substances, $p < 0.05$; # – the indicator is statistically significantly different from the control cultured with FBS, $p < 0.05$ ($n = 12$)

Discussion

The umbilical cord represents a promising source of cells, tissues and biological substances that could meet the needs of regenerative medicine [4–10]. The cryoextract and lyophilized umbilical cord sample is the most straightforward for further utilization [11–15]. The objective of this study was to investigate the properties of UCC and two forms of LUCC in an *in vitro* system, comparing their effects with a medium without biostimulants and with a medium containing a standard FBS stimulant. The standard cell line L929, which is commonly employed in pharmacological studies, was selected as the test culture. To comprehensively assess the effect of UCC and LUCC, a series of screening tests were selected to comprehensively assess the state of cells. These included a morphological assessment of cells and monolayer, an assessment of cell adhesion, an MTT test to assess metabolic activity, a neutral red absorbance test to assess lysosomal function, a scratch test to assess migration activity, and a population doubling test to assess proliferative activity [17–22].

The results of our study demonstrated that UCC and its lyophilized forms at –80 and –20 °C have a significant impact on L929 cells, highlighting their potential for application in regenerative medicine. At a concentration of 0.1 mg/ml, the UCC effectively promoted the formation of a confluent monolayer of cells, comparable to the effect of FBS, which is commonly used as a growth stimulant for cultivating of different cells. Similar outcomes have been observed when using lyophilized umbilical cord as an allograft for healing chronic wounds, supporting the notion that biologically active components – such as proteins, growth factors, cytokines, inflammation modulators, proteases, adhesion molecules and other regulators – remain preserved after lyophilization [13].

The umbilical cord is a known source of MSCs and growth factors, which play a key role in stimulating cell adhesion and proliferation. This is confirmed by our results, where the best effect on cell adhesion was achieved with a concentration of 0.1 mg/ml of the cryoextract and –80 °C lyophilized form. However higher concentrations (1.0 mg/ml) had a negative effect on adhesion and proliferation, likely due to cytotoxic effects caused by an excessive amount of proteins or other cryoextract components. Similar results have been reported in studies investigating the use of umbilical cord MSCs for wound healing and other tissue damage treatments [6, 23].

Interestingly, the lyophilized at –20 °C cord extract had a significantly lower effect on the cells compared to the –80 °C lyophilized form and cryoextract. This may be due to partial degradation of biologically active substances, such as proteins and growth factors, at higher freezing temperatures, which could diminish the product's effectiveness. Lyophilization, as a technique for preserving biological activity, plays a critical role for maintaining the functional properties of such biological materials [11, 12].

The cell migration test (scratch test) showed that the UCC and –80 °C lyophilized form did not significantly affect cell migration compared to the control. However, the stimulation of adhesion at low concentrations of the cryoextract and lyophilized cord suggests their potential for use in regenerative processes, particularly in tissue repair following injury or surgery. For instance, cryopreserved human umbilical cord patches have been effectively used for chronic wounds of the foot and ankle [11, 24], diabetic foot ulcers [24, 25], and diabetic ulcers with osteomyelitis [14, 15, 24, 26–30]. The ability of umbilical cord cryoextract to retain its properties after lyophilization increases its clinical appeal, as it simplifies storage and transportation by eliminating the need for extremely low temperatures [11, 13].

Furthermore, our MTT assay results showed no toxic effects from either the cryoextract or the –80 °C lyophilized form on L929 cells, which is an important consideration for potential clinical use, as toxicity is a key factor limiting the application of biomaterials in therapy. In contrast, the lyophilized cord stored at –20 °C stimulated pinocytotic activity, which may indicate reduced efficacy due to potential protein denaturation at higher freezing temperatures.

Despite the positive results, a limitation of this study is the lack of comparison with other cell lines, which would provide a broader understanding of the versatility of umbilical cord cryoextract and lyophilized products. Future research should focus on investigating the effects of these samples on different cell types to better assess their potential in various therapeutic applications.

Conclusions

The results of this study demonstrated that umbilical cord cryoextract possesses biostimulant properties, enhancing the adhesive and proliferative activity of L929 cells, leading to the formation of a monolayer comparable to that achieved with FBS. The cryoextract did not significantly affect cell migration or metabolic activity, with its most pronounced effects observed at a concentration of 0.1 mg/ml. Similar properties were noted with -80LUCC, while -20LUCC lost these biostimulant properties, instead stimulating pinocytotic activity and inhibiting cell proliferation.

Based on these findings, the optimal concentration for further studies is 0.1 mg/ml for both UCC and -80LUCC. The data suggest that -80LUCC is a feasible alternative to umbilical cord cryoextract, with the added advantage of easier storage and transport due to its lyophilized form.

Interests disclosure

The authors declare no conflict of interest prior to disclosure.

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ВПЛИВ ЛІОФІЛІЗОВАНОГО ТА ЗАМОРОЖЕНОГО КРІОЕКСТРАКТУ ПУПОВИНИ НА КУЛЬТУРУ КЛІТИН L929

Проблематика. Пуповина людини є перспективним джерелом біологічно активних речовин, які мають регенеративні властивості. Однак невивченою є можливість використання ліофілізованого кріоекстракту пуповини для застосування в регенеративній медицині, що полегшить зберігання та транспортування кріоекстракту. Для цього необхідним є дослідження впливу таких кріоекстрактів на клітинній моделі.
Мета. Оцінити вплив ліофілізованого та замороженого кріоекстракту пуповини на культуру клітин лінії L929 для визначення їхнього терапевтичного потенціалу.
Методика реалізації. Дослідження проводилось на культурі клітин L929. Отриманий кріоекстракт пуповини людини і його ліофілізовані форми за температур –80 і –20 °С додавали до середовища культивування Дульбекко (DMEM) у трьох концентраціях – 0,1, 0,5 та 1,0 мг/мл. Контролем були клітини, які культивували в середовищі DMEM без і з додаванням бичачої фетальної сироватки. Досліджували морфологію клітин і конфлюєнтність моношару. Для дослідження впливу кріоекстрактів проводили оцінку життєздатності клітин за показниками адгезії, міграційної активності за допомогою скретч-тесту, піноцитозної активності за тестом поглинання нейтрального червоного, рівня метаболічної активності – за допомогою МТТ-тесту, проліферації – вивчаючи час подвоєння популяції.
Результати. Виявлено, що додавання кріоекстракту пуповини та його ліофілізованої за –80 °С форми не є токсичним для досліджуваних клітин. Найбільш ефективною виявилася концентрація 0,1 мг/мл, яка суттєво стимулювала адгезію та проліферацію клітин порівняно з середовищем культивування без додавання фетальної сироватки. Ліофілізований за –20 °С кріоекстракт пуповини не проявляв стимулювального впливу на показники життєздатності клітин, але стимулював їхню піноцитозну активність.
Висновки. Отримані результати щодо кріоекстракту пуповини та його ліофілізованої за –80 °С форми вказують на те, що вони можуть бути використані як фактори росту для культивування клітинних ліній. При цьому саме ліофілізований зразок забезпечує свою ефективність за відсутності необхідного для зберігання та транспортування обладнання. Ліофілізована за –20 °С форма кріоекстракту суттєво стимулює піноцитозну активність досліджуваних клітин та пригнічує їхню проліферацію.

Ключові слова: кріоекстракт; кріоконсервація; лінія клітин L929; ліофілізація; пуповина; клітинна культура; життєздатність; метаболічна активність