

Effect of long-term storage on induced photon emission of boar spermatozoa

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ABSTRACT: Ultraweak photon emission measurements were found to be a useful tool for investigating the influence of stress factors on cells. In the present study, induced photon emission of boar spermatozoa was investigated using a luminometer during a 12-day storage at 15°C in Biosolwens extender. It was shown that the day of storage had a significant effect on some photon emission parameters. During storage, a significant increase in the Integral, Peak max. and Slope max. parameters and a decline in the T.-half (fall) parameter were observed. A significant correlation was observed between photon emission parameters and sperm motility. In conclusion, the results of the present study indicate that the measurement of induced photon emission can be an alternative, sensitive and relatively simple method for assessing the effect of preservation on oxidative damage to boar spermatozoa.

Keywords: boar semen; oxidative stress; luminescence; photon emission; sperm motility

The possibility of determining both fresh and preserved semen quality *in vitro* is of major importance to artificial insemination practice. The current methods used to evaluate semen quality allow only one characteristic, structural property or element of sperm metabolism to be investigated. Moreover, the results obtained by these methods correlate with the fertilizing capacity of spermatozoa only to a certain extent. This calls for new research methods that can be used to determine semen quality.

A promising methodological solution in this area is the use of photon emission (luminescence) measurements. It has been known for a long time that living cells emit light spontaneously without any external excitation. This ultraweak photon emission, attributable to oxidative metabolic reactions, is noticeably enhanced under oxidative stress conditions and is decreased by *in vitro* addition of antioxidants (Boveris et al., 1980; Gonzalez-Flecha et al., 1991; Sławiński et al., 1992). Earlier studies of this biophysical phenomenon showed an extremely

low emission intensity of bull, ram and boar spermatozoa and a possibility of inducing emissions of much higher intensity with Fe ions (Laszczka et al., 1995). Measurements of the spectral distribution of sperm emission and analysis of the relationships between the concentration of Fe ions and the intensity of induced luminescence show that it is strictly related to lipid peroxidation (Sławiński et al., 1998; Gogol, 2005), which is one of the basic biochemical processes accelerating the ageing of spermatozoa, especially during semen preservation (Cecil and Bakst, 1993; Cerolini et al., 2000). In lipid peroxidation, which is a chain free radical process, the generated reactive oxygen species, by initiating further peroxidation reactions, can cause serious damage to the cells and significantly change their structure and metabolism. Reactive oxygen species were shown to cause membrane deterioration, resulting in ATP depletion (De Lamiranda and Gagnon, 1992; Armstrong et al., 1999), decreased sperm movement (Aitken et al., 1993a; Armstrong et al., 1999), DNA damage (Aitken et al., 1998;

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Donnelly et al., 1999) and blocked sperm-egg fusion (Mammoto et al., 1996).

At present, the most widely used assay for lipid peroxidation involves the measurement of malondialdehyde (MA), a small molecular mass degradation product of peroxidative process that can be measured by virtue of its capacity to form adducts with thiobarbituric acid (Aitken et al., 1993b). Although the method is sensitive and can detect the end-point reaction product of lipid peroxidation, it is relatively elaborate and provides only an indirect measure of lipid peroxidation. Moreover, MA accounts for only around 5% of the products generated during lipid peroxidation (Marshall et al., 1985). Other extremely toxic lipid peroxidation products such as 4-hydroxynonenal, which are known to be present in semen and to have a powerful inhibitory effect on sperm function, are not accounted for in the MA assay (Selley et al., 1991).

As an alternative to the detection of end products such as malondialdehyde, chemiluminescence is a potentially sensitive method to assess the oxidation or autooxidation of lipids. Some lipid peroxidation products, particularly singlet molecular oxygen species and excited carbonyls, are chemiluminescent species (i.e. they produce light upon return to their ground state). Oxidation of lipids *in vitro* is accompanied by a luminescence signal (Miyazawa et al., 1994; Albertini and Abuja, 1998), the amplitude of which correlates well with the peroxide concentration (Miyazawa et al., 1994). Moreover, in several studies the luminescence signal was correlated with other indicators of lipid peroxidation, such as the malondialdehyde concentration (Doi et al., 2002) and the content of conjugated dienes (Albertini and Abuja, 1998) confirming that chemiluminescence can serve as an indicator of lipid peroxidative damage and oxidative stress.

The aim of this study was to evaluate changes in induced photon emission of boar spermatozoa during 12-day preservation of semen at 15°C and to determine the relationship between parameters of this emission and sperm motility.

MATERIAL AND METHODS

Semen collection and dilutions. Ten adult, healthy Polish Landrace boars were used in this study. Semen (1 ejaculate from each male) was collected by the gloved hand technique. After separation of the gel the sperm concentration was

determined with haemocytometer. Semen samples were then diluted to a final concentration of 60×10^6 sperm/ml in Biosolwens extender (Biochefa, Poland) and stored for 12 days at 15°C.

Luminescence was measured and sperm motility was assessed directly after dilution (day 0) and at day 6 and 12 of semen storage.

Luminescence measurements. Luminescence was measured at 20°C using an AutoLumat LB953 (Berthold) luminometer equipped with a cooled photomultiplier with a spectral response range from 370 to 620 nm. Prior to the measurement of luminescence, spermatozoa were separated from the seminal plasma and diluents by two-fold centrifugation ($700 \times g$ for 15 min) and resuspended in 0.9% NaCl to a concentration of 200×10^6 cells/ml.

10 μ l of 5mM luminol was added to 500 μ l of the washed sperm suspension at a concentration of 200×10^6 cells/ml. Using an automated injector system the emission was induced by adding 100 μ l of 0.3mM FeSO₄ solution (final concentration 0.05mM).

Immediately after injection the light emission kinetics was measured during 450 seconds. After complete measurements the results were automatically printed in a report giving the following values of luminescence parameters:

Integral – total integral of the measurement signals (counts/integration time)

Peak max. (cps) – height of the highest peak

Slope max. (cps) – maximum slope value of the curve

T.-slope max. – time at the maximum slope

T.-half (rise) – time at a half “peak max.” height in ascending direction

T.-max. (peak) – time at the peak maximum

T.-half (fall) – time at a half “peak max.” height in descending direction

Assessment of sperm motility. Samples of semen were incubated at 37°C for 30 min and then the percentage of motile spermatozoa was evaluated under a contrast phase microscope equipped with a heated plate at 37°C.

Statistical analysis. Results are expressed as means \pm SE. Data were subjected to analysis of variance according to the GLM procedure of the Statistical Analysis System (SAS). The significance of differences between means was tested at $P < 0.05$ and $P < 0.01$ by Duncan's multiple range test. The correlations between luminescence parameters and motility were performed using Spearman's rank method.

Table 1. The effect of storage time on luminescence parameters and sperm motility (mean, \pm SE; $n = 10$ ejaculates from different boars)

Parameters	Day 0	Day 6	Day 12
Integral	4.18 \pm 0.48 ^A	7.92 \pm 0.74 ^B	8.77 \pm 1.22 ^B
Peak max.	2.12 \pm 0.31 ^A	4.77 \pm 0.82	8.19 \pm 2.23 ^B
Slope max.	36.70 \pm 15.65 ^a	158.30 \pm 46.40	314.80 \pm 117.52 ^b
T.-slope max.	17.01 \pm 3.66	23.33 \pm 5.27	26.88 \pm 5.90
T.-half (rise)	49.90 \pm 5.39	42.00 \pm 5.59	42.40 \pm 7.49
T.-max. (peak)	133.40 \pm 11.58	103.60 \pm 12.79	94.60 \pm 15.14
T.-half (fall)	253.90 \pm 14.22 ^a	226.60 \pm 15.22	186.80 \pm 27.67 ^b
Motility (%)	70.00 \pm 6.54 ^{Aa}	46.50 \pm 9.10 ^b	24.00 \pm 7.02 ^{Bc}

^{abc} $P < 0.05$; ^{AB} $P < 0.01$

RESULTS

The effect of semen storage on luminescence parameters and sperm motility is shown in Table 1. During semen storage, a significant increase in the value of Integral, Peak max. and Slope max. parameters and a decline in T.-half (fall) parameter were observed. Particularly significant was the increase in the value of Integral after 6 days of preservation ($P < 0.01$). On day 12, the value of this parameter was only slightly higher than the value obtained on day 6 ($P > 0.05$). Changes in the values of Peak max., Slope max. and T.-half (fall) were more consistent over the entire 12 days of storage. A similar tendency was observed for sperm motility. The proportion of motile spermatozoa decreased gradually, by 23.5% between days 0 and 6 ($P < 0.05$) and by 22.5% between days 6 and 12 ($P < 0.05$). The semen storage time had no significant effect on the values of T.-slope max., T.-half (rise), and T.-max. (peak) parameters.

A significant correlation was observed between the percentage of motile spermatozoa and luminescence parameters except the parameter T.-slope max. (Table 2). The luminescence parameters most strongly correlated with sperm motility were Peak max. (-0.86), T.-half (fall) (0.77), Integral (-0.72) and T.-max. (peak) (0.70).

DISCUSSION

This study demonstrated that the long-term storage of boar semen has a significant effect on the parameters of luminescence from spermato-

Table 2. Correlations between luminescence parameters and sperm motility ($n = 30$)

Parameters	Motility
Integral	-0.71638 ($P < 0.0001$)
Peak max.	-0.86025 ($P < 0.0001$)
Slope max.	-0.44675 ($P = 0.0133$)
T.-slope max.	0.13100 ($P = 0.4902$)
T.-half (rise)	0.43441 ($P = 0.0165$)
T.-max. (peak)	0.69793 ($P < 0.0001$)
T.-half (fall)	0.77188 ($P < 0.0001$)

$P < 0.05$ was considered significant

zoa. Considering the association between induced luminescence and lipid peroxidation, this finding confirms the possibility of using the measurements of this biophysical phenomenon to assess the intensity of pathological oxidative processes in boar spermatozoa. It can be conjectured that similarly like the iron-promoted malondialdehyde assay (as an indicator of lipid peroxidation), the Fe ion-induced photon emission provides information on two aspects of sperm biochemistry: the extent to which an oxidative stress has led to the accumulation of lipid peroxides in the membrane which will respond to the presence of iron ions by stimulating the propagation of the peroxidative chain reaction, and/or the ability of the spermatozoon to withstand the propagation of lipid peroxidation through the presence of chain-breaking antioxidants, defensive enzymes and transition metal chelators. No matter which mechanism is involved, luminol-dependent

chemiluminescence not only allows to determine the level of reactive oxygen species (generated during the lipid peroxidation process) but also it permits the observation of the reaction kinetics. This makes it possible to obtain more detailed information about the processes that take place in the sperm cells and how to use it for semen quality evaluation.

The possibility of using induced luminescence measurements for the assessment of semen quality is shown by the relationships we obtained between the analysed parameters and sperm motility, which is the basic parameter evaluated in practical conditions. Although according to Hammerstedt (1996) no useful assay was developed to accurately predict male fertility, several reports indicated a positive relationship between sperm motility and fertilizing ability in the pig. Holt et al. (1997) demonstrated that porcine sperm motion parameters measured by computer-assisted semen analysis were correlated with the results of two fertility trials using on-farm inseminations of 1.5×10^9 sperm/dose. These authors showed that the parameters reflecting sperm velocity characteristics during incubation in capacitating conditions were related to conception rates. In other studies, Tardif et al. (1999) demonstrated that the percentage of motile spermatozoa as assessed by ordinary light microscopy was correlated with *in vivo* fertility in pigs when suboptimal sperm numbers were used for insemination.

The decreased motility of spermatozoa may occur due to the action of free radicals under oxidative stress. 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 (Hammerstedt, 1993). There are several possible mechanisms behind the decreased motility of spermatozoa connected with oxidative stress. The most often cited is peroxidation of membrane lipids (Aitken et al., 1989; 1993a,b). The attack of free radicals on the unsaturated fatty acid rich lipids of sperm cell membranes leads to an irreversible reduction of membrane fluidity and to the damage of cell membrane related ATP-ases, which are responsible for the regulation of the intracellular level of ions necessary to maintain normal sperm motility (Ohta et al., 1989).

The lipid peroxidation process in spermatozoa leads to the production of substances having cytotoxic properties, such as malondialdehyde and 4-hydroxynonenol (Aitken et al., 1995). Low concen-

trations of these substances were shown to inhibit a large number of cellular enzymes and functions, including anaerobic glycolysis limiting ATP generation by the sperm cell (Comporti, 1989).

De Lamiranda and Gagnon (1992) suggested that reactive oxygen species were responsible for the loss of sperm motility through decreased phosphorylation of axonemal proteins required for sperm movement.

The above free radical processes that occur under oxidative stress conditions can explain the relationships shown here between photon emission parameters and sperm motility. A similar relationship between the potential for iron-induced malondialdehyde generation as an indicator of lipid peroxidation and human sperm movement was reported by Kobayashi et al. (1991) and Aitken (1993a).

Our findings demonstrate that the measurement of induced photon emission can be an alternative, sensitive and relatively simple method for assessing the effect of preservation on oxidative damage to boar spermatozoa.

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