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ScienceDirect
Theriogenology
Theriogenology 66 (2006) 2025–2035
www.journals.elsevierhealth.com/periodicals/the

Chilled storage of semen from Atlantic halibut, *Hippoglossus hippoglossus* L. I: Optimizing the protocol

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Received 5 October 2005; received in revised form 9 June 2006; accepted 9 June 2006

Abstract

Asynchrony in gamete production between females and males, and decrease in semen quality towards the end of reproductive season make chilled short-term storage of Atlantic halibut, *Hippoglossus hippoglossus*, semen a desirable method to apply for artificial propagation of this fish species. The goal of the present study was to determine the critical physiochemical factors that affect the success of chilled storage of halibut spermatozoa, and to develop a reliable, simple and efficient protocol for the storage. The presence and type of gaseous atmosphere, dilution and type of diluent, dilution ratio, and additional factors including spermatozoa sedimentation and replenishing the storage medium were tested in relation to spermatozoa motility parameters. Also, fertilization tests were performed with stored semen. Normoxia (air atmosphere) conditions were superior to both hyperoxia (pure oxygen) and no gaseous atmosphere for chilled storage. Dilution of semen with a diluent was superior to incubating undiluted semen. A dilution factor of between 6 and 10 times the original semen volume resulted in the longest viability of stored spermatozoa. Preventing spermatozoa sedimentation through daily swirling of the samples was superior to weekly swirling, however the effect was negligible for the first month of storage. Replenishing the storage medium showed no advantage to incubating in unchanged medium. Semen diluted in modified Hanks' balanced salt solution 1:5–1:9, supplemented with antibiotics, and kept at 0–1 °C in Ziploc bag filled with air retained its viability for exceptionally long time. A decrease in the percentage of motile spermatozoa was observed after 43 days of storage, and a decrease in curvilinear velocity occurred after 15 days. Samples remained motile for at least 79 days of storage and the fertilization ability was retained for at least 70 days of storage. The results demonstrate a high potential of application of chilled storage of semen into reproduction programs in Atlantic halibut aquaculture.

Keywords: Atlantic halibut; CASA; Preservation; Fish; Sperm

1. Introduction

The Atlantic halibut, *Hippoglossus hippoglossus* L., is the world's largest flatfish, and because of its high market value is regarded as a coldwater marine species having a very high aquaculture potential. Halibut breed normally between January and April [1]. Males produce high quantities of semen, but its quality decreases towards the end of reproductive season, mostly because of dehydration and ageing processes to spermatozoa [2–5]. The presence of low quality of sperm at the end of reproductive season, at a time when high quality eggs are still being produced by females, represents a major problem in halibut aquaculture as it restricts the ability

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0093-691X/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2006.06.003

to obtain the desired quantity and quality of halibut embryos ([4,6], our own observations, unpublished).

Implanting of Atlantic halibut males with an agonist of gonadotrophin-releasing hormone (GnRHa) is one approach presently used to overcome the problem of poor quality of semen at the end of the season [4,7]. The treated fish show extended spermiation period, their semen is more hydrated and the percentage of motile spermatozoa is higher than in untreated males, although the effect of this treatment on fertilization ability of spermatozoa is not clearly advantageous [4]. Also, it remains unknown whether the GnRHa treatment reduces the problem of spermatozoa ageing, which is intensive at the end of reproductive season [5]. An alternative approach to overcome the problem of poor quality of sperm late in the season is to preserve sperm when it is at its best quality, to use it for fertilizations later on. This can be achieved through cryopreserving or chilled short-term incubating the sperm.

Short-term storage of sperm is an useful biotechnique that facilitates hatchery operations, for example, it reduces the need of frequent collections of milt from males, enables transportation of sperm to distant locations and prevents problems related to asynchrony in gamete production between males and females [8–10]. Generally, these operations are easy to perform, expensive equipment is not required, and no specific training is necessary to apply it to hatchery routines, making it a desirable tool for controlled reproduction. The interactions of multiple biological, physical and technical factors affect the efficiency of chilled storage of spermatozoa, in terms of maintaining spermatozoa viability. The most important factors include individual male variability, spermiation advancement, and storage conditions. Avoiding urine and/or feces contamination during collection, treatment with antibiotics to prevent microbial growth, proper gaseous atmosphere and storage temperature are considered as critical aspects of gamete management that prolong the viability of stored spermatozoa [9,10]. Under gaseous atmosphere, stored undiluted sperm becomes dehydrated in the course of storage because of water evaporation; also, bacterial growth is observed within several days of collection. On the other hand, storage of undiluted sperm without air atmosphere causes loss of viability because of oxygen depletion. Dilution of stored sperm with storage media helps to overcome the problems of desiccation and reduces bacterial growth, however, appropriate diluents are not developed for many fish species [10].

Most often, the reported procedures of short-term storage of fish sperm allow prolonging the spermatozoa viability for 1–2 weeks post-collection [9,10]. Atlantic halibut semen preserved in a supercooled state at −4 °C retained spermatozoa motility for 2 months [10] and good fertilization ability for 1 months of storage [11]. These results indicate that chilled storage of semen is a biotechnique of good potential to apply to routine hatchery operations in reproduction of Atlantic halibut.

The ultimate goal of the present study was to develop efficient, simple and reliable protocol for chilled storage of Atlantic halibut semen to be applied to mass-scale larvae production. In the first part of the study, we have focused on optimizing the protocol through investigating the effects of storage conditions: dilution of semen and its ratio; presence of gaseous atmosphere; and operations during storage, on spermatozoa motility parameters and fertilization ability.

2. Material and methods

Procedures for gamete collection, assessment of spermatozoa concentration and computer-assisted sperm analysis (CASA) followed the methodology described by Babiak et al. [5]. The final proportion of diluent with diluted sperm to activating solution (seawater) was approximately in range 1:10–1:50, depending on sperm dilution ratio. In CASA, records where the number of spermatozoa was lower than 50 or higher than 200 were discarded from analysis. Sperm motility was quantified for 0.5 s at 30 s post-activation [5]. Gametes were collected from the Atlantic halibut broodstock kept at the Bodø University College, Norway. For experiment I, spermatozoa motility was estimated under a microscope (×500) using 5 μL filtered marine water (salinity 34 ppt, 7 °C) as an activating solution. In experiment II, the following CASA parameters were investigated: percentage of motile spermatozoa (MOT); curvilinear velocity of spermatozoa (VCL), that is velocity of the movement along the actual trajectory; straight-line velocity of spermatozoa (VSL), that is the velocity of the movement from the start point to the end point along theoretical straight-line joining the two points; and linearity of the movement (LIN), that is percentage ratio VSL/VCL. Spermatozoa concentration was estimated using a Bürker's chamber. Osmolality was measured in the whole semen and in the tested diluents with a Fiske One-Ten Osmometer (Fiske Associates, Norwood, MA, USA). pH was measured using an Orion SA720 pH meter (Orion Research Inc., Boston, MA, USA). All measurements were repeated 2–5 times.