Arch. Pol. Fish.	Archives of Polish Fisheries	Vol. 12	Fasc. 1	13-21	2004

THE INFLUENCE OF SEMEN CRYOPRESERVATION ON THE RELEASE OF SOME ENZYMES FROM SIBERIAN STURGEON (*ACIPENSER BAERII*) AND STERLET (*ACIPENSER RUTHENUS*) SPERMATOZOA

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ABSTRACT. The milt of individual males of Siberian sturgeon (*Acipenser baerii*) and sterlet (*Acipenser ruthenus*) was frozen without cryoprotector (at –79°C) or cryopreserved with methanol as the cryoprotector. The activity of arylsulfatase (AS), acid phosphatase (AcP), β-N-acetylglucosaminidase (NAGase), and protein concentration was determined. The protein concentration and enzymatic activities in supernatant obtained after cryopreservation were higher than in milt plasma, but they were lower than that in the material obtained after freezing at –79°C. The protein and enzymatic leakage of sterlet spermatozoa was statistically higher in supernatants that had been frozen at –79°C than in those that had undergone cryopreservation. Differences in the protein and AS leakage the Siberian sturgeon supernatants were also observed.

Key words: SIBERIAN STURGEON (*ACIPENSER BAERII*), STERLET (*ACIPENSER RUTHENUS*), SEMEN, CRYOPRESERVATION

INTRODUCION

The cryopreservation of the semen of many teleost fish is widespread (Billard et al. 1995, Babiak et al. 1997a, b, Glogowski et al. 1997a, b). Unfortunately, there is little information available concerning the technology of sturgeon cryopreservation. The gamete biology of teleost fish spermatozoa differs from that of the sturgeon sperm, which possess an active acrosome (Cherr and Clark 1984). Some parameters are available to check the quality of semen following cryopreservation. For example, Ciereszko et al. (1996) measured the acrosin-like activity, Tsvetkova et al. (1996)

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observed early embryo development and Glogowski et al. (1999) checked the percentage of eyed eggs in bream (*Abramis brama* (L.)) following cryopreservation. No information was found regarding the enzymes present in sturgeon semen.

Arylsulfatase and β-N-acetylglucosaminidase are acrosomal enzymes and play an important role during the acrosomal reaction in mammalian spermatozoa. Both enzymes, in concert with others, may aid in the binding to the zona pellucida (Brandon et al. 1997, Carmona et al. 2002). Thus, the biochemical analysis of acrosomal enzymes is significantly important to the understanding of their physiological role in the process of fertilization. This paper represents the first description of the comparison of the amount of protein and enzymatic activities in fresh milt plasma, supernatants obtained after freezing at –79°C and after the cryopreservation of Siberian sturgeon (*Acipenser baerii* Brandt) and sterlet (*Acipenser ruthenus* L.) spermatozoa.

MATERIAL AND METHODS

Siberian sturgeon (n = 19) and sterlet (n = 10) were reared in cages situated in a warm water canal (17°C) supplied by the Dolna Odra power plant (Department of General Zoology, Szczecin University, Poland). All males were injected with one pellet of Ovopel per kg of body weight (Interfish Ltd, Hungary; Horvath et al. 1997), containing 18-20 μg of GnRH analog and 8-10 mg of metoclopramide. Spermiation occurred within 24 h after hormonal stimulation, and the milt was collected using a syringe equipped with rigid tubing that was inserted into the urogenital opening (to avoid contamination with urine).

ANALYTICAL PROCEDURES

The spermatozoa concentration was measured with the optical density method (Ciereszko and Dabrowski 1993) according to the following formula: Sperm concentration ($\times 10^9$ ml^{-1}) = Absorbance at 530 nm × 4.683 + 0.016.

Sperm motility was observed under a microscope (400× magnification) for activated semen according to Jähnichen et al. (1999). One technician evaluated the motility of the fresh semen. Protein was measured with the Lowry et al. (1951) method. The activity of arylsulfatase (AS) was measured using p-nitrocatechol sulphate (NCS, Sigma Aldrich) as a substrate in 0.5 M acetate buffer, pH 4.9 (Gadella et al. 1993). After 30 min of incubation at 37°C, the reaction was stopped with 1 M NaOH and absorbance at 515 nm was measured. The activity of acid phosphatase (AcP) was

measured using 5 mM p-nitrophenylphosphate (disodium salt) in a 20 mM citrate buffer (Glogowski et al. 1996). After 30 min of incubation at 37°C, the reaction was stopped with 0.1 M NaOH and absorbance at 410 nm was measured. β-N-acetylglucosaminidase (NAGase) activity was measured using 0.5 mM p-nitrophenyl β-N-glucosaminide as a substrate in a 0.1 M citrate buffer, pH 5.0 (Jauhiainen and Vanha-Perttula 1986). After 60 min of incubation at 37°C, the reaction was stopped with 0.1 M NaOH and absorbance at 410 nm was measured.

THE INFLUENCE OF SEMEN CRYOPRESERVATION ON THE RELEASE OF AS FROM SPERMATOZOA

After determining the concentration and motility of spermatozoa in Siberian sturgeon and sterlet milt, 1.5 ml of semen was centrifuged to obtain milt plasma, while 1.5 ml of milt was frozen at –79°C (without cryoprotector). Cryopreservation was done according to the method described by Glogowski et al. (2002) using methanol as the cryoprotector.

The protein concentration and enzymatic activities were measured in the fresh milt plasma and supernatants obtained after centrifuging the frozen and cryopreserved semen.

STATISTICAL ANALYSIS

The data were analyzed using the Wilcoxon paired rank test, the Mann-Whitney U-test, and the unpaired t-test. The linear regression coefficient was used to investigate correlations between semen characteristics. Significance of differences were inferred at $P < 0.05$. The GraphPad PRISM package was used for these calculations.

RESULTS AND DISSCUSSION

Table 1 shows that the quality of the Siberian sturgeon and sterlet milt differed, especially with regard to sperm concentration with its high SD value. The Siberian sturgeon sperm concentration was similar to that of sterlet, as was motility (there were no statistical differences between the species). These data indicate that there are individual differences in spermatozoa concentration between sturgeon males.

The effect of cryopreservation on Siberian sturgeon and sterlet milt was compared with the biochemical indicators in the material after freezing the milt without methanol at –79°C and fresh milt plasma. The results indicated that the protein concentration and

TABLE 1

Sperm parameters of Siberian sturgeon and sterlet males used for cryopreservation

	mean ($\bar{x}$)	min - max	SD
	Sperm concentration (10^9 ml^{-1})		
Siberian sturgeon (n=19)	0.64	0.03 - 1.57	0.42
Sterlet (n=10)	0.88	0.12 - 2.15	0.70
	Motility (%)		
Siberian sturgeon (n=19)	76	50 - 90	13.9
Sterlet (n=10)	67	40 - 90	16.4

enzymatic activities were highest in the supernatants obtained after freezing at –79°C and were statistically different from the results in fresh and cryopreserved materials (Table 2). This study indicated that the supernatants obtained after cryopreservation with methanol had a protein concentration and enzymatic activities between the levels established for seminal plasma (minimal damage of spermatozoa) and supernatants after freezing semen without cryoprotector (maximal damage).

TABLE 2

Protein concentration and enzymatic activity in milt plasma and supernatants obtained after freezing milt at –79°C and cryopreserved

	Siberian sturgeon $\bar{x} \pm$ SD	Sterlet $\bar{x} \pm$ SD
	Protein concentration (mg ml^{-1})	
Milt plasma	$0.39^{A} \pm 0.19$	$0.29^{A} \pm 0.12$
Supernatant after freezing at –79°C	$1.18^{B} \pm 0.66$	$1.33^{B} \pm 0.57$
Supernatant after cryopreservation	$0.80^{C} \pm 0.31$	$0.52^{C} \pm 0.19$
	AS activity (U l^{-1})	
Milt plasma	$319^{A} \pm 235$	$132^{A} \pm 101$
Supernatant after freezing at –79°C	$838^{B} \pm 436$	$779^{B} \pm 590$
Supernatant after cryopreservation	$529^{C} \pm 311$	$219^{C} \pm 121$
	AcP activity (U l^{-1})	
Milt plasma	$2.71^{A} \pm 1.97$	$1.63^{A} \pm 1.22$
Supernatant after freezing at –79°C	$11.22^{B} \pm 7.32$	$10.79^{B} \pm 7.69$
Supernatant after cryopreservation	$8.26^{B} \pm 5.74$	$4.16^{C} \pm 2.46$
	NAGase activity (U l^{-1})	
Milt plasma	$24.7^{A} \pm 17.9$	$28.3^{A} \pm 18.1$
Supernatant after freezing at –79°C	$34.1^{B} \pm 17.0$	$42.6^{B} \pm 21.6$
Supernatant after cryopreservation	$30.9^{B} \pm 20.1$	$32.9^{A} \pm 19.6$

Values with different superscript letters among milt plasma, supernatant after freezing at –79°C and supernatant after cryopreservation differ from each other, A, B, C – $p \leq 0.01$

The values of AS, AP activity, and protein concentration were several times higher in supernatants obtained after freezing with and without cryoprotector than in the fresh milt plasma of Siberian sturgeon and sterlet. Only the NAGase activity in sterlet milt plasma and supernatant after cryopreservation did not differ statistically. Thus, the presence of methanol caused a reduction in the amount of proteins. The activities of several enzymes could be an important factor for checking the stability of the spermatozoa membrane during cryopreservation. This parameter could provide useful information about changes in the structure of spermatozoa during freezing. High variation in enzymatic activity might be due to the varied quality of Siberian sturgeon and sterlet sperm.

The leakage of protein from the spermatozoa during freezing with and without cryoprotector was also recorded considering the amount of protein and enzymatic activities in the seminal plasma (Table 3). There were statistical differences in protein and enzymatic leakage between Siberian sturgeon and sterlet milt supernatants that had been frozen at –79°C and those that had been cryopreserved (except AP and NAGase activity for Siberian sturgeon semen).

Additionally, a statistically significant correlation between AS leakage and spermatozoa concentration if the milt was frozen without cryoprotector (Fig. 1) was also

TABLE 3

Enzymatic "leakage" of Siberian sturgeon and sterlet spermatozoa after freezing at –79°C or cryopreservation

	Siberian sturgeon		Sterlet	
	Leakage	Leakage on 10^9 spermatozoa	Leakage	Leakage on 10^9 spermatozoa
	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$
	Protein concentration			
Supernatant after freezing at –79°C	$0.79^{A} \pm 0.49$	1.76 ± 1.80	$1.04^{A} \pm 0.52$	2.12 ± 1.58
Supernatant after cryopreservation	$0.41^{B} \pm 0.23$	1.54 ± 2.37	$0.23^{B} \pm 0.16$	0.66 ± 0.97
	AS activity			
Supernatant after freezing at –79°C	$519^{A} \pm 305$	940 ± 496	$647^{A} \pm 549$	796 ± 341
Supernatant after cryopreservation	$210^{B} \pm 214$	427 ± 341	$87^{B} \pm 80$	196 ± 306
	AcP activity			
Supernatant after freezing at –79°C	8.51 ± 6.85	14.06 ± 6.83	$8.34^{a} \pm 7.60$	10.77 ± 3.29
Supernatant after cryopreservation	5.55 ± 4.86	12.25 ± 9.70	$3.23^{b} \pm 1.93$	3.98 ± 3.34
	NAGase activity			
Supernatant after freezing at –79°CC	9.45 ± 9.13	34.1 ± 53.3	$14.23^{A} \pm 12.17$	50.7 ± 79.7
Supernatant after cryopreservation	6.21 ± 8.00	16.3 ± 19.9	$4.65^{B} \pm 7.42$	21.1 ± 43.3

Values with different superscript letters between supernatant after freezing at –79°C and supernatant after cryopreservation differ from each other, A, B, C – $p \leq 0.005$; a, b – $p \leq 0.05$

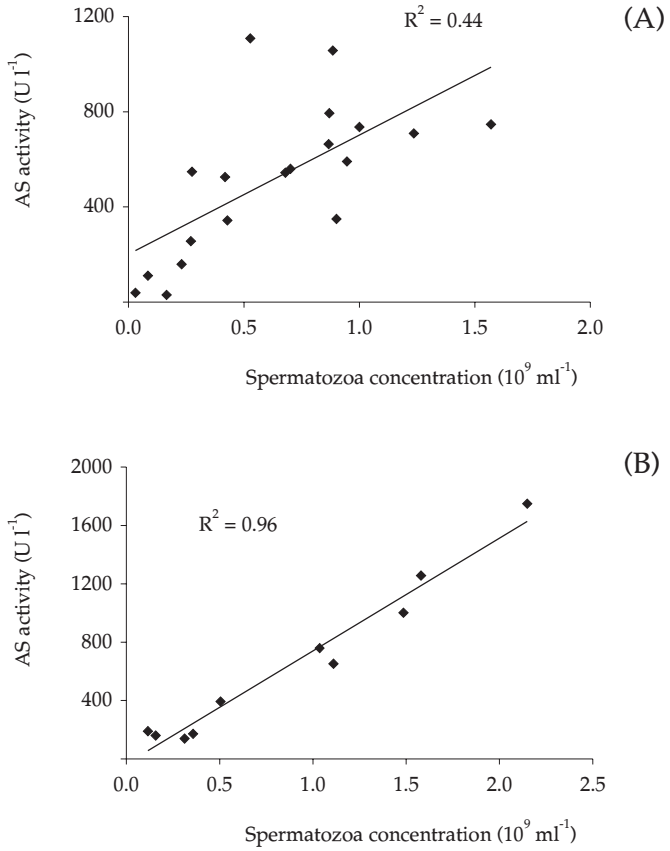

Fig. 1. Correlation between AS leakage and spermatozoa concentration after freezing Siberian sturgeon (A) and sterlet (B) milt without cryoprotector (-79°C).

detected. No correlation was observed when spermatozoa were frozen with methanol (Fig. 2). Additionally, there were high statistical correlations between AS activity, protein concentration in Siberian sturgeon and sterlet spermatozoa supernatants (obtained after freezing at –79°C and cryopreservation) and sperm concentration (data not shown). This fact was probably due to the localization of AS in sturgeon milt, where 70-80% of total AS activity is due to spermatozoa (Sarosiek et al., unpublished data). A correlation between AS activity and protein concentration in Siberian sturgeon milt plasma and spermatozoa concentration (R^2 = 0.61 and R^2 = 0.68, respectively) was also observed. The correlation coefficients for sterlet milt plasma were lower at R^2 = 0.15 and

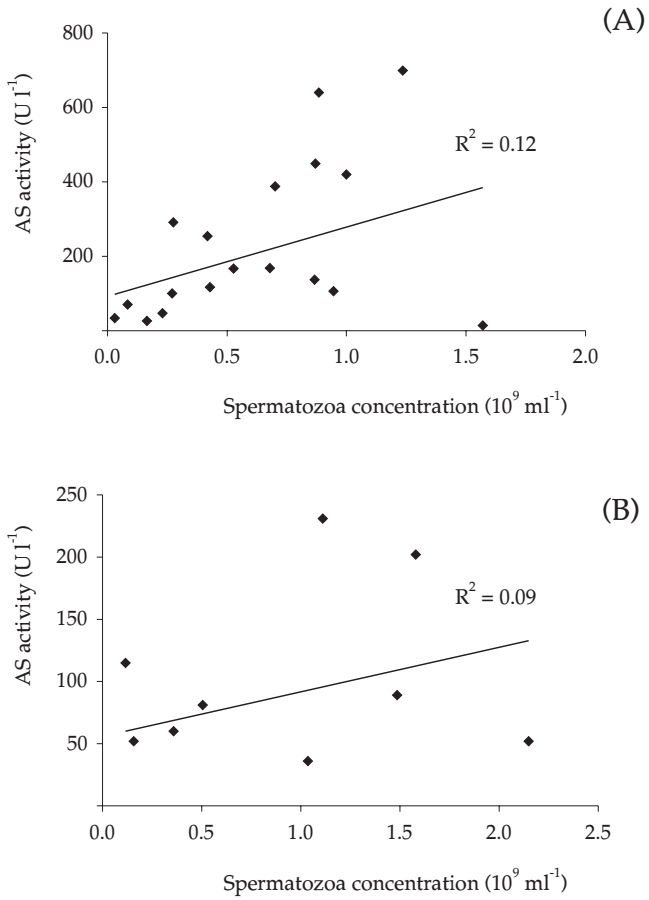

Fig. 2. Correlation between AS leakage and spermatozoa concentration after cryopreservation of Siberian sturgeon (A) and sterlet (B) milt.

R^2 = 0.30. This fact confirmed that the quality of sterlet gametes was better; thus, there was less leakage of protein from the spermatozoa to the milt plasma. This problem was observed earlier by Glogowski et al. (1999) who described a high correlation between sperm concentration and parameters in bream milt plasma.

The results obtained for NAGase are difficult to explain. The role of this enzyme in sturgeon spermatozoa may be similar to that of mammalian sperm NAGase, which is located in the acrosome and is released during the acrosomal reaction (Miller et al. 1993). NAGase has also been found in rainbow trout (*Oncorhynchus mykiss* (Walb.)) spermatozoa (the sperm of teleost fish do not possess an acrosome,

Jamieson 1991, data not presented). The authors of the current work feel that performing laboratory work on the biochemical characteristics of teleost and sturgeon NAGase could aid in understanding the role of this enzyme.

The cryopreservation procedure has been described and successfully designed for mammals. The artificial insemination of cows is done regularly using cryopreserved bull semen (Watson 2000). This method has been effective in the reproduction of several cyprinid fishes too, such as carp (*Cyprinus carpio* L.) (Babiak et al. 1997a) and northern pike (*Esox lucius* L.) (Babiak et al. 1997b, Glogowski et al. 1997a). Unfortunately, there have been rather few successful attempts to cryopreserve the milt of sturgeon, a species that is currently in decline (Beamesderfer and Farr 1997). The experiment described in this paper indicated that measurements of enzymatic activities might be useful in observations of the quality of and changes in sturgeon spermatozoa, and that they might be a good marker of cryoinjury.

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STRESZCZENIE

WPŁYW KRIOKONSERWACJI NA UWALNIANIE ENZYMÓW Z NASIENIA JESIOTRA SYBERYJSKIEGO (*ACIPENSER BAERII*) I STERLETA (*ACIPENSER RUTHENUS*)

Nasienie pobrane od 19 osobników jesiotra syberyjskiego (*Acipenser baerii*) i 10 – sterleta (*A. ruthenus*) zamrożono w –79°C bez obecności krioprotektora oraz poddano kriokonserwacji z metanolem jako krioprotektorem. Koncentrację oraz ruchliwość użytego w eksperymencie nasienia zamieszczono w tabeli 1. W świeżej plazmie nasienia oraz supernatantach uzyskanych po odwirowaniu zamrożonego materiału oznaczono zawartość białka oraz aktywność arylosulfatazy (AS), kwaśnej fosfatazy (AcP) i β-N-acetyloglukozoaminidazy (NAGase) (tabela 2). Oznaczone parametry w supernatancie uzyskanym po kriokonserwacji były statystycznie istotnie wyższe niż w plazmie nasienia i jednocześnie niższe niż supernatancie po mrożeniu bez obecności metanolu (tabela 2). Wykazano również istnienie korelacji pomiędzy „wyciekiem" arylosulfatazy i koncentracją plemników w materiale uzyskanym po mrożeniu w –79°C (rys. 1). W supernatancie po kriokonserwacji nie obserwowano istnienia takiej zależności (rys. 2).