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Original article

Use of biochemical markers to evaluate the quality of fresh and cryopreserved semen from the arctic fox (*Vulpes lagopus*)

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Abstract

The aim of this study was to use biochemical markers to evaluate the quality of fresh and cryopreserved semen from the arctic fox (*Vulpes lagopus*). Twenty-three manually collected ejaculates were analysed for the main indicators of semen quality (sperm concentration and ejaculate volume). Sperm motility and percentage of morphologically normal and abnormal spermatozoa were determined according to the stage of cryopreservation (fresh – measurement A; equilibrated – measurement B; frozen/thawed – measurement C). Furthermore, the seminal plasma and supernatants were analysed after equilibration and freeze/thawing for the activity of the enzymes alkaline phosphatase (ALP), acid phosphatase (AcP), lactate dehydrogenase (LDH) and aspartate aminotransferase (AspAT), and for the activity of acrosin inhibitors (AP). The mean concentration of sperm was 625.1 million/cm^3, and ejaculate volume averaged 1.6 cm^3. Seminal plasma was characterized by the highest activity of alkaline phosphatase (3.43×10^3 U/l) and lowest activity of acrosin inhibitors (4.55×10^3 U/l). After equilibration, the supernatants showed the highest activity of acid phosphatase (94.9 U/l) and after freeze-thawing, they showed a high activity of lactate dehydrogenase (535.8 U/l) and aspartate aminotransferase (577.1 U/l), which indicates that these proteins had leaked from spermatozoa into the extracellular medium during the biotechnique of semen cryopreservation. In addition, several significant relationships were found between some indicators of semen quality and plasma and/or supernatant enzyme activity.

Key words: cryopreservation, semen, arctic fox, biochemical markers

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Introduction

Reproduction of fur animals, including the arctic fox (*Vulpes lagopus*) is a complex phenomenon that depends on many genetic and environmental factors. In an effort to maximize the reproductive potential of males, insemination with both fresh and preserved semen is increasingly used. The cryopreservation process is detrimental to semen quality. According to Niżański and Bielas (2003), low cooling temperature and warming of semen (after freezing) are the main causes of structural and functional damage to male reproductive cells. To avoid cold shock damage to sperm, many studies have been performed to develop improved semen freezing methods and to modify sperm properties at different stages of preservation.

Most researchers focus their attention on assessing sperm motility and morphological normality, while the biochemical evaluation of fox semen has been of peripheral interest. In the available literature, little attention is paid to determining the activity of plasma enzymes during semen cryopreservation despite the fact that they provide information not only about semen quality but also about the condition of gonads. Therefore, finding the relationships between biochemical markers and the quality of fresh and cryopreserved semen may assist in determining the potential fertilizing capacity in males.

The aim of this study was to perform a qualitative and biochemical evaluation of arctic fox semen (fresh and at different stages of cryopreservation), and to determine the relationship between selected quality indicators and activity of enzymes in the extracellular environment.

Materials and Methods

The study used semen collected at one time point from 23 one-year-old arctic foxes (*Vulpes lagopus*), originating from a fur farm in Łachowo near Szubin, the Kujawsko-Pomorskie province, Poland. Semen samples (n = 23) were obtained manually in March. The experiment was approved by the Local Ethics Committee (1/2010). The study was financially supported by departmental sources (Faculty of Animal Breeding and Biology, University of Technology and Life Sciences in Bydgoszcz).

After preliminary assessment (measurement of volume, flow cytometric determination of sperm concentration), a part of each ejaculate was centrifuged (10 min., 8000 x g) to obtain seminal plasma, and the remainder was cryopreserved. Semen was cryopreserved using a modified version of the procedure described by Christiansen et al. (1985). Ejaculates were prediluted 1:2 with an extender (TRIS – fructose – citric acid) and cooled at 5°C for 75 min. Then, a second extender (TRIS, 12% glycerol and 20% egg yolk) was added at a 1:1 ratio and again cooled at 5°C for 2 h (equilibration period).

After equilibration, one-half of the semen was evaluated and centrifuged (as above), and the decanted supernatant was frozen until analysis of enzyme activities. The remainder of the semen was frozen (in 0.5 cm^3 straws in nitrogen vapour – 4 cm over nitrogen for 7 min). After 3 months of storage in liquid nitrogen (Dewary series HC, Taylor-Wharton, USA), frozen samples were thawed in a 37°C water bath, evaluated, and centrifuged, and the supernatant was used for enzyme assays. In addition, sperm plasma membrane status was assessed using a LIVE/DEAD kit (Molecular Probes). The fluorochromes (SYBR-14 and propidium iodide, PI) included in this kit made it possible to distinguish between live and dead spermatozoa depending on cell membrane status (Olympus BX51 fluorescence microscope; 200 spermatozoa evaluated in each preparation). To this end, 100 µl of thawed semen was supplemented with 1 µl of SYBR-14 (diluted 1:20 with DMSO) and incubated for 10 min at room temperature, after which 1 µl of PI was added. After further 10 minutes, the percentage of live spermatozoa (green fluorescence) was determined.

The other semen parameters were evaluated after each stage: fresh – immediately after collection (measurement A); post equilibration – just before freezing (measurement B); and post thaw (measurement C). Sperm motility was examined under a phase-contrast microscope at 400x magnification on a glass slide warmed to 37°C. Smears were used to determine the percentage of morphologically normal and abnormal spermatozoa as classified by Blom (1981). Seminal plasma was analysed for total protein content according to Lowry et al. (1951), seminal plasma and supernatants were analysed for the activity of acrosin inhibitors (AP) using the method of Geiger and Fritz (1983), while the activity of acid (AcP) and alkaline phosphatase (ALP), aspartate aminotransferase (AspAT) and lactate dehydrogenase (LDH) was determined using Alpha Diagnostics enzyme tests. Supernatant enzyme activity was calculated in terms of seminal plasma, taking account of semen dilution rate (x 6).

Correlations between the analysed parameters were determined based on Spearman's coefficients of correlation. Statistical analysis was performed using Statistica 8.0.

Table 1. Quality results and biochemical parameters of semen during three stages of cryopreservation

Biochemical parameter	Measurement A				Measurement B				Measurement C			
	$\bar{x}$	SD	min	max	$\bar{x}$	SD	min	max	$\bar{x}$	SD	min	max
Motility (%)	75.9	10.5	50.0	90.0	61.1	8.9	40.0	75	45.4	11.1	30.0	65.0
Percentage of spermatozoa of normal morphology (%)	96.5	1.0	94.0	97.8	96.7	0.9	94.9	97.8	96.6	0.8	94.9	97.7
Activity ALP x10^4 (U/l)	3.43	1.38	1.15	6.97	2.95	1.21	0.96	5.92	2.76	1.34	0.82	6.22
Activity AcP (U/l)	51.4a	16.2	19.2	85.8	94.9[b]	31.9	61.8	169.5	78.9[b]	31.6	39.0	191.8
Activity LDH (U/l)	300.7[a]	179.5	41.7	561.8	529.2[b]	200.8	199.0	923.9	535.8[b]	192.0	227.8	835.9
Activity AspAT (U/l)	200.3[a]	136.7	38.9	487.9	349.7[a]	211.5	81.7	824.2	577.1[b]	232.4	233.6	955.5
Activity AP x10^3 (U/l)	4.55	2.05	0.78	8.10	5.03	2.45	1.13	9.82	4.64	1.95	1.06	8.05
TEST LIVE/DEAD (%)	–	–	–	–	–	–	–	–	44.75	8.25	30.0	60.0

Notes: measurement A – fresh semen, measurement B – equilibrated semen, measurement C – frozen/thawed semen; "–" – not analysed, a, b – Means followed by different superscripts are different $p \leq 0.05$.

Table 2. Coefficients of correlation between analysed parameters in fresh, equilibrated and frozen/thawed semen.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Measurement A	1 Sperm concentration	1																	
	2 Ejaculate volume	-0.48 *	2																
	3 Motility	-0.21	0.22	3															
	4 Activity AcP	0.72 **	-0.48 *	-0.38	4														
	5 Activity ALP	0.80 **	-0.57 *	-0.23	0.83 **	5													
	6 Activity AP	0.67 **	-0.42	-0.28	0.88 **	0.82 **	6												
	7 Activity LDH	0.74 **	-0.38	-0.35	0.68 **	0.61 **	0.56 *	7											
	8 Activity AspAT	0.82 **	-0.40	-0.34	0.93 **	0.80 **	0.84 **	0.70 **	8										
Measurement B	9 Activity AcP	-0.02	0.10	0.22	-0.10	-0.17	-0.13	-0.04	0.01	9									
	10 Activity ALP	0.78 **	-0.54 *	-0.27	0.84 **	0.94 **	0.79 **	0.68 **	0.78 **	-0.19	10								
	11 Activity AP	0.86 **	-0.49 *	-0.28	0.86 **	0.87 **	0.91 **	0.66 **	0.85 **	-0.11	0.82 **	11							
	12 Activity LDH	0.80 **	-0.36	-0.33	0.72 **	0.63 *	0.61 *	0.94 **	0.82 **	0.05	0.66 **	0.70 **	12						
	13 Activity AspAT	0.74 **	-0.26	-0.15	0.74 **	0.68 **	0.68 **	0.54 *	0.85 **	0.13	0.64 **	0.83 **	0.65 **	13					
Measuremen C	14 Activity AcP	0.58 *	-0.44 *	-0.05	0.58 **	0.56 *	0.59 *	0.58 *	0.61 *	0.17	0.46 *	0.59 *	0.57 *	0.60 *	14				
	15 Activity ALP	0.73 **	-0.47 *	-0.31	0.86 **	0.94 **	0.80 **	0.59 *	0.82 **	-0.26	0.90 **	0.83 **	0.61 *	0.69 **	0.53 *	15			
	16 Activity AP	0.77 **	-0.45 *	-0.34	0.88 **	0.90 **	0.95 **	0.63 *	0.89 **	-0.14	0.87 **	0.94 **	0.67 **	0.73 **	0.48 *	0.87 **	16		
	17 Activity LDH	0.70 **	-0.31	-0.39	0.76 **	0.59 *	0.63 *	0.92 **	0.78 **	0.01	0.66 **	0.66 **	0.91 **	0.63 *	0.58 *	0.60 *	0.67 **	17	
	18 Activity AspAT	0.87 **	-0.39	-0.23	0.76 **	0.75 **	0.77 **	0.59 *	0.81 **	0.03	0.75 **	0.84 **	0.68 **	0.86 **	0.46 *	0.70 **	0.80 **	0.63 *	

* Notes: measurement A – fresh semen. measurement B – equilibrated semen. measurement C – frozen/thawed semen *$p \leq 0.05$; ** $p \leq 0.001$

Results

Ejaculate volume ranged from 1.1 to 1.95 cm^3 (1.61 ± 0.22), and sperm concentration per cm^3 of semen averaged 625.1 ± 310.3 million. The quality indicators and biochemical markers of the semen from arctic foxes, at different stages of cryopreservation, are presented in Table 1. The results of morphological analysis showed that an average of 97% spermatozoa were morphologically normal. Morphological analysis was performed concurrently with biochemical evaluation of semen. Total protein in the seminal plasma of arctic foxes averaged 16.1 ± 3.2 g/l. In fresh semen, protein content was found to be significantly correlated to AspAT activity and AcP activity ($r = 0.43$ and $r = 0.44$, respectively at $p \leq 0.05$). Seminal plasma (measurement A) was characterized by the highest activity of alkaline phosphatase and the lowest activity of acrosin inhibitors. The highest activity of acid phosphatase (94.9 U/l) was found in the supernatant after equilibration (measurement B). After freeze/thawing, the supernatants were rich in lactate dehydrogenase and aspartate aminotransferase.

Both in fresh semen and in supernatants after equilibration and freeze/thawing, the positive correlations found between sperm concentration, ALP, LDH and AspAT enzyme activity, and AP activity were statistically significant ($p \leq 0.001$). Semen volume was negatively correlated with AcP and ALP activity (measurement A), ALP and AP activity (measurement B), and AcP, ALP and AP activity (measurement C). No statistically significant correlations were found between percentage of spermatozoa with normal motility (in fresh and cryopreserved semen) and the analysed biochemical parameters. A significant relationship was found between sperm motility in fresh semen and sperm motility in equilibrated semen ($r = 0.51$ at $p \leq 0.05$) (Table 2).

Discussion

The decreasing sperm motility during successive stages of cryopreservation (about 40%) points to cryogenic damage to sperm substructures, including the motility apparatus. The LIVE/DEAD assay showed that the percentage of live spermatozoa in frozen/thawed spermatozoa was 44.75 ± 8.25%. The lowest percentage of spermatozoa with normal motility supports the opinion of other authors that the freezing-thawing process is the reason why this parameter deteriorates (Niżański and Bielas 2003). In turn, the constant percentage of morphologically normal spermatozoa (about 96%) in all three measurements is evidence of subcellular damage, which cannot be detected by simple microscopic observation. It is only under scanning electron microscopy (SEM) and transmission electron microscopy (TEM) that these microdamages become visible. According to Hofmo and Berg, the condition of the blue fox sperm plasma membrane considerably deteriorates after freezing (1989).

Seminal plasma, a complex mixture of secretions mainly from accessory sex glands, is a fluid especially rich in proteins. The main function of these proteins, through maintaining the optimum composition of lipoprotein membranes, is to protect male gametes against antiproteinases. Due to the high content of protein in hen's egg yolk (found in the second extender), the level of this indicator was only determined in seminal plasma. The average content of total protein in seminal plasma of the arctic fox was similar to the findings of other authors reported for canine semen (Souza et al. 2007, Mogielnicka-Brzozowska et al. 2012).

The seminal plasma of arctic foxes is a rich source of acrosin inhibitors (Stasiak and Janicki 2007), one function of which is to inactive acrosin released from damaged or dead embryos, which prevents a premature activation of proacrosin. The measurements of inhibitor activity at different stages of fox semen cryopreservation indicate that this activity increased considerably in supernatants after equilibration (5.03×10^3 U/l). Meanwhile, the positive correlations (at $p \leq 0.001$) between inhibitor activity and sperm concentration could point to immaturity or damage to sperm membrane lipoproteins. Probably as a result of temperature changes during semen cryopreservation (thermal shock), the continuity is broken and the discussed substances are released from the plasma membrane, or the inhibitors leak from the acrosome. Therefore it seems appropriate to conclude that the rate of increase in acrosin inhibitor activity in seminal plasma at different stages of cryopreservation shows the extent of damage to sperm membranes, and thus a reduction in sperm fertilizing capacity.

Two phosphohydrolases are predominant in seminal plasma: alkaline phosphatase and acid phosphatase. The lowest activity of alkaline phosphatase was characteristic of plasma after thawing. Similar changes in ALP activity for canine semen were obtained by Schafer-Somi et al. (2013), who reported that the activity of this enzyme in seminal plasma after 24-hour freezing decreased by about 10%, compared to a decrease of almost 19% in our study. It may be that such a high decrease in ALP activity was due to temperature denaturation of this protein. The high coefficients of correlation (between sperm concentration and ALP activity) support earlier opinions that in the case of canid semen, the highest alkaline phos-

phatase activity is characteristic of the secretion from epididymides, which serve important functions in the sperm maturation process (Kutzler et al. 2003). In turn, acid phosphatase, which is found in the sperm cell membrane, plays an important role in the conversion of proacrosin into acrosin (Fraser et al. 2006). The highest AcP activity was observed in the supernatants post equilibration. According to Niżański and Bielas (2003), a cold shock occurs when male reproductive cells are cooled to 0°C. This lowers the quality of semen through a change in plasma membrane permeability and "leakage" of the enzymes present in the acrosome.

Aspartate aminotransferase (AspAT) activity depends not only on the function of accessory sex glands, but also on the degree of sperm damage. This is why increased plasma activity of this enzyme is evidence of lowered semen quality (Borkowski and Strzeżek 1994). Our results clearly indicate that AspAT is released into the extracellular environment at the successive stages of cryopreservation. Other authors believe that there are several reasons for increased AspAT activity. One reason is the decreasing ATP content of sperm, in particular of the sperm mid-piece, during cryopreservation. According to Niżański and Bielas (2003), AspAT activity is closely related to sperm ATP content and sperm motility. Another reason for the increasing AspAT activity in the extracellular fluid is the improper concentration of glycerol added to the extended semen. Proper glycerol concentration in the extender (6% in final dilution) and the lack of a statistically significant relationship between the activity of this enzyme and sperm motility may suggest another reason for the increase in AspAT.

Another protein which indicates the degree of damage to plasma membrane is lactate dehydrogenase (LDH). LDH activities determined in the supernatants after both equilibration and freezing did not differ significantly ($p \leq 0.05$). This confirms earlier opinions that sperm structures are most vulnerable to cold shock when semen is cooled to 0°C. Changes in cell membrane permeability of male reproductive cells are due to the phospholipids found in the membranes. In response to low temperature, these compounds undergo a phase change (from liquid to gel), which causes protein and lipid membrane components to separate (Leeuw et al. 1990). In this way ion transport mechanisms are disrupted and the sperm plasma membrane permeability increases (Niżański and Bielas 2003). According to Watson and Plummer (1985), plasma membranes of spermatozoa resistant to a decrease in temperature have an approximately 1:1 quantitative ratio of polyunsaturated to saturated fatty acids. In turn, Miller et al. (2005), who compared the composition of sperm membranes in the silver fox and in the blue fox, found that in addition to the high ratio of unsaturated to saturated fatty acids and a higher level of DPA (22:5, n-6), the membranes must also have a high concentration of sterols (desmosterol and cholesterol).

The present study has confirmed that arctic fox spermatozoa are susceptible to cold shock. Because spermatozoa are the source of most enzymes in seminal plasma, the determination of their activity may provide valuable information about the stability of protein-lipid membranes in male reproductive cells. The increased activity of the analysed enzymes in the extracellular fluid during semen cooling is an indicator of the maximum cell membrane damage.

When evaluating semen quality for its suitability for insemination, the morphological analysis should be expanded to include biochemical markers. The evaluation of acrosome and mitochondria status by determining the activity of proper enzymes, seems useful for assessing the susceptibility of spermatozoa to cooling and freezing.

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