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A brief exposure to low pH prior to refrigerated storage reduces the motility and viability of goldfish sperm (*Carassius auratus*, Linnaeus, 1758)

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Summary

Optimal conditions of short-term storage of fish sperm are important for successful artificial reproduction protocols or incubation of sperm in a cryoprotecting medium for successful sperm cryopreservation. The influence of acid or alkaline incubating conditions on short term storage of goldfish sperm was investigated in a series of experiments. Goldfish sperm was diluted an immobilizing solution (1 : 3) and incubated for 1 h in acidic, pH 6.5 (AC group) or alkaline, pH 8.5 (AL group) conditions. Subsequently, the sperm of both groups was further diluted (1 : 3) in the immobilizing solution and the pH was adjusted to pH 7.51 (±0.02) and 7.56 (±0.07) in the AC and AL group respectively. The samples were kept in sealed bags and stored at 4°C. Viability (%) and motility (%) was estimated in samples obtained daily for the next 4 days. The results indicate that even a brief exposure of sperm to acid acidic conditions prior to storage accelerated the decline of sperm viability and motility during refrigeration.

Introduction

There are several published protocols for chilled storage of fish sperm (Mansour et al., 2004; Babiak et al., 2006; Peñaranda et al., 2010a,b). A successful sperm preservation protocol involves the dilution of semen in an immobilising solution, which protects the chemical and structural cellular integrity of spermatozoa. An activation solution is required for initiating the motility of the immobilised spermatozoa for the fertilisation of egg. The integrity of spermatozoa requires the preservation of membrane integrity and the safeguarding of cellular functions and capacity for the reactivation and motility of spermatozoa when kept in the immobilising solution.

Several motility parameters of sperm can be influenced by the pH of the immobilising solution (Perchec et al., 1995; Woolsey and Ingermann, 2003) or by the ionic composition and concentration, osmolarity, pH, and the dilution rate of sperm in an activating solution (Alavi and Cosson, 2005). An immobilizing medium can control the ionic environment and overcomes the problems of oxygenation and dehydration during chilled storage (Saad et al., 1988; Babiak et al., 2006).

Sperm viability and motility are important parameters for evaluating the quality of sperm. There are several studies that demonstrate the negative effect of low pH on mammalian and fish sperm motility (Arienti et al., 1999; Nynca et al., 2012). During sperm collection and handling, various parameters can influence the pH of sperm. For example, the pH of sperm may vary with season, diet and the level of contamination with urine (Dreanno et al., 1998; Bozkurt and Secer, 2005). A drop in the pH can result in low motility, fertilization and hatching rate (Nynca et al., 2012). The exact influence of extracellular pH on sperm motility and viability may vary with species and the acidic or alkaline range of the pH that the sperm is exposed (Dziewulska and Domagała, 2013; Gonzalez-Bernat et al., 2013). Exposure of sperm to acidic conditions may result in lethal or sub-lethal cellular damage and, in some cases, promote irreversible changes in the cell physiology that affect the motility of the spermatozoa (Peñaranda et al., 2010a,b; Santiso et al., 2012).

The intracellular pH of spermatozoa can be influenced by external pH with consequences for sperm motility. The effect of external pH on sperm motility, during storage or after activation of spermatozoa, involves the regulation of membrane ion channels and regulation of intracellular pH and protein posphorylation (Wang et al., 2003). As a result, extracellular acidic conditions can influence the intracellular pH, decrease sperm motility, the effect of which may be due to depletion of energy stores and decreased ATP hydrolytic activity of the outer arm of dynein (Perchec et al., 1995; Woolsey and Ingermann, 2003) with consequences for the capacity of motility of the spermatozoa.

In cyprinidae, sperm viability may not be affected in a wide range of pH, but motility may be compromised by extreme pHs; for example, the motility of carp spermatozoa incubated for 24 h at pH 6 or 10 was significantly lower compared to spermatozoa incubated at pH 7.8 or 9.0 (Saad et al., 1988). As a result, incubation of spermatozoa to extreme pH conditions may result in sublethal damages, which will result in compromised motility and even in reduced fertilisation ability. For example, the swimming

velocity and the percentage of motile spermatozoa of *A. alburnus* (Cyprinidae) varies according to the pH of the seminal plasma (Lahnsteiner et al., 1996), indicating that the spermatozoa may be sensitive to the pH conditions to which they are exposed, with consequences for motility of the cyprinid sperm. Unfavourable acidic conditions during sampling, handling, storage, or during activation of spermatozoa, can result in lethal and sublethal damages. For example, even a brief exposure of sperm to urine can result in a drop in the pH, the osmolality, the precautious mobilization of spermatozoa and the rapid depletion of energy stores, with consequences for the stored sperm quality (Nynca et al., 2012). As a result, sperm integrity may be compromised after exposure to unfavourable pH conditions, with consequences for the swimming velocity and duration of motility. Optimal short-term storage conditions of fish sperm are important for successful artificial reproduction protocols or incubation of sperm in a cryoprotecting medium for successful cryopreservation of sperm from commercially important fish species or phenotypes of ornamental fish such as the goldfish.

The purpose of the present work was to evaluate goldfish sperm motility and viability after a brief exposure to acidic conditions prior to refrigerated storage.

Materials and methods

In a pilot experiment, fresh undiluted goldfish sperm was diluted (1 : 250) in an activating solution with different pHs; the maximum duration of forward swimming spermatozoa was estimated visually using a light microscope at 400× magnification. The activating medium contained 45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl and the pH tested was 6.5, 7.0, 8.0 and 8.5.

Furthermore, fresh undiluted sperm was diluted (1 : 3) in immobilising solution with acid pH (6.8) and alkaline pH (8.5) and at the frequently reported optimal pH value of 7.8 for semen of goldfish (Zadmajid et al., 2013) and other cyprinidae. After incubation for 1 h at 4°C, the sperm was diluted (1 : 250) in an activating solution (45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8.0) and the maximum duration of forward swimming spermatozoa was estimated visually using a light microscope at 400× final magnification.

Based on the results obtained from the above experiments, the influence of acid and alkaline incubation on sperm viability and motility was further investigated. Sperm was collected (using capillary pipette tips as catheters) from 11 goldfish. Semen from eight fish was selected, as their sperm was dense and quiescent before dilution; after activation, their spermatozoa exhibited more than 90% motility and over 70 s minimum of forward swimming motion. Semen of the selected fish was pooled and subdivided in two equal portions for either the acid or alkaline treatment group. For the dilution of spermatozoa, the phosphate buffer used in previous works (Nathanailides et al., 2011) was replaced with Tris buffer, as we observed that phosphate interfered with the fluorescent microscopy used for the viability assay.

The two pooled samples of spermatozoa were diluted (1st dilution 1 : 3 v/v) in an acid (125 mM NaCl, 0.1 mM CaCl, 20 mM Tris pH 6.5, titrated with 1 M HCl) or an alkaline (125 mM NaCl, 0.1 mM CaCl, 20 mM Tris, pH 8.5) immobilizing solution (Saad et al., 1988). In the first dilution, cell density was about 6×10^6 ml^{-1}.

The diluted sperm was incubated for 1 h and then briefly centrifuged (at 1000 ***g*** for 3 min), the supernatant was partially discarded, diluted again (2nd dilution 1 : 3) in the immobilising solution, and the pH adjusted with 1 M HCL. In the second dilution, cell density was about 3×10^6 ml^{-1}. The second dilution preparations had pH 7.51 (±0.02) and pH 7.56 (±0.07) in the acid and alkaline group, respectively, and contained 200 mU ml^{-1} penicillin + streptomycin. Subsequently, the preparations of immobilised spermatozoa were kept in 1.0 ml Ziploc bags (1.9 × 1.9 cm), which were inflated with air, gently rotated daily (Babiak and Dabrowski, 2003) and stored at 4°C.

Viability and motility was estimated in samples obtained daily until reaching levels of <15%. The number of dead spermatozoa was assessed by eosin Y stain. A daily sample of stored semen (10 μl) from each preparation was mixed with 10 μl of 0.5% eosin Y stain on a glass microscope slide and viewed using light microscopy to determine the percentage of viable spermatozoa. Live spermatozoa remained unstained whereas dead spermatozoa were stained red (O'Connell et al., 2002).

Motility was estimated after a third dilution (1 : 250) in an activating solution (45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8.0, containing 1% BSA), as the percentage of spermatozoa exhibiting forward motion (Nathanailides et al., 2011). The final density of spermatozoa was about 4–5×10^6 ml^{-1}. Immediately after the third dilution, 10 μl of the activated sperm was placed in a Makler Chamber and viewed using light microscopy at a final magnification ×400. The % of forward-moving spermatozoa was estimated using a computer-aided sperm motility analysis (CASA, TEST SPERM 2.1; Videotest, St. Petersburg, Russia) software. Sperm motility was recorded with 'PINNACLE STUDIO' software (Pinnacle System, GmbH, Braunschweig, Germany). The system included a microscope with a phase-contrast device, a standard microcellular camera for sperm, a colour-analogous system to transfer images to the computer, and special software (VIDEOTEST-SPERM 2.1). Videotaping of sperm motility was conducted at a rate of 25 frames s^{-1}, and the files saved in AVI format. Duration of each single videotaping was 1 s, with three videotapings for each sperm sample. Equipment used for the analysis of sperm motility included a phase contrast microscope (Olympus BX41) and video camera (Sonny Exwave HHD) connected to a computer. Videotaping was conducted at a total magnification of 400×. The settings and procedures used in the present work were based on Pavlov (2006). Briefly, in the present work a Makler chamber (Sefi Medical Instruments, Israel) was used (10 μm depth, 20 μm sample volume). Within 20–30 s after mixing with activating solution, sperm were placed in the chamber and the preparation was focused; the time was considered as the initial swimming time for the present study. Recording of motility was initiated within 10 s of focusing. As a result, the exact time of the registration of motility was within 30–40 s after mixing the thawed sperm with an activating solution. Randomly selected microscopic fields were used (n = 6–7) and at

least 110 motile spermatozoa from each replica (n > 330 spermatozoa from each treatment) were observed for the estimation of the % motile spermatozoa of each sampling day.

Data present means ± SD for triplicate measurements. Percentages were arcsine transformed prior to statistical analysis. Statistical analysis was performed by ANOVA followed by Tukey's *post-hoc* test. Probability values $P < 0.05$ were considered statistically significant.

Results

Effect of the swimming medium pH on the duration of sperm motility of fresh sperm

Motility duration of fresh sperm was significantly reduced at pH 6.5 and 7.0 of the swimming medium when compared to pH 7.5, 8.0 and 8.5 (Fig. 1).

Effect of pH during incubation at 4°C, on duration of sperm motility of reactivated refrigerated sperm

Duration of motility was also lower in the sperm that was incubated at 4°C for 1 h at pH 6.5, when compared to spermatozoa incubated for 1 h at pH 7.8 or 8.5 (Fig. 2).

Effect of pH during incubation at 4°C, on viability and motility of sperm stored at 4°C and reactivated

Incubation of goldfish sperm at pH 6.5 or 8.5 resulted in significantly different viability and motility of spermatozoa after activation. Viability (Fig. 3) and motility (Fig. 4) of both the acid and alkaline group decline in the refrigerated sperm during storage, rapidly compared to viability in both the acid and alkaline incubation treatment. Compared to the alkaline incubation, the acid pre-incubated sperm exhibited a much faster rate of decline of both sperm viability and motility. As a result, on the fourth day of chilled storage, the acid pre-incubated sperm was neither motile nor viable, whereas the alkaline incubation exhibited 62.79 (±2.90) motility and 69.01 (±5.9) viability.

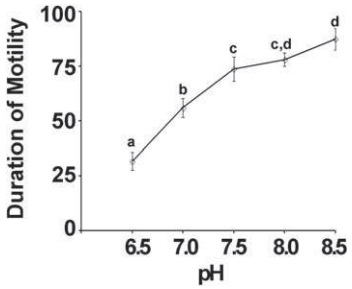

Fig. 1. Effect of the pH of the swimming medium on the duration of sperm motility of fresh goldfish spermatozoa. Sperm was diluted (1:100) in an activating swimming medium (45 mM NaCl, 5 mM KCl, 30 mMTris–HCl) with different pH (6.5; 7.0; 7.5; 8.0 and 8.5). Bars represent standard deviation of triplicates (n ≥ 150 spermatozoa in each replicate) Different letters indicate significant differences (ANOVA, $P < 0.05$)

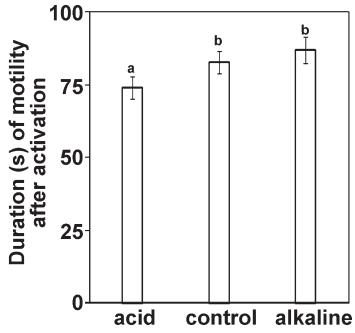

Fig. 2. Effect of the pH, during incubation at 4°C, on the duration of sperm motility of reactivated refrigerated sperm. The sperm was diluted (1:3) in an immobilising solution (125 mM NaCl, 0.1 mM CaCl, 20 mM) with pH 6.5; 7.8 and 8.5 and was activated after dilution (1:100) in an activating solution (45 mM NaCl, 5 mM KCl, 30 mM Tris–HCl, pH 8.0). Bars represent standard deviation of of triplicates (n ≥ 150 spermatozoa in each replicate). Different letters indicate significant differences (ANOVA, $P < 0.05$)

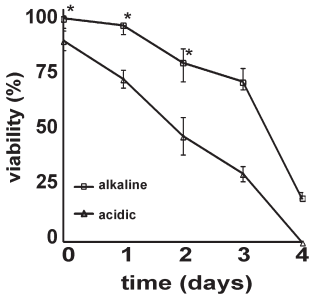

Fig. 3. Effect of the pH (pH 6.5: lines with triangles and pH 8.5: lines with squares), during incubation at 4°C, on the viability of goldfish sperm stored at 4°C and reactivated. Bars represent standard deviation of of triplicates (n ≥ 150 spermatozoa in each replicate)

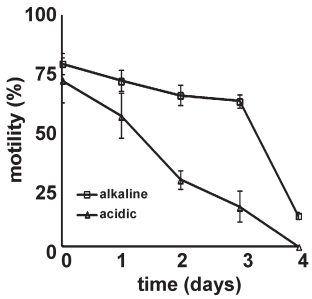

Fig. 4. Effect of the pH (pH 6.5: lines with triangles and pH 8.5: lines with squares), during incubation at 4°C, on the motility (%) of goldfish sperm stored at 4°C and reactivated. Bars represent standard deviation of of triplicates (n ≥ 150 spermatozoa in each replicate)

Discussion and conclusions

The results presented here indicate that acidic conditions during activation or during storage resulted in reduced motility of spermatozoa.

There is some evidence to suggest that the pH of several fish species may vary with season, diet, or level of contamination with urine (Dreanno et al., 1998; Bagheri et al., 2013;

Zadmajid et al., 2013). For example, the semen of common carp may vary from pH 6.5 to 8.0 (Bozkurt and Secer, 2005). Unfavourable acidic conditions can cause sublethal damages, which would compromise both sperm DNA integrity and motility (Santiso et al., 2012) and the fertilising ability of some fish species (Alavi and Cosson, 2005; Gonzalez-Bernat et al., 2013; Dadras et al., 2014). In the present work, sperm motility of goldfish was compromised in acidic conditions, which may be explained by the consequences of a possible reduction in the intracellular pH of sperm during incubation at pH 6.5. Furthermore, during storage in the sealed bags, anaerobic conditions may arise, which would result in elevation of CO_2 and a further decrease in intracellular pH, with consequences for the sperm motility capacity (Morisawa, 1994; Bencic et al., 2000; Woolsey and Ingermann, 2003).

A reduction in sperm quality over a period of chilled storage may be a result of sperm activation, oxidative damage, compromised integrity of cell structures and DNA. Such a reduction indicates lethal damage, whereas the sperm motility indicates the level of sublethal damage (Ciereszko et al., 2005).

The results indicate that even a brief exposure for 1 h to acid conditions can affect sperm viability and motility during short-term refrigerated storage. The correlation between sperm viability and motility in both the acid and alkaline conditions was good, but sperm incubated in alkaline conditions exhibited higher motility and for longer periods of time. The acid group exhibited a dramatic drop in viability and motility after 3 days of chilled storage.

In agreement with the literature, the results of the present work demonstrated a negative effect of low pH on goldfish sperm. In practise, semen may be exposed to an acidic environment due to dietary or seasonal factors or different sampling and handling protocols. In the present work, this negative effect was observed in the spermatozoa activated at acidic conditions or in spermatozoa exposed for 1 h in acidic conditions during storage. In conclusion, even a 1 h brief exposure of spermatozoa to an unfavourable pH environment may result in reduced motility and viability of goldfish sperm and reduce the length of successful storage at 4°C.

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Conflicts of interests

All authors state that they have no potential conflicts of interest.

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