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A comparative study on antioxidant systems in semen of species of the Percidae, Salmonidae, Cyprinidae, and Lotidae for improving semen storage techniques

Franz Lahnsteiner *, Nabil Mansour

Department of Organismic Biology, University of Salzburg, Hellbrunnerstr. 34, A-5020 Salzburg, Austria

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ABSTRACT

The present study investigated the antioxidant systems in semen of different teleost fish species (burbot — *Lota lota*, perch — *Perca fluviatilis*, bleak — *Alburnus alburnus*, brown trout — *Salmo trutta*) with the intention to define types and effective concentrations of antioxidants suitable for supplementation of sperm storage solutions and cryopreservation extenders.

Biochemical analysis revealed that in semen of *L. lota*, *P. fluviatilis*, *A. alburnus*, and *S. trutta* antioxidants (ascorbic acid, carnitine, glutathione, methionine, tocopherol, and uric acid) and oxidant defensive enzymes (catalase, gluthatione reductase, peroxidase, and superoxide dismutase) occurred in an almost similar qualitative and quantitative pattern whereby uric acid concentrations and superoxide dismutase activities were high while activities/concentrations of other enzymes and metabolites were low and/or fluctuating. Species-specific differences existed in the occurrence of catalase and carnitine.

Important antioxidants and oxidant defensive enzymes were tested on their sperm protective effect in *in vitro* experiments. Spermatozoa were incubated in sperm motility-inhibiting saline solutions containing the antioxidants or enzymes and thereafter motility was activated with distilled water and measured and membrane integrity and sperm lipid peroxidation were determined. The experiments demonstrated that uric acid is the major antioxidant of semen of the investigated species, as it improves the sperm motility and membrane integrity and decreases the sperm lipid peroxidation. Therefore, supplementation of sperm diluents with uric acid can be recommended to increase the quality of semen whereby the effective concentration was 0.5 mmol/l for all investigated species. Also methionine has importance as antioxidant in teleost fish semen whereby the oxidized form (methionine sulfoxide) was most effective to increase sperm motility and membrane integrity. The effective concentration was 1.5–3 mmol/l. Finally, catalase improved sperm motility and membrane integrity in all species with exception of *A. alburnus* and therefore it can be useful to protect spermatozoa from reactive oxygen species, too. The optimal activity was 2 kU/l for *P. fluviatilis* and *L. lota*, and 0.1 kU/l for *S. trutta*.

1. Introduction

Reactive oxygen species (ROS) can cause different kinds of damages to spermatozoa, as lipid peroxidation of membranes, damage in the midpiece, axoneme, and DNA, as well as loss of motility and fertility (Sikka, 2004; Tramer et al., 1998). The seminal plasma and spermatozoa of teleost fish contain different types of antioxidants (Liu et al., 1995; Ciereszko et al., 2000; Lahnsteiner et al., 2010) which have important meanings in maintaining the semen viability under *in vivo* conditions, and which could have also relevance in practice for supplementation of semen storage media and cryopreservation extenders to improve the quality of spermatozoa.

Ascorbic acid (Ciereszko and Dabrowski, 1995; Metwally and Fouad, 2009) and uric acid (Ciereszko et al., 1999) are considered important antioxidants in semen of teleost fish. In rainbow trout and carp semen methionine and methionine sulfoxide reductase could have antioxidative functions (Lahnsteiner, 2009) and in semen of the Percidae the occurrence of reduced glutathione was described (Stejskal et al., 2008). Oxidant defensive enzymes as catalase, peroxidase, and superoxide dismutase have been detected in seminal plasma and/or spermatozoa of salmonid species, too (Mansour et al., 2006; Lahnsteiner et al., 2010).

Unfortunately, most studies on antioxidative systems in teleost fish, in particular the experimental ones, focus on a limited number of model species, mainly of the family of the Salmonidae, while for other species only little information is available. However, comparative data would be of particular importance to get a more overall picture about sperm biology in teleost fish and to understand the species-specific differences in the occurrence and effectiveness of semen antioxidant systems. These data could also help to develop and improve semen handling and storage techniques for a broader number of species.

* Corresponding author.
E-mail address: Franz.Lahnsteiner@sbg.ac.at (F. Lahnsteiner).

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Table 1
Motility and membrane integrity of the investigated fresh semen samples of *Perca fluviatilis*, *Alburnus alburnus*, *Lota lota*, and *Salmo trutta* used for the experiments. Data are mean ± standard deviation ($n = 8$). The sperm velocity is the average path velocity. The percentage of linear, non linear and circular motile spermatozoa was evaluated for the motile spermatozoa only.

	Perca fluviatilis	*Alburnus alburnus*	*Lota lota*	*Salmo trutta*
Membrane integrity (%)	96.4 ± 6.9	91.1 ± 4.2	92.0 ± 7.3	95.1 ± 5.2
Immotile sperm (%)[1]	5.3 ± 9.8	8.3 ± 6.2	8.8 ± 11.8	8.5 ± 3.8
Locally motile sperm (%)[2]	3.3 ± 13.2	10.2 ± 6.8	15.2 ± 4.1	4.8 ± 8.5
Motile sperm (%)[3]	91.4 ± 12.4	81.5 ± 8.9	76.0 ± 14.2	86.7 ± 12.2
Sperm velocity (μm/s)[4]	121.1 ± 21.9	119.7 ± 15.2	92.0 ± 9.8	117.2 ± 14.3
Linear motile sperm (%)[4]	33.5 ± 14.8	55.8 ± 22.6	46.6 ± 24.6	60.6 ± 21.4
Non linear motile sperm (%)[4]	42.2 ± 13.8	25.8 ± 19.2	28.1 ± 1.7	13.01 ± 8.0
Circular motile sperm (%)[4]	24.3 ± 3.3	18.4 ± 14.3	25.4 ± 25.3	26.4 ± 17.7

[1]velocity < 5 μm/s, [2]velocity of 5–20 μm/s, [3]velocity > 20 μm/s, [4]reported for motile spermatozoa only.

To obtain more information about the antioxidant systems in semen of teleost fish the present study was conducted. Antioxidants (ascorbic acid, carnitine, glutathione, methionine, and uric acid) and oxidant defensive enzymes (catalase, gluthatione reductase, peroxidase, and superoxide dismutase) occurring in spermatozoa and seminal plasma of burbot (*Lota lota*), perch (*Perca fluviatilis*), and bleak (*Alburnus alburnus*) semen were investigated and compared quantitatively and qualitatively with those of brown trout (*Salmo trutta*). Some important antioxidants (ascorbic acid, glutathione, methionine, and uric acid) and oxidant defensive enzymes (catalase) were selected and tested on their sperm protective effect in *in vitro* experiments. Thereby, spermatozoa were incubated in SMIS containing the different antioxidants and thereafter motility was activated and measured and membrane integrity was determined. Antioxidants with a positive effect on sperm viability and their effective concentrations were determined to improve sperm storage solutions and cryopreservation extenders.

2. Material and methods

2.1. Collection of semen

Brown trout (*S. trutta f. fario*), burbot (*L. lota*), and perch (*P. fluviatilis*) semen was obtained from the fish farm Kreuzstein in Upper Austria, bleak (*A. alburnus*) semen from a wild population from Lake Mondsee whereby mature male fish were collected during their spawning time in May by electrofishing. Semen was stripped by abdominal massage. Sperm motility of the collected samples was evaluated by subjective estimations and semen samples with a motility rate <70% were excluded from the experiment. Eight samples were used in each species. The semen was centrifuged at 300 × *g* for 10 min at 4 °C to separate seminal fluid and spermatozoa. The supernatant seminal fluid was centrifuged a second time under similar conditions to exclude possible contamination with spermatozoa. Spermatozoa were diluted in sperm motility-inhibiting saline solution (SMIS), centrifuged a second time to remove remnants of seminal fluid, and finally diluted to concentrations of circa 5×10^8 cells/ml. For brown trout and burbot SMIS consisted of 103 mmol/l NaCl, 40 mmol/l KCl, 1 mmol/l $CaCl_2$, 0.8 mmol/l $MgSO_4$, 20 mmol/l hepes (pH 7.8) — Lahnsteiner et al., 1999; Lahnsteiner et al., 2002), for perch of 150 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgSO_4$, 1 mmol/l $CaCl_2$, and 10 mol/l hepes (pH 7.8) (Lahnsteiner et al., 1995) and for *A. alburnus* of 75 mmol/l NaCl, 70 mmol/l KCl, 2 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, and 20 mmol/l Tris (pH 8.0). Each sperm sample was divided in 3 aliquots. One part was used for the viability experiments, the other part was frozen at −70 °C for enzyme measurements, the third part was diluted with 3 mol/l perchloric acid at a ratio of 1:1 for analysis of antioxidants. Seminal plasma samples were stored at −70 °C, too.

2.2. Analysis of antioxidants and oxidant defensive enzymes in spermatozoa and seminal fluid

Sperm extracts were homogenized after thawing and centrifuged at 1000 × *g* for 10 min at 4 °C. The perchloric acid extracts were neutralized using 1 mol/l KOH. The concentrations of ascorbic acid, carnitine, gluthatione, methionine, and uric acid and the activities of catalase, glutathione reductase, superoxide dismutase, and peroxidase in spermatozoa and seminal plasma were measured with routine enzymatic assays as described in Bergmeyer (1985). Methionine sulfoxide reductase was determined with the modified colorimetrical method of Sagher et al. (2006) based on the reduction of DABS (4-N, N-dimethylaminoazobenzene-4-sulfonyl chloride)-methionine sulfoxide to DABS-L-methionine (Lahnsteiner, 2009). All enzymatic assays were performed at 20 °C. Methionine was analyzed with thin layer chromatography (TLC) using silica gel plates as stationary phase and a 4:1 (v:v) mixture of phenol and 60 mmol/l borate buffer (pH 9.3) as mobile phase. After the plates had been stained with a 0.5% ninhydrin solution in 96% ethanol, methionine was identified based on the RF value (migration distance of spot/migration distance of frontline × 100) and quantified based on the size and color intensity of the spots in comparison to an appropriate control using a ImageJ 1.38x program.

2.3. Influence of antioxidants and oxidant defensive enzymes on sperm motility and sperm membrane integrity

Based on the biochemical analysis, different antioxidants and oxidant defensive enzymes were selected. Washed spermatozoa from 8 semen samples were incubated in SMIS containing 0.5–2.0 mmol/

Table 2
Antioxidants and oxidant defensive enzymes in seminal plasma of *Perca fluviatilis*, *Alburnus alburnus*, *Lota lota*, and *Salmo trutta*. Data are mean ± standard deviation ($n = 8$).

	Perca fluviatilis	*Lota lota*	*Alburnus alburnus*	*Samo trutta*
Enzymes				
Catalase, μmol/min/l	0.14 ± 0.21	0.09 ± 0.07	< 0.001	0.79 ± 0.57
Glutathione reductase, μmol/min/l	15.50 ± 31.17	10.38 ± 6.03	12.11 ± 15.99	22.6 ± 13.5
Methionine reductase, nmol/min/l	4.70 ± 5.83	0.40 ± 0.80	6.22 ± 4.72	22.02 ± 18.21
Peroxidase, μmol/min/l	0.02 ± 0.03	0.03 ± 0.06	0.03 ± 0.01	0.17 ± 0.12
Superoxide dismutase, units/l*	630 ± 497	1006 ± 507	881 ± 148	1142 ± 232
Metabolites				
Ascorbic acid, μmol/l	11.81 ± 12.40	29.43 ± 28.55	8.34 ± 11.51	21.20 ± 19.10
Carnitine, μmol/min/l	0.00 ± 0.00	0.00 ± 0.00	1.4 ± 1.2	0.00 ± 0.00
Glutathione, μmol/l	3.6 ± 4.9	4.1 ± 1.6	3.0 ± 2.0	5.8 ± 1.9
Methionine, μmol/l	1.61 ± 2.06	3.85 ± 2.41	9.07 ± 7.82	32.8 ± 9.9
Uric acid, μmol/l	101.2 ± 37.6	66.1 ± 53.9	66.7 ± 6.8	96.3 ± 33.9

*1 unit inhibits the reduction of cytochrome C by 50% in a coupled system using xanthine and xanthine oxidase.