The effects of heat on the testes of mammals[1]

B.P. Setchell[2]

Discipline of Anatomical Sciences, University of Adelaide, Adelaide 5005, Australia.

Abstract

Advances since my last review in 1998 on our understanding of the effects of heat on the testis are summarized. Techniques used to study these effects have included exposing the whole animal to a hot environment, insulation of the whole scrotum or just its neck, immersing the testes in a warm water bath, surgically returning one or both testes to the abdominal cavity (induced cryptorchidism) or exposing them to microwave radiation. Most techniques have been shown to affect principally primary spermatocytes and early spermatids, but there is some evidence for effects on spermatogonia and Sertoli cells. The Leydig cells and androgen secretion do not appear to be directly affected.

There is considerable variation in susceptibility between individual animals and between different strains, which appears to be due to differences in the ability of the animals to maintain testis temperature as well as in inherent susceptibility of the different germ cells. The mechanism of cell death appears to be apoptosis, not necrosis, and may involve reactive oxygen species (ROS), the tumour suppressor protein p53, nitric oxide synthase (NOS), the translocation of the proapototic factor Bax from the cytoplasm to a perinuclear position, the release of cytochrome c from mitochondria and several caspases. Animals deficient in Fas and Fas ligand appear still to be susceptible, making it unlikely that these factors are involved.

Although many authors believe that the effects of heat are fully reversible, there is now evidence of long-term effects, following either locally applied heat or temporary induced cryptorchidism. These long-term effects appear to be different from those seen following irradiation, and may indicate a failure of Sertoli cell function. Sperm produced by mice which had been exposed to a hot environment bind to ova normally but are less able to fertilize in vivo and in vitro, even when motile sperm are selected by a swim-up procedure, and many of the resultant embryos do not develop normally. Blocking caspase activity or release of cytochrome c reduced the effect of heat. Mice lacking iNOS were more resistant to the effects of local testicular heating, and inhibitors of xanthine oxidase (an enzyme important in the production of ROS) reduced the effects of making the testis cryptorchid, while mice in which the gene for superoxide dismutase (SOD1, which is involved in the breakdown of ROS) had been knocked out were more sensitive. Treatment of rats with pregnant mare serum gonadotropin, thyroxin or low doses of testosterone increased the time at which infertility began following testicular heating. Treatment with vitamin E or PBN (N-tert-butyl-α–phenylnitrone, a free radical spin trap but also a possible source of NO) decreased the effects of local testicular heating on testis weight, while ascorbic acid had no effect; in contrast treatment with PBN increased the effect of temporary induced cryptorchidism. The most surprising finding was that induced cryptorchidism actually improved spermatogenesis in juvenile spermatogonial depletion (jsd) mice and to some extent in previously irradiated mice and rats treated with hexanedione.

Keywords: testis; heat effects.

Introduction

The literature on the effects of heat on the testis has already been recently reviewed (Kandeel and Swedloff, 1988, Setchell, 1998, Morgenthaler et al., 1999) but as a great deal of new information has been obtained since 2000, an update would seem to be justified. The testes of many species of mammals descend either during fetal life or shortly after birth into a scrotum, where the temperature is appreciably lower than in the abdomen. However, in some mammals, notably elephants, hyraxes, edentates, insectivores and cetaceans, the testes remain inside the abdomen (see Setchell and Breed, 2006), although in dolphins, the testes appear to be cooled by venous blood from the tail running into the abdominal cavity and next to the arterial supply for the testes (Rommel et al., 1992; 1994). In bulls, as in other animals with scrotal testes, the testes are cooled by countercurrent exchange of heat between the venous and arterial blood in the spermatic cord (Waites and Moule, 1961; Brito et al., 2004). Heat is lost from the testis and scrotum to the environment through the scrotal skin, which is well endowed with sweat glands (see Setchell and Breed 2006). The temperature on the surface of the scrotum is lower at its base than near the neck, but the temperature inside the testis is almost uniform, even slightly warmer at the base (Kastelic et al., 1996; 1997). In rams, the tunica dartos muscle is involved in moving the testes closer to the body by contracting under cold conditions and further by relaxing when hot. The body-scrotal temperature difference is greater in an environment of 6°C and less at 40°C, although during fever induced by lipopolysaccharide injection or during exercise, scrotal temperature remains constant while body temperature

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[2]Corresponding author: brian.setchell@adelaide.edu.au
Phone: +61(8) 8303 5965; Fax: +61(8) 8303 4398

rises (Maloney and Mitchell, 1996). Scrotal surface temperature in stallions is increased during exercise, particularly if the animal is fitted with a scrotal suspensory (Staempfli *et al*., 2005).

Techniques

The techniques for studying the effects of heat on scrotal testes are varied and include: (1) exposure of the whole animal to a hot environment; (2) intermittent (Mieusset *et al*., 1992; Arman *et al*., 2006) or continuous (Vogler *et al*., 1991; 1993; Fleming *et al*, 2004; Walters *et al*., 2005a; b; 2006) insulation of the scrotum, or just the neck of the scrotum (Kastelic *et al*., 1996); (3) surgically returning one or both testes to the abdomen, referred to here as induced cryptorchidism (Yin *et al*., 1997; 1998; 2002; Mu *et al*., 2000; Setchell and Wahab-Wahlgren, 2001; Zhang *et al*., 2002; 2004; Vigodner *et al*., 2003; Setchell and de Rooij, 2006); (4) immersing the scrotum in a warm water bath (Lue *et al*, 1999; 2000; 2002; Lee *et al*. 1999; Rockett *et al*., 2001; Setchell *et al*., 2001; 2002; Zhang *et al*., 2005); (5) exposing the testes to microwave radiation (Imig *et al*., 1948; Gunn *et al*., 1961; Saunders and Kowalczuk, 1981; Saunders *et al*., 1983; Kowalczuk *et al*, 1983). The results using this last technique were not included in my previous review, as it was thought then that the effects may not have be just due to the increase in testicular temperature, which rose to about 41°C during a 5 minute exposure and to 44°C during a 10 minute exposure, then cooled to 36°C over the next 6 and 15 minutes respectively. Damage to scrotal skin was also reported (Gunn *et al*., 1961) and this may have reduced the ability of the animal subsequently to keep its testes cool. Later work has shown that exposure to the microwave emission from a mobile phone had negligible effects on the testis, although rectal temperature was increased slightly (Dasdag *et al*., 1999; 2003).
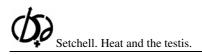
Systemic effects

It must be remembered that heating the scrotum may have systemic effects as well as direct effects on the testes. It has been known for some years that heating the scrotum in sheep increased respiration rate even to the extent of cooling the rest of the body including the hypothalamus, so that the animal began to shiver as soon as the scrotal heating ceased (Waites, 1962). More recent studies have shown that panting and reduction in body temperature following scrotal heating occurred in rams with a fever as well as under normal conditions (Maloney *et al*., 2003).

Cells in testis affected by heat

From histological studies, it has been concluded that pachytene spermatocytes and early spermatids are the cells in the testis which are most susceptible to heat (see Setchell 1998 for earlier references). These findings have since been confirmed using flow cytometry and confocal microscopy of isolated seminiferous tubules after making the testis cryptorchid in hamsters (Vigodner *et al*., 2003). However, even when these cells are not killed, there is now evidence that some of them may complete their development, but appeared as spermatozoa with damaged DNA, as revealed by COMET and sperm chromatin structure analyses (SCSA, Karabinus *et al*., 1997; Sailer *et al*., 1997; Banks *et al*., 2005). In bulls, these effects are most obvious during the period 12 to 21 days after a 48 hour scrotal insulation, so the affected sperm would have been from cells still inside the testis at the time of heating; however, some changes are seen between 3 and 9 days post-heating, indicating some sensitivity of sperm already in the epididymis (Karabinus *et al*., 1997). Likewise in mice, sperm from the cauda epididymidis show maximal changes between 10 and 14 days after heating, again these cells would have been still in the testis at the time of heating. There were however some changes at 3 days, and these cells again would have already been in the epididymis when heated (Sailer *et al*., 1997). Even clearer indications of an epididymal effect were seen by Banks *et al*. (2005), who showed that while COMET changes were apparent in caudal mouse sperm at 21, 24, 28 and 32 days post-heat, even greater rises were found between 1 and 24 hours, with normal values again at 7 and 14 days post heat, showing that sperm in the epididymis were affected, as well as cells in the testis. A similar pattern was seen with SCSA assays, which were elevated between 1 and 6 hours and between 14 and 32 days post-heat, but normal at 24 hours and 7 days. There was about a 30% reduction in the percentage of ram sperm immunoreactive for the post-meiotically expressed sperm surface protein PH20 between 17 and 31 days after 24 h scrotal insulation, although there were minimal effects on the distribution of the activity on the sperm head (Fleming *et al*., 2004). In a follow-up to this study, SCSA assays showed an increase in the proportion of sperm with DNA damage after either 24 or 48 scrotal insulation, but there was also a spectral shift in the total population of cells staining in the semen, possibly indicating an increase in immature cells (JF Smith and RM McDonald, personal communication). A similar shift was also seen in human sperm collected after a 24h fever (Evenson *et al*., 2000). Even ejaculated human sperm with poor capacitation characteristics contained fragile DNA after heat treatment (40°C for 4 hours) *in vitro* (Mann *et al*., 2002).

There is also some evidence that Sertoli cells may be affected, in terms of decreased secretion of androgen binding protein (Hagenas and Ritzen, 1976; Karpe *et al*., 1981) and expression of intermediate filaments in Sertoli cells is disrupted in cryptorchid

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monkeys (Zhang *et al*., 2004). Dedifferentiation of adult Sertoli cells has been induced in monkeys by heat treatment (Zhang *et al*., 2006b)

The boundary tissue surrounding the seminiferous tubules may also be affected (Kanwar *et al*., 1974), but it is not clear if this is a direct effect of the heat or a consequence of the disrupted spermatogenesis as the changes were apparent only after 7 days. Insulation of the whole scrotum or just the scrotal neck of *Bos indicus* bulls for 4 days was sufficient to produce falls in sperm production and motility without affecting the echotexture of the testis (Brito *et al*., 2003). The sensitivity of cells in the testis other than pachytene spermatocytes and early spermatids is apparent from the results of experiments in which testis weight was reduced after heating even when spermatogenesis had been arrested by treatment of rats with a GnRH agonist and an antiandrogen (Setchell *et al*., 2002). Spermatogonia are generally believed to be unaffected, but there is some evidence that their susceptibility to radiation is increased by heat treatment 30 minutes previously (Reid *et al*., 1981). Furthermore, the numbers of A spermatogonia are drastically reduced in rabbits made cryptorchid for 13 weeks but recovered partially 7 weeks after orchidopexy (Zhang *et al*., 2002)

In induced cryptorchid mouse testes, differentiation of A-spermatogonia was blocked, but could be restored if the testis was returned to the scrotum (Nishimune *et al*., 1978; Nishimune and Haneji, 1981) or if tubule fragments from cryptorchid testes were cultured at scrotal temperature (32.5°C, Nishimune and Komatsu, 1972; Nishumune and Aizawa, 1978, Aizawa and Nishimune, 1979; Haneji and Nishimune, 1982).
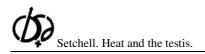
It has been generally accepted that the effects of heat on the testis are fully reversible, but some recent studies suggest that this is not so. In a number of studies reviewed previously, testis weight had not returned to control values even 60 days after a single heat exposure (Setchell, 1998). Rats, about 50 days old at the start of the experiment, were followed for about 6 months after a single exposure of their testes for 30 minutes to 43°C. There was an initial decline in testis weight and sperm numbers but then they showed a return of testis weight and sperm numbers to about 70 and 50% respectively of control after 97 days, followed by a second fall to about 50 and 5% at 182 days. This was accompanied by a decrease in the percentage of histologically normal tubules cross-sections from 51% to 6% (Setchell *et al*., 2001). In a second experiment, testis weight was still only 70% of control at 105 days after a single exposure of the testes to heat (Setchell *et al*, 2002). Making one rat testis cryptorchid for 48 hours reduces testis weight to about 60% of control after 21 days (Setchell and Wahab-Wahlgren, 2001), compared with 35% for a single 30 minute 43 °C exposure (Galil and Setchell, 1988), but the testes after both treatments remained at about the same size for up to 185 days. The percentage

of normal tubules also remained low, at about 20 % for the heated and 70% for the previously induced cryptorchid testes (Setchell and de Rooij, 2006). However, there is an important difference between the damage caused by heat and by irradiation; the latter is thought to result from an arrest of spermatogonial recruitment from A_{al} to A_1 (Porter *et al*., 2006). Of the affected tubules in the previously induced cryptorchid testes, about 35% contained no germ cells, and in the others, the most advanced cell types were A spermatogonia (35%), B gonia or preleptotene spermatocytes (19%), leptotene or zygotene spermatocytes (4%), pachytene spermatocytes (9%) or round spermatids (3%); the corresponding values for the heated testes were 22, 26, 33, 7, 12 and 1% respectively (Setchell and de Rooij, 2006). Similarly, in adult rabbits made cryptorchid for 13 weeks, the numbers of B spermatogonia, the various classes of spermatocytes and round spermatids were still below normal 7 weeks after orchidopexy, with still no elongated spermatids, although there had been a substantial rise in the numbers of A spermatogonia at this time (Zhang *et al*., 2002). These findings suggest that there are local factors, perhaps originating from the Sertoli cells, which allow spermatogenesis to proceed in some areas, but which are deficient in other sites where proliferation of the spermatogonia is inhibited, but not recruitment from A_{al} gonia.

Individual and strain variation

There is considerable variation between individual animals in their response to heat exposure. Of the six bulls subjected to scrotal insulation by Vogler *et al*., (1991; 1993), two showed a large increase in abnormal spermatozoa (to more than 60%) whereas others had as few as 23% abnormal cells. Likewise, 4 bulls used for semen collection for in vitro fertilization showed widely variable effects of 48h scrotal insulation on pronuclear formation, embryo development and apoptosis, with two bulls classed as severe responders, one a moderate responder and one showing no response to scrotal insulation (Walters *et al*., 2005a;b; 2006). There was also considerable individual variation in the percentage of PH-20 positive sperm between 4 rams following scrotal insulation for 24h (Fleming *et al*., 2004). In a study on the effects of intermittent scrotal insulation in rams, percent motile sperm at 21 days after the start of insulation varied from 9 to 35% in 4 animals. In a second study, two rams were subjected to intermittent scrotal insulation in two successive years; one showed a severe reduction in percent motile sperm on both occasions, while the other was much less severely affected, even though the temperatures produced in their testes were similar (Arman *et al*., 2006).

Bos indicus bulls are less sensitive to the effects of high temperatures than *Bos taurus* or crossbred bulls, but as they are actually more sensitive

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to the effects of scrotal insulation (Brito *et al*, 2003); this would appear to be due to the greater ability of *indicus* animals to keep their testes cool (Brito *et al*, 2002). *Bos indicus* bulls have greater testicular artery length to testicular volume ratios, and smaller testicular artery wall thickness and arterial to venous distances, which may be responsible for greater cooling of the arterial blood in the spermatic cord (Brito *et al*., 2004).
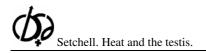
Differing capacity to keep their testes cool was also found in mice of two strains, one of which had been kept for many generations at 33 ^{o}C and bred successfully under these conditions, but their testes remained just as sensitive to the effects of direct heating (van Zelst *et al*., 1995). Mice of the MRL/MpJ and AKR strains are much more sensitive to the effects of cryptorchidism than those of A/J, BALB/c, C3H/He or C57BL/6 strain (Kon and Endoh, 2001; Kazusa *et al*, 2004; Kon, 2005; Namiki *et al*, 2005), but these authors did not present any evidence on the testicular temperatures achieved.

There is also some evidence for the development of thermotolerance in the testes of mice (Marigold *et al*, 1985) and rats (Shilkina, 1976). However, repeated exposure of rat testes to heat produced a progressive decrease in testis size and an increase in the percentage of severely damaged tubules (Bowler, 1972), which was attributed to an effect on spermatogonia.

Mechanism of cellular damage caused by heat

It was originally claimed that cell death following heating was not apoptosis but necrosis, although this was not accompanied by inflammation (Allan *et al*., 1987). However, subsequent studies have shown that apoptosis was involved (Shikone *et al*., 1994; Ohta *et al*, 1996; Yin *et al*, 1997; Lue *et al*., 1999; 2000; 2002; Rockett *et al*., 2001; Sinha Hikim *et al*., 2003a; b). Isolated cells from 40 day-old rats also showed apoptosis when the culture temperature was raised from 32.5^{o}C to 43°C for one hour, followed by a return to 32.5^{o}C for another 24h. Similar apoptosis could be induced in these cells by generating reactive oxygen species with xanthine and xanthine oxidase, and the effects of heat were reduced in the presence of catalase. A temperature-dependent increase in intracellular peroxide production by testicular cells was detected using the fluorescent probe DCFH/DA (Ikeda *et al*., 1999). Xanthine oxidase inhibitors suppress apoptosis induced by experimental cryptorchidism (Kumagai *et al*., 2002). It is also relevant that mice in which superoxide dismutase had been knocked out were more sensitive to the effects of induced cryptorchidism (Ishii *et al*., 2005).

The tumour suppressor protein p53 also appears to be involved. Mice deficient in this protein show a decreased response to induced cryptorchidism, although the fall in testis weight is only delayed, not eliminated (Yin *et al*., 1998). In the testis, p53 is confined to primary spermatocytes (Almon *et al*, 1993; Schwartz *et al*, 1993) and in cryptorchid monkey testes, p53 is thought to repress the expression of the orphan receptor TR2, which is localized in germ cells (Mu *et al*., 2000). However, the earliest observations in this study were made only after one month, when germ cell loss would have probably been quite extensive. Furthermore, following heating of monkey testes, p53 RNA is elevated 3 days later, but there is no change in TR2, while TR3 and TR4 are depressed (Zhang *et al*., 2006a). A germ cell-specific heat shock protein hsp 105 (Itoh and Tashima 1990; 1991) translocates from cytoplasm to nucleus within 2 days of the testis being moved to the abdominal cavity in rats and hsp105 binds to p53 in cultured testis cells at scrotal temperature (32.5^{o}C) but not at 37 or 42°C (Kumagai *et al*., 2000). Hsp105 mRNA and protein in spermatids fell within 3 days and rose again by day 30 after two exposures of monkey testes to 43 ^{o}C for 30 minutes, while hsp60 in Sertoli cells and spermatogonia rose about 5-fold over the same period. It was concluded that hsp105 and its complex with p53 might be involved in cell cycle arrest or the induction of apoptosis (Zhang *et al*., 2005). Other heat shock proteins (hsp 27 and 90, which are found in Sertoli cells, spermatogonia, spermatocytes and spermatids), also increase and move from cytoplasm to nucleus in isolated testis cells from mice when the temperature is increased from 37 to 42 ^{o}C (Biggiogera *et al*., 1996) although these authors seem to believe that the mouse testes are normally abdominal! Hsp 70-2, which is uniquely expressed in meiotic phase spermatocytes (Dix 1997; Dix *et al*., 1996; 1997) is not induced by increases in temperature, but an increase in the mRNAs for the related proteins hsp 70-1 and 70-3 could be detected within 4 hours and the proteins themselves were increased within 16 hours after exposure of mouse testes for 20 min at 43 ^{o}C, predominantly in spermatocytes (Rockett *et al*., 2001). These authors also employed DNA microarrays to measure expression of genes in the testes from heat-shocked mice. Of the 2208 genes studied, 27 were upregulated and 151 downregulated. Among those upregulated were genes involved in stress responses, DNA repair and cell adhesion, while those downregulated included genes involved in cell cycle regulation, both in mitosis and meiosis, protein folding, DNA repair, stress responses and apoptosis, giving an overall pattern of cellular shutdown. In particular, the meiotic cell cycle of the spermatocytes appears to be arrested at the G2/M transition, as indicated by changes in the G2/M-specific cyclins, A1, B1 and B2 and the M-phase inducer phosphatase CDC251 genes (Rockett *et al*., 2001). The activity of DNA polymerases α and β was decreased in induced cryptorchid rat testes, while DNA polymerase γ and topoisomerase did not change (Fujisawa *et al*., 1988). There is also cleavage of poly(ADP)ribose polymerase (Sinha Hikim *et al*.,

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2003a; b; Vera *et al*., 2005).

Apoptosis 4 or 6 h following heating of the mouse testis is greater if the clusterin/ApoJ gene is knocked out, but similar to wild type after 12 h and less at 24h, while testis weight falls earlier (48h vs 72h post-heat) in wild type than knockout mice, suggesting that clusterin delays the initial kinetics of the heat-stress pathway, but clears the damaged cells more quickly (Bailey *et al*, 2002).

It has been suggested that Fas is responsible for the second phase of apoptosis beginning about 10 days after the testis is made cryptorchid (Yin *et al*., 2002). Increased expression of Fas, but not Fas ligand RNA can be detected following heat exposure of rat testes (Lee *et al*., 1999). Fas expression in germ cells was increased following induced cryptorchidism, but testis weight was still decreased in Fas-deficient *lpr/lpr* mice, although not to the same extent as in BALB/c mice (Ogi *et al*., 1998). However, in Fas ligand-defective *gld* mice, heat-induced apoptosis is not blocked, casting more doubt on the involvement of the Fas system in this process (Sinha Hikim *et al*., 2003a; b; Vera *et al*., 2004). The pro-apoptotic factor Bax is translocated from a cytoplasmic to a paranuclear localization within half an hour of heating the testis, while total Bax levels in the testis remained constant. The relocation of Bax is accompanied by cytosolic translocation of cytochrome c and is associated with activation of the initiator caspase 9 and the executor caspases 3, 6 and 7 (Yamamoto et al., 2000; Vera et al., 2004). However, Rockett et al. (2001) detected a reduction in Bax expression following heating.

Inhibitors of caspases reduced apoptosis, independently of the cytosolic translocation of cytochrome c, and protection was also provided by minocycline, a second generation tetracycline that effectively inhibits cytochrome c release, and in turn caspase activation (Matsuki *et al*., 2003;Vera *et al*., 2005). Ectopic expression of testis-specific calpastatin (a naturally occurring inhibitor of calpain, a protease involved in apoptosis) in spermatocytes reduces apoptosis in heated mouse testes (Somwaru *et al*., 2004).
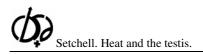
Nitric oxide synthase (NOS) appears to have a functional role in heat-induced apoptosis since in adult inducible NOS (iNOS) deficient mice, the testes were about one-third larger than normal, and furthermore showed much less apoptosis in early (I to IV) and late (XI to XII) stages of spermatogenesis following exposure of the testes to 43°C for 15 minutes (Lue *et al*., 2003). Endothelial NOS (eNOS) is increased in rat testes made cryptorchid between 20 and 34 days of age, and examined at 60 days old, and appears in the degenerating cells as well in its normal location in endothelial, Sertoli and Leydig cells (Zini *et al*., 1999). In Hoxa11 knockout mice, which have congenitally cryptorchid testes, treatment with L-NAME (an inhibitor of NOS) reduces the apoptosis and improves spermatogenesis (DeFoor *et al*., 2004). Overexpression of eNOS in transgenic mice accelerates germ cell

apoptosis induced by experimental cryptorchidism (Ishikawa *et al*., 2005).

Male-mediated effects induced by heat on fertility and embryonic development

There were a number of suggestions in the earlier literature that males exposed to heat can produce sperm which do not produce normal offspring in unexposed females (see Setchell, 1998). These findings have been extended in two further studies involving male mice exposed in a microclimate chamber to 36°C for 24 hours (Zhu and Setchell, 2004; Zhu *et al*., 2004) or 35 ^{o}C for two periods of 12 hours on consecutive days (Yaeram *et al*., 2006). In the former, there were no changes in the proportion of eggs showing two polar bodies as an indication of fertilization 14 to 16 hours after mating when this took place with oestrus-synchronized females on days 7 or 35 days after heating, although there was a fall at 21 days. However, the proportion of zygotes progressing to 2-cell stage by 34 to 39 h after mating was reduced when mating occurred 21 or 35 days after heating, and likewise, the proportion progressing to 4-cell or morulae by 61 to 65 hours was reduced when mating occurred 7 or 21 days after heating. The proportion reaching blastocyst stage by 85 to 90 hours after mating was reduced when mating occurred 7 or 21 days after heating, and the proportion of expanded blastocysts was reduced by mating on day 35. The proportion of abnormal embryos at 61 to 65 hours and 85 to 90 hours was increased with mating at 21 days post-heat (Zhu and Setchell, 2004). When embryos were collected 25 to 28 h after hCG from mated superovulated females and cultured *in vitro,* development to 2-cell stage after 24h of culture was normal with mating on day 3 or day 42, but reduced for days 7, 14, 21, 28 and 35; the 4-cell or morulae after 48h culture, the 8-cell or blastocysts after 72h, and blastocysts after 96 or 120 h culture were reduced with mating on the same days post-heat. The percentage of abnormal embryos was increased after 48, 72, or 96 h of culture with mating on days 14 to 35, and the proportion of degenerating embryos after 72, 96 or 120 h culture was increased with mating on days 7 to 28, 7 to 35 and 3 to 35 post-heat respectively (Zhu *et al*., 2004).

In the second study, the proportion of ova reaching 2-cell stage by 24h after mating with superovulated females had fallen slightly when mating occurred7 days post-heat and appreciably with mating after 10 or 14 days. Using *in vitro* fertilization, it was found that the same number of motile sperm from the epididymides of males heated 7, 10 or 14 days earlier were less effective in terms of the proportion of ova fertilized, with no effect 3 days after heating. Even when motile sperm were separated from immotile sperm in the sample by a swim-up procedure, a similar result was obtained. This was not due to a reduction in the number of sperm binding to the zona pellucida, but

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there were reductions in the proportion of ova with sperm in the perivitelline space and in the cytoplasm of the eggs (Yaeram *et al*., 2006).

In another study, bulls were subjected to scrotal insulation for 48 hours and semen collected and cryopreserved 2 or 3 weeks later. Following IVF with swum-up sperm from these samples, there were decreased rates of sperm penetration, pronuclear formation (Walters *et al*., 2006), embryo cleavage, development and blastocyst formation (Walters *et al*., 2005a) with semen collected from two of the bulls three weeks after the insulation, but not with semen from two other bulls, or with semen collected after two weeks. The same two bulls produced embryos with increased caspase activity after 8 days in culture, but there was no effect on apoptosis, as judged by percentage of cells positive in the TUNEL procedure (Walters *et al*., 2005a).

Further work is needed to determine whether these effects are due to changes in the DNA in the sperm or to a change in other factors, such as the cytosolic phospholipase C introduced into the egg at the time of fertilization (see Saunders *et al*., 2002).

Factors affecting sensitivity of testes to heat

The results of Elfving (1950) have already been summarised in my earlier review, but no-one seems to have followed up his observations that the period of infertility following local heating of the testes of rats can be delayed from about 10 days post-heat to more than 20 days by treating the animals with FSH-like gonadotropin or thyroxin, or with a low dose of testosterone; a higher dose of testosterone was without effect, while no treatments affected the time at which fertility returned at between 60 and 85 days after heating. The beneficial effects of caspase inhibitors and drugs which inhibit the release of cytochrome c have been mentioned above.

The effects of antioxidants and the free radical spin trapping agent PBN (α-phenyl-N-t-butylnitrone) on the testes were studied by treating rats before and during local heating of the testes or making one testis cryptorchid for 48 hours (Setchell and Wahab-Wahlgren, unpublished results). Antioxidants have been shown to reduce the damage to DNA in infertile patients (Kodama *et al*., 1997) and PBN reduces the free radical oxidative damage to DNA in thalidomide toxicity (Parman *et al*., 1999). Vitamin E and PBN significantly reduced the effect on the weight of the testes of heating 21 days earlier, while the effect if Vitamin C was not significant. In unilaterally induced cryptorchid rats, Vitamin E was ineffective, whereas PBN caused a greater fall in testis weight than in the controls (Table 1). This may have been because under hyperthermic conditions, PBN releases nitric oxide (Cui *et al*., 2006), which may have contributed to the apoptosis caused by the abdominal temperature.

Treatment of rats with adrenaline enhances the effect of heating the testes (Murashev, 1984), presumably by reducing testicular blood flow (see Setchell and Breed, 2006).

Table 1. The effects of antioxidants on the loss of weight of rat testes 21 days after local heating to 42°C for 20 minutes or of making one testis cryptorchid for 48 hours. Values are means $\pm$ SEM with numbers of rats in parentheses.

Treatment	Testis weight (mg)
Control unheated	1557 $\pm$ 38 (5)
Control heated	1143 $\pm$ 23 (8)###
Vitamin E heated	1239 $\pm$ 30 (8)**
PBN heated	1211 $\pm$ 28 (8)*
Vitamin C heated	1198 $\pm$ 35 (14)
	Testis weight as fraction of scrotal testis of same animal
Cryptorchid control	0.606 $\pm$ 0.016 (8)###
Cryptorchid + Vitamin E	0.599 $\pm$ 0.043 (8)
Cryptorchid + PBN	0.519 $\pm$ 0.015 (8)***

Doses used were 200 mg Vitamin E S/C before heating or at time of cryptorchidectomy (0900h), then every 12 hours, 16 mg PBN I/V before heating or I/P at the same times at Vitamin E in the cryptorchid rats and 200 mg Vitamin C intravenously at start and 10mg/min during testis heating.
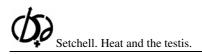*, **, ***: P< 0.05, 0.01 or 0.001 different from heated or cryptorchid controls;
###: P< 0.001 different from control unheated or contralateral scrotal testis.

Interaction between heat and other effects on the testis

Low doses of testosterone suppress spermatogenesis in rat, (Lue *et al*., 1999) and monkeys (Lue *et al*., 2006), paradoxically by suppressing LH secretion and thus reducing the concentration of testosterone in the testis. The detailed effects on spermatogenesis are different from those after heat, and it is interesting that a combination of the two treatments has a greater effects that either alone, leading to the concept of a "Two-hit" approach to male contraception.

Induced cryptorchid testes in rats are less affected than scrotal testes by treatment of the animals

with 2,5-hexanedione, but this appears to be due to a reduction in the formation in the testes of pyrroles, a required intermediate step in the hexanedione-induced injury (Boekelheide *et al.*, 2000). However, the most surprising finding is that spermatogonial differentiation arrest in juvenile spermatogonial depletion (jsd) mice is virtually eliminated by making the testes cryptorchid, whether this was done in animals 4, 12 or 62 weeks old. A similar but smaller effect was seen in improving the tubule differentiation index in irradiated rats from 0.1% in scrotal testes to 2.1% in those made cryptorchid for 8 weeks, beginning 4 weeks after irradiation. It appears that spermatogonial differentiation may be sensitive to reduced temperatures or is inhibited by testosterone only at scrotal but not abdominal temperatures (Shetty and Weng, 2004).

Conclusions

Recent studies on the effects of heat on the testis, either applied directly, by insulating the scrotum or by making the testis cryptorchid have provided much new information on the mechanism of the damage caused to spermatogenesis. They also provide hope that a treatment may soon be devised which may protect the testes by blocking the apoptosis.

However, other recent evidence has been obtained that there may be much more long-term effects of heat than previously thought. Furthermore, the effects of heat may not be confined to cell death in the testis and the consequent fall in sperm numbers in semen, but the sperm produced may be less capable of fertilization and the production of normal embryos. In these times of global warming, these effects may have more serious consequences for both the human population as well as for domestic and wild animals.

Nevertheless, the main question still remains unanswered. Why do the testes of some, but not all mammals require a temperature lower than body temperature for normal spermatogenesis to proceed.

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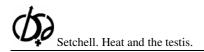
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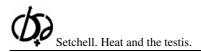
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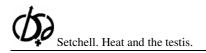
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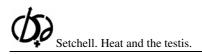
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