

A single, mild, transient scrotal heat stress causes DNA damage, subfertility and impairs formation of blastocysts in mice

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Abstract

Infertility represents a major clinical problem and 50% of cases are attributable to the male partner. Testicular function is temperature dependent, and in both man and mouse the position of the testes in the scrotum ensures that they are kept at between 2 and 8 °C below core body temperature. We used a mouse model to investigate the impact of a single, transient, mild, scrotal heat stress (38, 40 or 42 °C for 30 min) on testicular function, sperm DNA integrity and embryo survival. We detected temperature-dependent changes in testicular architecture, number of apoptotic cells and a significant reduction in testis weight 7 and 14 days after heat stress at 42 °C. We report for the first time that DNA strand breaks (γ -H2AX-positive foci) were present in spermatocytes recovered from testes subjected to 40 or 42 °C. Fertility of heat-stressed males was tested 23–28 d after treatment (sperm at this time would have been spermatocytes at time of heating). Paternal heat stress at 42 °C resulted in reduced pregnancy rate, placental weight and litter size; pregnancies from the 40 °C group had increased resorptions at e14.5. Abnormalities in embryonic development were detected at e3.5 and *in vitro* fertilisation with sperm recovered 16 h or 23 d after scrotal stress at 42 °C revealed a block in development between the 4-cell and blastocyst stages. This study has provided evidence of temperature-dependent effects on germ cell DNA integrity and highlighted the importance of an intact paternal genome for normal embryo development.

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Introduction

Damaged DNA originating in the male germ line may contribute to early pregnancy loss (recurrent miscarriage), birth and developmental defects and/or have consequences for their offspring later in life. DNA damage rates in sperm are higher in infertile individuals (Irvine *et al.* 2000), patients subjected to cancer therapies (Charak *et al.* 1990, Kenney *et al.* 2001), men who smoke (Zenzes *et al.* 1999, Zenzes 2000) and also possibly in older men (Wyrobek *et al.* 2006). Concerns have been expressed that using sperm with damaged DNA for intracytoplasmic sperm injection (ICSI) could have a long-term effect on the health of the children born to subfertile couples (Gosden *et al.* 2003). There is compelling evidence that there is a correlation between male infertility and testicular heat stress (Jung *et al.* 2001, 2002, Paul *et al.* 2008), which is supported by studies in a number of animal models (Setchell 1998, Ivell 2007, Paul *et al.* 2008).

It is well established that spermatogenesis and normal testicular function are both temperature dependent, and in most mammals the testes are kept between 2 and 8 °C below core body temperature by virtue of being situated

in the scrotum outside the main body cavity (Harrison & Weiner 1948, Ivell 2007). Testicular temperature is regulated by a countercurrent heat exchange system between incoming arterial blood and outgoing venous blood, the temperature of which is lower than that of arterial blood due to the loss of heat through the skin of the scrotum. The fact that the scrotum has no subcutaneous fat helps dissipate heat to the exterior (Glad Sorensen *et al.* 1991). Testicular temperatures are reported to be higher in men with clinical conditions (cryptorchidism and varicocele), certain occupations (bakers, welders and professional drivers) and in those with poor posture or tight clothing (Mieusset *et al.* 1987, 2007, Salisz *et al.* 1991, Lerchl *et al.* 1993, Thonneau *et al.* 1998). Experimental studies have suggested that a mild increase in testicular temperature could be a potential contraceptive method for men (Mieusset & Bujan 1994) and that nocturnal scrotal cooling could improve sperm count in patients with elevated testicular temperatures who have been diagnosed with oligoastheno-teratozoospermia (Jung *et al.* 2001).

Several rodent models have been used to study the impact of heat stress on the testis including transient exposure of the testes to elevated temperatures (typically

greater than 40 °C), surgical induction of cryptorchidism resulting in long-term exposure of the testes to core body temperature (37 °C) or housing of males at elevated temperatures (e.g. 35–36 °C) for several hours (Zhu & Setchell 2004, Zhu *et al.* 2004, Cammack *et al.* 2006). In these studies, the common features of disturbances in testicular function that have been recorded include decreased testicular weights, germ cell loss and increased rates of apoptosis (McLaren *et al.* 1994, Setchell *et al.* 1996, 1998, Lue *et al.* 1999, Banks *et al.* 2005, Perez-Crespo *et al.* 2008). Some studies have reported that pachytene spermatocytes and early spermatids are the cell types most susceptible to testicular heat stress (Setchell 1998). Localised scrotal heating of mice is also associated with detection of DNA damage in sperm and reduced sperm counts. When DNA damage was detected in sperm recovered from the epididymes within hours of heating this was taken as evidence that epididymal sperm are still susceptible to heat-induced DNA damage even though the DNA is tightly packaged with protamines (Sailer *et al.* 1997, Banks *et al.* 2005).

A link between paternal heat stress and embryo survival has been demonstrated in a few studies. For example, Jannes *et al.* (1998) reported that sperm counts were reduced 28 days after 20 min of heat stress at 42 °C and when these males were mated the resulting offspring were significantly smaller at embryonic day (e)14.5 than those sired by controls. When Rockett *et al.* (2001) performed heat stress at 43 °C for 20 min they noted reduced litter sizes in females mated to heated males 23–28 days after stress. An impact on litter size was also noted when males are housed at ambient temperatures of 35–36 °C for periods of 12 or 24 h (Yaeram *et al.* 2006) with reduced number of embryos developing beyond the 2-cell stage (Zhu & Setchell 2004, Zhu *et al.* 2004).

In this study, we have explored the link between heat stress, DNA damage within germ cells and embryo survival by comparing the impact of transient scrotal heat stress at three different temperatures, namely 38, 40 and 42 °C. Our results demonstrate a clear temperature-dependent impact on germ cell function and fertility, consistent with an important contribution from the paternal genome in embryo/foetal survival.

Results

Heat stress-induced chromatin abnormalities in sperm

The sperm chromatin structure assay (SCSA) was performed on sperm recovered from the epididymes of control and heat-stressed mice. This is a population assay that determines the occurrence of acid-induced chromatin damage within a sample of around 10 000 sperm. Sperm samples from control mice exhibited sperm with DNA damage of around 10–15%. There was a slight (not significant) increase in sperm with DNA damage in the

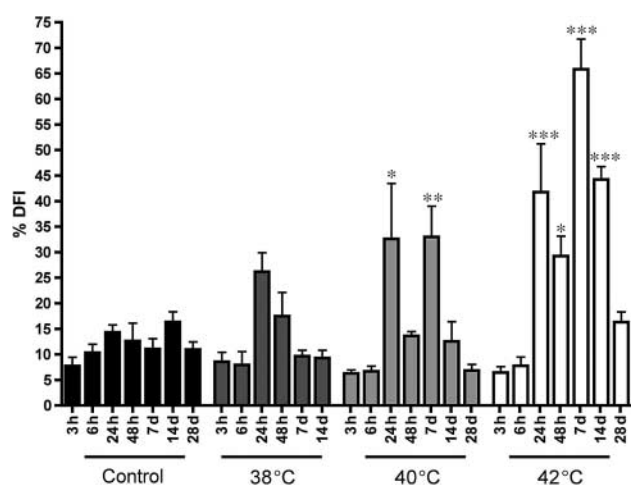


Figure 1 Susceptibility of epididymal sperm to acid denaturation detected by the SCSA. % DFI represents ratio of denatured sperm DNA (red) to total sperm DNA (red/(red+green)) fluorescence. ***($P < 0.001$), **($P < 0.01$) and *($P < 0.05$) indicate significant variation from time matched controls. For all time points, $n \geq 3$.

38 °C group at 24 h (Fig. 1) consistent with an effect on the sperm in the epididymis; however, there seemed to be no effect on the sperm recovered at later time points. Heat stress at higher temperatures resulted in increased number of damaged sperm, with peaks in the number of sperm with DNA damage at both 24 h and 7 d in the 40 °C group and at 24 and 48 h (epididymal sperm at time of heat stress) as well as 7 and 14 d (sperm derived from germ cells resident in the testis at the time of heating) in the 42 °C group (Fig. 1).

Testicular weights and gross histology of heat-stressed mice

Testes recovered from heat-stressed males were weighed and a gross estimate of overall testicular architecture was undertaken using sections stained with haematoxylin and eosin (Fig. 2). The impact of heat stress on testis weights 7 and 14 days after stress was temperature dependent, with a significant reduction at both time points in the 42 °C group (Fig. 2G). Testes from the 38 °C group appeared histologically indistinguishable from controls and only minimal changes were detected in the 40 °C group (not shown). By contrast, extensive changes were observed in those heated to 42 °C with multinucleated giant cells and degenerating germ cells observed within the seminiferous epithelium at 24 h (Fig. 2B and C arrows). By 48 h, germ cell depletion was evident and large areas of the seminiferous epithelium contained only Sertoli cells, spermatogonia and elongated spermatids (Fig. 2D). Germ cell loss was also obvious in testes recovered at 7 and 14 d and many tubules appeared to lack both spermatocytes and round spermatids causing the seminiferous epithelium to collapse (Fig. 2E and F). In the 40 °C group (where

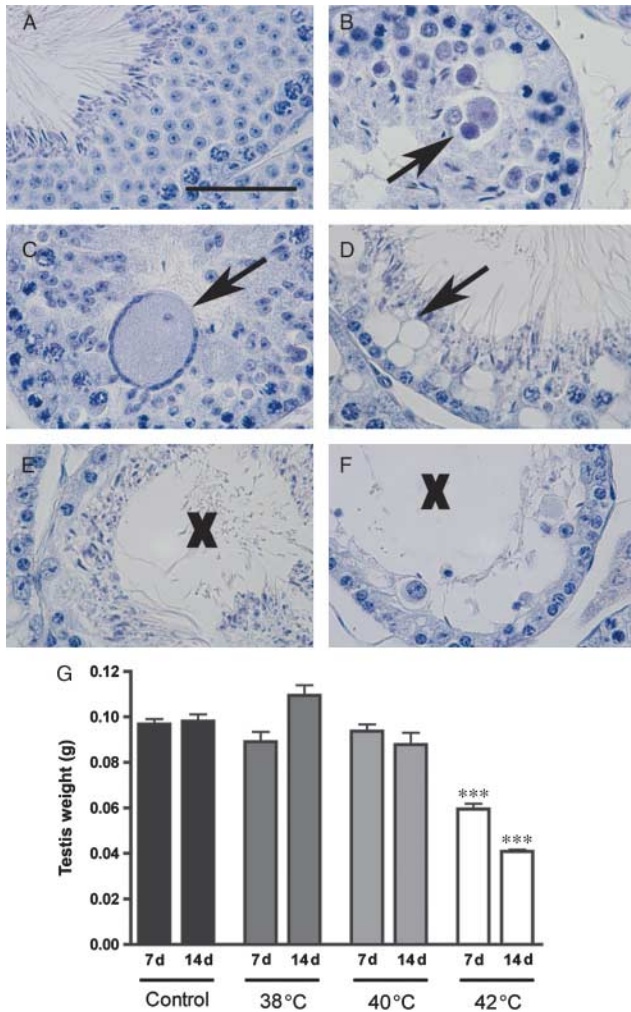


Figure 2 Histological evaluation of testes from mice stressed at 42 °C and testis weights at 7 d and 14 d after heat stress. (A) Haematoxylin and eosin-stained testes from control mice and (B and C) heated mice at 24 h with arrows highlighting degenerating cells and multinucleated giant cells, (D) 48 h with an arrow highlighting gaps in the seminiferous epithelium and absence of spermatocytes, (E) 7 d and (F) 14 d with X highlighting tubules with depleted germ cells and collapse of the seminiferous epithelium in (E). For (G) $n \geq 4$, ***($P < 0.001$). Scale bar represents 50 μm .

there was a slight, but non significant, reduction in testis weight), some nuclear condensation was observed in germ cells at 24 and 48 h suggesting that they were undergoing apoptosis and at later time points a few 'gaps' were observed in the seminiferous epithelium where these cells had been lost (not shown).

Impaired DNA repair in spermatocytes

TUNEL staining was used to detect cellular DNA fragmentation and as a marker of cell death. As expected, a few TUNEL-positive germ cells were found in control testes (Fig. 3) with the majority in stage XII tubules. Testes recovered from mice heated to 38 °C had

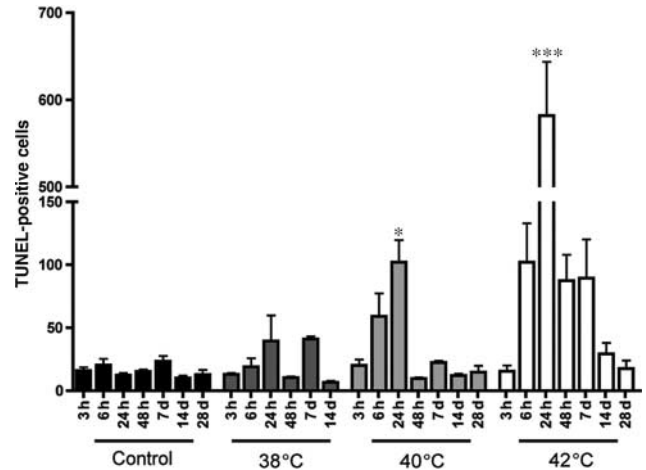


Figure 3 Quantification of cells with fragmented DNA using TUNEL staining. The total number of TUNEL-positive cells per 100 tubules was counted. All groups have $n \geq 3$. ***($P < 0.001$) and *($P < 0.05$) indicate significant variation from time matched controls.

similar number of positive cells to controls (Fig. 3). Testes from both the 40 and 42 °C groups contained significantly more TUNEL-positive cells at 24 h after heat stress with a striking 38-fold increase in the 42 °C-heated testes indicating heat-induced DNA fragmentation in germ cells. The vast majority of TUNEL-positive cells were spermatocytes.

To quantify the DNA damage occurring in spermatocytes, germ cell spreads were prepared from controls and heat-stressed testes and these were immunostained for synaptonemal complex protein (SCP3) and the phosphorylated form of H2AX (γH2AX) that binds specifically to unsynapsed DNA or double-strand breaks (DSBs) (Paull *et al.* 2000). Consistent with previous results (Hamer *et al.* 2003), the spermatocytes had intense immunopositive staining for γH2AX localised to the sex body, which contains the unpaired regions of the X and Y chromosomes (Fig. 4). In controls, γH2AX immunostaining of the autosomes was low/negative with never more than two to three foci on each spermatocyte (Fig. 4A and B). Results from mice heated to 38 °C were comparable with controls (Fig. 4G). However, spermatocytes obtained from mice heated to 40 °C had a significant increase in the number of γH2AX -positive foci by 24 h that was maintained at 48 h (Fig. 4C and D respectively). After heat stress at 42 °C, there was a dramatic increase in the number of γH2AX -positive foci as early as 3 h after stress and this was maintained for up to and including 14 d although number of foci were reduced to control levels by 28 d (Fig. 4G).

Reduced fertility in heat-stressed males

Females mated to heat-stressed (40 or 42 °C) males were assessed for fertility by determining pregnancy rate and foetus numbers at e14.5. The matings were set up for

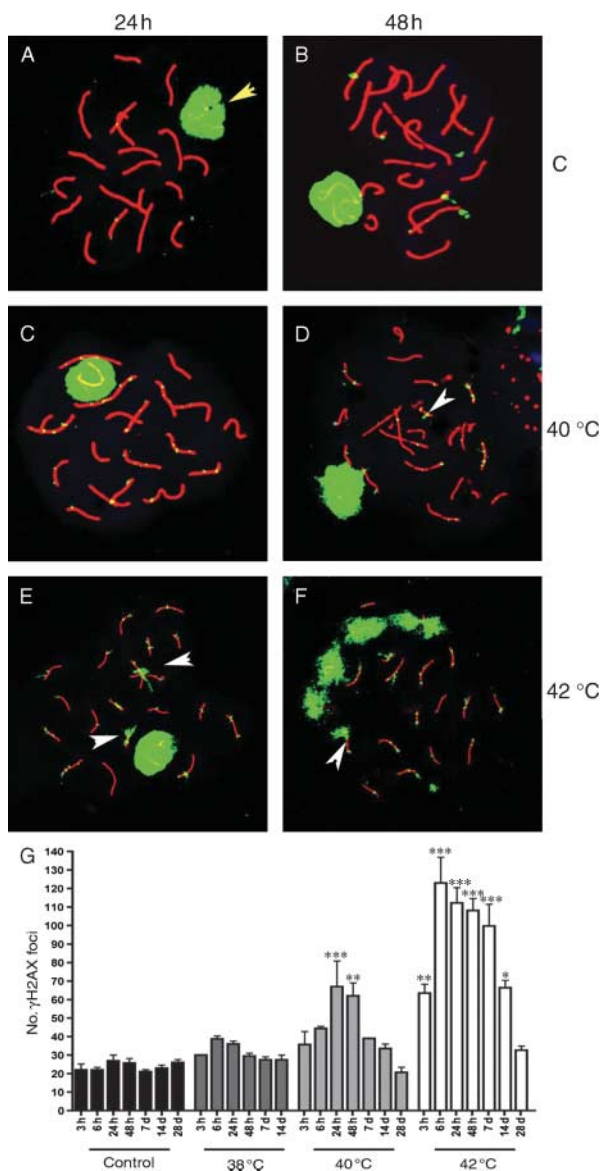


Figure 4 DNA lesions in pachytene spermatocytes detected using γ H2AX immunohistochemistry on germ cell spreads. (A and B) Immunodetection of SCP3 (red) and γ H2AX (green) 24 and 48 h after heat stress in controls, (C and D) the 40 °C group and (E and F) the 42 °C group. The sex body is highlighted with a yellow arrowhead in (A). Some foci are highlighted with white arrowheads. (G) Foci were counted on 50 pachytene spermatocytes per mouse with at least three mice per group.

5 days, 23–28 days after heat stress, to reflect an insult on the spermatocytes at the time of heating. The pregnancy rate in the 40 °C group was comparable with controls (Fig. 5A). The effects of a transient 42 °C heat stress were striking, though, with a drastic reduction (sevenfold) in pregnancy rate (Fig. 5A). The females in this group that did achieve a pregnancy had reduced number of foetuses (average one foetus per female; Fig. 5B). The number of resorptions per female was also quantified at e14.5 (Fig. 5B) and although there seemed to be no difference

between controls and the 42 °C group with 20 and 15% respectively, of females containing resorption sites almost 60% of the 40 °C group had evidence of foetal death (Fig. 5B and C).

Reduction in placental weights

Given that the paternal genome is reported to be involved in the development of extraembryonic tissue (Barton *et al.* 1984), we examined whether there was any difference in the placenta formation in the control and heated mice by recording placental weights. Placental weight in the 42 °C group was significantly reduced to around 80% of control values (Fig. 6).

Abnormal development in embryos produced by heat-stressed males

In order to assess whether paternal heat stress had an impact on early embryonic development, embryos were recovered by flushing at e3.5; at this stage they should have developed into blastocysts. The retrieved embryos were immunostained with antibodies directed against POU5F1 (OCT3/4) and TJP1 (previously zonula occludens-1, ZO-1). POU5F1 is a transcription factor that is expressed in early development and plays a role in pluripotency and is localised to the inner cell mass (ICM). TJP1 is a tight junction protein and its expression in trophoblast cells contributes to the formation of the blastocoelic cavity. The blastocysts obtained from females mated with control mice had a normal structure with a distinct POU5F1-positive ICM, a blastocoelic cavity and TJP1-positive junctions within the trophoblast (Fig. 7A and B). Although most of the embryos obtained from the 40 °C group appeared to have a normal morphology (Fig. 7C), the development of a small number of embryos was retarded (e.g. 32-cell stage; Fig. 7D), lacking a blastocoelic cavity. The embryos resulting from matings with males from the 42 °C group were all grossly abnormal (Fig. 7E and F). These embryos had fragmented nuclei, abnormal POU5F1 staining, an 'amorphous' appearance and no blastocoelic cavity.

IVF with sperm from heat-stressed males reveals failure of embryos between the 4-cell and blastocyst stages

In order to examine further what was happening to the embryos resulting from fertilisation with sperm from the 42 °C group, *in vitro* fertilisation (IVF) was carried out. This was performed using sperm retrieved from males that had been subjected to heat stress either 16 h (Fig. 8A) or 23 d previously (Fig. 8B) in order to determine the outcome of using sperm resident in the epididymis during heat stress or those which had developed from spermatocytes (testicular stress)

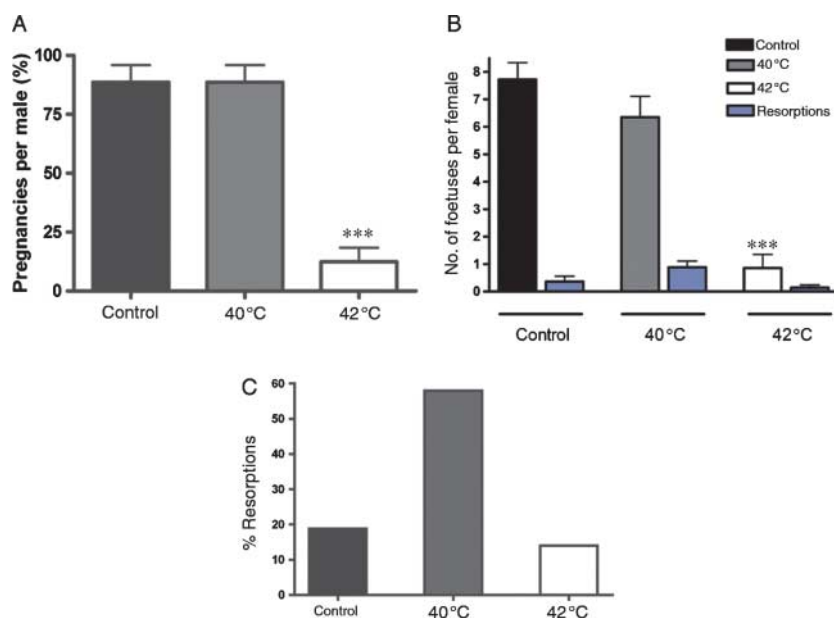


Figure 5 Pregnancy rate and litter sizes of females mated to control males or those that had been subjected to heat stress (40 or 42 °C). (A) Rates are expressed as percentage of females pregnant per male. (B) Litter size is expressed as the number of foetuses per female. Resorption number per female is also depicted in (B). ***($P < 0.001$) indicates significant variation from control mice, $n = 18$ females per temperature group and 6 males per temperature group. (C) Resorption rate expressed as percentage of females per group that exhibited one or more resorptions.

respectively. Embryo development was examined at the 4-cell, 4-cell and blastocyst stages. The results for both the 16-h and 23-d groups were similar, with no significant effect on the number of embryos developing to the 2-cell or the 4-cell stages as a result of fertilisation with sperm from controls or heat-stressed males. However, the number of embryos developing to the blastocyst stage was greatly reduced (~40% of controls) when sperm were obtained from mice 16 h after heating (epididymal insult) (Fig. 8A). Strikingly, none of the embryos generated from sperm retrieved from males 23 d after testicular heat stress progressed beyond the 4-cell stage and no blastocysts were formed (Fig. 8B).

Discussion

The link between increases in testicular temperature and impaired fertility is well established. Studies in mice have used a variety of techniques to determine the effects of heat stress on fertility and it is notable that similar disturbances in testicular function and reduced fertility have been recorded in animals housed at 36 °C for 12 or 24 h (Zhu & Setchell 2004, Zhu *et al.* 2004) and those subjected to a transient scrotal heat stress at 42–43 °C for 20–60 min (Lue *et al.* 1999, 2000, 2002, Rockett *et al.* 2001, Setchell *et al.* 2001, 2002, Banks *et al.* 2005, Zhang *et al.* 2005). Our study includes novel data on the origin of DNA damage found in sperm i.e. heat-induced DNA lesions detected in spreads of spermatocytes, and an apparent reduction in fertility of mice mated to males where only minimal testicular changes were detectable i.e. those heated at 40 °C.

In this study, the disturbances observed in testicular architecture and increased TUNEL staining were consistent with previous reports. For example, we found that the

extent of the disruption in testicular histology of the testis was temperature dependent with the highest temperature having the most drastic effects. Heat stress at 42 °C induced the formation of giant degenerating germ cells, multinucleated giant cells, and was accompanied by germ cell loss and ‘gaps’ within the seminiferous epithelium. The cells predominantly affected were the spermatocytes and round spermatids. The formation of multinucleated giant cells has been reported previously in cases of induced cryptorchidism and localised scrotal heating (Chowdhury & Steinburger 1970, Waldbieser & Chrisman 1986, Yin *et al.* 1997, Chaki *et al.* 2005). These cells, which were TUNEL-negative, may form as a result of the sloughing of groups of round spermatids. This aggregation of abnormal and apparently degenerating cells appears to be unique to the testis and may be related to the phagocytic properties of the Sertoli cells (Mizuno *et al.* 1996). TUNEL-positive spermatogonia were rare and these cells were therefore able to participate in the repopulation of the testis allowing recovery of full spermatogenesis by 32 days (Banks *et al.* 2005). Some of these changes were also seen in the 40 °C group albeit to a much lesser extent.

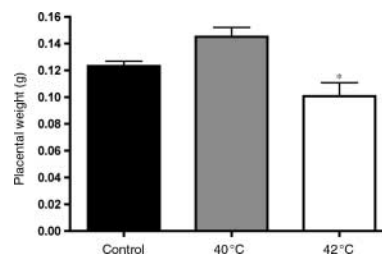


Figure 6 Placental weights from females mated to control and heated (40 and 42 °C) males. Placental weights are shown in grams. *($P < 0.05$) indicates significant difference from controls.

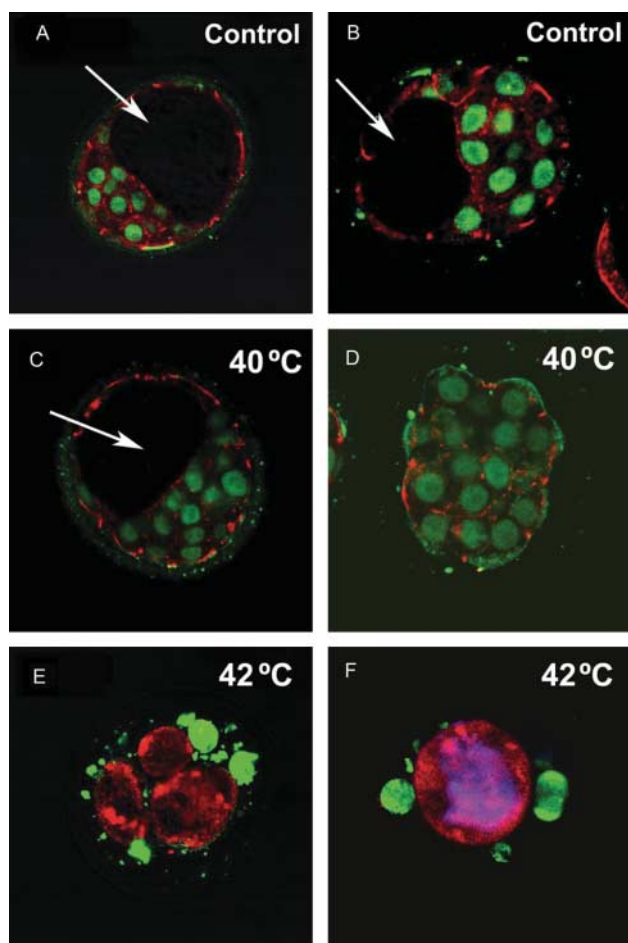


Figure 7 Blastocyst stage embryos immunostained with POU5F1 and TJP1 from female mice mated to either control or heated (40 or 42 °C) males. (A and B) In control blastocysts POU5F1 (green) is localised to the cells of the inner cell mass (ICM) and TJP1 (red) is localised to the tight junctions. White arrows highlight blastocoelic cavity. Examples of blastocysts retrieved from females mated to 40 °C-heated males are shown in (C and D) and those from the 42 °C group are shown in (E and F).

Further investigations on the impact of heat stress on the functional integrity of the spermatocytes revealed an increased incidence of DNA strand breaks i.e. γ H2AX foci, in pachytene spermatocytes. Phosphorylation of γ H2AX occurs following formation of DSBs (Rogakou *et al.* 1998) and a dramatic increase in DSBs was detected in germ cells in the 42 °C group (and to a lesser extent in the 40 °C group), which was consistent with the observed increase in cell death in this group compared with those subjected to lower temperatures. It is unclear, however, whether the heat stress applied in the current study is inducing chromosome aberrations or impairing their repair.

Although a large number of germ cells were eliminated by apoptosis, especially in the 42 °C group, this process does not appear to have been 100% efficient as the number of sperm with DNA fragmentation recovered from the epididymis was increased (at 40

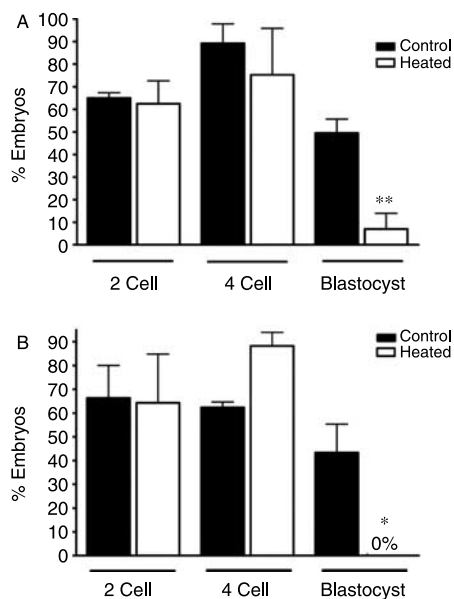


Figure 8 Embryo development following *in vitro* fertilisation with sperm recovered from control mice and mice subjected to heat stress at 42 °C (A) 16 h previously and (B) 23 d previously. % 2-cell embryos represent the percentage of total oocytes retrieved. % 4-cell embryos and blastocyst embryos represent the percentage of 2-cell embryos that developed to these stages, $n = 4$ IVF cycles. * ($P < 0.05$) and ** ($P < 0.01$) indicate significant difference from controls.

and 42 °C) compared with controls as assessed by the SCSA; these sperm had therefore completed meiosis I and II, spermiogenesis and release subsequent to heat treatment. Studies comparing the pregnancy outcome from patients whose partner had a high SCSA value reported that this assay can identify the likelihood of achieving a pregnancy and that this has proven to be more reliable than conventional measurements of sperm parameters (Larson-Cook *et al.* 2003, Virro *et al.* 2004). In this study, we found that the largest number of sperm with DNA damage following testicular heat stress were detected at 7 and 14 d after heating at 42 °C, which agrees with results from an earlier study by Sailer *et al.* (1997). These sperm would have been early or late spermatids at the time of heating which is consistent with the expectation that post-meiotic germ cells are only capable of limited DNA repair and prompts the question: do pre-meiotic (i.e. spermatogonia) germ cells have a greater capacity for repair, making them less susceptible to stress-induced abnormalities? Spermatids formed from secondary spermatocytes must have a minimal capacity for DNA repair (Sega 1979, Sotomayor & Sega 2000) as they are silenced both transcriptionally and translationally during the condensation of the chromatin during sperm head remodelling. Furthermore, as sperm cannot respond by inducing either apoptosis or DNA repair, any DNA damage induced in these post-meiotic spermatids could persist. A significant increase in the number of sperm with DNA damage was also observed at the 24-h

time point (42 and 40 °C). This would reflect the status of the DNA in sperm that were present in the epididymis at the time of the insult and may be due to an increased level of oxidative stress and thus a less favourable epididymal environment as has been suggested in previous studies (Banks *et al.* 2005). The ability of the epididymis to store sperm is impaired at deep abdominal temperatures (Bedford 1991) and increased temperature is also reported to cause changes in oxygen levels, water and ion transport mechanisms, protein synthesis and cell structure (Djakiew & Cardullo 1986, Seiler *et al.* 2000).

DNA damage in sperm caused by oxidative stress, e.g. in fathers who smoke, can be passed on to their children and has been linked with an increased incidence of childhood cancer in their offspring (Zenzes *et al.* 1999, Zenzes 2000). This has caused concern over the use of sperm from subfertile men in assisted reproductive technologies (IVF and ICSI). Thus, the final aim of the study was to determine whether the heat-induced DNA aberrations we observed (using the SCSA and determination of γ H2AX foci) culminated in problems with fertility and embryo development. Male fertility was tested using natural matings 23–28 days after heat stress (40 and 42 °C), a time chosen to reflect an insult to the spermatocytes at the time of heating. The 42 °C group exhibited a sevenfold reduction in the pregnancy rate and those that did achieve pregnancy had a reduced number of foetuses at e14.5, although an increase in resorption sites was not observed. Therefore, we hypothesised that either there was a reduction in fertilisation rate or embryos were failing to implant and we therefore went on to investigate the status of embryos recovered by flushing the oviduct on e3.5. The embryos recovered from mice mated to controls had developed into blastocysts but all of those resulting from matings with the 42 °C males were grossly abnormal and no blastocysts had formed. It was therefore concluded that the reduced pregnancy rate in this group was due to pre-implantation failure of embryos. Our data is consistent with a recent study by Perez-Crespo *et al.* (2008) which found a decrease in the number of pregnancies and foetuses but no change in resorption rate using a similar heat stress regime to our own.

No difference in pregnancy rate was observed in females mated to the 40 °C group as compared with controls; however, closer examination of the females revealed that ~60% had evidence of resorption sites at e14.5 (Fig. 5C). This suggests that in this group some embryos had developed past the 'block' that occurred in the 42 °C group only to fail at a later stage of pregnancy. Some evidence of a problem with early embryonic development was detected in the 40 °C group at e3.5 when a small number of the embryos appeared retarded (i.e. 32-cell stage) compared with controls (blastocysts). Although we cannot rule out the possibility that some variation may occur because not all oocytes will have been fertilised at the same time, these observations

suggest that some embryos generated using sperm from the 40 °C males developed at a slower rate than controls. Previous studies have shown that scrotal heat stress can cause transient growth retardation in early embryos though they appeared to catch up with controls at later stages in development (Setchell *et al.* 1998). Although there was no apparent change in testicular weights and no gross changes in testicular histology when we conducted a more detailed analyses of germ cells in the 40 °C group we detected increased number of γ H2AX-positive foci on meiotic spreads and a transient increase in the number of TUNEL-positive cells. Notably, although the SCSA did not detect a significant number of sperm with DNA damage in either the 40 or 42 °C groups at 28 days after stress, natural matings of animals at this time had an impact on foetal survival and blastocyst development respectively suggesting that this assay was not sensitive enough to detect subtle changes in the DNA of the sperm (as it detects the susceptibility of the chromatin to acid denaturation) that could still have an impact on fertility. Previous studies have demonstrated that the paternal genome is important for the development of extra-embryonic tissues including the trophoblast. For example, embryos with two female pronuclei can implant but die because their extra-embryonic tissues develop poorly (Surani *et al.* 1984). Following on from these early studies there is now an appreciation that differential imprinting of the maternal and paternal genomes occurs during gametogenesis and that this is important for the parent-specific expression of a subset of genes which have important roles in growth of the foetus and placenta (Gold & Pedersen 1994). In this study, placental weights were reduced following natural matings with male mice subjected to a 42 °C stress consistent with an impact on the paternal genome. This warrants further investigation of embryos from heat-treated fathers at later stages in development and on their imprint status. A recent study has reported aberrant imprinting in sperm from oligospermic patients (Kobayashi *et al.* 2007) and concerns have been expressed about the potential for increased rates of rare diseases due to imprinting disorders in children born as a result of assisted reproductive technologies (Ludwig *et al.* 2005).

It is clear that the length of time the mice are exposed to heat is also important as a recent study found scrotal heating at only 36 °C but for a period of 12 h reduced pregnancy rate and litter size and impaired embryo development in IVF (Yaeram *et al.* 2006), whereas we saw no impact of heat stress at 38 °C. Different studies looking at the effect of heating for 24 h at 36 °C have reported abnormal development in embryos recovered from females mated to heat-stressed males including reduced number of embryos and blastomeres and altered gene expression at the 2-cell stage (Zhu & Setchell 2004, Zhu *et al.* 2004, Zhu & Maddocks 2005).

We extended our fertility studies by performing IVF using sperm from males subjected to a 42 °C heat stress either 16 h or 23 d previously. Although there was no difference in the number of embryos developing to the 2-cell or 4-cell stages in these groups and controls, there was a significant reduction in those that developed to blastocysts in the 16-h group and no blastocysts developed in the 23-d group. This confirms the data from the natural matings and clarifies the stage at which the embryos were failing i.e. between the 4-cell and blastocyst stage. Many DNA repair and damage response genes are expressed in early mammalian embryos (Jaroudi & SenGupta 2007), but low levels of some indicate that the embryo's ability to repair DNA damage may be strictly limited (Zheng *et al.* 2005). Our data are consistent with other studies that have shown that human and rodent sperm with DNA damage are capable of fertilising eggs (Twigg *et al.* 1998) but suggests that the DNA originating in the sperm introduces genomic instability to the embryo and can have fatal effects on development.

In mice, reactivation of the paternal genome is thought to occur during early cleavage beginning around the one-cell stage (Matsumoto *et al.* 1994) and therefore any mutations/deletions of DNA in the male genome could result in the disruption of regulatory networks in early embryos. The fact that the embryos resulting from heated males were able to undergo the first two cleavages as normal but fail after this point further suggests that the paternal genome is not involved in these cleavages but becomes more active after the 4-cell stage. This is consistent with the reports that maternal *Pou5f1* is expressed until the 4- to 8-cell stage when it switches to embryonic *Pou5f1* (Yeom *et al.* 1996, Pesce *et al.* 1998); a block in embryonic development before the blastocyst stage is therefore consistent with activation of genes important to embryo survival at this time (Zeng *et al.* 2004). This agrees with a study using uniparental embryos that showed that the paternal genome is not required before the 8-cell stage in mice (Renard *et al.* 1991).

This study has confirmed that spermatogenesis is impaired if scrotal temperatures rise above the normal physiological range and shown that there may be a temperature threshold, which is also influenced by duration, above which germ cell degeneration is induced. It appears that although the testis contains an active apoptotic machinery it is not 100% efficient at eliminating germ cells with abnormalities and that many of these abnormal cells go on to develop into mature sperm with DNA damage. These studies also show that increased scrotal temperature causes subfertility in male mice by affecting pregnancy rate, resorption rate and embryo development in normal control females. This provides further evidence of male-mediated effects on embryo survival given the link between imprinting of paternal-derived genes and

foetal development; it would be of interest to determine whether heat-induced changes in germ cell development result in any trans-generational effects on offspring produced from these 'heated' fathers. These findings should be taken into consideration when using sperm from infertile men in IVF/ICSI treatments where the normal quality control processes involved in fertilisation are bypassed.

Materials and Methods

Animals

Male and female C57BL/6 mice were bred and maintained under standard conditions according to United Kingdom Home Office guidelines. Animals had free access to food and water. All mice were purchased from Harlan (Harlan Sprague-Dawley Inc., Oxford, UK).

Induction of transient heat stress

Males aged 8–9 weeks were subjected to a single heat stress of 38, 40 or 42 °C for 30 min. Each animal was anaesthetised and the lower third of the body (hind legs, tail and scrotum) was submerged in a water bath. Control animals were anaesthetised and left at room temperature. After 30 min each animal was administered an anaesthetic reversal agent (Antisedan; Pharmacia and Upjohn), dried and returned to their cages.

Histological evaluation

Three hours to 28 days (d) after heat shock, animals were killed by cervical dislocation and both testes from each animal were weighed. One testis was immersion fixed in Bouin's for 8 h before being cut in half; tissues were stored in 70% (vol/vol) ethanol and processed into paraffin wax using standard procedures. Tissue sections (5 µm) were stained with haematoxylin and eosin; images were captured from an Olympus microscope BH2 under a ×40 lens using a video camera (Hitachi HV-C20).

Preparation of meiotic spreads

The testes were dissected into PBS then quickly moved into 200 µl RPMI media (Sigma), warmed to 32 °C where the tunica albuginea was removed using forceps and discarded along with any large blood vessels. The remaining tubules were finely chopped using two scalpel blades to form a milky suspension. This was diluted to a final volume of 3 ml RPMI. The resulting cell suspension was transferred to a 15 ml falcon tube where the tubular remnants were allowed to settle. The non-remnant fraction was transferred to a fresh tube and centrifuged at 115 *g* for 5 min. The pellet containing germ cells was resuspended in 2 ml warm RPMI.

Glass slides (BDH, Merck) previously boiled in dH₂O and air-dried were coated in five drops of 4.5% sucrose solution in dH₂O using a Pasteur pipette. A glass pipette was filled

with the cell suspension and one drop was dropped onto the slides from a height of 20–30 cm. Two drops of 0.05% Triton X-100 (in dH₂O) were added to each slide for 10 min at room temperature followed by eight drops of fixative (2% formaldehyde, 0.02% SDS, pH 8.0) per slide and incubated for 1 h in a humidified chamber. The slides were dipped briefly six times in dH₂O and allowed to air dry for 5 min before storing at –70 °C until use.

Immunohistochemistry of spread spermatocytes

The slides were defrosted by washing in PBS for 5 min and blocked with blocking buffer (5% goat serum, 0.15% BSA and 0.1% Tween-20) for 1 h at room temperature. The primary antibodies (anti-SCP3 mouse monoclonal (1:400; Abcam, Cambridge, UK) and anti- γ H2AX rabbit polyclonal (1:200; Upstate Biotechnology, Millipore UK Ltd, Watford, Herts, UK) were diluted in blocking buffer and incubated overnight in a humidified chamber at 4 °C. After three 5-min washes in PBS, the secondary antibodies (goat anti-mouse Alexa-546, goat anti-rabbit Alexa-488 both 1:500; Molecular Probes, Invitrogen) were applied and incubated for 1 h at room temperature. Following three further washes in PBS, slides were incubated with DAPI nuclear stain (Sigma) at 1:1000 in PBS for 10 min before two final PBS washes. Finally, the slides were mounted in Permafluor aqueous mounting medium (Beckman Coulter Ltd, High Wycombe, Bucks, UK).

For γ H2AX quantification, at least 50 pachytene nuclei were analysed from each mouse and the number of foci falling on the synaptonemal complexes counted. Microscope analysis was performed on Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK).

Mating study

The C57BL/6 males were each mated with three C57BL/6 virgin females aged ~10 weeks. Mice were allowed to mate for 5 days (23–28 days after heat/anaesthetic only) and checked for post-coital plugs each morning. If a plug was detected the female would be removed to a separate cage and noted as being at gestation day e0.5. Females were killed on e14.5 and litters assessed for numbers and resorptions.

A second group of matings was set up as above but females were killed on e3.5 and embryos flushed from the uterine horns with RPMI media using a syringe. Blastocysts were washed several times in 3 mg/ml polyvinylpyrrolidone/PBS before being fixed in 2% paraformaldehyde (PFA) for 20 min.

Immunostaining of flushed embryos

Embryos retrieved at e3.5 from females mated with heated or control males were fixed in 4% PFA for 20 min and permeabilised in 0.25% Triton X/PBS. Blastocysts were washed thoroughly in PBT (PBS, 0.05% Tween-20) and blocked in 2% goat serum (30 min) before being incubated with primary antibodies directed against POU5F1 (1:200; Santa Cruz Biotechnology, Heidelberg, Germany) and

TJP1 (1:200; Zymed, Invitrogen) for 1 h at room temperature. After thoroughly washing in PBT, the blastocysts were incubated with fluorescently labelled secondary antibodies (goat anti rabbit Alexa-546 and goat anti mouse Alexa-488). Blastocysts were washed again and incubated in DAPI nuclear stain before four final washes in PBT. Blastocysts were mounted in a droplet of PBS on a glass-bottomed Petri dish for immediate visualisation of immunostaining on a Zeiss LSM 510 Meta Axiovert 100M confocal microscope (Carl Zeiss Ltd).

In vitro fertilisation (IVF)

IVF was performed using both sperm from control males and males that had been heated to 42 °C either 16 h or 23 d previously. F1 (CBA male \times C57BL/1 female) females were superovulated by i.p. injection of 5IU pregnant mare serum gonadotrophin followed by human chorionic gonadotrophin (hCG) 48 h later. Oocyte-cumulus complexes were collected 16 h after hCG administration, washed and held in T6 medium (Quinn *et al.* 1982). Sperm were isolated from the cauda epididymis and vas deferens and allowed to capacitate in drops of T6 medium overlaid with silicon fluid for 2 h at 37 °C in 5% CO₂. Sperm ($1\text{--}2 \times 10^6$) were added to the oocytes held in a 0.5 ml drop of T6 medium and incubated for 4–5 h at 37 °C in 5% CO₂ to allow fertilisation to occur. Oocytes were then transferred to 200 μ l drops of KSOM medium (Devreker & Hardy 1997) overlaid with silicon fluid and incubated at 37 °C in 5% CO₂ to allow development to proceed. The following day 2-cell embryos were scored as a measure of fertilisation and then transferred to smaller drops (2 μ l per embryo) of KSOM and left in the incubator to develop further. The number of embryos developing to the 4-cell and blastocyst stages was recorded.

Recovery of spermatozoa for SCSA and sperm counts

Epididymes were placed in 500 μ l fresh Biggers, Whitten and Whittingham medium (BWW; Biggers *et al.* 1971). The tissue was thoroughly minced with fine scissors and incubated at 34 °C for 30 min to allow the motile spermatozoa to 'swim up'. The motile spermatozoa fraction was removed leaving debris behind and made up to 1 ml in BWW. Aliquots were made for the SCSA (see below). For sperm counts, the sample was diluted in 4% PFA and counted using a haemocytometer. Two counts were made for each sample and the mean taken.

Sperm chromatin structure assay

A modified version of the SCSA method described by Evenson *et al.* (1999) was used. Comparison with other DNA strand break assays (TUNEL and COMET) has shown that the DNA denaturation measured in the SCSA is largely due to DNA strand breaks (Gorczyca *et al.* 1993, Chohan *et al.* 2004). The sperm samples in BWW were adjusted to a concentration of $1\text{--}2 \times 10^6$ cells/ml with TNE (0.15 M NaCl, 0.1 M Tris, 1 mM EDTA pH 7.4); 100 μ l of this sample was mixed with 200 μ l acid detergent solution (0.1% Triton X-100, 0.15 M NaCl,

0.08 M HCl). After 30 s, 600 µl acridine orange (AO; Sigma) diluted in AO staining solution (37 mM citric acid, 126 mM Na₂HPO₄, 1 mM EDTA, 0.15 M NaCl, pH 7.4 with AO added fresh to a final concentration of 6 µg/ml) was added to the sperm/ detergent mix, mixed again and incubated for 3 min at room temperature before analysing using a fluorescence assisted cell sorting machine.

The sperm samples were analysed on a Coulter Epics XL Flow cytometer (Beckman Coulter Ltd) with a 480 nm excitation laser. Green fluorescence (from double-stranded DNA) was detected using a bandpass filter (530 nm ±15) and red fluorescence (single-stranded DNA) using a longpass filter (>650 nm). The cytometer 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. Events (sperm cells) of up to 10 000 were read for each sample. Raw data were analysed using Flowjo Software (Tree Star Inc., Ashland, OR, USA). Background contamination (cells other than spermatozoa) was removed by gating. The extent of denaturation of the sperm DNA was determined by calculating the DNA fragmentation index (DFI), which is based on the ratio of denatured spermatozoa DNA (red) to total spermatozoa DNA (red/(red+green)) fluorescence, expressed as the percent of abnormal sperm with denatured DNA (%DFI).

Statistical analysis

Results are expressed as means and standard errors of the mean were analysed using one-way ANOVA followed by the Bonferroni *post hoc* test, using GraphPad Prism version 4 (Graph Pad Software Inc., San Diego, CA, USA).

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