Chromatin Structural Changes in Sperm After Scrotal Insulation of Holstein Bulls

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ABSTRACT: The reported effects on semen quality ascribed to testicular heat stress generally relate to traits impacting sperm transport and fertilizing ability but not to the genetic material contained by the sperm. To characterize the effects of testicular heat stress on sperm chromatin, susceptibility of DNA in sperm nuclear chromatin to *in situ* acid denaturation was measured by flow cytometry after staining with acridine orange using the sperm chromatin structure assay (SCSA). Semen was collected from Holstein bulls at 3-day intervals, before and after 48-hour scrotal insulation, until the morphologically abnormal sperm content in raw semen exceeded 50%. After cryopreservation in egg yolk–citrate extender, semen was thawed and sampled during incubation *in vitro* at 38.5°C. Overall, SCSA results showed that chromatin susceptibility to denaturation was increased for sperm collected post- vs. preinsulation and was more pronounced for sperm presumably in the testes during insulation than

for those sperm presumably in the epididymides. Increased susceptibility was detected as early as the first collection postinsulation; however, chromatin of sperm presumably in the proximal epididymis during insulation did not appear to have been detrimentally affected. Chromatin susceptibility to denaturation increased with increased incubation time *in vitro*, but the rate of change in susceptibility during incubation did not differ among pre- vs. postinsulation specimens. We conclude that elevated scrotal temperatures adversely affect both epididymal and testicular sperm by reducing sperm chromatin stability. The effects of heat stress on the chromatin of epididymal sperm were more subtle than those exhibited by testicular sperm but detectable within close proximity to the heat stress event.

Key words: Heat stress, bovine, sperm chromatin, semen quality.

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The male's contribution to fertility involves the fertilization of the oocyte such that normal survival of the conceptus is promoted (Courot and Colas, 1986). Male-related embryonic death has been linked to factors that result in reduced semen quality such as heat stress (Setchell et al, 1988) and season (Colas, 1983). For bulls, increased incidence of sperm abnormalities and reduced sperm output have been reported during the warmer seasons of the year (Igboeli and Rakha, 1971; Parkinson, 1987). Artificially elevated ambient temperatures result in increased levels of morphologically abnormal sperm and reduced sperm motility and viability (Skinner and Luow, 1966; Rhynes and Ewing, 1973). Artificially elevated scrotal temperatures have detrimental effects on testicular and epididymal sperm in the bull, as indicated by increased incidence of abnormal morphology and reduced post-thaw viability, (Vogler et al, 1991, 1993). It is not known whether the chromatin structural quality of those sperm is similarly affected.

Monitoring sperm viability and morphology can indicate the onset of semen quality reductions that may foretell changes in fertility. Chromatin condensation during spermiogenesis is thought to result in species characteristic sperm head morphology and it has been suggested that sperm nuclear morphology is an indicator of chromatin organization that is likely to be important to fertility (Ward and Coffey, 1991). Abnormally shaped sperm heads from subfertile bulls contain structurally abnormal chromatin (McCosker, 1969; Gledhill, 1971) resulting from abnormal chromatin condensation (Gledhill, 1971; Gledhill et al, 1971). The susceptibility of sperm nuclear DNA to heat or acid-induced denaturation *in situ* is inversely related to chromatin structural integrity (Evenson et al, 1980), heterospermic fertility ranking (Ballachey et al, 1988), sperm viability (Karabinus et al, 1990), and levels of morphologically normal sperm (Ballachey et al, 1986, 1987, 1988; Karabinus et al, 1990). The quality of chromatin carried by the sperm may affect initial cleavage or embryonic development.

The present study was conducted to investigate whether testicular heat stress induced by 48-hour scrotal insulation detrimentally affected the chromatin structure of subsequently ejaculated sperm. It extended earlier studies (Vogler et al, 1991, 1993) that reported the effects of 48-hour scrotal insulation on the viability and morphology of fresh and cryopreserved bull sperm.

Materials and Methods

This study evaluated the chromatin structure of sperm in the cryopreserved semen utilized in earlier investigations (Vogler et al, 1991, 1993). Experimental design and the procedures for scrotal temperature elevation, semen collection, processing, and freezing detailed by those authors are again described below.

Semen Collection

Semen was collected from each of six Holstein bulls, beginning at different times of the year to avoid the potentially confounding effects of high summer temperatures. Bulls ranged from 13 to 34 months of age at time of collection and were individually penned in a cold housing barn.

At 3-day intervals preceding and following scrotal insulation (SI), an artificial vagina was used to obtain two successive ejaculates from each bull. Semen was collected at this frequency for 3 weeks prior to the experiment in order to stabilize extragonadal sperm reserves. Data collection began on day 6 preceding SI (day −6). After semen collection on day 0, the scrotum was insulated with a sack tailored to enclose the entire scrotum up to the abdominal wall. Thirty minutes after installing the sack, it was readjusted to accommodate the scrotal response to insulation. Scrotal surface temperatures (reported in Vogler et al, 1991) were recorded immediately after adjusting the sack and before removal 48 hours later. The sack was made of polyester batting quilted between waterproofed nylon taffeta. Collections continued after SI until incidence of morphologically abnormal sperm exceeded 50% in a bull's semen. This extended to day +12 for all six bulls, to day +15 for four bulls, to day +18 for two bulls, and to day +21 for one bull.

Sperm obtained from the three collections prior to SI (days −6, −3, and 0; period 1) served as the baseline to which post-SI collections were compared. Sperm obtained from the first three collections following SI (days +3, +6, and +9; period 2) were presumed to be in the epididymis and/or rete testis at time of SI, based on an estimated epididymal passage time of 8–11 days in the bull (Orgebin-Crist, 1962; Amann and Schanbacher, 1983). Sperm collected during period 3 (days +12, +15, +18, and +21) were presumably located in the testes at the time of SI.

Semen Freezing

The two ejaculates obtained from a bull on a collection day were pooled, then diluted to a concentration of 50×10^6 sperm per ml in egg yolk–citrate extender containing glycerol (7%, v:v, final concentration). Extender was clarified prior to use by filtering through a glass prefilter attached to a 0.45-μm Acrodisc filter (Gelman Sciences, Ann Arbor, Michigan). Extended semen was packaged in 0.5-ml French straws, then frozen, and stored in liquid nitrogen.

Sperm Chromatin Evaluation

Sperm chromatin was evaluated by flow cytometry using the sperm chromatin structure assay (SCSA; Evenson, 1980; Evenson et al, 1980, 1989). For each bull, three straws of cryopreserved semen from the same collection were removed from liquid nitrogen, immediately thawed for 45 seconds in 35°C water,

then pooled. The pooled specimens were then incubated for 180 minutes at 38.5°C to determine the effects of incubation at physiological temperature (Loeffler, 1986) on chromatin of sperm collected pre- vs. post-SI. At thawing (time 0) and at 30, 60, 120, and 180 minutes of incubation, 70-μl semen was pipetted into a 250-μl microcentrifuge tube (precooled on ice), frozen at −20°C in air, and stored at −95°C.

For flow cytometric evaluation, each frozen sample of incubated semen was thawed for 30 seconds in 37°C water. Fifty microliters thawed semen was diluted in buffer (0.15 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris) HCl, 1.0 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4), to a final concentration of approximately 1×10^6 sperm/ml. Diluted semen was sonicated (Biosonik IV, VWR Scientific, San Francisco, California; 30 seconds, 50% low power setting, 1.27-cm probe) to separate heads from tails. Freezing and thawing sperm and sonication as described here does not alter SCSA-derived data (Ballachey et al, 1986; Karabinus et al, 1991).

Two hundred microliters sonicated semen was admixed with 400 μl ice-cold 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, Missouri) in 0.08 N HCl and 0.15 M NaCl, pH 1.2, for potential *in situ* denaturation of sperm nuclear DNA. The DNA in sperm having normal chromatin structure is resistant to this standardized level of physical stress; varying degrees of susceptibility to potentially denaturing conditions indicate abnormal chromatin structure (Evenson et al, 1985, 1989). After 30 seconds, 1.2 ml ice-cold acridine orange staining solution (0.2 M Na_2HPO_4, 1.0 mM disodium EDTA, 0.15 M NaCl, 0.1 M citric acid monohydrate, 6.0 μg/ml chromatographically purified acridine orange [Polysciences, Warrington, Pennsylvania], pH 6.0) was added (Darzynkiewicz et al, 1975). After 3 minutes, sperm were evaluated by flow cytometry at a rate of approximately 250/second. Acridine orange is a metachromatic dye that fluoresces green when intercalated into native, double-stranded (undenatured) DNA but fluoresces red when associated with single-stranded (denatured) DNA (Kapuscinski and Darzynkiewicz, 1984). In each of two replicates, the red and green peak mode fluorescence emitted by each of 5,000 sperm were quantified for two consecutive flow cytometric analyses performed on each semen sample.

The ratio of red fluorescence to total (red plus green) fluorescence (alpha t, α_t; Darzynkiewicz et al, 1975) was calculated for each sperm. The SCSA results are expressed as the mean and standard deviation of α_t ($\bar{x}\alpha_t$ and $SD\alpha_t$, respectively) and the percentage of cells lying outside the main population of α_t (%COMPα_t) for each sperm sample analyzed. The $\bar{x}\alpha_t$ describes the average α_t value for the sperm analyzed in a semen sample. The %COMPα_t indicates the percentage of sperm having increased α_t values (increased susceptibility to DNA denaturation, abnormal chromatin structure) relative to the main population of sperm as illustrated in Figure 1. The $SD\alpha_t$ is a measure of sperm-to-sperm variation for α_t within a semen sample.

Statistical Analysis

Data were analyzed by least-squares procedures using the general linear models procedure of the Statistical Analysis System (SAS Institute, Inc., 1988). Main effects were bull, period, collection day nested within period, incubation time, and replicate.

The interaction of bull with a main effect served as that effect's error term, whereas collection day nested within period was used to test period. Period effects were analyzed by pooling data across collection days within period. Data from each collection day post-SI were compared with data pooled across collection days in period 1. Incubation time was used as a covariate for regression analysis of incubation data. Slopes and intercepts of the incubation response lines for each period were compared using the procedure of Kleinbaum and Kupper (1978). A $P <$ 0.05 was considered significant.

Results

The SCSA results for unincubated (time 0) frozen-thawed sperm, pooled across collection days within period, are summarized in Table 1. Compared to period 1, values for $\bar{x}\alpha_t$ and %COMPα$_t$ were greater for periods 2 and 3 ($P <$ 0.001), whereas SDα$_t$ was elevated for period 3 only ($P <$ 0.001).

The SCSA results for unincubated (time 0) frozen-thawed sperm are presented by collection day in Figure 2. Relative to pooled period 1 values (see Table 1 for period 1 means), $\bar{x}\alpha_t$ and %COMPα$_t$ were elevated for the first collection (day +3) after SI ($P =$ 0.03) and for every other collection ($P <$ 0.001) after SI, except day +9. The SDα$_t$ was elevated ($P \leq$ 0.02) on every collection day after SI except days +3 and +9.

The results of 180-minute *in vitro* incubation of frozen-thawed semen, pooled across collection days within period are plotted in Figure 3. Regression analysis showed that incubation time had a significant linear effect on $\bar{x}\alpha_t$ and %COMPα$_t$ and had quadratic and cubic effects on SDα$_t$. The slopes of regression lines for any given SCSA variable did not differ among collection periods ($P <$ 0.05). For both $\bar{x}\alpha_t$ and SDα$_t$ the intercepts of the regression lines did not differ for period 1 and period 2, but were significantly greater for period 3 than for period 1. For %COMPα$_t$, the intercepts were significantly increased for each successive period. The sequential changes in sperm chromatin stability during 180-minute incubation of a representative frozen-thawed specimen are illustrated in Figure 1.

Discussion

In the present study, semen collected from Holstein bulls before and after 48-hour scrotal insulation was evaluated to determine the response of sperm chromatin to the effects of scrotal insulation. In most previous studies in the bull, the timing of appearance of semen quality reductions indicated that testicular sperm were affected by heat stress (Austin et al, 1961; Skinner and Luow, 1966; Igboeli and Rakha, 1971; Rhynes and Ewing, 1973; Ross and En-
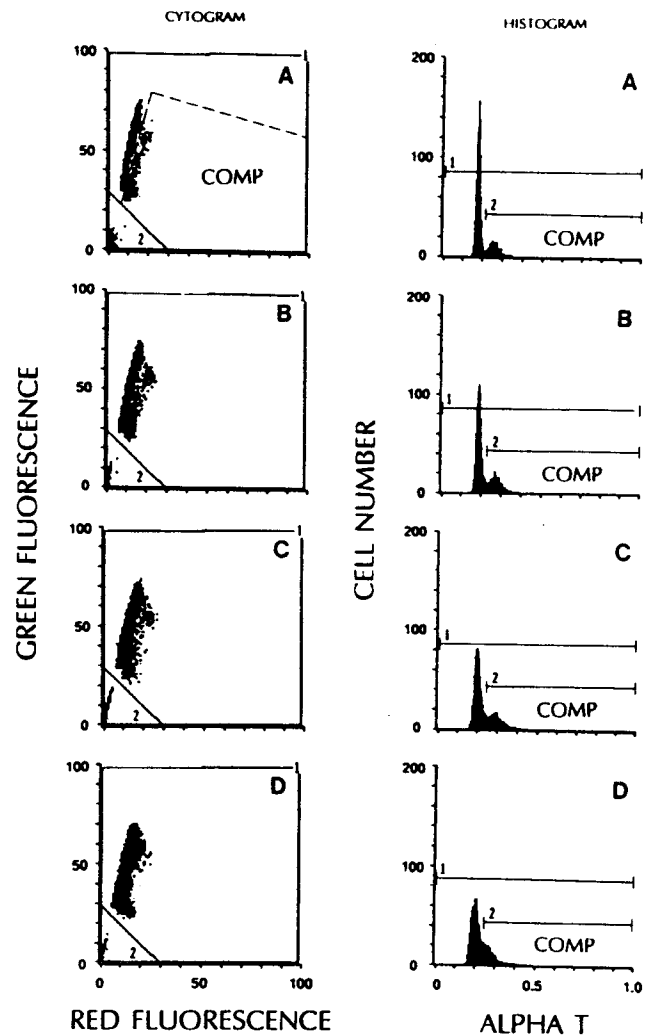

FIG. 1. Cytograms and frequency histograms generated from flow cytometric evaluation of acridine orange-stained bull sperm illustrating the progressive changes in chromatin structure of sperm in a semen sample from **(A)** thawing through **(B)** 60-, **(C)** 120-, and **(D)** 180-minutes of incubation at 38.5°C. In cytograms, region 2 excludes debris from the sperm population to be analyzed. The broken line separates cells outside the main population of α$_t$, %COMPα$_t$ from the main population. The expression alpha-t (α$_t$) is the ratio of red to total fluorescence emitted by sperm chromatin after staining. In frequency histograms, region 1 indicates entire sperm population, region 2 indicates %COMPα$_t$.

twistle, 1979; Wildeus and Entwistle, 1986; Parkinson, 1987). More recently, Vogler et al (1991) showed that the post-thaw motility and acrosome integrity values for sperm collected during the 9 days following 48-hour scrotal insulation did not differ from those of sperm collected during the period preceding insulation (55.3 ± 2.9% and 79.3 ± 1.6%, respectively) but were reduced for the period ≥12 days following SI (37.8 ± 2.9% and 54.3 ± 1.6%). Likewise, preinsulation levels of abnormal sperm (19.6 ± 5.7%) were exceeded on day +12 (47.5 ± 27.4%) and remained elevated through day +21 (Vogler et al, 1991, 1993). The present results are consistent with

Table 1. Least-squares means (±SEM) for sperm chromatin structure assay (SCSA) variables pooled across collections within period for frozen-thawed bovine semen collected before and after scrotal insulation (SI)

SCSA variable*	Collection period†		
	Period 1 (n = 18)	Period 2 (n = 18)	Period 3 (n = 13)
$\bar{x}\alpha_t$	189.3 ± 1.0ᵃ	193.2 ± 1.0ᵇ	201.7 ± 1.6ᶜ
SDα_t	30.9 ± 0.5ᵃ	32.1 ± 0.5ᵃ	41.9 ± 0.9ᵇ
%COMPα_t	7.4 ± 0.7ᵃ	11.0 ± 0.7ᵇ	20.7 ± 1.1ᶜ

* $\bar{x}\alpha_t$ = mean of α_t, where α_t = the ratio of red fluorescence to total fluorescence; SDα_t = standard deviation of α_t; %COMPα_t = percentage of cells outside the main population of α_t.

† Period 1 = the three collections prior to scrotal insulation (SI) (days −6, −3, 0); period 2 = the first three collections after SI (days +3, +6, +9); period 3 = the collections performed on days +12 through +21.

ᵃᵇᶜ Means within rows having different superscripts are different (P = 0.0001).

those reports, showing that the chromatin stability of testicular sperm was reduced by heat stress, as evidenced by significantly elevated overall values for the SCSA variables in period 3 (≥12 days post-SI, Table 1), the collection period during which ejaculated sperm were presumably in the testes at the time of SI. In addition, the present results show that heat stress adversely affected the chromatin of epididymal sperm but not to the degree exhibited by testicular sperm. The significantly elevated values for $\bar{x}\alpha_t$ and %COMPα_t for period 2 (≤9 days post-SI, Table 1) indicate that the effects of SI on sperm chromatin, which became fully evident in period 3, appear to have begun to manifest themselves in period 2, the collection period during which ejaculated sperm were presumably in the epididymis/rete testis at the time of SI.

Increased values for SCSA variables indicate greater susceptibility of sperm nuclear DNA to denaturation, correspond to increased chromatin heterogeneity, and are inversely related to bull fertility (Ballachey et al, 1987, 1988; Karabinus et al, 1990). Since SDα_t has been the SCSA variable most highly related to bull fertility potential (Ballachey et al, 1987, 1988; Karabinus et al, 1990), the elevated SDα_t value for period 3 (Table 1) indicates that sperm collected ≥12 days post-SI would be expected to have lower fertility potential relative to earlier collections; however, fertility trials were not conducted with this semen for confirmation. Although the SCSA variables $\bar{x}\alpha_t$ and %COMPα_t have not been shown to be as highly related to bull fertility potential as SDα_t, those variables do reflect chromatin changes in period 2 (Table 1) that, in retrospect, appear to have foretold the later changes in chromatin, characterized by elevated SDα_t in period 3, which have been related to bull fertility potential. The degree of change in %COMPα_t from period to period suggest that it was more sensitive than $\bar{x}\alpha_t$ as an indicator of changes in chromatin quality in response to SI. This ob-
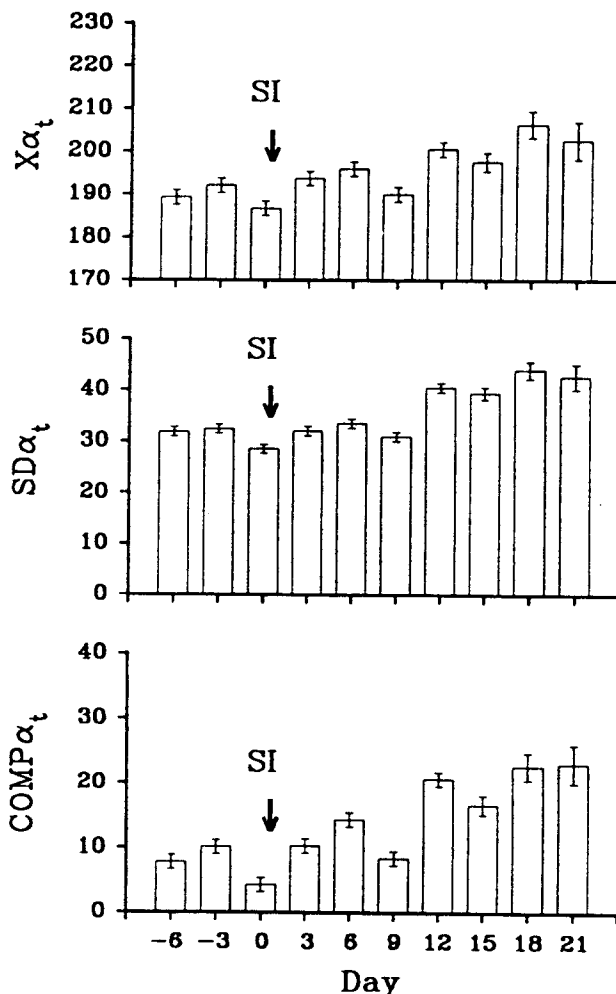

FIG. 2. Effects of 48-hour scrotal insulation (SI) on sperm nuclear chromatin resistance to denaturation. (A) The mean of α_t ($\bar{x}\alpha_t$) where α_t = the ratio of red to total fluorescence, (B) the standard deviation of α_t (SDα_t, and (C) the percentage of cells outside the main population of α_t (%COMPα_t). Sperm collections on days −6, −3, and 0 preceded scrotal insulation (SI) initiated on day 0 (arrow). Sperm collected or days 3, 6, and 9 were presumably in epididymis or rete testis during SI; sperm collected after day 9 were in the testes during SI. Elevated values for $\bar{x}\alpha_t$, SDα_t, and %COMPα_t indicate increased susceptibility of sperm nuclear DNA to denaturation and greater heterogeneity of sperm chromatin structure.

servation corresponds with other evidence (Karabinus and Evenson, unpublished) that shows that the onset of detrimental effects of naturally occurring heat stress on bovine sperm chromatin were also more readily detected by %COMPα_t than by $\bar{x}\alpha_t$ or SDα_t. Although %COMPα_t has been shown to have high positive relationships with measurements of sperm head morphometric variables obtained using computerized image analysis (Sailer et al, 1996), the elevated values for %COMPα_t in period 2 were not accompanied by increased levels of sperm head abnormalities during that period (Vogler et al, 1991, 1993).

A limited number of studies have shown that epididymal sperm are vulnerable to the effects of heat stress.
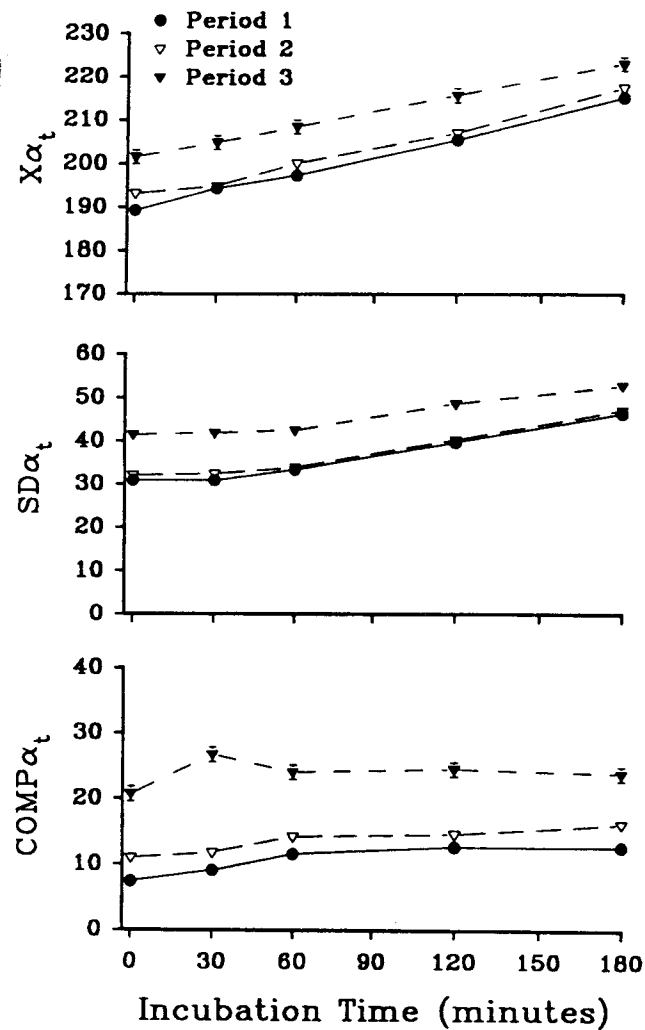
FIG. 3. Effects of 180-minute incubation at 38.5°C on sperm chromatin susceptibility to denaturation for sperm obtained in the three collections preceding scrotal insulation (period 1), the three collections immediately following scrotal insulation (period 2), and for sperm obtained in period 3, the collections following those of period 2. **(A)** The mean of α_t ($\bar{X}\alpha_t$) where α_t = the ratio of red to total fluorescence, **(B)** the standard deviation of α_t ($SD\alpha_t$), and **(C)** the percentage of cells outside the main population of α_t ($\%COMP\alpha_t$).

Wildeus and Entwistle (1983) reported that elevated levels of decapitated sperm were present in semen collected 6 days after scrotal insulation but were unable to repeat those results in a later study (Wildeus and Entwistle, 1986). Vogler et al (1991), on the other hand, demonstrated that sperm presumably in the epididymis during scrotal insulation exhibited reduced post-thaw acrosome integrity and percent motility but only after a 3-hour incubation. Unlike the results of that report, the *in vitro* incubation of frozen-thawed sperm in the present study, at a temperature approximating that of the female genital tract, did not reveal further differences among collection periods not already detectable in unincubated sperm. However, incubation results support an earlier observation

(Karabinus et al, 1990) showing that chromatin susceptibility to denaturation increased with increased post-thaw incubation time (Fig. 3) and that the rates of change during incubation were similar across collection periods despite initial differences in chromatin stability. The initial (time 0) differences in chromatin stability appeared dependent upon the proximity of sperm collection to SI and, therefore, the presumed stage of sperm maturation at the time of SI. Sperm at different stages of development are affected differently by heat stress (Chowdhury and Steinberger, 1964; Skinner and Luow, 1966; Waites and Ortavant, 1967). The present results support these observations, indicating that the sensitivity of chromatin to the effects of SI was greater in less mature sperm. Nonetheless, initial differences in post-thaw chromatin stability had little effect on subsequent changes during incubation *in vitro*, implying that chromatin may exhibit similar behavior *in vivo*.

To more sharply resolve the timing of appearance of heat stress effects on sperm chromatin, SCSA results for individual collection days post-SI were compared to data pooled across collection days in period 1. Values for the SCSA variables for days +3 and +6 and for day +12 of later were elevated, whereas those values for sperm collected on day +9 did not differ from pre-SI values. By comparison, sperm morphology and sperm motility values reported by Vogler et al (1991) remained stable through day +9. Sperm collected on days +3, +6, and +9 were probably in the epididymis/rete testis at the time of SI (Orbegin-Crist, 1962; Amann and Schanbacher, 1983). Published epididymal transit times (Amann et al, 1976; Orgebin-Crist and Olson, 1984) would place sperm collected on day +3 and day +6 in the more distal epididymis during SI relative to those collected on day +9, which were probably in the proximal epididymis/rete testis area. Thus, the chromatin of sperm in the more distal epididymis was apparently more susceptible to the effects of SI than that in the proximal epididymis. The progressive stabilization of sperm chromatin by disulfide bond formation (Calvin and Bedford, 1971) and the condensation of chromatin and loss of acridine orange stainability of DNA (Evenson et al, 1989) that normally occur during epididymal transit would imply greater chromatin resistance to denaturation in the distal vs. the proximal epididymis. The reason for this apparent paradox is not clear. It should be noted that the precise epididymal location of the sperm during SI may be difficult to pinpoint due to animal-to-animal variation, inexact transit times through the epididymal regions, and the continued progression of sperm through the epididymis, reported to be unaffected by scrotal insulation (Ross and Entwistle, 1979), during the 48-hour period of SI. On the other hand, the relatively high ejaculation frequency employed in this study, while not affecting epididymal sperm transit (Or-

gebin-Christ, 1962; Amann and Almquist, 1962; Amann, 1981; Amann and Schanbacher, 1983), should have resulted in less mixing of epididymal sperm and less masturbation than with a lower collection frequency. It could be speculated that differences among epididymal regions in terms of structure and function (Amann, 1987), the origin of vascular supply (Setchell, 1970), the origin and type of innervation (Hodson, 1970), and the degree and control of protein synthesis and the type of proteins synthesized and secreted (Cornwall and Hann, 1995) may have contributed to the apparent differential effects of SI on the chromatin of epididymal sperm. The response of these or other factors to SI may have directly interfered with normal chromatin stabilization in the distal epididymis. Alternatively, the response to SI may have resulted in some positive/protective effect(s) being imparted by the more proximal epididymis due to an effector not found, inactive, or present in an ineffectual level in the more distal duct or due to regional maturational events that resulted in differences in chromatin stability.

It is clear that heat stress can adversely affect semen quality in the bull and may result in reduced fertility potential. The present results show that heat stress in the form of 48-hour scrotal insulation adversely affected the chromatin of epididymal and testicular sperm. The effects on chromatin stability were detected sooner after scrotal insulation than the reported effects on conventional semen quality traits and appeared to vary depending on the presumed epididymal location of sperm during SI. Future studies employing shorter semen collection intervals and more numerous bulls will help further clarify the rapidity with which sperm chromatin manifests the effects of SI, verify the apparent differential effects of heat stress on the chromatin of extragonadal sperm, and identify differences (if they exist) among bulls in their response/resistance to heat stress. Such studies, coupled with fertility trials in the field and *in vitro,* should increase the understanding of epididymal function as well as give greater insights to the early detection of heat stress effects and their impact on sperm function.

Acknowledgments

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