Reprod. Fertil. Dev., 2000, **12**, 251–261

Changes in susceptibility of bovine sperm to *in situ* DNA denaturation during prolonged incubation at ambient temperature under conditions of exposure to reactive oxygen species and nuclease inhibitor

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Abstract. Sperm were incubated for up to 9 days in the presence or absence of exogenous hydrogen peroxide, phenylalanine, catalase and aurintricarboxylic acid to assess the influence of reactive oxygen species and inhibition of deoxyribonucleases on sperm chromatin stability. The assessment of sperm DNA susceptibility to *in situ* acid denaturation by the sperm chromatin structure assay did not detect any difference in chromatin stability between sperm incubated for 9 days under aerobic and anaerobic conditions in a diluent called 14G. Exposure to exogenous hydrogen peroxide under both aerobic and anaerobic conditions and to phenylalanine under aerobic conditions (which produces hydrogen peroxide by a reaction catalysed by the aromatic amino acid oxidase present in sperm) was detrimental to sperm chromatin stability, increasing its DNA susceptibility to *in situ* acid denaturation over the incubation time. This effect was eliminated if catalase was present in the diluent. Inclusion of the general deoxyribonuclease inhibitor aurintricarboxylic acid in the diluent severely decreased sperm chromatin stability under both aerobic and anaerobic conditions. Aurintricarboxylic acid was mildly cytotoxic, as revealed by viability assessment, under aerobic, but not under anaerobic, incubation conditions. Exogenous hydrogen peroxide, either directly added to the diluent or generated through the enzymatic oxidation of phenylalanine, was detrimental to sperm motility and the integrity of the plasma membrane.

Extra keywords: antioxidants, motility, nuclear chromatin integrity, ROS, sperm chromatin structure assay (SCSA), viability.

Introduction

The integrity of the sperm genome is vital. Any detrimental changes to the sperm cellular machinery and nuclear DNA could affect their oocyte-penetrating ability and the potential to both initiate and sustain the development of the embryo. During *in vitro* incubation at ambient temperature, sperm undergo damaging alterations that could affect the haploid genome (Salisbury and Hart 1970; Vishwanath and Shannon 1997).

Sperm nuclear chromatin integrity can be investigated using the DNA-binding properties of acridine orange (AO), which intercalates into double-stranded (ds) DNA as a green fluorescing monomer and binds to single-stranded (ss) DNA as a red fluorescing aggregate. The sperm chromatin structure assay (SCSA) was developed to measure sperm DNA susceptibility to *in situ* acid denaturation by quantifying the metachromatic shift from green fluorescence of AO bound to dsDNA to red fluorescence emitted by AO bound to ssDNA (Evenson *et al.* 1980). Reduced sperm chromatin stability as measured by SCSA correlates strongly with DNA strand breaks (Aravindan *et al.* 1997) and sub-fertility in bull, human and boar (Ballachey *et al.* 1987, 1988; Evenson *et al.* 1994, 1999). Sperm DNA fragmentation has also been correlated with reduced *in vitro* fertilization rates (Sun *et al.* 1997). A decline in chromatin integrity during incubation at ambient temperature has been detected by SCSA in mouse sperm (Estop *et al.* 1993) and human sperm (Ellington *et al.* 1998). Bull sperm incubated in milk or egg yolk extender have also shown increased SCSA values over time (Karabinus *et al.* 1991).

Reactive oxygen species (ROS) are harmful to sperm at elevated levels (Jones and Mann 1973; Alvarez *et al.* 1987; Aitken *et al.* 1989; D'Agata *et al.* 1990; Aitken and Fisher 1994; Cummins *et al.* 1994; Armstrong *et al.* 1999) and are a major source of damage to sperm DNA (Gagnon-*et al.* 1991). The major sources of ROS in diluted semen incubated at ambient temperature are oxidative deamination of aromatic amino acids by aromatic L-amino acid oxidase released from dead and damaged sperm (Shannon and Curson 1972, 1981), mitochondrial respiration (Aitken and

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10.1071/RD00081 1031-3613/00/050251

Clarkson 1987), and seminal leukocytes (Aitken *et al.* 1992; Kessopoulou *et al.* 1992). Because sperm are almost devoid of cytoplasm, they possess only very low amounts of the ROS-scavenging enzymes that protect somatic cells from oxidative damage. Moreover, DNA repair enzymes are apparently inactive in mature sperm, making these cells uniquely susceptible to oxidative attack (Hughes *et al.* 1998). Functional sperm rely on the tight packing of their DNA around protamines, which reduces exposure to free radicals, and on antioxidants present in the seminal plasma for protection from oxidative damage (Hughes *et al.* 1998). During *in vitro* manipulation of sperm samples, oxidative damage to sperm DNA can be alleviated by supplementing the diluent with antioxidants (Hughes *et al.* 1998), ROS-degrading enzymes and elimination of oxygen from the diluent (Shannon and Curson 1982).

Exchange of nuclear histones during spermatogenesis, first by transition proteins and subsequently by protamines, is characterized by scission and re-ligation of DNA by topoisomerases in order to accommodate the torsional stresses involved in these protein exchanges (Roca and Mezquita 1989; McPherson and Longo 1993*a*, 1993*b*). Some activity of these DNA hydrolysing enzymes may persist in mature sperm. DNA strand breaks in sperm may also be caused by endogenous deoxyribonucleases activated by external stimuli, which cleave both exogenous and genomic DNA, and this leads in some cases to cell death that in some respects resembles apoptosis (Spadafora 1998; Blanc-Layrac *et al.* 2000). This type of DNA damage in mouse sperm can be prevented by pre-incubation with the deoxyribonuclease inhibitor aurintricarboxylic acid (ATA) prior to *in vitro* fertilization, leading to increased efficiency of embryo production (Zaccagnini *et al.* 1998). It has been proposed that the ATA acts by protecting sperm nuclei from induced or spontaneous DNA damage and/or by priming sperm chromatin for the events of early embryogenesis (Zaccagnini *et al.* 1998).

There is significant interest in both the bovine and porcine industry to extend the fertile life of sperm stored in the unfrozen state (Vishwanath and Shannon 1997). The obvious advantage of storing sperm unfrozen is that far fewer sperm are required per insemination dose to achieve the same level of fertility compared with frozen semen. However, the short shelf life of unfrozen semen makes it a less flexible option. A better understanding of the biochemical changes occurring during extended incubation at ambient temperatures is necessary in order to extend the fertile life of unfrozen sperm. The current study was performed to investigate whether the stability of bull sperm chromatin deteriorates as a result of deoxyribonuclease activity during extended incubation at ambient temperature. Also investigated was whether the stability of the chromatin was affected by increased exposure to ROS under these incubation conditions. Instability of the chromatin was assessed as an increase in DNA susceptibility to *in situ* denaturation by acid. The influence of endogenous deoxyribonucleases on chromatin stability was investigated by the inclusion of ATA in the incubation medium. Increased exposure to ROS was achieved by storing sperm in the presence of H_2O_2 added directly to the diluent or L-phenylalanine, which is a substrate in an oxidation reaction catalysed by aromatic L-amino acid oxidase and results in generation of H_2O_2 as a by-product (Shannon and Curson 1972). Reduced levels of ROS were achieved by including catalase in and eliminating O_2 from the diluent.

Materials and methods

Materials

Analytical grade chemicals were used unless otherwise stated. Citric acid and D-glucose were obtained from Riedel-de Haën AG (Seelze, Germany). O_2, CO_2, oxygen-free N_2 and the gas mixture of 5% H_2 and 95% N_2 were purchased from British Oxygen Co. (Hamilton, NZ). EGTA, n-[p-aminobenzenesulfonyl] acetamide (sulfacetamide), streptomycin sulfate, penicillin, fatty acid free BSA, 2.0 N standardized HCl, Triton X-100, phenylalanine, and aurintricarboxylic acid (ATA) (practical grade) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris) was purchased from United States Biochemical Corporation (Cleveland, OH, USA). Catalase was extracted from livers of 18-month-old, disease-free bulls owned by Livestock Improvement Corporation (Hamilton, NZ) using a method described by Summer and Mybrack (1951). Sodium chloride was purchased from Scientific Supplies Ltd (Auckland, NZ). Chromatographically purified acridine orange (AO) was purchased from Polysciences (Warrington, PA, USA). Propidium iodide and SYBR-14 were purchased as FertiLight™ Sperm Viability Kit from Molecular Probes, Inc. (Eugene, OR, USA). All other chemicals were purchased from BDH Chemicals Ltd (Poole, UK).

Flow cytometer instrumentation

A Becton Dickinson fluorescence-activated cell sorter (FACScan) with built-in air-cooled argon ion laser operated at 488 nm emission spectra was used in this study. The FACScan was interfaced to a Macintosh Quadra 650 computer with CellQuest™ Becton Dickinson Software (Becton Dickinson, San Jose, CA, USA). A 515-nm band pass filter was used to collect green fluorescence (FL1) and a 630-nm band pass filter to collect red fluorescence (FL3).

Preparation of diluents

14G, a diluent commonly used for liquid semen storage, was prepared according to Shannon (1965). The medium also contained, di-potassium hydrogen orthophosphate 3-hydrate 35 mM, magnesium chloride 2.2 mM and calcium chloride 2-hydrate 2.5 mM, as well as penicillin and streptomycin sulfate, each 1.25 mega-units per litre, sodium sulfacetamide 0.1 μM and catalase 800–1000 U mL^{-1}. When anaerobic incubation conditions were required, the diluent was purged with oxygen-free N_2 for 30 min and placed inside an anaerobic chamber, as described below, to equilibrate overnight. When sperm were incubated under aerobic conditions, the diluent was not gassed at all.

For motility analysis, BSA was added to the diluent to a final concentration of 1% to prevent sperm from adhering to the glass slides.

Anaerobic conditions

The anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA) was filled with a pre-mixed oxygen-free gas blend of 5% hydrogen and 95% nitrogen and equipped with an air lock to allow

transfer of equipment and solutions without exchange of the internal atmosphere with air. A positive pressure was maintained inside the chamber, limiting the possibility of air access to the interior. Any residual oxygen inside the chamber was reduced to water by hydrogen in the gas mixture, aided by a palladium catalyst. Absence of oxygen in the atmosphere in the anaerobic chamber was confirmed by use of Anaerobic Indicator BR55 (Unipath Limited, Basingstoke, UK).

Semen collection and preparation

Semen was collected from mature dairy bulls (*Bos taurus*) using an artificial vagina, and ejaculates were assessed visually for percentage motile sperm under an Olympus BH2 microscope equipped with phase contrast optics, at ×125 magnification (Olympus Optical Co., Ltd, Tokyo, Japan). Only ejaculates containing more than 75% motile sperm were used in the experiments. The concentration of sperm in the semen was measured using a semen concentration photometer (Instruments Medecine Veterinaire, L'Aigle-Cedex, France). For all treatments, semen was initially diluted in an anaerobic diluent at 32°C, to a concentration of 240×10^6 sperm mL^{-1}, and allowed to cool slowly, to avoid cold-shock, over a period of 30 min to ambient temperature (18–20°C). It was then diluted again to a final concentration of 20×10^6 sperm mL^{-1}, in either aerobic or anaerobic diluent, at ambient temperature.

Sperm viability measurements

Aliquots of sperm (in triplicate) were stained with FertiLight™ Sperm Viability Kit, according to the manufacturer's instructions. Sperm with intact plasma membranes ('live') fluoresce green (515 nm) whereas sperm with damaged plasma membrane ('dead') fluoresce red (630 nm). Distributions of 10 000 green- and red-stained sperm sub-populations were displayed as two-parameter dot plot cytograms for log FL1 and log FL3, where FL1 is a peak intensity of green fluorescence and FL3 is a peak intensity of red fluorescence of a cell passing through the excitation laser beam (Johnson *et al.* 1996). The proportion of sperm with intact plasma membrane was quantified using quadrant analysis (Johnson *et al.* 1996) to delineate green only, red only and double green and red fluorescence sperm populations. Percentage of live sperm was calculated by dividing the number of cells in the green fluorescence quadrant by the sum of sperm cells in all of the quadrants.

Assessment of percentage of motile sperm

The percentage of motile sperm in samples was visually assessed by trained technicians using six observation fields under a ×10 negative phase contrast objective.

Sperm chromatin structure assay

SCSA procedures as described in detail by Evenson and Jost (1994) were used in this study. Samples, taken from incubation bottles at designated time intervals, were snap-frozen and stored in liquid nitrogen. SCSA of all samples from different treatments of the sperm from the same ejaculate were performed on the same day. The whole SCSA procedure was performed in triplicate for each thawed sample. To confirm the stability of the assay and reliability of the instrument, the reference bull samples were assayed after every 15 experimental samples. Reference samples were diluted aliquots of the same ejaculate frozen under identical conditions and used throughout the trial to set the photomultiplying tubes of the flow cytometer to the values that ensure that the mean red and green fluorescence of the same samples fall within ± 5 channels of each repeated measurement of the same sample.

Sample tubes were removed from liquid nitrogen and immediately thawed by immersion in a 37°C water bath. Immediately after thawing each sample was diluted with ice-cold TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl and 1 mM EDTA, pH 7.4) to a concentration of 8×10^6 mL^{-1} sperm cells and the tubes were placed on wet ice. Using a Falcon tube (Becton Dickinson), 200 µL of diluted sample was mixed with 400 µL of ice-cold acid detergent solution (0.1% v/v Triton X-100, 0.08 M HCl, 0.15 M NaCl). Exactly 30 s later, 1.2 mL of staining solution (0.1 M citric acid monohydrate, 0.2 M di-sodium hydrogen orthophosphate, 1 mM di-sodium EDTA, 0.15 M NaCl, 6 µg ml^{-1} AO, pH 6.0) was added. The sample tube was placed into the flow cytometer and sample flow started. Signal acquisition was started after 2 min equilibration.

Flow cytometry was performed using the slow flow rate setting (12 µL min^{-1} of sample) with 15 mW laser output. Prior to SCSA measurements, the instrument was calibrated using CaliBRITE™ calibration beads (Becton Dickinson), then a mixture of 75% AO staining solution and 25% acid detergent solution was passed through the instrument's sample lines for 20 min to equilibrate AO with the sample tubing. After the last sample was analysed, a 0.4% (w/w) solution of sodium hypochlorite was passed through the sample lines, followed by a H_2O rinse to remove all traces of AO. Green (515 nm) and red (630 nm) fluorescence of 5000 cells was acquired at approximately 75 events s^{-1}. List-mode data files were then analysed using WinList™ Software (Verity House, Inc., Topsham, ME, USA).

The level of DNA denaturation in each individual cell was quantified by alpha t (α_t) where α_t = red/(red + green) fluorescence. The following SCSA parameters were obtained from α_t distribution histograms: cells outside the main population (COMPα_t), reflecting the number of cells with increased DNA denaturability, and the mean and standard deviation of α_t, reflecting the degree of chromatin instability in the gated cell population.

Experimental design

Sperm samples from three bulls were used throughout this study and two incubation experiments were performed. In both experiments diluted sperm were incubated at ambient temperature in sealed 100-mL sterile Schott bottles (Schott Glaswerke, Mainz, Germany) wrapped in aluminium foil. Bottles in which sperm were incubated were opened daily and equilibrated with the gas phase by gentle shaking for 10 s. For incubation of sperm under anaerobic conditions, the sperm were suspended in an anaerobic medium and incubated inside an anaerobic chamber as described above.

In the first experiment, sperm were diluted in the following media: normal 14G buffer (containing catalase), 14G buffer without catalase, 14G buffer supplemented with 100 µM H_2O_2, 14G buffer without catalase supplemented with 100 µM H_2O_2, 14G buffer supplemented with 1 mM phenylalanine, and 14G buffer without catalase supplemented with 1 mM phenylalanine. The concentration of H_2O_2 was chosen based on the report by Aitken *et al*. (1998), where 100 µM H_2O_2 induced significant damage to sperm DNA as assessed by the COMET assay. Diluted semen was incubated for 9 days in each of the diluents under aerobic or anaerobic conditions at ambient temperature. Motility and viability of incubated sperm were assessed and samples for SCSA were removed and frozen in liquid nitrogen on Days 0, 3, 6, and 9 of incubation.

In the second experiment sperm were diluted in normal 14G buffer and in 14G buffer supplemented with 25 µM ATA. They were then incubated under aerobic or anaerobic conditions for 8 days. Motility and viability of incubated sperm were assessed and samples for SCSA were removed and frozen on Days 0, 2, 4, 6, and 8 of incubation.

Statistical analysis

Estimates of motility, viability and SCSA parameters were analysed using general linear model (GLM) procedures of SYSTAT® 8.0 Statistics (SPSS Inc. Chicago, IL, USA). At each time interval, matrices of *P* values for Tukey's pair-wise comparisons were obtained. Differences with the *P* value <0.05 were regarded as statistically significant for all analyses.

Results

The effects of exposure to ROS on sperm viability and motility

Sperm motility and viability declined over time of incubation under all treatments but not at the same rate (Table 1).

Motility was affected most severely by inclusion of exogenous H_2O_2 in the medium under anaerobic incubation conditions. Under aerobic incubation conditions, there was no significant difference between the detrimental effect on motility of H_2O_2 directly added to the diluent and that of H_2O_2 generated via oxidation of phenylalanine under aerobic conditions. Apart from the treatment where H_2O_2 was included in the diluent, motility was maintained better under anaerobic than under aerobic incubation conditions. Whether H_2O_2 was added directly to the medium or generated in the course of the oxidation of phenylalanine, catalase protected incubated sperm from the detrimental effects of H_2O_2 on motility.

Viability in the presence of exogenous H_2O_2 declined more sharply under aerobic conditions (Table 1). Where exogenous H_2O_2 was added to the diluent under anaerobic conditions, motility ceased on Day 3 of incubation without as marked a loss of plasma membrane integrity as observed under aerobic conditions in the presence of H_2O_2 or phenylalanine. In the treatments where H_2O_2 was present, loss of membrane integrity was more rapid than in sperm exposed to exogenous H_2O_2 under anaerobic conditions. There was no significant difference between the viability of sperm incubated in the presence or absence of catalase if no exogenous H_2O_2 was included or generated in the diluent on any of the incubation days within aerobic and anaerobic treatments.

H_2O_2, in the absence of catalase, caused a significant loss in membrane integrity under both aerobic and anaerobic incubation conditions, although the effect was stronger under aerobic conditions on Days 6 and 9. Phenylalanine did not have a significant effect on viability during incubation under anaerobic conditions (where H_2O_2 would not be produced from its oxidation) but caused membrane disintegration during incubation under aerobic conditions in the absence of catalase. Apart from where H_2O_2 was added, sperm viability was maintained better under anaerobic than under aerobic conditions.

The effects of exposure of sperm to H_2O_2 on the susceptibility of their DNA to in situ *acid denaturation*

The SCSA parameters of sperm incubated under conditions that exposed them to different levels of ROS are presented in Table 2. Sperm exposed to exogenous H_2O_2 that was either directly added to the diluent or generated by aromatic amino acid oxidase in the presence of phenylalanine under aerobic conditions suffered significant decline in the resistance of their DNA to *in situ* acid denaturation. From Day 6 onwards, catalase did not protect sperm chromatin from destabilization by H_2O_2 under aerobic incubation conditions: both mean α_t and mean of standard deviation of α_t were elevated. The effect of H_2O_2 generated via aromatic amino acid oxidase on sperm chromatin stability was most severe and was observed at the earliest time point during aerobic incubation. As expected, the presence of phenylalanine in the medium under anaerobic conditions did not affect chromatin stability because the H_2O_2-yielding reaction of aromatic L-amino acid oxidation could not take place in the absence of dissolved oxygen. Somewhat unexpectedly,

Table 1. Percentages of motile sperm and sperm with intact plasma membrane (mean ± SEM) during incubation at ambient temperature in diluents that allow exposure to different levels of ROS

n = 9; different superscripts within row denote significant differences (P<0.05).

	Aerobic						Anaerobic					
	Normal diluent		Diluent + H_2O_2		Diluent + phenylalanine		Normal diluent		Diluent + H_2O_2		Diluent + phenylalanine	
	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase
% motile												
Day 0	75 ± 1.8^{d}	75 ± 1.8^{d}	73 ± 2.1^{cd}	67 ± 2.8^{b}	73 ± 2.1^{cd}	75 ± 1.8^{d}	72 ± 1.1^{cd}	70 ± 0.0^{bc}	68 ± 2.1^{b}	60 ± 2.8^{a}	67 ± 4.2^{b}	73 ± 1.1^{cd}
Day 3	48 ± 3.8^{e}	48 ± 2.1^{e}	38 ± 2.8^{cd}	15 ± 1.8^{b}	47 ± 4.2^{e}	13 ± 4.6^{b}	40 ± 6.3^{d}	37 ± 4.6^{c}	33 ± 6.4^{c}	0 ± 0.0^{a}	45 ± 4.8^{de}	35 ± 1.8^{c}
Day 6	15 ± 4.8^{bc}	12 ± 2.8^{b}	17 ± 2.8^{c}	0 ± 0.0^{a}	22 ± 7.4^{cd}	0 ± 0.0^{a}	35 ± 6.3^{d}	32 ± 4.6^{d}	30 ± 6.6^{d}	0 ± 0.0^{a}	33 ± 6.9^{cd}	32 ± 2.8^{d}
Day 9	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}	0 ± 0.0^{a}	25 ± 4.8^{b}	18 ± 5.6^{b}	18 ± 5.6^{b}	0 ± 0.0^{a}	20 ± 6.6^{b}	18 ± 4.6^{b}
All days	35 ± 6.7^{bc}	35 ± 6.7^{bc}	32 ± 6.5^{b}	20 ± 6.5^{ab}	35 ± 6.5^{bc}	22 ± 7.3^{ab}	43 ± 4.1^{c}	39 ± 4.5^{bc}	38 ± 4.4^{bc}	15 ± 6.1^{a}	41 ± 4.0^{c}	40 ± 4.8^{c}
% live												
Day 0	79 ± 4.3^{ab}	82 ± 3.2^{b}	82 ± 3.4^{b}	75 ± 2.6^{ab}	78 ± 3.9^{ab}	78 ± 1.5^{ab}	81 ± 1.8^{b}	79 ± 2.8^{ab}	78 ± 6.1^{ab}	71 ± 2.1^{a}	75 ± 1.7^{ab}	82 ± 4.0^{b}
Day 3	68 ± 5.9^{bc}	67 ± 6.4^{bc}	67 ± 6.5^{bc}	43 ± 4.0^{a}	66 ± 7.6^{b}	58 ± 7.5^{ab}	67 ± 6.7^{bc}	71 ± 5.6^{c}	71 ± 6.2^{c}	46 ± 9.9^{a}	68 ± 6.6^{bc}	68 ± 6.6^{bc}
Day 6	58 ± 7.8^{c}	57 ± 7.6^{c}	59 ± 7.4^{c}	17 ± 4.7^{a}	53 ± 8.2^{c}	25 ± 5.3^{ab}	65 ± 6.7^{c}	63 ± 7.1^{c}	66 ± 7.5^{c}	35 ± 9.7^{b}	65 ± 6.5^{c}	65 ± 6.7^{c}
Day 9	45 ± 9.1^{c}	29 ± 5.8^{bc}	45 ± 9.2^{c}	3 ± 1.8^{a}	34 ± 9.9^{bc}	3 ± 1.4^{a}	58 ± 7.1^{d}	58 ± 7.4^{d}	59 ± 7.7^{d}	24 ± 5.9^{b}	57 ± 7.3^{d}	57 ± 7.0^{d}
All days	63 ± 2.4^{c}	59 ± 3.7^{c}	63 ± 2.6^{c}	35 ± 5.2^{a}	58 ± 3.1^{bc}	41 ± 5.6^{ab}	68 ± 1.6^{c}	68 ± 1.6^{c}	68 ± 1.4^{c}	44 ± 3.4^{ab}	66 ± 1.2^{c}	68 ± 1.7^{c}

Table 2. SCSA parameters (mean ± SEM) during incubation at ambient temperature in the diluents that allow exposure to different levels of ROS

n = 9; different superscripts within row denote significant differences (P<0.05).

	Aerobic						Anaerobic					
	Normal diluent		Diluent + H_2O_2		Diluent + phenylalanine		Normal diluent		Diluent + H_2O_2		Diluent + phenylalanine	
	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase	With catalase	Without catalase
Mean α_t												
Day 0	223 ± 1.9^{b}	220 ± 2.9^{ab}	222 ± 2.3^{b}	219 ± 0.9^{ab}	221 ± 1.2^{ab}	219 ± 0.7^{ab}	216 ± 0.6^{a}	222 ± 1.9^{ab}	221 ± 2.1^{ab}	222 ± 1.7^{ab}	222 ± 2.4^{b}	219 ± 1.5^{ab}
Day 3	221 ± 2.2^{a}	219 ± 0.8^{a}	222 ± 2.9^{a}	221 ± 0.9^{a}	222 ± 1.8^{a}	229 ± 3.3^{b}	220 ± 0.7^{a}	221 ± 0.9^{a}	218 ± 1.6^{a}	220 ± 2.7^{a}	220 ± 1.7^{a}	219 ± 1.5^{a}
Day 6	224 ± 1.4^{ab}	217 ± 0.4^{a}	229 ± 1.1^{b}	240 ± 2.2^{c}	220 ± 1.4^{a}	240 ± 3.5^{c}	220 ± 1.1^{a}	222 ± 1.8^{a}	220 ± 1.4^{a}	221 ± 2.4^{a}	221 ± 1.5^{a}	219 ± 1.7^{a}
Day 9	218 ± 2.3^{a}	217 ± 0.2^{a}	258 ± 7.2^{b}	286 ± 4.7^{c}	223 ± 1.4^{a}	259 ± 4.0^{b}	225 ± 1.2^{a}	224 ± 1.5^{a}	223 ± 1.6^{a}	226 ± 3.1^{a}	224 ± 0.8^{a}	222 ± 0.8^{a}
All days	221 ± 1.0^{a}	218 ± 0.8^{a}	233 ± 3.2^{b}	242 ± 4.7^{b}	222 ± 0.7^{a}	237 ± 2.9^{b}	220 ± 0.7^{a}	222 ± 0.8^{a}	220 ± 0.9^{a}	222 ± 1.3^{a}	222 ± 0.9^{a}	220 ± 0.7^{a}
SDα_t												
Day 0	27 ± 1.3^{a}	25 ± 1.5^{a}	22 ± 1.8^{a}	26 ± 1.9^{a}	26 ± 1.9^{a}	27 ± 1.7^{a}	23 ± 1.7^{a}	28 ± 3.0^{a}	22 ± 1.6^{a}	23 ± 1.3^{a}	24 ± 1.9^{a}	22 ± 1.6^{a}
Day 3	25 ± 1.7^{ab}	25 ± 1.8^{ab}	23 ± 1.9^{ab}	24 ± 1.7^{ab}	26 ± 1.8^{ab}	30 ± 2.1^{b}	27 ± 1.8^{ab}	25 ± 1.7^{ab}	22 ± 1.7^{a}	20 ± 1.5^{a}	23 ± 1.4^{ab}	21 ± 1.8^{a}
Day 6	25 ± 1.5^{a}	25 ± 2.0^{a}	60 ± 11.0^{b}	116 ± 5.3^{c}	26 ± 2.9^{a}	62 ± 10.9^{b}	23 ± 1.6^{a}	26 ± 1.9^{a}	24 ± 0.9^{a}	24 ± 1.4^{a}	24 ± 1.7^{a}	23 ± 1.7^{a}
Day 9	24 ± 1.7^{a}	28 ± 2.3^{a}	122 ± 25.9^{c}	190 ± 8.0^{d}	41 ± 6.5^{b}	104 ± 13.2^{c}	25 ± 1.8^{a}	27 ± 1.0^{a}	23 ± 1.9^{a}	24 ± 2.2^{a}	26 ± 2.6^{a}	25 ± 1.8^{a}
All days	25 ± 0.8^{a}	26 ± 0.9^{a}	57 ± 9.7^{b}	89 ± 11.9^{c}	30 ± 2.5^{a}	56 ± 6.7^{b}	25 ± 0.9^{a}	26 ± 1.0^{a}	23 ± 0.8^{a}	23 ± 0.8^{a}	24 ± 0.9^{a}	23 ± 0.8^{a}
COMPα_t												
Day 0	8 ± 0.4^{b}	5 ± 0.8^{a}	9 ± 0.6^{b}	7 ± 0.7^{ab}	7 ± 0.8^{ab}	6 ± 0.8^{ab}	5 ± 0.7^{a}	8 ± 0.4^{b}	7 ± 0.6^{ab}	6 ± 0.9^{ab}	9 ± 1.2^{b}	6 ± 0.4^{ab}
Day 3	8 ± 0.5^{a}	7 ± 1.0^{a}	8 ± 0.6^{a}	8 ± 0.9^{a}	8 ± 0.3^{a}	22 ± 3.8^{b}	7 ± 0.8^{a}	7 ± 0.9^{a}	6 ± 0.8^{a}	6 ± 1.5^{a}	7 ± 0.7^{a}	7 ± 0.4^{a}
Day 6	9 ± 1.8^{a}	6 ± 0.8^{a}	9 ± 0.4^{a}	10 ± 0.8^{a}	7 ± 0.5^{a}	27 ± 6.7^{b}	7 ± 0.7^{a}	9 ± 0.3^{a}	7 ± 0.6^{a}	7 ± 1.5^{a}	8 ± 1.0^{a}	7 ± 0.8^{a}
Day 9	11 ± 0.7^{ab}	6 ± 0.7^{a}	14 ± 1.3^{b}	18 ± 1.2^{b}	8 ± 0.6^{ab}	33 ± 6.8^{c}	11 ± 1.5^{ab}	10 ± 0.4^{ab}	10 ± 1.8^{ab}	14 ± 5.1^{b}	10 ± 1.2^{ab}	9 ± 0.9^{ab}
All days	8 ± 0.6^{ab}	6 ± 0.4^{a}	10 ± 0.5^{ab}	11 ± 0.8^{b}	7 ± 0.3^{ab}	22 ± 3.0^{c}	8 ± 0.6^{ab}	8 ± 0.3^{ab}	8 ± 0.6^{ab}	8 ± 1.4^{ab}	8 ± 0.6^{ab}	7 ± 0.4^{ab}

direct addition of H_2O_2 into the anaerobic diluent in the absence of catalase had no significant effect on sperm chromatin stability parameters apart from a slightly higher proportion of COMP on Day 9 of incubation.

There are some differences in the pattern of changes to chromatin stability of sperm that are not well reflected in the numerical parameters shown in Table 2. The DNA of a population of sperm exposed to H_2O_2 added directly to the diluent underwent a sudden and dramatic shift from high stability to almost complete denaturation and possible nuclear fragmentation, while the remaining sub-population was unchanged. Additionally, sperm that had been incubated in the presence of phenylalanine under aerobic conditions, and thus exposed to H_2O_2 generated by aromatic amino acid oxidase, exhibited a gradual shift in the chromatin stability of the entire population without a clear resolution of a sub-population of cells with highly unstable chromatin (Fig. 1).

The decline in DNA resistance to acid denaturation during incubation was generally concomitant with the decline in sperm motility and viability.

The effects of ATA on motility and viability of sperm incubated under aerobic and anaerobic conditions

The results of assessment of percentages of motile sperm and sperm with intact plasma membranes during incubation in the presence and absence of ATA under aerobic and anaerobic conditions are presented in Table 3. There was no significant difference in the percentage of motile sperm in any of the treatments on Days 0 and 8 of incubation. Unlike the results presented in Table 1, where motility of sperm incubated in normal 14G buffer was generally preserved better under anaerobic incubation conditions, the percentage of motile sperm was significantly lower under anaerobic than aerobic conditions on Days 2, 4, and 6 of incubation and was not significantly affected by the presence of ATA in the diluent.

Sperm viability in all treatments was not significantly different on Days 0 and 2 of incubation. On Days 4–8, the viability of sperm incubated in aerobic diluent containing ATA was significantly (P<0.05) lower than in all other treatments.

Mean viability on Day 8 and across all days of incubation was slightly but significantly higher under anaerobic than aerobic incubation conditions.

The effect of ATA on the susceptibility of DNA of sperm incubated under aerobic and anaerobic conditions to in situ *acid denaturation*

Inclusion of ATA in the sperm incubation medium had a strong destabilizing effect on sperm chromatin (Fig. 2) as measured by all parameters derived from SCSA (Table 3). This effect was observed almost immediately after dilution of the sperm in the medium containing ATA, with no change in viability and motility, and increased with the time of incubation. Mean α_t on Days 0 and 8 of incubation, as well as on average across all days of incubation, and the SD of α_t on

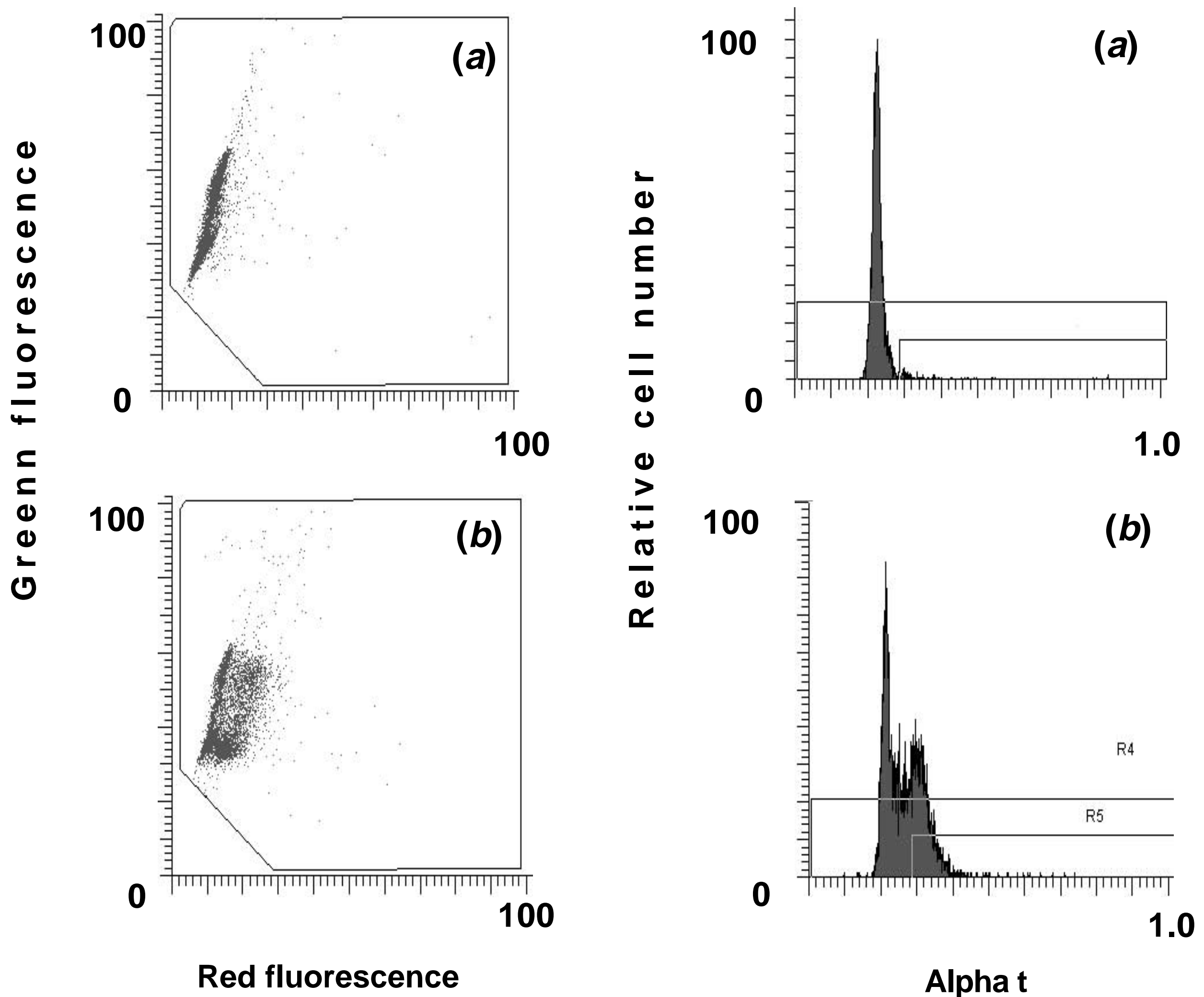

Fig. 1. Representative SCSA two-parameter cytograms and corresponding histograms of bovine sperm incubated for 9 days at ambient temperature under the following conditions: (*a*) sperm diluted in 14G buffer containing catalase, incubated under anaerobic conditions; (*b*) sperm diluted in 14G buffer devoid of catalase and containing 1 mM phenylalanine incubated under aerobic conditions. Dots represent individual cells. The position of the sperm cell dot along the Y-axis is the value of its green fluorescence intensity (dsDNA), and its X-axis position is the measure of its red fluorescence intensity (ssDNA). The heterogeneity of the green fluorescence intensity of DNA-associated chromophores in sperm cells results in an elongated spread of dots and is due to an optical artefact caused by their highly asymmetrical shape and high refractive index (Evenson *et al.* 1994). This does not present a problem for measuring SCSA parameters because they are derived from the ratio of green and total fluorescence values of individual cells (Evenson *et al.* 1994).

Days 2 and 6 were slightly but significantly higher ($P<0.05$) for sperm incubated under aerobic than anaerobic conditions in the medium containing ATA. All SCSA parameters of sperm incubated under aerobic and anaerobic conditions in the diluent that did not contain ATA were much lower than those of sperm incubated in the presence of ATA. All SCSA parameters of samples incubated in the medium without ATA remained stable during the incubation period. There was no significant difference ($P<0.05$) between sperm incubated under aerobic and anaerobic conditions in the absence of ATA on any of the incubation days.

Discussion

The effects of exposure to H_2O_2 on the motility and viability of sperm during incubation at ambient temperature were the same as has been observed in previous reports, where exogenous H_2O_2 was either added directly to the diluent (Aitken *et al.* 1998) or generated by aromatic amino acid oxidase (Shannon and Curson 1972, 1981, 1982, 1983). This study also confirmed the beneficial effect of catalase on sperm survival (Shannon and Curson 1982) during incubation, although total exclusion of oxygen from the medium was more effective in maintaining sperm motility and

Table 3. Percentages of motile sperm, sperm with intact plasma membrane and SCSA parameters of sperm (mean ± SEM) during incubation at ambient temperature in diluents containing no aurintricarboxylic acid (ATA) or 25 µM ATA under aerobic and anaerobic conditions

n = 9; different superscripts within row denote significant differences (P<0.05).

	Aerobic		Anaerobic	
	With ATA	Without ATA	With ATA	Without ATA
% motile				
Day0	66 ± 1.3[a]	65 ± 1.2[a]	66 ± 1.1[a]	66 ± 1.3[a]
Day 2	54 ± 1.0[b]	53 ± 1.2[b]	46 ± 1.1[a]	48 ± 1.5[a]
Day 4	48 ± 1.7[b]	51 ± 1.3[b]	37 ± 1.9[a]	44 ± 3.5[ab]
Day 6	33 ± 1.2[b]	35 ± 1.2[b]	25 ± 1.2[a]	27 ± 1.9[a]
Day 8	16 ± 1.0[a]	13 ± 1.5[a]	13 ± 1.2[a]	15 ± 1.9[a]
All days	43 ± 0.6[a]	43 ± 0.6[a]	37 ± 0.6[a]	40 ± 0.9[a]
% live				
Day0	78 ± 2.3[a]	78 ± 2.8[a]	78 ± 2.2[a]	80 ± 2.1[a]
Day 2	69 ± 2.4[a]	72 ± 2.8[a]	74 ± 1.4[a]	73 ± 2.1[a]
Day 4	64 ± 3.1[a]	69 ± 2.9 [b]	69 ± 1.4 [b]	69 ± 1.8 [b]
Day 6	57 ± 3.1[a]	64 ± 3.0[b]	66 ± 1.7[b]	67 ± 2.3[b]
Day 8	45 ± 3.2[a]	54 ± 3.6[b]	63 ± 1.1[c]	65 ± 2.4[c]
All days	63 ± 1.3[a]	67 ± 1.3[b]	70 ± 0.7[c]	71 ± 1.0[c]
Mean α_t				
Day0	219.0 ± 0.84[a]	217.2 ± 0.71[a]	357.6 ± 6.50[b]	391.6 ± 4.11[c]
Day 2	221.1 ± 0.77[a]	220.5 ± 1.06[a]	426.5 ± 4.96[b]	423.1 ± 4.95[b]
Day 4	221.1 ± 0.77[a]	218.8 ± 0.66[a]	438.1 ± 7.99[b]	459.0 ± 7.71[b]
Day 6	216.0 ± 0.67[a]	218.6 ± 0.69[a]	456.3 ± 4.08[b]	460.7 ± 6.56[b]
Day 8	216.6 ± 0.76[a]	219.2 ± 0.63[a]	458.0 ± 2.25[b]	492.1 ± 6.26[c]
All days	218.8 ± 0.15[a]	218.9 ± 0.15[a]	427.3 ± 1.03[b]	445.3 ± 1.18[c]
SDα_t				
Day0	21.7 ± 0.37[a]	23.4 ± 0.45[a]	114.0 ± 3.86[b]	110.7 ± 4.01[b]
Day 2	22.9 ± 0.42[a]	22.6 ± 0.43[a]	168.6 ± 1.58[b]	178.5 ± 3.77[c]
Day 4	28.0 ± 1.19[a]	25.3 ± 0.77[a]	186.2 ± 1.84[b]	182.5 ± 1.91[b]
Day 6	23.1 ± 0.74[a]	23.4 ± 0.35[a]	196.1 ± 2.98[b]	214.5 ± 3.15[c]
Day 8	22.8 ± 0.48[a]	21.7 ± 0.70[a]	217.6 ± 2.61[b]	212.3 ± 1.95[b]
All days	23.7 ± 0.13[a]	23.3 ± 0.11[a]	176.5 ± 0.51[b]	179.7 ± 0.59[b]
COMPα_t				
Day0	0.53 ± 0.27[a]	1.16 ± 0.47[a]	19.68 ± 2.65[b]	19.80 ± 2.42[b]
Day 2	0.44 ± 0.23[a]	1.82 ± 1.01[a]	27.53 ± 2.24[b]	29.63 ± 2.72[b]
Day 4	0.72 ± 0.11[a]	0.73 ± 0.08[a]	37.94 ± 4.51[b]	42.33 ± 5.39[b]
Day 6	0.47 ± 0.27[a]	0.94 ± 0.21[a]	41.72 ± 4.92[b]	39.58 ± 3.81[b]
Day 8	0.88 ± 0.19[a]	0.93 ± 0.19[a]	42.00 ± 4.78[b]	50.81 ± 3.99[b]
All days	0.61 ± 0.14[a]	1.12 ± 0.18[a]	33.78 ± 1.71[b]	36.43 ± 1.64[b]

viability during incubation than addition of catalase alone (Table 1). The detrimental effect of H_2O_2 on sperm motility observed during incubation in the diluent without catalase could be related to the inactivation of the glutathione peroxidase/glutathione reductase ROS-scavenging system demonstrated to be present in bovine semen (Bilodeau *et al.* 2000). This system has been previously shown to be susceptible to inactivation by H_2O_2 through the complete conversion of reduced glutathione to its oxidized form, rendering cells unprotected from lipid peroxidation (Alvarez and Storey 1989). The sharp decline in sperm motility when exposed to H_2O_2 under anaerobic conditions is probably caused by a different mechanism. The decline in motility observed in sperm exposed to H_2O_2 in a medium devoid of catalase, suggests that H_2O_2 is detrimental to glycolytic energy production and also causes rapid disintegration of plasma membrane. The glycolytic pathway is inhibited by exposure to H_2O_2 through damage to glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and the lowering of the essential cofactor nicotinamide adenine dinucleotide (NAD) (Cochrane 1991). Those cells heavily dependent upon mitochondrial oxidative phosphorylation will not be as subject to ATP loss, caused by inhibition of GAPDH, as those more dependent upon glycolysis; the reverse would be true for cells more dependent upon glycolysis (Cochrane 1991). Under anaerobic conditions, glycolysis is the sole energy source for the cell, whereas under aerobic conditions, sperm are also able to utilize respiration for ATP production; the oxidative damage to glycolytic pathway enzymes is more detrimental to cells incubated under anaerobic than aerobic conditions. In contrast plasma membrane integrity is more susceptible to oxidative damage by ROS under aerobic than anaerobic conditions, because ROS can initiate the lipid peroxidation chain reaction, which is sustained by oxygen and thus can proceed under aerobic but not anaerobic conditions. This effect could also be aggravated by the inactivation of glutathione peroxidase/reductase system (Alvarez and Storey 1989).

Our finding that exposure to H_2O_2 during incubation caused an increase in susceptibility of sperm DNA to *in situ* acid denaturation supports previous observations. DNA damage in sperm exposed to ROS *in vitro* has been demonstrated using the single cell electrophoresis (COMET) assay (Aitken *et al.* 1998) and the deoxynucleotidyl transferase-mediated end-labelling (TUNEL) assay (Lopes *et al.* 1998). Detection of DNA breaks by single cell electrophoresis (Aravindan *et al.* 1997) and the TUNEL assay (Evenson 1999) has also been shown previously to correlate well with the SCSA as used in this study. There is a time lag before the effects of H_2O_2 on chromatin stability are detected and this could be due to an indirect mechanism of damage caused by H_2O_2. It has been previously demonstrated that H_2O_2 is not intrinsically toxic to sperm; rather, it inactivates the glutathione oxidase/reductase scavenging system present in bovine semen (Alvarez and Storey 1989; Bilodeau *et al.* 2000). This would make sperm susceptible to the process of gradual oxidative chromatin damage caused by the reactive oxygen species produced during aerobic metabolism. The data presented here show remarkable stability of bull chromatin incubated in 14G diluent in the absence of exogenous reactive oxygen species, compared with changes previously observed in mouse sperm incubated in unsupplemented T6 diluent

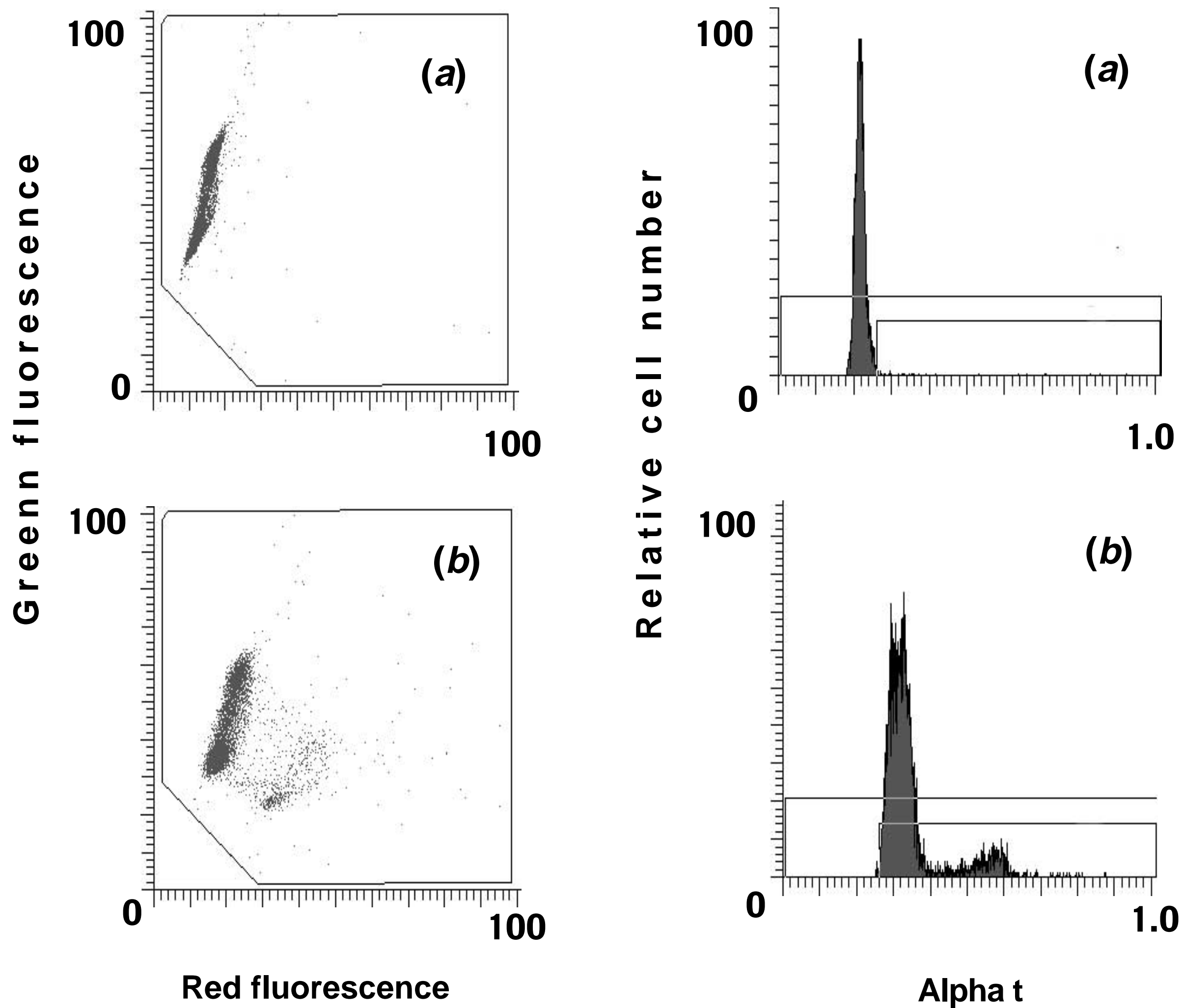

Fig. 2. Representative SCSA cytograms and corresponding histograms of bovine sperm incubated at ambient temperature for 15 min under the following conditions: (*a*) sperm diluted in 14G buffer; (*b*) sperm diluted in 14G buffer containing 25 M ATA.

(Estop *et al.* 1993), or human sperm incubated in human tubal fluid supplemented with human serum albumin (Ellington *et al.* 1998), where a significant decline in chromatin stability was detected after only one day of incubation. The high stability of bull sperm chromatin observed in our experiments may in part be an intrinsic property of the sperm of this species which, unlike sperm of numerous primates and rodents (Balhorn *et al.* 1991), including that of humans (Domenjoud *et al.* 1988) and mice (Maleszewski *et al.* 1998), contain only one type of protamine. However, since other studies have demonstrated SCSA alterations during incubation of bull sperm in commercial semen extender at a higher temperature (39°C) (Karabinus *et al.* 1991), it may be the function of the 14G diluent used here that provided protection to the genome under the present incubation conditions. This extender was experimentally developed in this laboratory over a number of years for maintenance of high viability and fertility of bovine sperm during *in vitro* storage at ambient temperature (18–22°C) (Shannon 1965).

In contrast to the deleterious effects of ROS on sperm chromatin, any endogenous nuclease activity in the sperm was seen to be of little effect under the present incubation regime. Sperm cells possess endogenous nucleases that according to some reports can, under stress conditions, be activated to cleave both exogenous and genomic DNA, leading to apoptosis-like cell death (Spadafora 1998). ATA is a general nuclease inhibitor, which has been reported to suppress endonuclease activity and promote long-term survival of some types of somatic cells under conditions that would otherwise lead to apoptotic cell death (Batistatou and Greene 1991; Okada and Koizumi 1995). Incubation of sperm with ATA prior to IVF has been shown to improve the yield of two-cell embryos (Zaccagnini *et al.* 1998). Since the susceptibility of sperm DNA to *in situ* acid denaturation as determined by SCSA has, in limited studies, been shown to be

strongly correlated with DNA strand breaks (Aravindan *et al.* 1997), we expected to detect a potentially protecting effect of ATA, exerted via nuclease inhibition, on sperm DNA stability.

A surprisingly high level of DNA instability that increased with the time of incubation was detected in all samples incubated in the presence of ATA, while virtually no change occurred in all SCSA parameters in samples incubated in the absence of ATA (Fig. 2). The destabilizing effect of ATA on chromatin was very rapid, as it was detected in all samples frozen approximately 15 min after dilution (Fig. 2). In this experiment, chromatin was destabilized by ATA before any change in motility or viability was observed (Table 3), thus showing that SCSA parameters were independent of classical semen measures. This observation supports numerous others that increased SCSA values are not due to cell death as defined by the loss of motility and plasma membrane integrity. The mechanism of chromatin destabilization by ATA may well be different from the mechanism of chromatin destabilization caused by exposure to H_2O_2, as the latter occurs in parallel with the loss of motility and viability (Tables 1 and 2).

Zaccagnini and colleagues (1998) did not offer a clear explanation of the fact that ATA was most effective in increasing IVF yields at concentrations below those required for complete inhibition of sperm nucleases. They speculated that at low concentrations (5 μM), ATA has an anti-apoptotic effect exerted through partial inhibition of nucleases, whereas higher concentrations (25–50 M) ensure complete endonuclease inhibition but also lower two-cell IVF yields. The results of the present study are contradictory to the hypothesis that ATA stabilizes the sperm nucleus. It is possible that ATA, while inhibiting sperm nucleases, causes changes to protamines and DNA packing within the nucleus leading to higher DNA susceptibility to acid denaturation. In turn, such partial decondensation of chromatin could have a positive effect on IVF yields by making sperm chromatin easier to fully decondense inside the oocyte during fertilization. An alternative explanation for the decreased chromatin stability in the presence of ATA observed in our study is that ATA somehow caused DNA damage.

If ATA caused limited DNA strand breaks it would not necessarily decrease *in vitro* fertility because, as demonstrated by Ahmadi and Ng (1999) using the mouse model, the oocyte can repair defective DNA contributed by sperm if the damage does not exceed 8%. Also, sperm with extensive DNA damage can fertilize an oocyte with the same efficiency as an undamaged sperm. Furthermore, cleavage can proceed after fertilization with sperm with damaged DNA up to the eight-cell stage, at which time paternal genomes are activated. Studies are needed that go up to and beyond the eight-cell stage. However, it is difficult to imagine that the DNA damage would actually increase IVF yields. It may be that ATA is acting in another way, as it has been reported to inhibit numerous enzymes other than nucleases (Zaccagnini *et al.* 1998).

The finding that sperm, incubated for 4 to 8 days in the presence of ATA under aerobic conditions, had lower viability than in all other treatments suggests that ATA is cytotoxic under aerobic but not anaerobic conditions.

In human sperm, various markers of apoptosis have been reported. Fas molecule and DNA fragmentation are present in some cells of sub-fertile males (Sakkas *et al.* 1999) and are thought to be the result of uncompleted apoptosis that was initiated in the testis rather than induced following ejaculation. Externalization of phosphatidylserine, detected with annexin-V, and DNA fragmentation were also observed in ejaculated human sperm (Blanc-Layrac *et al.* 2000). However, those markers may not necessarily be specific to apoptosis (Charriaut-Marlangue and Ben-Ari 1995; Glander and Schaller 1999). Since apoptosis is an active process requiring protein synthesis for its execution (Walker *et al.* 1988) and since sperm are devoid of ribosomes and thus can not synthesize proteins, classical apoptosis is unlikely in these cells. Also, apoptosis-specific morphological changes, such as budding of the whole cell and the production of membrane-enclosed apoptotic bodies, have not been reported in sperm. Even so, the possibility of a programmed or induced cell death mechanism in sperm that is different to apoptosis in somatic cells could not be excluded.

In conclusion, ATA makes sperm DNA more susceptible to *in situ* acid denaturation. Whether those changes are due to altered protamine/DNA structure or to DNA strand breaks is not known. Taking into account that ATA is a general nuclease inhibitor, the first explanation seems more likely. Further experiments involving SCSA together with estimation of DNA strand breaks through single cell electrophoresis (COMET assay) or *in situ* labelling of DNA breaks (TUNEL assay) of bovine sperm exposed to various concentrations of ATA would help clarify this question. An IVF trial using bovine sperm pre-incubated with ATA and assayed for chromatin stability would demonstrate if partial destabilization of sperm chromatin prior to IVF could be beneficial to the outcomes of IVF.

Acknowledgments

This research was financially supported by Livestock Improvement Corporation, Technology New Zealand through a Graduate Research in Industry Fellowship, Environmental Protection Agency Grant No. R827019, and United States Department of Agricultural Sabbatical Grant No. 9803911.

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Manuscript received 25 July 2000; revised and accepted 3 January 2001.