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PROTEIN-LIPID AND CHOLESTEROL-PHOSPHOLIPID RATIO AS AN INDICATOR OF CRYORESISTANCE OF GAMETES OF FARM ANIMALS

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Abstract

In our previous studies, it was shown that the sperm of farm animals differs in both species and individual composition, so the purpose of the research, the results of which are presented in this paper was to identify the resistance of gametes of various animal species to the action of cryopreservation factors. The content of proteins and lipids, cholesterol and phospholipids was determined by biochemical methods, and their quantitative ratio was also calculated. It was found that the resistance of diluted sperm to freezing-thawing is directly proportional to the protein-lipid ratio, while the relationship between cholesterol-phospholipid ratio and cryostability of the sperm is inversely proportional. The results of the conducted studies convincingly show that phospholipids undergo the greatest changes and that the molar ratio of cholesterol phospholipids in gametes changes towards one, especially after thawing of sperm.

Key words*: gametes, cryopreservation, protein-lipid ratio, cholesterol-phospholipid ratio.*

INTRODUCTION

A cell is a highly organized structure containing many functional units, characterized by the presence of plasma and internal membranes. The plasma membrane is the outer membrane of the cell, and the inner membranes are membranes of cell organelles. Biomembranes are characterized by extreme diversity and are able not only to separate the cell contents from the external environment and to ensure the separation of the internal cell volume into compartments, but also participate in the regulation of many processes (Ghennis, 1997). For example, plasma membranes provide a diffusion barrier, active transport, electrical excitability, intercellular communication, hormonal and immune responses etc. On the membranes of the endoplasmic reticulum are synthesized proteins, fats and carbohydrates. Membranes of nerve cells are able to transmit impulses in the form of changes in electric potential etc. The uniqueness of the functions of each membrane is largely determined by the properties of the membrane proteins that make up its composition. Proteins - various enzymes, transport proteins, receptors, pores, channels etc. - make a significant contribution to the formation of the structure of cell membranes (Ghennis, 1997). The average protein content in the membranes is approximately 60% (by weight of dry matter), while the composition of biomembranes also includes lipids - 30% and carbohydrates - 10%. Naturally, the ratio between these components can vary significantly depending on the nature of the membranes. Thus, the protein content in membranes can vary from 20% in myelin to 80% in mitochondria (Ghennis, 1997). Lipids-phospholipids, glycolipids, cholesterol - make up the backbone of the membrane and are responsible for the integrity of the membrane structure. Carbohydrates are found in the composition of membrane proteins (glycoproteins and proteoglycans) or lipids (glycolipids) (Ghennis, 1997). In addition, the membranes contain a relatively large amount (~30%) of bound non-freezing water. Despite the diversity of biomembranes, the basic principles of structural organization of all membranes of animal, plant and bacterial origin are the same. According to the widely accepted "liquid mosaic" model, originally proposed in 1972 by Singer and Nicholson (Singer et al., 1972), the biomembrane is represented as a

fluid phospholipid bilayer in which proteins are immersed. Subsequently, however, it became apparent that the molecular organization of the membranes is much more complex than it follows from the liquid mosaic model. In particular, it was shown that not all membrane proteins diffuse freely in a liquid lipid bilayer (Ghennis, 1997). Some parts of the membranes differ in structure from the classical lipid bilayer due to lipid polymorphism. Within the same membrane, regions with different lipid composition and functions can adjoin (Epand, 1998). At present, it is believed that the complex dynamic structure of biomembranes, which is characterized by curvatures, phase transitions, thickness variations and the formation of non-layer structures is determined by the specific interactions of membrane proteins with lipids (Epand, 1998). Such interactions in many respects ensure the effective fulfillment by membranes of various cellular functions arising during metabolism. The dynamic properties of biomembranes can be illustrated by the example of a "metamorphic" model, which includes the basic membrane processes.

MATERIALS AND METHODS

To separate the plasma membranes, a two-phase polymer system was used, consisting of dextran with a molecular weight of 500000 and polyethylene glycol-6000 D produced by Fluka A G Busch (Switzerland). The components of the polymer system were dissolved in 0.1 M phosphate buffer with a pH of 6.5. The resulting solution was transferred to a long funnel and kept for 12 hours, after which the upper and lower phases were separated, which were stored until use. All operations to isolate plasma membranes were performed at 4°C.

In contrast to the method (Ivanov et al., 1981), the membranes were separated using a dividing funnel, where the phases are not concentrated at the separation boundary. In addition, in connection with the detection of a color change in the samples and the construction of a calibration curve, an albumin working solution was prepared using Tris HCl (or EDTA at the determination of protein in gamete homogenate). The determination of the strength of gamete plasma membranes was performed by using the method of electrochemical surf of cell membranes due to the diffusion potential difference based on the registration of changes in the pH of the non-buffer environment. When cells are placed in a non-buffer isotonic medium, chlorine ions exit through the plasma membrane, which leads to the appearance of a diffuse potential on the membrane. This can be used to record changes in the transmembrane potential by determining the pH of the incubation medium. At a transmembrane potential exceeding the critical value, acidification is replaced by reverse alkalization, which is regarded as a consequence of the release of potassium ions from the cell as a result of electrical breakdown of the membranes.

Lipid extraction was carried out by the method of (Bligh et al., 1959). The method is based on the destruction of protein-lipid bonds by polar solvents, followed by extraction of lipids by a non-polar solvent combined into a single mixture containing water in a ratio of 1:2:0.8.

The content of total lipids was studied using the Brandon method described by Skorohod et al. (1983). The principle of the method is based on the ability of potassium dichromate to oxidize lipids. Spectrometry of the resulting chromic acid was carried out at a wavelength of 590 nm.

RESULTS AND DISCUSSIONS

The study of the role of membrane organization of proteins directly in a living organism is difficult due to the complex organization of living matter and the simultaneous occurrence of many interrelated processes. However, the possibility of carrying out of gene mutation that provides selective changes in the structure of expressed proteins, for example, the expression of only soluble forms of proteins, allows in some cases to show the importance of the functioning of membrane-bound proteins (Esther et al., 1997; Huang et al., 1992). Consider this using the example of a KIT-ligand, one of the membrane-bound growth factors of mammals.

Note that the importance of binding to the biomembrane is revealed not only for integral proteins, but also demonstrated in some cases for peripheral proteins, for example, pyruvate oxidase - a peripheral enzyme that catalyzes the

oxidation of pyruvate to acetic acid and the restoration of ubiquinone. The specified enzyme circulates in the body and binds to the plasma membrane only in the presence of a substrate and cofactor; in this case, a C - terminal lipid-binding domain is formed in the protein molecule.

Thus, the biological role of various membrane enzymes can be largely determined by their ability to bind to the membrane. Firstly, binding to a biomembrane provides localization (concentration) of enzymes in a certain part of the cell and/or in that region of the membrane where the substrate is concentrated. For example, acetylcholinesterase is fixed in the postsynaptic membrane, where the concentration of acetylcholine is high. Secondly, the adsorption of enzymes on the membrane makes it possible to interface the processes of catalysis and transmembrane transfer. Thus, during the functioning of membrane-bound enzymes involved in the hydrolysis of starch and proteins, a locally high concentration of soluble product molecules is created near the cell membrane, which contributes to their effective absorption by the cell. Thirdly, for many enzymes, at binding to the membrane ensures the availability of water-insoluble substrates. These can be integral enzymes involved in the processing of membrane proteins (for example, secretase of membrane proteins, see above), as well as peripheral enzymes: phospholipases (Burack et al., 1994), protein kinase C (Newton et al., 1998), pyruvate oxidase etc. Finally, optimal microenvironment is formed upon binding providing native conformation and catalytic activity of membrane enzymes.

Proteins of biological membranes make up a significant part in most living organisms - about a quarter of the genome of prokaryotes and eukaryotes encode these proteins (Fagerberg et al., 2010; Liu et al., 2001). Membrane proteins play a vital role in the implementation of many cellular functions, such as signal transmission, energy production and conversion, recognition and transport of substances through the membrane. According to the biophysical classification, membrane proteins are subdivided into integral ones, which penetrate the membrane through them, often several times, “sit” rigidly in it and can be extracted from it using, for example, non-polar organic solvents or detergents, and peripheral ones that are weakly bound to the membrane or associated with its surface due to non-covalent bonds and can be separated from it by an aqueous buffer solution without destroying the integrity of the entire biological membrane. There are also anchored membrane proteins that hold onto the membrane by immersed in the hydrophobic region of fatty acid or prenyl residues attached to the polypeptide chain as a result of posttranslational modification. Currently available information about the structure and mechanisms of action of membrane proteins is rather sparse - among all protein structures in Protein Data Bank, data on membrane proteins make up only 1-2% of the total number. This is due to the difficulty of conducting structural and functional studies of membrane proteins.

The lipids of a bilayer of biological membranes are amphipathic molecules consisting of a hydrophilic polar head and a hydrophobic tail. The classification of lipids is based on their chemical structure - depending on the structure of the polar head, they are charged (anionic) and uncharged (neutral and zwitterionic). Lipids also differ in the structure of the hydrophobic tail (the number of chains, the length of the hydrocarbon chain, with the presence or absence of double bonds, cycles etc.). The main molecules of the membrane matrix are glycerophospholipids. In addition to them, the membrane contains many other types of lipids, of which sterols (for example, cholesterol), sphingolipids, glycolipids, and others are worth mentioning separately (Sezgin et al., 2017). Due to the chemical structure and the uniqueness of the physicochemical properties of each type of lipid, the structure of associates into which they are able to spontaneously organize in aqueous mediums under different external conditions is also diverse. In 1925, Gorter and Gredel suggested (Cherezov et al., 2007) that lipids are able to form bilayer structures - in the aqueous medium, when the hydrophobic tails are densely packed to each other, while the hydrophilic head parts look outward, directly contacting with the water phase. Subsequently, this idea took shape in a two-dimensional fluid model with lateral diffusion of membrane

lipids, which was called the mosaic model of biological membranes (Nicolson, 2014).

The presence of cholesterol, which is an essential component of mammalian cell membranes, increases the packing density of lipids in the membrane, which leads to changes in the viscosity or permeability of the membrane and is involved in the formation of rafts (Burger et al., 2000; Sezgin et al., 2017). The lipid composition of cells of different organisms differs from each other, which determines both the structure of the bilayer formed by them and the variety of functions that lipids perform in membranes (Sezgin et al., 2017). For example, bacterial membranes mainly consist of phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) (Burack et al., 1994). Eukaryotic membranes are composed primarily of glycerophospholipids, sphingolipids and sterols. The main anionic lipid in them is phosphatidylserine (PS).

Our laboratory staff noticed that the biochemical composition of plasma membranes is of great interest in elucidating the causes for the different resistance of gametes to low temperatures and investigated the cryogenic changes in the ratio of protein/lipid plasma membranes of gametes of different animal species (Table 1).

As can be seen from the table, the highest ratio of protein/lipid in plasma membranes is observed in carp gametes and is somewhat lower, but still higher than in other animals - in the bull, whose gametes tolerate freezing well.

The results of the researches showed that the ratio of cholesterol/phospholipids was the same in the diluted gametes of bull and ram (Table 2). The lowest ratio of these components was found in the membranes of boar gametes, which are characterized by high sensitivity to cooling and freezing. The value of this indicator in the membranes of the rooster and ram gametes occupies an intermediate position.

Table 1. Cryogenic changes in the ratio of protein/lipid plasma membranes of animal gametes

Animal species	Protein/lipid ratio of membranes			Number of repetitions
	Native gametes	Refrigerated gametes	Thawed gametes	
Bull	0.43±0.014	0.3±0.11	0.25±0.019*	6
Ram	0.36±0.031	0.38±0.13	0.52±0.032*	6
Boar	0.17±0.009	0.17±0.09	0.20±0.004*	5
Rooster	0.40±0.042	0.4±0.08	0.52±0.041*	5
Carp	0.65±0.07	0.54±0.15	0.42±0.08*	3

*Statistically authentic cryogenic changes

Apparently, a decrease in the protein/lipid ratio during thawing is more advantageous for preserving functional usefulness than its increase, because the semen of bull and carp in the gamete membranes of which the indicated decreases is better preserved during cryopreservation than the semen of other animal species.

In the following studies, the goal was to study the dynamics of changes in the molar ratio of cholesterol/phospholipids in the process of gamete cryopreservation.

During cooling, freezing and thawing of the semen, there is a tendency to shift the molar ratio in the direction of increase. In bull gametes, this phenomenon is most pronounced. The observed changes are of certain interest, since the phase transition of lipids disrupts the plasticity of membranes, leading to the formation of a rigid membrane structure, and a decrease in the thermal stability of cells.

Table 2. Indicators of the impact of technological processing of semen on the ratio of cholesterol/phospholipids (C/Pl) in the gametes of bulls (n=7), rams (n=6) and boars (n=8).

Lipids	Dilution		Refrigeration		Thawing	
	Mol %	C/Pl	Mol %	C/Pl	Mol %	C/Pl
Bull						
Cholesterol	1.07±0.04	0.282	0.99±0.04	0.326	0.89±0.04*	0.382
Phospholipids	3.79±0.06		3.04±0.06		2.33±0.10*	
Ram						
Cholesterol	1.11±0.03	0.281	1.05±0.04	0.302	0.93±0.05*	0.293
Phospholipids	3.95±0.06		3.48±0.03		3.18±0.03*	
Boar						
Cholesterol	1.25±0.05	0.349	1.18±0.03	0.375	1.11±0.03*	0.381
Phospholipids	3.58±0.09		3.15±0.05		2.84±0.04	

*Statistically authentic cryogenic changes

The results of these studies convincingly show that phospholipids undergo the greatest changes and that the molar ratio of cholesterol/phospholipids in gametes changes in the direction of one, especially after thawing the semen, that is, in the direction of the ratio that eliminates the phase transition of lipids.
Consequently, changes in the ratio of cholesterol/phospholipids, as well as loosely bound cholesterol in gametes during cryopreservation, have a non-specific character, that is, the nature of their modification is the same for the gametes of all studied animal species.

CONCLUSIONS

Changes in the ratio of cholesterol/ phospholipids and the content of loosely bound cholesterol in gametes during cryopreservation are non-specific, that is, their modifications are the same for the studied animal species.
The observed changes are of particular interest, since the phase transition of lipids disrupts the plasticity of membranes, leading to the formation of a rigid membrane structure, and a decrease in the thermal stability of cells.
During cooling, freezing and thawing of the semen, there is a tendency to shift the molar ratio in the direction of increase. In bull gametes, this phenomenon is most pronounced.
It is proved that phospholipids undergo the greatest changes and that the molar ratio of cholesterol/phospholipids in gametes changes in the direction of one, especially after thawing of the seed, that is, in the direction of the ratio that eliminates the phase transition of lipids.

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