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OPTIMIZATION OF TECHNOLOGICAL PARAMETERS OF CRYOPRESERVATION OF BULL AND CARP SEMEN

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Abstract

Increasing the efficiency of reproduction of farm animals is possible through the use of progressive cryotechnology. Numerous studies have been devoted to this problem. However, a significant number of cells do not restore their functional activity after deconservation, which hinders a fuller use of the genetic potential of high-value breeding animals. Therefore, the purpose of the research was to determine the optimal technological parameters of cryopreservation. To achieve the intended goal, experiments were carried out to determine: the optimal rate of cooled semen up to 4°C, the temperature of the fluoroplastic plate, the duration of cooling of bull sperm after diluting it with mediums containing various cryoprotective agents. It has been established that when developing new mediums, it is necessary to use the optimal modes of cryopreservation of sperm of different types of farm animals. The results presented in this paper allow us to understand that cryotechnologies should be developed taking into account the features of the frozen object and the physicochemical properties of the components of protective mediums.

Key words*: cryoprotective mediums, cryoprotectants, cryotechnologies, gametes.*

INTRODUCTION

The cryobanks of reproductive cells and tissues benefits agriculture, livestock programs and biomedical research (Gunasena et al., 1997). Cryopreservation may even exclude the need to maintain a large number of breeding animals, since the genetic material is still available for future use. The cryobank of embryos, sperm, and oocytes is becoming very important both for reducing maintenance costs and for improving the dissemination of highly efficient farm animals (Agca et al., 2005; Oh et al., 1998).

The development of effective cryoprotective medium is one of the essential aspects of a successful solution to the issue of storing animal semen in a frozen state (Курбатов, 1988; Милованов, 1962; Наук, 1991; Шапиев, 1998; Balan, 2013; Watson, 1995). However, an equally important condition is the development of the optimal parameters of cooling, freezing and thawing, at which the protective effect of the components of the medium is maximally manifested (Борончук et al., 2003; Наук, 1991; Осташко, 1978; Шапиев, 1998; Balan, 2013; Roşca, 2000).

The procedure of sperm preservation differs among different species due to their inherent characteristics. There are noticeable species differences in the size and morphology of spermatozoa. In addition, there are more subtle differences in the composition of membrane phospholipids and sperm metabolism (Barbas et al., 2009). Despite the species differences, there are common stages in any sperm freezing procedure. All technologies include sperm collection and distribution, the addition of cryoprotective agents and cooling above 0°C, freezing below 0°C, storage and thawing (Curry et al., 1994). At all these stages, spermatozoa are exposed to a number of potentially destructive stresses, such as changes in temperature, osmotic and toxic stresses caused by exposure to high molar concentrations of cryoprotectants, as well as the formation and dissolution of ice crystals in the extracellular space (Watson, 2000). The success of cryopreservation depends on the resistance of spermatozoa to these influences (Salamon et al., 2000; Watson, 2000). Mediums, cryoprotectants, optimal cooling and thawing rates are essential for successful cryopreservation of sperm (Curry, 2007;

Hammerstedt et al., 1990; Mazur, 1984; Purdy, 2006). The composition of medium and the cooling rate have a significant effect on sperm viability, and there is a strong interaction between medium and the cooling rate (Woelders et al., 1997). If the cooling rate is lower or higher than optimal, it can cause irreversible damage of the spermatozoa (Fiser, et al., 1990; Koshimoto et al., 2000; Mazur, 1970). The optimal cooling rate should be low enough to allow water to leave the cells to avoid the formation of intracellular ice, and fast enough to avoid severe cell dehydration and cryo-damage (Mazur, 1970).

It is generally accepted that at least 50% of spermatozoa die during the freezing and thawing procedure (Watson, 2000), known as cold shock. Thus it may be necessary to eight times more cryopreserved sperm compared to fresh sperm to achieve *in vivo* fertilization (Shannon et al., 1995).

Since in previous studies (Борончук et al., 2003) a number of substances were identified that quite effectively stabilize the studied indicators of the functional-biochemical status of gametes in the process of conservation, naturally, the question arose about working out the optimal technological parameters for each of them.

MATERIALS AND METHODS

Special experimental studies were carried out at private and state breeding enterprises in compliance with the proper zoo-veterinary requirements. In the experiments used the semen of bulls of Black and White breed, as well as carp. Freezing of the experimental material was carried out in the form of open granules with a volume of 0.1-0.2 ml, in liquid nitrogen vapor. The temperature was measured using a thermocouple and mercury thermometers.

Statistical processing of digital material was performed using the Student's t-test.

RESULTS AND DISCUSSIONS

Essential in the cooling mode is the cooling rate in the critical temperature range, defined as the range in which the formation of ice crystals and subsequent dehydration of cells occurs. The cooling rate determines whether the cells remain in equilibrium with their extracellular environment or become increasingly super cooled with an increase in the possibility of intracellular ice formation (Kumar et al., 2003). The process of cell dehydration that accompanies slow freezing is potentially beneficial for cell survival, whereas fast freezing rates are thought to be more likely to cause cell death (Watson, 2000).

Freezing cells in medium induces ice formation in the extracellular space, creating an osmotic gradient across the plasma membrane between the initially isotonic intracellular solution and the frozen extracellular solution. Depending on whether the cooling rate is high or low, the intracellular water will either move through the cell membrane and join the extracellular ice phase, or freeze and form ice inside the cell, respectively. In most cases, cells undergoing the formation of intracellular ice become osmotically inactive or lysed due to the loss of the integrity of the cell membrane (Mazur, 1984). Likewise, cells that experience severe loss of intracellular water also become osmotically inactive due to prolonged exposure to high concentrations of solutes (Lovelock, 1953). Thus, too high or too low a cooling rate can be fatal to the cells. The optimal cooling rate exists between high and low speeds, which has been confirmed experimentally for a variety of celules. The cell survival curve, constructed as a function of the cooling rate, has a characteristic inverted U - shape (Mazur, 1972). Too low or too high a given cooling rate depends on the permeability of the cell membrane to water and on the likelihood that any water remaining in the cell at any given negative temperature will originate and turn into ice.

In the first series of experiments, the effect of the bull's semen cooling rate on the motility and longevity of thawed gametes was investigated. The cooling of the semen was carried out in the refrigerator at a rate of 0.52; 0.25 and 0.16°C/min to 4°C. As cryoprotectants were used: acrylamide, succinamide, glycerin, a mixture of acrylamide and polyacrylamide, a mixture of dextran, glycerol and globulin. The results are presented in Table 1.

Table 1. Influence of the rate of decrease in the temperature of bull semen from 30°C to 4°C on its quality when using various cryoprotectants

Cryoprotectants	Experimental variants	Gamete motility (points) after:			Longevity of gametes at 37°C (hour)
		Dilution	Cooling	Thawing	
		M ± m	M ± m	M ± m	M ± m
0,52°C/min					
Acrylamide	1	8.3 ± 0.13	7.7 ± 0.22	3.7 ± 0.37	4.0 ± 0.01
Succinamide	2	8.1 ± 0.21	7.6 ± 0.40	3.4 ± 0.21	4.0 ± 0.01
Acrylamide + polyacrylamide	3	8.1 ± 0.21	7.5 ± 0.35	3.3 ± 0.22	4.0 ± 0.01
Dextran + glycerol + globulin	4	8.2 ± 0.13	7.8 ± 0.13	4.0 ± 0.17	4.0 ± 0.17
Glycerol	5	8.1 ± 0.11	7.6 ± 0.07	3.8 ± 0.13	4.0 ± 0.01
0.25°C/min					
Acrylamide	1	8.3 ± 0.13	7.7 ± 0.40	4.0 ± 0.17	4.2 ± 0.22
Succinamide	2	8.1 ± 0.21	7.6 ± 0.40	3.4 ± 0.27	4.0 ± 0.01
Acrylamide + polyacrylamide	3	8.1 ± 0.21	7.3 ± 0.13	3.3 ± 0.13	4.0 ± 0.01
Dextran + glycerol + globulin	4	8.2 ± 0.13	7.6 ± 0.21	4.3 ± 0.13	5.4 ± 0.27*
Glycerol	5	8.1 ± 0.11	7.7 ± 0.13	4.2 ± 0.13	4.0 ± 0.01
0.16°C/min					
Acrylamide	1	8.3 ± 0.13	7.6 ± 0.2	3.7 ± 0.33	4.2 ± 0.22
Succinamide	2	8.1 ± 0.21	7.5 ± 0.17	3.2 ± 0.33	4.0 ± 0.01
Acrylamide + polyacrylamide	3	8.1 ± 0.21	7.6 ± 0.32	2.8 ± 0.22	4.0 ± 0.01
Dextran + glycerol + globulin	4	8.2 ± 0.12	7.7 ± 0.13	4.3 ± 0.13	5.2 ± 0.22*
Glycerol	5	8.1 ± 0.11	7.6 ± 0.11	4.2 ± 0.13	4.0 ± 0.01
Note: * The differences are statistically significant compared to the first cooling variant.					

It follows from the table that the change in the cooling rate within the limits of the speeds used by us does not significantly affect the motility and longevity of deconserved gametes (the exception is the 4th variant of the experiment).
A similar experiment was carried out on carp semen (Table 2). The cooling conditions are presented in the table.

Table 2. Influence of the cooling rate on the quality of deconserved carp semen

Experimental conditions	Temperature decrease rate, °C/min	Motility of thawed gametes, points
Cooling was carried out using pond water	0.56	4.7 ± 0.21
Cooling was carried out without using water	0.89	3.7 ± 0.18
Cooling was carried out using pond water and ice	1.07	4.2 ± 0.21

As can be seen from the table, cooling the carp semen at a rate of 0.56°C/min allows achieving the highest motility of thawed gametes. An increase of the cooling rate reduces its quality, which can be explained by the conditions of the experiment, under which the manifestation of a cold shock is possible (Ostachko et al., 2004).
Further studies were aimed at studying the effect of the surface temperature of the fluoroplast plate when freezing the sperm of breeding bulls in mediums containing various cryoprotectants on the motility and longevity of gametes after thawing (Figure 1). The data presented in figure 1 show that in the case of dilution of the semen with a medium containing acrylamide as a cryoprotectant, the protective properties of this substance are most fully manifested at a temperature of minus 100-120°C, while when using glycerin, the best results were obtained at a temperature of minus 120°C.
The next important issue that we have studied is the determination of the optimal duration of semen cooling at 4°C in the case of using various cryoprotectants (Figure 2). The physiological parameters of thawed semen were studied by cooling it for 0-7 hours with an interval of one hour. The figure shows the

optimal time for semen cooling in the case of using various cryoprotectants.

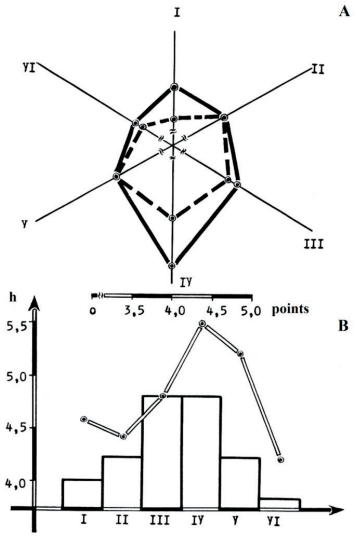

Figure 1. Influence of the surface temperature of the fluoroplastic plate on the quality of the bull semen:

A. Motility of gametes after frozen-thawing in a medium with: —— glycerin; - - - - acrylamide;
B. Longevity of gametes at 37°C, frozen in a medium with ═ glycerin, acrylamide;

I - 60°C; II - 80°C; III - 100°C; IV - 120°C; V - 140°C; VI - 160°C

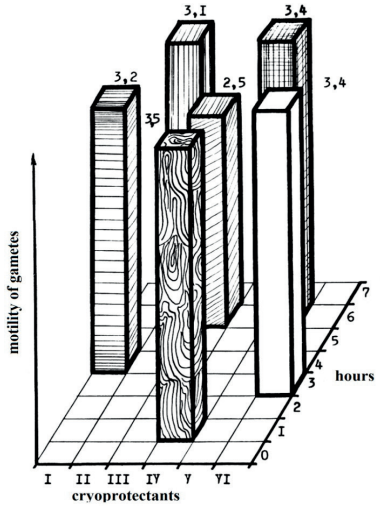

Figure 2. Duration of cooling of bull semen at 2-4ºC in case of using substances tested as cryoprotectants:

I - glycerin; II - polyacrylamide; III - polyethylene glycol 300; IV - acrylamide; V - acrylamide + polyethylene glycol 300; VI - acrylamide + polyacrylamide

The conducted studies have shown that the motility of thawed semen reaches its maximum value after 1 hour of cooling when using acrylic acid amide in the medium. In the case of using polyacrylamide and polyethylene glycol, the motility of gametes after thawing is higher when they are cooled for 7 and 6 hours, respectively. When using a cryoprotective mixture based on acrylamide and polyacrylamide, the best motility of the thawed semen was achieved when cooling lasted 3 hours, and when a cryoprotective mixture based on acrylamide and polyethylene glycol was used, the protective effect was most pronounced after 6 hours of cooling. The use of glycerin requires 3-4 hours of cooling.

In subsequent experiments was determined the optimal duration of exposure of bull and carp semen at 4°C, after reaching its specified temperature, in the case of using new cryoprotectants (Table 3).

Table 3. Optimal duration of exposure of bull and carp semen at 4°C in the case of freezing it with the use of various cryoprotectants

Cryoprotectant name	Exposure duration, min	Thawed semen indicators	
		Gamete motility, points	Longevity, hour
		M ± m	M ± m
Bull semen			
Acrylamide	0	4.1 ± 0.2	6.4 ± 0.76
Glycerol	60	4.5 ± 0.17	6.0 ± 0.61
Succinamide	40	3.8 ± 0.22	5.8 ± 0.65
Acrylamide + polyacrylamide	60	4.1 ± 0.21	6.2 ± 0.50
Dextran + glycerol + gamma globulin	25	4.7 ± 0.13	7.4 ± 0.80
Carp semen			
1,3-Butylene glycol	5	4.7 ± 0.21	-

It follows from the table that the optimal exposure for different cryoprotectants is not the same. Thus, the use of acrylamide as a cryoprotective agent in the composition of the medium for diluting and freezing bull semen makes it possible to exclude the period of its exposure at 4°C, while the use of glycerol, succinamide, mixtures of acrylamide with polyacrylamide and dextran, glycerol with globulin as a cryoprotectant requires holding the semen after cooling it to 4°C for 60, 40, 60

and 25 minutes, respectively. And when cryopreservation of carp semen under the protection of 1,3-butylene glycol, the exposure is only 5 minutes.

Thus, were determined the optimal rate of temperature reduction, semen exposure at 4°C, as well as the optimal duration of sperm cooling for the most promising cryoprotectants, as well as the optimal temperature of the fluoroplastic plate when freezing semen in the form of granules.

Summarizing the results of the studies presented in this article and previously published works (Борончук et al., 2003; Balan, 2013; Roşca, 2000), it can be concluded that the stabilization of the structural and functional parameters of gametes during cryopreservation of animal semen can be ensured by: maintaining the stability of bonds that determine intermolecular interactions by using in the composition of synthetic mediums of new cryoprotectants of both endo- and exocellular action; polar compounds capable of forming complexes of membrane components with elements of cryoprotective medium and membranotropes, maintaining the ionic potential and modifying biomembranes, as well as using specific parameters of technological methods of genome cryopreservation for each medium component and animal species.

CONCLUSIONS

Cryotechnologies should be developed taking into account the characteristics of the frozen object and the physicochemical properties of the components of protective mediums.

Cooling of frozen cells at 4°C is carried out for the purpose of penetration of protective agents into them, balancing the osmotic pressure in the cell-medium system, regulating biochemical processes aimed at preserving the functional state and implementing adaptive reactions.

The duration of exposure of the semen at 4°C depends on the physicochemical properties of the cryoprotectants used.

There is a corellative relationship between the duration of cooling of the semen at 4°C and the molecular weight of the tested substances.

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