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PHASE TRANSFORMATION AND PROTEIN-HORMONAL PROFILE OF HUMAN CORD BLOOD SERUM AT LOW TEMPERATURES

Key words: *cord blood serum, thermomechanical analysis, low temperatures, cryopreservation, lyophilization, growth factors, hormones.*

Cord blood serum (CBS) is currently considered a promising basis for the development of effective therapeutic agents for regenerative medicine. Cord blood is the blood of a newborn in the first minutes of life, which remains in the vessels of the placenta and umbilical cord. It contains high concentrations of placental nucleoproteins, exosomes, growth factors, hormones, and other biologically active molecules. These compounds act as potent immunomodulators and intercellular communicators; they activate regeneration and exert powerful anti-inflammatory, anti-apoptotic, angiogenic, neurotrophic, and hematopoietic effects, which determines the clinical value of CBS [1, 4, 5, 9].

The significant therapeutic potential of CBS determines its effectiveness in the treatment of traumatic injuries, particularly in cases of concussion (blast injury) and radiation damage. The modern conditions of military medicine require a transition from liquid product forms to lyophilized ones, as this form ensures the stability of biologically active molecules during transportation at positive temperatures and allows for their application without thawing or cryogenic support. However, unified approaches to CBS lyophilization are currently lacking, and the developed methodological strategies remain fragmented and inconsistent [3, 8]. This significantly increases the risks of structural disruption of the lyophilizate and the subsequent loss of biological activity of the serum components. Identifying the temperature intervals within which phase-structural transformations (PSTs) occur during freeze–thawing is a fundamental step in developing effective cryotechnologies. Given the above, the aim of this study was to determine the temperature intervals of phase-structural transformations in human cord blood serum to optimize its lyophilization parameters and ensure the preservation of its protein profile and key regulatory compounds.

Human cord blood was used in this study in accordance with the principles of the Declaration of Helsinki, adopted by the General Assembly of the World Medical Association. Blood was collected from the placental segment of the umbilical vein immediately following delivery and separation of the infant from the mother. The cord blood was collected into dry vials; after clot retraction, it was centrifuged at 785 g for 10 min (CM-3 MICROmed,

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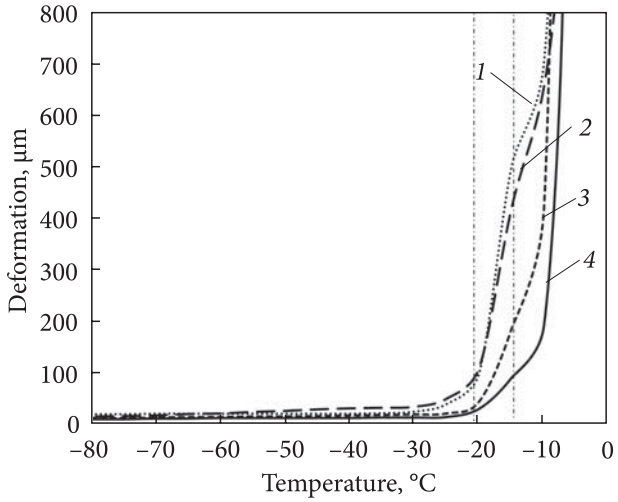

TMA curves of CBS under various cooling rates and a heating rate of 1 deg/min, indicating the temperature interval of PSTs (vertical lines): *1* — 1 deg/min; *2* — 4 deg/min; *3* — 20 deg/min; *4* — immersion in liquid nitrogen

China). The supernatant (serum) was then aliquoted into 0.5 mL cryovials (Nunc, USA). A total of 42 CBS samples served as the material for this study.

To determine the temperature intervals of PSTs occurring during the freezing and thawing of CBS, thermomechanical analysis (TMA) was performed using a tensodilatometric system developed at the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine [2, 6, 7]. For the TMA procedure, a CBS sample with a volume of 0.5×10^{-6} m^3 was placed into the deforming section of the device and cooled at rates of 1, 4 and 20 degrees/min to a temperature of –160 °C, followed by holding at this temperature for 10 min. Separately, an uncontrolled cooling regimen was applied by immersing the sample directly into liquid nitrogen. A constant external deforming stress of 0.66×10^5 kg/m^2 was then applied to the samples. Subsequently, the CBS samples were heated at a constant rate of 1 degree/min, and thermomechanical curves were recorded in "deformation-temperature" coordinates under pure shear mode. The temperature intervals of PSTs in the samples were determined by the deviation of the experimental curve from the tangents drawn to the sections of the TMA curve with a constant flow rate.

The protocols for cryopreservation, lyophilization, and low-temperature storage of CBS were designed taking into account the determined temperature interval of PSTs. The CBS samples were frozen at a rate of 1 °C/min to –40 °C, followed by storage at –40 °C for a month. Lyophilization of CBS was performed from an initial temperature of –40 °C to a final temperature of 30 °C for 10 hours at a pressure of 10 Pa. The residual moisture content was 2%. After freeze-drying, the CBS samples were rehydrated with distilled water to the original volume. The CBS samples were studied in their native state, depreserved — after freezing, low-temperature storage, lyophilization, and rehydration.

The total protein content in the CBS samples was determined by the biuret method using the "Filisit-Diagnostics" (Ukraine) reagent set on a "ULAB 102UV" spectrophotometer (Ulab, China) at a wavelength of 550 nm according to the manufacturer's instructions. The content of protein fractions (albumins, α1-, α2-, β- and γ-globulins) was determined photometrically based on the optical density of the samples, followed by calculating the percentage of each fraction. The levels of growth hormone (GH), human chorionic gonadotropin (hCG), alpha-fetoprotein (AFP), prolactin, cortisol, and noradrenaline were determined by enzyme-linked immunosorbent assay on microplate readers "ELx 808" (Bio-Tek Instruments, Inc., USA) and "SUNRISE" (TECAN, Austria) in accordance with the manufacturers› instructions.

Statistical analysis of the results ($n = 20$) was performed after verifying the normality of data distribution using the Shapiro-Wilk test. Paired Student's t-test was used to compare variables in related groups. Independent samples with heterogeneous variances were compared using Welch's t-test. Data analysis was carried out using the "Past 3.25" software package (University of Oslo, Norway). The critical level of statistical significance was set at $p \leq 0.05$.

As a result of the conducted studies, it was determined that for the specified cooling rates, the temperature interval of the CBS transition into the solid state was identical and ranged from –30 to –22 °C (Figure). Furthermore, the TMA curves revealed the presence of a recrystallization transformation process immediately preceding the melting process of the respective phase. According to the TMA curves, the boundary values for the recrystallization pro-cess were identical for all cooling rates and occur-red within the temperature interval from –15 to –10 °C. The figure presents the TMA curves of the CBS samples obtained under cooling conditions at the determined controlled (1, 4, and 20 deg/min) and uncontrolled (immersion in liquid nitrogen) rates during subsequent heating at a constant rate of 1 deg/min.

A compelling indicator of the correctness of the temperature intervals selected and applied in the presented cryotechnologies is the degree of preservation of the CBS composition. The obtained results indicate that the total protein concentration in the CBS samples following freezing, storage at –40 °C**,** as well as after lyophilization and reconstitution, did not undergo statistically significant changes compared to the native one and remained at a stable level (approximately 52 g/L). A similar trend was observed regarding the fractional composition of proteins, which was reproducible across all stages of the study (native samples, samples after freezing and lyophilization, respectively). The albumin fraction dominated (66.5%), while the content of globulin fractions ranged within: α1 — 2.14—2.42%, α2 — 4.95—5.04%, β — 11.84—11.97% and γ — 13.37—13.68%, with no statistically significant differences between the groups.

From the perspective of biotechnological evaluation, this is a fundamentally important result: the stability of the total protein and protein profile indicates that the selected technological chain is not accompanied by pronounced protein destruction, aggregation, or selective loss of individual fractions within the sensitivity limits of the applied methods. In the context of the PST analysis, this fact aligns with the conclusion that the selected temperature regimens are physico-chemically justified and sufficient to ensure the preservation of the key CBS components.

In the depreserved CBS samples, after storage at –40 °C for a month, the preservation of the studied components ranged from 88.0 to 99.2% of the native level: total protein — 99.2% (from 52.96 to 52.55 g/L), AFP — 97.7% (from 53.491 to 52.282 ng/mL), cortisol — 95.7% (from 4.17 to 3.99 μg/dL), hCG — 89.4% (from 113.0 to 101.0 mIU/L), GH — 88.0% (from 16.6 to 14.6 ng/mL), noradrenaline — 91.8% (from 267 to 245 pg/mL), and prolactin — 89.6% (from 8.778 to 7.861 mIU/L). In the CBS samples reconstituted after lyophilization, the preservation of these indicators was 76.0 to 100.0%: total protein — 97.8% (from 52.96 to 51.81 g/L), AFP — 100.0% (from 53.91 to 53.486 ng/mL), cortisol — 96.6% (from 4.17 to 4.03 μg/dL), hCG — 92.9% (from 113.0 to 105.0 mIU/L), GH — 92.2% (from 16.6 to 15.3 ng/mL), prolactin — 89.7% (from 8,778 to 7,877 mIU/L), and noradrenaline — 76.0% (from 267 to 203 pg/mL).

The presented results practically correspond to our previously obtained data regarding the composition preservation of lyophilized CBS from parturients with extragenital pathology [10]. The analysis of the obtained data indicates that following cryopreservation, storage at –40 °C, lyophilization, and reconstitution, the contents of AFP, hCG, cortisol, and GH did not differ from the corresponding values in native CBS, while for prolactin and noradrenaline, only minor trends without statistical significance were observed. In general, these data confirm the stability of the regulatory component of CBS under the selected lyophilization program.

Thus, the established temperature intervals of phase transformations enable the development of technological regimens for the cryopreservation and lyophilization of human CBS, ensuring a high degree of composition preservation in terms of biochemical parameters (total protein, protein fractions) and key regulatory components (AFP, hCG, cortisol, GH, prolactin, and noradrenaline). Consequently, human CBS lyophilized according to the developed methodology meets the demands of clinical and military medicine for innovative, effective therapeutic agents for wartime utilization.

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ФАЗОВІ ПЕРЕТВОРЕННЯ ТА БІЛКОВО-ГОРМОНАЛЬНИЙ ПРОФІЛЬ
СИРОВАТКИ КОРДОВОЇ КРОВІ ЛЮДИНИ ЗА НИЗЬКИХ ТЕМПЕРАТУР

***Ключові слова**:* сироватка кордової крові, термомеханічний аналіз, низькі температури, кріоконсервування, ліофілізація, фактори росту, гормони.