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Iron-induced luminescence as a method for assessing lipid peroxidation of frozen–thawed goat spermatozoa

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Freezing/thawing procedures induce enhanced reactive oxygen species (ROS) formation in mammalian sperm and these ROS may be a cause for the decrease in sperm function following cryopreservation. In the present study, we used a chemiluminescence method to detect ROS-induced damage in goat spermatozoa. Iron-induced luminescence of fresh and frozen/thawed sperm cells was assessed using a luminometer. It was shown that the freezing/thawing procedure had a significant effect on some luminescence parameters. Semen freezing significantly increased the values of integral, peak max, T.half (rise) and T.max (peak) parameters. A significant correlation was observed between the percentage of motile spermatozoa and integral, peak max and T.half (rise) parameters. In conclusion, the results of the present study indicate that measurement of induced luminescence can be an alternative, sensitive and relatively simple method for assessing the effect of cryopreservation on oxidative damage to spermatozoa.

Introduction

Freezing and thawing, two major steps in cryopreservation of spermatozoa, have a major effect on cell structure and function (Hammerstedt *et al.*, 1990). The freezing–thawing cycle causes damage to the plasma membrane (Hammerstedt *et al.*, 1990), reduces motility and the fertilising ability of spermatozoa (Hammerstedt, 1993) and induces premature capacitation and nuclear decondensation (Cormier *et al.*, 1997). Even in the presence of cryoprotectants such as glycerol, egg yolk and milk, significant structural alterations take place. In recent years, antioxidants have been tested in combination with basic common cryoprotectants to minimise the damage caused by freezing and thawing. The beneficial effects of antioxidants provide indirect evidence that an oxidative stress occurs during cryopreservation (Alvarez and Storey, 1992; Chen *et al.*, 1993; Sanchez-Partida *et al.*, 1997). This has been confirmed by studies which showed that reactive oxygen species (ROS) are produced during freezing and thawing of bovine (Chatterjee and Gagnon, 2001) and equine spermatozoa (Ball *et al.*, 2001).

One of the major biological processes associated with ROS is lipid peroxidation. Lipid peroxidation proceeds with the extraction of hydrogen and the formation of a number of reactive intermediates that can result in a chain reaction or propagation of peroxidation within the membrane (Aitken *et al.*, 1993a; Storey, 1997). Mammalian sperm cells are particularly sensitive to oxidative damage due to the high level of easily peroxidisable polyunsaturated fatty acids (Jones *et al.*, 1979) and fairly low activity of the enzymatic anti-oxidative system. 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.*, 1993b; Kodama *et al.*, 1996).

In view of the importance of lipid peroxidation in defective sperm function, quantification of this process is of some diagnostic significance. 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.*, 1993a). 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, MA only accounts for around 5% of

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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).

Recently, the application of a fluorescent fatty acid probe, C_{11}-BODIPY$^{581/591}$, has been described in a number of studies as a means to monitor lipid peroxidation in living cells (Borst et al., 2000; Pap et al., 2000; Ball and Vo, 2002). The probe shifts from red to green upon oxidation, and the ratio of red and green fluorescence has been used as a measurement of lipid peroxidation. The dye is relatively non-fluorescent in solution and has been evaluated by a fluorescence microplate reader, by epifluorescence microscopy, by confocal microscopy and by flow cytometry (Pap et al., 2000). The fluorescence changes in C_{11}-BODIPY$^{581/591}$ reflect indirectly the oxidation of unsaturated fatty acids (Borst et al., 2000).

Chemiluminescence is considered to be an alternative and potentially sensitive method to assess the oxidation or auto-oxidation of lipids (Miyazawa et al., 1994; Albertini and Abuja, 1998). In several studies, the luminescence signal has been correlated with other indicators of lipid peroxidation, such as the MA concentration (Doi et al., 2002), the concentration of exogenously added lipid hydroperoxides (Guajardo et al., 2002) and the content of conjugated dienes (Albertini and Abuja, 1998), confirming that low-level chemiluminescence can serve as an indicator of lipid peroxidative damage. 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 and Szczęśniak-Fabiańczyk, 2006) have shown that recording the iron ion-induced luminescence can be an alternative and relatively simple method of measuring sperm lipid peroxidation.

The aim of the present study was to evaluate the effect of the freezing/thawing process on iron ion-induced luminescence of buck sperm as an indicator of cell oxidative damage.

Material and methods

Semen
Five healthy, adult goat bucks were used in this study. Semen was collected from February to May using an artificial vagina. Semen was processed using a modified Corteel cryopreservation procedure (Kareta and Cegła, 1999) in a milk–glycerol extender, frozen in 0.25 ml plastic straws using liquid nitrogen vapour and stored in liquid nitrogen.

Frozen straws were thawed in a 37°C water bath for 30 s immediately before use. Freshly ejaculated semen as well as thawed semen samples from the same bucks were also evaluated for luminescence parameters and sperm progressive motility.

Luminescence measurements
Luminescence was measured at 20°C using a Berthold AutoLumat LB953 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 final concentration of 200×10^6 cells per ml.

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

Immediately after injection of $FeSO_4$ solution, light emission kinetics was measured for 300 s (Figure 1). After complete measurements, the following luminescence parameters were calculated: integral – total integral of the measurement signals (counts $\times 10^5$ per integration time); peak max (c.p.s. $\times 10^3$) – height of highest peak; slope max (c.p.s. $\times 10^2$) – maximum slope value of curve; T.slope max – time at maximum slope; 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.

Assessment of sperm motility
The percentage of progressive motile spermatozoa was evaluated under a contrast phase microscope equipped with a heated plate at 37°C.

Statistical analysis
Results are expressed as means ± s.e. Data were subjected to variance analysis according to the GLM procedure of the Statistical Analysis Systems Institute (2001).

The significance of differences between means was tested by the least squares method using the LS means procedure. The correlations between luminescence parameters and motility were calculated using Spearman's rank method.
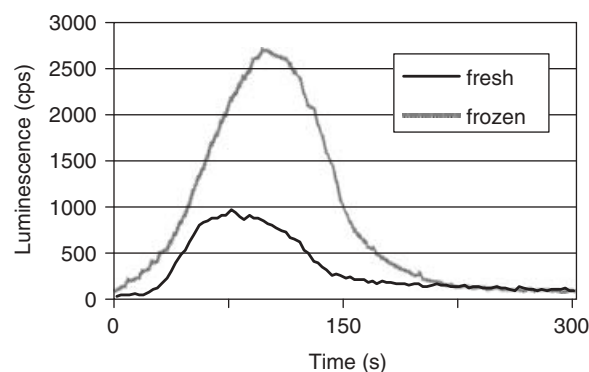

Figure 1 Kinetics of induced luminescence of goat spermatozoa.

Results

The effect of freezing/thawing on luminescence parameters and sperm motility is shown in Table 1. Semen freezing significantly increased the values of integral ($P < 0.01$), peak max ($P < 0.01$), T.half (rise) ($P < 0.05$) and T.max (peak) ($P < 0.05$) parameters. No significant differences between fresh and frozen sperm in the values of slope max, T.slope max and T.half (fall) parameters were observed.

Table 1 *The effect of freezing–thawing on luminescence parameters and motility of goat spermatozoa (mean ± s.e.)*

	Semen	
Parameter	Fresh	Frozen
Integral	$1.90^A \pm 0.16$	$6.43^B \pm 0.96$
Peak max	$2.03^A \pm 0.30$	$8.86^B \pm 1.76$
Slope max	1.46 ± 0.33	8.27 ± 4.14
T.slope max	5.96 ± 0.43	10.32 ± 2.20
T.half (rise)	$35.71^a \pm 3.51$	$54.29^b \pm 6.61$
T.max (peak)	$69.14^a \pm 4.67$	$92.00^b \pm 7.80$
T.half (fall)	131.40 ± 13.03	141.71 ± 7.74
Motility (%)	$82.3^A \pm 3.08$	$51.7^B \pm 4.57$

[a,b] Means within a row with different superscripts are significantly different at $P < 0.05$.
[A,B] Means within a row with different superscripts are significantly different at $P < 0.01$.

Table 2 *Correlations between luminescence parameters and sperm motility*

Parameter	Motility[†]
Integral	-0.79849 ($P < 0.0001$)
Peak max	-0.74061 ($P < 0.0001$)
Slope max	-0.25192 ($P = 0.1792$)
T.slope max	-0.14537 ($P = 0.4434$)
T.half (rise)	-0.44171 ($P = 0.0145$)
T.max (peak)	-0.31138 ($P = 0.0939$)
T.half (fall)	-0.04666 ($P = 0.8101$)

[†] $P < 0.05$ was considered significant.

The proportion of motile spermatozoa decreased after semen freezing by 30.6% ($P < 0.01$).

A significant correlation was observed between the percentage of motile spermatozoa and integral, peak max and T.half (rise) parameters (Table 2). Integral ($r = -0.80$) and peak max ($r = -0.74$) were luminescence parameters that were the most strongly correlated with sperm motility. No significant correlation was found between T.max (peak) and T.half (fall) parameters and the percentage of motile spermatozoa.

Differences between males in the luminescence parameters were observed (Tables 3 and 4). In both fresh and frozen semen, the sperm of buck C was characterised by the lowest, and the sperm of buck B by the highest, values of integral and peak max parameters.

Discussion

In the present study, induced luminescence (photon emission) measurements were used to determine oxidative damage to goat spermatozoa. Earlier studies covering measurements of the spectral distribution of sperm emission and analysis of the relationships between the concentration of Fe ions and intensity of induced luminescence show that this biophysical phenomenon is strictly related to lipid peroxidation (Sławiński *et al.*, 1998; Gogol, 2005). 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.*, 1993a; 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.*, 1993a). 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). Our study is evidence that the kinetics and intensity of induced lipid peroxidation of sperm can be observed based on changes in the intensity of luminescence recorded. Our

Table 3 *Results of luminescence measurement and sperm motility for fresh semen (mean ± s.e.)*

	Buck (no. of ejaculates)				
Parameter	A (6)	B (2)	C (5)	D (6)	E (2)
Integral	$2.4^A \pm 0.3$	$2.7^{aAC} \pm 0.8$	$1.3^{bB} \pm 0.2$	$1.5^{bcBC} \pm 0.1$	$2.4^{ac} \pm 0.1$
Peak max	$3.1^{aA} \pm 0.5$	$3.2^{ab} \pm 2.2$	$0.8^{cB} \pm 0.1$	$1.6^{bc} \pm 0.3$	2.2 ± 0.3
Slope max	$1.0^a \pm 0.3$	0.8 ± 0.1	$0.9^a \pm 0.5$	$2.9^b \pm 0.8$	0.9 ± 0.1
T.slope max	6.1 ± 1.0	4.1 ± 0.4	6.0 ± 0.9	5.9 ± 0.6	7.4 ± 2.4
T.half (rise)	$45.5^A \pm 7.1$	$48.0^a \pm 6.0$	$40.2^a \pm 3.1$	$19.5^{bB} \pm 5.2$	31.5 ± 1.5
T.max (peak)	$72.5^a \pm 7.9$	$87.0^A \pm 15.0$	$86.4^A \pm 5.3$	$47.0^{bB} \pm 6.1$	64.5 ± 1.5
T.half (fall)	$115.5^{ac} \pm 11.0$	$186.0^{ab} \pm 81.0$	$189.0^{bA} \pm 37.2$	$92.5^{cB} \pm 6.0$	126.0 ± 12.0
Motility (%)	$85.0^{aA} \pm 2.9$	$57.5^B \pm 2.5$	$91.7^{AC} \pm 1.7$	$88.0^{AC} \pm 2.0$	$75.0^{bAD} \pm 5.0$

[a,b,c] Means within a row with different superscripts are significantly different at $P < 0.05$.
[A,B,C,D] Means within a row with different superscripts are significantly different at $P < 0.01$.

Table 4 *Results of luminescence measurement and sperm motility for frozen semen (mean ± s.e.)*

Parameter	Buck (no. of ejaculates)				
	A (6)	B (2)	C (5)	D (6)	E (2)
Integral	7.4 ± 1.3	$12.1^{aA} \pm 5.5$	$2.8^{bB} \pm 0.9$	$5.5^{bc} \pm 1.3$	$9.7^{ac} \pm 4.7$
Peak max	10.3 ± 2.3	$17.6^{a} \pm 11.3$	$3.0^{b} \pm 1.4$	7.2 ± 2.4	15.6 ± 10.8
Slope max	5.7 ± 5.0	0.6 ± 0.1	0.8 ± 0.2	22.0 ± 12.7	1.2 ± 0.5
T.slope max	12.2 ± 5.6	5.7 ± 2.1	6.7 ± 0.8	13.2 ± 5.4	9.7 ± 1.5
T.half (rise)	$44.0^{A} \pm 4.8$	$121.5^{B} \pm 7.5$	$58.8^{A} \pm 7.6$	$36.5^{A} \pm 12.5$	$60.0^{A} \pm 0.0$
T.max (peak)	$77.0^{A} \pm 6.8$	$157.5^{aB} \pm 13.5$	$110.4^{b} \pm 4.1$	$70.5^{cA} \pm 17.4$	$90.0^{bc} \pm 3.0$
T.half (fall)	$120.0^{aA} \pm 7.8$	$196.5^{bB} \pm 25.5$	$160.8^{bc} \pm 7.0$	$131.0^{ac} \pm 17.0$	136.5 ± 22.5
Motility (%)	55.0 ± 7.64	$35.0^{A} \pm 15.0$	$75.0^{aB} \pm 7.64$	$47.0^{b} \pm 4.64$	$40.0^{b} \pm 10.00$

[a,b,c] Means within a row with different superscripts are significantly different at $P < 0.05$.
[A,B] Means within a row with different superscripts are significantly different at $P < 0.01$.

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 flatter kinetic curve. This means the lower peak max and slope max values and the bigger difference between T.half (rise) and T.half (fall). Dissection of the luminescence signal into more parameters then only the integral one makes possible to obtain more detailed information about the processes that take place in the sperm cells.

The rapid increase in luminescence intensity after freezing/thawing of semen, which was accompanied by a decrease in sperm motility, demonstrates that the phospholipids present in goat spermatozoa readily undergo peroxidation.

The decreased motility of spermatozoa may occur due to the action of free radicals under oxidative stress. 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 and 1993b). The attack of free radicals on the unsaturated fatty acid-rich lipids of sperm cell membranes leads to irreversible reduction of membrane fluidity and to the damage of cell membrane-related ATPases, which are responsible for 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 creation of substances having cytotoxic properties, such as MA and 4-hydroxynonenol (Aitken *et al.*, 1995). Low concentrations of these substances have been 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) suggest that ROS are responsible for the loss of spermatozoal 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 between photon emission parameters and sperm motility.

A similar relationship between the potential for iron-induced MA generation as an indicator of lipid peroxidation and human sperm movement was reported by Kobayashi *et al.* (1991) and Aitken *et al.* (1993a).

Analysis of the effect of freezing on the values of particular luminescence parameters and the correlation between sperm motility and luminescence parameters indicate that integral and peak max are the luminescence parameters particularly useful for determining the ROS-induced damage at the level of the sperm plasma membrane.

The large individual differences shown between luminescence parameters (which determine the sperm sensitivity to lipid peroxidation) show the possibility of using luminescence measurements when selecting males whose semen is highly suitable for freezing.

In conclusion, our findings confirm that an oxidative stress occurs during semen cryopreservation and demonstrate that measurement of induced luminescence can be a sensitive and relatively simple method for assessing the effect of freezing and thawing on oxidative damage to spermatozoa.

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