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Ferrous Ion Induced Photon Emission as a Method to Quantify Oxidative Stress in Stored Boar Spermatozoa*

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The aim of the study was to evaluate the effect of semen storage on ferrous ion induced luminescence of boar spermatozoa and to determine the relationship between parameters of this luminescence and lipid peroxidation as measured by malondialdehyde (MDA) contents. Boar semen samples were diluted in Biosolwens extender and stored for 12 days at 15°C. Luminescence and MDA were measured directly after dilution (day 0) and at 6 and 12 days of semen storage. Luminescence was measured at 20°C using a luminometer equipped with a cooled photomultiplier with a spectral response range from 370 to 620 nm. Emission was induced by adding $FeSO_4$ solution (final concentration 0.05mM). MDA content was measured by the HPLC method. The day of storage had a significant effect on some luminescence parameters and MDA content in spermatozoa. A significant correlation was observed between luminescence parameters and MDA concentration. The results of the study confirm that induced luminescence is strictly related to lipid peroxidation in spermatozoa that occur during boar semen storage. Parameters of luminescence treated as a holistic response of cells to oxidative stress can be useful for monitoring spermatozoa quality during semen preservation.

Key words: Boar semen, lipid peroxidation, luminescence, photon emission, sperm motility.

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Free radicals and especially reactive oxygen species (ROS) can initiate peroxidation of polyunsaturated fatty acids in biological membranes. Boar spermatozoa, like those of other mammals, are particularly susceptible to oxidative damage because of their high content of polyunsaturated fatty acids (PARKS & LYNCH 1992; JOHNSON *et al.* 2000). The attack of free radicals on unsaturated fatty acid-rich lipids of sperm cell membranes leads to an irreversible decrease in membrane fluidity (BORST *et al.* 2000), alteration in membrane permeability and metabolism (JONES *et al.* 1979; OHYASHIKI *et al.* 1988; OHTA *et al.* 1989) and reduced sperm ability to penetrate the egg (AITKEN *et al.* 1993; KODAMA *et al.* 1996).

Lipid peroxidation may be one of the basic mechanisms responsible for the reduction in sperm fertility during semen preservation. Sperm isolated from fresh boar semen (ROCA *et al.* 2004), semen stored in hypothermic liquid (CEROLINI *et al.* 2000), and frozen-thawed semen (BREININGER *et al.* 2005) are susceptible to $FeSO_4$ and ascorbate catalyzed lipid peroxidation, as measured by malondialdehyde (MDA) formation. Measurement of MDA, an product of oxidation breakdown derived from arachidonic acid (SPITELLER 2006), is a widely used assay for lipid peroxidation in spermatozoa (STOREY 1997). 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 (PAP *et al.* 2000). Moreover, MDA only accounts for 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 MDA assay (SELLEY *et al.* 1991).

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Chemiluminescence is considered to be an alternative and sensitive method to assess the oxidation or autooxidation of lipids (MIYAZAWA *et al.* 1994; ALBERTINI & ABUJA 1998). In several studies, the luminescence signal has been correlated with other indicators of lipid peroxidation, such as the MDA concentration (DOI *et al.* 2002), the concentration of exogenously added lipid hydroperoxides (GUAJARDO *et al.* 2002), and the content of conjugated dienes (ALBERTINI & ABUJA 1998). In the case of spermatozoa, however, the intensity of spontaneous luminescence is extremely weak and thus difficult to measure. Earlier studies (LASZCZKA *et al.* 1995; SŁAWIŃSKI *et al.* 1998; GOGOL 2005; GOGOL & SZCZĘŚNIAK-FABIAŃCZYK 2006) suggest that recording the ferrous ion induced luminescence can be an alternative and relatively simple method of detecting and quantifying lipid peroxidation damage and oxidative stress in spermatozoa cells. However, the relationship between induced luminescence and sperm MDA production has not been investigated.

The aim of the study was to evaluate the effect of semen storage on ferrous ion induced luminescence of boar spermatozoa and to determine the relationship between parameters of this luminescence and lipid peroxidation as measured by MDA contents.

Material and Methods

Semen collection and dilutions

Fresh semen from eight adult crossbred boars was used in the experiment. Semen (3 ejaculates from each male) was collected using the gloved hand technique. After separation from the gel, sperm concentration was determined using a 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 and MDA concentration were measured 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 measurement of luminescence, spermatozoa were separated from the seminal plasma and diluents by two-fold centrifugation (700 g for 15 min) and resuspended in 0.9% NaCl to a concentration of 200×10^6 cells/ml.

To 500 μl of the washed sperm suspension at a concentration of 200×10^6 cells/ml, 10 μl of 5mM luminol was added. Emission was induced by adding (using an automated injector system) 100 μl of 0.3 mM $FeSO_4$ solution (final concentration 0.05 mM).

Immediately after injection light emission kinetics was measured during 450 s (Fig. 1). Afterwards, the following luminescence parameters were calculated:

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

– Peak max (cps) – height of highest peak

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

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

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

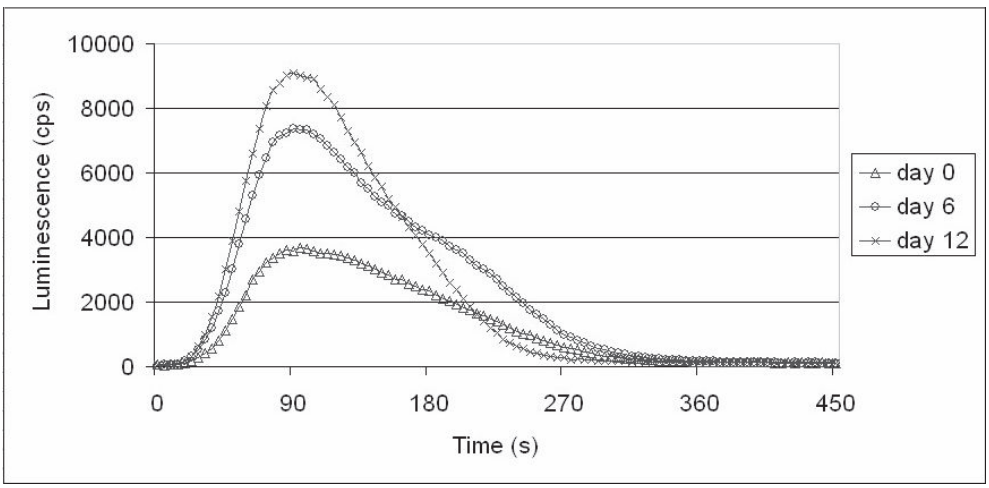

Fig. 1. Kinetics of induced luminescence from boar spermatozoa after different times of storage.

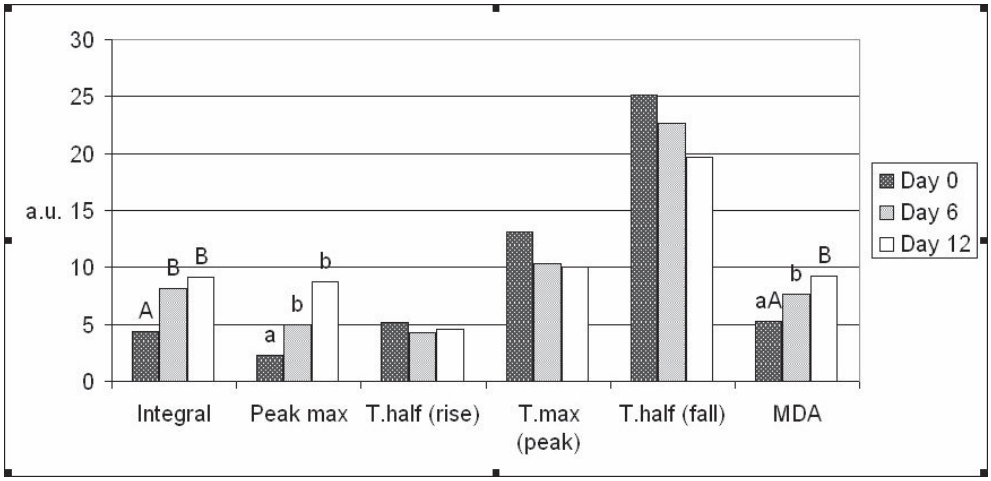

Fig. 2. Effect of semen storage on luminescence parameters and MDA content in boar spermatozoa (a,b – significantly different at P<0.05; A, B – significantly different at P<0.01).

Lipid peroxidation as measured by MDA contents

Spermatozoa were separated from the seminal plasma and diluents by two-fold centrifugation (700 g for 15 min) and resuspended in 0.9% NaCl to a final concentration of 100×10^6 cells/ml. The sperm suspension was incubated at 37°C for 1 h in the presence of the Fe(II)/ascorbate system (AITKEN *et al*. 1989). The concentration of MDA was determined by the HPLC method (NIELSEN *et al*. 1997).

Statistical analysis

Data were subjected to variance analysis according to the GLM procedure of the Statistical Analysis System Institute (2001). 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 and sperm MDA content were performed using the Spearman's rank method.

Results

The day of storage had a significant effect on some luminescence parameters and MDA content in the spermatozoa (Fig. 2). During semen storage, a significant increase in the value of Integral and Peak max parameters was observed. Particularly significant was the increase in the value of Integral after 6 days of storage (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). A similar tendency was observed for Peak max and sperm MDA content. The content of MDA increased from 5.22 to 9.21 μmol/10^8 spermatozoa from day 0 to day 12 of storage (P<0.01). The semen storage time had no significant effect on the values of T.-half (rise), T.-max (peak) and T.-half (fall) parameters.

Table 1

Correlations between luminescence parameters and MDA content

Parameter	MDA
Integral	0.6264 (P = 0.0011)
Peak max	0.5704 (P = 0.0036)
T.half (rise)	-0.4921 (P = 0.0146)
T.max (peak)	-0.6109 (P = 0.0015)
T.half (fall)	-0.5001 (P = 0.0128)

P<0.05 was considered significant.

A significant correlation was observed between luminescence parameters and MDA concentration (Table 1). The luminescence parameter most strongly correlated to MDA concentration was Integral (r = 0.63).

Discussion

These results support previous studies suggesting that measurement of ferrous ion induced luminescence is an effective method of monitoring lipid peroxidative damage and oxidative stress in spermatozoa (SŁAWIŃSKI *et al.* 1998; GOGOL 2005; GOGOL *et al.* 2007).

The minimal spontaneous lipid peroxidation observed in boar (CEROLINI *et al.* 2000; GUTHRIE & WELCH 2007), bovine (BROUVERS & GADELLA 2003), equine (BAUMBER *et al.* 2000) and human (STOREY 1997) spermatozoa incubated in the absence of a promoter suggests that if transition ele-

ments are not available, lipid radical formation is maintained at a very low level in these cells. This reflects the fact that spermatozoa possess an active enzymatic defense system against lipid peroxidation (ALVAREZ & STOREY 1989; WILLIAMS & FORD 2004; TRAMER *et al*. 2004). In addition to the enzymatic system, lipid peroxides may be stabilized in the sperm plasma membrane by chain-breaking antioxidants such as vitamin E (SHEWEITA *et al*. 2005; DREVET 2006). However, in the presence of a ferrous ion promoter, these peroxides are induced to break down.

Ferrous ions have been used extensively to induce rapid lipid peroxidation in a variety of cell types including spermatozoa (JONES *et al.* 1979; AITKEN *et al.* 1993; STOREY 1997; GOMEZ *et al.* 1998). The ferrous ion promotes the catalysis of lipid peroxides to alkoxyl and peroxyl radicals, which appear to be important in the propagation of the chain reaction of lipid peroxidation in the sperm membrane (AITKEN *et al.* 1993; ALVAREZ & STOREY 1995). In these radical chain reactions, electron-excited molecules are generated and then radiatively deactivated, which manifests itself as an emission of light (chemiluminescence, ultraweak photon emission).

Chemiluminescence (CL) assay is not per se a quantitative reflexion of lipid peroxidation. However, it provides instant and direct information on the interactions of ROS with constituents of spermatozoa (lipids, phospholipids, perhaps proteins etc).

It has to be realized that the CL method is multiparametric as it provides instantaneous information on the kinetics of the reaction, energetics of exergonic (exothermic) reactions, the rate of the total peroxidation reaction, an instant effect of a variety of inhibitors or catalysts as well as physiological and physical factors such as e.g. pH, temperature, ionic strength of the medium, enzymes, etc. This information is given in real time and can be observed on the monitor as a kinetic curve, i.e. the light intensity I vs. reaction time t, I = f(t) from the very beginning to the end of the reaction.

Our method enables the total level of ROS (generated during the lipid peroxidation process) to be determined (Integral parameter) as well as the observation of the reaction kinetics probably related to the antioxidant capacity of sperm. It is supposed that higher sperm antioxidant activity gives a flatter kinetic curve. This means a lower Peak max and greater difference between T.half (rise) and T.half (fall). Dissection of the luminescence signal into more parameters than only the Integral one makes it possible to obtain more detailed information about the processes that take place in sperm cells.

Chemiluminescent determination of lipid peroxidation is more selective than chemical methods, such as e.g. MDA. This follows from the nature and mechanism of CL. It has to be realized that during the generation of light, a photon of energy (hni) is restricted only to exothermic chemical reactions which produce free energy, which are energetic enough to promote a molecule to an electronic excited state, singlet or triplet. The intensity (I) of chemiluminescence is: I = FW where F is the total quantum yield of the light emission and W is the rate of the elementary reaction rate. Thus, the intensity of chemiluminescence vs. reaction time, i.e. the kinetics of CL, reflects the rate of photon production.

The second rule is that the energy level of products' molecules is lower than that of reacting substrates. Because of these selection rules fulfilling the CL process, only formation of relatively stable carbonyl, alcohol and oxygen molecules (the Russel reaction) in the process of lipid (or other substrate) peroxidation can be taken into account. Thus, multiple side-reactions accompanying lipid peroxidation do not play a major role in CL assay (unless they do not quench CL or change the transfer of excitation energy).

In summary, this study shows that induced luminescence is strictly related to lipid peroxidation that occurs during boar semen storage. Parameters of luminescence treated as a holistic response of cells to the oxidative stress can be useful for monitoring spermatozoa quality during semen preservation.

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