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THE IMPACT OF DIFFERENT LYOPHILIZATION REGIMES ON THE PROTEIN COMPOSITION OF HUMAN CORD BLOOD SERUM

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Background: Modern medicine is confronted with the challenge of minimizing the side effects of drug treatment of serious diseases, including oncological pathologies. One of the most perspective concepts is the use of cord blood serum as a source of regenerative components to support the patient's health. The serum contains a variety of growth factors, cytokines and immunosuppressive cells that promote tissue repair and regulation of the immune response. An important goal is to preserve the biological activity of cord blood serum protein fractions during long-term preservation. Lyophilization is considered one of the most effective methods of stabilizing biological substances. However, the optimal temperature regime for the preservation of cord blood proteins during lyophilization requires further investigation.
Objectives: To evaluate the effect of different lyophilization regimes on the composition and stability of cord blood serum proteins.
Materials and methods: Cord blood serum was examined after freezing to -20°C and lyophilization with previous cooling to -20°C and -80°C. The total protein content was calculated using a standardized determination kit, and the remaining dry mass was weighed on an analytical balance. The protein composition was examined by spectrofluorimetry and polyacrylamide gel electrophoresis. The residues of aromatic amino acids (tyrosine-tryptophan, tryptophan) were analyzed by spectrofluorimetry. The peaks in the protein profiles of the analytical samples were electrophoretically examined and the preservation of the biomaterial was compared.
Results: It has been shown that freezing and lyophilization at -80°C provide high stability of protein fractions without significant loss of total protein or its structural changes. On the contrary, lyophilization at -20°C was accompanied by a significant decrease in the total amount of protein and protein fractions, changes in protein structure, indicating aggregation, denaturation, and degradation of protein molecules. The densitogram of lyophilized cord blood serum when cooled to -80°C was significantly close to the result of the frozen sample, while the analysis of the peaks of lyophilized serum cooled to -20°C showed a significant decrease in parameters.
Conclusions: Lyophilization of human cord blood serum with preliminary cooling at -80°C allows preserving the protein concentration, the number of fractions and the structure of proteins according to electrophoresis and spectrofluorimetry. Lyophilization of cord blood serum with preliminary cooling at -20°C significantly reduces the protein concentration, the number of fractions and changes the structure of proteins compared to frozen serum.

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KEYWORDS: cord blood serum, spectrofluorimetry, electrophoresis, lyophilization, freezing, storage, cryopreservation, regenerative medicine.

The challenges of modern medicine require minimizing the possible negative effects of drug treatment of many serious diseases on the general condition of the organism [1]. It is well known that many patients with severe diseases, in particular cancer patients, have problematic consequences due to the general negative effect of chemotherapy and radiation therapy on the condition of the immune system, liver, kidneys, etc. [2, 3]. One of the modern areas in which regenerative medicine is developing is the inclusion of cord blood serum (CBS) to reduce the harmful effects of drugs on patients' organisms and improve post-treatment recovery and rehabilitation [4].

It is known that human cord blood is a source that is rich in myeloid suppressor cells, which are a perspective object of clinical research related to their immunosuppressive properties, as well as the ability to affect autoimmune and inflammatory diseases as an immunomodulator [5, 6]. Modern scientific investigations of the components of human cord blood are aimed at finding ways to use all components of the feto-placental complex, which will further allow to evaluate the regenerative properties of the components of the CBS and successfully include them in treatment protocols [7, 8].

CBS are a source of cytokines and growth factors that have anti-inflammatory, antiapoptotic and angiogenic effects and can be used in various fields of regenerative medicine [9]. In addition, CBS can be added to the culture medium to increase proliferative activity, colony-forming effect, and the number of cell generations. That means that culture medium supplemented with CBS supports the proliferation and differentiation of conjunctival and limbal epithelial cells. It has been determined that CBS contains a higher concentration of growth factors and cytokines than fetal bovine serum or adult serum [10]. Thus, the addition of CBS to the culture medium may have a better overall effect on the functioning of the cellular medium and is important for further clinical research [11].

The question of how to preserve cord blood, namely freezing, lyophilization, and the correct temperature regime, which will help to maximize the preservation of cord blood components for further use in medical practice, is relevant. The informational biomarkers in the circulating low molecular weight serum proteome were investigated using continuous elution electrophoresis. The results of the research showed that sequential concentrated fractions of serum proteins demonstrate a clear high-resolution ability of 1–2 kDa below 20 kDa [12].

The issue of the preservation of cord blood serum proteins under different lyophilization regimes is still uncertain. Thus, the effective lyophilization of proteins [13, 14], test system [15], plasma [16] and probiotics [17, 18] was demonstrated.

It has been shown that the efficiency of lyophilization depends on the selected protocol, primarily on the initial cooling temperature, which should ensure complete freezing. According to various authors, this temperature ranges from -20 °C and -80 °C [13–19], which corresponds to the refrigeration equipment found in most laboratories.

Thus, the aim of the research was to investigate the effect of different lyophilization temperatures (-20°C and -80°C) on the composition of the protein component of human CBS.

MATERIALS AND METHODS

CBS was obtained during labor with the informed consent of women in labor without extragenital, infectious pathology and gestosis.

We analyzed frozen to -20ºC CBS and lyophilized in two modes (with preliminary cooling at -20ºC and -80ºC). CBS were lyophilized using an Alpha 1-2 LD plus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany).

For lyophilization, the serum was poured into glass vials, 3 ml per vial. The first part of the serum was cooled to -80°C, the samples were transferred to the freeze-drying module, where the temperature was -35°C, the pressure was reduced to 30 Pa for 6 hours. After that, the temperature was then increasedat a rate of 1–3º per hour to 35°C, after which lyophilization was stopped.

The second part of the serum was cooled to -20°C, the samples were transferred to the freeze-drying module, where the temperature was -20°C, the pressure was reduced to 30 Pa for 6 hours. After that, the temperature was then increasedat a rate of 1–3º per hour to 35°C, after which lyophilization was stopped.

Total protein was measured using the «Total Protein Concentration Test Kit for Human Serum» («Phyllisit», Ukraine). Before starting the analysis, the Biuret reagent was prepared according to the kit instructions, calibration and physiological solutions were made, 1.0 ml of the reagent was added to each test tube with the test sample, mixed and left for 30 minutes at room temperature. Measurements were performed using a SM600 spectrophotometer (Utrao, China) at a wavelength of 550 nm [20].

The dry residue was weighed on an analytical balance AD50 (Axis, Ukraine) at the calculation of 5 ml of liquid CBS after the lyophilization process in two regimes (-20°C and -80°C).

Spectrofluorimetric analysis and polyacrylamide gel electrophoresis were used to study the preservation of the protein composition.

Spectrofluorimetric analysis of aromatic amino acids (tyrosine and tryptophan) in the protein fractions of CBS was carried out using a fluorescence spectrometer FL 8500 (Perkin Elmer, USA). For the research, the samples of the protein fractions of CBS were dissolved in phosphate-salt buffer (0.01 M, pH 7.4) in a ratio of 1:10. The samples were mixed thoroughly, and the supernatant was transferred to quartz cuvettes for analysis. Excitation was performed at a wavelength of 280 nm for tyrosine-tryptophan and 295 nm for tryptophan. The emission was recorded in the range of 300–400 nm [21].

Denaturing electrophoresis in a polyacrylamide gel (NuPAGE™ Bis-Tris Mini Protein Gels, 4–12%) was performed using the XCellSureLock Mini-Cell Electrophoresis System (Thermo Fisher Scientific, USA) with the addition of appropriate buffers and reducing agents. For electrophoretic separation, 10-cell cassettes with a gradient polyacrylamide gel "NuPAGE™ Bis-Tris Mini Protein Gels, 4–12%" (Invitrogen) were used. Samples were mixed with reagents in eppendorf tubes (total volume 20 μL per cell), heated at 70°C for 10 minutes in a dry thermoblock Eppendorf ThermoStat plus. After that, the solutions were loaded into the gel using Gel Loading Tips (Invitrogen, LC1001). Electrophoresis was performed at 200 V for 50 minutes [22].

Statistical processing of the data was performed using the Graph Pad Prism program (Graph Pad, USA). The data were compared using the nonparametric Mann-Whitney U test. The results of concentrations were presented as the median (Me) range. Differences at $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

After calculating the total protein, it was determined that the -80°C CBS lyophilizate showed a slight increase in total protein concentration of 4% from the frozen sample. The CBS lyophilized at -20°C demonstrated a 16% decrease in total protein levels compared to the frozen CBS. Lyophilization at -80°C allows almost complete preservation of the protein concentration, and a slight increase may be due to the loss of some water or changes in the phase state of proteins. Lyophilization at -20°C is accompanied by a noticeable decrease in total protein

content, which may indicate damage of protein structures due to possible denaturation and aggregation processes (Fig. 1).

The dry residue was 0.3 g per 5 ml or 0.06 g per 1 ml.

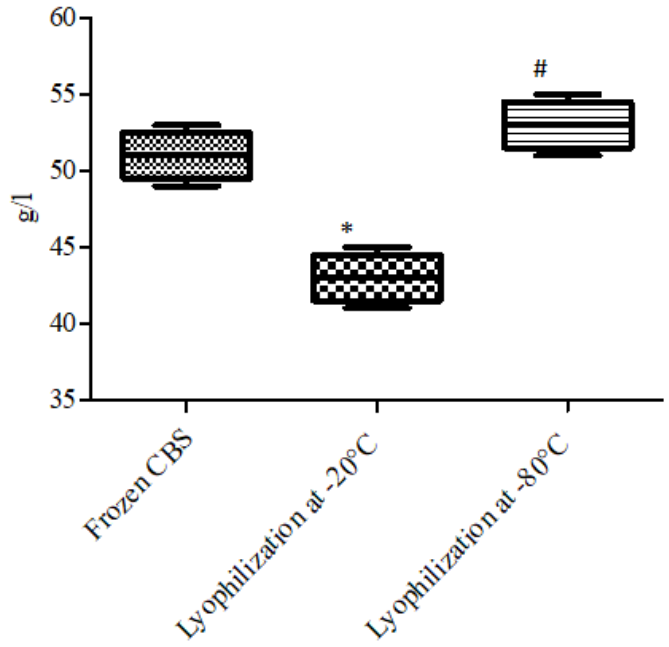

Fig. 1. Changes in the concentration of total protein in frozen and lyophilized CBS at different temperature conditions (-20°C and -80°C); * — he index is statistically different from frozen CBS, $p<0.05$; # — the index is not statistically different from frozen CBS, $p>0.05$. Data are presented as m ± SD (n=6).

The fluorescence analysis showed that the main contribution to the emission spectrum when excited at tyrosine wavelengths (280 nm) is made by tryptophan residues. This is due to the effective energy transfer from tyrosine to tryptophan, which is typical for proteins containing both types of amino acids. The observed fluorescence maximum at 335–336 nm indicates the dominance of the tryptophan signal even upon tyrosine excitation. Comparison of the spectra upon excitation of tryptophan and tyrosine shows a similar shape and intensity ratio, which confirms the presence of both residues in the proteins. At the same time, the higher total fluorescence intensity upon tyrosine excitation indicates the presence of tyrosine residues in a certain number of CBS proteins. The observed small shift of the emission maximum upon excitation of different amino acid residues may be related to conformational changes of proteins, which result in a change in the polarity of the serine surrounding the indole chromophore of tryptophan amino acid residues.

Spectrofluorimetry revealed that in frozen CBS the maximum emission is at 340 nm, which corresponds to the values for tryptophan located on the protein surface, and is assumed to be in contact with bound water molecules and other polar groups [23]. The mentioned maximum value occupies an intermediate position between the corresponding values for tryptophan fluorescence in a rather hydrophobic environment inside the protein globule (330 nm) and on the protein surface in contact with free water molecules (350 nm) [23]. The CBS lyophilizate at -20°C demonstrated a shift of the maximum to 337 nm, which may indicate changes in the structure of proteins that lead to a shift of the indole chromophore of tryptophan amino acid residues to a more hydrophobic environment, for example, closer to the middle of the protein globule. Usually, a decrease in the polarity of the microenvironment of tryptophan amino acid residues is accompanied by an increase in the intensity of their fluorescence maximum [24], however, the absence of a corresponding increase in the fluorescence intensity in the case of CBS lyophilized at -20°C may be due to two factors: (a) the aforementioned decrease in the level of total protein under these conditions; (b) a possible increase in the fluorescence

suppression of tryptophan amino acid residues by closely spaced electron acceptor groups (carboxyl, protonated amino group), which occurs as a result of the discussed change in the structure of proteins. The CBS lyophilizate at -80°C gives a maximum fluorescence intensity at 339 nm, indicating a lower degree of structural changes compared to the temperature regime of -20°C, and is also closer to the frozen CBS in terms of emission intensity (Fig. 2).

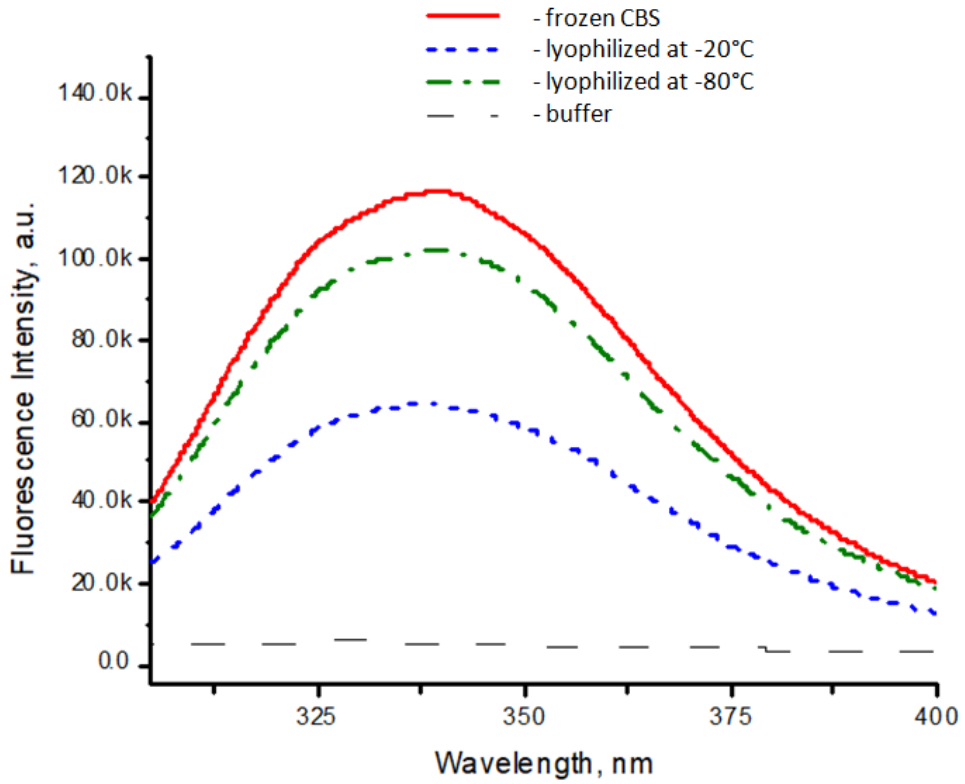

Fig. 2. Fluorescence intensity of tryptophan in human serum and cord blood lyophilizate at different temperature conditions -20°C and -80°C. Data are presented as m ± SD (n=6).

The method of tyrosine-tryptophan spectrofluorimetry also allowed us to assess the change in the fluorescence intensity of aromatic amino acids in frozen human CBS and in lyophilized serum obtained at different temperature regimes (-20°C and -80°C). The data obtained indicate that the highest fluorescence intensity with a maximum of about 340 nm is observed in frozen CBS, but the serum lyophilizate when cooled to -80°C demonstrates a slight decrease in intensity, but retains the profile. This indicates the high stability of the protein structure of this sample. The CBS lyophilizate when cooled to -20°C is characterized by a significant decrease in the fluorescent signal, which indicates damage or changes in the state of protein molecules. The probable cause of this condition is denaturation and aggregation of the protein component. As a result of the first process mentioned above, the fluorescence suppression of tryptophan amino acid residues by closely spaced electron acceptor groups (carboxyl, protonated amino group) may increase, and as a result of the second process, the availability of aromatic residues for excitation and emission will decrease (Fig. 3).

The highest level of fluorescence is observed in the frozen sample of CBS, indicating that the natural structure of proteins is preserved. After the lyophilization process at -20°C, the fluorescence decreased by 45% relative to the frozen CBS, which can be explained by changes in the spatial structure of proteins (even their denaturation) and subsequent protein aggregation. On the other hand, lyophilization at -80°C demonstrates the preservation of intensity at a level

closer to the frozen material, which may indicate minimal damage to the protein structure (Fig. 4).

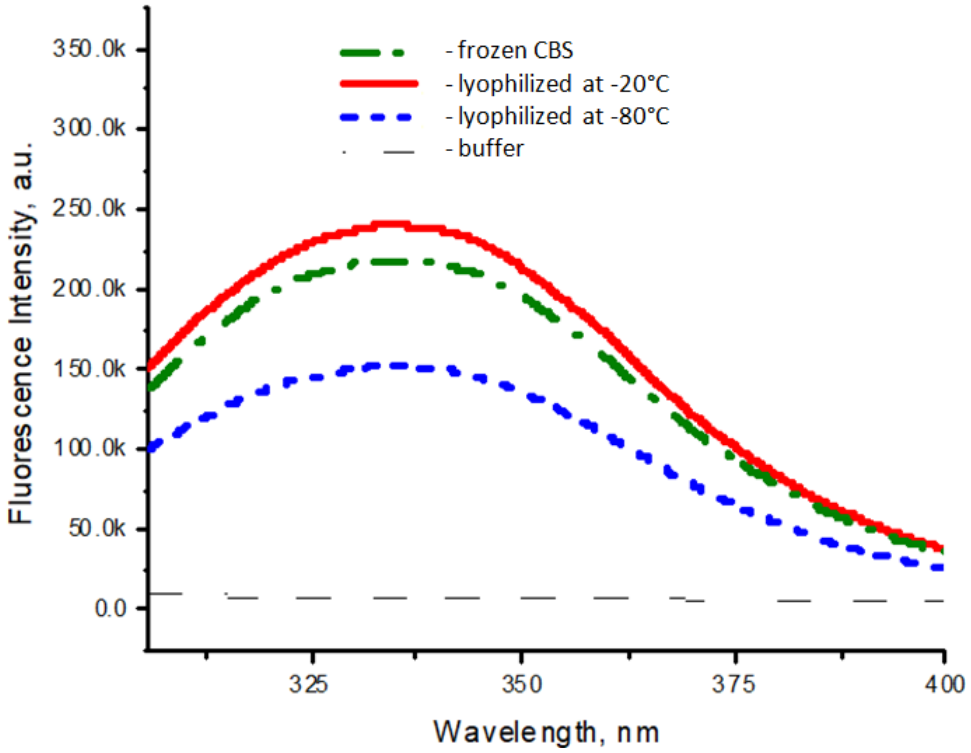

Fig. 3. Fluorescence intensity of tyrosine-tryptophan in human serum and cord blood lyophilized at different temperature conditions -20°C and -80°C. Data are presented as m ± SD (n=6).

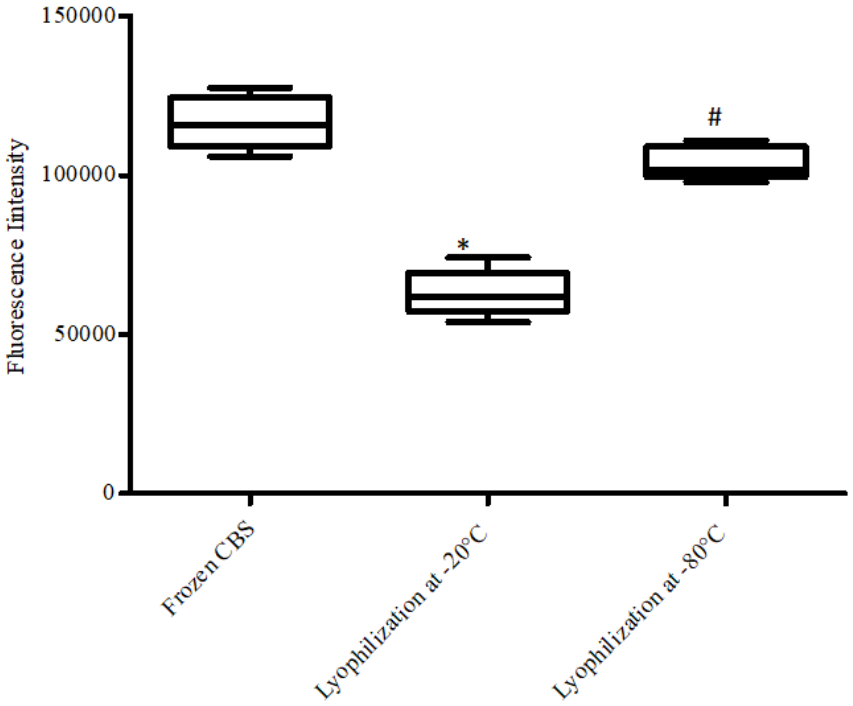

Fig. 4. Results of tryptophan fluorescence of protein fractions of frozen CBS and CBS lyophilizate at -20°C and -80°C; * — the index is statistically different from frozen CBS, $p<0.05$; # — the index is not statistically different from frozen CBS, $p>0.05$. Data are presented as m ± SD (n=6).

Therefore, it was discovered that lyophilization at -20°C causes significant structural changes in the protein components of CBS, which is expressed in a decrease in the fluorescence

intensity of tryptophan amino acid residues and a shift in the maximum of their fluorescence band. In contrast, lyophilization at -80°C better preserves the native structure of proteins, as evidenced by the almost complete preservation of the emission intensity of tryptophan amino acid residues and a slight shift in the maximum of their fluorescence band.

The densitograms of frozen CBS and lyophilized CBS at -80°C were found to be similar by electrophoresis, both samples demonstrate a complex protein profile with several distinct peaks, indicating the presence of a significant number of protein components of different molecular weights. The densitogram showing the analysis of CBS lyophilizate at -20°C revealed a much smaller number of intense peaks, which may indicate a reduced protein concentration, possible degradation and denaturation of protein components, as well as a reaction to the effect of specific conditions of sample obtaining and preparation (Fig. 5, 6, 7).

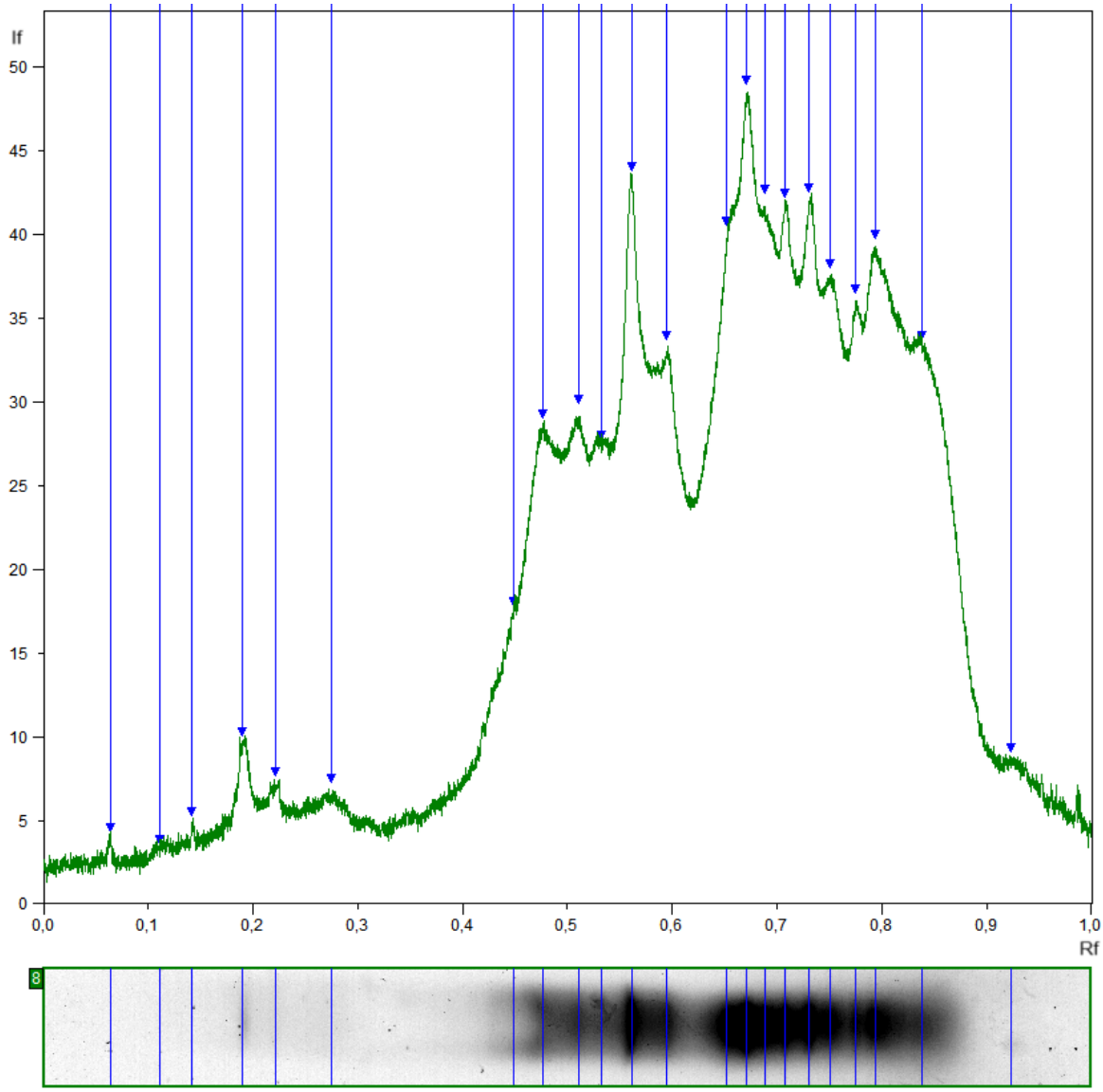

Fig. 5. Graphic representation of the densitogram of electrophoretic separation of proteins of frozen human CBS by denaturing electrophoresis in polyacrylamide gel. Rf (horizontal axis) reflects the relative migration of protein components in the polyacrylamide gel; signal intensity (vertical axis) indicates the degree of protein staining, which correlates with the concentration in the sample. Data are presented as m ± SD (n=6).

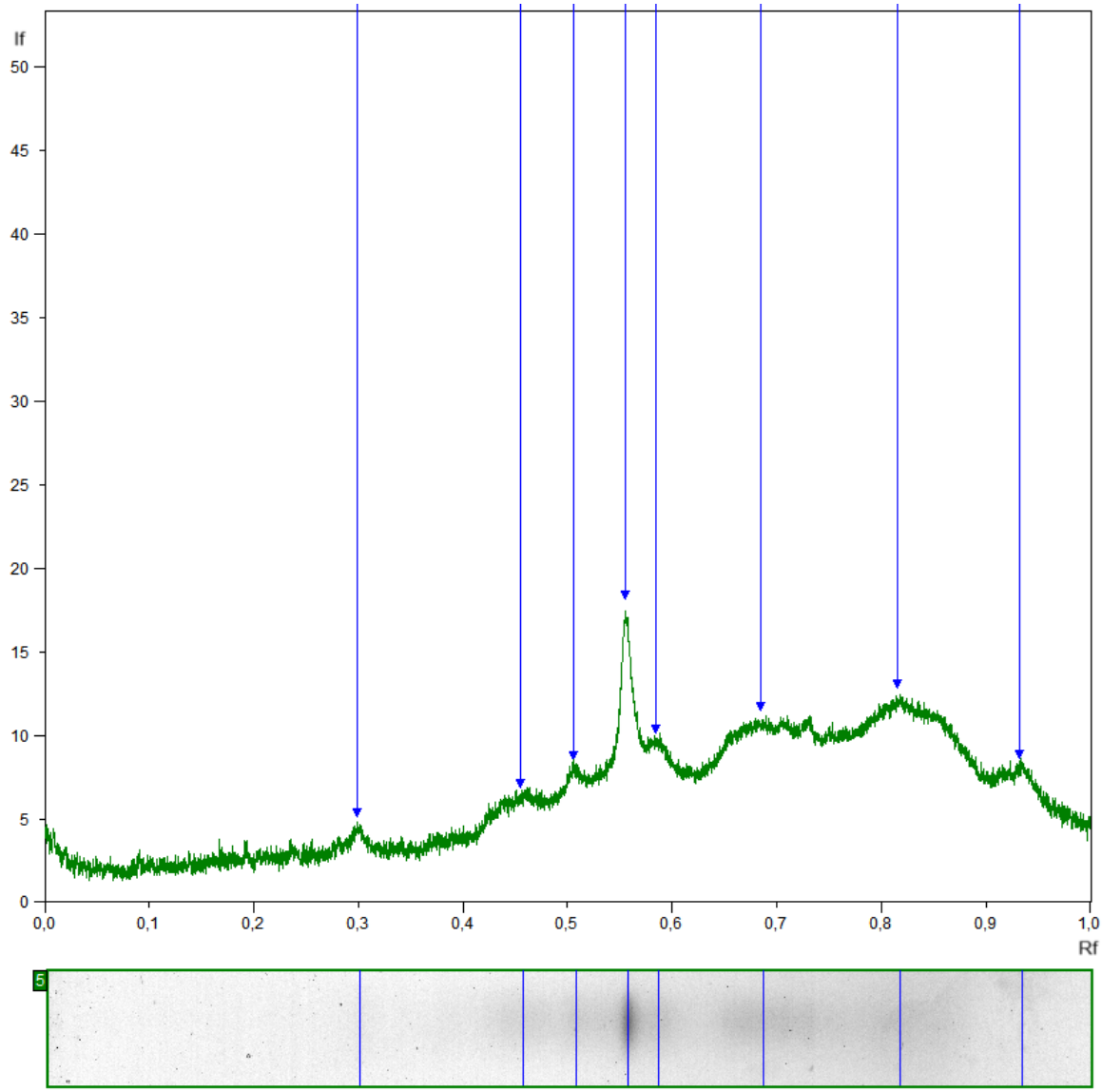

Fig. 6. Graphic representation of the densitogram of electrophoretic separation of proteins of lyophilized CBS at -20°C by denaturing electrophoresis in polyacrylamide gel. Rf (horizontal axis) reflects the relative migration of protein components in the polyacrylamide gel; signal intensity (vertical axis) indicates the degree of protein staining, which correlates with the concentration in the sample. Data are presented as m ± SD (n=6).

In summary, the results obtained indicate that lyophilization is a perspective method of long-term storage of human CBS with minimal loss of its protein composition and properties. In particular, the analysis of the total protein concentration showed that in samples lyophilized at a cooling temperature of -80°C, the protein content was practically the same as in frozen control serum. This is confirmed by the results of spectrofluorimetric analysis, where the fluorescence intensity of the aromatic amino acids tyrosine and tryptophan in lyophilized samples at -80°C remained almost at the level of the frozen sample. Instead, lyophilization at a cooling temperature of -20°C was accompanied by significant losses of protein fractions, a decrease in total protein concentration, and a decrease in fluorescence intensity. This may be due to partial denaturation of protein molecules.

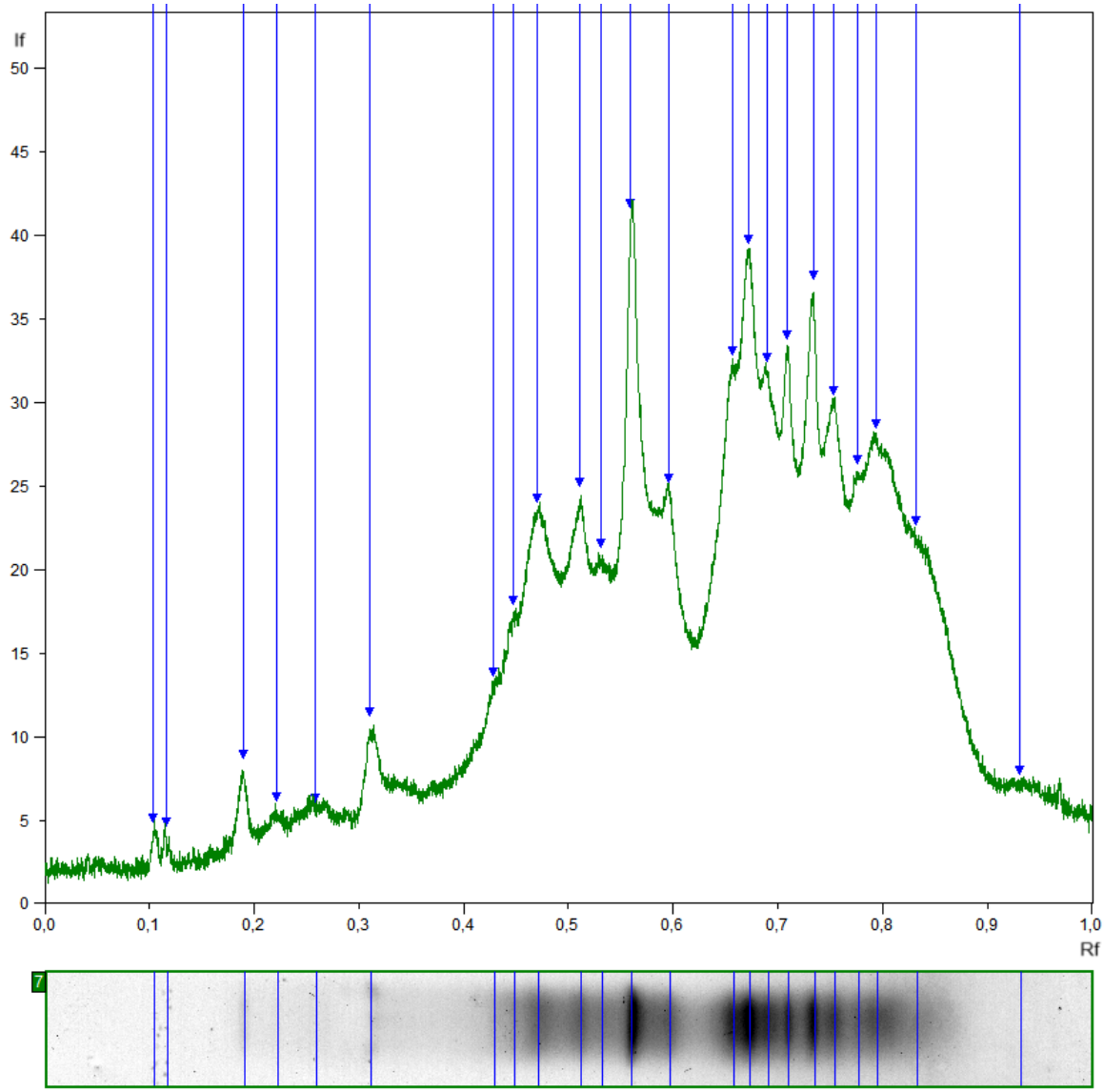

Fig. 7. Graphic representation of the densitogram of electrophoretic separation of proteins of lyophilized CBS at -80°C by denaturing electrophoresis in polyacrylamide gel. Rf (horizontal axis) reflects the relative migration of protein components in the polyacrylamide gel; signal intensity (vertical axis) indicates the degree of protein staining, which correlates with the concentration in the sample. Data are presented as m ± SD (n=6).

The data obtained are consistent with the literature, which indicates the benefits of lyophilization over traditional storage at higher temperatures for biomaterials containing proteins [13–19]. For example, a study of freeze-drying for extracellular vesicle preparations stored at -80°C showed better results than just freezing [25]. The high level of preservation of protein components in lyophilizates at -80°C allows us to consider this method as optimal for the preparation of stable CBS products for further use in research, in particular in the area of regenerative medicine [26, 27].

CONCLUSIONS

Research into the preservation of serum composition is important for the further use of CBS in various areas. Storage of the lyophilized product is less costly than freezing, which significantly affects the choice.

Lyophilization of human CBS with pre-cooling at -80°C allows to maintain protein concentration, number of fractions and protein structure according to electrophoresis and spectrofluorimetry. Lyophilization of CBS with pre-cooling at -20°C significantly reduces the protein concentration, number of fractions and changes the structure of proteins compared to frozen CBS.

It is important to study the composition of serum to understand the effect of storage. The effect of such serum on the organism remains unexplored and requires further research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ВПЛИВ РІЗНИХ РЕЖИМІВ ЛІОФІЛІЗАЦІЇ НА БІЛКОВИЙ СКЛАД СИРОВАТКИ КОРДОВОЇ КРОВІ ЛЮДИНИ

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Актуальність. Сучасна медицина стикається з необхідністю мінімізації побічних ефектів медикаментозного лікування важких захворювань, зокрема онкопатологій. Одним з перспективних підходів є застосування сироватки кордової крові як джерела регенеративних компонентів для підтримки організму пацієнтів. Сироватка містить широкий спектр факторів росту, цитокінів та імуносупресивних клітин, що сприяють відновленню тканин та регуляції імунної відповіді. Важливим завданням є збереження біологічної активності білкових фракцій сироватки пуповидної крові при тривалому зберіганні. Ліофілізація розглядається як один із найбільш ефективних методів стабілізації біологічних речовин. Проте оптимальний температурний режим для збереження білків СКК під час ліофілізації потребує подальшого дослідження.

Мета — оцінити вплив різних режимів ліофілізації на склад і стабільність білків сироватки кордової крові.
Матеріали і методи. Досліджували сироватку кордової крові після заморожування до -20 °С та ліофілізації з попереднім охолодженням до -20 °С та до -80 °С. Визначали загальний вміст білку стандартним набором для визначення, залишок сухої речовини зважували на аналітичних вагах. Білковий склад вивчали методами спектрофлуориметрії та електрофорезу в поліакриламідому гелі. Спектрофлуориметрично аналізували залишки ароматичних амінокислот (тирозин-триптофан, триптофан). Електрофоретично досліджували піки у протеїнових профілях аналітичних зразків та порівнювали збереженість біоматеріалу.
Результати. Було показано, що заморожування та ліофілізація при -80 °С забезпечують високу стабільність кількості білкових фракцій без значної втрати загальної кількості білку або її структурних змін. Натомість ліофілізація при -20 °С супроводжувалася достовірним зниженням загальної кількості білку та білкових фракцій, зміною структури білку, що свідчить про агрегацію, денатурацію та деградацію білкових молекул. Денситограма ліофілізованої сироватки кордової крові при охолоджені до -80 °С була значно наближеною до результату замороженого зразка, у той час, як аналіз піків ліофілізованої сироватки при охолоджені до -20 °С показав значне зниження показників.
Висновки. Ліофілізація СКК людини з попереднім охолодженням -80°С дозволяє зберегти концентрацію білку, кількість фракцій та структуру білків за даними електрофорезу та спектрофлуориметрії. Ліофілізація СКК з попереднім охолодженням -20°С значно знижує концентрацію білку, кількість фракцій та змінює структуру білків у порівнянні з замороженою СКК.
КЛЮЧОВІ СЛОВА: сироватка кордової крові; спектрофлуориметрія; електрофорез; ліофілізація; заморожування; зберігання; кріоконсервування; регенеративна медицина.