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PRESERVATION OF PROTEIN FRACTIONS AND HORMONES OF PLACENTAL BLOOD SERUM AFTER FREEZE-DRYING

The preservation of placental blood serum (PBS) following freeze-drying was studied, factoring in the course of pregnancy in parturient women. Placental blood was obtained from women with a physiological pregnancy, premature births, and concomitant autoimmune thyroiditis or hypertension. Serum was obtained by separating the supernatant after centrifugation of placental blood. After freeze-drying, the concentration of total protein and the ratio of protein fractions in the PBS were determined by the biuret method, and the levels of prolactin, human chorionic gonadotropin (hCG), alpha-fetoprotein (AFP), cortisol and somatotropin (STH) were measured by the enzyme-linked immunosorbent assay. It was found that the composition of the PBS depended on the course of pregnancy. Extragenital pathology is accompanied by a redistribution of protein fractions and changes in prolactin, AFP, STH and hCG levels. After freeze-drying the PBS from healthy parturients, the studied parameters did not differ from those in native serum, whereas in cases of complicated pregnancy, changes in the ratio of protein fractions were observed.

Key words: *placental blood serum, freezing, freeze-drying, protein fractions, hormones.*

Modern medicine, especially in military and emergency settings, requires effective and logistically convenient dosage forms suitable for use at all stages of hospitalization. Lyophilized preparations meet these requirements, as they are characterized by high physical and chemical stability, a long shelf life, no need for special storage conditions; their minimal weight and volume facilitate transportation, while their rapid rehydration ensures ease of use [32].

Traumatic brain injury (TBI) complicated by hypothermia is a common problem among military personnel and civilians in combat zones, particularly in Ukraine. This is due to the fact that injuries often occur in conditions of low temperatures, the absence of adequate shelter, and complicated or delayed evacuation [6, 30]. A significant proportion of victims sustain injuries against the background of general hypothermia, as evidenced by the consistent incidence of hypothermia among the wounded [6, 10, 25, 30]. Hypothermia can significantly exacerbate the consequences of TBI, contributing to the development of inflammatory processes, coagulopathy, microcirculatory disorders, and an increased risk of secondary brain injury, making this combination one of the most dangerous clinical conditions in modern warfare. Furthermore, hypothermia is an independent risk factor for mor-

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tality following combat-related injuries, being associated with increased mortality [6, 30] and poorer clinical outcomes in trauma patients [25, 29, 31].

Given the above, the current task is to improve existing treatment approaches and search for innovative multitargeted therapy methods. One of the modern biomedical approaches to the effective restoration of damaged organs and regulatory systems is the use of placental derivatives, particularly placental blood components such as cells, plasma, serum, *etc*. These components possess unique therapeutic potential and play a critical role in the regeneration of damaged tissue by suppressing inflammatory reactions and apoptosis, inducing angiogenesis, and activating regenerative processes [19, 22, 23, 27]. Placental derivatives have been shown to possess immunomodulatory properties [16] and demonstrate efficacy in the treatment of heart failure [21]. They have also been found to improve the course of experimental encephalomyelitis [26], ischemic stroke, experimental Parkinson's disease, and spinal cord injuries [13—15], stimulate the regeneration of nervous tissue and exhibit neuroprotective properties [11]. These studies underscore the potent multitargeted therapeutic effect of placental derivatives, which is mediated through reparative and regulatory processes.

The unique profile of biologically active substances of placental blood serum (PBS), including proteins, hormones, cytokines, growth factors, neurotrophic factors and immunomodulators, determines its regenerative, anti-inflammatory, immunosuppressive, anti-apoptotic and neuroprotective properties [17, 23]. This provides a rationale for the potential efficacy of PBS in mitigating the consequences of concussive injury complicated by hypothermia. Given the significant therapeutic potential of PBS, ensuring the long-term preservation of its biological properties remains a primary objective. Freeze-drying, as one of the most effective methods for preserving PBS, can potentially ensure the maximum stability of its components. However, the efficacy of this process largely depends on the initial composition of the source material. It is well established that PBS composition is characterized by high variability and is influenced by several factors, particularly the clinical features of pregnancy and labor [5, 8, 20, 24]. This necessitates a rigorous selection of placental blood samples for further experimental and clinical use, taking into account the maternal medical history, the course of pregnancy and childbirth, and the presence of gestational complications.

Thus, given that both the course of pregnancy and freeze-drying can affect the content of biologically active compounds in the PBS, which are potentially capable of having a corrective effect in TBI against the background of hypothermia, the aim of the work was to study the safety of placental blood serum after freeze-drying, taking into account the course of pregnancy in parturient women.

MATERIALS AND METHODS

In the study, human PBS samples were used. The experimental design was approved by the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. Human placental blood we used, was procured from the umbilical vein of postpartum placentas at 32–40 weeks of gestation, with informed consent from the mothers, and in accordance with the principles of the Declaration of Helsinki adopted by the General Assembly of the World Medical Association.

Placental blood was obtained from women in labor in the following groups: with a physiological course of pregnancy, women in labor with concomitant extragenital pathology (autoimmune thyroiditis (AT) or hypertension); and also in case of premature labor. Placental blood was collected using a closed system without interference in the management of labor, the postpartum period, or the medical care of the newborn. Placental blood was drawn from the umbilical vein after complete placental expulsion and cessation of umbilical cord pulsation, then collected into dry sterile 50 ml vials. To allow clot formation, the vials were kept at room temperature for 4 hrs, followed by storage at 4 °C for 10 hrs. After clot retraction, the samples were centrifuged for 10 min at 785 g (TsLMN-R10-01-Elekon, RF), and the supernatant (PBS) was collected. A total of 32 PBS samples were obtained. During sampling, gestational age, maternal medical history, gestational complications, the course of labor, and the newborn's condition were recorded.

The serum was aliquoted into 1.0 ml cryovials (Nunc, USA) and frozen at a cooling rate of 1 °C/min to a temperature of –40 °C. The temperature interval of –40…–35 °C, corresponding to the phase transition of the serum into a solid state, was previously established by the method of thermomechanical analysis [28]. This interval constitutes the optimal range of final freezing temperatures for PBS, theoretically ensuring the maximum preservation of bioactive compounds during cryopreservation and subsequent freeze-drying. Frozen PBS samples were stored for one month at –40 °C and subsequently subjected to freeze-drying. The drying process started at an initial temperature of –40 °C, reaching a final temperature of 30 °C over duration of 10 hrs, with an ultimate (residual) pressure of 10 Pa. The residual moisture content of the serum samples did not exceed 2%. Reconstitution of the PBS was performed immediately before the study by adding distilled deionized water to a final volume of 1.0 ml.

To evaluate the stability of bioactive compounds in native PBS samples compared to those subjected to cryopreservation and subsequent freeze-drying, we determined the concentrations of components which, due to their biological properties, could potentially contribute to the mitigation of damage caused by blast-induced concussive injury.

The levels of prolactin, hCG, AFP, cortisol, STH, and noradrenaline were determined by enzyme-linked immunosorbent assay according to the instructions of commercial kits, using an "ELx 808"microplate reader (Bio-Tek Instruments, Inc., USA) and the "SUNRISE" microplate photometer (TECAN, Austria). The total protein content and the ratio of protein fractions in the PBS samples were determined via the biuret method using a commercial kit "Philisit-Diagnostica" (Ukraine) and a ULAB 102UV spectrophotometer "ULAB 102UV" (Ulab, China).

Statistical analysis was performed based on the sample size and data distribution. To compare paired samples (*n* = 20; PBS samples of 38—40 weeks of gestation), the paired Student's t-test was used. For small sample sizes (*n* = 3 for PBS samples from parturients with extragenital pathology and *n* = 6 for PBS samples of 32 weeks of gestation), the Wilcoxon signed-rank test was applied. Welch's t-test was used to compare independent samples with unequal variances. Data analysis was conducted using Past software, version 3.25 (University of Oslo, Norway). Results are presented as the mean ± standard error of the mean (SEM). The critical level of significance was set at $p \le 0.05$.

RESULTS AND DISCUSSION

It was found that the total protein concentration in PBS samples of 38—40 weeks of gestation was 52.96 ± 1.46 g/L (Table 1), which was lower than the reference values for adult peripheral blood serum [1]. In PBS samples obtained from parturients with preterm labor or concomitant extragenital pathology (EP), the total protein concentration was also lower compared to adult peripheral blood (Table 1). The reduced total protein concentration in the blood is physiological for pregnant women, which, to some extent, explains its correspondingly lower concentration in PBS.

The ratio of protein fractions in PBS samples obtained from healthy parturients was consistent across different gestational ages. However, a significant increase in the relative content of the α-2-globulin fraction was observed in PBS samples from parturients with EP. This may be attributed to a physiological demand for the correction of pathogenetic changes. The α-2-globulin fraction contains acute-phase proteins, which are involved in the inhibition of various non-specific plasma proteases, the transport of cytokines, growth factors, and hormones, as well as the development of immune and inflammatory reactions, exhibiting predominantly immunosuppressive effects.

The content of hormones in native PBS samples from healthy parturients was consistent with the literature data [4, 7, 33]. High levels of AFP, STH, hCG, prolactin, and noradrenaline were determined. At the same time, the AFP concentration declined as gestational age increased, which is attributed to the slowing of fetal and placental morphogenesis in late pregnancy. Thus, the mean AFP concentration in PBS samples at 32 weeks of gestation was 154625.0 ± 2463.0 ng/ml, whereas in samples at 38—40 weeks, it was 53491.0 ± 808.9 ng/ml (Table 1). These findings align with data indicating a 30% weekly reduction in the mean AFP levels in neonatal placental blood between 35 and 42 weeks of gestation [4]. A decrease in STH levels was also observed — from 31.3 ± 1.5 ng/ml in PBS samples at 32 weeks of gestation to 16.6 ± 0.9 ng/ml in those at 38—40 weeks. This reflects a gradual decline in STH levels starting mid-term, which is associated with a reduced functional demand for high growth factor levels prior to delivery [7]. Conversely, prolactin levels increased during this period, rising from 4642.0 ± 61.0 mIU/l to 8778.0 ± 105.6 mIU/l, consistent with research on umbilical cord blood in newborns from 23 to 43 weeks of gestation [33].

In PBS samples obtained from parturients with AT, a significant reduction in the levels of hormones associated with proliferative and regenerative processes — specifically prolactin, AFP, and STH — was observed against a background of elevated cortisol. In PBS samples from parturients with hypertension, a significant decrease in AFP levels and an increase in cortisol were also noted, while the level of hCG — a key reproductive hormone with immunosuppressive effects — was reduced.

Following storage at −40 °C and subsequent freeze-drying, the total protein concentration and the ratio of protein fractions in PBS samples from healthy parturients showed no significant difference compared to native serum (Table 2).

In contrast, in PBS samples from parturients with EP, changes in the ratio of protein fractions were observed after freeze-drying (Table 2). Specifically, in PBS samples from parturients with AT, the relative content of β-globulins decreased by 1.3 times, and α-2-globulins by 1.7 times, while the content of α-1-globulins increased by 3.1 times. Freeze-drying of PBS from parturients with hypertension led to a 5.3-fold decrease in the relative amount of β-1-globulins and a 1.6-fold increase in the proportion of α-globulins. The changes observed are likely associated with the combined effect of physical stress factors during freezing and freeze-drying, which may lead to alterations in the tertiary and quaternary structures of proteins, as well as their partial aggregation or degradation [2]. The varying sensitivity of specific proteins (structural, transport, regulatory, *etc.*) and protein fractions (albumins, α-/β-/γ-globulins) to such stresses may also account for the shifts in their ratios following freeze-drying.

The levels of the studied hormones in PBS samples from parturients with EP, following cryopreservation and freeze-drying, did not differ significantly from the corresponding values in native serum (Table 2). In PBS samples from parturients with AT, a slight increase in prolactin, STH, and cortisol levels was observed; however, these changes did not reach statistical significance. According to literature data, low-temperature storage of certain hormones (hormones of the insulin-like growth factor family, steroid hormones) can lead to specific alterations in their levels, which are attributed to modifications in molecular structure, progressive dissociation of hormone-protein complexes in frozen serum, or proteolysis [3, 12, 18]. At the same time, studies have demonstrated no changes in the levels of hCG, placental growth hormone, and AFP (as well as their associated binding proteins) during long-term serum storage. While the levels of some of these hormones and binding proteins decreased or increased during storage, these changes did not exceed the inter-assay variation of the enzyme-linked immunosorbent assay and remained within the clinical reference range [9]. Cur-

Table 1. **Total protein concentration, protein fraction ratios, and hormone levels in human placental blood serum**

Indicators (UOM)/Norm	PBS of healthy parturients		PBS of WL with AT, $n = 3$	PBS of WL with hypertension, $n = 3$
	Gestation period 32 weeks, $n = 6$	Gestation period 38—40 weeks, $n = 20$		
Total protein (g/L)/65—85	49.24 ± 0.84	52.96 ± 1.46 #	53.12 ± 0.54	62.1 ± 2.02 #*
Albumin (%)/56—66	65.91	66.98	59.19	61.44
α-1-globulins (%)/3—6	2.86	2.42	1.81	1.42
α-2-globulins (%)/7—10	6.21	4.95	12.43 #, *	8.94*
β-globulins (%)/7—12	11.44	11.97	15.22	14.47
γ-globulins (%)/13—19	13.58	13.68	11.35	13.73
Prolactin (mIU/L)/86—496	4642.0 ± 61.0	8778.0 ± 105.6	5148.0 ± 66.1 *	9974.0 ± 17.5
hCG (mIU/L)/less than 5	104.0 ± 6.3	113.0 ± 6.4	98.3 ± 0.9	39.1 ± 0.7 *
AFP (ng/ml)/less than 5	154625.0 ± 2463.0	53491.0 ± 808.9 #	32822.0 ± 413.7 *	27952.0 ± 61.4 *
Cortisol (mcg/dL)/4.82—19.7	3.99 ± 0.36	4.17 ± 0.21	9.82 ± 0.19 *	8.17 ± 0.11 *
STH (ng/ml)/less than 2.1	31.3 ± 1.5	16.6 ± 0.9 #	5.8 ± 0.2 *	13.6 ± 0.1
Noradrenaline (pg/ml)/less than 600	302.0 ± 11.9	267.0 ± 23.2	238.0 ± 19.3	296.0 ± 7.6

The difference is significant compared to the PBS of healthy parturients at 32 weeks of gestation; * the difference is significant compared to the PBS of healthy parturients at 38—40 weeks of gestation, $p \leq 0.05$.

Table 2. **Total protein concentration, protein fraction ratios, and hormone levels in PBS samples before and after storage at –40 °C followed by freeze-drying**

Indicators (UOM)/Norm	Native PBS			PBS after storage at –40 °C			PBS after freeze-drying		
	Healthy parturients (n = 20)	Parturients with AT (n = 3)	Parturients with hypertension (n = 3)	Healthy parturients (n = 20)	Parturients with AT (n = 3)	Parturients with hypertension (n = 3)	Healthy parturients (n = 20)	Parturients with AT (n = 3)	Parturients with hypertension (n = 3)
Total protein (g/L)/65—85	52.96 ± 1.46	53.12 ± 0.54	62.1 ± 2.02	52.55 ± 1.50	53.72 ± 0.58	61.51 ± 1.46	51.81 ± 1.65	53.85 ± 0.54	65.30 ± 2.02
Albumin (%)/56—66	66.98	59.19	61.44	66. 53	65.17	59.86	66.91	62.45	59.19
α-1-globulins (%)/3—6	2.42	1.81	1.42	2.14	5.34	1.46	2.41	5.58 *	7.59*
α-2-globulins (%)/7—10	4.95	12.43	8.94	4.01	6.84	8.72	5.04	7.43 *	9.78
β-globulins (%)/7—12	11.97	15.22	14.47	11.84	10.90	13.22	11.97	11.34 *	8.94 *
γ-globulins (%)/13—19	13.68	11.35	13.73	13.37	11.75	16.74	13.67	13.20	14.50
Prolactin (mIU/L)/86—496	8778.0 ± ± 105.6	5148.0 ± ± 66.1 *	9974.0 ± ± 17.5	8721.0 ± ± 253.3	5038.0 ± ± 62.5	9931.0 ± ± 21.8	8737.0 ± ± 250.6	5214.0 ± ± 36.1	9925.0 ± ± 14.4
hCG (mIU/L)/less than 5	113.0 ± 6.4	98.3 ± 0.9	39.1 ± 0.7	111.0 ± 8.4	96.8 ± 1.0	38.3 ± 1.2	110.0 ± 5.6	97.6 ± 0.8	37.5 ± 0.9
AFP (ng/ml)/less than 5	53491.0 ± ± 809.0	32822.0 ± ± 413.7	27952.0 ± ± 61.4	52282.0 ± ± 407.3	32798.0 ± ± 327.0	27255.0 ± ± 492.0	53486.0 ± ± 823.2	32674.0 ± ± 211.9	27820.0 ± ± 293.9
Cortisol (mcg/dL)/4.82—19.7	4.17 ± 0.21	9.82 ± 0.19	8.17 ± 0.11	4.07 ± 0.18	9.88 ± 0.19	7.92 ± 0.29	4.05 ± 0.15	10.12 ± 0.17	7.85 ± 0.45
STH (ng/ml)/less than 2.1	16.60 ± 0.90	5.83 ± 0.21	13.58 ± 0.19	16.10 ± 0.72	5.54 ± 0.21	13.23 ± 0.21	16.20 ± 0.66	6.04 ± 0.19	14.02 ± 0.29
Noradrenaline (pg/ml)/less than 600	267.0 ± 23.2	238.0 ± 19.3	296.0 ± 7.6	261.0 ± 15.6	232.0 ± 14.5	301.0 ± 4.2	258.0 ± 11.2	229.0 ± 4.6	291.0 ± 2.7

* The difference is significant compared to the native PBS, $p \leq 0.05$.

rently, the impact of low-temperature storage on overall hormonal stability and the underlying causes of their degradation in serum/plasma after storage remain poorly understood and warrant further investigation.

The findings indicate that EP in parturients, as well as preterm labor, may be accompanied by deviations from physiological total protein concentrations, imbalances in α-1-/α-2-/β-globulin ratios, and alterations in the levels of prolactin, AFP, STH, hCG, and cortisol in PBS. These changes result from pathological states and adaptive stress within the mother-placenta-fetus regulatory systems. Following freeze-drying, reconstituted samples of such serum exhibit certain quantitative and qualitative changes in their component profile, which could potentially reduce the physiological activity of proteins, peptides, and hormones, thereby diminishing the therapeutic efficacy of PBS in subsequent applications.

CONCLUSIONS

The decisive criteria for selecting placental blood serum samples for freeze-drying and clinical application are the gestational age and the absence of pre-existing or pregnancy-related extragenital pathology. The cryotechnological approaches employed in this study ensure a high level of preservation for regenerative and regulatory compounds in the reconstituted placental blood serum, which confirms the promising potential of its use in clinical and military medicine.

Future studies will be aimed at assessing the efficacy of lyophilized placental blood serum in mitigating the consequences of mild blast-induced traumatic brain injury complicated by hypothermia.

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ЗБЕРЕЖЕНІСТЬ БІЛКОВИХ ФРАКЦІЙ І ГОРМОНІВ СИРОВАТКИ ПЛАЦЕНТАРНОЇ КРОВІ ПІСЛЯ СУБЛІМАЦІЙНОГО ВИСУШУВАННЯ

Досліджено збереженість сироватки плацентарної крові (СПК) після сублімаційного висушування з урахуванням перебігу вагітності у породіль. Плацентарну кров отримували від жінок із фізіологічним перебігом вагітності, при передчасних пологах, а також за наявності супутніх аутоімунного тиреоїдиту або гіпертонічної хвороби. Сироватку одержували шляхом відокремлення надосадової рідини після центрифугування плацентарної крові. Після сублімаційного висушування визначали концентрацію загального білка та співвідношення білкових фракцій у СПК біуретовим методом, рівні пролактину, хоріонічного гонадотропіну людини (ХГЛ), альфафетопротеїну (АФП), кортизолу та соматотропного гормону (СТГ) — методом імуноферментного аналізу. Встановлено, що склад СПК залежить від перебігу вагітності. Екстрагенітальна патологія супроводжується перерозподілом білкових фракцій та зміною рівнів пролактину, АФП, СТГ і ХГЛ. Після сублімаційного висушування СПК здорових породіль досліджувані показники не відрізнялися від нативних, тоді як за ускладненого перебігу вагітності виявлено зміни у співвідношенні білкових фракцій.

***Ключові слова*:** сироватка плацентарної крові, заморожування, сублімаційне висушування, білкові фракції, гормони.