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CHANGES IN BIOLOGICAL ACTIVITY OF PLACENTA EXTRACTS DEPEND ON ITS STORAGE TEMPERATURE

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Abstract

BACKGROUND: Cryopreservation is a critical method for the long-term storage of biological tissues, including human placental extracts (HPEs). Preserving the biological activity of these tissues during storage is essential for medical applications, particularly for their anti-inflammatory and anti-aggregation properties. The impact of storage temperature on the biological efficacy of HPEs remains a crucial area of study. **OBJECTIVE**: To investigate the effect of different cryopreservation temperatures on the preservation of biological activities in human placental extracts and their fractions, with a particular focus on their effects on erythrocyte thermal hemolysis and ADP-induced platelet aggregation. **MATERIALS AND METHODS**: Human placentas were collected after normal pregnancies from healthy parturients and cryopreserved at -20°C, -80°C, and -196°C for up to six months. Placental extracts and fractions were analyzed for their impact on erythrocyte thermal stability and platelet aggregation using spectrophotometric methods. Calorimetric studies were conducted to assess the phase state of placenta tissues at low temperatures. **RESULTS**: Extracts from placentas stored at -20°C for six months lost their ability to reduce erythrocyte hemolysis and platelet aggregation. Storage at -80°C and -196°C preserved these activities for at least six months. Calorimetric analysis revealed the presence of unfrozen water in placenta tissues at -20°C, contributing to the degradation of biological activity. **CONCLUSION**: Cryopreservation at -80°C or lower effectively preserves the biological activity of placental extracts for extended periods, making these temperatures ideal for long-term storage in medical applications.

Keywords: anti-inflammatory activity; cryopreservation; erythrocyte thermal hemolysis; non-crystallized water; platelet aggregation.

INTRODUCTION

One of the most important challenges in modern cryobiology is finding optimal temperature regimes for long-term storage of biological materials that ensure maximum preservation of their properties. In many cases, the storage temperature is crucial for the maximum preservation of both animal and plant tissues at low temperatures (1, 2, 3, 4). For example, lowering the temperature enables longer preservation of essential amino acids, reduces protein oxidation dynamics, and increases carbonyl content during bull muscle tissue storage (1, 2, 4). The storage temperature of biological tissues can also affect enzyme activities (3). It has been shown that when rice tissues were stored at -80°C for up to 12 weeks, superoxide dismutase activity did not decrease, while at -20°C it was maintained only for a short

period. After a week of storage at -20°C, ascorbate peroxidase activity significantly decreased, whereas it increased at -80°C compared to the control. This study also revealed that storing intact rice tissue led to an increase in ascorbate peroxidase activity, while storing extracts resulted in a significant decrease compared to fresh samples (3).

Among various biological materials, significant attention is given to natural extracts. Due to the presence of bioactive substances, they are widely used in medicine, pharmacology, veterinary medicine, cosmetology, and the food industry (5, 6, 7, 8, 9, 10). Human placental extracts contain many biologically active substances (11, 12, 13). However, hypothermic storage of the placenta for even a day leads to certain morphological changes in its tissues, and storage for two days results in pronounced destructive changes in all its layers (14). Overall, the storage term of placental tissue at 4°C is limited to 48 hours (15). A decrease in antioxidant activity is observed after the first week of hypothermic storage of derived HPEs (16). Clinical use of HPEs requires a sufficient amount of the extract at a certain time. Therefore, preserving the unique biological properties of HPEs during long-term storage remains an urgent task.

When examining the biological activity of natural extracts, studies focusing on the relationship between antioxidants and degenerative diseases associated with inflammation are essential (17). It has been established that the presence of uracil, tyrosine, phenylalanine, and tryptophan in the human placenta ensures its extracts have pronounced antioxidant and anti-inflammatory properties (11, 13, 18).

In vivo studies of extracts' therapeutic efficacy are usually preceded by in vitro studies carried out on cell models (7, 19). Erythrocytes are often used due to their availability and relatively simple structure; they are also important health indicators. During an inflammatory response, erythrocytes, as part of the haematological system, are exposed to circulating inflammatory mediators and associated oxidative stress (20). Early reports established a correlation between drugs' anti-inflammatory activity and their ability to reduce erythrocyte thermal hemolysis (21, 22). Indices of erythrocyte thermal hemolysis have since been widely used as a cellular test system in studying the potential anti-inflammatory activity of various natural substances (7, 8, 9, 19, 23).

Another widely used index of anti-inflammatory effect is the ability of biologically active substances to reduce platelet aggregation, induced in particular by the well-known platelet agonist adenosine-5′ diphosphate (ADP) (10, 24, 25). Platelets play a key role in most stages of inflammation in the human body, confirming their direct participation in pathophysiological states associated with inflammation (26). Anti-platelet therapy is crucial in treating most cardiovascular diseases, and dual (aspirin and clopidogrel) therapy significantly reduces the risk of recurrent ischemic events (27). However, traditional therapy is not effective in all patients and/or has significant adverse effects, particularly in the elderly (28, 29). A significant number of patients exhibit platelet resistance to commonly used antiplatelet agents. These facts drive research into the causes of such resistance and the search for new antiplatelet agents (10, 25). HPEs and their fractions, which possess anti-inflammatory and anti-aggregation activities (6, 24), may help solve the problem of selecting drugs to reduce platelet aggregation levels in aspirin-resistant patients.

The goal of this study was to analyze the effect of cryopreservation temperature on the preservation of biological activity in individual fractions of human placenta extracts (HPEs) relative to erythrocytes and platelets and how the presence of non-freezing water in placenta tissues at low temperatures can affect it.

MATERIALS AND METHODS

Ethics, biological materials, inclusion-exclusion criteria

Human placentas weighing between 400–600 g were collected after normal pregnancies from healthy parturients (23–28 years old) with their Informed Consent at full-term spontaneous delivery (38–40 weeks gestation, n=8) and immediately placed on ice. Subjects were excluded if there was evidence of fetal abnormalities, intrauterine growth restriction, diabetes, hypertension, anemia, tobacco or drug use, or other medical or obstetric complications, as described previously (33). Blood was obtained from healthy adult volunteers with their informed consent at the Kharkiv Regional Center of Blood Service.

All studies were approved by the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine NAS of Ukraine (Prot. N1/2023) and performed in accordance with the ethical standards of the Declaration of Helsinki.

Placenta preparation

Placental fragments were separated from connective tissues and washed several times with 0.15 M sodium chloride (NaCl) solution until mucus and blood were visually removed. Fetal membranes (amniotic and chorion) were then removed. A thin layer from the cotyledonary region of the placenta was sliced into pieces (3 x 2 x 0.5 cm). These pieces were mixed with physiological saline solution in a 1:5 weight ratio and stirred for 2 to 3 min. The supernatant was removed, and a fresh portion of physiological saline was added. This procedure was repeated 3 or 4 times. Washed placenta pieces/fragments were then exposed to low-temperature conditions.

Placenta fragments were frozen-thawed as follows: freshly obtained fragments were frozen without any solution in closed plastic bags down to -20°C (in a freezer) at a rate of ~1 to 2°C/min, down to -80°C at a rate of ~3 to 4°C/min, and down to -196°C with a high cooling rate of approximately 100°C/min (Fig. 1). After freezing, the fragments were either thawed or stored for 1, 3, or 6 months. Thawing was carried out in a water bath at 37°C.

Preparation of placental extracts and their fractions

Aqueous-saline extracts of human placenta and their individual fractions were prepared from each placental fragment mentioned above using the method described in detail earlier (30). Briefly, washed placenta fragments were homogenized with physiological saline solution in a 1:1 (v/v) ratio, followed by 12-h exposure at 4°C and centrifugation for 15 min at 1500g. The supernatant was collected and filtered through a 0.45 μm membrane filter (Millipore Corp; Cork, Ireland). The obtained filtrate was the aqueous-saline human placental extract (HPE). Separate HPE fractions (3 mL volume) were obtained by gel-chromatography on a 21 x 2 cm column with Sephadex G-200 gel (Loba Feinchemie; Fischamend, Austria). Protein content in the extracts and fractions was measured spectrophotometrically at 280 nm. Placentas from eight parturients were analyzed in the study. The following HPEs were studied: derived from fresh placenta, from the same placenta fragments subjected to one-time freeze-thawing (-20°C, -80°C, -196°C), and from fragments stored for 1, 3, and 6 months at -20°C, -80°C, or -196°C (Fig. 1). Individual fractions were prepared from all obtained HPEs using the gel chromatography method described above. The following groups of fractions were chosen for study: > 150 kDa, 45–75 kDa, 7–13 kDa, < 4 kDa (Fig. 1).

Calorimetric study

The phase state of the placenta at low temperatures was studied by differential scanning calorimetry (DSC). Placenta samples weighing 1 g were cooled by immersion in liquid nitrogen, with an average cooling rate of 200°C/min. Thermograms were recorded upon heating at a rate of 0.5°C/min. This enabled the observation of all possible phase changes in a wide temperature range, confirming or denying the presence of non-crystallized liquid at low temperatures.

Platelet aggregation study

Platelet aggregation was studied to determine the ability of agonists to induce in vitro platelet activation and platelet-platelet binding (31, 32). Traditionally, platelet aggregation is studied in platelet-rich plasma (PRP) (32), as it allows for the evaluation of platelets under gentler conditions compared to a suspension of washed platelets.

PRP was obtained using a standard method (24) by centrifuging donor blood for 15 min at 100–150g. The obtained supernatant was PRP, which was collected in a separate test tube and used for the platelet aggregation study. To study the effect of HPE on platelet aggregation, PRP was mixed with HPE in a 1:1 (v/v) ratio and incubated at +37°C for 15 min (Fig. 1). ADP (10^{-5} mol/L) (Sigma-Aldrich Chemical) was used as an aggregation inducer. The ADP solution was added to PRP samples at 37°C. The degree of platelet aggregation was determined 5 min after the addition of ADP (5) using the spectrophotometric method (31). The aggregation degree was calculated as a percentage of the difference in optical density between control samples and those with ADP.

Erythrocyte thermal hemolysis

Erythrocytes of donor blood were separated from the plasma and blood cells by a single centrifugation at 1500g for 5 min. The next two

washings were carried out in an excess of phosphate-buffered saline (PBS), pH 7.4.

The effect of HPE and individual fractions on erythrocyte thermal hemolysis (Fig. 1) was evaluated by their impact on the level of hemolysis at hyperthermia (9, 19). For thermal hemolysis studies, we used the method described earlier (33). Briefly, washed RBCs were re-suspended with an equal volume of PBS, pH 7.4, and a total HPE or its fraction was added in a ratio of 1:1 (v/v). After 1 h of incubation, RBCs were re-suspended in PBS, and incubated at 55°C for 20 min in a regulated water thermostat. After the incubation, the heated samples were centrifuged at 1500g for 5 min and the hemoglobin content of the supernatant was determined by measuring optical density using the spectrophotometric method. The hemolysis was expressed as a percentage relative to the fully hemolyzed cells.

Data collection and statistics

Statistical analysis was performed using Statistica 7.0 (StatSoft). Data are presented as mean ± standard error. In order to compare the measured parameters with control, the Wilcoxon matched pairs test was used.

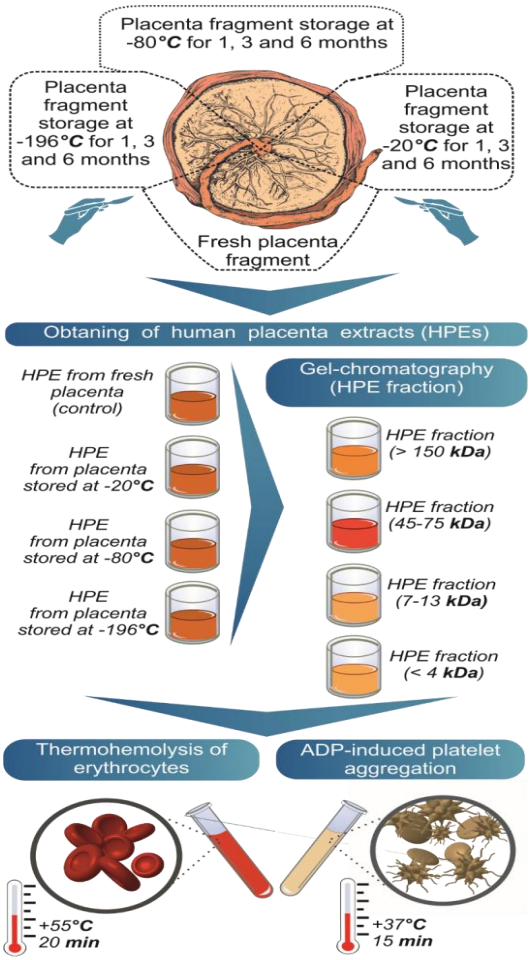

Figure 1. Experimental protocol.

RESULTS

Effect of low-temperature storage of placenta on HPEs and their fractions' ability to modify erythrocyte thermal stability

Incubating erythrocytes with HPE significantly reduced the level of erythrocyte thermal hemolysis (Fig. 2). Storage of the placenta at low temperatures for up to one month did not affect the ability of HPE to reduce erythrocyte thermal hemolysis, irrespective of the storage temperatures (-20°C, -80°C, -196°C). However, after three months of storage, the temperature's effect on the biological activity of HPE became detectable. Notably, storing the placenta at -20°C for six months resulted in a significant increase in thermal hemolysis of erythrocytes incubated with HPE from it (Fig. 2). This increase was significantly higher not only compared to freshly obtained HPE but also compared to the control. This indicates that extracts from placenta stored for six months at -20°C not only lose their protective properties but also acquire components that induce hemolytic effects.

A decrease in the efficacy of HPE from placenta stored for six months at -80°C compared to freshly obtained HPE was observed (Fig. 2). No correlation was found between the HPE effect on thermal hemolysis and the storage duration at -196°C (Fig. 2). To identify which HPE components affect erythrocyte thermal hemolysis and are sensitive to low-temperature storage, the whole HPE was separated into fractions using gel chromatography. The fraction >150 kDa was not effective in increasing erythrocyte thermal stability and showed a hemolytic effect after six months of storage at -20°C. The activity of the 45–75 kDa fraction significantly decreased after three months of storage and was completely lost after six months. Despite a reliable decrease, the biological activity of low-molecular-weight HPE fractions was not completely lost even after six months of storage at -20°C (Fig. 3). Molecular weights of the studied fractions are shown in Fig. 1. Low-molecular-weight fractions of 7–13 kDa and <4 kDa most effectively reduced erythrocyte thermal hemolysis (Fig. 3). The high-molecular-weight

fraction >150 kDa was not effective in increasing erythrocyte thermal stability and showed a hemolytic effect after six months of storage at -20°C. The activity of the 45–75 kDa fraction significantly decreased after three months of storage and was completely lost after six months. Despite a reliable decrease, the biological activity of low-molecular-weight HPE fractions was not completely lost even after six months of storage at -20°C (Fig. 3).

Differences in the effectiveness of HPE fractions of various molecular weights can be explained by their different abilities to modify the erythrocyte membrane and exert a stabilizing effect. Whole HPEs from placenta stored for six months at -80°C and -196°C maintained the ability to increase erythrocyte heat resistance. In contrast, placenta storage at -20°C for six months resulted in a complete loss of HPE effectiveness in increasing erythrocyte heat resistance.

Effect of low-temperature storage of placenta on HPEs and their fractions' ability to alter ADP-induced platelet aggregation

The addition of HPEs from fresh placenta to PRP significantly decreased platelet aggregation compared to the control (Fig. 4). Storage of the placenta at low temperatures for one month did not impair the antiaggregant efficacy of its extracts, regardless of the storage temperature. However, longer storage durations highlighted

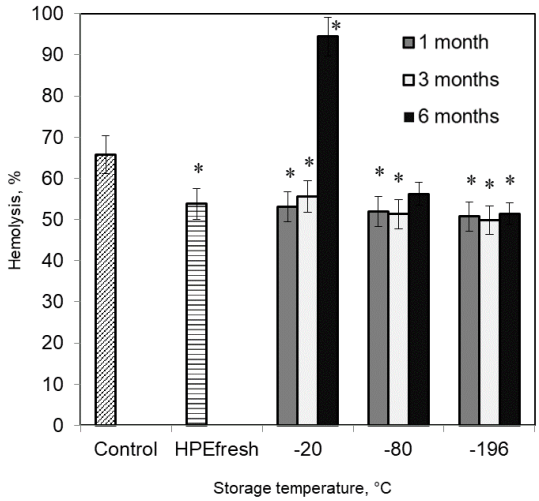

Figure 2. Influence of placenta storage temperature on extracts ability to reduce erythrocyte thermal hemolysis. Control: erythrocytes with phosphate-buffered saline. HPEfresh: erythrocytes with freshly obtained human placental extract (HPE). *: significant differences compared to the control erythrocytes ($p<0.05$, $n=6$)

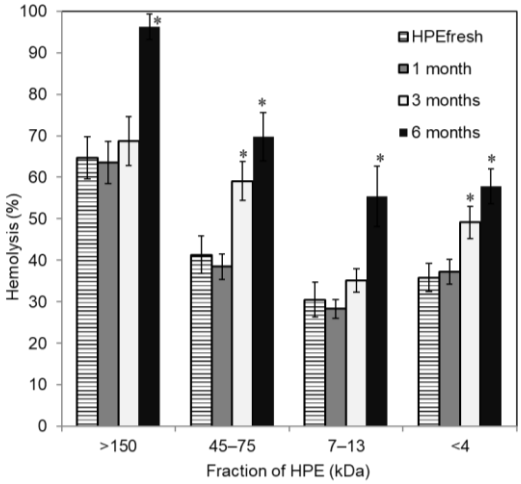

Figure 3. Decrease in biological activity of different HPE fractions (ability to low erythrocytes thermal hemolysis) under placenta storage at -20°C.*: differences are significant relative to corresponding HPE fractions from fresh placenta ($p<0.05$, $n=6$).

the importance of storage temperature for preserving the anti-platelet aggregation activity of placental extracts. After three months of storage at -20°C, a decrease in the efficacy of the extracts was observed, and after six months, their ability to reduce ADP-induced platelet aggregation was completely lost (Fig. 4). At -80°C, a tendency to decrease the HPE antiaggregant ability was noted after six months, but no significant differences were found compared to freshly obtained HPE. Storage at -196°C preserved the HPE antiaggregant activity regardless of the duration (Fig. 4).

Comparative analysis of the ability of different HPE fractions to reduce ADP-induced platelet aggregation and changes in their antiaggregant efficacy under placenta storage at -20°C revealed that all investigated fractions from freshly obtained placenta possessed anti-platelet activity (Fig. 5). Storage at -20°C for one month did not decrease the ability of HPE fractions to reduce ADP-induced platelet aggregation, and some fractions even showed a tendency to increased efficacy after low-temperature exposure (Fig. 5). However, the dynamics of antiaggregant activity decrease varied among individual HPE fractions with prolonged storage

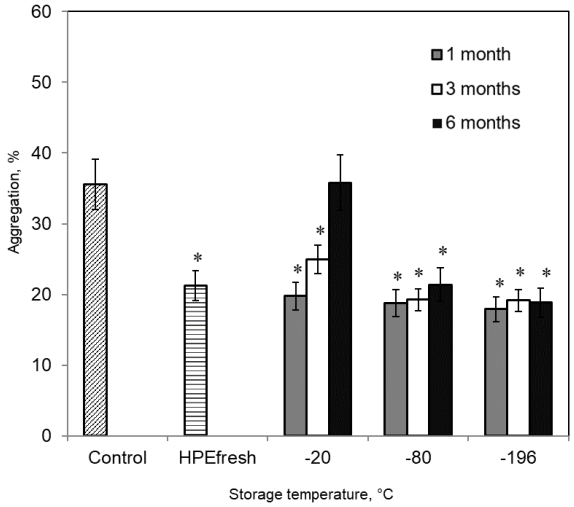

Figure 4. Influence of placenta storage temperature on HPE ability to inhibit ADP-induced platelet aggregation. Control: platelet-rich plasma (PRP) with phosphate-buffered saline. HPEfresh: PRP with freshly obtained human placental extract (HPE).*: significant differences compared to the control group ($p<0.05$, $n=6$).

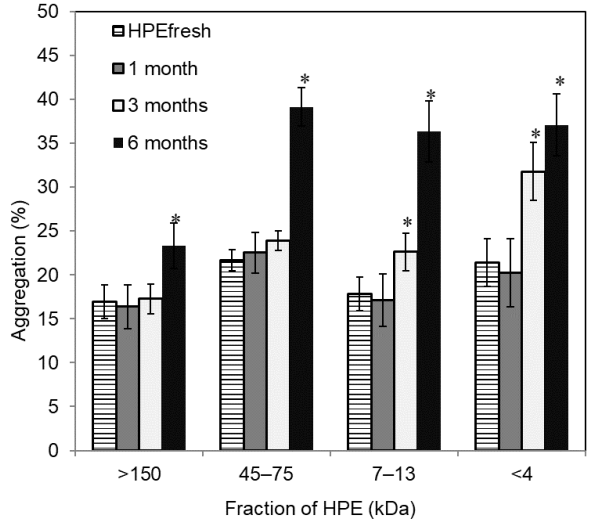

Figure 5. Changes in biological activity of different HPE fractions (ability to inhibit ADP-induced platelet aggregation) under placenta storage at -20°C.*: significant differences relative to corresponding HPE fractions from fresh placenta ($p<0.05$, $n=6$).

at -20°C. After three months, the efficacy of the high-molecular and medium-molecular-weight fractions did not change significantly, while the antiaggregant activity of the 7–13 kDa fraction decreased, and the <4 kDa fraction almost completely lost its effectiveness (Fig. 5). After six months of storage at -20°C, almost all fractions lost their ability to reduce platelet aggregation, with only the high-molecular fraction (>150 kDa) partially retaining its effectiveness.

Phase state of placental tissue at low temperatures

The significant decrease in the biological effect of placental aqueous-saline extracts and their individual fractions under storage at -20°C may be primarily related to the presence of non-crystallized liquid in placenta tissues at this storage temperature. Therefore, the phase behavior of human placenta samples at low temperatures was investigated using DSC. The placenta was cooled at high rate for the DSC study to maximize the amount of metastable phase and determine the lowest temperature at which supercooled liquid could be present during cooling. Conversely, it was heated at low rates because the sample masses were 1 g, which is large for calorimetric studies. When cooling at low rates during real cryopreservation, less supercooled liquid will be present in the tissues. And it is better to heat the tissues at high rates to avoid recrystallization during heating.

The placenta sample thermogram obtained during the heating stage at a rate of 0.5 °C/min is shown in Fig. 6. No significant features were observed in the thermogram up to -108°C. In the temperature range of -108°C to -97°C, a non-monotonic change in the sample heat capacity was observed, likely corresponding to the devitrification of the phase vitrified during the cooling stage. This process is probably related to the high cooling rates used (~200 °C/min). An insignificant portion of liquid with dissolved substances did not have time to crystallize during the cooling stage. Further heating revealed temperature-diffused peaks of low intensity in the range of -67°C to -54°C, likely corresponding to changes in the phase state of biomacromolecule hydration water and/or completion of crystallization during heating. Water that did not crystallize at high cooling rates can crystallize at low temperatures after the appearance of a liquid phase during slow heating. At -29°C, melting of the crystallized mixture begins. Near the beginning of the main intense endothermic peak of ice melting, a minor peak (T = -23.3°C) is observed, possibly corresponding to the melting of bound water or eutectic compounds. A portion of water and salt molecules can bind to organic components of placenta tissues and not participate in eutectic compound formation, resulting in low peak intensity. The melting peak of placental tissue water is wide with a diffused top (Fig. 6), indicating that water bound to organic components melts gradually over a wide temperature range.

DISCUSSION

Our in vitro study confirms that HPEs from fresh placenta possess significant anti-inflammatory and anti-aggregation properties. This was evaluated through their ability to reduce erythrocyte thermal hemolysis and ADP-induced platelet aggregation. Further research into

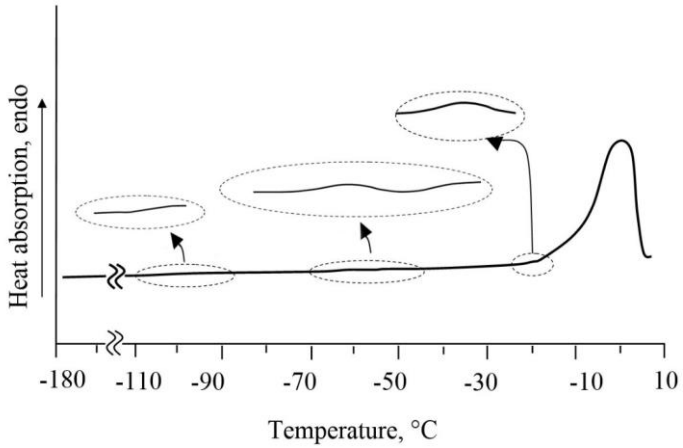

Figure 6. DSC-thermogram of human placenta (mass = 1 g). Heating stage (0.5°C/min) after fast cooling (200°C/min).

individual HPE fractions revealed the effectiveness of fractions with molecular weights >150 kDa, 45–75 kDa, 7–13 kDa, and <4 kDa from freshly obtained HPEs in reducing these indicators.

The efficacy of the 7–13 kDa fraction in reducing thermal hemolysis and platelet aggregation, thereby demonstrating anti-inflammatory properties, aligns with our previous findings on the antioxidant actions of fractions in this molecular weight range. This was established based on the effects of HPE fractions on erythrocytes under oxidative stress induced by sodium nitrite (34, 35). This correlation between inflammation and oxidative stress is well-documented in the literature (17, 20).

Placental tissue contains several groups of important anticoagulants of various molecular weights, likely explaining why all HPE fractions studied exhibit anti-platelet aggregation activity. The high efficacy of the high-molecular-weight fraction suggests the presence of heparin-like anticoagulants and phospholipid-binding protein anticoagulants, which increase in concentration following placenta freezing. Thrombomodulin (74 kDa), found in the placenta, probably lies within the middle-molecular-weight fraction and reduces platelet aggregation, though to a lesser extent. The high anti-platelet aggregation efficiency of the HPE fraction of less than 4 kDa is likely due to low-molecular-weight acidic peptides, while the 7–13 kDa fractions may contain fibronectin-like peptides (36). A clinical study by T.K. Sur and colleagues suggests possible mechanisms for reducing platelet aggregation, including the inhibition of prostaglandin synthesis pathways or the release of 5-HT receptors (24).

The presence of numerous membrane-bound intracellular mechanisms of platelet activation accounts for the variety of biologically active compounds capable of modulating platelet function. The leading role in the mechanisms of antiaggregant drug action on platelets is primarily mediated by changes in the receptor sensitivity of platelet membranes. Different HPE fractions interact with platelet membranes via different mechanisms, which explain the varying effects on ADP-induced platelet aggregation.

Analyzing results on the HPE effect on erythrocyte heat resistance (33) and on antiplatelet action mechanisms (37) suggests that the target of bioactive substances action for both cells is a modulating of their cytoskeleton rearrangements.

Our examination of the impact of placenta storage temperature on the preservation of the beneficial properties of its extracts and individual fractions shows that storage at -80ºC and -196ºC better preserves the properties of HPEs in reducing thermal hemolysis and platelet aggregation compared to storage at -20ºC. The influence of the storage term is also more pronounced at -20ºC. After just three months of storage at -20ºC, the biological efficacy of medium- and low-molecular-weight fractions diminishes, and after six months, they completely lose their effectiveness.

Previously, we also observed a negative effect of six-month storage at -20ºC on the low-molecular fraction (less than 5 kDa). Exposure of erythrocytes to such fractions resulted in decreased erythrocyte acid resistance and modified the erythrocyte cytosol (30). Furthermore, extracts from placenta stored for six months at -20°C showed no antioxidant activity against erythrocyte oxidative stress, with their antiradical activity decreasing by more than 50% (35).

It is known that the rate and final cooling temperature significantly influence the quality of animal tissues, such as meat from various agricultural animals during frozen storage (38). However, our study found no impact of placenta cooling temperatures and storage for up to one month on the quality of its extracts, indicating that crystallization processes, which can destructively affect placenta tissues, do not influence the anti-inflammatory activity of its extracts. We have found that the storage temperature of the placenta plays a crucial role in preserving its biological activity only during long-term storage.

The negative effect of placenta storage at -20°C on HPE properties can be largely associated with the presence of a liquid phase at this temperature, as confirmed by DSC analysis. Uncrystallized water at low temperatures is common in various biological tissues. For example, in mouse skeletal muscles and tuna muscle tissue, multiple water crystallization phases have been observed, indicating complex interactions within these tissues (39, 40). Similar behavior has been noted in plant tissues and other animal tissues, where water forms hydration shells around proteins, preventing aggregation and crystallization at lower temperatures (41, 42). Moreover, the shape, size and temperature of the crystallization peak strongly depend on the cooling rate (43).

Placenta freezing itself does not significantly impact the preservation of biologically active components. However, negative changes may occur due to protein conformational modifications and biochemical processes in the liquid phase at moderately low storage temperatures. For instance, protein denaturation and oxidation are common under frozen storage, often triggered by high ionic strength and pH changes in the remaining liquid phase (1, 44).

Reducing the storage temperature can enhance the preservation of biological tissues by minimizing the liquid phase and inhibiting negative processes. Lower storage temperatures tend to reduce protein denaturation, oxidation, and lipid hydrolysis, which can otherwise progress during long-term storage at higher temperatures (1, 4). For example, the content of free fatty acids in fish increases at -20°C but remains stable at -30°C or -80°C (1).

Thus, the presence of a liquid phase in placenta tissues at -20°C can lead to protein denaturation, lipid oxidation, and other biochemical processes that alter the composition of placenta extracts and their fractions. This can result in the loss of their biological activity, particularly their ability to increase the heat resistance of erythrocytes and reduce ADP-induced platelet aggregation. Changes in protein composition and potential lipid peroxidation can lead to a complete loss of the unique properties of the placenta, causing not a decrease but an increase in erythrocyte thermal hemolysis.

The biological activity of extracts obtained after placenta storage at -196°C did not differ from that of extracts from fresh placenta, as evaluated on cellular models of erythrocyte thermal hemolysis and platelet aggregation. The effectiveness of these extracts was demonstrated in experiments on burn wound healing, showing high efficacy (45, 46). These experiments confirmed that placenta storage at -196°C preserves the ability of HPEs to accelerate the reparative process in cases of burn injury in laboratory animals (45, 46).

Thus our investigations have shown that placenta freezing and storage for up to 1 month at -20°C, -80°C, or -196°C do not affect the activity of HPEs and their fractions. However, placenta storage at -20°C for three months leads to a significant decrease in the biological activity of its extracts. After 6 months of storage at -20°C, the ability to reduce erythrocyte thermal hemolysis and platelet aggregation is completely lost. Nonetheless, the 7-13 kDa fraction retains its ability to reduce erythrocyte thermal hemolysis after 3 months and partially after 6 months of storage at -20°C. The anti-platelet efficacy of the high-molecular fraction is partially preserved after 6 months at -20°C.

In contrast, placenta storage at -80°C and -196°C maintains a high level of preservation of its extract properties for at least 6 months. The complex phase behavior observed in placenta tissues cooled rapidly to low temperatures indicates the possibility of partial preservation of molecular mobility at temperatures down to -108°C. The presence of a minor melting peak at -23.3°C suggests the existence of unfrozen water, underscoring the necessity to store the placenta below this temperature.

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