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Original article

Boar variability affects sperm metabolism activity in liquid stored semen at 5°C

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Abstract

Metabolic activity of boar spermatozoa, liquid stored for three days at 5°C, was measured using bioluminescence for ATP content, fluorescent assay (JC fluorochrome) of mitochondrial activity and oxygen consumption. Sperm motility and plasma membrane integrity (PMI) were simultaneously analyzed. Apart from the statistically significant effect ($P < 0.001$) of semen storage time, the importance of the individual source of the ejaculate for the analyzed parameters of metabolic efficiency of spermatozoa was shown. This phenomenon was manifested in the interaction of the individual source of the ejaculate with spermatozoa motility, integrity of their membranes and metabolic activity with the passing time of semen preservation. Recorded results indicate that the individual factor may have a significant influence on the technological usefulness of boar spermatozoa for liquid storage. Quality analyses conducted on boar semen stored at 5°C may be used for pre-selection of boars producing sperm with an enhanced tolerance to cold shock.

Key words: boar, spermatozoa, individual variation, metabolic activity

Introduction

Boar spermatozoa are particularly sensitive to cold shock, which induces changes in the plasma membranes of spermatozoa, resulting in a reduction in their biological properties (Johnson et al. 2000). The effect of cold shock on spermatozoa is dependent on the rate of their cooling, the time and temperature range of storage, as well as the type of applied extender (Paulenz et al. 2000). Substances serving a protective role in relation to spermatozoa plasmalemma, used in the composition of extenders, may inhibit the effect of cold shock on the biological quality of spermatozoa. Such an action was found in relation to the lipoprotein fraction isolated from egg yolks of hens and of the African ostrich (Strzeżek et al. 1999, 2004, 2005, Fraser et al. 2002, Dziekońska et al. 2009).

The deterioration of biological properties of spermatozoa is manifested in their reduced motility. One of the mechanisms involved in the control of spermatozoa motility is modulation of their mitochondrial activity (Flores et al. 2009). Many authors have shown that metabolic efficiency is necessary to maintain spermatozoa motility (Nevo and Polge 1970, Halang et al. 1985, Ruiz-Pesini et al. 1998). Certain authors indicated that spermatozoa of mice (Mukai and Okuno 2004, Flores et al. 2009) and boars (Marin et al. 2003) are capable of maintaining motility after a complete inhibition of mitochondrial function. In such a case spermatozoa of these species produce energy through glycolysis, which indicates their adaptation to changing environmental conditions. Nonetheless, mitochondrial metabolism plays a key role in the regulation of many spermatozoa functions (Flores et al. 2009).

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The energy demand of the motility apparatus of spermatozoa requires a higher level of respiratory activity and a loss of plasma membrane integrity may result in metabolic disorders (Fraser et al. 2002). In our earlier studies it was shown that the evaluation of plasma membrane integrity and mitochondrial activity using different combinations of fluorochromes (PI/JC-1) may facilitate a simple identification of dysfunctional spermatozoa during liquid semen storage (Dziekońska et al. 2009).

Numerous studies conducted on cryopreserved spermatozoa have supplied information that individual variation may have a significant effect on the biological quality of boar semen after thawing, i.e. when subjected to the action of cold shock temperatures (Thurston et al. 2002, Fraser et al. 2007, 2008). In the opinion of Paulenz et al. (2000) boar spermatozoa are subjected to the influence of the above mentioned process at temperatures below 15°C and particularly in the range of 2 to 12°C (Watson 1981, 1995, Watson and Plummer 1985). Thus the aim of our study was to determine the effect of the individual source of ejaculate on metabolic activity of boar spermatozoa stored at 5°C. It is our assumption that studies on the metabolic efficiency of boar spermatozoa at this temperature could prove useful in the detection of individual differences in spermatozoa resistance to the cooling process and in this way be useful in the pre-selection of individuals producing spermatozoa with an enhanced tolerance to cold shock.

Materials and Methods

Semen collection procedure

This study was conducted on 50 ejaculates collected from 5 boars (with 10 ejaculates from each), aged from 8 to 44 months and of the Large White Polish breed. Boars were kept under standard management conditions and fed according to the standard feeding regime. Semen collection were carried out from each boar using gloved-hand technique; once a month over a 10-month period (from September to June). Prior to semen dilution, samples of fresh semen were subjected to micro- and macroscopic analyses. Dilutions were performed on samples of semen, in which motility was at least 70% and in which the percentage of morphologically normal spermatozoa was more than 85%. Motility of spermatozoa was evaluated with a phase-contrast light microscope (Olympus C011) equipped with a heated stage (37°C). Sperm morphology was assessed using the Giemsa staining method (Watson, 1975). The concentration of spermatozoa was counted in a hemocytometric chamber. Permission to conduct this study was granted by the Local Ethics Committee.

Technology of liquid boar semen preservation

Semen samples for preservation were diluted to 30 x 10^6 spermatozoa/ml with the Kortowo-3 extender (69.3 mM fructose, 64.6 mM sodium citrate, 8.0 mM Na_2-EDTA, 14.2 mM potassium acetate) at pH 6.8. Gentamycin was added to the extender at 0.25 g/L.

Diluted semen was subjected to preliminary incubation for 2 h at room temperature and next refrigerated at 5°C.

Evaluation of metabolic efficiency of preserved boar spermatozoa

Metabolic efficiency of preserved boar spermatozoa was evaluated by analyzing motility of spermatozoa, integrity of spermatozoa plasma membrane and mitochondrial activity (PI and JC-1 staining), ATP content and oxygen consumption by spermatozoa (ZO_2). The above mentioned parameters were analyzed in fresh semen and at different time intervals of the preservation process, i.e. after semen dilution (D0), after 48 h (D2) and after 72h (D3).

Evaluation of motility and plasma membrane integrity

Prior to the motility analysis samples of diluted semen were placed in a water bath for 10 min at a temperature of 37°C. In order to analyze spermatozoa motility a sample of semen (6 µl) was spread onto a slide placed on a heated microscopic plate (37°C) and next covered with a cover glass (20 mm x 20 mm), with the percentage of motile spermatozoa being assessed at a 200 x magnification under a light microscope (Olympus BX 40, Tokyo, Japan).

Sperm plasma membrane integrity (PMI) was assessed using propidium iodide, PI (Sigma, St. Louis, MO, USA), as described in a previous study (Fraser et al. 2002). Membrane-damaged PI-stained spermatozoa were examined under an epifluorescence microscope (Olympus CH 30, Tokyo, Japan). PMI was defined as the percentage of intact spermatozoa. Two slides were assessed per sample and 200 spermatozoa were counted per slide.

Fluorescent method to determine mitochondrial activity

The sperm mitochondrial function was assessed using dual staining with fluorescent probes, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, JC-1 (Molecular Probes, Eugene, USA) with propidium iodide (PI, Sigma Chemi-

cal Co., St. Louis, MO, USA), according to a previously described method (Thomas et al. 1998), with some modifications (Dziekońska et al. 2009).

For analyses a volume of 1 ml diluted semen (30 x 10^6 spermatozoa/1 ml) and 3 µl of basic JC-1(1 mg JC-1/ml dimethyl sulfoxide, DMSO) solution were mixed and incubated for 15 min at 37°C. Next 12 µl of the basic PI solution (0.5 mg PI /ml phosphate buffered solution) were added to the sample and it was incubated for 10 min at 37°C. After incubation samples were centrifuged (500 x g, 5 min), the supernatant was removed and the precipitate of spermatozoa was suspended in 1 ml buffer containing 130 mM NaCl, 4 mM KCl, 14 mM fructose, 10 mM Hepes, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.1% BSA, at pH of 7.4. Next 10 µl of spermatozoa suspension were placed on a microscope slide, covered with a cover glass (22 mm x 22 mm) and assessed under a fluorescence microscope (Olympus CH 30, Tokyo, Japan) applying appropriate filters (DMG and DMB). Viable spermatozoa with functional mitochondria emitted orange-red fluorescence. A minimum of 200 cells on the slide were evaluated in randomly selected fields of the slide for each sample.

Determination of ATP contents

The content of ATP was determined in accordance with the protocol of the ATP Bioluminescence Assay Kit CLSII (by Roche Molecular Biochemical). In order to extract ATP from boar spermatozoa 100 µl of a diluted semen sample were supplemented with 900 µl of 0.1 M Tris buffer, containing 4 mM EDTA (pH 7.75). Next samples were placed for 5 min in a water bath at 100°C. Afterwards cooled samples were stored (until further analyses) at a temperature of -20°C.

Bioluminescence of samples was read using a Junior bioluminometer (Berthold Technologies, GmbH & Co. KG, Germany). For this purpose 100 µl luciferase and 100 µl seminal extract were added to the analyzed sample. Next the ATP content in the sample was determined using a standard logarithmic curve for ATP. Results are given in nmol/10^8 spermatozoa.

Determination of oxygen consumption

Oxygen consumption was measured using a YSI Model 5300 Biological Oxygen Monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, USA), with the application of Clarke's electrode following the manufacturer's protocol.

Oxygen consumption determined as coefficient ZO_2 was expressed in µlO_2 consumed by 10^8 spermatozoa in the course of 1 h at 37°C.

Statistical analysis

Computer statistical analysis of results was performed using Statistica 6.0 (StatSoft Incorporation, Tulsa, OK, USA). Recorded results were given as arithmetic means with the statistical error (mean ± S.E.M.). The effect of two factors and interactions between them on analyzed parameters of metabolic efficiency of spermatozoa were calculated using the ANOVA two-way analysis of variance. Differences between arithmetic means were calculated with the use of a multiple test (the Neuman-Keuls test) taking into consideration individual sources of ejaculate. Statistically significant differences between arithmetic means were calculated at the level of significance $p \leq 0.05$.

Results

Table 1 presents the results of the ANOVA two-way analysis of variance, the Fisher test (F) and the significance test (P) concerning the effect of the individual source of ejaculate and storage time and dependencies between these factors on analyzed indexes of metabolic efficiency of stored boar spermatozoa. It needs to be stressed that values of all analyzed parameters were dependent on the individual source of ejaculate and preservation time. The individual factor had a statistically highly significant effect ($P < 0.001$) on the percentage of motile spermatozoa, the percentage of spermatozoa with intact plasma membrane, the percentage of spermatozoa with active mitochondria, oxygen consumption and ATP content. Moreover, an interaction was found between the individual source of ejaculate and storage time, which had a statistically significant effect ($P < 0.001$) on motility of spermatozoa, integrity of plasma membranes and mitochondrial activity.

Table 2 presents the analysis of motility and integrity of plasma membranes of spermatozoa coming from fresh ejaculates and those stored in Kortowo-3 extender at D0, D2 and D3 time points at a temperature of 5°C. Among the 5 analyzed boars (A, B, C, D, E) boar D was characterized by the lowest percentage of motile spermatozoa and the percentage of spermatozoa with intact plasma membranes (PMI) irrespective of storage time. The highest percentages of motile spermatozoa and of spermatozoa with intact plasma membrane were recorded for boars A and B. Statistically significant differences ($P < 0.05$) were found between the analyzed boars.

Table 1. ANOVA sources of variation in sperm quality characteristics following storage of boar semen in Kortowo-3 extender at 5°C.

Source	df	Motility		Plasma membrane integrity (PMI)		Mitochondrial energy status (JC-1)		Oxygen consumption (ZO_2)		ATP content	
		F-test	P-value	F-test	P-value	F-test	P-value	F-test	P-value	F-test	P-value
Boar	4	729.160	0.001	216.090	0.001	259.720	0.001	77.930	0.001	41.860	0.001
Storage time	2	274.360	0.001	48.110	0.001	21.100	0.001	20.700	0.001	80.710	0.001
Boar × storage time	8	7.055	0.001	5.150	0.001	5.160	0.001	1.126	0.345	2.882	0.004

ANOVA was used to analyze interactions of main effects: boar (A;B;C;D;E) and storage time (Day 0; Day 2; Day 3); df – degree of freedom; F – Fisher test

Table 2. Sperm motility (%) and sperm plasma membrane integrity-PMI (%) in fresh and stored boar semen in Kortowo-3 extender at 5°C. Values represent means (± SEM) of 10 ejaculates from 5 boars each. Within rows values with different superscripts (a-c) are significantly different at $P < 0.05$ (Neuman-Keuls *post hoc* test).

Boar (age-month)	Fresh semen		Preserved semen					
			Day 0		Day 2		Day 3	
	Motility	PMI	Motility	PMI	Motility	PMI	Motility	PMI
A (12-22)	76.0 ± 1.0^{a}	95.5 ± 0.4^{a}	72.0 ± 2.4^{a}	94.2 ± 2.1^{a}	40.5 ± 5.1^{a}	67.0 ± 3.5^{a}	32.5 ± 4.5^{a}	62.0 ± 4.2^{a}
B (34-44)	75.5 ± 0.5^{a}	93.0 ± 1.9^{a}	66.5 ± 4.7^{a}	93.3 ± 1.4^{a}	40.0 ± 4.1^{a}	61.7 ± 1.5^{ab}	29.5 ± 3.9^{a}	55.6 ± 6.1^{ab}
C (20-30)	72.5 ± 1.3^{a}	91.5 ±1.4^{ab}	65.0 ± 5.6^{a}	87.3 ± 4.3^{ab}	14.0 ± 5.5c	64.8 ± 4.4^{ab}	11.3 ± 3.6^{b}	52.7 ± 5.7^{bc}
D (8-18)	70.0 ± 2.0^{a}	85.1 ± 4.8^{b}	50.0 ± 4.4^{b}	72.3 ± 6.1^{b}	13.0 ± 3.3^{c}	47.0 ± 5.1^{b}	05.0 ± 1.7^{c}	39.2 ± 5.7^{c}
E (8-18)	73.0 ± 2.8^{a}	92.6 ± 0.9^{a}	66.0 ± 2.9^{a}	86.7 ± 4.4^{ab}	26.0 ± 3.8^{b}	55.0 ± 4.5^{ab}	16.0 ± 3.3^{b}	46.8 ± 5.8^{bc}

Table 3. Sperm mitochondrial function %, ATP content (nmol/10^8 spz) and oxygen consumption (µl O_2/10^8 spz/1h/37°C) in fresh and stored boar semen in Kortowo-3 extender at 5°C. Values represent means (± SEM) of 10 ejaculates from 5 boars each. Within rows values with different superscripts (a-c) are significantly different at $P < 0.05$ (Neuman-Keuls *post hoc* test).

Boar (age-month)	Fresh semen			Preserved semen								
				Day 0			Day 2			Day 3		
	JC-1	ATP	ZO_2	JC-1	ATP	ZO_2	JC-1	ATP	ZO_2	JC-1	ATP	ZO_2
A (12-22)	95.5±0.4^{a}	14.3±1.1^{ab}	29.2±2,9^{ab}	94.2±0.6^{a}	11.8±0.9^{a}	24.3±2.2^{a}	67.0±3.5^{a}	11.3±1.4^{a}	20.8±2.6^{a}	60.0±3.3^{a}	7.5±1.0^{a}	15.5±2.2^{a}
B (34-44)	92.9±1.9^{a}	11.9±0.9^{b}	22.6±2.3^{ab}	93.3±1.4^{a}	11.6±0.9^{a}	26.2±1.6^{a}	61.7±4.5^{a}	9.5±0.7^{a}	18.6±1.7^{ab}	52.6±6.2^{ab}	7.6±0.8^{a}	14.4±2.1^{a}
C (20-30)	91.5±1.4^{ab}	11.5±0.7^{b}	20.7±1.8^{b}	87.3±4.3^{ab}	12.0±0.7^{a}	24.6±2.3^{a}	55.8±5.8^{ab}	7.1±1.2^{ab}	12.6±3.1^{bc}	51.4±5.6^{ab}	7.1±0.8^{a}	12.5±2.4^{ab}
D (8-18)	84.7±4.8^{b}	12.5±1.5^{ab}	17.5±0.6^{b}	77.5±2.7^{b}	9.6±0.9^{a}	14.2±0.9^{a}	47.1±5.1^{b}	3.8±0.5^{c}	10.1±1.2^{c}	39.5±5.8^{b}	2.8±0.5^{b}	7.4±1.1^{b}
E (8-18)	92.9±0.8^{a}	15.2±0.9^{a}	29.1±3.5^{a}	89.7±1.9^{ab}	11.7±0.9^{a}	20.6±1.9^{a}	53.9±5.1^{ab}	9.6±1.1^{ab}	15.9±2.5^{ab}	45.8±6.2^{b}	7.4±1.6^{a}	12.2±3.1^{ab}

Table 3 presents changes in the percentage of spermatozoa with active mitochondria, ATP content and oxygen consumption (ZO_2) in fresh ejaculates and those which were stored (D0, D2, D3). Within the analyzed parameters considerable differentiation was shown, determined by the individual source of ejaculate. On the first day of storage the lowest percentage of spermatozoa with active mitochondria was found for boar D. Between boar D and the other boars statistically significant differences were observed ($P < 0.05$). In the case of the analysis of ATP content the lowest values were recorded for boars B and C, with the highest for boar E. In terms of oxygen consumption the highest values were found also for boar E, while the lowest were recorded for boars C and D. On the second and third day of storage (D2 and D3)

the highest values of analyzed parameters were obtained for boars A and B, with the lowest for boar D, whereas in the case of the analysis of mitochondrial activity on the third day of storage (D3) it was also the lowest for boar E. Between the analyzed boars statistically significant differences ($P < 0.05$) were observed in the analysis of mitochondrial membrane potential between boar A, and boars D and E; in ATP content between boar D, and the other boars (A, B, C and E), while in terms of oxygen consumption (ZO_2) it was between boar D, and boars A and B.

Discussion

Results of investigations indicate that individual variation plays a significant role during semen storage at a temperature of 5°C. In the presented investigations it was observed that all the analyzed parameters of semen quality (i.e. the percentage of motile spermatozoa, plasma membrane integrity and mitochondrial activity, ATP content and oxygen consumption by spermatozoa) were dependent on the individual source of ejaculate.

The effect of individual variation on the quality of boar spermatozoa has mainly been noted in reference to spermatozoa subjected to cryopreservation (Larsson and Einarsson 1976, Thurston et al. 2002, Saravia et al. 2005, 2009, Roca et al. 2006 a,b, Fraser et al. 2007). Investigations conducted by Roca et al. (2006a) indicated that individual differences are the main factor explaining differences in the viability of cryopreserved spermatozoa. Thurston et al. (2002) and Fraser et al. (2008) showed that differences in the quality of cryopreserved boar sperm are determined genetically. The authors – using AFLP technology (amplified fragment length polymorphism) – identified markers connected with genes affecting freezing capacity of semen. AFLP technology was used to select boars producing sperm characterized by "good quality" after thawing. The basic mechanisms of individual genetic differences connected with the susceptibility to damage induced by cryopreservation remain unknown, but they may be manifested in the varied biochemical composition of semen (Holt et al. 2005). For example, Waterhouse et al. (2006) stated that differences in individual sensitivity of boar spermatozoa to cryopreservation are connected with the amount of long-chain polyunsaturated fatty acids (PUFAs) contained in plasma membranes. Since boar spermatozoa are characterized by a high level of long-chain polyunsaturated fatty acids (PUFAs) and a low cholesterol level in membranes, they are particularly sensitive to cooling processes (Park and Lynch 1992, Penny et al. 2000).

Individual differences in semen quality are connected with the composition of seminal plasma (Strzeżek et al. 2005, Rath et al. 2009). Flowers and Turner (2001) showed a relationship between different protein profiles of seminal plasma and the fertilizing capacity of spermatozoa produced by different individuals. Proteins are major components of seminal plasma and they have a positive or negative effect on the functioning of highly diluted fresh spermatozoa (Caballero et al. 2004). Some of them are capable of protecting spermatozoa against cold shock (Pursel et al. 1973) and destabilization of plasma membranes (Maxwell and Johnson 1999). Hernández et al. (2007) showed the protective effect of an addition of seminal plasma to the extender used in cryopreservation against cryogenic damage to spermatozoa, which was determined by the origin from the so-called "poor", "average" or "good" boars. An addition of seminal plasma coming from "good" boars considerably improved motility and viability of cryopreserved spermatozoa and their fertilizing capacity. In our investigations whole ejaculate was used for liquid storage. Thus individual differences in the biochemical composition of seminal plasma could have affected observed changes in terms of the analyzed parameters, i.e. motility or integrity of plasma membranes, as well as mitochondrial efficiency (mitochondrial activity, the rate of ATP synthesis and oxygen consumption) of stored boar spermatozoa.

As our results indicate, viability of spermatozoa subjected to the action of a temperature of 5°C was dependent on the individual source of ejaculate. Thus, boars used in this experiment produced spermatozoa with varying resistance to cold shock. Cold shock is one of the most important factors in explaining the irreversible decrease in permeability and integrity of all plasma membranes, which lead to disturbed functioning of cells and cell death (Watson and Plummer 1985, de Leeuw et al. 1990, 1991, Watson 1995). Cooling of diluted semen promotes maintenance of spermatozoa viability thanks to the reduction of metabolic activity of cells, which leads to a considerable decrease in energy demand and product formation (Althouse et al. 1998). Thus, in our study changes in the motility of spermatozoa were correlated with a reduction in the percentage of spermatozoa with intact plasmalemma (data not presented). Preservation of plasma membrane integrity of spermatozoa is required for the maintenance of the efficiency of metabolic processes. Motility of spermatozoa is connected with an adequate amount of ATP, and thus the efficiency of processes generating this compound, primarily oxidative phosphorylation, taking place in spermatozoa mitochondria (Halang et al. 1985). Aalbers et al. (1985) showed that the concentration of ATP is closely connected with the frequency of movements of the tails of spermatozoa after cryopreservation; a similar phenomenon was observed in stored liquid boar semen. Juonala et al. (1998) reported that during

storage of boar semen for 7 days the observed lower percentages of motile spermatozoa and spermatozoa with progressive movement were accompanied by a decrease in ATP content. The reduction of motility observed in spermatozoa in our investigations was accompanied by a similar decrease in ATP content and oxygen consumption.

To sum up we have shown that the individual factor has a significant effect on values of analyzed metabolic efficiency indexes. Boars producing semen with higher values of analyzed parameters were also characterized by their higher values during long-term liquid preservation using a critical liquid preservation temperature.

Conclusions

On the basis of our results we may conclude that the individual factor may have a significant effect on the suitability of boar spermatozoa for preservation. Causes of individual variation in metabolic efficiency remain unclear.

The application of a temperature of 5°C for semen storage may be used as a test in the preliminary selection of individuals producing spermatozoa with an enhanced cold shock resistance and thus their potentially higher viability after the cryopreservation process.

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