REPRODUCTIVE BIOLOGY

ORIGINAL RESEARCH

The photon emission, ATP level and motility of boar spermatozoa during liquid storage

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SUMMARY

Changes were studied in induced photon emission (as an indicator of oxidative stress), ATP level and sperm motility during seven day-storage of boar semen at 15°C extended with the use of BTS extender. Photon emission was measured using a luminometer equipped with a cooled photomultiplier with a spectral response range from 370 to 620 nm. The time of storage had a significant effect on luminescence parameters (integral and peak max), intracellular ATP level and percentage of motile spermatozoa. The increase in luminescence parameters was paralleled by a decrease in ATP level and sperm motility. A significant correlation was found between the percentage of motile spermatozoa and integral (r=-0.27) and peak max (r=-0.31). ATP level was correlated with integral (r=-0.25) but not with peak max. Our results suggest that reactive oxygen species and products of cell membrane lipid peroxidation have a negative effect on ATP level and sperm motility. Induced luminescence assessment in combination with sperm motility and

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ATP level can give valuable information about the status and function of spermatozoa which may be relevant for predicting the fertilizing potential of the semen. *Reproductive Biology, 2009, **9**, 1: 39-49.*
Key words: boar, semen, photon emission, ATP level, spermatozoa motility, lipid peroxidation

INTRODUCTION

In commercial practice, boar semen used for artificial insemination is stored in a liquid state after dilution in an appropriate extender. Insemination with frozen boar semen generally results in low fertility and fecundity. Therefore, liquid preservation in pigs remains the preferred . In practice, semen is usually stored for only three days, but attempts have been made to extend the time of semen storage without reducing fertility indices [16, 20]. Every extender should provide an energy source for stored sperm cells and ensure a proper pH and osmotic pressure. It also protects sperm against thermal shock and inhibits bacterial growth.

The selection of the optimum semen preservation method depends on accurate and objective sperm evaluation which enables identification of structural and biochemical parameters related to decreased quality of extended semen. The classical method of semen evaluation is based on the application of numerous tests measuring progressive sperm motility, the percentage of viable cells and sperm morphology. The classical laboratory methods are usually insufficient for predicting fertility because only samples with markedly poor quality can be detected, although the use of combined tests can provide additional information for evaluating sperm quality [9, 25].

The luminescent method evaluating sperm oxidative damage is among the promising methods of semen quality evaluation that have emerged over recent years [12, 13, 14, 21]. Previous studies show that induced photon emission (chemiluminescence) is strictly related to lipid peroxidation of spermatozoa [10, 11, 24]. The aim of this study was to determine photon emission parameters of boar spermatozoa stored in a liquid state. Changes in induced photon emission (as an indicator of oxidative stress) were moni-

tored and compared with adenosine triphosphate (ATP) spermatozoa level and motility of boar spermatozoa during seven day-storage at 15°C in BTS extender.

MATERIALS AND METHODS

Semen collection and dilutions

The study was conducted between July and December. We used boar semen obtained from the Pig Research Center in Żerniki Wielkie, Poland. Semen (22 ejaculates) from five adult crossbred boars aged from two to four years was used in the experiment. Semen was collected using the gloved hand technique. After separation of the gel, sperm motility and concentration was determined. Ejaculates used in the experiment had 150-380 ml of volume, 177-580×10^6 sperm/ml of concentration and 60-80% of motility. Semen samples were diluted to a final concentration of 30×10^6 sperm/ml in BTS extender (Minitub, Tiefenbach, Germany), which is claimed to preserve semen for three days, and stored for seven days at 15ºC. Luminescence, ATP concentration and motility of spermatozoa were measured on 1st, 4th and 7th day of semen storage.

Chemiluminescence (CL) measurement

Chemiluminescence was measured at 20ºC using an AutoLumat LB953 (Berthold, Bad Wildbad, Germany) luminometer equipped with a cooled photomultiplier with a spectral response range from 370 to 620 nm. Prior to the measurement of CL, spermatozoa were separated from the seminal plasma by two-fold centrifugation (700×g, 15 min) and resuspended in 0.9% NaCl to a concentration of 200×10^6 spermatozoa/ml. 10 µl of 5 mM luminol (purity≥97%; Sigma-Aldrich, USA) was added to 700 µl of the washed sperm suspension (concentration of 200×10^6 spermatozoa/ml). Emission was induced by adding (with the use of an automated injector system) 100 µl of 0.4 mM $FeSO_4$ (Sigma-Aldrich, USA) solution (final concentration 0.05 mM).

Immediately after injection, light emission kinetics was measured during 600 seconds (fig. 1) and the following CL parameters were calculated: integral i.e. total integral of the measurement signals (counts/integration time) and peak max (cps): height of highest peak.

ATP measurement

ATP was extracted from spermatozoa and assayed using the firefly luciferase bioluminescent assay kit (Cambrex Bio Science, Rockland, USA). This ATP assay is based upon the quantitative measurement of a stable level of light produced as a result of an enzyme reaction catalyzed by firefly luciferase. The amount of light generated by this enzymatic reaction is then measured in a luminometer and is directly related to the amount of ATP in the sample.

Briefly, a 10 µl aliquot of semen sample was mixed with 100 µl Cell Lysis Reagent and incubated at room temperature for five minutes to extract ATP from cells. Following the addition of 100 µl ATP Monitoring Reagent,

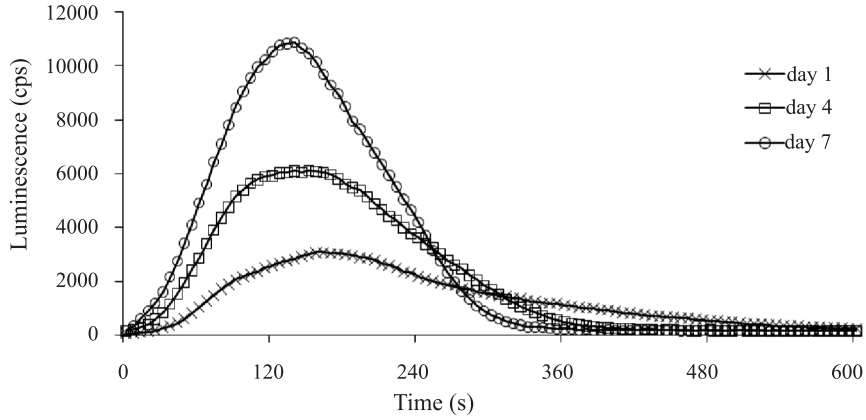

Figure 1. Kinetics of induced luminescence (cps: counts per second) from boar spermatozoa after different time of storage (integral: total integral of the measurement signals for each sample = area under curve; peak max: height of the highest point of curve)

luminescence was measured using an AutoLumat LB953 luminometer. The generated signal was compared to standard ATP dilutions. Sperm ATP content from each probe was assessed in duplicates.

Assessment of sperm motility

Samples of semen were incubated at 37°C. After 15 minutes of incubation, motility was determined using a phase contrast microscope (100× magnification) equipped with a heating plate (37°C). The motility was assessed throughout the study by a trained technician and expressed as percentage of progressively motile spermatozoa.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) according to the GLM procedure of the Statistical Analysis System (SAS Institute, Inc., Cary, USA). The model included day of storage, boar and the interaction (day of storage by boar) as possible sources of variation. When ANOVA revealed a significant treatment effect, means were compared using Duncan's multiple range test. The significance of differences between means was tested at $p<0.05$ and $p<0.01$ using Duncan's multiple range test. The correlations between luminescence parameters, sperm ATP content and motility were performed using Pearson's rank method.

RESULTS

The time of storage had a significant effect on CL parameters, intracellular ATP level and percentage of motile spermatozoa (tab. 1). A significant increase in integral and peak max was observed during semen storage. The increase in CL parameters was paralleled by decreases in ATP level and sperm motility. Particularly significant was the increase of integral during the initial period of storage (between day 1 and day 4); the increase between day 4 and day 7 was not so marked. A similar tendency was observed for

Table 1. The effect of storage time on luminescence parameters (integral and peak max), ATP concentration and sperm motility (mean±SEM) of semen diluted in BTS extender

Parameters	Day 1	Day 4	Day 7
Integral	13.9 ± 1.2^{a}	$17.5\pm1.4^{a,b}$	19.6 ± 1.8^{b}
Peak max	8.5 ± 1.2^{a}	$11.5\pm1.8^{a,b}$	14.6 ± 1.8^{b}
ATP concentration (nmol ATP/10^8 spermatozoa)	17.1 ± 5.3^{A}	11.9 ± 5.3^{B}	10.8 ± 5.1^{B}
Motility (%)	56 ± 3.2^{a}	$48\pm3.1^{a,b}$	45 ± 3.0^{b}

n=22 ejaculates from 5 boars; values within a row with different superscripts are significantly different at $p<0.05$ (a, b) or $p<0.01$ (A, B)

ATP level and sperm motility. The decrease in ATP level and sperm motility was 25% and 8% between day 1 and day 4, and 8% and 3% between day 4 and day 7, respectively. During 7 day-storage, ATP level decreased markedly by over 33%, while sperm motility decreased only by 11%. The increase in the peak max values was more consistent over the entire seven days of storage.

A significant correlation was found between the percentage of motile spermatozoa and integral ($r=-0.27$, $p<0.05$) and peak max ($r=-0.31$, $p<0.05$). The ATP level was significantly correlated with integral ($r=-0.25$, $p<0.05$) but not with peak max. We observed differences among males in the CL parameters (tab. 2). The sperm of B and E boars was characterized by the lowest, and the sperm of D boar by the highest values of integral and peak max. On day 1 of storage, ATP concentration in sperm ranged from 13.4 nmol ATP/10^8 spermatozoa in the semen of boar D to 20.5 nmol ATP/10^8 spermatozoa in the semen of boar C. On day 7 of storage the highest decrease in ATP level was observed for the semen of boar C and the lowest for the semen of boar E. The lowest motility on day 1 of storage was observed for the semen of boar E (36%) and D (49%). The sperm motility of boar E was the lowest but it almost did not change during storage. On day 1, the highest sperm motility was observed for boars A, B and C, it ranged from 63 to 67%. The highest decrease in motility during storage was noticed in the semen of boar B.

Table 2. Luminescence parameters (integral and peak max), ATP concentration and sperm motility (mean±SEM) in semen stored in BTS extender for seven days at 15ºC

Items	Day	Boar (no. of ejaculates)				
		A (5)	B (4)	C (4)	D (5)	E (4)
Integral	1	12.8±2.7^{a,b}	11.7±1.9^{a,b}	16.3±2.0^{a,b}	18.4±1.0^{a}	9.5±3.4^{b}
	4	16.1±2.3^{a,A,B}	14.4±1.6^{a,A,B}	19.4±2.2^{a,b,A,B}	24.5±2.2^{b,B}	11.9±4.4^{a,A}
	7	19.6±3.3^{a}	14.9±3.0^{a}	22.5±4.4^{a}	25.3±3.1^{a}	14.2±5.5^{a}
Peak max	1	6.5±1.9^{a}	4.6±1.5^{a}	10.7±2.1^{a,b}	14.3±1.7^{b}	5.6±3.5^{a}
	4	8.4±2.1^{a,A}	6.5±1.2^{a,A}	13.7±2.5^{a,b,A,B}	20.9±2.4^{b,B}	6.5±3.8^{a,A}
	7	12.3±3.2^{a,A,B}	7.1±2.0^{a,A}	17.7±4.1^{a,b,A,B}	23.0±2.1^{b,B}	11.2±4.7^{a,A,B}
ATP concentration (nmol ATP/10^8 spermatozoa)	1	17.8±0.8^{a,b}	16.2±1.0^{a,b}	20.5±3.6^{a}	13.4±2.4^{b}	18.0±3.0^{a,b}
	4	13.6±1.7^{a,b}	9.4±1.6^{a}	9.2±2.7^{a}	11.9±2.3^{a,b}	16.5±3.1^{b}
	7	11.1±1.9^{a}	9.5±1.3^{a}	9.1±2.9^{a}	9.9±2.1^{a}	15.1±3.2^{a}
Motility (%)	1	67±3.7^{a,A}	63±1.4^{a,b,A}	65±2.9^{a,A}	49±7.5^{c,b,A,B}	36±4.7^{c,B}
	4	57±3.4^{a}	46±8.0^{a,b}	56±4.7^{a}	45±9.1^{a,b}	35±2.9^{b}
	7	55±3.2^{a}	40±7.1^{a}	49±5.5^{a}	41±9.1^{a}	39±5.9^{a}

n=22 ejaculates; values within a row with different superscripts are significantly different at $p<0.05$ (a, b, c) or $p<0.01$ (A, B)

DISCUSSION

An important indicator of storage-related membrane damage is a change in membrane permeability manifested by increased permeability to stains and a release of intracellular substances [17]. This phenomenon could be caused by lipid peroxidation due to the relatively high content of unsaturated fatty acids in phospholipids of the boar sperm membrane [17]. The lower resistance of stored sperm to lipid peroxidation, which we observed in this study by means of luminescent method, agrees with the increased production of malondialdehyde during boar semen storage reported in literature [5].

The CL assay used in our study is not *per se* a quantitative reflection of lipid peroxidation. However, it provides instant and direct information about interactions of reactive oxygen species (ROS) with constituents of spermatozoa (lipids, phospholipids, possibly proteins, etc; [11]). Our method enables us to estimate the total level of ROS (integral) generated during lipid peroxidation [24] and the reaction kinetics is probably related to the antioxidant capacity of sperm. It is assumed that higher sperm antioxidant activity gives flatter kinetic curve and consequently a lower peak max [11].

Our data show that stored boar spermatozoa are very sensitive to lipid peroxidation and confirm the possibility of using induced sperm CL to evaluate their resistance to oxidative stress. Spermatozoa with damaged membranes do not show any spontaneous luminescence [14]. However, reactive oxygen species induced by the ferrous ions likely lead to a further increase of oxygen damage to lipids and proteins which may manifest itself as induced photon emission. This ferrous-induced effect can be believed to be evoked by the previously initiated highly spermicidal lipid peroxidation followed by disturbances in motility and energy metabolism. Reactive oxygen species have been shown to cause membrane deterioration, resulting in ATP depletion [4, 7] and decreased sperm movement [4].

Spermatozoa are particularly vulnerable to oxidative damage during *in vitro* storage when the production of free radicals could be significantly enhanced as a result of metabolic changes [15]. The attack of free radicals on the unsaturated fatty acid of sperm cell membranes leads to irreversible reduction of membrane fluidity and to the damage of cell-membrane related

ATPases which are responsible for regulating the intracellular level of ions necessary to maintain normal sperm motility [22]. The lipid peroxidation process in spermatozoa leads to the creation of substances having cytotoxic properties, such as malondialdehyde and 4-hydroxynonenol [3]. Low concentrations of these substances have been shown to inhibit numerous cellular enzymes and metabolic processes including anaerobic glycolysis, and thus, limiting ATP generation by the sperm cell [6]. De Lamirande and Gagnon [7] suggest that ROS are responsible for the loss of spermatozoal motility through decreased phosphorylation of axonemal proteins required for sperm movement.

The free radical-formation that occurs under oxidative stress conditions explains the relationships between photon emission parameters and sperm motility found in our study. Our data suggest that the loss of motility may be a consequence of reduced ATP production in stored spermatozoa. A similar relationship between the potential for ferrous-induced malondialdehyde generation as an indicator of lipid peroxidation and human sperm movement was reported by Aitken et al. [2] and Kobayashi et al. [18].

In our study, boar spermatozoa stored up to seven days retained a good progressive motility (45%) although Estienne et al. [8] obtained better results (58%) for BTS extender. A significant variability between boars in the motility of stored semen is in agreement with other studies [8, 19] which suggests that a variability exists among boars with regard to the ability to maintain sperm quality during storage. The large individual differences concerning luminescence parameters suggest the possibility of using CL measurements for the identification of males whose semen is highly suitable for long-term storage.

After insemination, sperm cells are transported within the uterus towards the oviduct. During this process they face new offensive peroxidation environments especially in the uterus, where an invasion of leukocytes adds massive amounts of radicals into the uterine fluid [1, 23]. Probably, because of reduced antioxidants or scavenging activity, sperm cells stored in a liquid state are less able to withstand this hostile environment and will deteriorate due to excessive lipid peroxidation. In conclusion, this study demonstrated that parameters of induced CL and sperm ATP level are sensitive indica-

tors of the effect of storage time on boar spermatozoa and therefore may be useful as an additional objective laboratory tests of boar semen quality. Further studies are necessary to ascertain whether the results of these tests are correlated with semen fertility.

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