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RESEARCH PAPER

Effective Technology of Carp (*Cyprinus carpio*) Sperm Cryopreservation for Use in a Large-Scale Cryobank

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Abstract

Preliminarily developed technology of cyprinids sperm cryopreservation was improved by optimization of protective medium composition. Effectiveness of this medium was confirmed by examination of trial media as well as by freezing sperm samples for storage in cryobank. Protective medium for cyprinid fishes sperm is aqueous solution consisting of 0.1% sucrose, 0.35% sodium chloride, 20-24% methanol and 3-6% ethylene glycol. When used, this medium stably ensured cryoprotection of sperm cells of different quality and conservation of its fertility at the level of native sperm. Results of evaluation of quality of cryopreserved cyprinid sperm samples before putting them in a low-temperature gene bank of VNIIPRKh are presented.

Introduction

Cryopreservation of fish sperm as a universally recognized tool for conservation and reproduction of genome from endangered and extinct species, and also species of aquaculture, allows to solve various selection and different scientific problems. Specifically, preserved in cryobanks Cyprinidae genome collections may be used for genetic certification, conservation of breed standard, supporting genetic variety of aquaculture fish by means of introduction wild fish genes, as well as obtaining of progeny with no mature males, exchange of frozen sperm between fish farms etc.

Methods of fish sperm cryopreservation have been developing for more than half century and are for many species known. They usually include dilution of the sperm with solutions of specific protectors, freezing of obtained suspension in a certain regime, storage in liquid nitrogen, thawing in optimal conditions, and estimation of results by motility of frozen-thawed sperm and its fertilization of eggs. Cryopreservation success, i.e. preservation of viability and fertility of frozen cells, depends on many factors: native sperm quality, choice of optimal protective medium composition for specific fish species, sperm dilution ratio, freezing and thawing regimen, method of thawed sperm activation and other technical details. Therefore, the need for improvement of technology always remains actual.

To achieve successful results by any method of fish sperm cryopreservation, choice of suitable protective medium is extremely important. This medium must be non-toxic for the sperm, a little hypertonic due to exact concentrations of dissolved substances and having certain ionic composition. Among the components of the medium, permeable (intracellular) and unpermeable (extracellular) cryoprotectants play the main part in the protection of cells. During slow decrease of temperature, the effect of intracellular cryoprotectants (low-molecular carbohydrates, alcohols, dimethyl-sulfoxide, aminoacids) is explained

by the prevention of excessive cell dehydration (osmotic shock) and denaturation of proteins thanks to reaction to them. During rapid decrease of temperature this is explained by the prevention of intracellular crystallization because of freezing-point reduction. Extracellular cryoprotectants (high-molecular carbohydrates, polyols, certain aminoacids, lecitins), interacting with membrane phospholipids, promote membrane stabilization and overcoming temperature shock by cells.

Since the middle of last century research of cyprinids sperm cryopreservation methods has been conducted in several countries of Europe and Asia. Up to the end of the twentieth century cryopreservation attempts allowing to retain fertility with 50-70% frozen-thawed spermatozoa were considered successful. Most of the times various aqueous solutions of mineral salts and sugars (sometimes with adding other substances) as extenders and ethylene glycol (Kopeika et al., 1986; Tsvetkova, Karanova, 1994) and dimethyl-sulfoxide (Chen et al., 1992; Kurokura, et al., 1984; Linhart et al., 2000; Withler, 1982) as permeable cryoprotectant were used. Freezing techniques and correspondingly cooling rates in described methods differed, various containers (pellets, straws, cryotubes) were used. Thawing of containers with cryopreserved sperm was carried out in a water bath of 25-40ºC. Activation of thawed sperm before the insemination of eggs was accepted.

Ukrainian scientists from Institute for Problem of Cryobiology and Cryomedicine were the first to achieve the most stable great results of carp eggs fertilization with cryopreserved sperm (50% fertilized cells) (Kopeika et al., 1983). Further improvement of proposed method by this research group led to an elaboration of a rather effective industrial technology of carp sperm cryopreservation that involved the use of compound cryomedium on the basis of Tris-HCl with adding ethylene glycol and egg yolk, and the three-stage regime of freezing of sperm in cryotubes in the vapour of the liquid nitrogen (Kopeika et al., 1986). Fertilization of large eggs batches with cryopreserved by this method sperm was 40-60%, for individual batches – more 70%. This method was used when forming collections of cryopreserved carp sperm in low-temperature gene banks of Institute for Problem of Cryobiology and Cryomedicine and All-Russian Research Institute of Freshwater Fisheries (VNIIPRKh) (Kopeika et al., 1989; Tsvetkova et al., 1990; Tsvetkova et al., 1996). But further research conducted in 2001-2003 in the Laboratory of Cryobiology of VNIIPRKh allowed to create an original technology of cyprinids sperm cryopreservation (Tsvetkova et al. 2004), which has been used for addition to the collection of cryobank.

In published works concerning cyprinids sperm cryopreservation methods in the last 20 years there have been given descriptions of a large number of various protocols, among which choosing the most effective protocol is extremely difficult. Modified Kurokura's extender has often been used as the diluent of sperm (aqueous solutions of NaCl, KCl, $CaCl_2$, $MgCl_2$, $NaHCO_3$, the composition of which is differently interpreted by authors) (Horvath et al., 2002; Magyary et al., 1996; Linhart et al., 1999; Linhart et al., 2000; Horvath et al., 2003; Magyary et al., 2011; Ogretmen & Inanan, 2014; Ogretmen et al., 2014; Ogretmen et al., 2015; Yavas et al., 2014). Also, another physiological salts solutions have been tested, such as aqueous (Akcay et al., 2004; Babiak et al., 1997; Bernath et al., 2016; Horokhovatskyi et al., 2016; Irawan et al., 2010; Magyary et al., 2011; Sultana et al., 2010; Tsai et al., 2010), tris-buffer (Akcay et al., 2004; Bernath et al., 2016; Bozkurt & Yavas, 2017; Bozkurt et al., 2014; Bozkurt et al., 2016; Horokhovatskyi et al., 2016; Horvath et al., 2003; Horvath & Urbanyi, 2002; Lahnsteiner et al., 2000; Lahnsteiner et al., 2003), sometimes supplemented sugars (Akcay et al., 2004; Bernath et al., 2016; Bozkurt et al., 2016; Horvath et al., 2003; Horvath et al., 2002; Irawan et al., 2010; Tsai et al., 2010), also simply aqueous or tris-buffer sugars solutions (Bozkurt et al., 2014; Bozkurt et al., 2016; Horvath et al., 2003; Horvath et al., 2007; Horvath & Urbanyi, 2002; Ogretmen & Inanan, 2014; Yildiz et al., 2013). A comparison of various diluents and permeable cryoprotectants added to them has been conducted by many researchers.

As permeable cryoprotectants, the most often used were dimethyl-sulfoxide in v/v concentration 5-15% (Alvarez et al., 2003; Bozkurt et al., 2014; Bozkurt et al., 2016; Horokhovatskyi et al., 2016; Irawan et al., 2010; Lahnsteiner et al., 2000; Lahnsteiner et al., 2003; Linhart & Rodina, 1999; Linhart et al., 2000; Magyary et al., 1996; Ogretmen & Inanan, 2014; Ogretmen et al., 2014; Ogretmen et al., 2015; Tsai et al., 2010; Yavas et al., 2014; Yildi et al., 2015), methanol in v/v concentration 10% (Bernath et al., 2016; Bozkurt & Yavas, 2017; Horvath et al., 2003; Horvath & Urbanyi, 2002; Horvat et al., 2007; Sultana et al., 2010) and dimethyl-acetamide in v/v concentration 5-20% (Akcay et al., 2004; Babiak et al., 1997; Bozkurt et al., 2016; Warnecke & Pluta, 2003; Yavas et al., 2014). Sometimes glycerol in v/v concentration 5-15% was added to diluents (Akcay et al., 2004; Bozkurt et al., 2014; Bozkurt et al., 2016; Tsai et al., 2010). Some researchers still use Kopeika's medium (Kopeika, 1986) with ethylene glycol in concentration 16% (Boryshpolets et al., 2009; Boryshpolets et al., 2017; Dietrich et al., 2017; Dzyuba et al., 2013; Li et al., 2010) or its modifications (Bekh, 2004; Gorbunov et al., 2010; Matishov et al., 2012; Cherepnin, 2016; Horokhovatskyi et al., 2016).

In many cryomedia, hen egg yolk in concentration 5-20% was used as additional unpermeable protectant that stabilizes cell membrane (Babiak et al., 1997; Betsy & Kumar, 2015; Boryshpolets et al., 2017; Bozkurt et al., 2014; Ogretmen & Inanan, 2014; Ogretmen et al., 2014; Ogretmen et al., 2015; Yavas et al., 2014). Its influence was considered the best, in comparison with turkey and quail egg yolks, which differ in cholesterol, fatty acid and phospholipid contents (Bozkurt et al., 2014). Although,

in the same researchers' opinion, soybean lecithin in concentration 10% is more preferable than yolk (Yildiz et al., 2013).

Positive influence of other additions to protective media, namely: glycin (Lahnsteiner et al., 2000), vitamin B_{12} (Matishov et al., 2012), honey (Ogretmen & Inanan, 2014); propolis (Ogretmen et al., 2014), cysteine (Ogretmen et al., 2015), taurine (Kutluyer et al., 2016), cholesterol-loaded cyclodextrin (Yildiz et al., 2015), antifreeze glycoproteins (Tsvetkova & Karanova, 1994; Tsvetkova et al., 2009; Cherepnin, 2015) was revealed.

Sperm dilution ratio varies significantly by different authors: from 1:1 to 1:9. Recommended equilibration time for sperm-medium suspension also varies: from 0 to 60 minutes.

Most protocols are united by use of 0.25 or 0.5 ml straws for freezing of diluted sperm. It is reported too about successful attempts of use of large straws: 1.2 ml (Lahnsteiner et al., 2003), 1.5 ml (Bozkurt & Yavas, 2017), 1.8 ml (Alvarez et al., 2003), 2 ml (Linhart & Rodina, 1999; Linhart et al., 2000), 1.2 ml and 5 ml (Horvath et al., 2007). In most technologies, freezing of straws is realized by their horizontal station on a styrofoam rack that floats on the surface of liquid nitrogen in a styrofoam box. The straws are frozen in liquid nitrogen vapour 3-4 cm above the surface of liquid nitrogen for 3-20 min with subsequent plunge into the liquid nitrogen. Some authors give description of various three-stage regimes of freezing, including the use of programmable freezers (Irawan et al., 2010; Linhart & Rodina, 1999; Linhart et al., 2000; Magyary et al., 1996; Sultana et al., 2010; Tsai et al., 2010).

It is usually recommended that thawing procedure should be carried out in a water bath at 30-40ºC during 10-30 s. The best activation media for frozen-thawed sperm of cyprinids were considered the solution composed of the following: 45 mM NaCl, 5 mM KCl, 30 mM Tris-HCl pH 8-8.2 and 0.35% NaCl solution.

Admired by many authors, the idea of standardization of carp sperm cryopreservation protocols based on widely-spreading common technique of sperm freezing in straws seems still far from practical realization. In considered works the highest results on eggs fertilization of cryopreserved sperm: 84% (Horvath & Urbanyi, 2002), 85% (Yildiz et al., 2013), 90% (Tsai et al., 2010), 94% (Ogretmen & Inanan, 2014; Ogretmen et al., 2015), 99% (Bozkurt et al., 2016; Yavas et al., 2014), 100% (Magyary et al., 2011) were achieved by use various extenders and cryoprotectants. Conducted in a number of works (Akcay et al., 2004; Bernath et al., 2016; Bozkurt et al., 2016; Horvath et al., 2003; Horvath et al., 2007; Horvath & Urbanyi, 2002; Irawan et al., 2010; Magyary et al., 2011; Ogretmen & Inanan, 2014; Warnecke & Pluta, 2003) comparison of cryopreservation results in different protective media within the same protocol on the same sperm have come to different conclusions too. Besides, the reproducibility of cryopreservation results, which are mainly determined on native sperm quality, still remains low.

Our research originally was concerned with freezing of large volumes of sperm in cryotubes. The comparison of effect of described protective media within proposed method conducted earlier in the laboratory of cryobiology of VNIIPRKh showed undoubtable advantage of the developed cryoprotective medium as on effectiveness as on ease of preparation. Optimization of two important factors determining effectiveness of cryoprotection: composition of salts and cryoprotectants of different effect allowed to give up traditional complex mixtures.

Nevertheless, the need for improvement of cryopreservation technology and correction of cryomedia composition to increase its protective quality remains actual on account of changeability of native sperm quality. Cryoresistance of fish sperm is conditioned not only by genetic particularity but to a considerable extent depends on the influence of the environment on spermatogenesis and on conditions of spawners maintenance.

Cryoprotective effect of medium is usually estimated by two indicators: percentage of post-thaw motility and fertilization rates. Since correlation between these indicators is not always observed (Pronina, 2004; Akcay et al., 2004), the final conclusion about the suitability of specific medium may be drawn only on stable reiteration of fertilization results in the series of experiments with the sperm and eggs samples of different quality.

The proposed technology of carp sperm cryopreservation has been developed in the course of a large number of experiments (unpublished data) and is intended for use in a large-scale cryobank. The primary purpose of the present study was to improve this technology by optimization of composition of cryomedium that can provide protection of cells of different quality sperm.

Materials and Methods

Gamete Collection and Evaluation of Sperm Quality

Adult carp individuals of different breeds (18 males and 5 females, age 6-7 years, average length 58 cm and body weight 5,5 kg in both sexes) were obtained from Experimental selection-pedigree fish farm Jakhot, Branch for Freshwater Fisheries of Russian Federal Research Institute of Fisheries and Oceanography ("VNIIPRKh"), Rybnoye, Russia in the middle of the spawning period in 2017-2018. Gamete were collected by manual abdominal stripping (males: 12 h after an one-time injection of carp pituitary extract (cPE, "Aquasystem", Kaliningrad, Russia) in a dose of 2 mg body weight kg $^{-1}$; females: 12 h after a double injection of cPE, the first injection in a dose of 0.5 mg kg $^{-1}$, and 24 h after – the second injection in a dose of 4.5 mg kg $^{-1}$) at 22ºC water temperature. Fish abdomens and urogenital papillas were dried before stripping. Semen was collected into 50 ml calibrated test tubes. Samples

contaminated with feces or urine were discarded. Transportation of gamete was carried out at 1-5ºC in a thermo-bag. Only well-rounded and transparent eggs were used for fertilization. Eggs samples were collected from one fish.

Sperm motility of each male was determined individually and after combining into a common pool. Sperm motility was estimated using light microscope (Micromed 3 LED M, Micromed, Russia) at 400× magnification and expressed as the percentage of cells with progressive motility. Samples were activated by mixing on a glass slide 1 µl of sperm with 20 µl hatchery water at 18-20°C (Since native sperm is well activated by ordinary hatchery water, additional stimulation of cell movement is not required). For cryopreservation experiments sperm samples with 90-100% motility were used. For the storage in cryobank sperm samples with 100% motility were sorted (sperm of lower quality was cryopreserved in exceptional cases).

Cryopreservation Procedure

Semen and each experimental protective media were kept at 10-12ºC prior to dilution. Sperm was diluted at ratio of 1:1 (v/v) (sperm/protective media) by slow adding of media, a drop at a time, to sperm by continuous stirring. The diluted samples were poured during 5-10 min into 1.5 ml cryotubes that immediately (without equilibration) were installed on the disk of freezer provided with temperature sensor and a connected recorder which indicates temperature of suspension in cryotube. Freezing was conducted by following program: from 10ºC to -15ºC at a rate 1-2ºC min^{-1}, from -15ºC to -70ºC at a rate increasing to 15-20ºC min^{-1} and then plunged into the liquid nitrogen.

Thawing of Semen and Fertilization

The frozen cryotubes were thawed in a water bath at 38-40ºC for 1 min. The motility of thawed sperm was tested using microscope after activation in 0.1% $NaHCO_3$ solution (This solution was chosen based on the results of our preliminary research and has shown its effectiveness over many years of practice). The thawed semen was used immediately for fertilization.

For fertilization eggs from one female were used. Egg batches about 100-150 eggs (three replicates per treatment) were inseminated in dry Petri dishes with frozen or fresh (control) sperm. Thawed sperm diluted with activation solution at ratio of 1:100 (v/v) (one 1.5 ml cryotube contained 0.75ml sperm was diluted 75 ml activation solution) was added over the eggs and gently mixed. Fresh (control) sperm were activated by mixing with hatchery water at the same ratio. In one minute the eggs were rinsed with hatchery water and left for incubation at 18-22ºC on the table or in the thermostat. The fertilization rates were counted in the gastrula stage. In the industrial trials incubation of the eggs was carried out in a vertical glass incubator with running water.

Statistical Analysis

Data of parameters observed for sperm fertility were presented as mean percentages. Overall differences between means were considered significant when $p<0.05$. Regression analysis to assess the strength and type of relationship between composition of protective medium and sperm fertility was carried out using the MS Excel statistical data analysis.

Results

Preliminarily developed by us technology of cyprinids sperm cryopreservation (Tsvetkova et al., 2004) has been used at 2003-2016 for setting of sperm samples in cryobank of VNIIPRKh. In that technology the basic cryoprotective carp medium (later indicated medium N1) was used. Sperm samples set for storage in 1989-2000 were frozen in Kopeika's medium (Kopeika, 1986). In 2017-2019 for the adding of the collection of cryobank was used improved protective medium (Dokina et al., 2019). The total volume of cryopreserved sperm of Cyprinid fishes in low-temperature gene bank of VNIIPRKh is 10460 ml from 1029 males of 43 species, breeds and breed groups. The monitoring of preserved sperm quality shows that fertility of sperm samples frozen in medium N1 from 2001 in average 2-2.5 times as high as fertility of sperm samples frozen before.

The results of the experiments on optimization of protective media composition carried out to improve technology of cyprinids sperm cryopreservation are summarized in Table 1.

Our preliminary research in 2001-2003 during elaboration of basic carp medium showed that there was no need to use tris-buffer with different value of pH, and as a result of comparison of influence on spermatozoa cryoprotection of compound salt solutions, the important role and optimal concentration of sodium chloride in this medium was revealed.

Experiment N1 compared the influence of different concentrations of permeable cryoprotectants: methanol (MeOH), ethylene glycol (EG) and propylene glycol (PG) by themselves and in their different combinations. High protective effect once again was confirmed for two cryoprotectants combination applied in basic carp medium: methanol and ethylene glycol, even by reduced concentrations, which was displayed in fertilization of more than 90% of eggs with sperm frozen in corresponding experimental media N 1, 5 and 7. Besides, it was found that reduction of methanol concentration is permissible only to 20%. Methanol in itself has shown twice lesser effectiveness than its combination with ethylene glycol (the comparison of media N 1, 3 and 7), but ethylene glycol and propylene glycol by themselves have not ensured cryoprotection of sperm cells at all. Protective effect of propylene glycol and methanol combination was much lower than that of ethylene glycol and methanol combination in the same concentrations.

Table 1. The effect of protective medium compounds on the results of carp sperm cryopreservation

N of trial, breed	N of medium	Concentration of cryoprotectants in basic diluent, % (w/w) (basic diluent is aqueous solution of 0.1% sucrose, 0.35% NaCl)				Post-thaw motility, %*	Fertilization rate, %
		MeOH	EG	PG	DMSO		
1 Carp Zagorsky (pooled sperm of 4 males, motility 100%)	1	24	18			10-20	91.3±4.3
	2	15	10			10-15	70.7±7.9
	3	24				10	42.1±0.8
	4		18			0	0
	5	20	10			20-30	90.2±1.6
	6	18	18			20-30	82.1±2.8
	7	24	10			30-50	92.0±4.5
	8	24		18		5-10	19.2±3.6
	9	15		10		5-10	60.5±2.1
	10			18		1	0
	11	20		10		10	52.4±17.4
	12	18		18		5	7.5±1.8
	13	24		10		5-10	64.7±10.1
	control						95.8±1.2
2 Carp ZU-NK (pooled sperm of 5 males, motility 60%)	1	24	18			10-15	76.1±8.2
	7	24	10			30-35	89.5±2.6
	19	24	6			20-30	89.8±3.9
	5	20	10			15-25	88.9±1.1
	20	20	6			20	85.4±2.7
	control						97.6±1.4
3 Carp Moscow (pooled sperm of 4 males, motility 90%)	1	24	18			20-30	94.6±1.8
	7	24	10			30-40	93.4±4.8
	19	24	6			30-40	95.4±2.3
	31	24	3			20	94.4±1.2
	control						98.0±0.9
4 Carp ZU-NK (pooled sperm of 3 males, motility 90%)	1	24	18			15-20	89.8±2.6
	7	24	10			10-15	92.6±4.6
	19	24	6			20	95.1±3.3
	31	24	3			10-15	94.0±4.2
	39	24	1			5	89.5±5.2
	37	20	18			5-10	86.4±7.3
	5	20	10			10-15	94.1±2.7
	20	20	6			10-15	93.9±1.0
	38	20	3			10	95.6±0.8
	40	20	1			5-10	93.0±2.9
	control						99.1±0.2
5 Carp Zagorsky (pooled sperm of 2 males, motility 100%)	1	24	18			10	67.6±0.9
	3	24				10	29.4±7.1
	7	24	10			10	95.5±1.7
	19	24	6			20	97.1±2.8
	31	24	3			25	96.0±2.8
	46	20				5	28.5±6.9
	5	20	10			15	89.8±2.3
	20	20	6			25	93.8±1.9
	38	20	3			30	92.0±4.9
	47	24			10	25	80.4±12.7
	48	24			3	5	83.0±5.8
	49	20			10	25	80.1±1.4
	50	20			3	10	81.7±8.3
	51				20	1	2.6±0.8
	control						96.2±0.9

* In some experimental variants, differences were observed among three replicates of motility determinations.

In experiment N2 different percentage ratio of methanol and ethylene glycol in the basic diluent of carp media was compared. It was found that reduction of methanol concentration up to 20% and ethylene glycol concentration up to 10% and 6% has not decreased cryoprotective action of medium but even has slightly increased it.

The second tests of media with reduced methanol and ethylene glycol content in experiments N3 and N4 have confirmed the need to decrease methanol concentration up to 20% and ethylene glycol concentration up to 6% and even up to 3%.

Experiment N5 brought a closer definition of methanol and ethylene glycol concentrations in media with reduced content of these cryoprotectants and combined action of methanol and dimethyl-sulfoxide (DMSO) in several concentrations in comparison with methanol and ethylene glycol combination was also examined. This experiment once again confirmed high effectiveness of protective media on elaborated by us base with concentrations of usually used protectants in range: 20-24% for methanol and 3-10% for ethylene glycol. Fertilization of eggs with sperm frozen in these media was at the level of native sperm fertilization in control. Among of them media N19 and N31 with methanol concentration of 24% and ethylene glycol concentration accordingly of 6% and of 3% had the highest cryoprotective action.

Methanol and dimethyl-sulfoxide combination in revealed optimal ranges of concentrations (media N47-N50) have ensured significant improvement of media cryoprotective properties in comparison with each of these protectants in itself (media N3, 46, 51). But combined action of methanol and ethylene glycol was much more effective, which is in agreement with published information (Li et al., 2010) about preferability of ethylene glycol (in comparison with DMSO) for carp sperm freezing because it decreases osmotic and oxidative stress caused by cryopreservation technique.

Regression analysis revealed statistical significant parabolic relationship between ethylene glycol concentration in medium and eggs fertilization percentage by methanol concentration of 24%. The character of the curve confirms the conclusion that the greatest development of fertilized eggs is provided by ethylene glycol concentration of 3-8%. Analytical maximum of development percentage was reached by ethylene glycol concentration of 6.2%.

The absence of toxicity of these cryoprotectants at the indicated concentrations is beyond doubt, since no signs of deviations from the physiological norm were found in the offspring obtained using cryopreserved sperm. However, the materials from this study were not intended for inclusion in this article.

Moreover, these experiments estimated the influence of several amides (formamide, dimethyl-formamide, acetamide, dimethyl-acetamide, carbamide) as main or additional cryoprotectants in carp medium on cells survival after cryopreservation.

In experiment N1, the addition of amides to the basic carp medium in concentrations of 0.7% led to decrease in its protective action: sperm frozen in media with amides ensured egg fertilization of 70-80% only in comparison with 91.3% for medium N1.

In experiment N2 test of amides in basic diluent in concentrations of 15% as main cryoprotectants turned out to be useless (all cells were dead) and test of them in concentrations of 10% in combination with 20% of methanol only in cases of dimethyl-formamide and dimethyl-acetamide showed a small protective effect (eggs fertilization accordingly 36.3 and 33.8%).

The addition of amides in concentrations of 3% to media N 19 and N 20 (in experiments N 3 and N 4 accordingly) did not promote the increase of their protective action. Dimethyl-formamide, dimethyl-acetamide and carbamide practically did not alter the protective action of media, the other amides noticeably changed it for the worse.

The additional tests of several effective media revealed at the different stages of work (N 5, 7, 19, 20, 31, 38) have also been carried out by cryopreservation of sperm samples for the adding to the collection of cryobank. The analysis of obtained data showed that preservation of the highest fertility of thawed sperm was achieved by use of media contained in basic diluent cryoprotectants in concentrations range: 20-24% for methanol and 3-6% for ethylene glycol (Table 2).

Discussion

Improvement of preliminarily developed technology of cyprinids sperm cryopreservation (Tsvetkova et al., 2001; Tsvetkova et al., 2004) was carried out by the optimisation of protective medium composition. In our opinion, it is the protective medium composition that determines principal cryopreservation success. Tests of another freezing regime and freezing technique with the use of developed protective medium proved to be practically equally successful. The absence of influence of medium composition on the freezing rate was shown by some authors too (Boryshpolets et al., 2017). Earlier, many researchers have observed that simple carbohydrate diluents and seminal plasma imitated complex mineral compositions were equally effective (Babiak et al., 1998). Our preliminary research showed that there was no need to use tris-buffer diluents and complex salt compositions, and also there was no need for separate dilution of sperm first with extender then with cryoprotectant, and for equilibration time. Moreover, in our experiments jelly-like agglutination of sperm after thawing was not observed, while it occurred when using of sugar and tris based extenders (Bernath et al., 2016; Horvath et al., 2003).

Among the components of the medium, cryoprotectants play the main part in the protection of cells. Many authors have reported having used methanol for cryoprotection of cyprinids sperm (Lubzens et al., 1993; Horvath et al., 2003; Horvath et al., 2007; Horvath & Urbanyi, 2002; Sultana et al., 2010;

Table 2. Evaluation of quality of cryopreserved cyprinid sperm samples before putting in cryobank

N of male	N of medium	Motility, %		Fertilization rate, %		
		native	frozen-thawed	native (control)	frozen-thawed	% of control
Carp Inversant F_2 (June 2017)						
2	7	90	30	91.5	92.5	101.1
3		70	30	96.8	78.3	80.9
4		70	30	94.4	84.4	89.4
5		90	30	93.9	85.1	90.6
6		70	20	97.7	88.3	90.4
Carp Butterfly (June 2017)						
1	7	90	25	94.3	77.1	81.8
2		90	20	96.2	79.2	82.3
3		90	25	91.3	84.9	93
4		90	20	94.5	82.6	87.4
1	1	90	25	94.3	74.4	78.9
2		90	25	96.2	73.5	76.4
Carp Zagorsky (June 2018)						
3	7	100	15	95.7	58.6	61.2
	19		15		90.6	94.7
	5		25		91.8	95.9
	20		10		92.5	96.7
4	19	100	10	99	79.0	79.8
	20		10		92.0	92.9
5	19	100	7	98.7	86.4	87.5
	7		5		46.7	47.3
Carp Volzhsky (June 2018)						
7	20	100	30	96.4	87.4	90.7
	38		25		88.4	91.7
10	31	100	5	100	84.7	84.7
0	19	100	8	97.3	87.7	90.1
	31		10		77.8	80.0
	20		30		95.3	97.9
	38		20		72.6	74.6
3	19	90	25	97.2	87.3	89.8
6	31	90	10	85.6	86.6	101.2
8	20	90	20	91.0	97.0	106.6
2	19	100	30	98.3	89.0	90.5
5	20	100	3	96.7	91.5	94.6
9	38	70	3	97.9	86.7	88.6
Carp Amursky (June 2018)						
8	20	-	-	83.1	84.6	101.8
0	19	-	-	88.5	63.8	72.1
Carp B/A (June 2018)						
2	20	80	15	96.1	94.6	98.4
3	31	70	20	97.7	86.8	88.8
5	31	70	10	95.9	86.0	89.7
Carp Stolin XVIII (May 2019)						
3	19	90	20	-	79.0	-
Carp Tri prim (May 2019)						
1	19	85	30	-	88.9	-
4	19	90	30	-	96.0	-
Carp Moscow scales (May 2019)						
4	20	-	-	87.3	82.6	94.6
3	20	-	-	92.6	84.5	91.3
5	20	-	-	88.0	89.1	101.3
7	19	-	-	94.1	82.4	87.6

Bernath et al., 2016; Bozkurt & Yavas, 2017). The success of high fertilization results was linked to the rapid penetration of small molecules of methanol through cell membranes (Bozkurt & Yavas, 2017). However, the particularities of its effect remain unclear (Horvath et al., 2003). Results of our experiments showed that significant increase of protective effect of the basic carp medium was ensured by optimal ratio of two most suitable cryoprotectants for carp sperm freezing of similar action: methanol and ethylene glycol, which may be related to the display of sinergy effect. Combined use of methanol and ethylene glycol probably promote extending of medium protective effect due to the different rate of molecule diffusion through membrane and the interaction with different cell structures.

Amides (formamide, dimethyl-formamide, acetamide, dimethyl-acetamide, carbamide), which were tested as main or additional cryoprotectants in the basic carp medium and its modifications, have not displayed positive influence on cell survival during cryopreservation, contrary to expectations based on reports of many authors (Akcay et al., 2004; Babiak et al., 1997; Bozkurt et al., 2016; Warnecke & Pluta, 2003; Yavas et al., 2014).

Improved protective medium for cyprinid fishes sperm, which ensures cell cryoresistance of different quality sperm, is aqueous solution consisting of 0.1% sucrose, 0.35% sodium chloride, 20-24% methanol and 3-6% ethylene glycol. Effectiveness of this composition was confirmed by examination of more than 50 experimental media as well as by freezing sperm samples for storage in cryobank. Use of this medium ensured the preservation of thawed cells fertility at the level of native sperm. More precise calculation of concentrations of medium components allowed to obtain a light hypertonic solution ensuring gentle cell dehydration, which rules out the possibility of osmotic shock and decreases the risk of intracellular crystallization.

Further improvement of developed medium is possible by addition to it of other substances such as amino acids, phospholipids, vitamins etc., which increase cell cryoresistance. Among the additions tested by us in the carp media, antifreeze glycoproteins had the most protective effect (Tsvetkova & Karanova, 1994; Tsvetkova et al., 2009).

Objective difficulties prevent the realization of the idea of standardization of carp sperm cryopreservation protocols. The large number of various protocols make it difficult to choose the most efficient method (Bernath et al., 2016; Martinez-Paramo et al., 2017), and the reproducibility of cryopreservation results remains low. Our practical experience shows that the tests of different protocols on high quality sperm often give similar results. The use of the most effective protocols on the lower quality sperm does not ensure stable reproduction of results. Significant variability of sperm quality observed in different seasons allows us to suggest that the principal influence on sperm cryoresistance is made by the conditions of breeders' maintenance. The evaluation of native sperm quality is the main problem when predicting its cryoresistance. The high motility of fresh sperm often does not guarantee the achievement of high fertility of cryopreserved sperm. Researchers' conclusions about the link between cryopreserved sperm motility and fertilization rate differ: some authors detect the precise correlation (Linhart et al., 2000), the others – its absence (Akcay et al., 2004; Pronina, 2004). The determination of the connection between biochemical parameters of sperm and its cryoresistance is laborious work, which is very time-consuming. The reproducibility of cryopreservation results strongly depends on rapid and accurate evaluation of eggs quality too.

In our opinion, cryopreservation success is ensuring stable acceptable rate of eggs fertilization with cryopreserved sperm by use of a simple, suitable for practical application, protocol. In aquaculture there is need for simple protocols (Babiak et al., 1998) and freezing of a large volume of sperm for industrial use. Our research was directed to achieve these aims. Suggested technology of cyprinids sperm cryopreservation is intended for use in a large-scale cryobank.

Ethical Statement

The study was approved by the Ethics Committee for the Protection of Animals in VNIIPRKh, Moscow region, Russia.

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Author Contribution

Conceptualization: OD, Data Curation: OD, KK, Formal Analysis: OD, NP, Investigation: OD, KK, Methodology: OD, NP, Project Administration: KK, Resources: KK, Supervision: KK, Visualization: OD, Writing-original draft: OD, Writing-review and editing: OD, KK.

Conflict of Interest

The authors does not have any conflict of interest.

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