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# CRYOGENIC CHANGES OF PROTEINS DURING CRYOPRESERVATION OF BULL AND ROOSTER SPERM

**Ion BALAN, Vladimir BUZAN, Gheorghe BORONCIUC, Nicolae ROŞCA, Iulia CAZACOV, Melania BUCARCIUC, Ion MEREUŢA, Alexandru DUBALARI, Irina BLÎNDU, Nicolai FIODOROV**

Institute of Physiology and Sanocreatology, 1 Academiei Street, MD 2028, Chişinău, Republic of Moldova

Corresponding author email: vladimirbuzan@yahoo.com

***Abstract***

*In the field of cryobiology, there are a large number of publications devoted to the study of the most labile lipid composition of gametes and their membranes. However, less attention is paid to the state of the protein component of cells. Therefore, the purpose of the presented paper was to study the protein spectrum and its changes in the process of cryopreservation of bull and rooster sperm. The presence of albumins, alpha, beta and gamma globulins was determined by the spectrophotometric method. Most of the proteins are contained in the seminal plasma, in gametes the total protein content is slightly lower. Of the seminal plasma proteins, most contain albumins, and of the gamete proteins, most contain gamma globulins (in the sperm of both animal species). With the passage of technological stages, the number of some proteins increases, while of others it decreases. This indicates about the destruction of protein structures, violation of the permeability of biological membranes and changes in the chromatographic mobility of proteins. From the data obtained, it was found that the protein spectrum of bull and rooster sperm is very different. If alpha globulins (18.2±1.84 mg/billion) prevail in the plasma of the bull`s sperm, and gamma globulins (16.8±3.27 mg/billion) prevail in the gametes, then in rooster sperm, both in plasma and gametes, most of all is albumin (6.0±2.45 and 11.8±0.47 mg/billion, respectively).*

***Key words****: proteins, cryopreservation, gametes, seminal plasma.*

## INTRODUCTION

Living organisms are characterized by a wide variety of proteins, which form the basis of the structure of the body and provide many of its functions. It is believed that in nature there are approximately $10^{10}$–$10^{12}$ different proteins, which explains the great variety of living organisms. In unicellular organisms, there are about 3 thousand different proteins, and in the human body - about 5 million (Artemuk et al., 2010).

Protein molecules consist of one or more polypeptide chains organized in a characteristic three-dimensional structure. Individual proteins have a certain chemical composition. Their molecular weights span from 6,000 to over a million. The metabolism, structures, and function of each cell are decisively determined by proteins. Chemical reactions in a cell that would proceed extremely slowly in vitro are accelerated by special catalytic proteins, enzymes, hundreds of thousands of times. It goes without saying that this does not affect the equilibrium state of the reaction, but the speed of its onset. Other proteins perform external or internal protective functions (Iakubche et al., 1985).

## MATERIALS AND METHODS

The following methods of protein research were used in the investigations: the method for determining the total protein, the determination of protein fractions by the express method and the solubilization of proteins. Quantitative determination of the protein was carried out by Lowry et al., which is based on the ability of the protein in combination with copper to restore the Folin solution with the subsequent formation of colored reaction products. The protein content was determined spectrophotometrically at a wavelength of 750 nm.

The determination of protein fractions by the express method was determined by McCord in the modification of Karpiuk described by Golban et al. (Golban et al., 1988). The principle of the method is based on the property of

phosphate solutions of different concentrations to precipitate proteins. The optical density of the solutions was determined on an SF-46 spectrophotometer at a wavelength of 750 nm. The content of a single fraction was determined in gram percent. Taking the amount of total protein for 100 and knowing the amount of each fraction, the percentage of protein fractions is calculated.

Gamet protein solubilization was carried out by the method described by (Meddi, 1979). In this case, a fraction of hydrophilic proteins was obtained after shaking the gametes in double-distilled water for 20 minutes. The fraction of alkali-soluble protein was obtained by solubilization of them in a 0.1 molar solution of sodium hydroxide, and acid-soluble proteins by solubilization in a 0.1 M solution of hydrochloric acid under analogical conditions.

## RESULTS AND DISCUSSIONS

Albumin is the main blood protein of mammals, where its concentration is 500-700 μM. The albumin molecule is not coated with a carbohydrate shell and can bind a variety of molecules and atoms: water and metal cations ($Ca^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Ni^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Pt^{2+}$, $Au^{+}$), free fatty acids and fat-soluble hormones, unconjugated bilirubin, and bile salts, transferrin, nitric oxide, aspirin and other compounds (Fasano et al., 2005). The first publications devoted to albumin appeared at the end of the 19th century, and the three-dimensional structure of human serum albumin (HSA) was studied only at the end of the 20th century. A similar structure of bovine serum albumin (BSA) was obtained in 2012, and the three-dimensional structure of rat serum albumin (RSA) has not yet been obtained. The serum albumin molecule is formed by a single polypeptide chain consisting of 585, 584 and 583 amino acid residues for human, rat, and bovine albumin, respectively (Consortium T.U., 2015; Majorek et al., 2012; Strauss et al., 1977). The structure of albumin is conservative for all mammals: the molecule consists of three homologous domains, each of which consists of ten helices and can be divided into two subdomains, A and B, containing six and four helices, respectively; these two subdomains are connected by a long loop (Bujacz, 2012). The human and bovine albumin molecule has 17 disulfide bonds and one cysteine residue with a free SH- group (Bujacz, 2012; Ghuman et al., 2005). In the primary sequence of rat albumin, there are as many cysteine residues as there are in the human and bull sequences, and they are located at the same positions (Strausberg et al., 2002), so there is every reason to assume that RSA also has 17 disulfide bonds and one single cysteine in its three-dimensional structure. Alignment of the primary sequences of amino acid residues of the HSA, RSA, and BSA molecules using Clustal Omega (Sievers et al., 2011) showed that the HSA and BSA molecules have the highest identity – 75.6%. However, the percentage of primary structure identity in the molecules of HSA and RSA is 73.0%, while in BSA and RSA it is 69.9%. If we compare directly the amino acid residues of the Sadlow I site (the Sadlow II site is conservative in all three types of albumin), then here the HSA and RCA molecules have the highest identity - 75%, while the BSA shows only 57% identity with both the RCA and the HSA. In addition, BSA is dominated by substitutions of arginine for lysine residues at the Sadlow I site (Lys195 → Arg195, Lys199 → Arg199), which probably affects its configuration, since the side chains of arginine are more branched. The three-dimensional structure of albumin is quite labile, so that when interacting with the albumin molecule of different substances, there are such effects as cooperativity and allosteric modulation, usually inherent in 6 multimeric proteins (Ascenzi et al., 2010). However, due to the above differences in primary structures, according to the results of a study of the functional characteristics of albumin of one animal species, it cannot be a priori asserted that albumin in another species will have the same characteristics. For example, HSA when interacting with fatty acids in solution has high plasticity and flexibility, while the characteristics of BSA in solution with respect to fatty acids are not very different from the calculated data obtained for its crystal structure (Reichenwallner et al., 2013).

From the variety of physico-chemical processes occurring in the cell and consequently in the plasma membrane during freezing and thawing (Belous et al., 1982; Gulevschii et al., 1988; Moiseev, 1984), it is shown that decisive of these is the occurrence of transmembrane

defects in the plasma membrane, the development of which depends on the degree of modification of the protein skeleton and changes in the physico-chemical properties of lipids and proteins in the membrane at different stages prior to freezing. A specific feature of the biological effect of cold is that primary damage in gametes is localized only in extremely small volumes of molecular size. If injuries of gametes refers to the unique structures of the cell (chromosomes, nucleus) and affect the composition of the nuclear or cell membrane, these injuries may secondarily cause large irreversible functional impairments (Nauc, 1991; Novikov et al., 1992). Damage of membranes can significantly disrupt the stationary state in gametes by changing intermolecular interactions, their diffusion parameters, the course of enzymatic reactions and other vital processes (Ortman et al., 1994; Zamfirescu et al., 2010; Katkov, 1985). It follows that the efforts of researchers should be aimed at stabilizing primary, reversible changes, while irreversible ones are not subject to regulation. Damage of biological objects are among to the fundamental problems of modern cryobiology. Interest in this problem increases for a number of reasons, and first of all – it is stimulated by practical tasks. At present, there are more and more questions about the possibility of life for people, animals and plants in conditions unusual for normal functioning – at high and low temperatures and pressure, increased radiation, high accelerations, with a lack of water, high salt content, etc.

Cryo-damage occurs at all stages of sperm processing. However, there are critical zones on the temperature scale (Malinovskaia, 1983). Thus, studies of temperature-induced structural transitions on the water-lipid surface of membranes (Timbal et al., 1995) revealed the rearrangement of water-protein interactions at temperatures of 30, 25, 17 and 7ºC. Moreover, these interactions occur both on the outer surface of the proteins accessible to the free solvent, and in the cavities of biomacromolecules due to the tertiary structure, as well as the contacts of subunits in oligomeric ensembles of proteins. Similar temperature zones were determined (Jukovschii et al., 1987) when studying the mechanisms of stabilization of the native conformation of water-soluble proteins.

In our laboratory was studied the content of plasma and gametes proteins in the native and thawed sperm of bull, ram and boar (Table 1).

Table 1. Protein content in native and thawed sperm of farm animals

| Indicators | Animal species | | |
|---|---|---|---|
| | Bull (n=8) | Ram (n=4) | Boar (n=3) |
| Native sperm | | | |
| Proteins of plasma, mg/ml | 52.1±4.28 | 20.5±1.26 | 14.1±1.98 |
| Water-soluble protein of gametes, mg/billion | 5.6±0.87 | 4.6±1.22 | 3.6±0.43 |
| Alkali-soluble protein of gametes, mg/billion | 2.2±0.22 | 3.1±0.62 | 2.9±0.46 |
| Acid-soluble proteins of gametes, mg/billion | 3.8±0.38 | 5.0±1.20 | 7.2±1.23 |
| Total gamete protein, mg/billion | 11.6±1.02 | 12.7±2.93 | 13.8±0.49 |
| Thawed sperm | | | |
| Proteins of plasma, mg/ml | 48.4±5.11 | 18.9±1.47 | 13.6±2.27 |
| Water-soluble protein of gametes, mg/billion | 5.0±0.65 | 4.5±0.38 | 2.8±0.43 |
| Alkali-soluble protein of gametes, mg/billion | 2.5±0.40 | 2.5±0.46 | 2.1±0.03 |
| Acid-soluble proteins of gametes, mg/billion | 3.8±0.23 | 5.0±0.83 | 9.6±1.31 |
| Total gamete protein, mg/billion | 11.3±0.74 | 12.2±1.83 | 14.5±1.40 |

As can be seen from the table, the most protein is contained in the plasma of bull semen (52.2±4.28 mg/ml) and the least in boar semen (14.1±1.98 mg/ml). An analogous trend is also observed in the study of the content of water-soluble gamete proteins. If alkali-soluble proteins are contained at the same level, the content of acid-soluble proteins is specific for each species. The latter are most detected in boar gametes (7.2±1.23 mg/billion) and least in bull gametes (3.8±0.38 mg/billion). The total protein content in gametes is almost at the same level.

Technological processing of the sperm does not have a significant effect on the content of the

studied proteins in the bull and ram semen, and only in the boar there is a tendency to decrease alkali-soluble proteins.

Thus, the total protein content of animal spermatozoa during cryopreservation does not undergo significant changes. The data are presented in Table 2.

Table 2. Protein spectrum of native and thawed bull sperm

| Name of proteins | Native sperm | % | Thawed sperm | % |
|---|---|---|---|---|
| Plasma, mg/ml | | | | |
| Albumin | 3.6±0.26 | 13.5 | 13.7±2.84* | 35.8 |
| Alpha globulins | 18.6±1.84 | 68.7 | 17.2±0.84 | 44.9 |
| Beta globulins | 2.0±0.40 | 7.6 | 4.0±1.48 | 10.5 |
| Gamma globulins | 2.7±0.18 | 10.2 | 3.4±1.48 | 8.8 |
| Total | 26.5±1.91 | 100 | 38.3±3.63 | 100 |
| Gametes, mg/billion | | | | |
| Albumin | 5.1±1.48 | 14.4 | 12.8±7.92 | 49.7 |
| Alpha globulins | 7.6±4.0 | 21.6 | 4.0±1.13 | 15.6 |
| Beta globulins | 5.8±1.27 | 16.4 | 2.5±1.15 | 9.7 |
| Gamma globulins | 16.8±3.27 | 47.6 | 6.5±1.73* | 25.0 |
| Total | 35.7±5.52 | 100 | 25.8±8.27 | 100 |
| Total, plasma + gametes | 61.8±5.84 | | 64.1±9.03 | |

*Statistically authentic differences compared with native sperm

It is possible that although the total protein content during freezing and thawing does not significantly change, there may be conformational changes in individual protein fractions (Tables 2 and 3).

Table 3. Protein spectrum of native and thawed rooster sperm

| Name of proteins | Native sperm | % | Thawed sperm | % |
|---|---|---|---|---|
| Plasma, mg/ml | | | | |
| Albumin | 6.0±2.45 | 47.8 | 4.8± 1.90 | 46.3 |
| Alpha globulins | 2.8±0.88 | 22.1 | 1.5±0.21 | 14.5 |
| Beta globulins | 3.8±1.08 | 30.1 | 2.6±0.50 | 25.0 |
| Gamma globulins | 0 | 0 | 1.5±0.23 | 14.2 |
| Total | 12.6±2.82 | 100 | 10.4±1.99 | 100 |
| Gametes, mg/billion | | | | |
| Albumin | 11.8±0.47 | 54.3 | 2.1±0.91* | 9.6 |
| Alpha globulins | 2.7±1.53 | 12.4 | 1.2±0.12 | 5.5 |
| Beta globulins | 1.9±1.85 | 9.0 | 0.6±0.08 | 2.8 |
| Gamma globulins | 5.3±0.70 | 24.3 | 17.9±1.08* | 82.1 |
| Total | 21.7±1.72 | 100 | 21.8±1.42 | 100 |
| Total, plasma + gametes | 34.3 ±3.30 | | 32.2±2.44 | |

*Statistically authentic differences compared with native sperm

From the data of these tables it follows that the protein spectrum of the bull and rooster sperm is very different. If alpha globulins (18.6±1.84 mg/billion) prevail in the plasma of the bull's sperm, and gamma globulins (16.8±3.27 mg/billion) prevail in the gametes, then the rooster's sperm has the most albumins in both plasma and gametes (6.0±2.45 and, respectively 11.8±0.47 mg/billion).

A distinctive feature of the protein spectrum is that the rooster gametes contain much fewer globulins than those of a bull (the total content of alpha, beta and gamma globulins in a rooster was 9.9±1.89 mg/billion, and in a bull – 30.2±5.32 mg/billion).

Changes in the protein spectrum during cryopreservation are specific for each species. And only the amount of alpha and beta globulins in bull and rooster gametes during thawing has the same character - decreases.

Thus, although the total protein content of the animal sperm during cryopreservation does not change (see Table 1), its protein spectrum underwent quite pronounced modifications. In the process of cryopreservation of a sperm in its plasma, changes in the content of alpha, beta and gamma globulins are specific for each animal species, while in gametes, the dynamics of alpha, beta and gamma globulins are specific.

## CONCLUSIONS

The total protein content of animal sperm does not change during cryopreservation, while the protein spectrum undergoes quite pronounced modifications.

In the process of cryopreservation of a sperm in its plasma, changes in the content of alpha, beta and gamma globulins are specific for each animal species, while in gametes the dynamics of alpha and beta globulins is non-specific.

The process of cryopreservation of rooster sperm, unlike that of a bull, causes a decrease in the amount of albumins. This can be explained by the denaturation of proteins, namely albumins.

The appearance of gamma globulin fraction in thawed rooster seed plasma, a sharp decrease in albumin content in thawed rooster seed plasma, and a sharp decrease in albumin content in gametes allows us to note that low temperatures

can initiate translocation, aggregation, and disaggregation of proteins.

A specific manifestation of these disorders may be a decrease in the viscosity of the phospholipid bilayer, which leads to a violation of the barrier properties of plasma membranes in the cryopreservation cycle.

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