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The use of the acridine orange test and the TUNEL assay to assess the integrity of freeze-dried bovine spermatozoa DNA

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ABSTRACT. The ability to detect nuclear damage is an important tool for the development of sperm preservation methods. We used the acridine orange test (AOT) and the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL) assay to assess the DNA status of sperm cells preserved with different lyophilization media. The AOT did not detect any differences between different lyophilization media. However, differences in DNA integrity were observed among treatments with the TUNEL assay, suggesting that TUNEL is a more sensitive method to evaluate sperm DNA. The use of TCM 199 and 10% FCS as a lyophilization medium resulted in 14% of the cells with DNA fragmentation in TUNEL test. The AOT indicated only 4% of the cells with chromatin damage, with this same treatment, with no significant differences when compared to the other treatments. The degree of DNA fragmentation was negatively related to fertilizing potential, as sperm DNA damage was inversely correlated with pro-nucleus formation. The

TUNEL assay was found to be an efficient method to detect DNA damage in sperm, and it could be used as a tool to predict male fertility.

Key words: Bovine, Freeze-drying sperm, DNA, TUNEL, Acridine orange test, Conservation

INTRODUCTION

Lyophilization or freeze-drying is a procedure developed to preserve bioactive molecules (DNA, enzymes, and proteins), pharmaceutical products (antibiotics) and other delicate solvent-impregnated materials (Keskintepe et al., 2002; Kusakabe and Kamiguchi, 2004).

Recently, freeze-drying has been proposed as an alternative method to preserve mammalian spermatozoa, instead of cryopreservation (Wakayama et al., 1998; Kusakabe et al., 2001; Kaneko et al., 2003a,b). Preservation of the sperm cells is obtained by water removal due to ice sublimation, under a strong vacuum.

Although freeze-drying of sperm is a useful technique, especially for animal conservation programs, it causes more damages than the conventional sperm cryopreservation procedures. When mammalian spermatozoa are freeze-dried, they lose motility and therefore, they are unable to fertilize oocytes, both *in vivo* and *in vitro* (Liu et al., 2004). Nevertheless, if an intact nucleus is present in the sperm, they can be used to fertilize oocytes through intracytoplasmic sperm injection (ICSI) and are able to produce live offspring, as has been demonstrated in mice and rabbits (Kwon et al., 2004).

Therefore, for freeze-dried sperm to produce live offspring, the main requirement is the integrity of the nucleus (Wakayama et al., 1998; Wakayama and Yanagimachi 1998; Kusakabe et al., 2001). However, accurate evaluation of sperm DNA status is necessary to evaluate the efficiency of using freeze-dried sperm in assisted reproduction. Several methods have been used to assess the chromatin structure and DNA status of sperm (Erenpreiss et al., 2004). In general, the available assays can be classified into three categories, chromatin structural probes, direct assessment of DNA fragmentation, and nuclear matrix assays (Erenpreiss et al., 2006).

Among these methods, the acridine orange test (AOT) and the TUNEL (terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling) assay have been the most widely used to evaluate the sperm quality. The AOT, which uses a chromatin structural probe, measures the DNA susceptibility to the acid-induced conformational helix-coil transition *in situ*. On the other hand, the TUNEL assay, which is a direct method for the assessment of fragmented sperm DNA, quantifies the incorporation of dUTP at breaks in double-stranded DNA in a reaction catalyzed by terminal deoxynucleotidyl transferase.

It has been suggested that DNA damage can be induced by mechanical stress throughout the freeze-drying procedure, or by oxidative stress, which occurs after thawing or rehydration, during the holding period before ICSI (Kusakabe et al., 2001). If that is true, all the steps, from the beginning of the sperm freeze-drying procedure to its use to fertilize an oocyte, can affect the status of the DNA. We evaluated DNA damage in bovine spermatozoa submitted to different lyophilization treatments and examined how it affected fertilization potential. We also compared two methods of DNA evaluation, AOT and the TUNEL assay.

MATERIAL AND METHODS

Animals

All semen samples were obtained from a Nelore bull; its fertility had been previously tested *in vitro* in our laboratory. Semen was collected by electroejaculation.

Solutions for freeze-drying spermatozoa

All reagents, unless otherwise stated, were purchased from Sigma Chemicals (St. Louis, MO, USA). The different freeze-drying media that were tested: medium 1: TCM 199 with Hank's salts (Gibco Life Technologies Inc., Grand Island, NY, USA) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco Life Technologies Inc.); medium 2: TCM 199 with Hank's salts supplemented with 10% (v/v) FCS and 0.2 mol/L trehalose; medium 3: 10 mmol/L Tris-HCl buffered supplemented with 50 mmol/L of each of NaCl and EGTA [ethylene glycol-bis (β-aminoethyl ether)-N,N,N'N'-tetraacetic acid]; the pH of the final solution was adjusted to 8.2. Media 1, 2 and 3 were considered as treatments 1, 2 and 3, respectively.

For the control group, a portion of the semen was separated before freezing-drying, and was cryopreserved with egg yolk Tris glycerol extender (Cormier et al., 1997).

Sperm freeze-drying

In all treatments, the concentration was adjusted to 10×10^6 spermatozoa/100 µL freeze-drying solution. Samples were diluted and kept at room temperature for 30 min, cooled down with liquid nitrogen vapor for 1 h and then, plunged into liquid nitrogen. Frozen samples were immediately inserted into the freeze-drying machine (Thermo Savant, USA), previously stabilized at -40°C and 350×10^{-3} Mbar pressure. After 12-16 h of freeze-drying the tubes containing the samples were completely covered with aluminum foil and stored for three months at 4°C.

Rehydration

Freeze-dried sperm samples were rehydrated by adding 100 µL Milli-Q water at room temperature.

Evaluation of chromatin stability with the acridine orange test

Three smears from each sample were prepared on glass slides and air-dried. Each smear was fixed overnight in Carnoy's solution, freshly prepared with methanol and glacial acetic acid in a 3:1 proportion. The slides were air-dried again, and incubated in tampon solution (80 mmol/L citric acid and 15 mmol/L Na_2HPO_4, pH 2.5) at 75°C for 5 min to test chromatin stability. Subsequently, slides were stained with acridine orange stain (0.2 mg/mL). They were washed with water to remove background staining; while still wet, they were covered with coverslips and evaluated with an epifluorescence microscope (Axiophot Zeiss; 490/530 nm excitation/barrier filter). One hundred cells were analyzed in each treatment slide. Sperm with normal DNA content present a green fluorescence, whereas sperm with abnormal DNA con-

tent emit fluorescence in a spectrum varying from yellow-green to red. The percentage of sperm with intact chromatin was calculated by dividing the number of green-stained sperm by the total number of sperm and multiplying the result by 100.

Detection of DNA fragmentation by TUNEL assay

For TUNEL technique, we used the *In Situ* Cell Death Detection Kit, with fluorescein, according to manufacturer's (Roche Diagnostics GmbH, Mannheim, Germany) protocol, with slight modifications.

Spermatozoa were washed in 100 µL phosphate-buffered saline (PBS), supplemented with 0.1% polyvinylpyrrolidone (PVP). The sperm suspension was fixed with 100 µL 4% paraformaldehyde for 1 h at room temperature. Cells were washed again in PBS supplemented with 0.1% PVP and permeabilized with 0.5% Triton X-100 in 0.1% sodium citrate for 1 h on ice. The permeabilized spermatozoa were washed once in PBS supplemented with 0.1% PVP, and incubated with TUNEL reaction mixture, containing terminal deoxynucleotidyl transferase (TdT) plus dUTP label, in the dark at 37°C for 1 h. After labeling, the cells were washed in PBS with 0.1% PVP and counterstained with 5 µg/mL Hoechst 33342 to visualize total DNA. Negative (omitting TdT from the reaction mixture) and positive (using only DNAse I, 1 mg/mL for 30 min at room temperature) controls were tested for each sample. At least 100 cells of each sample were analyzed with a fluorescence microscope. Each spermatozoon was assigned to contain either normal (blue nuclear fluorescence due to Hoechst 33342) or fragmented DNA (green nuclear fluorescence). The final percentage of sperm with fragmented DNA was referred to as % TUNEL positive.

Gamete preparation for intracytoplasmic sperm injection

Oocytes were matured for 22 h in media composed of TCM 199 with Earle's salts, supplemented with 10% (v/v) FCS, 24 IU/mL LH, 10 µg/mL FSH, and antibiotics (50 IU/mL penicillin and 50 µg/mL streptomycin). Matured oocytes were then exposed to 1 mg/mL bovine hyaluronidase for 5 min to remove the cumulus cells. Denuded oocytes with evident polar bodies were transferred back to maturation medium and maintained in the incubator until microinjection.

Freeze-dried sperm samples were rehydrated and washed twice by centrifugation for 3 min at 700 *g* in TALP medium, where they remained until their use for ICSI. The frozen semen samples that were used as control were prepared by centrifugation for 20 min at 700 *g* in a 45-90% Percoll gradient (Parrish et al., 1995). The selected sperm obtained after centrifugation were capacitated by incubation with 200 µg/mL heparin for 1 h, before being used for ICSI.

Sperm microinjection

Sperm microinjection was performed with a micromanipulator (Narishige Instruments, Tokyo, Japan) connected to an inverted microscope (Nikon, Japan) with a 200X magnification. Dishes for microinjection were prepared by adding 10 drops of 20 µL TCM 199 with Hank's salts and 25 mM HEPES, 10% (v/v) FCS and antibiotics at the edge of the dish; four drops of 10% PVP solution (Irvine Scientific, Santa Ana, USA) containing the spermatozoa were placed

in the center of the dish. The selected spermatozoon had its tail broken by pressing the injection pipette against the bottom of the Petri dish. The spermatozoon with a broken tail was then aspirated into the injection pipette and transferred to the drop containing the oocytes. The tip of the pipette was introduced into the perivitelline space and the oolemma was ruptured by gently aspirating small amounts of the ooplasm into the pipette. Then, the spermatozoon and the small amount of aspirated cytoplasm were inserted back into the oocyte with a minimal volume of the PVP solution. Sham injections were performed in a similar manner for the parthenogenetic control.

Oocyte activation and embryo culture

For activation, oocytes were exposed to a 5-µM ionomycin solution for 5 min and then were transferred to SOF media supplemented with 6 mg/mL fatty acid-free BSA, in which they remained for 5 min to stop activation. After activation, oocytes were cultured in SOF medium for 3 h for extrusion of the second polar body and finally incubated in a solution of 1.9 mM 6-dimethylaminopurine for 4 h. Activated oocytes were cultured in SOF media, using a co-culture with cumulus cells under mineral oil at 39°C, with 5% CO_2 in air.

Evaluation of fertilization

A total of 165 oocytes were removed from culture at 16-18 h post-ICSI, fixed overnight in methanol:acetic acid (3:1, v/v) and stained with 1% acetic acid lacmoid. Then, the oocytes were examined with a phase-contrast microscope to check for decondensed sperm head and pronucleus formation.

Statistical analysis

Analysis of the data was performed using Sigma Stat for Windows, version 3.11 (Systat Software, Inc.). The results of the AOT and TUNEL assays of the lyophilization treatments were compared with the Mann-Whitney rank sum test. A difference of $P < 0.05$ was considered statistically significant. A linear regression analysis was performed to evaluate the relationship between DNA integrity and fertilization potential of freeze-dried sperm.

RESULTS

Based on the AOT, all treatments, including the control (frozen semen), resulted in stable chromatin (green fluorescence) in most of the cells (Figure 1A). The spermatozoa lyophilized with TCM 199 and FCS had 4% chromatin damaged cells (orange fluorescence; Figure 1B). Although, this was the highest rate (Table 1), it was not significantly different from the other groups ($P = 0.120$).

The TUNEL assay was also used to assess differences in DNA damage among treatments (Figure 2). The treatment using EGTA (T3) resulted in the lowest rate of DNA fragmentation (2%), being similar to the control ($P = 0.248$). Treatment with trehalose (T2) gave 5% of the cells with DNA fragmentation, which was higher than T3 and the control, but was lower than T1. The highest rate ($P < 0.05$) of DNA damage (14%) was observed in T1 (Table 1).

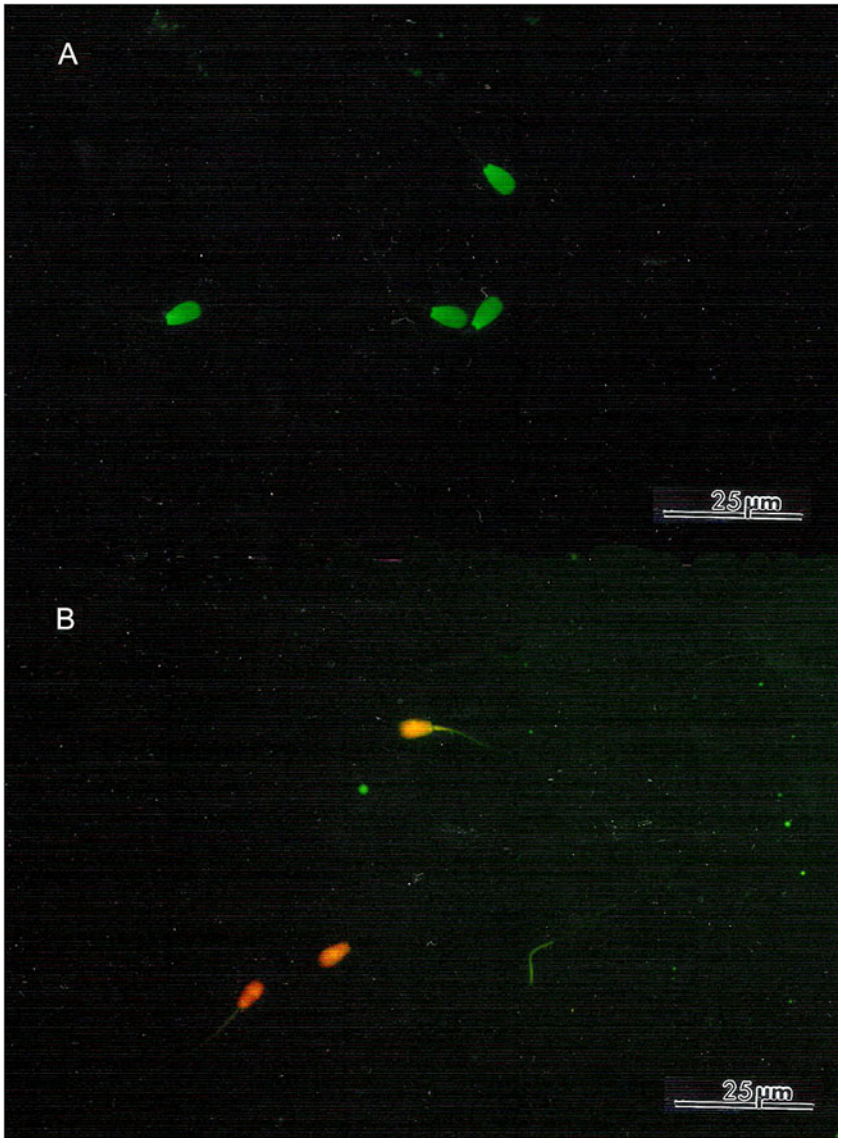

Figure 1. Acridine orange test applied to freeze-dried bovine spermatozoa, using TCM 199 with Hank's salts. **A.** Spermatozoa with intact chromatin (green); **B.** spermatozoa with damaged chromatin (orange).

The TUNEL assay was able to detect a higher percentage of cells with DNA injuries than was AOT (Table 1). In most of the treatments, the percentage of cells that were injured was low; however, in T1 in which the percentage of damage was increased, the TUNEL assay demonstrated a significant difference (P = 0.004), while AOT did not.

Table 2 depicts results of the freeze-dried spermatozoon nucleus functionality, for the different treatments, after ICSI. When matured oocytes were microinjected with spermatozoa lyophilized in T2 and T3, the proportion of oocytes presenting sperm head decondensation and

Table 1. Comparison between the acridine orange test (AOT) and the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL) assay to assess DNA damage in bovine sperm submitted to lyophilization with different freeze-drying media.

Method	Percentage of cells with damaged DNA			
	T1	T2	T3	Control
TUNEL assay	14%[a]	5%	2%	1%
AOT	4%[b]	2%	1%	0.5%

[a,b]Percentages with different superscripts, within columns, differ significantly ($P < 0.05$).
T1: TCM 199 with Hank's salts supplemented with 10% FBS;
T2: TCM 199 with Hank's salts supplemented with 10% FBS and 0.2 M trehalose;
T3: EGTA solution;
C: control (frozen semen).

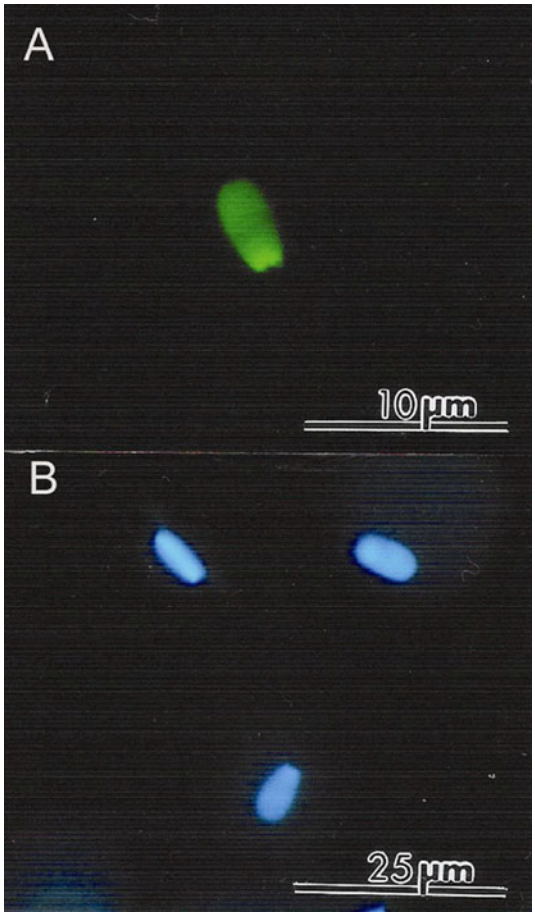

Figure 2. Bovine spermatozoa after freeze-drying with TCM 199 with Hank's salts, 10% FCS and 0.2 M trehalose presenting DNA fragmentation (A) and intact DNA (B), detected by the TUNEL assay.

pronucleus formation, was similar (P = 0.187); but both were superior to T1 (P < 0.05). As expected, the control treatment gave the highest percentage of pronucleus formation.

Table 2. Percentage of bovine oocytes presenting sperm head decondensation and pronucleus formation (PN) after intracytoplasmic sperm injection with spermatozoa lyophilized with different freeze-drying media.

Treatments	No. of oocytes	MII + ISH	ISH + 1PN	DH + 1PN	2PN
T1	40	9 (22.5%)	17 (42.5%)	6 (15.0%)[b]	8 (20.0%)[a]
T2	40	5 (12.5%)	6 (15.0%)	11 (27.5%)[a]	18 (45.0%)[b]
T3	40	10 (25.0%)	2 (5.0%)	13 (32.5%)[a]	15 (37.5%)[b]
Control	45	11 (24.4%)	4 (8.8%)	5 (11.1%)[b]	25 (55.5%)[c]

[a,b,c]Percentages with different superscripts, within columns, differ significantly (P < 0.05).
MII + ISH: MII oocyte and intact sperm head; ISH + 1PN: intact sperm head and female PN; DH + 1PN: sperm head decondensed and female PN; 2PN: male and female PN.
T1: TCM 199 with Hank's salts supplemented with 10% FBS;
T2: TCM 199 with Hank's salts supplemented with 10% FBS and 0.2 M trehalose;
T3: EGTA solution;
C: control (frozen semen).

A positive relation between DNA integrity, assessed by the TUNEL assay, and pronucleus formation after ICSI, was observed for all lyophilization treatments (Figure 3).

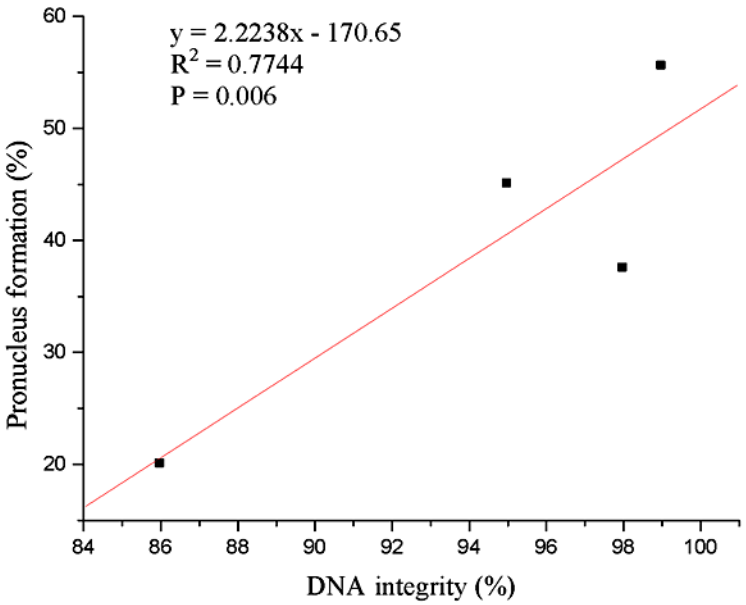

Figure 3. Linear regression between DNA integrity of freeze-dried sperm, based on the TUNEL assay, and pronucleus formation after intracytoplasmic sperm injection.

DISCUSSION

Freeze-drying is an alternative method for the preservation of sperm cells. However, it can cause irreversible injury to sperm structure, especially if suitable protection is not provided during the process itself and during storage. Considering these aspects, we examined how well two different approaches, AOT and TUNEL assays, detect injuries to DNA when bovine spermatozoa are lyophilized with distinct protective substances. Based on these results, it became clear that the composition of the lyophilization media affects sperm DNA integrity. However, this effect was only significant when the TUNEL assay was used; evaluation by AOT did not detect any increase in abnormality. Spermatozoa lyophilized with EGTA solution had the lowest DNA fragmentation rate, which was not significantly different from the control treatment. In the samples preserved with trehalose, only 4% of the sperm cells had fragmented DNA. The treatment with 10% FCS and TCM 199 conferred the lowest protection for sperm cell DNA, giving 14% of the cells with fragmentation.

The contradictory results obtained with AOT and TUNEL raise some questions about the sensitivity of these tests. The treatment using TCM 199 and FCS, which gave the highest rate of DNA fragmentation, also gave a high percentage of oocytes with intact heads in the cytoplasm and a low rate of pronucleus formation. These results agreed with the TUNEL technique findings, but not with AOT. Although the pattern of chromatin integrity was similar to that found with TUNEL, no significant differences were found among treatments.

Even though AOT is an easy test to perform, the results are suspect, as was seen in our study. According to Duran et al. (1998) and Evenson et al. (1999), the main reasons for the weakness of this test are subjectivity in reading and interpretation of the results, problems with rapidly fading fluorescence and heterogeneous slide staining. Those difficulties have limited the application of AOT in andrology clinics (Evenson et al., 2002; Perreault et al., 2003). To overcome these problems, Evenson et al. (1980) developed the sperm chromatin structure assay (SCSA), which is similar to AOT, but gives better results since the evaluation is made with a flow cytometer. The SCSA has been used with high efficiency with mouse, equine, bovine, and porcine sperm to evaluate sperm chromatin structure. Additionally, it has been used in humans as a routine measurement of sperm quality and fertility (Evenson and Jost, 1994; Love and Kenney, 1998).

In contrast, since the TUNEL assay detects endogenous DNA damage it has been considered a more suitable method to quantify DNA breakage in sperm cells (Young et al., 2003). In the analysis of human spermatozoa, the TUNEL assay has been shown to give similar results to SCSA (Larson-Cook et al., 2003) and single-cell gel electrophoresis and the Comet assay (Collins et al., 1997). We demonstrated by linear regression analysis that sperm DNA fragmentation is inversely related with fertilizing potential, since DNA fragmentation increased as pronuclear formation decreased.

In conclusion, previous evaluation of the sperm nucleus status is needed before using assisted reproduction techniques. Freeze-drying sperm is a new methodology, in which the composition of the lyophilization medium is one of the factors that can improve protection of the spermatozoa. Evaluation of the freeze-dried sperm DNA should be a routine procedure, given that the possibility of damage is greater than with other sperm preservation methods. We found the TUNEL assay to be appropriate for evaluating DNA status of lyophilized spermatozoa than AOT. Given that the DNA damage detect by TUNEL assay was related to fertilization potential, we conclude that it should be the method of choice to predict sperm fertility.

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