Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa

Stephen Banks, Sasha A King, D Stewart Irvine and Philippa T K Saunders

MRC Human Reproductive Sciences Unit, The Chancellor's Building, 49 Little France Crescent, Edinburgh, EH16 4SB, UK

Correspondence should be addressed to P Saunders, MRC Human Reproductive Sciences Unit, 49 Little France Crescent, Edinburgh EH16 4SB, UK; Email: p.saunders@ed.ac.uk

Abstract

An increase in scrotal temperature can lead to the production of poor quality spermatozoa and infertility. In the present study we have used mice to examine the impact of mild, scrotal heat stress (42 °C for 30 min) on numbers of spermatozoa as well as on the integrity of their DNA. Spermatozoa recovered from the epididymides hours (1 to 24) or days (7 to 32) after treatment were analysed using COMET and sperm chromatin structure (SCSA) assays. The treatment induced a stress response in both the testis and the epididymis that was associated with reduced expression of the cold inducible RNA binding protein (Cirp) and an increase in germ cell apoptosis (Apotag positive cells). Although spermatozoa present in the epididymis at the time of heating contained correctly packaged DNA, its integrity was compromised by heat stress. In addition, although some germ cells, which were present within the testis at the time of heat stress, were removed by apoptosis, many germ cells completed their development and were recovered as motile spermatozoa with damaged DNA. In conclusion, these data demonstrate that scrotal heat stress can compromise the DNA integrity of spermatozoa and this may have clinical implications for patients undergoing IVF and intra-cytoplasmic sperm injection (ICSI).

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Introduction

In most mammals, scrotal temperature is 2–8 °C below core temperature and this lower temperature is required for normal spermatogenesis (Harrison & Weiner 1948, Ulberg 1958). The impact of raised scrotal temperature in the rat (Lue *et al.* 2000), mouse (Jannes *et al.* 1998) and human (Mieusset *et al.* 1987) includes decreased testis weight, reduced viability, morphology and motility of spermatozoa, and reduced testicular blood flow and altered vasomotion (Setchell *et al.* 1995). In the mouse direct effects of scrotal heating on the developing germ cells are reported to include altered synthesis of DNA, RNA and proteins, as well as protein denaturation and abnormal chromatin packing (Steinberger 1991, Sailer *et al.* 1997).

Previous studies in the mouse have examined the effects on spermatogenesis of exposure of testes to a range of temperatures (38–42 °C) and exposure times (20 min to 1 h). In an extensive study by De Vita *et al.* (1990), it was shown that exposure to mild heat stress (38 °C) for a short time (20 min) caused cytotoxic effects in some germ cell types and that these effects were more pronounced in spermatocytes than in differentiated spermatogonia. Following heat

stress, the epididymis was reported to lose its ability to store and maintain viable spermatozoa, resulting in the gradual and progressive accumulation of dead, decapitated and immotile spermatozoa (Bedford 1991, 1994); the region most affected is reported to be the cauda.

In man, raised scrotal temperature may occur as a result of occupational exposure, lifestyle or a clinical disorder, such as cryptorchidism (Mieusset *et al.* 1987, Hjollund *et al.* 2000, 2002). It has been shown that men with a past history of cryptorchidism are likely to exhibit scrotal temperatures above the normal range and are often subfertile or infertile. It is also notable that the spermatozoa in their ejaculates exhibit an increased incidence of abnormalities such as tapered/elongated forms. In two separate studies on normal men, Hjollund *et al.* (2000, 2002) recorded the diurnal scrotal temperature using continuous monitoring over a 72 or 24 h period. They found evidence that mean scrotal temperature (median 33.3 °C) was increased during sedentary work periods (average 0.7 °C higher) and at night (median 34.8 °C) (Hjollund *et al.* 2000). In both studies a negative correlation was found between high scrotal temperature and sperm output with sperm concentration being decreased 40% per 1 °C increment of median day time

scrotal temperature in a study of 99 men (Hjollund *et al.* 2002). Studies of infertile males have shown increased scrotal temperature compared with fertile controls (Mieusset *et al.* 1987, Mieusset & Bujan 1995), but it has not yet been clearly established whether raised scrotal temperature is the cause of the infertility in these men or simply a concomitant symptom of their disorder. Treatment of couples with male factor infertility by IVF/intra-cytoplasmic sperm injection (ICSI) is now common place in clinical practice (Campbell & Irvine 2000). There is some evidence of a loss of genetic integrity within the spermatozoa used for these procedures and this may contribute to reduced fertilization rates, development of fewer blastocysts and poor embryonic development all of which have been reported (Lopes *et al.* 1998, Evenson *et al.* 1999).

The aims of the current study were first to investigate the effects of heat stress on the process of spermatogenesis, secondly to identify the cell types in the testis and epididymis susceptible to heat stress and thirdly to examine the integrity of the DNA in spermatozoa which developed from cells exposed to heat either during spermatogenesis or during epididymal transit. The response of the testicular and epididymal cells to a mild heat was monitored using immunohistochemical evaluation of known stress markers and Apotag. The integrity of the DNA in motile spermatozoa at various time points after heat stress was investigated using COMET and sperm chromatin structure assays (SCSA).

Materials and Methods

Animals and heat treatment

Wild type mice (MF1 strain) were bred and maintained at the Medical Faculty Animal Accommodation, University of Edinburgh. All animals were maintained under standard animal house conditions and treated in accordance with Government guidelines as stated in the Animals and Scientific Procedures Act 1986.

Animals were anaesthetised with an i.p. injection of Hypnorm (fentanyl citrate 0.315 mg/ml, fluanisone 10 mg/ml; Research Diagnostics Inc. Flanders, NJ, USA) and Hypnovel (midazolam hydrochloride; Roche, Lewes, East Sussex, UK). Following anaesthesia, the lower third (hind legs, tail and scrotum) of each male was passed through a hole in a polystyrene ‘raft’ which was then placed in a circulating water bath at 42 °C for 30 min. Control animals received anaesthetic only. All animals were returned to their cages, which were placed on a warm mat (20–25 °C) to maintain body temperature, until fully recovered from the anaesthetic.

Animals, three per treatment group plus three controls at each time point, were killed at 1, 2, 4, 6, and 24 h, and 7, 14, 21, 24, 28 and 32 days after treatment. Each time-point was chosen either to allow study of the spermatozoa present within the epididymis at the time of heating (1 to 24 h) or those which developed from the different germ

cell types that were present in the testis at the time of heat stress (7 to 32 days); these are summarized in Table 1. For the latter, calculations were based on the stage durations of mouse spermatogenesis as defined by Oakberg (1956) and Russell *et al.* (1990) and a 7-day epididymal transit time as defined by Cornwall *et al.* (1990).

Recovery of tissues

Testes were fixed in Bouins for 6 h then transferred to 70% ethanol and processed into paraffin wax for histological analysis. Epididymides were either fixed in Bouins or cleaned of fatty tissue and placed in 1 ml Biggers, Whitten, Whittingham media (BWW; Biggers *et al.* 1971) containing 20 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethan sulphonic acid] (HEPES; Gibco, Paisley, UK) and 0.3% human serum albumin (HSA; Gibco). In order to recover the motile spermatozoa the tissues from each of the treated animals (*n* = 3 per group) as well as the controls (*n* = 3 at each point) were minced with fine scissors and incubated at 34 °C (95% air, 5% CO₂) for 30 min prior to careful removal of all the buffer. Samples were diluted to a final volume of 1 ml in BWW, aliquots were taken for the sperm counts and the remainder of the sample stored at – 20 °C.

Concentration of spermatozoa

Aliquots (three per sample) prepared from minced epididymides from each of the treated animals (three per group) as well as from two groups of controls (1 h and 7 days, *n* = 6) were diluted 1:10 with BWW and a 10 µl aliquot placed into the chamber of an improved Neubauer haemocytometer (CamLab, Cambridge, UK). Sperm were counted according to the techniques described by the World Health Organisation (WHO 1999).

Immunohistochemistry

Slides were washed twice in PBS (5 min each) before incubation in a bath of 3% hydrogen peroxide in methanol at room temperature for 30 min to block endogenous peroxidases. After further washes in PBS, slides were blocked with normal swine serum (NSS; 5% BSA, 10% swine serum; Diagnostics Scotland, Carlisle, UK) for 1 h at room

Table 1 Summary of the germ cell types predicted to be affected by heat stress at the time points chosen post heat stress for analysis (timings based on Oakberg 1956).

Time point	Cell type at time of treatment
1, 2, 4, 6, 24 (h)	Mature spermatozoa in epididymis
7 days	Step 11 spermatid – mature spermatozoa
14 days	Step 1 spermatid – step 11 spermatid
21 days	Stage IV pachytene – step 1 spermatid
24 days	Stage XII pachytene – stage X pachytene
28 days	Preleptotene – stage III pachytene
32 days	A spermatogonia – zygotene

temperature. A rabbit polyclonal antibody raised against the cold inducible RNA binding protein (Cirp; Nishiyama *et al.* 1997) was diluted 1:20 000 in NSS and incubated on the sections for 2 h at room temperature; sections were washed in PBS as before. For fluorescent detection (testis sections) slides were incubated with swine anti-rabbit FITC (1:50 in NSS; DAKO, Ely Cambridgeshire, UK) for 1 h at room temperature and washed in PBS before mounting using Permafluor (Coulter-Immunotech, Hamburg, Germany). For colour detection (epididymis) sections were washed twice in tris-buffered saline (TBS) (5 min each) and incubated with swine anti-rabbit biotinylated secondary antibody (DAKO, 1:500 in BSA/TBS) at room temperature for 30 min. Following washes in TBS, single stained sections were incubated with avidin–biotin–HRP complex (DAKO) according to the manufacturer's instructions. Bound antibody was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB, DAKO); sections were counterstained with Mayer's haematoxylin. Sections from the testes and epididymes from each of the treated animals were stained on three separate occasions; staining intensity was compared with sections from the controls killed at the 1 h and 7 day time points ($n = 3$ per group). Negative controls, omitting the primary antibody, were included in each experiment and the results were negative (not shown). Sections were photographed using a Provis microscope (Olympus Optical, London, UK) and a Kodak DCS330 digital camera (Eastman Kodak, Rochester, NY, USA); images were assembled using Photoshop 6 (Adobe, Mountain View, CA, USA).

Detection of apoptotic cells using Apotag

Sections (three from each animal) were pretreated by incubation with 100 µg/ml proteinase K (Sigma, Gillingham, Dorset, UK) in buffer (3.5 ml 1 M Tris, pH 8, 0.7 ml 0.5 M EDTA, pH 8, made up to 35 ml with dH₂O) for 10 min at room temperature, and then washed twice in PBS (5 min each). The reaction mix (30 mM Tris/HCl, pH 7.2, 140 mM Na cacodylate, 1.5 mM CoCl₂) containing 1 µl/ml terminal d-transferase (TdT, Roche, Lewes, Sussex, UK), 5 µl/ml digoxigenin (DIG, Roche) was added and sections were covered with cover-slips which were sealed with cow gum/hexane before they were incubated at 37°C for 30 min.

Following the removal of cover-slips and washes in PBS, sections were blocked with 20% normal rabbit serum (NRS) in PBS for 10 min at room temperature. Sheep anti-DIG IgG (Boehringer, 1:100 in NRS/PBS) was added and the slides incubated at room temperature for 90 min before further washes in PBS. Rabbit anti-sheep IgG biotinylated (1:500 in NRS/TBS) was added for 30 min at room temperature before washing twice in TBS (5 min each). Bound antibodies were detected using DAB and sections were counterstained with Mayer's haematoxylin. In order to assess the numbers of Apotag-positive germ cells at each time-point (control and heated animals), all sections

were examined using a $\times 20$ objective on an Olympus BH2 microscope fitted with a Prior automatic stage (Prior Scientific Instruments, Cambridge, UK). The number of immuno-positive cells in three fields selected using the software provided with the stage was counted and the average value calculated for each animal.

COMET analysis of spermatozoa

Spermatozoa (5 µl) recovered from the epididymis (as above) were mixed with 25 µl Low Melting Agarose (LMA, 37°C; Trevigen, Gaithersburg, Maryland, USA). This gel/sample mix was dropped onto a 'COMET' slide (Trevigen) which was covered with a clean, warm (37°C) cover-slip, placed horizontally and chilled at 4°C. After the gels were set cover-slips were removed and the slides equilibrated in lysis buffer (0.75% SDS, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sodium lauryl sarcosinate, 0.01% Triton X-100) for 3 h at 37°C. Electrophoresis was performed by submerging slides in a horizontal gel electrophoresis tank containing alkaline electrophoresis buffer (3 M NaOH, 1 mM EDTA, pH 12.3). Slides were pre-equilibrated for 20 min at room temperature before current was applied (25 V, 300 mA for 10 min). Slides were transferred to ice-cold methanol (100%, 5 min) then ethanol (100%, 5 min) and allowed to dry overnight at room temperature before staining with 50 µl ethidium bromide (1:1000 in dH₂O). For each sample, 100 cells were analysed using the Comet Image Analysis System, version 4.0 (Kinetic Imaging Ltd, Liverpool UK). The percentage of head DNA, tail DNA and the COMET moment was calculated for each cell. COMET moment is a measurement that takes into account both the length of the COMET tail and the amount of DNA present in the tail and is expressed as an arbitrary unit (the greater the value, the higher the level of DNA damage). The Mann–Whitney U test was used to compare the results from each heated group against the controls ($n = 3$ in each group).

Sperm chromatin structure assay (SCSA)

A modified method of the SCSA described by Evenson *et al.* (1999) was employed. Briefly, motile spermatozoa recovered from the epididymis in BWB were defrosted at room temperature and adjusted to a concentration of 2×10^6 cells/ml with TNE (0.15 M NaCl, 0.1 M Tris, 1 mM EDTA pH 7.4). 100 µl of this sample was mixed with 200 µl of acid detergent solution (0.1% TritonX-100, 0.15 M NaCl, 0.08 M HCl, pH 1.2). After 30 sec at room temperature 600 µl acridine orange staining solution was added (37 mM citric acid, 126 mM Na₂HPO₄, 1 mM EDTA, 0.15 M NaCl, pH 7.4 with acridine orange (Sigma) added fresh to a final concentration of 6 µg/ml) and left at room temperature for an additional 150 s after which cells were immediately analysed in a Coulter Epics XL Flow Cytometer (Beckman Coulter (UK) Ltd. High Wycombe, Bucks, UK). Red fluorescence (single-stranded DNA) was

measured using a 675 nm detector and green fluorescence (double-stranded DNA) measured using a 525 nm detector. The Epics XL was calibrated for each run by adjusting the wild-type control samples to give a mean fluorescence value (arbitrary units) of 145 ± 10 at 675 nm and 445 ± 10 at 525 nm. 10 000 events were collected for each sample and each was run in triplicate. Raw data were exported as Static Dimension data files (LMD File Format) and analysed using FlowJo (Tree Star Inc., Ashland, Oregon, USA). Background contamination (bacterial contaminants and cells other than spermatozoa) were removed by gating and the results expressed in terms of α t (red/[red plus green] fluorescence, expressed as percentage). COMP α t (cells outside the main population) was calculated as the percentage of cells in the population exhibiting an α t value greater than 26% according to the methods described by Evenson *et al.* (1999).

Results

Expression of the cold-inducible RNA binding protein (Cirp) following scrotal heating

In testes from control mice, Cirp was immunolocalised to spermatocytes (Fig. 1a). Following *in vivo* heat treatment, a decrease in immunoexpression of Cirp was detected at 2, 4, 6 and 24 h; this was most marked in the samples recovered at 6 h (Fig. 1c) and expression remained low at 24 h (not shown). Expression appeared identical to that of controls in testes recovered 7 (Fig. 1d) or more (not shown) days after treatment. Cirp was immunolocalised to all regions of the epididymis in controls (Fig. 2 a–d). Six hours after heat stress immunoexpression of Cirp was markedly reduced in the initial segment, corpus and cauda but only slightly reduced in the caput (Fig. 2 e–h).

Apotag labelling of testicular germ cells

Consistent with other data obtained from our laboratory from studies on murine testes (Hsai *et al.* 2003, De Gendt *et al.* 2004) the number of Apotag-positive germ cells in control animals was low (Fig. 3, open bars). An increase in Apotag-positive germ cells was detectable 2 h after scrotal heating. The number of positive cells was increased further in samples recovered at 24 h, reached a peak at 14 days and thereafter declined to control levels (Fig. 3, shaded bars).

Changes in the number of motile spermatozoa following heat stress

The concentration of motile spermatozoa recovered from the epididymides of control animals ($n = 6$) was in the range of 5.7 to $7.4 \times 10^6/\text{ml}$ (mean $6.4 \times 10^6/\text{ml}$). The average number of motile spermatozoa retrieved from the epididymides was decreased 1 h after scrotal heat stress ($4.9 \times 10^6/\text{ml}$; $P < 0.05$). The number of motile spermatozoa was further reduced at subsequent time points reaching

Figure 1 Immunolocalisation of Cirp to murine testes removed from controls (a) and at the following times after scrotal heating b) 2 h, c) 6 h and d) 7 days. Note that expression of Cirp is intense in spermatocytes in controls (a) but that expression is markedly suppressed in the testes 6 h after a transient heat stress (c).

a nadir at 4 h post-heating ($1.1 \times 10^6/\text{ml}$; $P < 0.05$); numbers remained low (maximum $2.12 \times 10^6/\text{ml}$) for the duration of the experiment (32 days).

Levels of DNA damage in motile spermatozoa following heat stress

COMET Assay

DNA damage in motile spermatozoa recovered from the epididymides showed a significant increase 1 h following heat treatment (Tail DNA 37.8%; $P < 0.05$, COMET Moment 15.8, $P < 0.05$) compared with the control (25.3% COMET tail DNA, 6.9 COMET moment; Fig. 4). COMET values continued to increase until 4 h post heat

Figure 2 Immunoeexpression of Cirp in epididymides from control (a–d) and heat-treated (e–h) males recovered 6 h after heat treatment. Expression of Cirp was detected in the epithelial cells of the initial segment (a), caput (b), corpus (c) and cauda (d) of control animals. Immunoeexpression was reduced to undetectable in all but the caput (f) following scrotal heating.

treatment (Tail DNA 57.8%, $P < 0.05$, COMET moment 27.7, $P < 0.005$) and stayed above control levels until 7 days post heat treatment when they were indistinguishable from controls.

In addition there was a second rise in recovery of spermatozoa with COMET values above controls at 21 days after treatment (COMET Tail DNA 34.9%; $P < 0.05$, COMET Moment 19.4, $P < 0.05$). Values also remained above controls in samples recovered at 24, 28 and 32 days (Fig. 4; 28 days COMET Tail DNA 41%; $P < 0.05$, COMET Moment 19.4, $P < 0.05$).

Sperm chromatin structure assay (SCSA)

SCSA results were expressed as COMP α t which was a measure of the number of spermatozoa exhibiting abnormal DNA integrity (Fig. 5). Compared with control (COMP α t 5.9%), the spermatozoa from heat-treated animals showed a significant increase in COMP α t 1 h following treatment (COMP α t 31.1%; $P < 0.05$); highest values were obtained 6 h after heat stress (COMP α t 58.2%; $P < 0.05$). COMP α t values returned to control levels after 24 h. A second increase in COMP α t was seen at day 7 (COMP α t 16.9%; $P < 0.05$). Significant levels of

Figure 3 Apotag-positive germ cells in testes from controls (open bars) and those subjected to a transient scrotal heat stress (shaded bars). Bars show average values for each group ($n = 3$ animals per group) and points show the values for individual mice.

Figure 4 Levels of DNA damage in motile spermatozoa following scrotal heating as determined by the COMET assay. Values for tail moment (bars) and COMET moment (points) for each group are shown. d, days.

DNA abnormality were detected in spermatozoa recovered from 14 days to 32 days after treatment (Fig. 5).

Additional information relating to the status of the DNA within the spermatozoa was obtained by evaluating the numbers of spermatozoa in which the level of green fluorescence was over 800 ('High Green') as a measure of whether they contained DNA which was not fully condensed and therefore more accessible to acridine orange. Although most samples included 2 to 5% of spermatozoa with High Green values, a significant peak was observed at 14 days post heat stress when 18.3% ($P < 0.05$) of spermatozoa had a High Green value.

Discussion

In the present study we have shown that subjecting the testis to a transient heat stress results in an increased level of apoptosis in germ cells, but that those cells that do survive can complete spermatogenesis to form spermatozoa that carry damaged DNA. The DNA integrity of epididymal spermatozoa was also compromised if scrotal temperature was raised. We have shown for the first time that the temperature sensitive RNA binding protein Cirp is expressed in the murine epididymis and that its expression is reduced when scrotal temperature is raised. The heating regime (30 min at 42 °C) used in this study was chosen to mimic that of a hot bath. Jannes *et al.* (1998) had previously reported that subjecting mice to scrotal heating at a temperature of 42 °C for 20 min resulted in the production of poor quality spermatozoa, reduced embryo weight *in vivo*, and reduced fertilization rates *in vitro*, a situation that is reminiscent of that associated with some types of human sub-fertility.

Changes in the level of expression of the temperature-regulated RNA binding protein Cirp were consistently detected in both the testis and the epididymis following treatment, confirming that the transient heat stress had had an impact on cell function in both organs. The maximal decrease in Cirp immunoexpression was detected 6 h after treatment. These findings are in agreement with those of Nishiyama *et al.* (1998) who reported that expression of Cirp in germ cells was reduced 6 h after increasing testicular temperature in mice using a water bath (42 °C) or by placing the testes in the abdomen. Furthermore, these authors also reported that expression of Cirp was reduced in the germ cells of testes from men with varicocele and they proposed a role for Cirp in the regulation and

Figure 5 Percentage of motile spermatozoa exhibiting DNA damage expressed in terms of Comp α t as determined by the sperm chromatin structure assay (SCSA) following heat stress. Values are means $\pm$ s.d. ($n = 3$ animals per group); d, days.

co-ordination of germ cell function during spermatogenesis (Nishiyama *et al.* 1998). RNA binding proteins, some of which are expressed on the Y chromosome, have been identified as important regulators of male germ cell function in mammals (Cooke & Saunders 2002) and it appears that Cirp also contributes to normal germ cell function by regulating the availability of mRNAs. Studies *in vitro* have suggested that Cirp expression may have an impact on cell cycle regulation (Hamid *et al.* 2003) and it is therefore possible that loss of Cirp expression contributed to the increased levels of germ cell apoptosis observed because regulation of mitosis and/or meiosis is disturbed. Other studies have reported that heat stress (via experimental cryptorchidism and scrotal heating) results in increased apoptosis of testicular germ cells (Yin *et al.* 1997, Lue *et al.* 1999, Yamamoto *et al.* 2000, Rockett *et al.* 2001). In this study we observed both an increase in the numbers of Apotag-positive germ cells as well as an increase in the expression of the pro-apoptotic marker Bax (unpublished observations by S King and P Saunders in the testes after scrotal heating, a finding consistent with a role for Bax in heat-induced apoptosis of germ cells, reviewed in Sinha Hikim *et al.* 2003). We also observed that the numbers of apoptotic germ cells remained markedly elevated above controls from 2 h to 24 days after the transient heat stress suggesting that the treatment had induced changes in germ cell and/or somatic cell function that had a long term impact on testicular function and further studies are planned to elucidate what these changes might be.

The epididymis has four main functions; the transportation, maturation, storage and protection of spermatozoa. In most mammals, including humans, the epididymis is located within the scrotum that is generally maintained at several degrees below body temperature. In the present study the number of motile spermatozoa retrieved from the heat-stressed epididymis rapidly decreased and remained low for the duration of the experiment. The initial decrease maybe be attributed to increased removal of spermatozoa by the basal cells in the epididymal epithelium which have the ability to act as tissue-fixed macrophages and may also be involved in the detoxification of the epididymal epithelium and lumen (Yeung *et al.* 1994, Seiler *et al.* 1999, 2000).

The effect of scrotal heating on DNA integrity in the motile spermatozoa was assessed using a modified alkaline COMET assay as well as the SCSA. The COMET assay, which was originally developed to study the DNA integrity of somatic cells, has been adapted for use in spermatozoa (Singh *et al.* 1988, Haines *et al.* 1998, Irvine *et al.* 2000) and gives a measure of the single and double stranded DNA breaks present within the cell nucleus. In contrast, the SCSA measures the susceptibility of sperm chromatin to acid denaturation (Evenson *et al.* 1999). This assay is considered to give a measure both of what proportion of spermatozoa contain incorrect packaged/remodeled DNA as well as the prevalence of DNA strand breaks, as both situations will result in DNA more

susceptible to acid denaturation (Larson *et al.* 2000, Evenson *et al.* 2002). We found that both the mature spermatozoa in the epididymis and the spermatozoa that had developed from germ cells within the testis at the time of the heat stress contained DNA exhibiting higher SCSA values than those from control animals. Evenson *et al.* (2000) reported that in a fertile patient who had experienced a one day fever of 39.9°C, 36% of spermatozoa recovered 18 days after the fever contained denatured DNA but that this had decreased to 23% by 39 days. Results obtained comparing pregnancy outcome from patients in which the spermatozoa of the male partner have high SCSA values have reported that this assay can identify the likelihood of achieving a pregnancy more reliably than measurement of conventional semen parameters (Larson-Cook *et al.* 2003, Virro *et al.* 2004).

A number of studies have shown that DNA damage in spermatozoa can occur as a result of oxidative stress (Aitken *et al.* 1989, Aitken *et al.* 1998, Twigg *et al.* 1998, Aitken & Krausz 2001). Oxidative stress, which has been shown to affect spermatozoa in the testis and the epididymis, can be caused by factors such as cancer treatments and cigarette smoke (Hinton *et al.* 1995, Shen *et al.* 1997). We suggest that the increase in epididymal temperature could have resulted in an increased level of oxidative stress in the epididymis and this induced an increase in DNA strand breaks within the spermatozoa. This would be consistent with reports that raised temperature causes changes in oxygen levels, water and ion transport mechanisms, protein biosynthesis and secretion, and in the cellular structure of the epididymal epithelium (Djakiew & Cardullo 1986, Seiler *et al.* 2000). It has been proposed that the availability of oxygen in the epididymis is of significant importance in the maintenance and storage of maturing spermatozoa (Djakiew & Cardullo 1986) with lower scrotal temperatures increasing the solubility of oxygen and decreasing the respiration rate of spermatozoa, thereby ensuring that there is sufficient oxygen to sustain large numbers of spermatozoa. An increase in scrotal temperature could therefore result in an unfavorable epididymal environment.

Motile spermatozoa retrieved from the epididymides 7 to 32 days after scrotal heating originated from cells located within the testis at the time of treatment (see Table 1). In the current study, levels of DNA damage in spermatozoa, as measured by COMET and SCSA, were the same in controls and those recovered 7 days after heating. At the time of heat stress these cells would have been developing within in the testis as stage 11 elongating spermatids or more mature elongating spermatids and this suggests that the DNA within these cells was resistant to the effects of treatment. However in contrast, the SCSA revealed that the populations of motile spermatozoa recovered 14 days post heating contained 18% with a High Green value and that 60% had poor DNA integrity (Comp α t value greater than 26%). At the time of heating these cells would have been developing as step 1 to

step 11 spermatids. Marcon & Boissonneault (2004) have reported that during the normal process of spermiogenesis strand breaks are introduced in step 9–11 spermatids and that these are associated with the condensation of the germ cell DNA and replacement of histones by protamines, but that after stage 11 all DNA breaks within the normal spermatid have been repaired and the histones have been completely replaced by protamines. Zhao *et al.* (2004) have shown that transition proteins (TP) involved in the histone to protamine transition are also required for normal chromatin condensation, for reducing the number of DNA breaks and preventing a loss of genetic integrity. In the mouse transition proteins are expressed in step 10 to step 14 spermatids and the onset of protamine expression has been detected in step 10 spermatids using immunohistochemistry. Our results suggest spermatids undergoing DNA remodelling (up to and including step 11 spermatids) are vulnerable to the effects of heating, resulting in a disruption to the histone to TP to protamine transition and in incorrect packaging of DNA within the head of the spermatozoa.

Previous studies have identified pachytene spermatocytes and early spermatids as being susceptible to heat stress (Collins & Lacy 1969, De Vita *et al.* 1990) and this is not surprising as during meiotic prophase substantial rearrangements of DNA occurs within the nuclei of the leptotene, zygotene and pachytene spermatocytes. Collins & Lacy (1969) identified two critical periods in spermatogenesis (leptotene-pachytene and maturation division) through which cells were unable to progress following heating. Our results extend these earlier findings and have demonstrated that spermatozoa recovered 24, 28 and 32 days after testicular heating, which would have been developing as type A spermatogonia to stage X pachytene spermatocytes, were susceptible to heat-induced alteration in cell function and that this was associated with reduced numbers of motile spermatozoa being recovered up to 4 weeks later. Moreover, although the DNA damage within many of these cells was not fully repaired, they were able to complete their maturation and be released into the epididymis. Evenson and colleagues (Evenson *et al.* 1999, Larson *et al.* 2000) have shown that a COMP α value in human spermatozoa of greater than 27% would predict that pregnancy would not be achieved in the context of IVF and ICSI. Rockett *et al.* (2001) reported that male mice that had undergone heat stress (43 °C for 20 min) showed a significant decrease in the viable fetuses that they could produce. In the current study, the fertilizing capacity of the spermatozoa was not directly tested by performing IVF or mating experiments and further experiments are therefore required to determine if there is any loss of fertility following the transient heat stress we applied.

It has been shown that raised temperature can lead to oxidative stress within the rat testis; this in turn can trigger apoptosis (Ikeda *et al.* 1999). The effects of oxidative stress on the genomic integrity of spermatozoa have been

studied by inducing oxidative stress in the testis and in mature spermatozoa (Aitken *et al.* 1989, Lucasoli & Fraga 1995). Within the testis a number of cell types produce high levels of reactive oxygen species (ROS) that may result in oxidative damage to DNA, proteins and cell membranes and to combat this, the testis has developed a very complex antioxidant system (Bauche *et al.* 1994, Fisher & Aitken 1997). Therefore decreased expression of oxidative stress response genes, including extracellular superoxide dismutase (SOD) 3, manganese SOD 2 and genes involved in the metabolism of glutathione, which has been reported to occur following heating, may leave cells more susceptible to oxidative DNA damage (Barroso *et al.* 2000, Rockett *et al.* 2001).

A number of DNA repair mechanisms have been documented to be active during spermatogenesis (Richardson *et al.* 2000, Aguilar-Mahecha *et al.* 2001, Aitken & Krausz 2001, Rockett *et al.* 2001, Hsai *et al.* 2003). These are responsible for the repair of the DNA strand breaks known to occur accidentally during spermatogenesis, particularly during meiosis, and also those nicks and breaks that facilitate the transition of DNA from the somatic cell histone complex to the tightly packed protamine complex of the mature spermatozoa by relieving the torsional stress created during this process and aiding in remodelling (Ward & Coffey 1991, McPherson & Longo 1992). Normally these strand breaks and nicks are re-ligated before the completion of spermiogenesis (Sakkas *et al.* 1995, Marcon & Boissonneault 2004). Following heating, the expression of a number of genes in the testis is down-regulated, these genes include several involved in DNA repair and recombination (Exonuclease III, G/T mismatch binding protein, DNA topoisomerase, 8-oxohuanine DNA glycosylase), cell cycle regulation and stress response (Rockett *et al.* 2001). The reduced levels of expression of these gene products may mean that the testis is less able to deal with the damage caused to the DNA within the germ cells. Alternatively, the level of DNA damage induced by heat stress may simply overwhelm the repair mechanisms in the testis and while a certain amount of DNA damage is repaired some may persist into the mature spermatozoa; further studies are required to test these possibilities.

In summary, it appears that the loss of DNA integrity in mature spermatozoa resulting from heat stressed germ cells may be explained in two ways, first, DNA damage that occurs in the cells at the time of heating is not repaired during spermatogenesis, or secondly, the physiology of the testis is disrupted by the heat stress resulting in sub-optimal support for germ cell development which is detected as a loss of DNA integrity in mature spermatozoa. Alterations in generation of reactive oxygen species or reduced activity of DNA repair proteins could contribute to the persistence of DNA breaks in maturing spermatozoa.

In conclusion, we have demonstrated that mature spermatozoa in the epididymis are not protected from heat-induced DNA damage and that following a transient

heat stress the environment of the epididymis itself may contribute to the deterioration of DNA integrity of spermatozoa. Studies involving *in vitro* heating and culturing of spermatozoa and epididymis could be employed to explore this hypothesis further. Furthermore, we have identified cell types within the testis, which following heat stress, develop into motile, but DNA-damaged, spermatozoa. These findings suggest the need for additional criteria to be taken into consideration when selecting spermatozoa for IVF/ICSI treatment; in particular, the effect on DNA integrity may play a significant role in determining the viability of spermatozoa for use in an ICSI/IVF procedure.

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