



Short-Term Storage of Diluted Fish Sperm in Air Versus Oxygen

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Abstract

Herein we demonstrated the effect of oxygen and air atmosphere on the success of short-term storage of sperm obtained from rainbow trout (*Oncorhynchus mykiss*), sterlet (*Acipenser ruthenus*) and common carp (*Cyprinus carpio*) diluted in immobilizing media (100 mM NaCl, 40 mM KCl, 3 mM CaCl₂, 1.5 mM MgCl₂, 50mM Tris, pH 8.2 for salmonid fish, 17 mM NaCl, 1.7 mM KCl, 6 mM NaHCO₃, 0.5 mM CaCl₂, 0.5 mM MgSO₄ x 7 H₂O, 1.1 mM NaH₂PO₄ x 2H₂O, 1.0 mM Na₂HPO₄, 50 mM glucose, pH 7.5 for sturgeon, 100 mM NaCl, 3.1 mM KCl, 2 mM CaCl₂, 0.4 mM MgCl₂, 25 mM NaHCO₃, 0.3 mM NaH₂PO₄; pH 8.64 for cyprinids). Sperm motility of rainbow trout was observed up to 33 days, and percentage of motile spermatozoa was high (70%) up to 14 days after the start of the experiment. In the case of sperm samples which were stored with oxygen, sperm motility (30%) was observed for only two weeks; after this time, sperm motility decreased to 1%. Diluted sturgeon sperm quickly lost their movement potential when stored with oxygen. Samples kept in aerobic conditions maintained 80% of motile spermatozoa after 10 days of short-term storage. In the case of the carp, both, oxygen and aerobic conditions gave similar results within 6 days of the experiment. We assume that short-term storage of diluted fish sperm can be carried out in aerobic conditions which is easier and cheaper in comparison to the pure oxygen atmosphere.

Keywords: short-term storage, sperm motility, rainbow trout, carp

Introduction

Short-term storage of semen at reduced temperature above the freezing point of water is an old method used in for "artificial" reproduction of fish (McNiven *et al.*, 1993). It is useful and requires easy-to-use technology that reduces broodstock manipulation and workload during spawning, the busiest season in fish production process. It also allows spawning to be carried out, even when the period of females maturation is not synchronized (occurs later) with males (Scott and Baynes, 1980).

In the case of salmonids, there are many methods for maintaining high quality semen for more than one month, and only a fridge and a suitable diluent are required. The replacement of the aerobic atmosphere with pure oxygen allowed for rainbow trout (*Oncorhynchus mykiss*) to maintain their sperm's fertilizing ability up to 34 days after collection (Stoss and Holtz, 1983). Also, the use of fluorocarbon as an oxygen carrier allowed for the short-term storage of more than 50% of motile sperm for up to 37 days following stripping (McNiven *et al.*, 1993). In the case of

common carp (*Cyprinus carpio*), 30% of motile sperm managed to retain motility up to 85 hours from the time of collection due to the dilution of semen and storage in an atmosphere of oxygen (Ravinder *et al.*, 1997).

There are some reports in the literature regarding favorable air conditions compared to oxygen in the short-term storage of turbot (*Scophthalmus maximus*) sperm (Chereguini *et al.*, 1997) and salmonids sperm (Bencic *et al.*, 2000). Herein we demonstrate the effects of oxygen and air atmosphere on successful short-term storage of diluted sperm obtained from rainbow trout, sterlet (*Acipenser ruthenus*) and common carp. The aim of this study was to determine whether a pure oxygen atmosphere is necessary for the efficient storage of diluted male gametes of fish.

Materials and Methods

Origin of Sperm and Immobilizing Solutions

For the experiment sperm samples from three species were used: rainbow trout, sterlet and common carp. Sperm was immediately diluted in an

immobilizing solution after stripping. All immobilizing solutions were chosen in preliminary experiments from several solutions for each species proposed in the literature. For the rainbow trout sperm an ASP buffer was used (Morisawa and Morisawa, 1988) containing 100 mM NaCl, 40 mM KCl, 3 mM CaCl₂, 1.5 mM MgCl₂, and 50 mM Tris (pH 8.2). For the sterlet sperm, a buffered solution was used as given by Chulhong and Chapman (2005) which consisted of 17 mM NaCl, 1.7 mM KCl, 6 mM NaHCO₃, 0.5 mM CaCl₂, 0.5 mM MgSO₄ x 7 H₂O, 1.1 mM NaH₂PO₄ x 2H₂O, 1.0 mM Na₂HPO₄, and 50 mM glucose (pH 7.5). For the common carp sperm a TLP buffer was used as an immobilizing solution (100 mM NaCl, 3.1 mM KCl, 2 mM CaCl₂, 0.4 mM MgCl₂, 25 mM NaHCO₃, and 0.3 mM NaH₂PO₄ (pH 8.64) (Bavister, 1989) supplemented with 200 UI ml⁻¹ penicillin (Table 1).

Sperm Dilution and Short-term storage

The sperm of the rainbow trout and sterlet was diluted in 1:3 ratio, whereas common carp sperm was diluted in a 1:30 ratio. Diluted sperm samples (1 ml each) were kept in open vials placed in bags filled with oxygen or in closed vials at 4°C. Oxygen was changed every 24 h, and all samples were gently mixed twice a day to prevent the sedimentation of spermatozoa. The layer of diluted sperm in 0.5 ml eppendorf tubes was approximately 2 mm thick.

Sperm Motility Analysis

For rainbow trout sperm the activation buffer used contained 1 mM CaCl₂, 20 mM Tris, 30 mM glycine and 125 mM NaCl, (pH 9.0) (Billard, 1977), supplemented with 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA). For sterlet sperm activation, a buffer containing 10 mM Tris-HCl, 20 mM NaCl and 2 mM CaCl₂ (pH 8.5) (Jähnichen *et al.*, 1999) supplemented with 0.5% BSA was used. For common carp, a sperm activation buffer containing 68 mM NaCl and 50 mM urea (pH 7.7) (Woynarovich and Woynarovich, 1980) supplemented with 0.5% BSA was used.

Sperm motility was recorded in three-day intervals (rainbow trout), one-, four-, seven- and ten-day intervals (sterlet), and one-day intervals (common carp). Diluted sperm samples were mixed with activation media (between 1:100 to 1:400, depending on sperm concentration). Activated sperm was placed on Teflon-coated slide glasses (Tekdon, Inc., Myakka City, FL, USA) and covered with standard cover slips. Sperm movement was recorded six seconds after activation. The evaluation of sperm motility was performed by subjective method by one observer with the use of an Olympus BX51 microscope.

Statistical Analysis

The results were statistically analyzed using GraphPad Prism software (GraphPad Prism Software Inc., San Diego, CA, USA). A two-way ANOVA was applied to compare sperm motility percentage at different time points. Differences between groups were identified using a Bonferroni *post hoc* test.

Results

Rainbow trout sperm maintained a high motility percentage (70%) up to 14 days after the start of the experiment. Motility of about 20% spermatozoa was observed up to 33 days when stored in atmospheric air conditions (Figure 1A). Sterlet sperm preserved in a pure oxygen atmosphere rapidly lost their motility potential, but in atmospheric air conditions, almost 80% of spermatozoa remained motile up to ten days from sperm sampling (Figure 1B). About 40% of common carp sperm stored in both oxygen and air conditions remained of motile on day 4 of short-term storage (Figure 1C).

Discussion

In present study we tested the hypothesis that a pure oxygen atmosphere is beneficial during short-term storage of diluted fish sperm. We found that samples of fish sperm diluted with species-specific extenders had better capabilities to be stored in aerobic conditions versus a pure oxygen atmosphere.

Table 1. Immobilizing solutions used for rainbow trout (ASP), starlet (SB) and common carp (TLP) sperm short-term storage

| Compositions | Immobilizing solutions | | |
|--------------------------------------|--------------------------------------|------------------------------------|--|
| | ASP (Morisawa and Morisawa, 1988) | SB (Chulhong and Chapman, 2006) | TLP (Bavister <i>et al.</i> , 1989) |
| NaCl | 100 mM | 17 mM | 100 mM |
| KCl | 40 mM | 1.7 mM | 3.1 mM |
| CaCl ₂ | 3.0 mM | 0.5 mM | 2.0 mM |
| MgCl ₂ | 1.5 mM | - | 0.4 mM |
| NaHCO ₃ | - | 6.0 mM | 25 mM |
| NaH ₂ PO ₄ | - | 1.1 mM | 0.3 mM |
| Na ₂ HPO ₄ | - | 1.0 mM | - |
| MgSO ₄ x7H ₂ O | - | 0.5 mM | - |
| Tris | 50 mM | - | - |
| Glucose | - | 50 mM | - |
| pH | 8.2 | 7.5 | 8.64 |

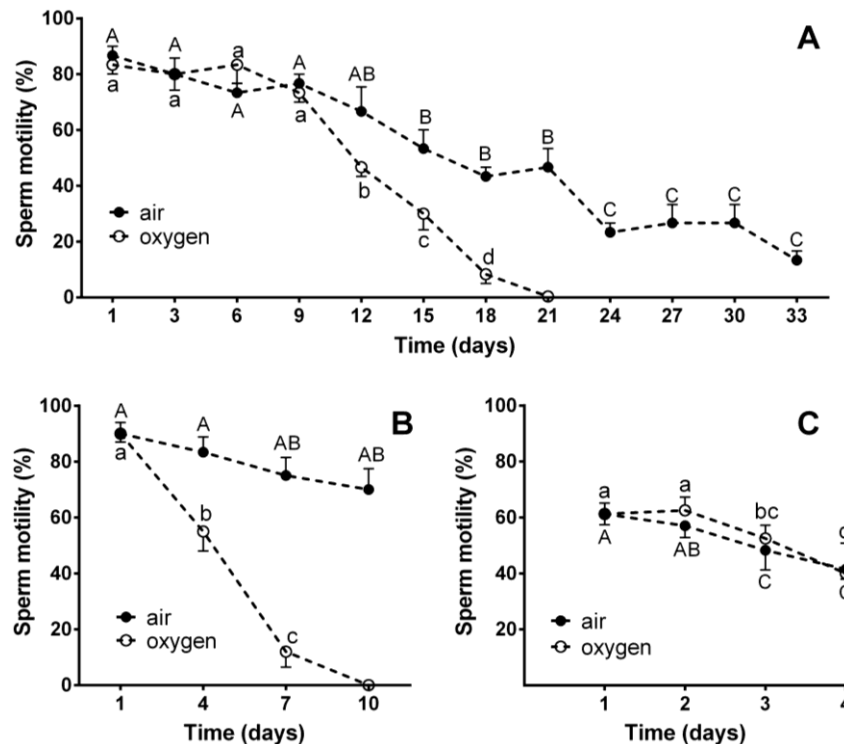


Figure 1. Effect of the pure oxygen atmosphere and aerobic atmosphere on rainbow trout (A), sterlet (B), and common carp (C), sperm motility. Upper-case letter indicate statistical difference for samples maintained in air atmosphere (●) and lower-case letter indicate difference for samples maintained with use of oxygen atmosphere (○).

This assumption was confirmed for two species from two different orders of fish—rainbow trout (*Salmoniformes*), sterlet (*Acipenseriformes*). In case of common carp (*Cypriniformes*) we found that an oxygen atmosphere was not superior in comparison to natural aerobic conditions. This is an important step towards developing efficient and easy methods for fish sperm handling in hatcheries.

Undiluted sperm of rainbow trout have a higher survival rate when stored in oxygen conditions (Billard, 1981). In our study we found that when diluted with ASP (artificial seminal plasma, Morisawa and Morisawa, 1988) rainbow trout sperm has a better motility rate when stored in anaerobic conditions versus a pure oxygen atmosphere. However, during the first ten days there was no difference in sperm motility between both storing conditions. Aerobic conditions allows about 50% of spermatozoa to stay motile at up to twenty days from sperm sampling, whereas sperm preserved in oxygen conditions was immotile at this time. Our results support the data of McNiven *et al.* (1993) and Bencic *et al.* (2000) who found that sperm maintained in limited air conditions can survive longer than sperm stored in an oxygen atmosphere. Free radicals derived from an oxygen atmosphere probably cause oxidative damages to sperm and therefore reduce their survival rate, as is known for mammalian sperm (du Plessis *et al.*, 2008).

Similarly, sterlet sperm motility was reduced when a pure oxygen atmosphere was provided, and the spermatozoa became completely immotile after ten days of storage in oxygen. At the same time,

samples preserved in aerobic atmospheric conditions consisted of more than 70% motile spermatozoa. Our data support the findings of Chulhong and Chapman (2005) who showed that the short term storage of sturgeon sperm can be successfully accomplished without the use of a pure oxygen atmosphere. Our data indicate that negative effects due to an oxygen atmosphere occurred more rapidly in sterlet sperm than in trout sperm. Further studies are necessary to explain why sturgeon sperm is more sensitive to the negative influence of a pure oxygen atmosphere.

In our study, we found that an oxygen atmosphere was not beneficial for the short-term storage of common carp sperm within four days. In both pure oxygen and atmospheric conditions, sperm motility decreased from about 60% to about 40%. However, common carp sperm can be stored up to sixteen days with a loss of only 20% of sperm motility, and the beneficial effects of an oxygen atmosphere could be seen only for the first six days of storage (Saad *et al.*, 1988). This data together with our results suggest that, for practice, the short-term storage of diluted common carp sperm can be carried out without the use of a pure oxygen atmosphere. Our data also suggest that fish sperm diluted with a species-specific extender does not need an oxygen atmosphere for the prolongation of their lifespan. We postulate that the short-term storage of diluted samples of fish sperm can be carried out in aerobic atmospheric conditions, which are easier and cheaper in comparison to pure oxygen conditions.

Conclusions

Short-term storage of diluted fish sperm can be successfully performed without the use of a pure oxygen atmosphere. In the case of common carp sperm, although the addition of oxygen had no negative effect on short-term sperm storage, the natural aerobic conditions produced the same results after four days of storage.

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