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Aquaculture 224 (2003) 203–212
Aquaculture
www.elsevier.com/locate/aqua-online

Sperm cryopreservation of sex-reversed rainbow trout (*Oncorhynchus mykiss*): parameters that affect its ability for freezing

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Received 19 December 2002; received in revised form 4 March 2003; accepted 4 March 2003

Abstract

Sex control profits the aquaculture industry allowing the obtaining of "all female" rainbow trout populations. Female production is highly profitable since they become sexually mature 1 year later than males, reaching their marketable size before maturation. Sex-reversed rainbow trouts have similar external morphology to normal males but lack sperm ducts, meaning that the animals must be sacrificed to obtain the milt. The peculiarities of the sperm, obtained directly from the testicle, make necessary the development of a specific cryopreservation protocol. In this study, several factors that could affect the freezability of these spermatozoa have been studied: the season of sperm extraction, the method of sperm extraction, and the activation with motility stimulators have been considered. Our results showed that seasonality clearly affects the success of the cryopreservation process, which should always be carried out with sperm obtained in winter season, the natural breeding period. The development of a clean sperm extraction method improved significantly the fertility rates obtained with cryopreserved sperm. The addition of methylxanthines as motility stimulators usually increased motility and fertility rates, but they did not provide significant improvements.

Keywords: Sex-reversed; Trout; Sperm; Cryopreservation

1. Introduction

The production of sex-reversed females of rainbow trout (*Oncorhynchus mykiss*) is of great importance in aquaculture. Females of rainbow trout reach their sexual maturity later

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doi:10.1016/S0044-8486(03)00221-7

than males, reaching their commercial size before becoming sexually matured. Thus, the production of "all female populations" is an important objective to be achieved for fish farms and reports important economical benefits in commercial rainbow trout culture (Panadian and Sheela, 1995).

The first step to obtain an entire offspring of females is the creation of sex-reversed females. One way to produce these individuals is by means of hormonal treatment, usually immersing eggs or fry into solutions containing steroids or feeding the animals with diets, which contain these hormones (Johnstone et al., 1979; Olito and Brock, 1991). These procedures allow the growing of functional testicles in individuals, which are genetically females, and consequently, all produced spermatozoa carry X chromosome. Sex-reversed females lack sperm ducts (Tsumura et al.,1991; Geffen and Evans, 2000) making necessary the sacrifice of animals when the obtaining of sperm is required. Sperm is extracted directly from the testicle and does not suffer modifications that occur in the sperm duct in normal males, such as reabsorption of spermatozoa (Billard and Takashima, 1983), regulation of seminal fluid ionic composition (Morisawa and Morisawa, 1990), and hormonal secretions (Van den Hurk et al., 1978; Schulz, 1986). These hormones increase the pH of the seminal plasma and cause this way an increase in the AMPc content inside the spermatozoa, allowing them to be motile once they get in contact with an hipoosmotic solution (Miura et al., 1992). Spermatozoa of sex-reversed females are almost immotile when collected from the testicle and need an exogenous maturation before activation. In the sperm duct, big amounts of tryglycerides are present in the seminal plasma (Piironen and Hyvarinen, 1983). It is well known that these compounds are used by spermatozoa of normal males to obtain energy. Trout spermatozoa have a single mitochondrion in the basis of their head, which has a low ability for oxidative phosphorilation (Le Lay et al., 1999). This fact provides an explanation for the short period of movement of these cells. Therefore, deficiencies in energy substrates such as tryglycerides or in AMPc content could represent a handicap for the energetic metabolism of the spermatozoa of sex-reversed individuals.

On the other hand, the lack of sperm ducts make necessary to maintain a higher number of animals in fish farms, since animals must be killed to obtain their sperm. Farmed animals are usually kept under photoperiod controlled conditions to have reproduction all through the year (Bromage et al., 2001). At this point, sperm cryopreservation of these individuals seems to be greatly useful since it would allow the sperm availability during all year, reducing the necessity of a big male stock. The possibility of cryopreserving sperm has many other applications, including the development and maintenance of gene banks (McAnrew et al., 1995), transportation of gametes (Lubzens et al., 1993), and facilitation of artificial fertilization when males and females have distant periods of maturation, allowing the availability of gametes for long periods of time (Van der Bank and Steyn, 1992).

The peculiarities of these sperm samples due to the testicular origin must be considered with detail to formulate a specific cryopreservation protocol suitable for sex-reversed trout females. The objective of this study was to determine the parameters, which could play an important role in the aptitude for cryopreservation of this milt. The effect of seasonality and the method of sperm extraction in the freezability of the sperm, as well as the addition of motility stimulators, were considered as possible keys for the optimisation of sex-reversed trout sperm cryopreservation.

2. Material and methods

2.1. Collection of sperm and eggs

Sperm and eggs were obtained from the fish farm Los Rigales (Huesca, Spain).

Sperm proceed from sex-reversed females produced by hormonal treatment with androgens and maintained under photoperiod controlled conditions.

Sex-reversed females were decapitated and their testicles were surgically extracted and carefully cleaned, removing the main blood vessels. Milt was obtained by two different methods. The first one, usually employed in the fish farm to collect sperm for fertilization, consists on the homogenization of the testicle. The second one was performed practicing cuts with a scalpel in the testicles and collecting the dripping sperm. Pools of milt from three males were done, and each experiment was carried out using four different pools. Samples were diluted 1:9 (v/v) in a commercial solution (MATURFISH®, IMV, France) to promote sperm maturation, and they were kept in that solution 2 h at 4 °C with oxygen supply.

Eggs were obtained from normal rainbow trout females by stripping. Pools of eggs from 10 females were used to perform the fertility trials.

Gametes were collected in spring (May) and in winter (December and January), and experiments were performed in parallel in both periods.

2.2. Sperm cryopreservation

The cryoprotectant dimethyl sulfoxide (DMSO, SIGMA, Madrid, Spain) (7%) was combined with two membrane stabilizers, egg yolk (SIGMA) (10%), and Dan Pro S760® (soybean protein complex, Central Soya Protein Group, Denmark) (7.5 mg/ml) in the mineral solution #6 from Erdahl and Graham (1980).

Before freezing, sperm was diluted (1:3, v/v) in the extender and maintained in that solution for 15 min to allow the proper penetration of DMSO into cells. French straws (0.5 ml) (IMV) were filled during the equilibration time, and then were placed 2 cm above the liquid nitrogen (LN_2) surface in a Styrofoam box during 10 min (freezing rate, 63 °C/min) to be finally plunged in it. The straws were stored in LN_2 tanks until their utilization and thawed in a water bath at 25 °C for 30 s (thawing rate, 666 °C/min). To establish freezing and thawing rates, temperatures inside the straw were recorded with a thermocouple placed in the middle of the straws during the freezing and thawing process.

2.3. Sperm motility assessment

Motility of fresh and frozen spermatozoa was tested using DIA532 (Billard, 1977) as activation solution. Two methylxanthines, caffeine, 5 mM, and theophylline 5 mM (SIGMA) were also added to DIA to check their ability as motility stimulators. Motility evaluation was performed placing 2 μl of sperm diluted 1:9 in MATURFISH® on a glass slide and immediately adding 12 μl of the activator (DIA, DIA-caffeine 5 mM, or DIA-theophylline, 5 mM). Motility was determined by

light microscopy (400 ×) always by the same evaluator. Values from 0 to 5 were attributed to the samples, being 0, 0% of spermatozoa with progressive motility, and 5, 100% of spermatozoa with progressive motility. Three observations were done per sample.

2.4. Fertility assessment

Eggs pooled from 10 females were divided in batches of 100 eggs and placed in Petri dishes. Fertilization experiments were carried out with fresh and frozen milt, using four pools of sperm in each period of the year. All fertilization trials were done in triplicate. The sperm dose used was 8.5×10^6 spermatozoa/egg with fresh sperm, and 17×10^6 with frozen one (the spermatozoa concentration was determined using a Neubauer chamber, three lectures were done per sample, and for each lecture, three different field of the chamber were recounted). Sperm was poured in a homogeneous way over the eggs, and after a quick and gentle mixing with a bird feather, sperm activator was added. After 10 min, eggs were gently washed with water and transferred into incubation baskets where it remained for 30 days at 10 °C with a water flux of 2 l/min. The number of embryos at eyed stage was counted and the percentage of fertilized eggs was established.

2.5. Statistical analysis

All data expressed as a percentage were normalized via angular transformation, and the results were analyzed by a multiple way analysis of variance (ANOVA). For the parameters, which displayed a significant influence, differences between treatments were detected using the LSD multiple range ($p < 0.05$).

3. Results

3.1. Motility

Motility of fresh sperm in the spring ranges from 88% to 100% (Fig. 1). In the winter, these values range between 69% and 94% (Fig. 2) showing a slight but significant decrease when compared to the spring results. In both seasons, the sperm extracted with scalpel provides significantly higher motility (99–100% in spring and 91–94% in winter) than the obtained by homogenization (88–93% in spring and 69–77% in winter). No differences were detected related to the use of different activators.

After freezing-thawing, sperm motility showed a significant drop, which was particularly important in spring. In this season, frozen spermatozoa displayed a very low motility after thawing (from 1% to 6%) (Fig. 1). Spermatozoa frozen in winter provided significantly higher motility after thawing (from 18% to 29%) (Fig. 2). The collection of sperm with the scalpel provided higher motilities than the homogenization in both seasons, winter and spring. No significant effect of motility stimulators was observed.

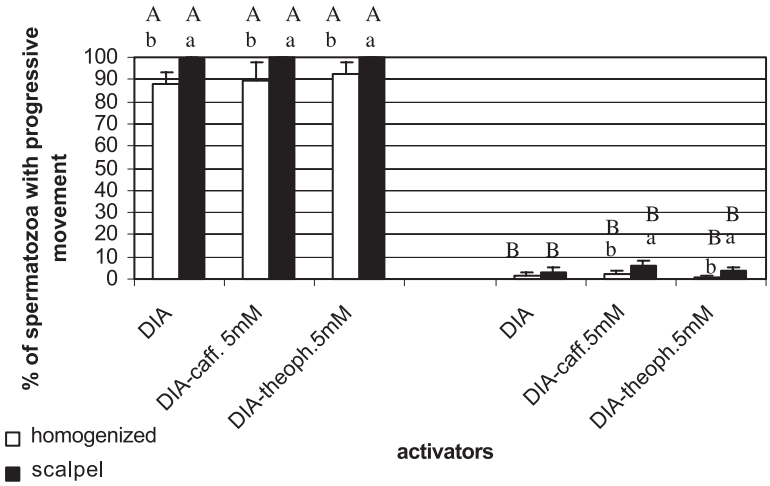

Fig. 1. Motility of fresh and cryopreserved sperm obtained in spring with both extraction methods and activated with three different activation solutions. Letters show differences between sperm extraction methods and capital letters between fresh and cryopreserved sperm.

3.2. Fertility

Fertilizing ability of fresh sperm was slightly but not significantly higher in winter than in spring. Fertility rates range from 74% to 84% and from 72% to 78%, respectively (Figs. 3 and 4) Neither the method of semen collection nor the use of methylxanthines affects the fertilizing ability of fresh sperm.

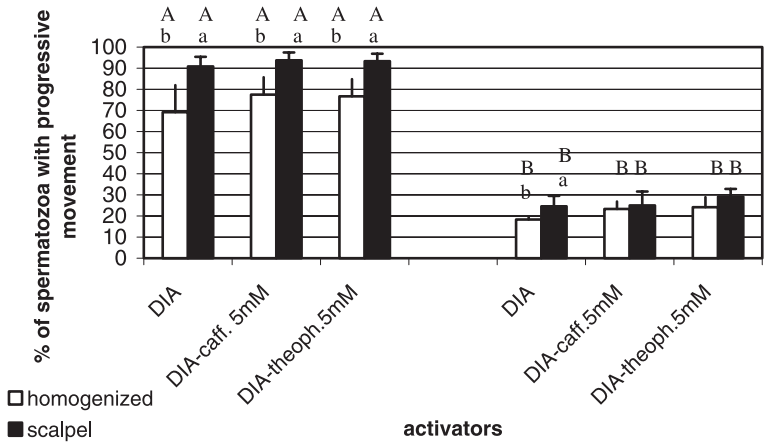

Fig. 2. Motility of fresh and cryopreserved sperm obtained in winter with both extraction methods and activated with three different activation solutions. Letters show differences between sperm extraction methods and capital letters between fresh and cryopreserved sperm.

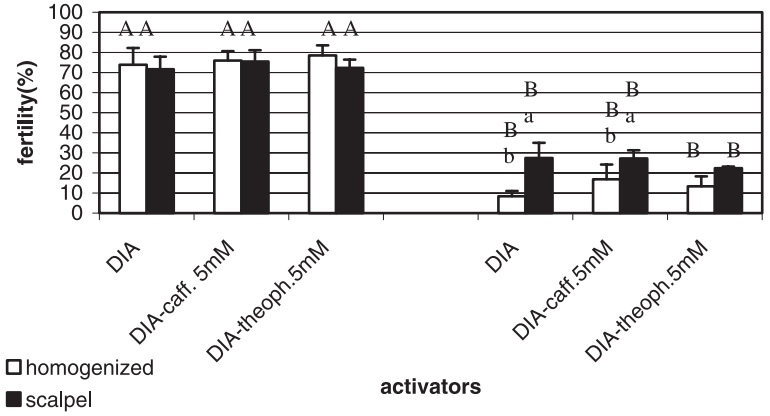

Fig. 3. Fertility rates using fresh and cryopreserved sperm obtained in spring by both extraction methods and using three different activation solutions. Letters show differences between sperm extraction methods and capital letters between fresh and cryopreserved sperm.

After freezing-thawing, fertility decreases, but important differences are related to the season of sperm collection. Sperm frozen in winter provides significantly higher fertility rates than the sperm frozen in spring. In winter, the minimum percentage of eyed eggs was obtained using sperm extracted by homogenization and activated with DIA (42%) and the maximum with sperm extracted with scalpel and activated with DIA-theophylline (65%). In winter, the minimum percentage of eyed eggs was obtained using sperm extracted by homogenisation and activated with DIA-caffeine (42%) and the maximum with sperm extracted with scalpel and activated with DIA-caffeine (65%). The last conditions provided similar fertility rates using fresh and frozen sperm. Nevertheless, in spring, the

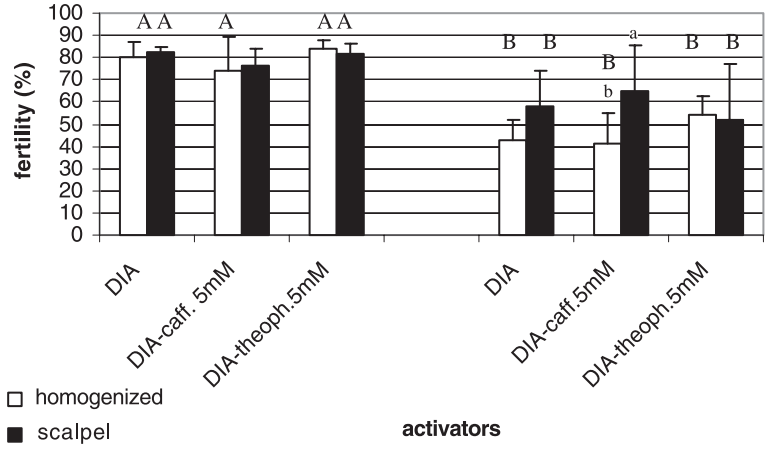

Fig. 4. Fertility rates using fresh and cryopreserved sperm obtained in winter by both extraction methods and using three different activation solutions. Letters show differences between sperm extraction methods and capital letters between fresh and cryopreserved sperm.

maximum fertility rate only reached 27% (scalpel extraction and DIA activation) and just 8% was obtained with homogenized sperm activated with DIA.

In both seasons, collection of sperm with the clean method (scalpel) improves the fertilizing ability of frozen-thawed sperm.

The use of the methylxanthines as motility stimulators did not significantly improve fertility rates.

4. Discussion

4.1. Effect of seasonality on sperm quality

The control of photoperiod is needed for maintaining mature males along all year. According to some authors, this manipulation does not affect fresh sperm quality (Bromage et al., 2001), but other studies suggest that sperm quality could be altered by the temperature at which gametogenesis takes place (Labbe and Maisse, 1996).

In this study, experiments were carried out in winter, the natural reproduction period for rainbow trout, and spring. Motility of fresh sperm was, in many cases, higher in spring than in winter, but those differences were not reflected in fertilizing capacity of fresh semen neither in motility of frozen sperm, since after freezing-thawing, motility was significantly higher in winter than in spring. Concerning to fertility, results also showed that after cryopreservation, sperm collected in winter produced significantly higher fertility rates than in spring period, showing a better aptitude for cryopreservation of the sperm obtained in the natural breeding season.

These data clearly demonstrated that seasonality strongly affects sperm quality in relation to freezability. Differences in freezability could be probably due to differences in plasma membrane composition, as consequence of the different temperature at which gametogenesis occurred. The durability of gametogenesis varies between 6 and 9 months (Barnabe, 1994), henceforth, animals that are ripe in winter have suffered their gametogenesis under conditions of relatively high temperature, whilst gametogenesis of animals mature in spring took place at low temperatures. Labbe and Maisse (1996) showed that trouts, which performed their gametogenesis under low temperatures, presented higher content of cholesterol/phospholipids in the plasma membranes of spermatozoa than the animals kept at higher temperatures. They also noticed that this fact correlated with low rates of fertility with cryopreserved sperm. The season of sperm extraction is subsequently a parameter of great importance that clearly affects the success of the cryopreservation process, which should always be carried out with sperm obtained in the natural reproductive period.

4.2. Effect of sperm extraction method

It is well known that contaminants reduce the seminal quality and the aptitude for cryopreservation in salmonid sperm (Rana et al., 1992). Obtaining the sperm directly from testicles by homogenization of the organ, urine and mucus contamination (which are the most common contaminants when the milt is obtained by stripping) are avoided, but some

contamination with blood and testicle tissues is present in samples. Results showed an improvement in motility as well as in fertility rates when a cleaner sperm extraction method was employed. Extraction by practicing incisions with scalpel in the testicle reduce contamination but also selects for mature spermatozoa, leaving the immature cells in the testicular cysts. An improvement was specially noticed in sperm extracted in spring and in frozen-thawed sperm, and thus, the importance of developing a clean extraction method is even greater for low quality samples or cryopreserved ones.

4.3. Effect of motility stimulators

Energetic metabolism is a key factor in the proper functionality of spermatozoa. Alterations in it will lead to a detriment in motility and a decrease or disappearance of fertility rates. There are tow main factors that can cause alterations in the energetic metabolism in these spermatozoa, one is its own testicular origin, and the absence of the important modifications that take place in the sperm duct of normal males. The second factor is the process of cryopreservation itself, which could cause cellular damages, including alterations in the single mitochondrion of these spermatozoa or loss of some enzymatic activities. Lahnsteiner et al. (1996) observed a decrease in ATPase activity in frozen sperm reducing the metabolism velocity of this nucleotide.

To improve the energetic metabolism of these spermatozoa, two methylxanthines were added to the activation solution DIA: caffeine and theophylline. Both substances are AMPc phosphodiesterase inhibitors, and thus prolong the effect of this second messenger. Trout sperm motility initiation is under AMPc-dependent phosphorilation control of some axoneme proteins (Morisawa and Okuno, 1982), which allow flagellar movement to take place by ATP hydrolysis mediated by dinein-ATPase. Considering the way of action of methylxanthines, we can assume that their addition in the activation solution should be similar to the addition of AMPc. This second messenger increases the number of spermatozoa able to initiate the flagella movement in trout (Morisawa and Ishida, 1987; Lahnsteiner et al., 1993; Cosson et al., 1995). It is known that methylxanthines have been used in mammal sperm providing good results in motility and fertility (Ronen and Marcus, 1978; Dodds and Seidel, 1983), and in more recent studies, it has been also confirmed in fishes. Ciereszko et al. (1996) reported that an improvement in lineal velocity of *Acipenser fulvescens* spermatozoa was observed when theophylline was used for activation in fresh and frozen sperm. Wilde et al. (1996) stated that the caffeine effect is specially noticed in sperm of animals with reduced fertility rates, which are more sensitive to any process to improve its quality. Motility and fertility have not been significantly improved in our study with the use of these stimulators, but it was observed that adding caffeine 5 mM to DIA to activate scalpel obtained sperm extracted in winter, a similar fertility rate as the respective fresh milt control was obtained (65.18% and 76.12%, respectively).

5. Conclusions

The results from this study suggest that sperm extraction method and season of sperm extraction present a huge importance to develop a proper cryopreservation protocol for

sex-reversed trout sperm. Scalpel provided a clean extraction method that allows contamination reduction in samples, as well as retention of immature spermatozoa in testicular cysts. The use of this method provides better results of motility and fertility, and thus it is strongly recommended when cryopreservation of this kind of milt was required. Seasonality seems to affect the quality of sperm and its resistance to cryopreservation; therefore, sperm obtained during the natural reproduction period definitively is a key factor for the success of the process.

Finally, the addition of methylxanthines as motility stimulators have not reported any significant improvement, but their use should not be discarded since an increase in motility and fertility rates could be obtained in particularly bad quality samples.

Acknowledgements

This work was supported by FEDER project 1FD97-0521-CO2 and by a PhD grant from Junta de Castilla y León. Authors would like to thank Los Rigales fish farm staff (Huesca) for their collaboration.

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