Theoretical and Experimental Cryobiology

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ANTIOXIDANT AND ANTIHYPOXIC ACTIVITY OF HUMAN CORD BLOOD EXTRACTS OBTAINED WITH VARIOUS TEMPERATURE REGIMENS OF DESTRUCTION OF CELLULAR ELEMENTS AND EXTRACTION MEDIA

The research analysed the dependence of antioxidant and antihypoxic activity of human cord blood (HCB) extracts on the temperature regimen of destruction, salt composition and pH of the extraction solution. Using the adrenaline autooxidation model, it was found that the antiradical activity of all the cryoextracts exceeded this index of those obtained using the same extraction solutions in combination with incubation at 70 °C for 30 min, or with the use of hypotonic lysis. The content of malondialdehyde and lipid hydroperoxides in the brain of animals exposed to normobaric hypoxia, which were injected with a low-molecular fraction (up to 10 kDa) isolated from HCB cryoextracts, was found to be significantly lower than when being injected with a low-molecular fraction of extracts obtained using high temperature or hypotonic lysis. During hypotonic lysis, enzyme molecules and low-molecular biologically active peptides also undergo significant destructive effects of endogenous proteases and lose their specific activity. It has been proven that cryoextraction, regardless of the applied freeze-thawing rates and the composition and pH of the medium, allows obtaining final products with significantly higher anti-radical and anti-hypoxic properties than after holding at high temperature and hypotonic lysis. Our findings prove the prospects of using cryotechnologies in the processing animal and human tissues and blood to obtain raw materials enriched with biologically active substances to produce medicines.

***Key words**: cord blood, extraction, low-molecular fraction, anti-radical properties, anti-hypoxic action.*

The development of methods for obtaining biologically active low-molecular compounds of natural origin is one of the promising areas of modern biology, biotechnology and pharmacology [2, 10, 20, 22]. In clinical practice, the effectiveness of the use of cellular elements, plasma, serum and individual high-molecular substances of human cord blood (HCB) has been confirmed for many years [4, 18, 19, 23]. As evidenced by the results of numerous studies, during disintegration of HCB cells and tissues of the fetoplacental complex, substances with pronounced properties of biogenic regulators and stimulants are released, with the help of which it is possible to influence the intensity of the inflammatory process, the direction of the immune response in infectious diseases, as well as the speed and quality of reparative regeneration [8, 9, 11]. Unfortunately, the chosen methods of decellularization of the initial raw material, extraction of biologically active substances (BAS) from it, and sterilization of the final product can significantly affect its composition, leading to variability in the concentration of active substances [5]. Most often, hypotonic lysis is used to obtain extracts of fetoplacental complex tissues [15], heating

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to high temperatures within the range of 50—100 °C and higher to improve hydrolysis, enzyme inactivation, sterilization, *etc*., [3, 14], ultrasound treatment [12], and exposure to low temperatures [21]. Of the entire spectrum of technologies for obtaining BAS from natural material, the most promising and prevailing in terms of several qualitative characteristics are cryotechnologies [2, 21]. The use of low temperatures during the processing of biological raw materials contributes to a more complete destruction of cellular structures and the release of BAS into the extraction solution [1, 2, 6]. However, to date, there is very little experimental data in the scientific literature on the influence of various low-temperature regimens, composition, and pH of extraction media on the qualitative and quantitative arrangement of the final products. The aim of this research was to compare the effect of different temperature regimens of destruction and extraction, salt composition and pH of the extraction medium on the antioxidant and antihypoxic properties of human cord blood extracts.

MATERIALS AND METHODS

Everything concerning the obtaining of human cord blood was done in accordance with the recommendations of the Declaration of Helsinki of the World Medical Association "Ethical principles of scientific medical research involving human subjects", and the manipulations with animals were carried out in accordance with the "General principles of animal experiments", adopted by the First National Congress in Bioethics (Kyiv, 2001) and agreed with the provisions of the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Strasbourg, 1985). The HCB was procured during physiologically normal childbirth at the 38^{th}—40^{th} week of pregnancy in maternity hospitals of the city of Kharkiv with the prior consent of women in labour. Blood sampling was performed by a medical team by freely flowing blood from the umbilical cord into a sterile container without anticoagulant until the placenta was separated. The volume of one sample ranged from 30 to 100 ml.

The obtained HCB samples were mixed with several variants of extraction medium of different salt composition and pH in a ratio of 1:4 and incubated for 30 min at room temperature (18—20 °C). The following extraction media were used: 150 mM NaCl and KCl with pH 5.0 and 7.4 and distilled water with pH 5.6. HCB cryoextracts were obtained by freezing and warming the samples at different rates. Two low-temperature decellularization and extraction modes were used: rapid freezing-rapid thawing and slow freezing-slow thawing. The samples were frozen using a ZP10 programmable freezer (SDTB with EU at the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine, Kharkiv) with a function of recording temperature values during the freezing. The samples were rapidly frozen at an average rate of 30 °C/min to a final temperature of –196 °C. The slow freezing of samples placed in cassettes was performed at a slow rate of 1 °C/min in liquid nitrogen vapor. Rapid warming of samples was performed using a water bath at a temperature of 38 °C, slow warming — at a temperature of (18—20) °C. For compari-son, the HCB extracts obtained by incubation in a water bath at 70 °C for 30 min and subsequent cooling to room temperature [17] and those obtained by hypotonic lysis induced by incubation in distilled water with pH 5.6 for 30 min at room temperature were used.

After the extraction, the samples were centrifuged for 10 min at 10,000 g. The supernatants obtained were used for further studies.

The low-molecular fraction (up to 10 kDa) of the HCB extracts was isolated by ultrafiltration using a membrane module "Vivaflow200" (Sartorius, Germany) [17].

Quantitative determination of total protein content was performed by the Lowry colorimetric method [13].

The antioxidant activity of human cord blood extracts was studied by determining their antiradical activity (ARA) using the adrenaline autoxidation model [7]. The method for assessing the inhibition of adrenaline autoxidation is based on the reaction of non-enzymatic oxidation of adrenaline to adrenochrome in an alkaline medium, which is accompanied by the accumulation of superoxide anion radical. Since antioxidant activity is the ability of BAS to eliminate or inhibit the formation of free radicals, the ability of extracts to inhibit the autoxidation of adrenaline to adrenochrome in an aqueous medium can be used to quantify their antioxidant activity.

Two ml of 0.15 M sodium carbonate buffer (pH 10.2) was suplemented to the test tubes, then 20 µl of the studied extracts were added. The reaction was started by adding 0.4 ml of 0.1% adrenaline solution to the system. After 10 min, the optical density of the solutions was measured using a spectrophotome-

ter «UNICO 2100" (UNICO, USA) at a wavelength of 480 nm.

The antiradical activity of the studied products was expressed in percentage and calculated by the formula:

$$APA = \frac{(E_c - E_t)}{E_c} 100\ \%,$$

where E_c is the optical density of the control solution; E_t is the optical density of the solution containing the extract being tested.

The antihypoxic activity of the low molecular weight fraction (up to 10 kDa) of the HCB extracts *in vivo* was examined by determining the content of malondialdehyde (MDA) and lipid hydroperoxides in the brain of mice subjected to normobaric hypoxia-hypercapnia [16]. The principle of the method for determining the content of MDA is its reaction with thiobarbituric acid to form a colored trimethine complex with an absorption maximum at 532 nm. The relative content of lipid hydroperoxides was determined by measuring the optical density at 480 nm. The experiments were performed in white nonlinear male mice weighing 29.5–30.5 g, which were kept on a standard diet. The studied fractions were administered intraperitoneally daily for 5 days and 30 min before the experiment at a dose of 4 mg (in terms of dry matter) per 1 kg of animal body weight. Mice were placed in a 250 ml flask, which was tightly closed with a sealed glass stopper. At the 20th minute of exposure to hypoxia/hypercapnia, the animals were removed from the flask, decapitated, the brain was removed, minced with scissors, transferred to a glass tube of a mechanical homogenizer, which was cooled by immersion in a mixture of water and ice, and homogenization was carried out by ten passes of a pestle at 400 rpm.

Statistical analysis of experimental data was performed using the software package "StatGraphics Plus 2.1" (Statgraphics Technologies, Inc., USA) by analysis of variance ANOVA using Post hoc tests like Scheffe, Bonferroni, Fisher's LSD, Tukey-Kramer and Kruskal-Wallis. The significance of the influence of a factor on the studied parameter was determined by the p-value criterion. Differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

The results of the study showed that cryoextracts, regardless of the used freezing and thawing rates, composition and pH of the extraction solutions, have a higher ARA compared to extracts obtained using

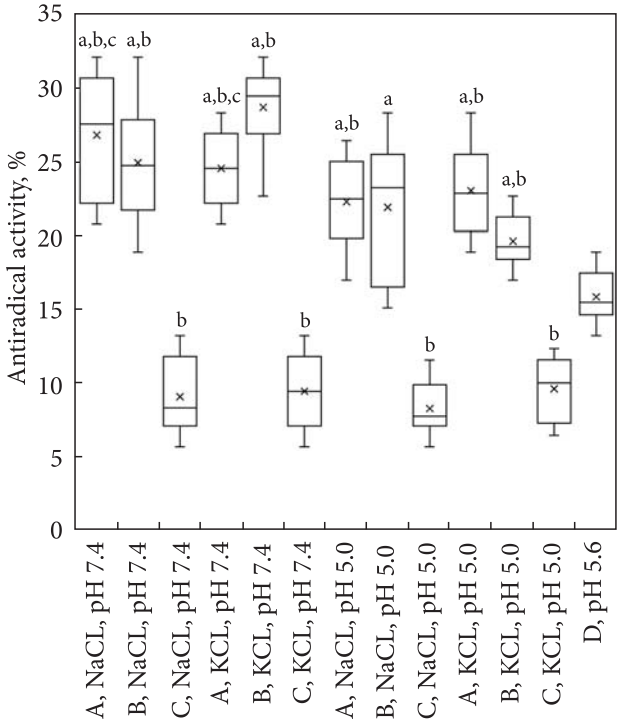

Fig. 1. ARA of low molecular weight fractions of HCB extracts: A — rapid freezing-rapid thawing; B — slow freezing-slow thawing; C — holding at 70 °C for 30 min; D — hypotonic lysis; (a — significant differences compared to the extract obtained by method C using the same solution, $p \leq 0.05$; b — significant differences compared to the extract obtained by method D, $p \leq 0.05$; c — significant differences compared to the extract obtained by method A using the same solution with pH 5.0, $p \leq 0.05$)

the same solutions in combination with holding at 70 °C for 30 min and by hypotonic lysis (except for the one obtained using 150 mM NaCl with pH 5.0 in combination with slow freezing-slow thawing). It should be noted that the use of hypotonic lysis also allows to obtain an extract with a significantly higher ARA index compared to extraction at 70 °C, but not as high as in the case of cryoextracts. This indicates that cryotechnologies provide more effective preservation of the antioxidant properties of BAS contained in the extracts compared to extraction at 70 °C and using osmotic lysis. (Fig. 1).

The dependence of the ARA of the HCB cryoextracts on the freezing-thawing regimen and on the salt composition of the decellularization and extraction medium was not revealed. As for the dependence of this index on the pH of the medium, significant differences occurred between cryoextracts obtained using 150 mM NaCl with pH 5.0 and 7.4 in combination with rapid freezing-rapid thawing, and between cryoextracts obtained using 150 mM KCl

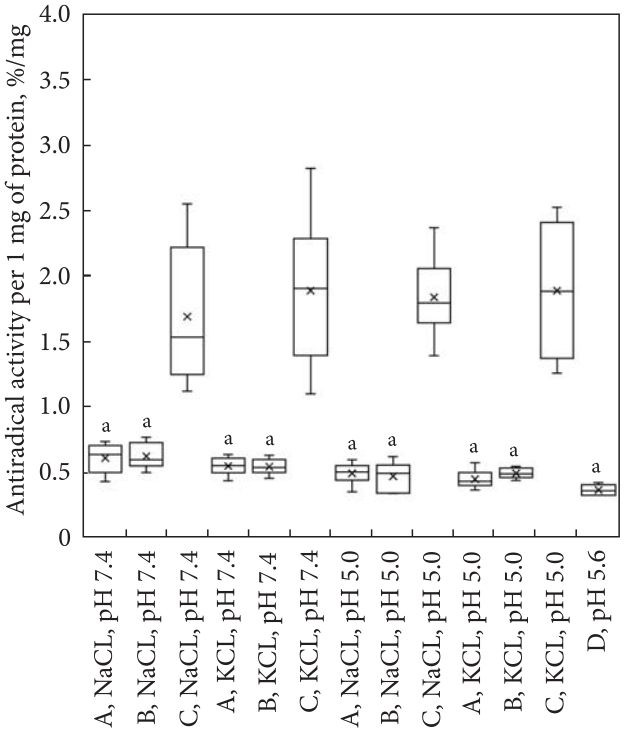

Fig. 2. ARA of HCB extracts per 1 mg of protein: A — rapid freezing-rapid thawing; B — slow freezing-slow thawing; C — holding at 70 ºC for 30 min; D — hypotonic lysis; (a — significant differences compared to the extract obtained by method C using the same solution, $p \leq 0.05$)

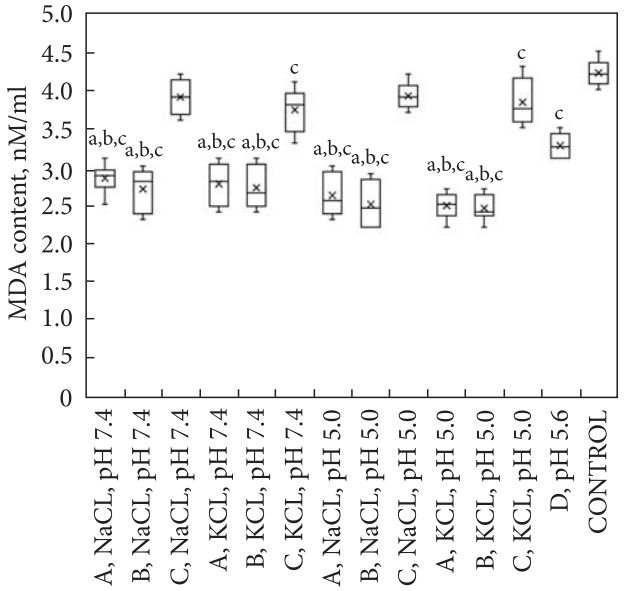

Fig. 3. MDA level in brain homogenate of mice subjected to normobaric hypoxia-hypercapnia after a 5-day course of injections of low molecular weight fractions of HCB extracts: A — rapid freezing-rapid thawing; B — slow freezing-slow thawing; C — holding at 70 ºC for 30 min; D — hypotonic lysis; Control — 0.9% NaCl; (a — significant differences compared to the extract obtained by method C using the same solution, $p \leq 0.05$; b — significant differences compared to the extract obtained by method D, $p \leq 0.05$; c — significant differences compared to the Control, $p \leq 0.05$)

with pH 5.0 and 7.4 in combination with slow freezing-slow thawing. In both cases, the cryoextract obtained with a more acidic medium had a lower ARA (Fig. 2).

The inhibition of adrenaline autooxidation used by us enables us to assess the overall antioxidant effect of BAS in extracts. However, as it is known, the antioxidant system consists of enzymatic and non-enzymatic links. For extracts of animal origin, it is important to preserve the activity of enzymes of the antioxidant system (primarily superoxide dismutase, catalase and peroxidase).

The low ARA of extracts obtained using heating may indicate that under the influence of high temperature, on the one hand, aggregation of a significant number of proteins occurs, which leads to their precipitation, and on the other hand, the structure of proteins, in particular enzymes, changes significantly, as a result of which the enzymes lose their specific activity. Low temperatures are a less damaging factor for macromolecules during decellularization and extraction and, in addition, they inhibit the action of proteases and allow preserving the activity of enzymes of the antioxidant system of cryoextracts.

Since the studied extracts had different protein contents, their ARA was analyzed per 1 mg of protein. This index of extracts obtained by holding at 70 ºC for 30 min, regardless of the salt composition and pH of the extraction solution, was significantly higher ($p \leq \leq 0.05$) than after using cryoextraction and the same solution or hypotonic lysis (Fig. 2). This may indicate that ARA of extracts obtained by heating is not provided by enzymes of the antioxidant system, but by some non-protein components or low molecular weight peptides. It was previously shown that the low-molecular (up to 5 kDa) fraction of cattle cord blood has antioxidant and antihypoxic effects, which indicates the presence of low-molecular substances with antioxidant properties in it [8, 11]. The task of determining the content in cord blood of such well-studied biologically active substances as trace elements, vitamins, steroid and peptide hormones, *etc.*, with a molecular weight below 5 kDa, is not difficult, but far from all low-molecular substances in the composition of cord blood have been isolated, identified and studied for biological activity. To confirm the involvement of their low-molecular components into ensuring the antioxidant effect of cord blood extracts, the antihypoxic effect of fractions up to 10 kDa of the HCB extracts was studied in an *in vivo* experiment. The results of determining the content of

MDA(Fig. 3) and lipid hydroperoxides (Fig. 4) in the brain of animals exposed to normobaric hypoxia-hypercapnia after a 5-day course of injections of low molecular weight fractions of the HCB extracts indicate a higher antihypoxic activity of cryoextracts, regardless of the temperature regimen, pH and salt composition of the decellularization and extraction medium compared to those obtained using high temperature or hypotonic lysis.

Extraction by hypotonic lysis also allowed to obtain extracts with higher antihypoxic activity compared to the use of high temperature ($p \leq 0.05$). The antihypoxic activity of extracts obtained with the use of incubation at 70 °C was at the control level (instead of low molecular weight fractions of extracts, mice were administered saline), although there was a noticeable tendency to reduce the level of MDA and lipid hydroperoxides in the supernatant of the brain homogenate of experimental animals.

In addition, it was found that the content of MDA (Fig. 3) and lipid hydroperoxides (Fig. 4) in the brain of animals exposed to normobaric hypoxia-hypercapnia after a 5-day course of injections of the low molecular weight fraction of human cord blood extract obtained by hypotonic lysis, although significantly lower compared to that which occurred with the administration of low molecular weight fractions of extracts obtained with the use of high temperature, was simultaneously significantly higher than in the case of the use of low molecular weight fractions of cryoextracts, regardless of the method of their preparation. This fact can be explained by the fact that, since hypotonic lysis occurs at positive temperatures, the destruction of low-molecular peptides and their loss of biological activity under the action of endogenous proteases is possible. The advantage of cryogenic technology is that the action of endogenous enzymes on BAS during the extraction is suppressed, and therefore more substances particularly with antioxidant and antihypoxic properties, enter the extract from cord blood cells with a preserved activity.

Thus, we established that the antiradical activity of cryoextracts, regardless of the temperature regimens used to obtain them, the salt composition and pH of the extraction solutions, was higher than the one for the extracts obtained using the same extraction solutions in combination with holding at 70 °C for 30 min, or with the use of hypotonic lysis. The results of the study of the antihypoxic effect of fractions up to 10 kDa of the HCB extracts *in vivo* also showed that this index of cryoextracts was significantly higher compared to those obtained using heating or hypotonic lysis.

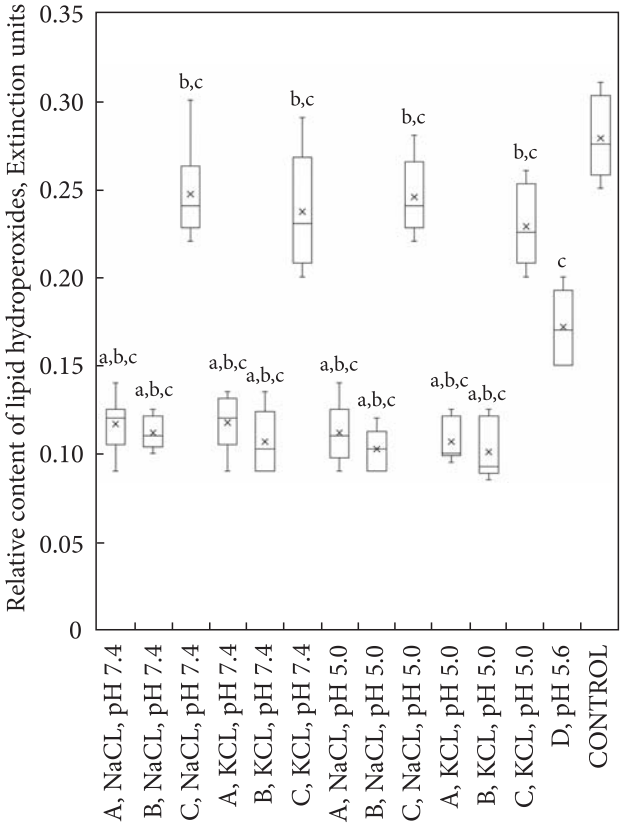

Fig. 4. The content of lipid hydroperoxides in the brain homogenate of mice subjected to normobaric hypoxia-hypercapnia after a 5-day course of injections of low-molecular fractions of HCB extracts: A — rapid freezing-rapid thawing; B — slow freezing-slow thawing; C — holding at 70 °C for 30 min; D — hypotonic lysis; Control — 0.9% NaCl; (a — significant differences compared to the extract obtained by method C using the same solution, $p \leq 0.05$; b — significant differences compared to the extract obtained by method D), $p \leq 0.05$; c — significant differences compared to the Control, $p \leq 0.05$)

The performed studies prove the prospects of implementing cryotechnologies for the products based on BAS of natural origin.

CONCLUSIONS

Our findings showed that low temperature decellularization and extraction allowed obtaining human cord blood extracts with higher antiradical and antihypoxic activity compared to the use of exposure at 70 °C for 30 min or hypotonic lysis.

The content of biologically active substances with antioxidant/antiradical activity in cryoextracts is practically independent of the medium pH used for decellularization and extraction and does not depend at all on its salt composition and temperature regimen (slow freezing-slow thawing or rapid freezing-rapid thawing).

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АНТИОКСИДАНТНА ТА АНТИГІПОКСИЧНА АКТИВНІСТЬ ЕКСТРАКТІВ КОРДОВОЇ КРОВІ ЛЮДИНИ, ОТРИМАНИХ З ЗАСТОСУВАННЯМ РІЗНИХ ТЕМПЕРАТУРНИХ РЕЖИМІВ ДЕСТРУКЦІЇ КЛІТИННИХ ЕЛЕМЕНТІВ ТА СЕРЕДОВИЩ ЕКСТРАГУВАННЯ

У роботі проведено аналіз залежності антиоксидантної та антигіпоксичної активності екстрактів кордової крові людини (ККЛ) від температурного режиму деструкції, соляного складу і pH розчину екстрагування. На моделі аутоокиснення адреналіну встановлено, що антирадикальна активність всіх кріоекстрактів перевищувала даний показник екстрактів, отриманих із використанням тих самих розчинів екстрагування у поєднанні з витримуванням при 70 °С протягом 30 хв, або застосуванням гіпотонічного лізису. Виявлено, що вміст малонового діальдегіду та гідропероксидів ліпідів в мозку підданих впливу нормобаричної гіпоксії тварин, яким вводили низькомолекулярну фракцію (до 10 кДа), виділену з кріоекстрактів ККЛ, значуще нижчий, ніж після введення низькомолекулярної фракції екстрактів, отриманих із застосуванням високої температури або гіпотонічного лізису. Під час гіпотонічного лізису молекули ферментів та низькомолекулярних біологічно активних пептидів також зазнають суттєвого руйнівного впливу ендогенних протеаз і втрачають свою специфічну активність. Доведено, що кріоекстрагування, незалежно від застосованих швидкостей заморожування-відігрівання і складу та pH середовища, дозволяє отримати кінцеві продукти із значно більш високими антирадикальними та антигіпоксантними властивостями, ніж після витримування при високій температурі та гіпотонічного лізису. Одержані результати доводять перспективність використання кріотехнологій в процесі переробки тканин та крові тварин і людини з метою отримання збагаченої біологічно активними речовинами сировини для виробництва лікарських засобів.

***Ключові слова*:** кордова кров, екстрагування, низькомолекулярна фракція, антирадикальні властивості, антигіпоксична дія.