CryoLetters **40 (3)**, 187-192 (2019)

HUMAN GAMETES CRYOPRESERVATION WITH CRYOPROTECTANT MODIFIED BY EGG YOLK

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Abstract

BACKGROUND: The modification of cryoprotectants with an egg yolk is an effective method to improve cell survival. **OBJECTIVE:** This study is dedicated to the comparison of four variants of cryoprotectants modified with egg yolk (commercially available and custom developed with yolk emulsion at 5.6 mg ml^{-1}) and with lecithin. **METHODS:** Cryoprotectant effectiveness was evaluated by cytotoxicity and vital screen tests, and by the determination of sperm motility index. **RESULTS:** A simple analytical model has been created to determine an approximate concentration of cholesterol in the solution sufficient to stabilize cellular membranes. It was shown that the average fractions of living cells, motility and membrane integrity were higher for our modification. **CONCLUSION:** We conclude that phosphatidylcholine (1.5 mg ml^{-1}) contributes to the cryoprotective action of egg yolk, but doesn`t define it entirely. The cryoprotective effect of egg yolk on plasma membranes is complex, and isn`t caused by the action of one of its components.

Keywords: cryopreservation, egg yolk, sperm quality, fertilization, model of cholesterol concentration.

INTRODUCTION

Gamete cryopreservation remains one of the most important methods in reproductive medicine. Despite a large amount of studies on cryoprotectants, the main characteristics of spermatozoa, such as motility and morphology, decrease by about 30 to 70% after cryopreservation with modern cryoprotectants (5, 9). Most of the commercial cryoprotectants consist of penetrating compounds (such as glycerol, DMSO) and non-penetrating compounds (sucrose, trehalose, egg yolk).

It is known that the main lipids of the outer monolayer in animal cells are phosphatidylcholines and sphingomyelin, and the inner monolayer is formed predominantly by phosphatidylserine and phosphatidylethanolamine that have smaller sizes of polar fragments (8). In spite of the rather ordered structure of the bilayers, several types of motility are recognized in phospholipids (3). Thus, one of the types of lipid phase transitions in the membrane is the formation of hexagonal structures (HII) (7), which is associated with low hydration of the membrane, an increase in ionic strength at alkaline pH, and a decrease of the saturated fatty acids in the membrane structure. The formation of hexagonal structures leads to a disturbance of the integrity of the membrane, the formation of permeability channels and other defects. The incorporation of cholesterol molecules causes a change in the shape of the membranes as a result of significant deformation of both sides of the lipid bilayer. Cholesterol molecules are oriented to the bilayer so that the

hydroxyl group is located near the polar groups of phospholipid molecules, and the rigid steroid rings interact and partly immobilize parts of the hydrocarbon chains (12). Some studies have shown that the addition of cholesterol leads to the stabilization of the hexagonal phase (HII) (10). Due to increased cholesterol concentration, the liquid crystal structure of the membranes becomes more plastic during phase transitions. Plasticity determines the resistance of membranes to damage and deformations during cryopreservation. Thus, a strict correlation of the molar percentage of cholesterol with the cryotolerance of spermatozoa of various mammalian species was found. Rabbit spermatozoa have the maximum of cryotolerance when their membranes have a molar percentage of cholesterol of 62; values are 50% for human spermatozoa and 38% for ram spermatozoa (2). Adding egg yolk to the cell culture reduces the intensity of osmotic processes by creating a hydrophobic phase from the lipoid components on the cell surface. Lipoid components (lecithin, phospholipids, lipoproteins) have a chemical affinity with the lipoproteins that cover of the cells; they layer on the membrane surface and form a hydrophobic phase in which the osmotically active substances and water dissolve. Therefore the osmotic and diffusion exchange between the cell and the extracellular medium slows down.

Thus, lipid non-penetrating compounds, such as egg yolk, appear to be very efficient as cryoprotectants. Egg yolk is known to increase the rate of viable and motile cells after cryopreservation in experiments on mammal spermatozoa (1, 5, 9, 13). The WHO laboratory manual for the examination and processing of human semen suggests a protocol for semen cryopreservation with the addition of fresh egg yolk (11). Whilst some commercial cryoprotectants contain egg yolk, it is rarely combined with saccharide components due to a decrease in efficiency. Moreover, as a product of animal origin, egg yolk always introduces a risk of viral infection. To minimize this risk, but keep the benefits of lipid compounds, we previously developed a protocol of commercial cryoprotectant modification with processed egg yolk powder (5.6 mg ml^{-1}) (6). It showed an increase in past-thaw motility by 20%, a 6 % decrease in the amount of cells with neck or middle part defects ($P \leq 0.05$) and 2.6% decrease in tail defects ($P \leq 0.1$).

In this study we compared cryoprotectant modified with egg yolk by our protocol with commercially available analogs and investigated an impact of egg yolk components on its cryoprotective effect. We also mathematically estimated the concentration of cholesterol in the external environment that is required for the equilibrium state of the cholesterol content in the plasma membrane.

MATERIALS AND METHODS

Ejaculate samples of 16 healthy donors (WHO criteria for normozoospermia) were used in the study. All samples were cryopreserved by a slow freezing protocol, by gradual cooling in liquid nitrogen vapour with subsequent storage in liquid nitrogen.

The following reagents were used: egg yolk emulsion (Sigma); SpermFreeze (LifeGlobal); Test Yolk Buffer (TYB) (Irvine Scientific); egg yolks from chicken (SIGMA), SpermWash Medium (SAGE), and VitalScreen (FertiPro).

Motility was evaluated according to “WHO Manual for Examination and Processing of Human Semen” and divided into different groups: progressively motile (PR), non-progressively motile (NP), immotile (IM). The motility index was calculated using the following formula: (post-thaw PR/ pre-freeze PR)*100. The results are shown in relative units (RU).

Spermatozoa vitality was determined by the VitalScreen method, using the eosin-nigrosin staining technique to establish the percentage of live spermatozoa.

In the results all values are shown as mean ± SEM. A two sample t-test with different variances was used to determine statistical significance of differences between observed groups. Different a, b, c indexes mark the groups that statistically differ from each other (5% significance level).

This study was approved by the Ethics Committee of IVF Clinic AltraVita and informed consent was obtained from all subjects.

RESULTS

In our previous studies we developed a protocol of commercial cryoprotectant modification with processed egg yolk powder (6). To determine an optimal egg yolk

Table 1. Cytotoxicity test – percentage of normally motile spermatozoa after incubation with different diluents

Incubation time (h)	Before freezing	TYB	ME-SF	EY-SF
00:00	54±3	-	-	-
00:30	64±3	51±3	55±3	54±3
01:00	66±3	44±2	49±2	46±2
02:00	47±2	39±2	47±2	48±2

concentration we defined a range from the minimum effective concentration (based on experiments with mammal spermatozoa) to 20 time's higher concentration (~0.5-10 mg ml^{-1}) and tested post-thaw motility after cryopreservation with egg yolk concentrations in this range. An optimal concentration (5.6 mg ml^{-1}) was determined, above which an increase in egg yolk concentration did not result in an increase of cryoprotective effect. Morphological and motility tests after cryopreservation with the addition of egg yolk in selected concentration showed an increase in past-thaw motility by 20% and a 6% decrease in amount of cells with neck or middle part defects decreased, and 2.6% decrease with tail defects.

In the current study we compared the developed modification with commercially available analogs. Four variants of cryoprotectants were chosen: the egg yolk containing cryoprotectant Test Yolk Buffer (TYB); a cryoprotectant without egg yolk, SpermFreezing (SF); SF modified by commercial microbiological emulsion (ME-SF); and our modification of SF with 5.6 mg ml^{-1} of egg yolk (EY-SF). All media were tested for cytotoxicity without an addition of SF.

Results showed that after 2 h of incubation the percentage of spermatozoa with normal motility types are almost equal in samples incubated without media and with our suggested modification, i.e., EY-SF (Table 1). The observed lower percentage in the sample incubated with TYB may be explained by the presence of cytotoxic compounds, like glycerol.

Ejaculate samples from 16 healthy donors were divided into four aliquots and frozen with chosen variants of cryoprotectants to assess their effectiveness. Pre-freeze and post-thaw spermatozoa motility was evaluated and corresponding motility indexes were calculated. The results are shown on Figure 1.

The results showed statistically significant differences in average motility indexes after cryopreservation with EY-SF and commercial cryoprotectants (unmodified SF and egg yolk containing TYB). The percentage of progressively motile spermatozoa remained higher in samples frozen with EY-SF than in samples frozen with unmodified SF by 10% ($P \leqslant 0.05$) and then in samples frozen with TYB by 7% ($P \leqslant 0.05$). The ME-SF variant of cryoprotectant did not show any statistically significant differences with other cryoprotectant variants.

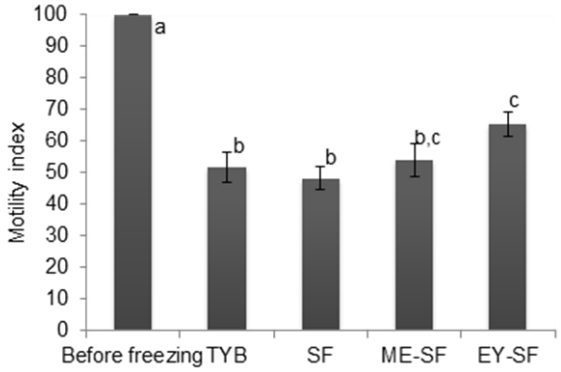

Figure 1. Pre-freeze and post-thaw motility index for samples cryoconserved with different variants of cryoprotectant.

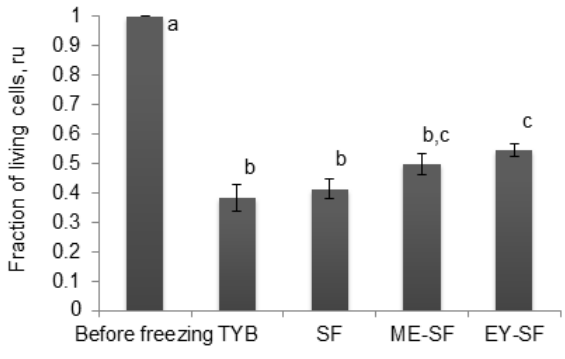

Figure 2. Pre-freeze and post-thaw fractions of living cells in samples cryoconserved with different variants of cryoprotectant.

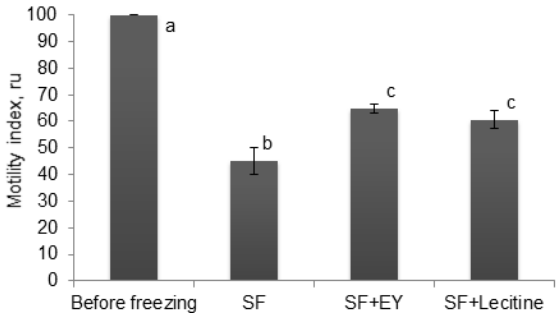

Figure 3. Motility index for samples before freezing and past-thaw after cryoconservation in presence of egg yolk of soy bean lecithin.

We can see higher values of the motility index in all samples than in previously obtained results. It could be explained by the use of different objects: ejaculate samples purified from seminal fluid in density gradient (in previous study) and unpurified native ejaculate samples (in present study).

To access sperm vitality in the tested samples, the eosin-nigrosin staining technique (VitalScreen test) was used. VitalScreen provides an accuracy check of the motility evaluation since the percentage dead spermatozoa should not exceed the percentage immotile spermatozoa.

The results (Figure 2) showed a similar picture to the motility evaluation. There were statistically significant differences in the average fractions of living cells after cryopreservation with EY-SF and commercial cryoprotectants

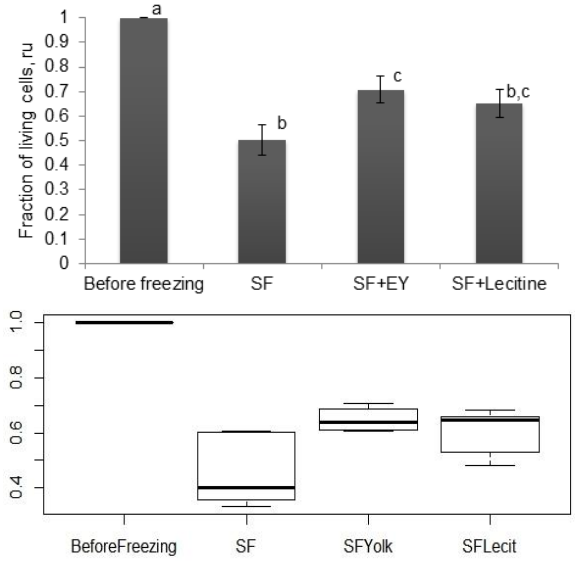

Figure 4. Pre-freeze and post-thaw fractions of living cells in samples cryoconserved with or without egg yolk or soy bean lecithin.

(unmodified SF and egg yolk containing TYB). Average fraction of living cells remains higher in samples frozen with EY-SF than in samples frozen with unmodified SF by 13.5% ($P \leq 0.05$) and then in samples frozen with TYB by 16% ($P \leq 0.05$).

At the 5% significance level, no differences in average fractions of living cells were observed between samples frozen with ME-SF and with the other cryoprotectant variants.

Egg yolk substitution

According to the literature, which is mostly based on experiments on animal sperm (4, 9, 13), to decrease a risk of infection due animal origin of egg yolk it could be replaced with soy bean lecithin.

We developed a protocol of SF modification with lecithin to a final concentration 1.5 mg ml^{-1} and conducted experiments to evaluate motility indexes after cryopreservation with SF, SF-lecithin and SF-EY. Lecithin containing cryoprotectant didn't show results as good as EY-SF (Figure 3).

We also conducted vitality tests for these samples (Figure 4) that showed that there were no statistically significant differences between samples frozen with cryoprotectant modified with lecithin and unmodified cryoprotectant. However, there was a significant difference with samples frozen with egg yolk modified cryoprotectant.

We suggest that better results with egg yolk modification could be explained by the presence of low density lipoproteins and cholesterol in its composition, which could have a positive effect. Also present in egg yolk is L-α-phosphatidylcholine, the main compound of soy bean lecithin.

Cholesterol in egg yolk could affect the plasticity of plasmatic membranes. During incubation in seminal liquid or buffer solution, constant desorption of cholesterol out of plasmatic membranes occurs. This is a natural mechanism of capacitation preparation. However, significant decreases in cholesterol concentration in membranes could cause increased damage during cryopreservation.

A simple analytical model was built to establish the minimal cholesterol concentration in the extracellular medium necessary for an equilibrium state of cholesterol inside and outside of plasmamembrane. This model describes the water diffusion of lipids between monolayer vesicles. As follows:

$$\left[Ch^M\right] \leftrightarrow [Ch^F] \quad [1]$$

$$\frac{d[Ch^M]}{dt} = k_+[Ch^F] - k_-[Ch^M] = 0 \quad [2]$$

$$\left[Ch^F\right] = \frac{k_-}{k_+}[Ch^M], \quad [3]$$

where [ChF] – is a cholesterol concentration in the extracellular medium, [ChM] – is the cholesterol concentration in cellular membranes, k_- – 1st order cholesterol desorption constant into extracellular media, k_+ – 1st order cholesterol adsorption constant from the extracellular medium into membranes.

A halftime of cholesterol desorption from spermatozoa membranes is 1 h (τ-1/2 = 1 h), and equilibrium constant for desorption/adsorption processes is $k = \frac{k_-}{k_+} = 1 * 10^{-3}$. Thus,

$$k_- = \frac{\ln 2}{\tau_{1/2}} = 0{,}69, \quad [4]$$

$$k_+ = \frac{0{,}69}{0{,}001} = 690, \quad [5]$$

$$\tau_{1/2} = \frac{\ln 2}{690} \approx 3{,}6 \text{ s} \quad [6]$$

To evaluate membrane cholesterol concentration the following approximations were made: spermatozoa concentration in tested samples was around $1*10^8$ cells ml^{-1}, after dilution according to the protocol concentration becomes $0.25*10^8$ cells ml^{-1}, cholesterol concentration in membranes of human spermatozoa is around 133 nm 10^8 $cells^{-1}$. Thus, membrane cholesterol concentration is:

$$\left[Ch^M\right] = 0{,}25 * 133 \approx 33{,}3 \ \frac{\text{nm}}{\text{ml}}. \quad [7]$$

Using the equilibrium constant we can calculate the extracellular cholesterol concentration needed for an equilibrium state:

$$\left[Ch^F\right] = \frac{k_-}{k_+}\left[Ch^M\right] = 1 * 10^{-3} * 33{,}3 = 3{,}3 * 10^{-2} \frac{\text{nm}}{\text{ml}} \quad [8]$$

In our experiments egg yolk was added in concentration 5.6 mg ml^{-1}; as cholesterol is ~1.55% (w/w) of egg yolk, then the cholesterol concentration in the test samples was $8.7*10^{-2}$ mg ml^{-1} or 224 nm ml^{-1}. This significantly exceeds the minimal concentration necessary to maintain initial membrane cholesterol concentration.

DISCUSSION

No cytotoxic effect of egg yolk was observed. The protocol of cryoprotectant modification with processed egg yolk powder we developed earlier not only allows the preservation of a higher amount of normally motile and vital spermatozoa than commercially available analogs, but also minimizes the risk of viral infection. We suggest that a possible mechanism by which egg yolk cryoprotects could be through the stabilization of the cellular membrane. That is indirectly confirmed by VitalScreen test.

L-α-phosphatidylcholine was suggested as the main active compound of egg yolk, which protects cells during freeze-thaw cycle. To test this hypothesis, a protocol of cryoprotectant modification with lecithin was developed. Experiments showed higher effectiveness of modification with egg yolk, i.e. L-α-phosphatidylcholine is not the only compound of egg yolk with a positive effect on spermatozoa cell integrity.

Cholesterol was suggested as another cryoprotective compound of egg yolk. A simple diffusion model was used to evaluate the minimal cholesterol concentration required to maintain the initial membrane cholesterol concentration. These approximations allowed us to conclude that cholesterol's cryoprotective effect was fully utilized in experiments with cryoprotectant modified by egg yolk.

The proposed mechanisms of cryoprotective influence of the egg yolk are associated with the effect of phosphatidylcholine and cholesterol on the parameters of plasma membranes. There is also a theory that the egg yolk weakens the osmotic and diffusion exchange between the outer and inner cell environments, due to the chemical affinity of its lipoid components and the lipoprotein cover of the cells. The cryoprotective effect of egg yolk on plasma membranes is complex, and is not caused by the action of one of its components. To determine and explain all possible mechanisms of egg yolk function as a cryoprotectant, further experiments are planned.

Acknowledgements: This research was funded by the Russian Science Foundation (project no. 14-50-00029).

Declaration: The authors report no financial or commercial conflicts of interest.

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