The use of nanosized liposomes from vegetable phospholipids in combination with albumine and some polysaccharides as cryoprotective agents in the course of cryopreservation

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Abstract. It is known that nanosized liposomes from vegetable and animal phospholipids promote survival and functional adequacy of animal cells in the course of cryopreservation. It is also known that albumine and some polysaccharides have membrane-protective properties. In this paper we investigated whether there is a synergism of cryoprotective action between soybean liposomes and macromolecular compounds: albumine, sodium alginate, methylcellulose, carrageenan and hyaluronic acid. To determine the cryoprotective effect we carried out cryopreservation of bull semen using a standard procedure in the presence of the substances under investigation. Albumine and sodium alginate demonstrated the most considerable cryoprotective effect in the presence of liposomes. In addition we determined dimensional characteristics of nanoparticles in a liposome suspension without and with sodium alginate and studied the influence of autoclave treatment on the nanoparticle size and cryoprotective effect of the complex. Autoclaving caused enlargement of a small-sized fraction of nanoparticles, supposedly, sodium alginate aggregates. Besides, the autoclaving did not demonstrate any influence on the cryoprotective efficiency of the mixture.

1. Introduction

The use of cryopreserved seed from elite producers is currently an integral part of agricultural production. In dairy cattle breeding in Russia, about 60% of the population is seeded with frozen-thawed seeds, for which more than 11 million sperm doses [1] of cryopreserved seed of producers are used annually. Most yolks for cryopreservation of sperm include egg yolk [2]. Egg yolk is highly effective in protecting sperm from cold shock both during cooling and during freezing. However, having high cryoprotective efficacy, the yolk has a number of undesirable properties: inconstancy of...
composition, difficulty in sterilization, rapid loss of cryoprotective properties during storage (within several hours), and the ability to induce immune sensitization when a female is introduced into the genital tract [2]. The search for an effective substitute for egg yolk in media for cryopreservation of sperm of farm animals is recognized as relevant worldwide. Due to the fact that lecithin is considered the main carrier of the cryoprotective properties of the yolk, plant phospholipids are the most promising substitute for the yolk.

It is known that nanosized liposomes from phospholipids of plant or animal origin contribute to the preservation of the viability and functional usefulness of animal cells during cryopreservation. A number of substances were selected as potential lecithin modifiers, which, on the one hand, affect the properties of phospholipid liposomes and their interaction with the aquatic environment and living cells, and, on the other hand, do not have toxicity and, moreover, can increase survival cells in extreme conditions. In particular, albumin and some polysaccharides have membrane-protective properties and can also help maintain cell integrity during freezing and thawing [3, 4].

In this paper, we investigated the effect of liposomes from soybean phospholipids, albumin, and a number of polysaccharides on the physical and cryoprotective properties of plant lecithin when introduced into cryoprotective media for bovine sperm. The aim of the work was to select promising polysaccharides that, when interacting with liposomes, have an effective cryoprotective effect. The following polysaccharides were tested: sodium alginate, methyl cellulose, carrageenan and hyaluronic acid.

2. Materials and methods

To prepare a suspension of liposomes from a complex of soybean phospholipids (azolectin, Sigma), a weighed portion of phospholipids of 0.36 g was placed in 10 ml of buffered sucrose solution (per 100 ml of sucrose solution 30 mM, HEPES 1 mM), phospholipid was left to hydrate for a day, then the obtained lipid particles were crushed to obtain nanosized liposomes. In experiments to determine cryoprotective properties, ultrasonic crushing was used. The resulting suspension to prevent oxidation of phospholipids was purged with nitrogen, then cooled in an ice bath to 4 ° C and processed for 5 minutes on an ultrasonic disintegrator UZDN-2T (NPP Akadempribor, Russia; 22. kHz, 60 Wcm2-1) using immersed probe with an end diameter of 2 mm. The average size of liposomes from azolectin in this treatment is 75-80 nm. For experiments to determine the effect of autoclaving on liposome size, liposome crushing was used on a high-pressure homogenizer DONOR-1 (Russia), because this method gives more aligned liposome sizes, which facilitates the interpretation of the results of the study.

To determine the cryoprotective properties of liposomes in the presence of albumin and polysaccharides, bovine spermatozoa were cryopreserved in a cryoprotective medium for bovine sperm. The experiment used the seed of bulls kept in the Head Center for the reproduction of farm animals, OJSC “GCV”.

Sperm was obtained on an artificial vagina according to the generally accepted method (ss). A tris citrate cryoprotective medium was used as the base solution (per 100 ml of a solution of tris (hydroxymethyl) aminomethane 20 mM, citric acid 7 mM, fructose 5 mM, glycerol 65 mM, pH = 6.7-6.9). On the basis of the basic cryoprotective medium, uterine solutions of albumin (fraction V, PanEco) 5%, medium viscosity methylcellulose (Sigma, M0262) 1%, hyaluronic acid (Acros organics) 0.5%, sodium alginate (Sigma, A2033) 0.5 were prepared %, carrageenan (Sigma, C1138) 1%. Albumin or polysaccharides were added to the basal medium based on the final concentrations shown in Tables 1-4 and a liposome suspension in a basal medium / liposome suspension ratio of 4:1. Freezing was carried out according to the method of freezing in open granules.

After cryopreservation, sperm viability was assessed using the following parameters: sperm motility 5 minutes after thawing (data were obtained using a Biola-500-1 sperm analyzer, Russia), average sperm movement speed (Biola-500-1), thawed sperm motility after cultivation at 38 °C for 5 hours (visually, using a Carl Zeiss Axio Scop Fl microscope, magnification * 280).
For experiments on the effect of autoclaving on the dimensional characteristics of liposomes in a cryoprotective solution without macromolecular compounds and in combination with sodium alginate, a cryoprotective medium with liposomes with or without sodium alginate was autoclaved and the particle size in suspension was determined before and after autoclaving. For autoclaving, a low temperature regime (115 °C, 1.5 Atm) was used, because higher temperatures cause caramelization of sugars and are not suitable for the treatment of media containing sugars. Liposome sizes were measured by dynamic light scattering on a Beckman Coulter submicron particle size analyzer.

Additionally, an experiment was carried out to test the cryoprotective effect of a cryoprotective medium with liposomes and sodium alginate before and after autoclaving, as described above.

3. Results and discussions
The cryoprotective effect of albumin and polysaccharides in combination with nanosized liposomes from soybean phospholipids in relation to bovine spermatozoa.

It is known that for the successful cryopreservation of spermatozoa in cryoprotective media, it is necessary, in addition to cryoprotectants, to introduce high molecular weight substances that help protect cell membranes from cold shock. In practice, such substances can be whole egg yolk, purified yolk or soybean phospholipids, albumin, milk casein, and others. Moreover, the introduction of complex protein components into the composition of the media, and even more complex products, such as egg yolk, significantly complicates theoretical understanding cryoprotection mechanisms, as well as manipulations with such media in practice (sterilization, storage). In our work, we conducted experiments to identify the cryoprotective properties of albumin, a natural surfactant that simultaneously has antioxidant properties, and non-toxic, biocompatible polysaccharides that can potentially have a membrane-protective effect similar to albumin. In addition, the long-term stability of liposomes can be improved by applying various polysaccharides to them [5, 6]. The cryoprotective effect of albumin and polysaccharides was observed against the background of liposomes of soybean phospholipids.

The results on the effect of albumin on the survival of bovine frozen spermatozoa in the presence of liposomes and glycerol are presented in table 1.

| Table 1. The effect of albumin in combination with nanosized liposomes from soybean phospholipids in a cryoprotective medium on sperm characteristics after cryopreservation |
|---------------------------------------------------------------|
| Mobility after defrosting, % | The average rate of sperm. Mkm sec⁻¹ | The mobility of frozen thawed sperm after 5 hours of cultivation at 38°C, % |
|---------------------------------|---------------------------------|---------------------------------|
| liposomes 8/5000                | 35.0±6.9                        | 50.5±4.8                         | 3.3±1.6                        |
| liposomes 8/5000 +albumin 0.5%  | 34.5±6.2                        | 61.5±4.0                         | 17.3±2.1                       |

From the data presented in the table, it is clear that albumin significantly improved the qualitative characteristics of cryopreserved sperm. Sperm frozen with the addition of albumin had a significantly higher sperm movement rate and significantly higher survivability (motility after 5 hours of cultivation).

Tables 2–4 provide data on the characteristics of cryopreserved bull sperm frozen in the presence of hyaluronic acid, methyl cellulose, and sodium alginate.
Table 2. The effect of hyaluronic acid in combination with nanosized liposomes from soybean phospholipids in a cryoprotective medium on sperm characteristics after cryopreservation

|                          | Mobility after defrosting, % | The average rate of sperm, Mkm sec\(^{-1}\) | The mobility of frozen thawed sperm after 5 hours of cultivation at 38°C, % |
|--------------------------|-------------------------------|-----------------------------------------------|--------------------------------------------------------------------------|
| Liposomes                | 27.0±5.5                      | 58±5                                         | 3±1.0                                                                    |
| Liposomes + hyaluronic acid, 0.5% | 28.0±6.0                      | 54±5                                         | 4±1.4                                                                    |

Table 3. The effect of methyl cellulose in combination with nanosized liposomes from soybean phospholipids in a cryoprotective medium on the characteristics of sperm after cryopreservation

|                          | Mobility after defrosting, % | The average rate of sperm, Mkm sec\(^{-1}\) | The mobility of frozen thawed sperm after 5 hours of cultivation at 38°C, % |
|--------------------------|-------------------------------|-----------------------------------------------|--------------------------------------------------------------------------|
| Liposomes                | 22.5±2.9                      | 54±7                                         | single                                                                   |
| Liposomes + cellulose 0.03% | 21.5±2.9                      | 51±7                                         | single                                                                   |
| Liposomes + cellulose 0.1% | 20.4±3.6                      | 53±3                                         | 1.5±1.3                                                                 |

Table 4. The effect of sodium alginate in combination with nanosized liposomes from soybean phospholipids in a cryoprotective medium on sperm characteristics after cryopreservation

|                          | Mobility after defrosting, % | The average rate of sperm, Mkm sec\(^{-1}\) | The mobility of frozen thawed sperm after 5 hours of cultivation at 38°C, % |
|--------------------------|-------------------------------|-----------------------------------------------|--------------------------------------------------------------------------|
| Liposomes                | 33.5±4.8                      | 56±8                                         | 3.3±1.1                                                                  |
| Liposomes + alginate Na 0.002% | 30.5±2.6                      | 56±6                                         | 11.0±6.7                                                                |
| Liposomes + alginate Na 0.01%  | 29.5±2.1                      | 65±3                                         | 11.0±6.3                                                                |
| Liposomes + alginate Na 0.05%  | 32.0±5.2                      | 63±3                                         | 12.5±5.6                                                                |
The presented results show that hyaluronic acid and methyl cellulose, despite the presence of membrane-protective properties, did not have a noticeable protective effect during cryopreservation. In contrast to these two substances, sodium alginate showed certain cryoprotective properties, although weaker than albumin. Sperm frozen in the presence of alginate had significantly higher survival rates (motility after 5 hours of cultivation) compared with sperm frozen without alginate. The experiments performed did not allow us to identify the most optimal concentration of alginate in a cryoprotective medium. It can be assumed that the optimum concentration of alginate has wide limits.

In addition to the substances indicated in the tables, we conducted experiments on the effect of carrageenan in concentrations of 0.01-0.1% on sperm survival during cryopreservation. The addition of carrageenan to the cryoprotective medium caused strong sperm agglutination and sharply reduced the characteristics of the frozen thawed sperm.

**Determination of the hydrodynamic diameter of unmodified and modified liposomes.**

Most culture and cryoprotective media are sterilized by filtration through filters with pores less than 200 nm. However, autoclaving sterilization in some cases seems to be a more reliable and affordable sterilization method. Both alginate and phospholipids are resistant to temperature effects of 100-120 °C. At the same time, exposure to high temperature can contribute to the fusion of liposomes and a change in the interaction of liposomes with macromolecular substances. We investigated the effect of autoclaving on the dimensional characteristics of liposomes. In the experiment, liposomes obtained by crushing on a high-pressure homogenizer DONOR-1 were used, because this method allows one to obtain more uniform liposomes with one peak in size distribution, while ultrasonic fragmentation of liposomes from a complex mixture of phospholipids often gives a size distribution with two and three peaks [7]. The average size of liposomes before autoclaving was 86 ± 65 nm, after autoclaving 84 ± 55 nm. Therefore, autoclaving does not change the average particle size and the nature of their size distribution, from which we can conclude that autoclaving does not initiate adhesion or fusion of liposomes with enlargement of sizes.

When sodium alginate was added to the cryoprotective medium containing liposomes, 2 peaks were observed in the particle size distribution. The first peak is in the range of 6-12 nm, the second peak is in the range of 60-150 nm (average size is 90 nm). The first peak corresponds quite accurately to the size of the associates of sodium alginate molecules. From the data obtained, it can be concluded that some of the molecules of sodium alginate are not distributed over the surface of liposomes, as expected, but are self-organizing in the form of independent globules. According to published data, during the self-organization of alginate into globules in weak solutions, a trimodal particle distribution is formed, formed by various associations of molecules with peaks of 6-13 nm, 29-70 nm and 130-170 nm [8]. The peak at 29-70 nm, obviously, merges with the peak characteristic of liposomes (25-70). In addition, there is a small amount of particles in the range of the third peak (130-370 nm).

After autoclaving, the character of particle size distribution changed. The maximum of the first peak shifted from 10 to 35 nm, the second peak shifted less significantly from 90 to 98 nm. From this we can conclude that in the process of autoclaving, aggregation of alginate aggregates occurs. In addition, it can be assumed that autoclaving contributes to an increase in the number of sodium alginate molecules adsorbed on the surface of liposomes.

When nonionic methylcellulose was added to the cryoprotective medium containing liposomes in the particle size distribution, 1 peak was observed both before and after autoclaving. The size of modified liposomes after autoclaving decreased, but in all cases they exceeded the size of unmodified liposomes. Perhaps this is due to the fact that the interactions between liposomes and the neutral polysaccharide are quite unstable.

Thus, experiments on the effect of autoclaving on the cryoprotective properties of a complex of nanosized liposomes with sodium alginate did not reveal significant differences. The cryoprotective efficacy of the cryoprotective medium, which underwent and did not undergo autoclaving, was identical. The size distribution of all polymer coated liposomes was wider than that of unmodified liposomes.
References

[1] Eskin G V, Kombarova N A, Kornilin R A Stabilization of sperm productivity in young bulls during prolonged heat stress with the help of BAXIN-VET // Problems of biology of productive animals 2011, № S4, C. 40-743.

[2] Barbas J P, Mascarenhas R D Cryopreservation of domestic animal sperm cells. Cell Tissue Bank. 2009 Feb;10(1):49-62.

[3] Dalimata A M, J K Graham, 1997, Cryopreservation of rabbit spermatozoa using acetamide in combination with trehalose and methyl cellulose: Theriogenology, v. 48, p. 831-841.

[4] Thirumala S, Gimble J M, Devireddy RV. Evaluation of methylcellulose and dimethyl sulfoxide as the cryoprotectants in a serum-free freezing media for cryopreservation of adipose-derived adult stem cells. Stem Cells Dev. 2010 Apr;19(4):513-22.

[5] G Smistad, S Bøyum, S J Alund, A B C Samuelsen, M Hiorth The potential of pectin as a stabilizer for liposomal drug delivery systems //Carbohydr. Polym., 90 (2012), pp. 1337-1344

[6] T Klemetsrud, H Jonassen, M Hiorth, A L Kjøniksen, G Smistad Studies on pectin-coated liposomes and their interaction with mucin //Colloids Surf. B, 103 (2013), pp. 158-165

[7] Shishova N V, Kombarova N A, Davydova G A, Seraya O Yu, Mironova E A, Abilov A I, Pashovkin T N, Gakhova E N, Study of the cryoprotective effect of plant lecithins lecipro-s and lecipro-90 // biological membranes: journal of membrane and cell biology (biologicheskie membrany), 2017. t. 34, № 3. pp. 223-230

[8] I A. Oberyukhtina, K G. Bogolitsyn, N R. Popova, L N. Parfenova. Application of the method of laser correlation spectroscopy in the study of the hydrodynamic properties of dilute solutions of sodium alginate. All-Russian Conference "Chemistry and Technology of Plant Substances", oral report, Kazan, June 24-27