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Relation of Mammalian Sperm Chromatin Heterogeneity to Fertility

Abstract. *Flow cytometry of heated sperm nuclei revealed a significant decrease in resistance to in situ denaturation of spermatozoal DNA in samples from bulls, mice, and humans of low or questionable fertility when compared with others of high fertility. Since thermal denaturation of DNA in situ depends on chromatin structure, it is assumed that changes in sperm chromatin conformation may be related to the diminished fertility. Flow cytometry of heated sperm nuclei may provide a new and independent determinant of male fertility.*

The factors that affect male fertility are poorly understood, although it is known that sperm cell count, shape, and motility are important. Since sperm nuclear morphology is related to chromatin condensation and other nuclear phenomena occurring during spermiogenesis, we hypothesized, as have others (*1*), that misshaped sperm nuclei have an altered chromatin structure. Furthermore, since the resistance of in situ DNA to thermal denaturation is related to counterion and protein interactions with DNA (*2*), it seemed likely that an altered chromatin structure would be reflected in an abnormal DNA denaturation profile. We report here that not only does the in situ DNA of misshaped sperm nuclei have a significantly decreased resistance to thermal denaturation, many morphologically normal nuclei derived from subfertile donors are also abnormally susceptible to in situ thermal denaturation of their DNA. We suggest that the structure of sperm chromatin, as reflected by its sensitivity to thermal stress, may be an additional determinant of fertility.

Resistance of sperm nuclear chromatin to heat denaturation was determined by heating isolated sperm nuclei (*3*) and then staining with acridine orange (AO) prior to analysis by flow cytometry. The differential staining of native versus denatured DNA is due to the metachromatic properties of AO; when intercalated into native double-strand DNA the dye fluoresces green (F_{530}); when stacked on single-strand DNA it fluoresces red (F_{600}) (*2*). Therefore, the level of DNA denaturation in situ can be determined by measuring the ratio of red fluorescence to total nuclear fluorescence (red + green); this ratio varies experimentally from about 0.1 (undenatured) to 0.9 (highly denatured) and is termed α_t (*2*). Figure 1 shows the data for sperm nuclei from a bull of high fertility and from one of low fertility. Note that the position of the main cluster of nuclei from the fertile bull was changed very little by heat, indicating that the DNA was resistant to thermal denaturation. The peak value of the α_t histogram (inset) (*2*) is low, near 0.1. In contrast, a very marked difference in AO fluorescence was induced by heating the nuclei

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of subfertile bull sperm, indicating that the chromatin of many nuclei was very sensitive to heat denaturation. In the α_t plot, only 24 percent of the nuclei are in the resistant population, compared to 83 percent for sperm from the highly fertile bull. The ratio of heated to unheated α_t is inversely related to fertility level (Table 1). Data for 12 other bulls confirmed these findings.

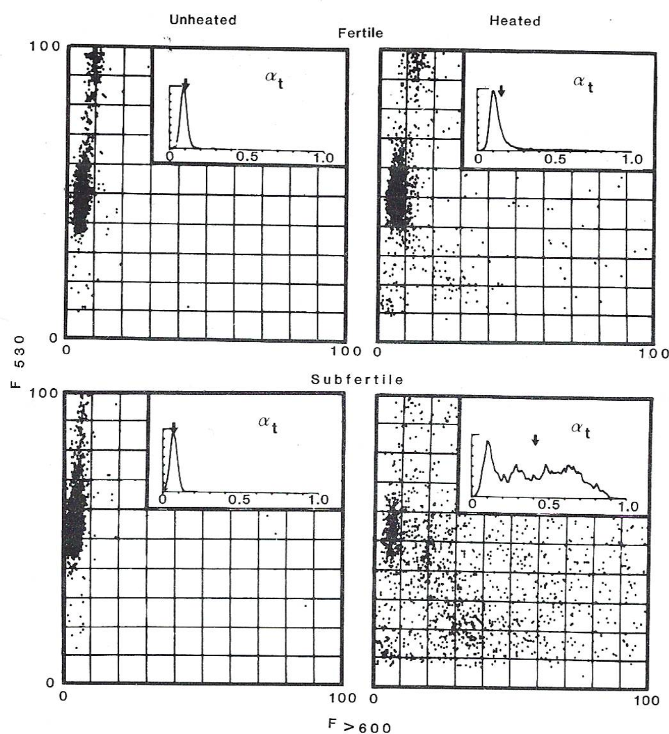

Fig. 1. Relation between fertility of Holstein bulls and resistance of isolated sperm nuclei to thermal denaturation. Holstein bull semen, extended in whole milk and frozen in plastic straws with liquid nitrogen, was obtained from Eastern Artificial Insemination Cooperative. Sperm nuclei were isolated, heated, and stained with AO as described (*3*). The green (F_{530}) fluorescence and red ($F_{>600}$) fluorescence emitted as each nucleus passed through the 488-nm argon-ion laser beam were separated optically and quantitated by separate photomultipliers. The data are based on a total of 5000 cells per sample. Each dot gives the result for a single nucleus: its position on the *y*-axis corresponds to the intensity of the green fluorescence, proportional to the amount of native DNA, and its position on the *x*-axis corresponds to the intensity of the red fluorescence, proportional to the amount of denatured DNA (*2*). The computer-generated α_t histogram (*10*) is shown as an inset in each scatterplot. The arrow indicates the mean.

Table 1. Relation between sperm from mammals of different fertility and the resistance of the sperm DNA to in situ thermal denaturation. Fresh human semen from four healthy males of recently proven fertility and 16 samples from a human fertility clinic were admixed 1:1 with redistilled, ultrapure glycerol (Bethesda Research Laboratories) and stored at −20°C. Within 2 to 3 weeks, the samples were prepared for analysis as described (*3*). The values refer to differences between the individuals in each group. The range of variations among patients from the fertility clinic was relatively high, and 3 of the 16 samples were not significantly different from the individual samples of the control group. The mice (strain C57BL/KS, Sloan-Kettering Breeding Colony) were fed a diet of Purina laboratory Chow or a zinc-deficient diet (Tekland Test Diets) from 6 weeks of age until 12 weeks, at which time they were killed. Sperm was obtained from their epididymides, and the nuclei were prepared and measured as described (*3*). The values refer to intercellular variations within individual samples containing 5000 cells. The experiment was repeated twice, with similar results. Values for the bulls refer to intercellular variations within samples from one highly fertile bull (73 percent conception rate) and one subfertile bull (47 percent conception rate), both represented in Fig. 1. Sperm from 12 other bulls classified as having high fertility levels had lower α_t values than that of bulls classified as having low fertility when measured under identical conditions. All the bulls were healthy, and the causes of the lower fertility levels are not known. The apparent differences in mean α_t values of unheated sperm from humans, mice, and bulls are due to differences in photomultiplier gain settings of the instrument on different days, and do not affect the α_t ratio of heated and unheated samples, which were always measured under identical conditions.

Species	Condition	Mean α_t ± standard deviation		Heated/unheated ratio
		Unheated sperm	Heated sperm	
Human	Proven fertility	0.18 ± 0.01	0.29 ± 0.03	1.16 ± 0.11
	Clinical samples	0.20 ± 0.02	0.45 ± 0.11	2.25 ± 0.12
Mouse	Control diet	0.07 ± 0.03	0.15 ± 0.12	2.1
	Zinc-deficient diet	0.07 ± 0.03	0.28 ± 0.18	4.0
Bull	High fertility	0.10 ± 0.04	0.16 ± 0.12	1.6
	Low fertility	0.07 ± 0.04	0.41 ± 0.23	5.8

The flow cytometry data for the subfertile bull sperm were confirmed by fluorescent light microscopy. Washed bull spermatozoa were smeared onto a glass slide, air-dried, and fixed overnight in a 1:1 solution of acetone and 70 percent ethanol. The samples were denatured with heat, stained with AO, and observed with fluorescent light microscopy. Almost invariably, the visibly misshaped nuclei fluoresced red; however, many that did not appear to have an abnormal morphology also fluoresced red.

Sixteen samples of semen from patients attending a fertility clinic were analyzed in the manner described above and compared to samples from four males of proven fertility. Although the reasons for diminished human fertility are complex, the same pattern of susceptibility to thermal denaturation observed for subfertile bulls was evident in 13 of the 16 patients; 3 had a normal pattern of resistance (Table 1).

Previous studies showed that dietary zinc plays an important role in human and animal growth and sexual development (*4, 5*). Miller *et al.* (*5*) found that testes development in male rats fed a zinc-deficient diet was retarded, with atrophy of the tubular epithelium and resulting infertility. As Table 1 shows, DNA in sperm nuclei from mice fed a zinc-deficient diet was less resistant to in situ thermal denaturation.

These data and studies of other mammalian species (boars, subfertile mice with autoimmune disease, and rabbits) indicate that resistance of sperm nuclear DNA to heat denaturation in situ is an important parameter of fertility, although the molecular mechanisms of the phenomenon are unclear. However, other studies indicate no difference between normally and abnormally shaped sperm heads in the resistance of their DNA to thermal denaturation (*6*); these studies, however, were complicated by the use of formaldehyde during denaturation, a procedure shown to introduce an artifact into measurements of in situ DNA denaturation (*7*). The observation (*1*) that chromatin of abnormally shaped bull spermatozoa binds up to 16 times more [^{3}H]actinomycin D than normal spermatozoa, together with our observation that misshaped spermatozoa are usually very susceptible to heat denaturation, suggests that an abnormally shaped nucleus contains chromatin with an altered conformation. Whether this phenomenon is related to the level of chromatin condensation is not yet clear. Some clues may come from somatic cell studies showing that DNA in mitotic cells under-

goes in situ thermal denaturation more readily than DNA in interphase cells. This suggests that tightly condensed chromatin is more easily disrupted by thermal stress (8). It has also been observed that DNA in pyknotic (hyperchromatic) nuclei of somatic cells denatures in a fashion similar to that of DNA in sperm from subfertile mammals (8). However, chromatin in mature mammalian sperm is even more condensed than that in interphase or mitotic somatic cells, yet the DNA in fertile sperm is not readily denatured by heat. It should be emphasized that the nature of condensed chromatin in metaphase chromosomes and mature sperm is very different, the latter having a different protein composition and substantial disulfide bonding.

Although the mechanism behind the differential sensitivity of sperm chromatin to thermal denaturation is not known, this method offers numerous advantages for studying fertility problems. The analysis is objective, and cell selection is unbiased. Statistical significance is easily attainable, since 10^5 cells can be analyzed in less than 2 minutes. We expect this assay to have application in many research areas, including animal husbandry, human infertility, and environmental and public health.

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3. Semen samples, either frozen and thawed, or obtained fresh were diluted ten times in 0.01*M* tris, 0.15*M* NaCl, and 0.001*M* EDTA (*p*H 7.4) and washed three times by centrifuging (2500*g*) through the buffer. The sperm was resuspended in 2.6 ml of the same solution and sonicated for 1 minute with a Branson Sonifier (model 185, Branson Sonic Power). The sonicate was mixed with one-third of an equal volume of 60 percent (by weight) sucrose in 0.01*M* tris-HCl (*p*H 7.4) and 2 m*M* EDTA, layered on 8 ml of the sucrose buffer solution, and centrifuged at 37,000*g* for 60 minutes (9). After aspiration of the supernatant, the pellet was resuspended in 1 ml of 0.15*M* NaCl, 5 m*M* $MgCl_2$, and 20 m*M* tris-HCl (*p*H 7.4) and then forcefully pipetted into 9 ml of a 1:1 mixture of 70 percent ethanol and acetone. All of the above operations were done at 4°C. After overnight fixation at 4°C, the cells were pelleted and resuspended in 2 m*M* cacodylate, $10^{4}M$ EDTA, and 40 percent (by volume) ethanol (*p*H 6.0). Portions (0.5 ml) containing about 2×10^5 cells per milliliter were either not heated or heated at 100°C for 5 minutes, admixed with 2 ml of staining solution consisting of 0.15*M* NaCl, 5 m*M* $MgCl_2$, 20 m*M* tris-HCl (*p*H 7.4), and $2.67 \times 10^{-5}M$ AO (2). After 1 minute the fluorescence of individual nuclei was measured with an FC 200 Cytofluorograf (Ortho Diagnostic Instruments) interfaced with a Data General minicomputer.
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Fluorescence photomicrograph of spermatozoa obtained from a subfertile bull. Cells were heated and subsequently stained with a metachromatic fluorochrome, acridine orange. The orthochromatic green fluorescence represents AO intercalation into native, nondenatured DNA while red metachromasia reflects stainability of DNA denatured in situ. The proportion of metachromatically stained cells was increased in subfertile sample (about × 2000). See page 1131. [D. P. Evenson *et al.*, Memorial Sloan-Kettering Cancer Center, New York]