Sperm DNA damage after scrotal insulation in rams

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ABSTRACT

Human male fertility is in decline and one reason given for this is the increased incidence of sperm showing chromosomal or DNA damage. Previous work in laboratory and farm animals as well as humans has shown that elevated testicular temperatures increases the incidence of sperm with DNA damage. As part of a study into the effect of temperature on mutation rate in spermatozoa using rams as a model, measurements of sperm DNA damage were also recorded. Twelve mature Dorset x Romney rams, were trained to AV semen collection. Scrotal insulation harnesses were applied (plastic bag with cotton wool insulation inside a rubberised fabric protection cover) to two groups of 6 rams for either a 24 or 48 hour period at day 0. Semen was collected on days -4 and 0 as control samples for each ram before treatment and days 3, 7, 10, 14, 17, 21, 24, 28, 31, 35, 38, 43, 45 and 49 post treatment. Semen was diluted in RSD-1 + TNE and snap frozen in liquid nitrogen. Samples were thawed and assessed by the Sperm Chromatin Structure Assay (SCSA) on the flow cytometer. This measures sperm susceptibility to '*in situ*' acid denaturation by staining with acridine orange fluorochrome, which fluoresces differentially when binding to double stranded DNA (green) or single stranded DNA (red). The data indicate an increase in the percentage of sperm that have DNA damage, appearing about 21 days post treatment and reaching a maximum at 35 days. The proportion of sperm with moderate to high levels of DNA damage was significantly ($P<0.001$) greater in the 48 hour treatment group and was still observed at 49 days while most of the 24 hour treated animals had recovered by this stage. Of particular interest was the shift of the entire sperm population, in DNA fragmentation index (ratio of red fluorescence:total (red + green) fluorescence), indicating increased susceptibility of DNA denaturation in all cells. This was accompanied by an increase in DNA stainability (increase of green fluorescence) which is interpreted as being due to an increase in the number of immature sperm cells with less compacted chromatin which results from abnormal or unprocessed protamines. The sperm damage seen after a relatively mild heat treatment indicates possible mechanisms for the adverse effects of modern life styles on male fertility.

Keywords: rams; scrotal insulation; sperm; DNA damage; chromatin assay.

INTRODUCTION

Human male fertility is in decline and one reason given for this is the increased incidence of sperm showing chromosomal or DNA damage. Previous work in laboratory and farm animals as well as humans has shown that elevated testicular temperatures increases the incidence of sperm with DNA damage (Smith, 1971; Evenson *et al*., 2000; Evenson *et al*., 1980).

Spermatozoa are produced by the process called spermatogenesis which is divided into 3 phases. During the last phase, spermiogenesis, the sperm nucleus condenses and the sperm head is formed. Sperm protamines replace histones giving rise to highly packaged chromatin structure with apparently no DNA transcription or RNA translation taking place. The maturing sperm cells' DNA repair is greatly reduced and ability to respond to damage by undergoing programmed cell death is progressively lost (Boe-Hansen *et al*., 2006; Aitken *et al*., 2004). Evenson *et al*. (2000) reported "studies have shown that nuclear protein composition of sperm produced by an individual can change with time which indicates environmental factors likely affect the level or fidelity of expression of nuclear protein genes and the organization of sperm chromatin". The data show that "the effects of influenza/fever were manifested as an increased susceptibility to DNA denaturation *in situ*, increased DNA stainability, decreased number of free-SH groups and alteration in nuclear protein composition of ejaculated sperm. Each of these changes appears to reflect an effect by fever that occurred early in spermiogenesis". Evenson *et al*. (2000) also reported "the presence of abnormal, unprocessed protamines does not cause an increased susceptibility to DNA denaturation but does cause an increase in DNA stainability which is likely the result of less compacted chromatin".

As part of a study into the effect of scrotal

temperature elevation on the mutation rate of ram sperm (Yu, 2003; Fleming *et al*., 2004) samples were taken to estimate sperm DNA damage.

There are several techniques to determine sperm chromatin status or DNA damage *e.g*. TUNEL, Comet assay and Sperm Chromatin Structure Assay (SCSA) (Evenson *et al*., 2002). We selected the SCSA as described by Evenson & Jost (1994) to determine DNA damage in sperm subjected to scrotal warming to body heat for 24 or 48 hours.

MATERIALS AND METHODS

Approval was granted by the AgResearch Ruakura Animal Ethics Committee and all experiments were performed in accordance with the New Zealand Animal Welfare Act (1999).

Reagents

Analytical grade chemicals were used unless otherwise stated. 2.0N standardized HCl, Triton X100, EDTA, Tris HCl were purchased from Sigma Chemical Co. (St Louis, MO, USA), chromatographically purified acridine orange (AO) purchased from Polysciences (Warrington, PA, USA). All other chemicals were purchased from BDH Chemicals Ltd (Poole, UK).

Flow Cytometer

A Becton Dickinson FACScan with built in air cooled argon-ion laser, (excitation 488nm) was used in this study. The green fluorescence (FL1) was collected through a 530/30 nm band pass filter and the red fluorescence through a 650 nm long pass filter.

Scrotal Heating

Twelve mature Dorset x Romney rams, were trained to AV semen collection (Evans & Maxwell, 1987). Semen was collected from each animal on days -4 and 0 as control samples for each ram before treatment. Scrotal insulation harnesses consisting of a plastic bag with cotton wool insulation inside a rubberised fabric protection cover, were fitted to groups of 6 rams for either a 24 or 48 hour period. The scrotal surface temperature was monitored and reached 39°C for both treatments. Further semen samples were collected from each animal at days 3, 7, 10, 14, 17, 21, 24, 28, 31, 35, 38, 43, 45 and 49 post treatment.

Semen Collection

One ejaculate from each ram was collected on each day and immediately placed in a water bath at 35°C. A range of sperm function parameters were measured on these samples and this has been reported elsewhere (Yu, 2003). Raw semen was diluted with warmed Ram Semen Diluent (RSD-1) (Upreti *et al*., 1995) to 100 $x10^6$/mL. A further sub aliquot of diluted semen was extended to a final concentration of 10 $x10^6$ per mL in TNE buffer (0.01M Tris-HCl, 0.15M NaCl, 1mM EDTA, pH 7.4) snap frozen and stored in liquid nitrogen until analysis.

Sperm DNA damage

Semen samples were assessed using SCSA as described by Evenson & Jost (1994) and Krzyzosiak *et al*. (2000). SCSA defines the changes in chromatin structure as the susceptibility of sperm chromatin to acid-induced denaturation by the metachromatic fluorescent shift of Acridine Orange (AO) fluorochromes emission of fluorescence from green (double stranded [ds DNA] or native DNA stainability) to red (single stranded [ss DNA] or fragmented DNA). This is expressed as DNA fragmentation index (DFI formerly termed alpha-t (α_t)) which is based on the calculation of the ratio of red:total (red + green) fluorescence. Sperm cells that fall outside the main non detectable DFI population (main population) are defined as having a high DFI. Each sample was assayed in duplicate (2 aliquots from the one sample) with 2 acquisition files per aliquot. Snap frozen samples in liquid nitrogen were thawed in a 37°C water bath and transferred immediately onto ice. A sub-aliquot of the thawed sample diluted with ice cold TNE to approximately 1 $x10^6$/200µL was mixed with 400 µL of ice cold acid detergent solution (0.1% v/v Triton X-100, 0.08N HCl, 0.15M NaCl). Exactly 30 seconds later, 1.2 mL of ice cold AO staining solution (0.1M Citric acid, 0.2M Na_2HPO_4, 1mM di-sodium EDTA, 0.15M NaCl, 6 ug mL^{-1} AO, pH 6.0) was added. The sample tube was then placed on to the flow cytometer. The sample was equilibrated for 2 minutes before acquisition of 5000 events.

Reference samples

A reference sample for the SCSA assay was prepared as a pool of ejaculates from all 12 rams prior to the commencement of the trial. Raw semen was diluted to 10 $x10^6$/mL in TNE, pooled and then frozen into 250 µL pellets on dry ice and stored in liquid nitrogen until SCSA analysis. These samples were run at the start and at regular intervals during and at end of the analysis on each assay or acquisition day. Reference samples are essential for the standardisation of the instrument and a measure of reproducibility, repeatability and accuracy for within and between days of the SCSA assay (Evenson & Jost, 1994; Boe–Hansen *et al*.,

2005). Data were acquired by Cell Quest™ Becton Dickinson Software. List mode data were then analysed using the software program FCS Express V2 and V3 (De Novo Software, Ontario, Canada).

Statistical analysis

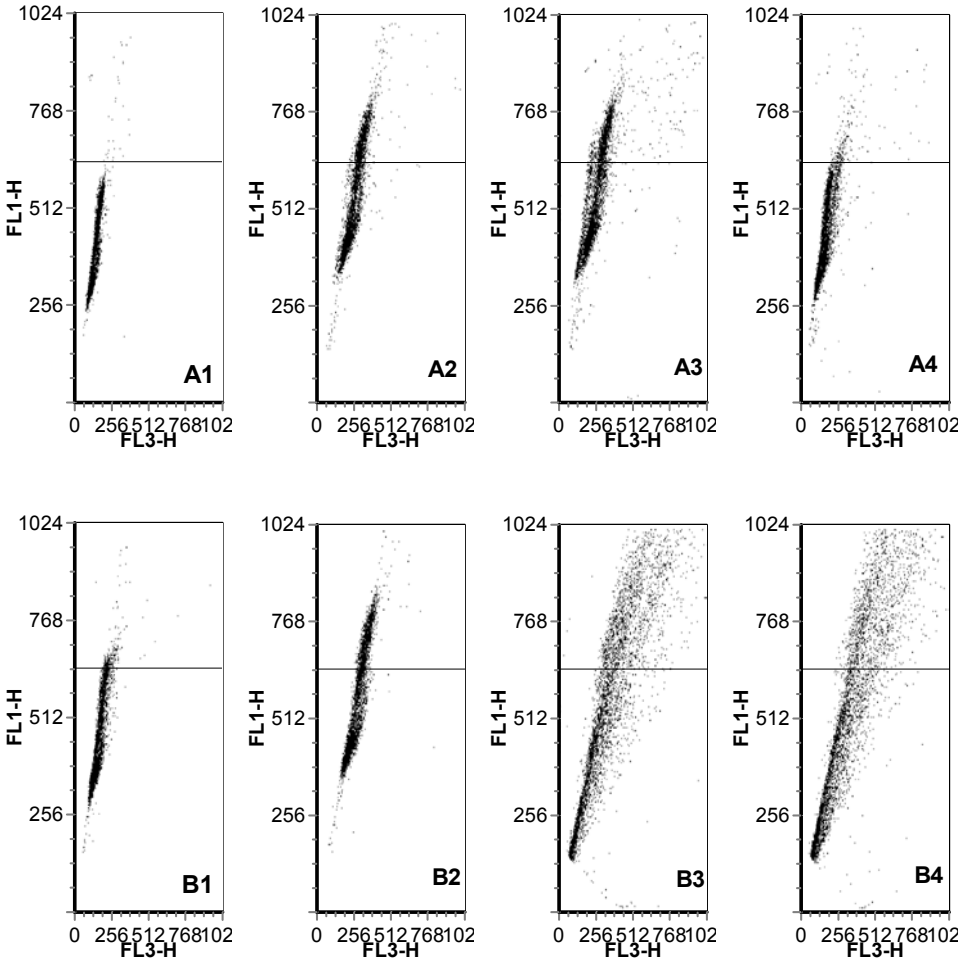
An adjustment for each ram was calculated by using the reference data, for variation due to instrumentation, sequence of data, assay time and day of assay (acquisition day) effects. A line was fitted with separate intercept and slope for each acquisition date, but a common curvature for assay sequence within day. The deviation from the overall mean of the reference data (across all dates) was calculated for every possible sequence number. This deviation represents the best estimate of the bias at each sequence number on each acquisition date. An adjusted value for each of the semen parameters was calculated for each ram obtained by subtracting this bias (sequence of data, assay, assay time and day effects), from the reference data provided. Analysis of variance of the bias adjusted response variables at each collection date, or averages across collection dates and differences from the pre-treatment dates was performed on the derived SCSA parameters:

a) Average peak channel – which represents the mean DFI for the cells in that sample;

b) Percent high DFI (%HDFI) which represents the cells having elevated DFI values that fall outside the normal distribution of cells which are regarded as having non detectable DFI, and

c) Percent cells with high DNA stainability (% HDS) representing those cells with green fluorescence higher than that seen in the control samples prior to treatment.

Figure 1: Representative SCSA cytograms (FL1 (green fluorescence) vs. FL3 (red fluorescence) for individual rams subjected to 24 hour (A) or 48 hour (B) scrotal heating. (*1) day -4, (*2) day 21, (*3) day 38 (*4) day 49 after treatment. The region above the line represents sperm cells with above normal High DNA stainability (HDS). The spread of dots to the right indicates cells with increased DNA damage (HDFI).



RESULTS

Analysis of the reference samples indicated statistically significant effects of acquisition date and assay sequence. Therefore as indicated in the statistical analysis section the data from the experimental samples was adjusted for this assay bias. However, while significant, the shifts in the reference sample parameters were inconsequential when compared to the changes produced by the scrotal insulation treatments.

The experimental data indicate a significant increase in the average of the overall DFI or DNA denaturation ($P<0.001$) as indicated by adjusted average peak channel values appearing about 21 days post treatment and reaching a maximum at 35 days (Figures 1 and 2). The proportion of sperm exhibiting a high DNA Fragmentation Index (%HDFI) was significantly greater ($P<0.001$) in the 48 hour treatment group and this was still observed at 49 days post treatment, while most of the 24 hour treated animals had recovered by this stage (Figure 3).

Figure 2: Mean values (±sem) for average peak channel (average DFI) of rams subjected to 24 or 48 hours of scrotal insulation at Day 0.

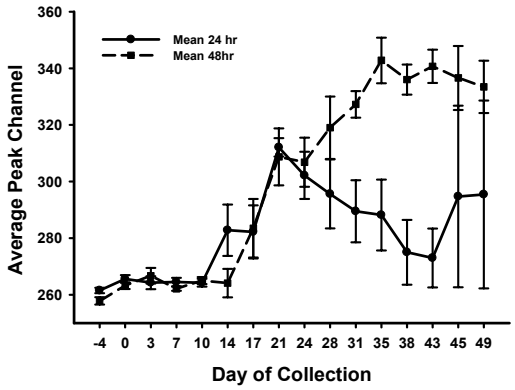
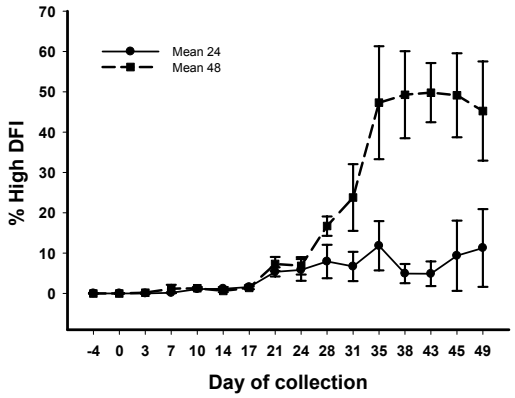
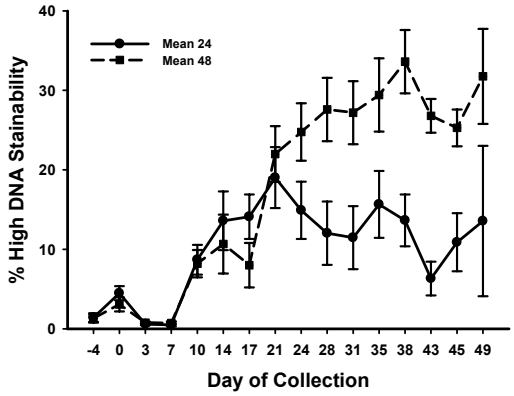


Figure 3: Mean values (±sem) for % High DNA Fragmentation Index (HDFI - cells outside main population of non-detectable DFI) of rams subjected to 24 or 48 hours of scrotal insulation at Day 0.



Of particular note was the shift of the level of DFI for the entire sperm population of samples, indicating increased susceptibility of DNA denaturation in all cells. This was still observed at 49 days in the rams treated for 48 hours, while most of the 24 hour treated animals had recovered by this stage (Figures 1 & 4). This was accompanied by an increase in cells with high DNA stainability (increase of green fluorescence), which was again significantly ($P<0.001$) greater in the 48 hour treatment group (Figure 4).

Figure 4: Mean values (±sem) for percentage of High DNA Stainability (HDS) of rams subjected to 24 or 48 hours of scrotal insulation at Day 0.



DISCUSSION

It has been documented that physiological and environmental stresses, gene mutations and chromosomal abnormalities can disrupt the biochemical events of spermatogenesis, which ultimately can lead to abnormal chromatin structure that is incompatible with fertility (Evenson *et al*., 2002; Aitken *et al*., 2004). Increased susceptibility to denaturation corresponds to the heterogeneity of the chromatin structure. Studies in humans and bulls show an increase of heterogeneity of the chromatin structure, is associated with disturbances in spermatogenesis and infertility (Ballachey *et al*., 1987; Evenson *et al*., 1980).

A study of scrotal heating in the stallion by Love & Kenny (1999) showed that DNA damage was dependent on the spermatogenic cell stage/phase that sperm was undergoing during the time of heat stress. This was also evident in our study and for other semen characteristics as reported by Smith (1971). Scrotal heating did not appear to dramatically alter the sperm chromatin structure of sperm in the ejaculate until approximately days 21 post treatment onwards. Sperm collected on day 3 to 17 post-treatment would have been in either the epididymis or the

rete testis at the time of heating (Love & Kenney, 1999; Smith, 1971; Setchell, 1998). Love & Kenney (1999) showed that the increased susceptibility of DNA denaturation (DFI) was associated with disulfide bonding in the absence of protamine changes and that the changes occurred before the sperm entered the epididymis.

We observed DNA damage (increase in DNA denaturation and increased DNA stainability) occurring in both 24 hour and 48 hour treatments. Variation between individual rams for DNA fragmentation was more evident amongst those treated for 48 hours than for 24 hours. The majority of the 24 hour treated animals had recovered by the end of the experimental collection period. However most animals treated for 48 hours did not recover within 49 days due to increased damage occurring at earlier stages of spermiogenesis. Heat stress for a period of 24 to 48 hours clearly demonstrated adverse effects on the sperm DNA. It is hypothesised that in the animals that did not recover by day 49, damage had occurred to the spermatogonial stem cells. A study into scrotal insulation of bulls by Karabinus *et al*. (1997) reported similar findings.

A human case study (Evenson *et al*., 2000) has shown that high fever, from influenza, caused changes in the sperm chromatin structure (increased susceptibility to DNA denaturation *in situ*, increased DNA stainability, decreased number of free-SH groups and alteration in nuclear protein composition) and these effects would appear to have occurred early in spermiogenesis. Furthermore, sperm protamine studies in humans have revealed protamine concentrations are inversely correlated with the DNA fragmentation index. Low P1/P2 ratios indicate elevated DNA fragmentation levels (Aoki *et al*., 2005). In contrast to the human the ram has only one type of protamine (Evenson *et al*., 2002). The results from our study show an increase in HDS after day 21 post treatment indicating damage at the DNA condensation stage. This has been interpreted as being due to an increase in the number of immature sperm cells with less compacted chromatin, which results from abnormal or unprocessed protamine (Evenson *et al*., 2000).

The susceptibility of high DNA stainability in our experiment varied for individual rams in both treatments. The sperm damage seen after a relatively mild heat treatment suggests possible mechanisms for the adverse effects of high environmental temperatures on male fertility in our domestic livestock species, especially when the natural scrotal cooling compensatory mechanisms are impaired or compromised. In addition it also suggests possible mechanisms for the adverse effects of modern life styles on human male fertility (Setchell, 1998). As reported by Love & Kenney (1999), human chromatin is more susceptible to DNA denaturation than that of other species. Therefore mild stresses may have a more dramatic effect of fertility. In vitro studies have shown primary spermatocytes exposed to temperatures of 44°C for as little as 10 minutes produced heat shock proteins. Love & Kenny (1999) suggest the human testis may be more vulnerable to heat stress because of recreational activities such as saunas and hot baths. In addition, insulating clothing, including use of plastic disposable nappies for infant males, sporting activities or stress related to disease causing fever may all contribute to the noted decline in human fertility over the past century (Setchell, 1998).

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