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Motility parameters of perch spermatozoa (*Perca fluviatilis* L.) during short-term storage with antioxidants addition

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Abstract In a natural environment, seminal plasma provides spermatozoa with protection against reactive oxygen species. Storing semen in cooling conditions requires diluting it with various buffer solutions. Therefore, the protective role of seminal plasma is not sufficient enough. Semen obtained from five male specimens was diluted with the Kobayashi buffer solution at a 1:9 ratio. To determine the influence of antioxidants on semen storage, a buffer solution was used, as before, with the addition of 1 % albumin, 1 mM vitamin C, 1.5 mg ml^{-1} vitamin E, 5 mM sodium citrate, 5 mM glutathione and 5 mM cysteine. After the preparation of such tests, the parameters of spermatozoa motility were measured every 3–5 days, using the CASA system (Image House CRISMAS Company Ltd.). Among all used antioxidants, the best effects were observed after the addition of glutathione to semen. After 17 days of storage, the percentage of motile spermatozoa in the samples preserved with glutathione addition was 57 %, while without antioxidant addition, it was 44 %. Furthermore, the addition of cysteine and albumin also resulted in the lengthening of the life span of perch sperm cells. The presence of the remaining antioxidants (vitamins C and E, and sodium citrate) did not have any positive influence on spermatozoa viability, and in these samples, no motile spermatozoa were observed after 12 days of storage. Our data show that dilution of perch sperm with buffered solution might be a promising method for short-term storage.

Keywords Antioxidants · CASA · Motility · Perch · Spermatozoa

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Introduction

Short- and long-term semen storage has recently become important for artificial reproduction in fish. The production of reactive oxygen species that occurs during semen cryoconservation and refrigerated storage (+4 °C) is responsible for lowering the stability of cell membranes, impairing mitochondria work and DNA fragmentation. These processes influence motility parameters of spermatozoa and thus decrease their fertilizing ability (Sanocka and Kurpisz 2004).

In a natural environment, seminal plasma provides spermatozoa with protection against reactive oxygen species. However, the storing of semen in cooling conditions requires a dilution with various buffer solutions, as the protective role of seminal plasma is insufficient (Martinez-Paramo et al. 2009). Research by Bucak et al. (2007) and Thuwanut et al. (2008) indicates that the addition of antioxidants to the buffer solution used during the semen cryopreservation process helps minimize spermatozoa damage due to the presence of free radicals.

For the percid fish, the best recognized species in term of their sperm preservation is walleye (*Stizostedion vitreum*). It was shown that using a simple extender (Moore 1987), it is possible to conduct 10 days of storage without evident loss in their fertilization potential (Satterlield and Flickinger 1995). In the case of pikeperch (*Sander sander*), another percid fish, preservation of their sperm without dilution brings satisfactory results up to only 7 days of preservation (Telea et al. 2008). The results obtained by Glogowski et al. (2008) showed a negative influence of oxygen on the survival rate of rainbow trout (*Oncorhynchus mykiss*) spermatozoa that were stored in vitro. The addition of albumin, proteins with antioxidant properties, allowed the lengthening of the period of rainbow trout semen storage (Kowalski et al. 2009). This study is an attempt to improve the composition of the buffer solution used for perch (*Perca fluviatilis*) semen storage by the addition of selected antioxidants to the extender solution, such as vitamins A and E, sodium citrate, glutathione, cysteine and albumin.

Materials and methods

Fish were wild caught from Sasek Wielki Lake (N/E Poland) and were transported to University of Warmia and Mazury in Olsztyn in April 2012. Average fish weight was 249 g, ±71; length: 21.3 cm, ±5.4. Water temperature was 12 °C. Perch semen ($n = 4$) was obtained in the Department of Lake and River Fisheries, University of Warmia and Mazury. At first, fish were anaesthetized, and then semen, was collected by the massaging of abdominal surfaces. Milt was collected using a syringe attached to a catheter inserted into the urogenital opening (to avoid contamination by urine). Semen from each individuals was diluted with the Kobayashi buffer solution consisting of 7.6 g NaCl, 2.98 g KCl, 0.37 g $CaCl_2 \times 2H_2O$, 0.31 g $MgCl_2 \times 6H_2O$, 0.21 g $NaHCO_3$, 1,000 ml, pH 9.5 (Kobayashi et al. 2004) at a 1:9 ratio. To determine the influence of antioxidants on semen storage, the Kobayashi buffer solution was used, with the addition of 1 % albumin, 1 mM vitamin C, 1.5 mg ml^{-1} vitamin E, 5 mM sodium citrate, 5 mM glutathione and 5 mM cysteine. The pH of each buffer was adjusted to 9.5, as was the control sample, to exclude the influence of pH on the semen storage. The volume of stored diluted semen samples was 100 µl (10 µl of semen with 90 µl of appropriate buffer). Semen that was not diluted (20 µl) with any buffer solution served as the control sample. After 2 days of refrigerated storage (+4 °C), antibiotics were added to each test sample (penicillin and streptomycin,

percentage concentration: 100 IU ml^{-1} and 100 μg ml^{-1}, respectively). All test samples were mixed daily in order to prevent the negative results of spermatozoa sedimentation. After the preparation of such tests, the parameters of spermatozoa motility were measured every 3–5 days, using the CASA system (Image House CRISMAS Company Ltd.). Sperm motion was documented in 2–3 s after the activation in two replicates, with the Basler 202K digital camera integrated with an Olympus BX51 microscope. In order to activate spermatozoa motion, the following fluid was used: 40 mM $NaHCO_3$, 20 mM Tris–HCl, 0.5 % albumin, pH 8.5 (Kowalski et al. 2010). The following sperm motility parameters were analyzed: VCL (total spermatozoa velocity; μm/s), VSL (spermatozoa velocity in a straight line; μm/s), LIN (linearity; %), STR (straightness; %), ALH (amplitude of lateral head displacement; μm), BCF (sperm beat cross-frequency; Hz), MOT (percentage of motile spermatozoa; %) and PRG (percentage of spermatozoa with forward progression; %). Statistical analysis was made using the GraphPad Prism program (GraphPad Software Inc., USA), incorporating the ANOVA two-way analysis of variance. The differences between particular test subjects were established by the Bonferroni posttest.

Results

Perch semen was characterized by the high percentage of motile sperm cells (MOT). The motility parameter of sperm diluted with the Kobayashi buffer solution reached 97 % at the beginning of the experiment. The addition of one of the antioxidants (vitamin C) proved to be the least beneficial the very first day, as semen diluted with the buffer solution containing vitamin C caused a significant decrease in motile spermatozoa (77 %). Perch semen that was not diluted with any buffer solution maintained motility up to the seventh day of testing (Table 1), even though the percentage of motile spermatozoa (MOT) was only 13 %. The addition of vitamins C and E did not result in the lengthening of the life span of sperm cells—on the twelfth day of the experiment, all motility parameters equaled zero, similarly to the control sample. Semen diluted with the buffer solution containing 1 % albumin or 5 mM cysteine showed 22–24 % of motile sperm cells after 17 days of storage. The dilution of perch sperm with pure Kobayashi buffer solution proved to be more beneficial—the percentage of motile spermatozoa was 44 % on the seventeenth day of the experiment. The best results were obtained after the addition of 5 mM of glutathione to the Kobayashi buffer solution—after 17 days of storage, the sperm motility was about 57 %.

Curvilinear sperm speed was decreasing at the end of storage time; however, VSL of the samples preserved with glutathione addition showed the highest value (193 μm s^{-1}) on the twelfth day of storage. At the same time, the LIN value of the samples also reached the highest value (64.7 %).

Discussion

Sperm of European perch is possible to preserve with the use of simple buffer originally prepared for sperm of rainbow trout by Kobayashi et al. (2004) for more than 2 weeks. After 17 days from sperm collection, we noted 56.9 % of motile sperm in the samples 10 times diluted with extender supplemented with 5 mM glutathione. According to our knowledge, this is one of the longest reported periods of perch sperm's successful chilled storage.

Table 1 Perch sperm motility parameters during 17 days of storage at +4 °C

Time (day)	Control	Extender	GSH 5 mM	BSA 1 %	Vit. C 1 mM	Vit. E 1.5 mg ml^{-1}	Sodium citrate 5 mM	Cysteine 5 mM
MOT (%)								
0	93.4^{ax}	97.1^{ax}	92.3^{ax}	92.7^{ax}	77.5^{ax}	82.6^{ax}	94.9^{ax}	96.0^{ax}
4	26.3^{ay}	85.3^{bx}	87.7^{bxy}	57.2^{ay}	31.2^{ay}	25.9^{ay}	56.9^{aby}	75.7^{bxy}
7	11.9^{ay}	75.4^{cxy}	75.9^{cxy}	46.4^{ayz}	10.5^{ay}	23.4^{aby}	46.6^{abcy}	62.5^{bcy}
12	0.0^{ay}	63.6^{byz}	72.7^{bxy}	31.5^{byz}	0.0^{ay}	0.0^{ay}	42.1^{by}	47.4^{byz}
17	0.0^{ay}	43.9^{cz}	56.9^{cy}	22.3^{abcz}	0.0^{ay}	0.0^{ay}	0.0^{az}	24.3^{bcz}
VCL (μm s^{-1})								
0	254^{ax}	269^{ax}	250^{ax}	237^{ax}	191^{ax}	267^{ax}	258^{ax}	261^{ax}
4	93^{ay}	260^{cx}	226^{bcx}	215^{bcx}	151^{abx}	202^{bcx}	254^{cx}	257^{cx}
7	88^{ay}	257^{bx}	216^{bx}	87^{ay}	107^{ay}	197^{bx}	221^{bxy}	260^{bx}
12	0^{ay}	228^{cx}	152^{bx}	0^{ay}	0^{ay}	132^{byz}	203^{bcy}	273^{cx}
17	0^{ay}	214^{cx}	114^{bx}	0^{ay}	0^{ay}	0^{az}	99^{bz}	221^{cy}
VSL (μm s^{-1})								
0	129^{ax}	138^{ax}	147^{ax}	145^{ax}	136^{ax}	112^{ax}	155^{ax}	123^{axy}
4	44^{axy}	170^{bcx}	162^{bcx}	162^{bcx}	172^{bcx}	96^{cx}	128^{cx}	172^{bcx}
7	46^{axy}	162^{bx}	153^{bx}	158^{bx}	33^{ay}	43^{ay}	121^{abx}	141^{bxy}
12	0^{ay}	168^{bx}	193^{bx}	96^{abxy}	0^{az}	0^{az}	74^{abxy}	158^{bx}
17	0^{ay}	157^{bx}	161^{bx}	55^{ay}	0^{az}	0^{az}	0^{ay}	64^{ay}
LIN (%)								
0	47.7^{ax}	48.1^{ax}	52.1^{ax}	51.4^{ax}	52.9^{axy}	47.8^{ax}	52.3^{ax}	45.1^{axy}
4	41.7^{ax}	58.9^{abx}	57.5^{abx}	62.4^{abx}	69.3^{bx}	46.8^{abx}	56.4^{abx}	61.8^{abxy}
7	43.1^{abx}	54.9^{abx}	53.2^{abx}	62.8^{ax}	30.9^{by}	31.6^{bx}	51.3^{abx}	54.7^{abxy}
12	0.0^{ay}	62.9^{bx}	64.7^{bx}	48.9^{bx}	0.0^{az}	0.0^{ay}	44.1^{bx}	67.1^{bx}
17	0.0^{ay}	61.3^{bx}	62.2^{bx}	40.4^{bx}	0.0^{az}	0.0^{ay}	0.0^{ay}	40.6^{by}
ALH (μm)								

Table 1 continued

Time (day)	Control	Extender	GSH 5 mM	BSA 1 %	Vit. C 1 mM	Vit. E 1.5 mg ml^{-1}	Sodium citrate 5 mM	Cysteine 5 mM
0	2.36^{abx}	2.53^{bx}	2.35^{abx}	2.10^{abx}	2.11^{abx}	1.69^{ax}	2.28^{abx}	2.55^{bx}
4	0.90^{ay}	1.93^{bxy}	2.07^{bxy}	1.66^{abxy}	1.28^{abxy}	0.94^{axy}	1.63^{abx}	1.84^{bxy}
7	0.70^{aby}	1.98^{cxy}	2.04^{cxy}	1.41^{axy}	0.51^{byz}	0.87^{ay}	1.48^{axy}	1.76^{cy}
12	0.00^{az}	1.42^{bcy}	1.89^{cxy}	0.96^{by}	0.00^{az}	0.00^{az}	0.98^{by}	1.43^{bcyz}
17	0.00^{az}	1.39^{dy}	1.51^{dy}	0.98^{by}	0.00^{az}	0.00^{az}	0.00^{az}	0.82^{cz}
BCF (Hz)								
0	12.1^{ax}	12.6^{ax}	11.9^{ax}	11.3^{ax}	10.6^{ax}	11.5^{ax}	11.6^{ax}	12.1^{ax}
4	7.8^{ay}	9.8^{axy}	10.5^{ax}	10.4^{ax}	8.2^{ax}	8.3^{ax}	8.7^{ax}	10.0^{axy}
7	6.7^{aby}	9.6^{axy}	10.0^{ax}	10.7^{ax}	3.4^{by}	8.0^{ax}	7.1^{abx}	9.1^{axy}
12	0.0^{az}	9.4^{bxy}	10.0^{bx}	8.0^{bx}	0.0^{ay}	0.0^{ay}	7.6^{bx}	9.6^{bxy}
17	0.0^{az}	8.7^{by}	9.7^{bx}	9.3^{bx}	0.0^{ay}	0.0^{ay}	0.0^{ay}	7.6^{by}

Control sample is undiluted, extender—samples diluted 1:9 with buffer (Kobayashi et al. 2004), GSH—diluted samples with glutathione addition, BSA—diluted samples with bovine serum albumin addition, Vit. C—diluted samples with vitamin C addition, Vit. E—diluted samples with vitamin E addition, sodium citrate—diluted samples with sodium citrate addition, cysteine—diluted samples with cysteine addition. Data shows the percentage of motile spermatozoa (MOT), curvilinear velocity (VCL), a straight linear velocity (VSL), linearity (LIN), amplitude of lateral head displacement (ALH), sperm beat cross-frequency (BCF). Data represent mean values ($n = 4$)

Different letter (a, b, c, d) indicate statistically significant differences between the buffers at the same time; the letters x, y, *z* indicate statistically significant differences between time points for each buffer ($p \leq 0.05$)

At the beginning of preservation, we found the differences in ALH value between the treatments. The highest ALH value we observed in the glutathione and cysteine supplemented samples. The changes in sperm motility after dilution with glutathione were reported for bovine semen (Triwulanningsih et al. 2008). As measurements were done after dilution with extender solution, it is possible that glutathione and cysteine might influence the sperm motility pattern immediately after dilution.

During the preservation, we observed the fluctuation of CASA parameters such as VCL, VSL, LIN, STR and PRG. These changes might be result of individual variation in the preserved samples as individual variation is one of the factors affecting the results of the sperm cryopreservation (Holt 2000). It probably also influenced the chilling storage of sperm in our study. This fluctuation might be also caused by decreasing in percentage of sperm motility. Most probably, the subpopulation of sperm, which exhibits lower speed, at day 12 becomes immotile (VCL less than 15 $\mu m\ s^{-1}$). That might be the cause of increased VSL and LIN value as well as STR and PRG as the remaining sperm most probably predominantly represent the cells of highest speed and linearity of movements.

The positive effect of using fish feed enriched with vitamins C and E in order to enhance spermatozoa antioxidant protection has been known for years (Ciereszko and Dabrowski 1995—rainbow trout; Mansour et al. 2006—arctic char (*Salvelinus alpinus*); Metwally and Fouad 2009—grass carp (*Ctenopharyngodon idellus*). Our previous experiments on the influence of antioxidant addition on the survival rate of ide (*Leuciscus idus*) spermatozoa after cryoconservation proved that not only vitamins C and E, but also glutathione and cysteine, improve sperm motility parameters (Sarosiek et al. 2011). Our tests showed that adding antioxidants such as vitamins C and E or cysteine does not have any beneficial influence on perch semen during its storage. It seems that the storage of perch semen does not require antioxidant supplements, as dilution with a pure Kobayashi buffer solution yielded good results. After almost 2 weeks of storage (12 days), 64 % of sperm was motile and statistically did not differ from the samples preserved with glutathione addition (72 %).

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