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Relationship between sperm nuclear protamine free -SH status and susceptibility to DNA denaturation

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Data from the sperm chromatin structure assay (SCSA), a flow cytometric measurement of susceptibility of sperm nuclear DNA to denaturation, show strong correlation with the fertility potential of bulls, boars, men and stallions. Previous studies showed a strong relationship between stallion spermatozoa with denatured DNA and the presence of DNA strand breaks. In the present study, the relationship between stallion sperm DNA denaturation and the redox status of -SH groups on the cysteine residues of sperm nuclear protamines that are thought to stabilize chromatin was investigated. Semen samples from 30 stallions were evaluated by the SCSA. Aliquots of the same samples were sonicated to liberate sperm nuclei, purified through a 60% sucrose gradient, stained with an -SH specific fluorochrome (CPM (7-diethylamino-3-(4'-maleimidylphenyl)-4-methyl-coumarin)), and the blue fluorescence of 5000 cells per sample was measured. If S=S bonds stabilize chromatin to inhibit DNA denaturation under the imposed low pH conditions, a low blue intensity would correlate with a low level of DNA denaturation. However, this study showed no correlation ($r = -0.199$, $P = 0.31$) of -SH stainability with the extent of DNA denaturation. Thus, other parameters, possibly DNA strand breaks, play a more significant role in susceptibility to DNA denaturation than the extent of S=S bonding within and between protamine molecules. These results also imply that rate of passage through the epididymis may not have significant effects on sperm fertility potential with regard to disulphide bonding status.

Introduction

Previous work in this laboratory found a significant relationship between fertility status and sperm nuclear susceptibility to DNA denaturation *in situ* as measured by the sperm chromatin structure assay (SCSA; Evenson and Jost, 1994), a flow cytometric procedure using the metachromatic fluorochrome acridine orange (AO). Correlation of fertility status with nuclear chromatin structure has been demonstrated in several studies: (1) use of heterospermic insemination for (a) nine phenotypically different bulls ($r = -0.94$, $P < 0.01$; Ballachey *et al.*, 1988) and (b) six phenotypically different boars ($r = 0.92$, $P < 0.01$; Evenson *et al.*, 1994); (2) fertility ratings for bulls based on non-return rates adjusted for various environmental effects (r ranged from –0.58 to 0.65, $P < 0.01$; Ballachey *et al.*, 1987); (3) time to pregnancy for 183 human couples ($r = 0.86$, $P < 0.001$; Evenson *et al.*, 1999); and (4) 106 stallions in commercial breeding operations correlating seasonal pregnancy rate with SCSA data ($r = 0.40$, $P < 0.01$; Kenney *et al.*, 1995).

A significant correlation of stallion sperm SCSA data with the presence of DNA strand breaks was reported in a previous study ($r = 0.65$, $P < 0.001$; Evenson *et al.*, 1995). An even stronger correlation was reported for human spermatozoa ($r = 0.97$, $P < 0.001$; Aravindan *et al.*, 1997). These results indicate that altered chromatin structure and DNA strand breaks may influence fertility and early embryo development. The current study investigated further the aetiology of susceptibility to

low pH-induced DNA denaturation *in situ*. It was hypothesized that the lack of oxidation of cysteine -SH groups on chromatin protamine during passage of the spermatozoa through the epididymis would prevent formation of the disulfide bonds (S=S) that are thought to provide stability to the sperm nuclear chromatin structure. Kosower *et al.* (1992) reported that the status of -SH groups in several mammalian species was related to susceptibility to DNA denaturation *in situ* as determined by AO staining.

Materials and Methods

Stallion semen processing

Semen was collected from thirty stallions using an artificial vagina (four animals were sampled on two different occasions) and aliquots of the 34 raw ejaculates were placed in cryotubes or 0.5 ml straws and frozen (–70°C). These samples were shipped by overnight express on dry ice to the flow cytometry laboratory at South Dakota State University and stored at –110°C in an ultracold freezer.

Sperm chromatin structure assay

In this assay, spermatozoa are subjected to low pH conditions to potentially denature DNA *in situ* followed by staining with AO (number 04539; Polysciences, Warrington, PA). Stallion samples were thawed for 30 s in a 37°C waterbath, diluted in TNE buffer (0.01 mol Tris l^{-1}, 0.15 mol NaCl l^{-1}, 1 mmol EDTA l^{-1}, pH 7.4) to a concentration of 1–2 × 10^6 spermatozoa ml^{-1}. A 0.2 ml aliquot was mixed with 0.4 ml low pH buffer (0.1% Triton-X100, 0.15 mol NaCl l^{-1} and 0.08 M HCl, pH 1.2). After 30 s the sample was stained with 6 μg ml^{-1} of the metachromatic dye AO and was measured by SCSA.

AO fluoresces green when intercalated into native double-stranded DNA and red when associated with denatured single-stranded DNA. A flow cytometer with a laser emitting 488 nm light was used to measure the amount of green and red fluorescence emitted from each of 5000 cells per sample. The extent of DNA denaturation is quantified by the expression alpha t (α_t), the ratio of red : red + green fluorescence. SCSA variables of interest are Meanα_t, SDα_t and the percentage of cells outside the main population (COMPα_t) that demonstrate DNA denaturation. Normal spermatozoa fluoresce green, whereas abnormal sperm chromatin produces variable amounts of red fluorescence.

Free chromatin -SH assay

This assay also used flow cytometry to determine relative -SH stainability of 5000 nuclei per sample. Quantification of -SH groups was determined by measuring the blue fluorescence intensity of the free -SH specific coumarin fluorochrome, CPM (7-diethylamino-3-(4'-maleimidylphenyl)-4-methyl-coumarin; number D-346; Molecular Probes, Eugene, OR) with mercury arc lamp UV excitation in an epi-illumination ICP22A flow cytometer (Evenson *et al.*, 1989). The TK450 excitation filter assembly with UG1 black filter was paired with the TK510 collection assembly using a fluorescein isothiocyanate special blue filter.

Frozen (–110°C ultracold freezer) semen samples were thawed at 37°C as described above and sonicated with a Bronwill Biosonik IV probe sonicator (VWR Scientific Inc., Minneapolis, MN) at a low-power setting of 50% of maximum power in a 4°C ice–water slurry for 30 s on, 30 s off for cooling, and a further 30 s at 50% of maximum power to remove all tails, membranes and cytoplasmic components from the sonication resistant nuclei. A 0.8 ml aliquot of sonicate was mixed with 0.2 ml of 60% sucrose solution (w/w in 0.01 mol Tris–HCl l^{-1} and 2 mmol EDTA l^{-1}, pH 7.4) to minimize 'sugar' shock. This sample was layered over 0.9 ml of 60% sucrose solution in a 2 ml tube and centrifuged using a TLS-55 swinging bucket rotor in a tabletop Beckman TL-100 Ultracentrifuge (Beckman Instruments, Palo Alto, CA) for 7 min at 214 200 *g* at 4°C. The pellet was resuspended in approximately 0.1–0.5 ml Hepes buffer (25 mmol Hepes l^{-1}, 0.15 mol NaCl l^{-1}, pH 7.4). A 15 μl aliquot of processed sperm nuclei was mixed with 685 μl Hepes buffer and stained with 5 μl CPM stock (2.4 mg CPM

diluted in 1.3 ml acetone, made fresh each day). Samples were mixed thoroughly with a Pasteur pipette, placed in a dark box and measured by flow cytometry after 30 min to allow time for staining equilibrium to be achieved.

Results

The SCSA values obtained were very heterogeneous (Table 1). Representative SCSA cytograms and corresponding alpha t (α_t) frequency histograms are shown (Fig. 1). Spermatozoa represented in the first row (stallion A) are of excellent quality as measured by SCSA and very few cells have denatured DNA (COMPα_t). In contrast, spermatozoa from stallion E (last row) have poor quality chromatin and 50% of the population have denatured DNA. However, the number of free -SH groups is highest for stallion A and that of stallion E is close to the lowest value (Fig. 2). Six samples were eliminated as their mean free -SH stainability was < 50 channels and could not be separated from noise. There were poor correlations of the SCSA variables Meanα_t, SDα_t and COMPα_t with CPM staining of free -SH groups; $r = -0.215$, -0.128 and -0.199, respectively, all $P > 0.27$. The relationship between the percentage of cells with DNA denaturation (COMPα_t) and mean stainability of free -SH groups by CPM for each stallion is shown (Fig. 3).

Discussion

Previous SCSA data showed that susceptibility to DNA denaturation in mammalian spermatozoa, including stallion spermatozoa, is correlated with fertility potential. This study tested the hypotheses that the number of free protamine -SH residues is related to the extent of DNA denaturation, and the more extensive the disulphide bonding, the more protected the DNA is from agents that may influence its susceptibility to denaturation. However, the results of this study indicate that the number of free -SH groups is not correlated with susceptibility to DNA denaturation. The assumption was made that the stainability of -SH groups reflects directly the relative amount of free -SH groups and thus, inversely, the number of S=S bonds in the chromatin of spermatozoa.

These results appear to be in contrast to a study on small mammals by Kosower *et al.* (1992) which indicated that the extent of acid-induced DNA denaturation in hamster spermatozoa is related to the extent of disulphide bonding, as is also implied by the stainable amount of free -SH groups. However, in the study of Kosower *et al.* (1992), the redox status of -SH and S=S was manipulated by oxidizing and reducing agents and the resulting data may not, as suggested by the authors, represent a true physiological phenomenon. The denaturation conditions were also different and light microscope analysis of AO fluoresence of spermatozoa on glass slides was used. Such analysis only permits classification of spermatozoa emitting green, red and yellow fluorescence. Furthermore, glass slides absorb AO, which causes artifactual shifts from red to green fluorescence (J. L. Weiss, K.L. Larson, D. M. Marshall, L.K. Jost and D. P. Evenson, unpublished).

Recent work in our laboratory revealed a strong relationship between SCSA measured

Table 1. Statistics for the sperm chromatin structure assay variables for stallion spermatozoa

	Minimum	Maximum	Mean	SD
Meanα_t	178.4	326.4	227.3	31.8
SDα_t	52.6	123.5	78.5	21.4
COMPα_t	6.6	78.2	21.6	17.9

$n = 34$ (semen from 34 stallions was measured by the sperm chromatin structure assay).
α_t: ratio of red : red plus green fluorescence.
COMPα_t: percentage of cells outside main population (percentage of cells showing DNA denaturation).

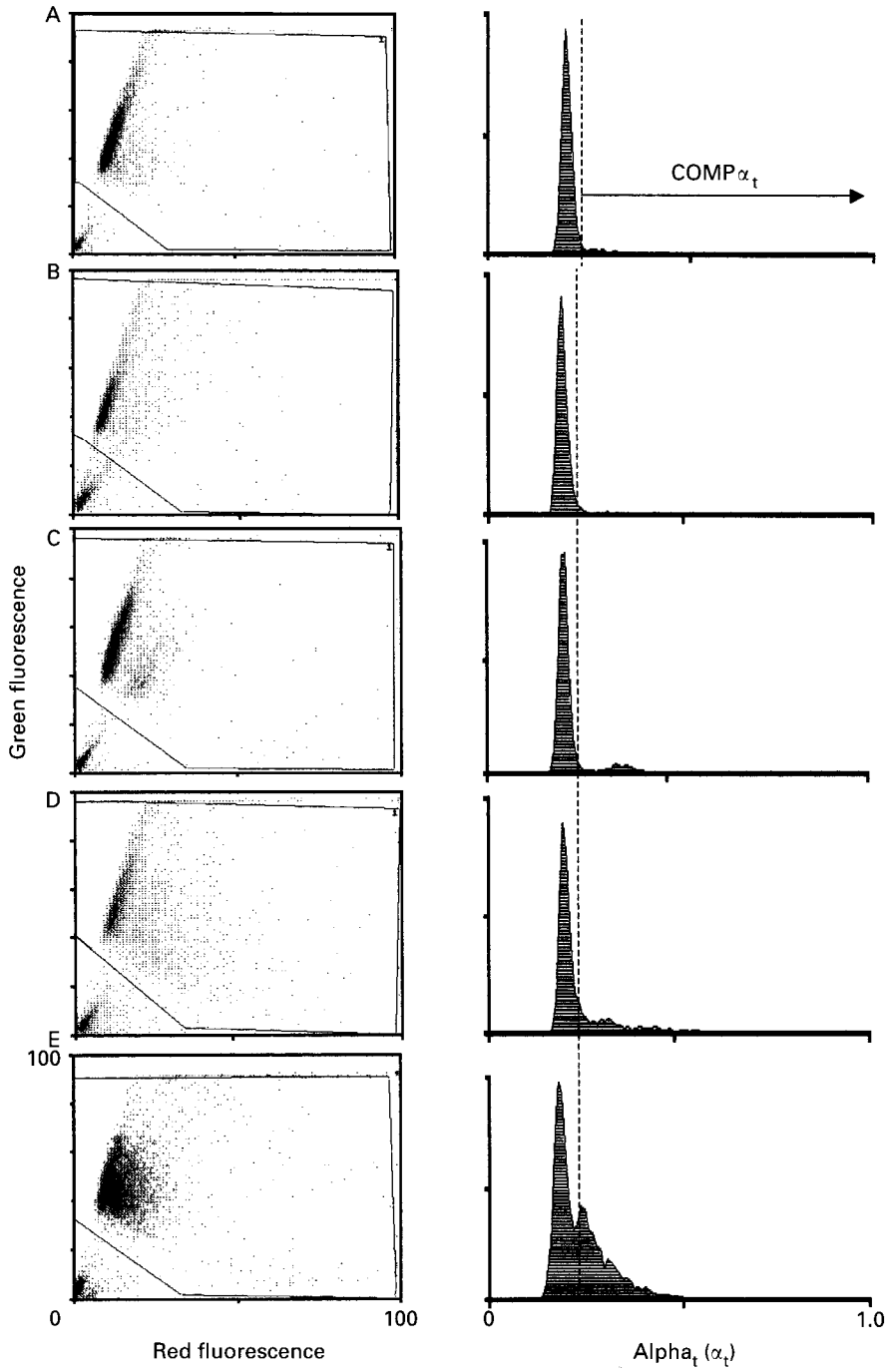

Fig. 1. Sperm chromosome structure assay (SCSA) data for spermatozoa from five individual stallions (A–E) in descending order with the best at the top. Red versus green fluorescence cytograms of acridine orange stained spermatozoa (left column) with corresponding alpha t (α_t) frequency histograms (right column). Spermatozoa from stallion A are of excellent quality and about 7% of cells outside main population (COMPα_t) have denatured DNA. In contrast, spermatozoa from stallion E (bottom row) have poor quality chromatin and 50% of the population have denatured DNA.

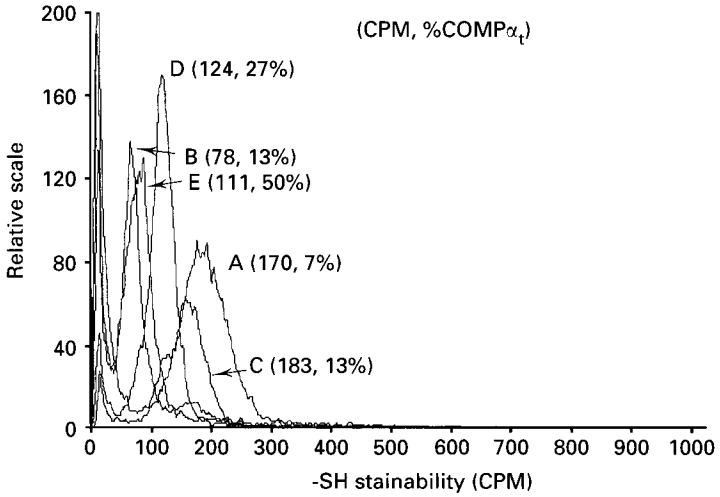

Fig. 2. Frequency histograms of CPM (7-diethylamino-3-(4′-maleimidylphenyl)-4-methyl-coumarin) stainability of free -SH groups from the same five stallions as in Fig. 1. Stallion A has the greatest number of free -SH groups, whereas the number in stallion E is one of the smallest values.

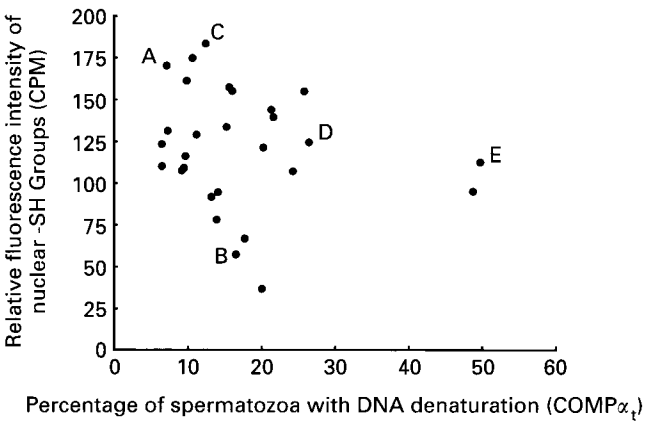

Fig. 3. A plot of the coordinates for each stallion semen sample related to the percentage of cells outside main population (COMPα$_t$) with DNA denaturation and mean blue fluorescence of -SH groups measured by CPM (7-diethylamino-3-(4′-maleimidylphenyl)-4-methyl-coumarin) stainability. Results from the same five stallions as in Fig. 1 are shown. Note the heterogeneity in values of both biomarkers and the lack of correlation between them (r = –0.199, P = 0.31; n = 28).

susceptibility to DNA denaturation and the presence of DNA strand breaks, as demonstrated by both *in situ* labelling of 3′OH strand breaks and by single cell electrophoretic identification of nuclear DNA fragments (Aravindan *et al.*, 1997). It seems likely that spermatozoa with extensive DNA strand breaks and fragments would not support early embryonic development. However, these DNA strand breaks may have been derived by one or more apoptotic mechanisms and thus, in line

with preliminary data, these spermatozoa may remain viable and motile. Therefore, they may be capable of fertilization but due to lack of chromatin integrity are not capable of normal fertilization and embryonic development. This situation may be analogous to the 'uncompensable semen factor' for bulls described by Saacke *et al.* (1994), that is, spermatozoa with slightly abnormal heads and abnormal nuclear craters (diadem defect) can fertilize the oocyte *in vivo*, but the recovered embryos are of poor quality and may not result in successful pregnancy outcome.

Substantial heterogeneity of stainable -SH groups has also been observed in bull spermatozoa (D. P. Evenson and L. K. Jost, unpublished). Whether a stearic hindrance prevents their proper oxidation or whether they are oxidized and subsequently reduced is not known. Storage in a frozen state over a long period of time will favour oxidation (D. P. Evenson, K. L. Larson and L. K. Jost, unpublished). Fresh and short-term frozen samples have the same pattern, so the freeze–thaw cycle does not influence the outcome. Another possibility for heterogeneity is the concentration of zinc in the reproductive tract. This mineral is known to aid the stabilization of -SH groups. Previous work in this laboratory on mice (Evenson *et al.*, 1980) and rats (Evenson *et al.*, 1993) showed that a zinc deficient diet resulted in extensive chromatin damage to caudal spermatozoa.

In conclusion, the extent of DNA denaturation in stallion spermatozoa was not significantly correlated with the amount of stainable -SH groups. This endpoint is likely to be inversely proportional to the extent of intra- and inter- molecular disulphide bonds. Thus, the susceptibility to DNA denaturation is likely to remain, at least in part, at a different molecular level. Cumulative data indicate that the extent of DNA denaturation is more likely dependent on the status of DNA integrity, for example, strand breaks, than on the presence of S=S bonds within and between adjacent protamine molecules that are complexed with the genomic DNA.

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