Available online at www.sciencedirect.com
ScienceDirect
Aquaculture 259 (2006) 403–414
Aquaculture
www.elsevier.com/locate/aqua-online

Post-thaw amendment of cryopreserved sperm for use in artificial insemination of a viviparous fish, the green swordtail *Xiphophorus helleri*

Qiaoxiang Dong [a,b], Changjiang Huang [a,b], Terrence R. Tiersch [a,*]

[a] *Aquaculture Research Station, Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, Louisiana 70803, USA*
[b] *School of Environmental and Public Health, Wenzhou Medical College, Wenzhou 325035, P. R. China*

Received 7 April 2006; received in revised form 28 April 2006; accepted 10 May 2006

Abstract

Sperm cryopreservation protocols have been developed for live-bearers such as the green swordtail *Xiphophorus helleri* and the platyfish *Xiphophorus couchianus*. Despite the high post-thaw motility (~75%) obtained in both species, the requirements of sperm storage within the female reproductive tract coupled with the process of internal fertilization place functional demands upon cryopreserved sperm samples far beyond those of oviparous species. The purpose of this study was to facilitate the artificial insemination process with cryopreserved sperm of *X. helleri* through evaluation of parameters related to sperm quality after thawing. Specifically, this study evaluated the effects on motility for fresh and thawed sperm samples of centrifugation (for concentration of sperm and washing for removal of cryoprotectant), ionic composition, and additions of glucose and fetal bovine serum (FBS) in extender solutions. Centrifugation at 1000 ×*g* for 10 min at 4 °C was found to have no adverse effects on sperm motility of fresh samples, and for cryopreserved samples, the removal of glycerol by washing yielded higher and longer post-thaw motility (e.g., 168 h vs. 48 h for the controls). Suspension of fresh sperm samples in magnesium-free Hanks' balanced salt solution (HBSS) did not affect motility; however, HBSS prepared with the absence of potassium or calcium, and the use of unsupplemented saline (NaCl alone) as extenders significantly reduced sperm motility. The presence of glucose in HBSS yielded higher and longer motility for fresh and thawed samples, but addition of glucose at greater than 2 g/L were unnecessary. Addition of 20% FBS prior to freezing was found to increase the post-thaw motility significantly compared to control treatment with 14% glycerol alone. Also addition of 20% FBS after thawing and centrifugation was found to induce the formation of sperm bundles, which may be beneficial for internal fertilization success. In conclusion, concentration of sperm and the removal of cryoprotectant (through centrifugation), and the addition of 20% FBS in the extender is recommended for future insemination trials with cryopreserved samples.

Keywords: *Xiphophorus helleri*; Sperm motility; Cryopreservation; Centrifugation; Ionic composition; Glucose; Fetal bovine serum

1. Introduction

The green swordtail *Xiphophorus helleri* is a viviparous teleost of the family Poecilliidae. Members of this genus are valuable as ornamental fish as well as biomedical research models for cancer genetics (Schartl,

* Corresponding author. Tel.: +1 225 765 2848; fax: +1 225 765 2877.
E-mail address: ttiersch@agctr.lsu.edu (T.R. Tiersch).

0044-8486/$ - see front matter © 2006 Published by Elsevier B.V.
doi:10.1016/j.aquaculture.2006.05.011

1995; Nairn et al., 1996; Tamaru et al., 2001; Walter and Kazianis, 2001). Due to internal fertilization, the sperm of *Xiphophorus* are analogous to those of mammalian species, and possess atypical features such as elongated sperm heads (Jamieson, 1991), well-developed mitochondrial sheaths in the midpiece (Stoss, 1983), and glycolytic activity comparable to that of mammalian sperm (Gardiner, 1978). Despite study of sperm cryopreservation in some 200 species of freshwater and marine fishes (Leung and Jamieson, 1991; Rana, 1995; Tiersch, 2000), sperm cryopreservation has just begun in live-bearers. Cryopreservation protocols have been developed to address their small body size and limited sperm volume (e.g., 5–10 μL per fish) (Huang et al., 2004b). In previous studies of *X. helleri* and *Xiphophorus couchianus*, sperm were suspended in Hanks' balanced salt solution at 300 mOsm/kg with 14% glycerol, loaded in 0.25-ml straws, equilibrated for 10 min, cooled at 20–25 °C/min, stored in liquid nitrogen, and thawed at 40 °C in a water bath for 7 s with motility after thawing on average as high as 78% (Huang et al., 2004a,c).

Despite this high post-thaw motility of *X. helleri* sperm; preliminary artificial insemination trials with cryopreserved sperm did not produce live young (Dong et al., 2005). However, these initial attempts suggested that the collection method (crushing of testis), which was different from routine insemination practice (stripping of sperm by abdominal massage), and the presence of cryoprotectant, per se, were not responsible for reproductive failure because live young were produced in fresh sperm samples collected by crushing of testis and suspended in 14% glycerol (Dong et al., 2005). Other hypotheses included: 1) insufficient numbers of sperm were used for insemination; 2) glycerol may have been toxic to thawed sperm samples; 3) the sperm had reduced motility or viability after cryopreservation that rendered them incapable of internal fertilization.

Thus, the purpose of this study was to facilitate the artificial insemination process with cryopreserved sperm through evaluation of parameters related to sperm quality after thawing, and development of amendment strategies to prepare sperm for insemination. Specifically, this study evaluated the effects on motility for fresh and thawed sperm samples of centrifugation, and extender components including ionic composition, and addition of glucose and fetal bovine serum. Centrifugation was evaluated for the purpose of concentrating sperm as well as for removing cryoprotectants after thawing, and the other factors were studied for the purpose of increasing sperm motility after thawing to improve sperm effectiveness within the female for internal fertilization. Recent artificial insemination trials that utilized sperm concentrated and washed by centrifugation has yielded live young and is reported elsewhere (Tiersch et al., 2005).

2. Materials and methods

2.1. Sperm collection and sample preparation

A total of 22 male *X. helleri* were used in this study. All fish were from inbred lines maintained by the *Xiphophorus* Genetic Stock Center, Texas State University, San Marcos, TX 78666 (www.xiphophorus.org). Fish were anesthetized in 0.01% tricaine-methane sulfonate (Western Chemical Inc., Ferndale, WA) for 2 min, and sperm were collected by surgical removal of the testis. Adherent tissue was dissected away and testes were placed into tared, resealable plastic bags (NASCO whirlpak, MBCOCT, New Haven, CT) and weighed. The number of testes used was based on experimental design considerations and in general 2–4 fish were used for each experiment. Only mature testes (with a creamy white appearance) and samples with an initial motility above 80% for fresh sperm were used for experiments. Hanks' balanced salt solution (HBSS) was added before crushing of the testis to release sperm. Dilutions with HBSS were based on the testis weight, and a ratio of testis to HBSS (mass:volume) of 1:100 generally yielded a sperm density of $\sim 5 \times 10^7$ cells/ml. Based on preliminary research (Huang et al., 2004b,c), HBSS at 300 mOsmol/kg was used for sperm suspension after collection. Unless otherwise specified, the osmolality of the HBSS used in this study was 300 mOsmol/kg.

2.2. Motility estimation

The motility characteristics of *Xiphophorus* sperm are distinct from those of most other teleosts. The sperm are motile upon collection and remain continuously motile after suspension in HBSS, and therefore activation solutions are not necessary for motility estimates. For estimation, a 5-μl aliquot was removed from each sample and placed on a glass microscope slide. In the cases when the ratio of sperm to HBSS (or HBSS-glycerol mixture) was below 1:100, 2 μl of sperm suspension were diluted 1:100 with HBSS before estimation. Sperm motility was estimated visually at 200× magnification using darkfield microscopy (Optiphot 2, Nikon Inc., Garden City, New York) and was expressed as the percentage of cells moving in a forward direction. Sperm vibrating in place were not considered to be motile. Unless specified, samples were always stored at 4 °C after treatment, but samples were warmed to room temperature (23 °C) prior to motility estimation.