The Effect of whole body heating on testis morphology and fertility of male mice

By

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DECLARATION

This thesis contains no material that has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the text of the thesis. I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Jakrit Yaeram

ABBREVIATIONS

ad libitum	without restriction
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CASA	computer aided sperm analysis
CO_2	Carbon dioxide
COC	cumulus/oocytes clumps
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
FSH	follicle stimulating hormone
GVBD	germinal vesicle breakdown
hCG	human Chorionic Gonadotropin
HCL	Hydrochloric acid
Hepes	(*N*-2-hydroxy-ethylpiperazine-*N*-2-ethane sulphonic acid
HTF	human tubule fluid
HTM	Hamilton-Thorn motility analyzer
ICM	inner cell mass
IGF	insulin-like growth factor
IVF	in vitro fertilization
LF	lactoferrin
LH	luteinizing hormone
PMSG	pregnant mare serum gonadotropin
RNA	ribonucleic acid
ROS	reactive oxygen species
SOD	superoxide dismutase
TEM	transmission electron microscopy

TGF	transforming growth factor
VAP	path velocity
VCL	curvilinear velocity
VSL	straight line velocity
ZP	zona pellucida

SUMMARY

Heat stress has been known to be associated with reduced fertility in male animals in a number of species. In the natural situation the harmful effects of heat stress have been observed in cryptorchid animals including humans, animals exposed to summer heat and men whose occupations involve exposure to high temperature. Due to these observations, a number of experiments have been undertaken over the years to investigate the associations between heat stress and male fertility. However, most of the experiments conducted have involved severe heat stress, exposing the whole body of animals or only their testes to either high temperatures or long durations. The results have demonstrated clearly that this type of heat treatment can cause severe damage to the testis and subsequent low fertility or sterility.

The current study was focused on the effects of more subtle heat stress, which is much closer to the natural situation, and involved heating the animals intermitently for short periods of time at a range of temperatures which are lower than core body temperature. The objectives of this study were therefore, firstly to establish the levels of ambient temperatures that can harmfully affect the testis, and to then find out the minimum temperature that influences subsequent fertility, and finally to investigate the possible mechanisms involved.

Crossbred F1 mice (C57BL male x CBA female) were used throughout this study. In the preliminary investigation, the testes of mature male mice (aged between 80 and 110 days) were heated in a water bath at 42°C for 5, 10, 20 or 30 minutes. However, in all subsequent experiments the entire body of the male was heated in a heating cabinet at various combinations of temperature, heating duration and heating frequency. Four temperatures of 33°C, 34°C, 35°C and 36°C, were employed and at each temperature mice were heated

either once for 8h or 24h, for 2 consecutive days at 12h per day, or for 3 consecutive days at 8h per day. Five to six-week-old female mice were mated to the males in a series of fertility trials. Control males were not heated but were maintained in an air-conditioned room at 21°C.

Spermatogenesis was greatly influenced by the heat load from localized heating of the testis at 42°C for 20 or 30min, as indicated by the marked reductions in testis weight and the number of sperm heads per testis. Heating the whole body of male mice at 33°C or 34°C at any combination of time of exposure had little effect on the testis. Heating at 35°C significantly reduced testis and epididymis weights and the number of sperm heads per testis at all durations and frequencies of exposure, except for heating once for 8h. At 36°C, all parameters measured were significantly lower in the heated groups.

When normal females were mated at different days after males were heated at 35°C and their fertility was examined, it was found that heating for 24h continuously resulted in marked decreases in pregnancy rates and litter sizes with males used between days 14 and 28 after heating. These harmful effects on fertility when heated males were mated to normal females, however, were not clear when the 24-hour heating period was split into 12h per day for 2 days. The heating temperature was then increased by 1°C to 36°C. It was found that at this temperature even at 12+12 hours heating, heat stress had a marked impact on fertility. Pregnancy rates and litter sizes were significantly reduced when heated males were used between days 7 and 20 after heating.

Both in vivo and in vitro fertilization experiments showed that male mice whose whole body had been heated at 36°C for 2 consecutive days at 12h per day, produced spermatozoa with a significantly reduced capacity to fertilize eggs from normal females.

Additionally, fertilization rate was not improved following use of the swim-up method, even though the concentration of motile sperm was increased by this procedure.

Swim-up spermatozoa were used in subsequent experiments to test the acrosome status and the capacity of spermatozoa to bind to the zona pellucida of eggs. It was clearly shown in this study that spermatozoa from heated males still retained their capacity to bind to the zona. It was also found that most (88-92%) live spermatozoa, regardless of treatment, had intact acrosomes. A sperm-zona penetration assay was then conducted and this revealed that the zona penetration rate was substantially reduced with spermatozoa from heated males.

Light microscopic investigation of sperm morphology showed that heat-stress adversely affected spermatogenesis of male mice, as suggested by the large proportion of defective and immature spermatozoa produced. The effect of heat stress on spermatogenesis was confirmed by electron microscopic investigation, which showed an abnormal morphology of spermatids in the testis and also of spermatozoa in both the testis and epididymis of heated males. The abnormalities observed were curled-shape acrosome, broken or partly lost acrosome, deformed manchette and nucleus, expanded subacrosomal space, bent flagellum and retention of proximal cytoplasmic droplet.

The mechanism by which heat stress affects the ability of spermatozoa to penetrate the zona pellucida of the egg is not known. Since sperm-zona binding is not inhibited by heat stress, as applied in this study, the results presented in this thesis suggest that subtle heat effects otherwise induce damage to or changes in the sperm plasma membrane, which could contribute to low sperm-zona penetration, with zona-bound spermatozoa not responding to the signals released from the zona pellucida to undergo the acrosome

reaction. A number of possible molecular mechanisms are discussed that could contribute to such effects, and further investigations of these phenomena are warranted.

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1. General introduction

To function normally the mammalian testis requires a certain range of temperatures that are normally lower than core body temperature. Increased testicular temperature in mammals with a scrotal testis, including humans, is generally accepted to be associated with reduced fertility. Cryptorchidism, for example, a state where one or two testes fail to descend normally to the scrotum outside the body cavity, has long been known to cause a rise in testicular temperatures and subsequently to disrupt spermatogenesis (Clegg, 1963; Davis and Firlit, 1966; Moore, 1924; Steinberger, 1955) and lead to infertility (Moore and Chase, 1923). The exposure of scrotal mammals to high ambient temperatures during summer months has also been reported to adversely affect reproductive performance of a number of species in various regions of the world including Australia (Table 1.1).

Table 1.1. Heat stress effect on fertility of some domestic animals

	No. of service per conception		Percentages of Conception		Percentages of females giving birth		Weaning-to-estrous Interval (day)	
	Summer	Winter	Summer	Winter	Summer	Winter	Summer	Winter
Buffalos (India) Bahga and Gangwar(1989)	2.4	1.6	40	70	-	-	-	-
Cows (Arizona, USA) Ray *et al.*, (1992)	2.3	1.9	-	-	-	-	-	-
Pigs (SE-Australia) Love *et al.* (1995)	-	-	-	-	74.1	87.5	-	-
Pigs (USA) Armstrong *et al.* (1986)	-	-	-	-	-	-	9.3	3.8
Pigs Clark *et al.* (1986)	-	-	-	-	-	-	10.7	3.8
Cows (Italy) Alnimer *et al.* (2002)	-	-	-	-	56.3*	81.0*	-	-

* Pregnancy rate

The significance of heat stress to the testis in primarily and secondarily testicond mammals (eg. elephants and cetaceans, respectively) is not so well understood although the unique vascular anatomy in the dolphin, by which arterial blood to the testis is cooled by counter-current exchange with the cooler venous blood returning from the dorsal fin, suggests a physiological need also to regulate testis temperature in such species (Rommel et al, 1992).

In South Australia, where this study was undertaken, it is not unusual for the summer temperatures to reach above 40°C (Figure 1.1). This high ambient temperature can induce a rise in body and/or testicular temperature. Hence, it has the potential to damage testis function and subsequently reduce male reproductive efficiency.

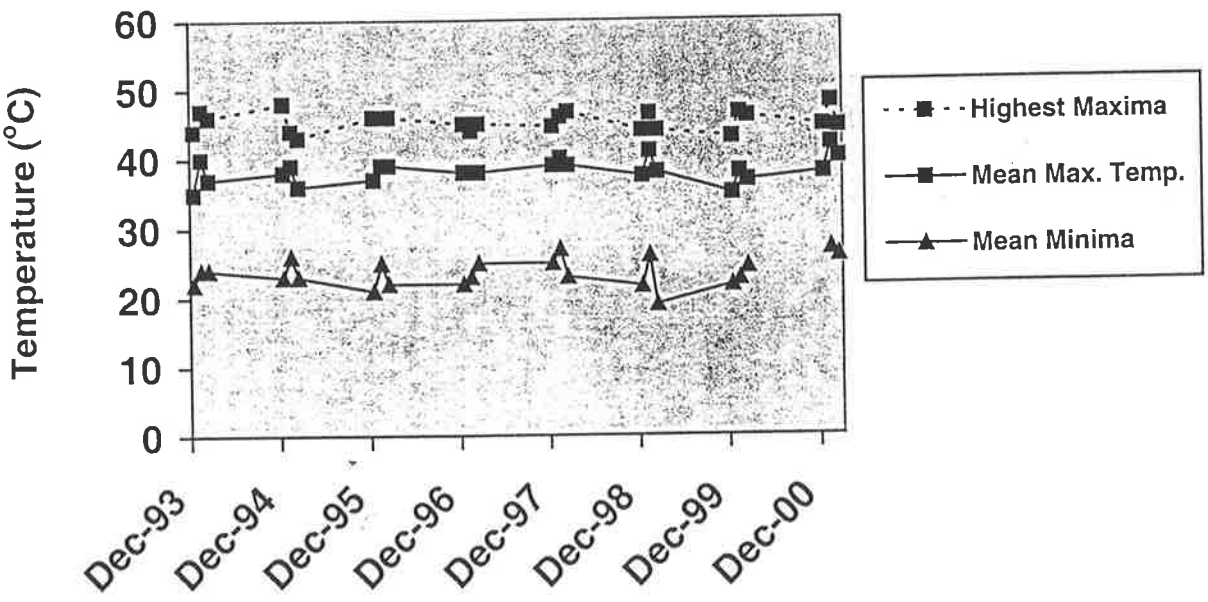


Figure 1.1. South Australian summer temperature in pastoral areas in the Northern region (Source: Monthly weather review, Bureau of Meteorology, South Australia).

At present, there are several situations that are commonly observed in modern domestic animal production that can lead to a rise in body and/or testicular temperatures. For example, animals (pigs and chicken) housed densely for the best use of modern and expensive housing, animals of European origin (pigs, cattle) raised in high ambient temperatures of tropical or sub-tropical areas, and animals, regardless of origins, exposed to extreme summer temperatures in the field (cattle and sheep) or kept in sheds during the summer months. The male animals are, as a consequence, more likely to reproduce poorly. In addition, it is possible that ambient temperatures will increase in the future due to global warming from the greenhouse effect. If this occurs, then it may have the potential to contribute to an adverse effect of heat stress on male reproduction.

The adverse effects of heat stress on male reproduction have long been recognized and have attracted a great deal of attention from animal scientists over several decades (refer to Anderson, 1945; Casady *et al.,* 1953; Dutt and Simpson, 1957; Pennycuik, 1967; Waites and Ortavant, 1967; Howarth, Jr, 1969; Bellve, 1972, 1973). In the early studies, it was doubtful whether heat-stress-related poor reproduction of the male was a result of the direct effect of heat. This is because in natural situations the entire body of the animal is heated, hence some other systems that may be involved in male reproduction (e.g. endocrine) can also be affected. In an attempt to resolve this question and to investigate if heat can directly adversely affect testis functions, heat treatment was applied to the testis locally without affecting other parts of the body. This was usually performed by one of three methods, namely the immersion of testes in warm water baths (Chowdhury and Steinberger, 1970; Galil and Setchell, 1988; van Zelst *et al.,* 1995; Setchell *et al.,* 1996, 1998; Sailer *et al.,* 1997), the use of scrotal insulation (Malmgren, 1990; Prabhakar *et al.,* 1990; Freidman *et al.,* 1991; Mieusset *et al.,* 1992; Karabinus *et al.,* 1997), and experimentally-induced cryptorchidism (Davis and Firlit, 1966; Setchell and Thorburn,

1970; Meistrich *et al.,* 1973; Sharpe, 1983; Nishiyama *et al.,* 1998). Heating the testis locally has been reported to effectively disrupt spermatogenesis (Reid *et al.,* 1981; Mieusset *et al.,* 1992; Hochereau-de Reviers, 1993) and subsequently reduce fertility (Setchell *et al.,* 1988; Mieusset *et al.,* 1992; Kirsten *et al.,* 1997) or even induce sterility (Sailer *et al.,* 1997).

It is now accepted that heat stress can cause fertility problems in animals through its direct effect on testis. However, inducing heat stress by applying heat directly to the testis is far from the normal situation. A number of experiments have been conducted using whole body heating, to investigate the effect of heating the entire body of male animals on their capacity to produce sperm and on their fertility. In the laboratory environment, large animals are normally exposed to heat in a hot chamber or room (Moule and Waites, 1963; Rathore, 1968, 1970; Meyerhoeffer *et al.,* 1985), while a hot cabinet is preferable for small animals (Burfening *et al.,* 1970; Bellve, 1972, 1973).

These experiments can be classified broadly into two groups, long period and short period heat exposure. Long exposure to heat at at least 35°C continuously for more than 24h effectively caused disruption of spermatogenesis and reduced fertility (Burfening *et al.,* 1970; Bellve, 1972; Garriott and Chrisman, 1981; Waldbieser and Chrisman, 1986). However, this application of continuous heat is somewhat unrealistic as in the real situation, the temperature usually cools down during the night time, hence the period of heat exposure is likely to be far less than 24 h per day. A short period heat exposure, on the other hand, is probably much closer to the natural situation. To be even closer, heat treatment should also be applied repeatedly for a few days (once per day) because during summer time heat is normally seen coming in as a "wave" over a period of a few days or more before subsiding.

Experiments involved in short period heat exposure can be classified further into two groups according to the level of heat used; repeated heating at a temperature above core body temperature, and repeated heating at a temperature below core body temperature.

While the former category produced a clear effect on male reproduction (Rathore, 1968, 1970), the latter produced mixed effects. It seems that the combination of the temperature used and the frequency of heating is a major factor determining the effect of heat on male reproduction. It is clear that a low frequency of heating requires a high temperature in order that adverse effects occur, while a high frequency of lower temperatures can be equally detrimental. The question remains as to the minimum temperature at any given frequency that will induce changes of testis function and subsequent fertility.

The present study involved a series of experiments designed to gather as much information as possible to address this. Firstly, a preliminary study employing local heating and a number of experiments using whole body heating with different temperatures, exposure durations and heating frequencies (Chapter 4) were undertaken to investigate and establish the effects of heat stress on the mouse testis. Then, the temperature-duration-frequency combinations clearly shown to have effects on testis were used to test aspects of male fertility (Chapter 5). Finally, factors likely to be responsible for male infertility (Chapter 6) were investigated. Possible mechanisms for the results obtained are summarized in Chapter 7.

CHAPTER 2.

REVIEW OF LITERATURE

Chapter 2. Review of literature

2.1 Gametogenesis

Spermatozoa of mature male animals and eggs of mature females are derived from primordial germ cells formed during fetal life. Primordial germ cells originate in the extragonadal tissues (Clark and Eddy, 1975; Eddy *et al.,* 1981; Byskov, 1982; Hogan *et al.,* 1986). In mice, they can be found in day-7.5 (presomite-stage) embryos in the wall of hindgut when they are in the yolk sac near the allantois (Ginsberg *et al.,* 1990). They subsequently migrate through different sites from the endodermal epithelium of the hindgut to the dorsal mesentery, the coelomic angles and finally arrive in the genital ridges. During the migration their number increases markedly due to the continuous mitotic activity. Upon arriving at the surface epithelium of the gonad they move into the cortex and together with supporting epithelial cells give rise to the cortical sex cords (Gondos, 1978; Jones, 1978). In a mouse embryo, primordial germ cells reach the genital ridges 10-11 days postcoitus. At this stage germ cells are called gonia. The gonia in the developing gonads then undergo a high rate of mitosis to form a large number of cells (De Felici and McLaren, 1982). After gonadal sex differentiation male gonia are termed spermatogonia and female gonia are called oogonia. In a male embryo, after a high rate of mitotic division germ cells arrest as T-prospermatogonia (McLaren, 1984) and will not enter meiosis until at least a week after birth. In a female embryo, on the other hand, germ cells enter prophase of the first meiosis before birth.

2.1.1 Oogenesis

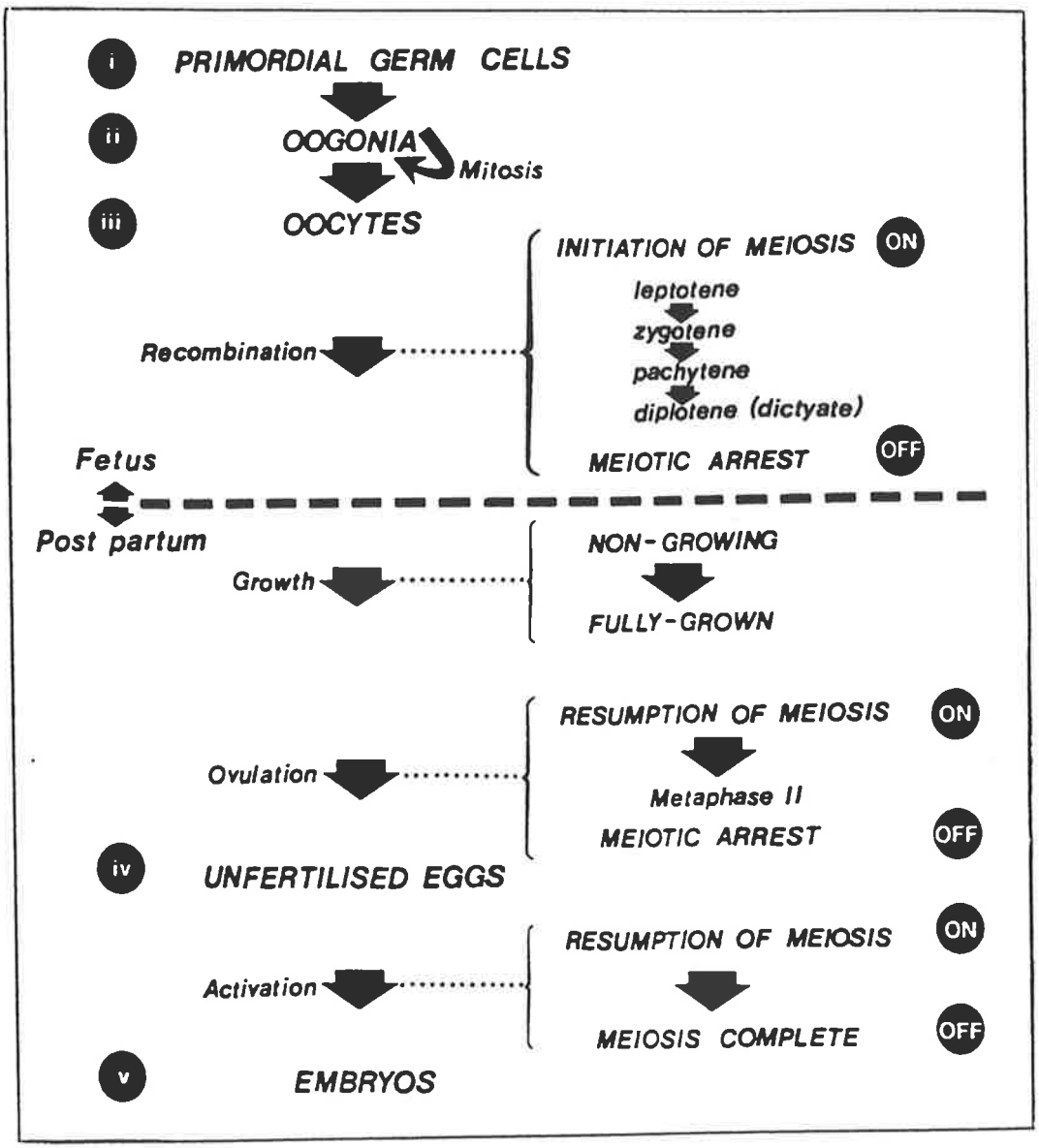
Oogenesis is a process of female germ cell differentiation starting with oogonia in the gonad of embryos and terminating with fully differentiated cells called eggs. In mouse embryos after a number of mitotic divisions and at about day 12 of embryogenesis, some oogonia enter the

preleptotene stage, in which the final DNA replication takes place, and then enter the leptotene stage of the first meiotic prophase (Baker, 1982) to become oocytes. At day 14 of embryogenesis about half of oogonia are transformed into oocytes, and by day 17 all oogonia become oocytes at different stages of the first meiotic prophase (Baker, 1982; Speed, 1982). During the first meiotic prophase oocytes progress through leptotene, zygotene, pachytene and diplotene. Homologous chromosomes pair and synapse during the zygotene stage, and this is followed by crossing over and recombination in the pachytene stage. By day 18 of embryogenesis some oocytes are seen in diplotene with their chromosomes exhibiting chiasmata that result from crossing over. The progression of meiosis in oocytes, however, is arrested at the diplotene stage. In most mammals, oocytes reach the late diplotene stage, the dictyate stage of meiotic prophase at birth or shortly postpartum, and remain at this stage throughout prepubertal stages of ovarian development. A schematic diagram showing oogenesis in the mouse is demonstrated in Figure 2.1.

After birth the process of oocyte growth is initiated and takes place in follicles within the ovary. The number of oocytes entering the growth phase is determined by the size of the pool of nongrowing oocytes prior to parturition (Krarup *et al.,* 1969). The oocyte and its surrounding follicles grow coordinately. As the follicle cells continue to divide, the oocytes too show a steady increase in size (Pedersen, 1969). The follicles of non-growing oocytes contain a single layer of granulosa cells. During the oocyte growth period the granulosa cells proliferate and differentiate to form multiple layers around the oocyte and develop numerous gap junctions with neighbouring cells. Granulosa cell differentiation is controlled by steroids produced in the follicles (MacCalman *et al.,* 1995). The changes in granulosa cell shape are accompanied by changes in cytoskeletal protein distribution (Ben-Ze'ev and Amsterdam, 1986), and expression of cell surface molecules.

Figure 2.1 Landmarks of oogenesis in the mouse. Progression from primordial germ cells to nongrowing oocytes during fetal development, as well as from nongrowing oocytes to fertilized eggs in sexually mature adults.

Source: Wassarman (1988)


i
PRIMORDIAL GERM CELLS
ii
OOGONIA
Mitosis
iii
OOCYTES
INITIATION OF MEIOSIS
ON
leptotene
zygotene
pachytene
diplotene (dictyate)
Recombination
Fetus
MEIOTIC ARREST
OFF
Post partum
NON-GROWING
Growth
FULLY-GROWN
RESUMPTION OF MEIOSIS
ON
Ovulation
Metaphase II
MEIOTIC ARREST
OFF
iv
UNFERTILISED EGGS
RESUMPTION OF MEIOSIS
ON
Activation
MEIOSIS COMPLETE
OFF
v
EMBRYOS

In mice and other species where a follicular antrum is formed, oocyte growth stops just before antrum formation, but antrum and follicle growth continue until the formation of mature Graafian follicle is completed. As the oocytes grow, there are both qualitative and quantitative changes in the patterns of proteins synthesized (Schultz *et al.*, 1979). The end result of oocyte growth is a mature oocyte endowed with a full complement of maternally supported factors needed to support early embryonic development. In the mouse, oocytes grow from 20 to 80μm in 3 to 4 weeks, with a 125-fold increase in oocyte protein (Schultz *et al.*, 1979). During all this period the oocyte nucleus remains arrested in the diplotene or dictyate stage of meiotic prophase, and meiosis is only resumed with the onset of oocyte maturation, which is initiated by the preovulatory LH (Luteinizing hormone) surge.

2.1.2 Oocyte maturation and ovulation

Fully grown oocytes in Graafian follicles resume meiosis and complete the first meiotic reduction division just prior to ovulation. Resumption of meiosis can be mediated by a hormonal stimulus *in vitro* or simply by the release of oocytes from the ovarian follicles into a suitable culture medium *in vitro* (Eppig, 1985; Tsafriri, 1985). The relationships between the secretion of pituitary hormone, oocyte maturation, and ovulation in mammals have been known for many years. In response to the release of LH, mammalian oocytes begin the process of germinal vesicle breakdown (GVBD) within a few hours, and the mature oocytes are ovulated some hours later (Donahue, 1972). Some events following GVBD are formation of a metaphase I spindle, movement of the spindle accompanied by large numbers of mitochondria to the cortex (Wassarman *et al.*, 1979; Albertini, 1987), separation of homologous chromosomes with emission of the first polar body, and arrest at metaphase II (Schultz, 1991). Eggs remain at this stage of meiosis in the oviduct, or in culture, until stimulated to complete meiosis by fertilization.

Progression from the dictyate stage (oocyte) to metaphase II (egg) of meiosis is called meiotic maturation. Oocyte maturation consists not only of germinal vesicle breakdown and chromosomal events culminating in the first meiotic division, but also of cytoplasmic processes such as changes in protein synthesis (Van Blerkom and McGaughey, 1978) and ultrastructural changes in cytoplasmic organelles (Cran *et al.,* 1980). Although nuclear and cytoplasmic events of oocyte maturation may be separated experimentally (Schultz and Wassarman, 1977), it is believed that the regulatory mechanisms operating to control maturation work in concert to provide chromosomally and cytoplasmically mature oocytes for ovulation and fertilization (McGaughey, 1983).

2.1.3 Spermatogenesis

In male animals the process of spermatogenesis transforms germ cells, spermatogonia, into the fully differentiated highly specialized motile cells called spermatozoa. Spermatogenesis can be divided into phases depending on stages of germ cell development, namely the replicative phase, meiotic phase, acrosomal phase, nuclear condensation/elongation phase and cytoplasmic elimination and release phase (Sharpe, 1994).

At the onset of puberty, non-differentiated spermatogonia migrate to the periphery of seminiferous tubules and start to differentiate at random (Gier and Marion, 1970), whereas supporting cells differentiate into the Sertoli cells. Spermatogonia are the cells within the testis that initiate the process of spermatogenesis. During the replicative phase they undergo mitotic activities many times and their numbers greatly increase. Various types of spermatogonia are found in the premeiotic lineage of rats and mice. A_s spermatogonia are regarded as the stem cells of the testis. They are located around the periphery of the seminiferous tubules and have no intercellular bridge connections with other spermatogonia (De Rooij and Russell, 2000) Chiarini-Garcia and Russell, 2001), while spermatogonial cells at later stages are joined by

intercellular bridges (Weber and Russell, 1987). The mitotic division of a stem cell (A_s) gives rise to a pair of A_{pr} spermatogonia, and after several divisions A_{pr} spermatogonia become A_{al-4}, $A_{al-8,}$ and A_{al-16} (Chiarini-Garcia and Russell, 2001). Subsequent divisions of A_{al} spermatogonia result in A_1, A_2, A_3, A_4 (van Beek, 1993), intermediate (In) and type B spermatogonia (Clermont, 1972). The final mitotic division of B spermatogonia produces preleptotene spermatocytes (Courot *et al.,* 1970; Clermont, 1972) which then enter the next phase, the meiotic phase.

All of the early-stage germ cells from A_s spermatogonia to preleptotene spermatocytes are situated near the basement membrane of the seminiferous tubules in the so-called basal compartment (outside the Sertoli cell blood-testis barrier). As development progresses germ cells move away from the basement membrane towards the lumen of the seminiferous tubules, and upon entering the meiotic phase they have moved inside the blood-testis barrier into the adluminal compartment.

During the meiotic prophase there is no further cell division. However, germ cells continue to develope sequentially from preleptotene to leptotene, zygotene, pachytene and diplotene spermatocytes. The last-stage primary spermatocytes then enter the first meiotic division and become secondary spermatocytes. There are a number of events which happen during the period of meiotic prophase; with DNA synthesis during the preleptotene and chromosome pairing during the leptotene stage (Parvinen *et al.,* 1991), RNA synthesis in pachytene spermatocytes (Monesi *et al.,* 1978), synapsis during the zygotene and pachytene stage, and chromosome condensation and segregation during the diplotene stage.

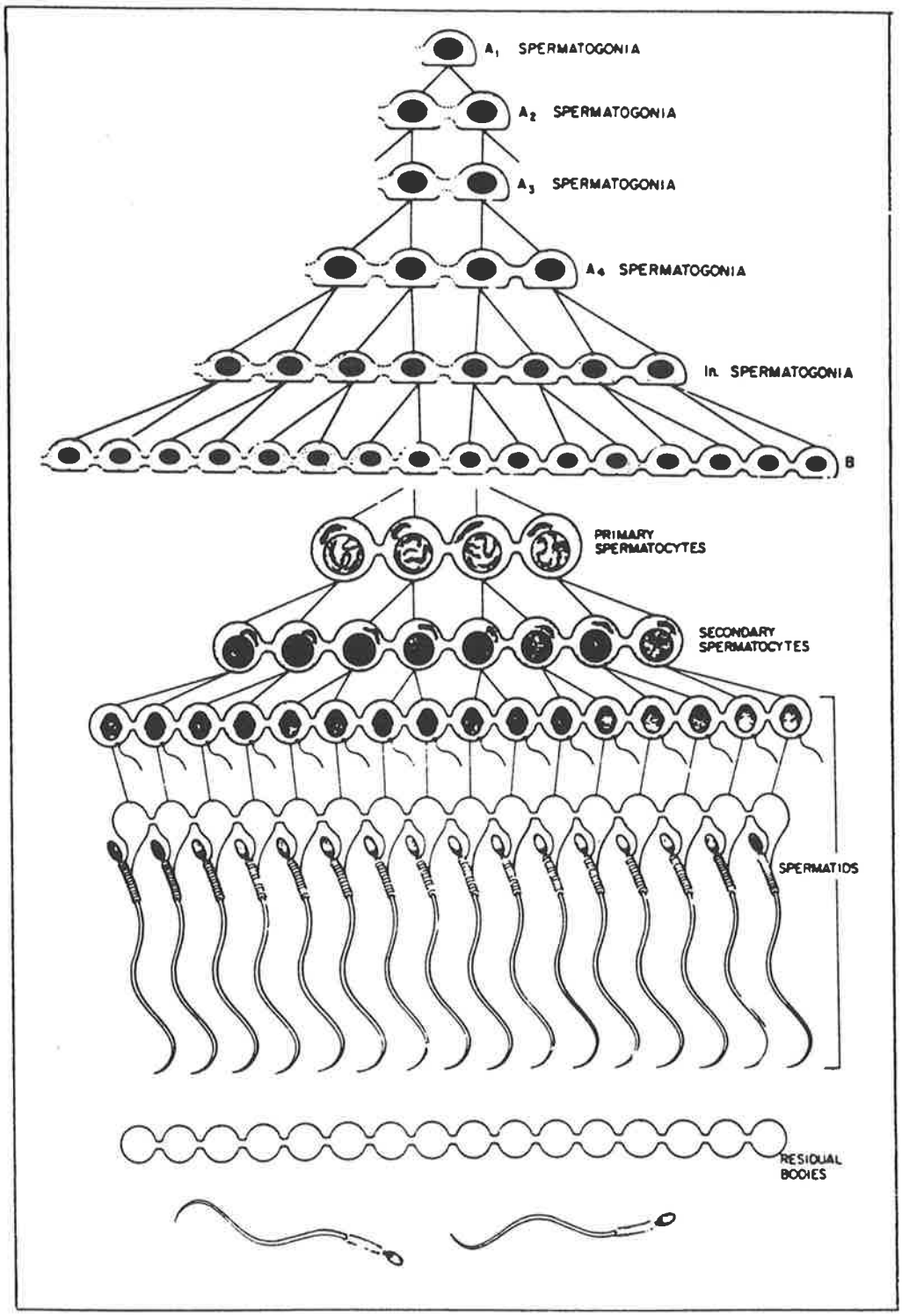
A secondary spermatocyte undergoes the second meiotic division which is in many ways similar to mitosis and gives rise to two round spermatids. Therefore, one spermatocyte after 2 meiotic divisions produces 4 spermatids. The process whereby round spermatids differentiate

into the mature spermatozoa involves a number of steps: 16 in the mouse and 19 in the rat, and is termed spermiogenesis. During spermiogenesis spermatids undergo morphological changes together with a change in position in the seminiferous tubule. Newly formed spermatids have spherical shapes and are located in the adluminal compartment of the seminiferous tubules. At the completion of the differentiating process the resultant spermatozoa have an elongated shape and protrude into the central lumen of the seminiferous tubules. The morphological changes during spermiogenesis include acrosome formation from acrosomic granules in the Golgi complex, condensation of the nuclear chromatin with the replacement of histones by protamines, formation of the sperm tail or flagella apparatus with the migration of mitochondria to the midpiece region, cytoplasmic elimination to get rid of most of the cytoplasm and finally release of elongated spermatids from the Sertoli cells. A diagram demonstrating germ cell types is illustrated in Figure 2.2.

The stem cells in the testis are able to replenish themselves via either self-renewal divisions or may undergo differentiation divisions to ensure that there are sufficient numbers of the stem cells for normal spermatogenesis to proceed (Chiarini-Garcia and Russell, 2001). The molecular control of self-renewal and differentiation of stem cells is not known. However, de Rooij and Russell (2000) suggested that a glial cell line-derived neurotrophic factor (GDNF), a substance produced by the Sertoli cells, may be involved. The duration of one cycle of the seminiferous epithelium (spermatogenic cycle) is approximately 10 days in monkeys (Miller *et al.,* 2000), 16 days in humans (Heller and Clermont, 1964), 8.6 days in mice and 12.9 days in rats (Franca *et al.,* 1998), and the entire time-span a stem spermatogonium needs to produce spermatozoa is about 34 days in mice, almost 2 months in rats, rabbits, bulls and rams (Amann *et al.,* 1965) and more than 2 months in humans (Heller and Clermont, 1964).

Figure 2.2 A diagram of the various cell types of the germinal line. (B refers to B spermatogonia). The diagram also illustrates the intercellular bridges formed by these cells, although because of the large numbers of cells involved, only the progeny of 2 of the possible 4 A3 spermatogonia and 2 of the possible 32 B spermatogonia are shown.

Source: Dym and Fawcett (1971)

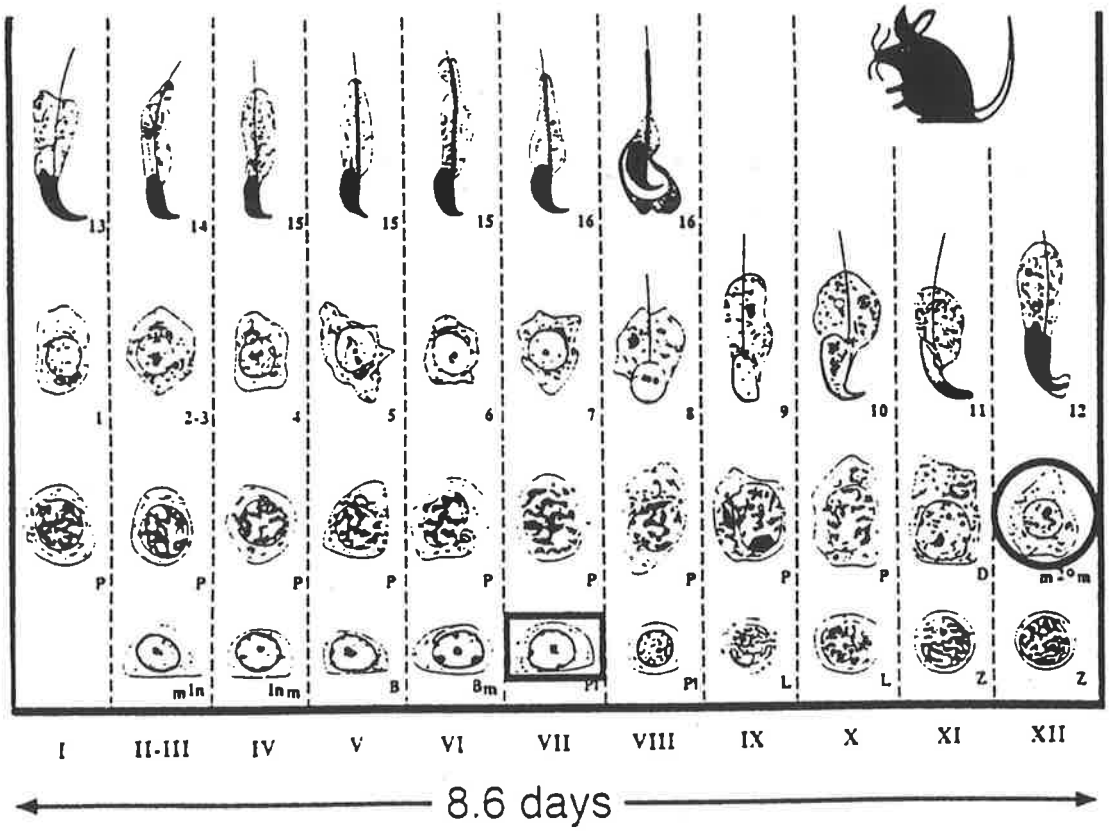

A1 SPERMATOGONIA
A2 SPERMATOGONIA
A3 SPERMATOGONIA
A4 SPERMATOGONIA
In. SPERMATOGONIA
B
PRIMARY SPERMATOCYTES
SECONDARY SPERMATOCYTES
SPERMATIDS
RESIDUAL BODIES

Because of precise timing of the differentiation process, the same cell types are always aligned from the basement membrane to the lumen and in association within a cross section of a seminiferous tubule. In the rat, there are 14 stages (cell association) that repeat at the same location in the tubule at a 12.9-day interval, while in the mouse there are 12 stages that repeat at an 8.6-day interval (Franca *et al.,* 1998). Cell association in the mouse is demonstrated in Figure 2.3.

It is known that several endocrine products, for example testosterone from Leydig cells and gonadotropins from the anterior pituitary gland, Luteinizing hormone (LH) and Follicle stimulating hormone (FSH) play a vital role in regulating spermatogenesis (Lee *et al.,* 1974; Amory and Bremnera, 2002; McLachlan *et al.,* 2002). There are receptors for FSH or testosterone on the Sertoli cells, but not on germ cells. It is, therefore, likely that these hormones exert their influence on germ cells by modulating Sertoli cell function. LH stimulates steroidogenesis, particularly testosterone synthesis, from Leydig cells in the male while FSH stimulates spermatogenesis (McLachlan *et al.,* 1995). LH also exerts a partial stimulatory effect on spermatogenesis in the male, probably mediated through increases in intratesticular levels of testosterone. Both gonadotropins act through classic protein hormone receptor mechanisms, involving a G-protein associated seven-channel transmembrane domain receptor (Simoni *et al.,* 1997). FSH binds to the Sertoli cell and spermatogonial membranes in the testis, and is the major stimulator of seminiferous tubule growth during development. It is, therefore, of major importance in determining testicular size (McLachlan *et al.,* 1995). Although FSH has a key role in the development of the immature testis, particularly by controlling Sertoli cell proliferation (Orth, 1988), it is now still uncertain whether FSH is essential for spermatogenesis in the adult.

Figure 2.3 Spermatogenic cycle of the mouse. The diagram shows the histological relationship of cells in the mouse seminiferous tubules of the testis during spermatogenesis. The lower row of cells rests closer to the basement membrane, and the upper row of cells is generally closer to the lumen of the seminiferous tubule (undifferentiated and young differentiated type A spermatogonia are not depicted). Because of precise timing of the differentiation process, the same cell types are always aligned from the basement membrane to the lumen and in association within a cross section of a seminiferous tubule. In the mouse, there are 12 stages that repeat at an 8.6-day interval. When a preleptotene (Pl) spermatocyte is labelled during S-phase of mitosis with [^{3}H]thymidine (box) and the testis is examined 13 days later, the radioactivity is found in the diplotene meiotic ($_m2°_m$) spermatocyte of the mouse (circle). Roman numerals I to XII indicate spermatogenic cycle stages.

Source: Franca *et al.* (1998)



Stages of the Mouse Cycle

2.2 Sperm maturation

2.2.1 Epididymal maturation

Testicular spermatozoa of mammals are incapable of fertilizing eggs unless injected into the cytoplasm of the oocyte. Their fertilizing capacity develops as they pass through the epididymis (Bedford, 1966; Orgebin-Crist, 1967). During this migrating period they acquire the potential to express coordinated movement and the competence to undergo capacitation (Lewis and Aitken, 2001).

Epididymal maturation involves several functional changes, including development of the potential for sustained motility, progressive loss of water, and the distal migration and eventual loss of the cytoplasmic droplet. The maturation process during which epididymal spermatozoa attain the capacity for progressive motility involves progressive changes in the flexibility and patterns of movement of their flagella. Rapid forward progression appears first in a few spermatozoa in the middle of the corpus epididymis and becomes the predominating motility pattern in spermatozoa from the cauda and vas deferens (Bedford, 1975). During epididymal transit, the droplet migrates from the neck region to a position near the annulus. Presence of the droplet on a significant number of ejaculated spermatozoa is a sign of incomplete maturation. During the passage through the epididymis a reduction in acrosome dimensions, the change associated with maturation of the acrosome, has been observed in most species (Bedford, 1975).

This maturation also includes various morphological, physiological and biochemical changes in the sperm components (Bedford, 1975; Hamilton, 1977). The plasma membrane is believed to be the site of the most prominent changes. Some of the changes which occur on or in the plasma membrane during the maturation are the absorption of antigens, glycoproteins, sialic acid (Romrell and O'Rand, 1978; Lewin *et al.*, 1979; Moore, 1980), carnitine, acetylcarnitine,

glycerylphosphoryl choline, lipids (Brooks, 1979), the changes in net negative surface charge (Hammerstedt *et al.,* 1979; Moore, 1979), in lipid composition and integrity (Evans and Setchell, 1979; Hammerstedt *et al.,* 1979), in activity of surface ATPase (Chulavatnatol and Yindipit, 1976), and the reduction in surface SH groups (Reyes *et al.,* 1976). In addition, epididymal maturation is also associated with a progressive loss of tyrosine phosphorylated proteins from the acrosomal domain of the sperm head, and an acquisition of competence to respond to high levels of intracellular cAMP by phosphorylating tyrosine residues on the sperm tail (Lewis and Aitken, 2001).

2.2.2 Capacitation

Although considered to be mature in the epididymis, spermatozoa are still incapable of penetrating the cellular and acellular vestment of the oocyte when they first leave the male reproductive tract. They require an additional phase of maturation in the female reproductive tract before they are able to fertilize eggs (Austin, 1951). During a specific-time dependent period in the tract, the plasma membrane of the spermatozoa is modified by the process known as capacitation. This process can be defined as a series of events that enables the sperm to bind with the oocyte and accomplish the acrosome reaction in response to the zona pellucida (Tardif *et al.,* 2001). Capacitation is a reversible process and Chang (1957) demonstrated that capacitated spermatozoa could lose the ability to fertilize an egg if they were re-exposed to seminal plasma. The reversal of capacitation is known as decapacitation, which can be reversed again (Weinman and Williams, 1964; Fraser, 1984; Fraser *et al.,* 1990; Lynn *et al.,* 1990; DasGupta, 1994). Numerous decapacitation factors have been detected in a variety of species, e.g. an "acrosome-stabilizing factor" from rabbit seminal plasma (Eng and Oliphant, 1978), an "antifertility factor" from human seminal plasma (Reddy *et al.,* 1979) and an “acidic

seminal fluid protein" (aSFP) in the bull seminal plasma (Dostalova *et al.,* 1994). This decapacitation factor can be removed from uncapacitated spermatozoa by gentle centrifugation, resulting in immediately highly fertile gametes, and it can be added back to capacitated spermatozoa, resulting in poorly fertile cells in which the acrosome reaction has been blocked (Fraser *et al.,* 1990).

The sites where capacitation begins and where it is completed varies from species to species. In some rodents, the dog and pig in which semen is deposited directly into the uterus at coitus, capacitation may start in the uterus, but the principal site for capacitation is believed to be the oviduct (Hunter and Hall, 1974). In species in which semen is deposited in the vagina (e.g. the rabbit and human), capacitation may start in the vagina or as soon as the spermatozoa migrate to the cervix. It has been reported that the alterations that occur in the sperm plasma membrane during capacitation include modifications in the structure, organization, and concentration of proteins, phospholipids, and sterols (Yanagimachi, 1988; Zaneveld *et al.,* 1991; Lin and Kan, 1996), a reduction of the superficial negative charge on the spermatozoa, the alteration of linking patterns to lectins (Cross and Overstreet, 1987) and an increase in membrane fluidity (Wolf *et al.,* 1986; Benoff *et al.,* 1993; Smith *et al.,* 1998; Harrison and Miller, 2000). Other molecular events that coincide with capacitation are the activation of ion channels (Fraser, 1990) and calcium uptake (Babcock *et al.,* 1979; Fraser, 1982; Handrow *et al.,* 1989; Okamura *et al.,* 1993) the generation of cAMP (White and Aitken, 1989; Fraser and Monks, 1990), and the production of reactive oxygen species (de Lamirande and Gagnon, 1993; Aitken *et al.,* 1995).

Although capacitation has been studied extensively, its process is still incompletely understood. During this period the masking lactosaminoglycan, one of the decapacitation factors, is lost from the outer surface of the sperm plasma membrane. This loss may be due to

the displacement of the capacitation factors. One such factor may be the glycosaminoglycan heparin, which facilitates capacitation in bovine spermatozoa (Handrow *et al.,* 1986; First and Parrish, 1987).

The increases in intracellular calcium and adenylate cyclase activity have also been associated with capacitation (Neil and Olds-Clark, 1987; Yanagimachi, 1988; White and Aitken, 1989). Cyclic adenosine monophosphate (cAMP) is believed to play an important role in capacitation (Tash and Means, 1983; Fraser and Ahuja, 1988). However, mechanisms regulating sperm adenylate cyclase activity and hence cAMP biosynthesis are poorly understood. Stein and Fraser (1986) reported that adenylate cyclase activity increases and phosphodiestrase activity decreases during capacitation *in vitro* of mouse spermatozoa. Such changes could provide increased availability of cAMP. As it is cAMP-dependent (Visconti *et al.,* 1995b; Leclerc *et al.,* 1996; Aitken *et al.,* 1998), protein phosphorylation is also believed to play an important role in sperm capacitation (Aitken *et al.,* 1995, 1996; Visconti *et al.,* 1995a, 1995b; Kalab *et al.,* 1998).

2.2.3 Sperm motility

Sperm in the testis and caput epididymis do not display full motility. The capacity for motility is acquired during the transit through the epididymis. Sperm concentrated in the cauda epididymis are capable of motility but are maintained in a state of quiescence in most mammals (Bedford, 1975; Carr and Acott, 1984). Some proteins, the so-called "forward motility protein", secreted from the epididymal epithelium in conjunction with elevated cellular cyclic adenosine monophosphate (cAMP) levels prime the flagellar apparatus for progressive motility (Brandt *et al.,* 1978; Stephens *et al.,* 1981). On ejaculation the sperm are

activated to full motility. They swim by the propulsive action of a single flagellum with linear swimming. The factors regulating sperm motility are calcium ions (Ca^{2+}), cAMP, adenosine, and intracellular pH (Tash and Means, 1987).

Sperm motility behavior changes during incubation in the female genital tract. After spending several hours in the tract and acquiring the capacity to fertilize (capacitation), sperm motility converts from linear swimming to a vigorous pattern of less progressive movement (Cooper, 1984; Katz and Yanagimachi, 1980; Suarez and Osman, 1987). This motility is referred to as "hyperactivation". The conversion in pattern is dependent on Ca^{2+} availability (Fraser, 1987; Suarez, 1987). Hyperactivation is characterized by sharply curved flagellar beats and a circular or erratic swimming trajectory (Yanagimachi, 1994). It has been observed in many species *in vitro*, including guinea pigs (Yanagimachi and Usui, 1974), mice (Fraser, 1977), rabbits (Cooper *et al.,* 1979), bats, dolphins, dogs, sheep, cattle, pigs (Yanagimachi, 1988), rhesus monkeys (Boatman and Bavister, 1984), and humans (Mortimer *et al.,* 1983; Burkman, 1984).

Several biological functions have been proposed for hyperactivation. These include increasing flexibility for moving sperm out of pockets created by mucosal folds (Suarez *et al.,* 1983; Suarez and Osman, 1987), increasing the chance that sperm will encounter the egg in the oviductal lumen (Suarez *et al.,* 1983), disengaging sperm from adherence to oviductal epithelium (DeMott and Suarez, 1992), facilitating the penetration of sperm through viscous and viscoelastic substances such as oviductal mucus and the cumulus matrix (Suarez *et al.,* 1991; Suarez and Dai, 1992), and facilitating the penetration of sperm through the zona pellucida (Drobnis *et al.,* 1988).

2.3. Fertilization and embryo development

2.3.1 Introduction

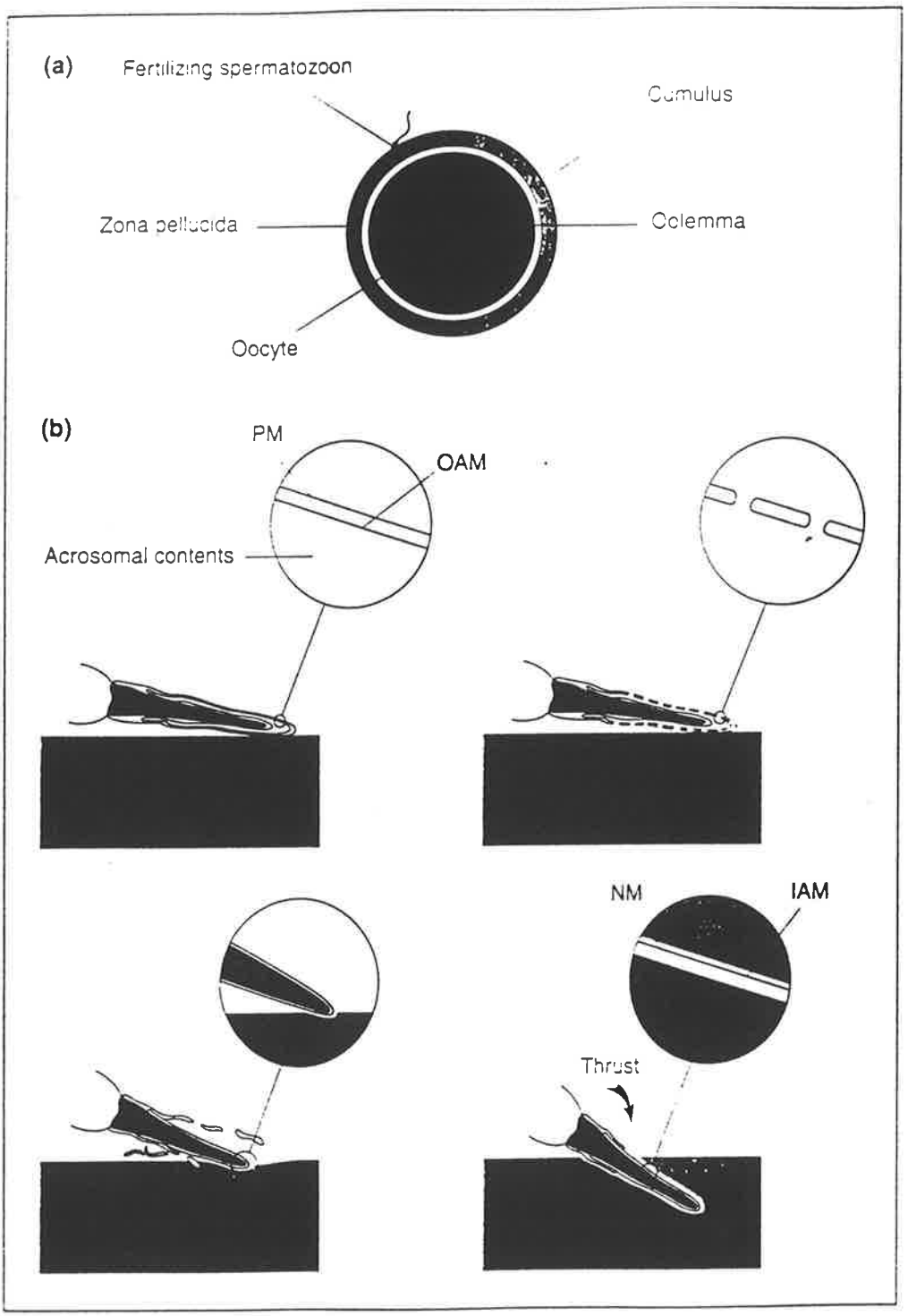
Mammalian fertilization may be defined as the process of union of two germ cells, egg and sperm, whereby the somatic chromosome number is restored. The fertilization process, taking place in the oviduct includes several ordered steps (Wassarman, 1987; Yanagimachi, 1994; Snell and White, 1996). Capacitated sperm must first penetrate the cumulus cells of the egg, then bind to the egg extracellular coat, the zona pellucida (ZP). The sperm-zona binding initiates sperm crossing the perivitelline space to bind to and fuse with the egg plasma membrane (oolemma). Within a short period of time the sperm penetrates the egg cytoplasm (Bronson *et al.,* 1999). Normally, only one spermatozoon manages to penetrate the egg cytoplasm. This is because of the block to polyspermy taking place at the zona pellucida and plasma membrane of the egg. This block to polyspermy is thought to be mediated by the contents of the egg cortical granules (cortical granule reaction) (Barros and Yanagimachi, 1972). A schematic diagram of early mammalian gamete interaction is illustrated in Figure 2.4.

2.3.2 Sperm-zona binding and penetration

An essential step in the process of mammalian fertilization is the recognition and binding between the spermatozoa and the egg's extracellular coat, the zona pellucida (ZP). The ability of spermatozoa to bind to the ZP is related to protein deposition on their surface during transit through the epididymis (Hinrichsen and Blaquier, 1980; Moore *et al.,* 1983; Tezon *et al.,* 1985). These surface proteins meditate gamete recognition by specific and irreversible binding of spermatozoa to complex glycoconjugates of the zona (Oehninger *et al.,* 1991). Three major sulphated glycoproteins, i. e. ZP1, ZP2 and ZP3 found in the zona pellucida have been

Figure 2.4 Schematic diagram of early mammalian gamete interactions. (a) After passing through the cumulus, the fertilizing spermatozoon reaches and binds to the zona pellucida. (b) Initial (primary) binding of the intact sperm head to the zona pellucida (ZP3) initiates the sperm acrosome reaction (1). The plasma membrane (PM) and outer acrosomal membranes (OAM) undergo multiple point fusions leading to membrane vesiculation (2). Release of the acrosomal contents (3) enables secondary binding between the sperm inner acrosomal membrane and the zona (ZP2). This interaction orientates the thrusting spermatozoon and allows penetration through the zona (4). NM, nuclear membrane.

Source: Brewis and Wong (1999)


(a)
Fertilizing spermatozoon
Cumulus
Zona pellucida
Colemma
Oocyte
(b)
PM
OAM
Acrosomal contents
NM
IAM
Thrust

identified in a number of mammalian species including mouse (Bleil and Wassarman, 1980; Shimizu *et al.,* 1983), rat (Araki *et al.,* 1992), hamster (Shabanowitz and O'Rand, 1988; Moller *et al.,* 1990), pig (Hedrick and Wardrip, 1987; Yurewicz *et al.,* 1987), and human (Shabanowitz and O'Rand, 1988). It is believed that complementary molecules present on the surface of opposite gametes (the sperm's plasma membrane and the egg's ZP) are responsible for sperm-egg interaction. In the mouse, mZP3 is believed to serve as a receptor (primary binding) for sperm (Bleil and Wassarman, 1983) while ZP2 may mediate the subsequent binding (secondary binding) of acrosome-reacted sperm to the zona pellucida (Bleil *et al.,* 1988). It has been demonstrated that only purified mZP3 binds exclusively to heads of capacitated acrosome-intact sperm and thereby prevents sperm from binding to ovulated eggs *in vitro* (Bleil and Wassarman, 1986; Wassarman, 1990; Mortillo and Wassarman, 1991; Wassarman and Litscher, 1995). Furthermore, preventing expression of the mouse gene encoding ZP3 results in production of eggs lacking a ZP (Tong *et al.,* 1995; Liu *et al.,* 1996) and infertility in female mice (Liu *et al.,* 1996). However, Wassarman (1999) suggested that mZP3 oligosaccharide, but not mZP3 polypeptide, played a direct role in sperm receptor function.

A number of sperm proteins believed to bind to ZP3 of the eggs have been identified, 95 kDa Tyrosine kinase receptor (Leyton and Saling, 1989; Burks *et al.,* 1995), β-galactosyltransferase (GalTase) (Shaper *et al.,* 1988; Miller *et al.,* 1992; Lu and Shur, 1997), sperm protein-56 (sp 56) (Bleil and Wassarman, 1990; Bookbinder *et al.,* 1995), zona receptor kinase (ZRK) (Burks *et al.,* 1995; Bork, 1996; Tsai and Silver, 1996), spermadhesin (Sanz *et al.,* 1991, 1992), and Zonadhesin (Hardy and Garbers, 1994, 1995).

The acrosomal status of spermatozoa that bind to the zona pellucida varies according to species. In guinea pigs, acrosome-reacted spermatozoa bind to ZP (Yanagimachi, 1981),

whereas in mice and humans, acrosome-intact spermatozoa bind to ZP, which then induces the acrosome reaction (Wassarman, 1988; Liu and Baker, 1990). After binding, a hydrolytic enzyme, acrosin, is released from the acrosomal compartment and acts on the zona pellucida to facilitate penetration of the zona pellucida by the sperm. Snell and White (1996) have suggested that zona penetration is mediated by an interaction between the zona protein ZP2, and one or more molecules on the inner acrosomal membrane.

2.3.3 Acrosome reaction

Mammalian sperm must undergo the acrosome reaction in order to fertilize eggs. The acrosome is a membrane-bound organelle containing a variety of hydrolytic enzyme. Some of these enzymes, most notably acrosin (Urch *et al.,* 1985), assist in the penetration of the zona pellucida by weakening its structure. In most species, freshly ejaculated sperm will not acrosome-react. They must be washed free of seminal plasma and spend some time, generally hours, to be capacitated, in either the female reproductive tract or in an appropriate medium *in vitro* in order to develop the ability to acrosome react. During capacitation a few sperm acrosome-react but a large number become responsive to more specific inducers of the acrosome reaction.

The acrosome reaction involves fusion of the outer acrosomal membrane with the plasma membrane at several sites, and release of the acrosomal contents to the extracellular space (Meizel, 1984; Wassarman, 1987). An early event involved in the acrosome reaction is an increase in free cytosolic calcium (Blackmore *et al.,* 1990). It has been reported that pre-ovulatory human follicular fluid enhances the influx of calcium through the sperm membrane (Thomas and Meizel, 1988), and increases the number of spermatozoa undergoing the acrosome reaction (Suarez, 1986 *et al.*; Sitteri *et al.,* 1988; Siegel *et al.,* 1990). At the

completion of the acrosome reaction, the hybrid membrane complex detaches from the head, exposing the inner acrosomal membrane, which then binds the zona pellucida glycoprotein ZP2 and maintains zona adhesion (Bleil *et al.,* 1988; Mortillo and Wassarman, 1991). The acrosome reaction is believed to be regulated by intracellular signalling systems. Although the precise signal transduction pathways have not been fully elucidated, it is known that the acrosome reaction is characterized by increases in intracellular concentrations and phosphorylation of tyrosine residues in proteins (Leyton and Saling, 1989; Carr and Acott, 1990; Naz *et al.,* 1991; Duncan and Fraser, 1993; Burks *et al.,* 1995; Luconi *et al.,*1995; Visconti *et al.,* 1995; Luconi *et al.,* 1996).

2.3.4 Sperm-oolemma binding

Having penetrated the zona pellucida, the sperm then crosses the perivitelline space and binds to the oolemma. The molecular basis of sperm-oolemma binding has yet to be fully elucidated. Almeida *et al.,* (1995) found that when oocytes were treated with monoclonal antibodies against the egg surface integrin, $\alpha 6\beta 1$, mouse sperm-oolemma binding was reduced. It has been proposed that fertilin is a sperm surface ligand for $\alpha 6\beta 1$. Fertilin contains a domain homologous to a family of integrin ligands known as disintegrins (Blobel *et al.,* 1992), which suggest a cell adhesion function for the molecule. Also, recombinant fertilin is known to bind to the oolemma (Evans *et al.,* 1997), and both monoclonal antibodies to fertilin (Primakoff *et al.,* 1987) and fertilin peptide analogs (Almeida *et al.,* 1995; Evans *et al.,* 1995) block sperm-oolemma binding.

2.3.5 Sperm-egg fusion

In an *in vitro* study, human sperm were found to adhere to the hamster oolemma within 10 min of their insemination, while the interval between sperm-oolemma binding and sperm-oolemma

fusion varied considerably (15-140 min) (Bronson *et al.,* 1999). The gamete membrane fusion has been shown by ultrastructural studies to be initiated in the equatorial segment of acrosome-reacted sperm (Yanagimachi, 1994). As P-selectin has been localized in this region, it has been proposed to have a role in sperm-oolemma fusion (Fusi *et al.,* 1996). Sperm fertilin is also thought to be involved in gamete fusion (Blobel *et al.,* 1992; Bigler *et al.,* 1997; Evans *et al.,* 1998). It has been suggested that binding of acrosome-reacted sperm to egg plasma membrane is supported by interactions between fertilin's disintegrin domains and integrin receptors (Hynes, 1992; Almeida *et al.,* 1995).

2.3.6 Egg penetration

Following sperm-oolemma binding and fusion, the process of sperm incorporation by the oocyte takes place. It has been reported that the time between sperm-oolemma fusion and sperm penetration of the cortical ooplasm is relatively short. It occurs over an interval of approximately 10 min for mouse sperm penetrating zona-free mouse eggs (Wolf and Armstrong, 1978), and human sperm penetrating zona-free hamster eggs (Bronson *et al.,* 1999).

The process of sperm incorporation by the oocytes appears to be quasi-phagocytic in nature (Sathananthan and Chen, 1986). It was observed that during sperm incorporation the egg surface in contact with the sperm exhibited ultrastructural changes. Oolemmal microvilli appeared to elongate and wrap around the equatorial segment of the acrosome of some individual sperm but not all sperm adherent to the oolemma (Bronson *et al.,* 1999). The sperm membrane in the postacrosomal region then fuses with the surface or vitelline membrane of the oocyte and the sperm is incorporated into the oocyte (Yanagimachi and Noda, 1972).

2.3.7 Egg activation

Upon fusion with the spermatozoon, the egg is activated. The most easily recognizable indications of egg activation in mammals are the exocytosis of cortical granules, and the resumption of meiosis. The egg arrested at metaphase of the second meiosis before fertilization completes meiosis after sperm-egg fusion. The haploid complement of the egg's chromosomes then transform into an egg pronucleus. Meanwhile, the sperm nucleus decondenses and transforms into a sperm pronucleus (Austin, 1965). DNA synthesis (chromosome duplication) begins in both the egg and sperm pronuclei several hours after sperm-egg fusion (Krishna and Generoso, 1977; Luthardt and Donahue, 1973). The fully developed sperm and egg pronuclei come into close approximation at the centre of the egg, their nuclear envelopes disintegrate, and their chromosomes mingle prior to the first mitotic division (cleavage) (Longo, 1973). The mingling of chromosomes (syngamy) can be considered as the end of fertilization and the beginning of embryonic development.

Fertilization of mouse eggs results in an egg-induced modification of both glycoproteins ZP3 and ZP2, such that ZP3 loses both its sperm receptor and acrosome reaction-inducing activities, and ZP2 is converted to a form called $ZP2_f$, which no longer binds acrosome-reacted sperm. These changes are thought to constitute the zona pellucida block to polyspermy (Ducibella *et al.,* 1990).

It has also been suggested that the fertilizing spermatozoon activates the egg by triggering a rise and oscillations in intracellular calcium concentration within the egg. This effect has been attributed either to the injection of calcium from the sperm, a surface interaction between the sperm and the egg leading to the entry of calcium from the extracellular medium or a release of calcium from internal stores within the egg, or most likely the introduction into the egg of a soluble sperm factor which triggers egg activation (Swann, 1990, Swann and Parrington, 1999,

Parrington, 2001). The last-mentioned factor was thought to be a phospholipase C from the sperm, since it also generates inositol 1,4,5-trisphosphate, but it does not seem to be identical to any of the recognized forms of this enzyme. It does appear to be a protein with a molecular weight between 30 and 70 kDa (Parrington et al, 2002).

2.3.8 Early embryonic development

In most mammals, fertilization occurs in the infundibulum of the oviduct, and after fertilization the zygote initiates cell division. The zygote is transported through the ampulla and, at the early blastocyst stage enters the uterus where it will eventually implant. During its period in the oviduct, the conceptus continues its cellular division. Each cell or blastomere undergoes a series of divisions called cleavage, during which the total size of the conceptus remains much the same. During the mid- to late cleavage stages in placental mammals, the cleaving conceptus changes its morphology by undergoing the process of compaction to yield a morula. During the compaction, individual blastomeres seem to lose their identity and merge into a single coherent mass of cells. This early morphogenetic event is marked by junctional differentiation in which gap junctions and incipient tight junctions form between outer blastomeres and initiate the process of epithelial differentiation, culminating in the formation of trophectoderm at the blastocyst stage (Goodall and Johnson, 1984; McLachlin *et al.,* 1983). The events of compaction are crucial for the continuation of preimplantation development as they contribute to the onset and progression of the formation of the blastocyst cavity, which is the second major morphogenetic event of preimplantation development.

The blastocyst contains two distinctive types of cell, an outer rim of trophectoderm cells surrounding a blastocoelic cavity containing blastocoelic fluid and an inner cell mass (ICM), which is eccentrically placed within the blastocoelic cavity against the trophectoderm. The trophectoderm cells will grow to form the trophoblast, eventually a portion of the embryonic

membranes, and finally part of the placenta. The inner cell mass is the portion that develops into the embryo and eventually the fetus. Throughout the development from fertilization to the blastocyst, the conceptus remains enclosed within the zona pellucida. In the mouse, hatching commences at 4.0-4.5 days postcoitus, and the blastocyst escapes from the zona pellucida. By this time, the inner cell mass has differentiated into an outer layer of primitive endoderm surrounding an inner core of primitive ectoderm (Pedersen and Burdsal, 1994). After hatching from the zona pellucida, the free-living blastocyst derives nutrients by absorption across the trophoblast membrane and through the fluid-filled blastocoele, or blastocyst cavity. The blastocyst enlarges rapidly and may press against the endometrial lining of the uterus, thereby initiating an implantation reaction.

Development of the preimplantation embryo is the result of a delicate interaction between the genetic program in the chromosomes of each zygote and the female genital tract. Progression of the fertilized ovum through cleavage, compaction and blastocyst formation is dependent on the successful implementation of the genetic and developmental program contained within the oocyte and embryo itself. The genetic and development program is initiated during the process of oogenesis, when there is active synthesis of both RNA and proteins. The oocyte thus accumulates a stockpile of gene transcripts and translation products that are used to direct the initial stages of embryogenesis (Wassarman and Kinloch, 1992). RNA synthesis ceases when the fully-grown oocyte is induced to ovulate, and, in mice, resumes during the 1-cell to 2-cell transition period in the early embryo. By the mid-2-cell stage most of the maternal mRNA is destroyed or inactivated (Paynton *et al.,* 1988) and by the late 2-cell stage all classes of RNA are transcribed from the embryonic mouse genome (Clegg and Piko, 1983). Thus, there is a major turnover of mRNA populations during this phase of early development, and the changes are both qualitative and quantitative. However, marked changes in protein synthesis patterns occur at different stages of embryo development depending on the species; these are evident at

the 2-cell stage in rodent embryos (Norris *et al.,* 1985), 4- to 8-cell stage in human (Braude *et al.,* 1988) and pig embryos (Jarrell *et al.,* 1991), and 8- to 16-cell stage in both sheep and cattle embryos (Crossby *et al.,* 1988; Frei *et al.,* 1989). Many of the transcripts that are expressed at the 2-cell stage of mouse development continue to be expressed continuously and accumulate in abundance as development proceeds to the blastocyst stage (Taylor and Piko, 1987).

Culture experiments have demonstrated that development of the preimplantation mouse embryo is promoted by autocrine and paracrine factors (Pollard, 1990). It is thought that polypeptide growth factors play a central role in these events (Schultz and Heyner, 1993). In this regard, insulin-like growth factor-I (IGF-I) (Harvey and Kaye, 1991), members of the epidermal growth factor (EGF) family of growth factors (Wood and Kaye, 1989), and members of the transforming growth factor (TGF) ß family (Paria and Dey, 1990) have been shown to influence preimplantation embryonic development. Additionally, receptors for these growth factors have been demonstrated on preimplantation embryos (Paria *et al.,* 1992; Wiley *et al.,* 1992). Although expression of several growth factor genes by the preimplantation embryo (Schultz and Heyner, 1993) suggests an autocrine role for these factors, the contribution of oviductal factors to embryonic development has been demonstrated in many mammalian species. Coculture of mouse embryos with explants from the oviduct can overcome the 2-cell block associated with development *in vitro* and promote embryonic development to the blastocyst stage (Minami and Iritani, 1993)

Expression of several growth factor genes has been documented in the mouse uterus, and ovarian steroids play key roles in regulating their expression (Kapur *et al.,* 1992; Das *et al.,* 1992). In this regard, lactoferrin (LF) is a major uterine secretory protein in the mouse, and

expression of this gene is activated by estrogens, whereas progesterone antagonizes this effect (McMaster *et al.,* 1992).

2.3.9 Implantation

Details of implantation vary in different species. However, in all animals implantation involves a direct interaction of the trophoblast with the luminal epithelium of the uterus. To implant successfully the embryo and the uterus must be synchronized, i.e the embryos have reached the expanded blastocyst stage and the endometrium has undergone certain hormone-dependent changes that causes it to become receptive to the embryo (Weitlauf, 1994)

On entering the uterus, the conceptus is positioned for implanting at a site (or sites) within the uterus that is characteristic for each species. Uterine muscular activity may be important in this process, as its inhibition leads to abnormal sites of implantation (Johnson and Everitt, 1995). The first stage of implantation involves the attachment of embryos to the uterine epithelium. In rodents and most of the primates whose implantation is classified as *invasive*, the conceptus breaks through the uterine mucosa by penetrating and phagocytizing the uterine luminal epithelium as they migrate into the uterine stroma (Perry, 1981). In mice and rats, the uterine lumen closes down around the embryos in the earliest phase of implantation, and thus the uterus has the appearance of 'clasping' the blastocysts (Enders and Schlafke, 1967). In the rabbit, the blastocyst enlarges to fill the uterine lumen and hence brings the trophoblast into apposition with the epithelium without general obliteration of the lumen (Hedlund *et al.,* 1972). The term apposition denotes the progressively increasing intimacy of contact between the trophoblast and the uterine epithelium. This invasive process is accompanied by transformation and proliferation of uterine stroma cells, referred to as 'decidualization' (Bazer *et al.,* 1993). The decidual tissue releases massive quantities of primary metabolic substrates (lipids, carbohydrates, nucleic acids and proteins) and, thus functions as a large 'yolk

reservoir' equivalent to the yolk of a bird's egg. With the formation and invasion of the decidua, implantation is completed, a physical hold and a nutritional source of decidual 'yolk' is established, and the basis of placental development, leading to adjacent circulations and exchange of nutrients, is initiated (Johnson and Everitt, 1995).

In domestic animals, implantation is superficial and classified as *non-invasive* (King *et al.,* 1982), and involves phases of trophoblast-uterine epithelial cell apposition and adhesion (Enders, 1972). In the horse and pig, attachment occurs at multiple sites over most of the external surface of the conceptus, but in ruminants, attachment is limited to uterine caruncles, distinct areas of projecting aglandular uterine mucosa, which in sheep and goats may be up to 90 in number. Between the sites of attachment, uterine glands continue to secrete a nutrient 'milk' for the conceptus, especially in the pig and horse (Johnson and Everitt, 1995).

2.3.10 Genomic imprinting

Genomic imprinting is an epigenetic phenomenon by which the two parental alleles of a gene are differentially expressed (Efstratiadis, 1994; Solter, 1988). It has been suggested that the differential imprinting of nuclear genes takes place during gametogenesis (Surani, 1986; Solter, 1988). A disruption of a normal imprinting is believed to be one of the many factors contributing to embryonic death. Investigations of the early development of parthenogenetic, biparental diploid gynogenetiç embryos (containing two female pronuclei) and biparental androgenetic embryos (containing two male pronuclei) has shown that neither of these types of embryos is viable, dying either before implantation or in early postimplantation stages (McGrath and Solter, 1984; Surani *et al.,* 1984). It has been proposed that the lethality is due to the absence or over expression of one or more gene products as the result of the unequal expression of genes in maternal and paternal genomes.

A number of imprinted genes have been discovered over years. In 1996, Leighton *et al.* reported that there were twelve imprinted genes in mammals, while in 2001 more than 50 imprinted genes were reported (Table 2.1) in the mouse (Moore, 2001). Of these genes, 18 are maternally expressed (paternally depressed) and 30 are paternally expressed. Moore and Haig (1991) reported that paternally expressed genes would promote embryonic growth whereas maternal genes would act to restrain the use of maternal resources. The insulin-like growth factor II (*Igf-2)* gene is known to be paternally expressed (Giannoukakis *et al.,* 1993; Ohlsson *et al.,* 1993; Leighton *et al.,* 1996; Moore, 2001). In the mouse, as the embryos are exquisitely sensitive to the levels of the paternally expressed growth factors, a complete loss of function of the *Igf-2* gene leads to a 40% reduction in birth weight (DeChiara *et al.,* 1991).

2.3.11 Embryonic mortality

It has been accepted that low fertility in animals can be influenced by a number of factors, e.g. low ovulation, fertilization and blastocyst implantation rates, and high embryonic and fetal mortality. Of these factors embryonic loss is considered to be the major component. Embryonic mortality has been estimated to be about 20-40% in cows (Lopez-Gatius *et al.,* 1996), 10-40% in sows (Lambert *et al.,* 1991), 10-30% in goats and 15-60% in mares (Allen, 1992; Bergfelt and Ginther, 1992). It is therefore obvious that embryonic mortality is an important source of economic loss for livestock producers. Although many factors responsible for embryonic wastage have been disclosed, it is not well understood how some of them affect embryos. In addition, it is believed that there are still other factors that have not been elucidated. Some of the known factors thought to contribute to embryonic mortality are now discussed.

Table 2.1 Imprinted genes in mice

Gene	Chromo-some	Repressed Allele	Imprinted In human?	Gene	Chromo-some	Repressed Allele	Imprinted In human?
Nnat	2	M		Igf2as	7	M	+
Gnas	2	P	+	Ins2	7	M	
Gnasxl	2	M	+	Mash2	7	P	+
Nesp	2	P	+	Kvlqt1	7	P	+
Nespas	2	M		Kvlqt1as	7	M	+
Sgce	6	M		Tapa1/Cd81	7	P	
Peg1/Mest	6	M	+	$P57^{kip2}$	7	P	+
Copg2	6	P	+	Orctl2	7	P	+
Copg2as	6	M		Itm	7	P	+
Mit1/1b9	6	M		Impt1	7	P	+
Peg3/Pw1	7	M		Ipl/Tssc3	7	P	+
Zim1	7	P		Rasgrf1	9	M	
Snrpn	7	M	+	Zac1	10	M	+
Snurf	7	M	+	Meg1/Grb10	11	P	+
Magel2	7	M	+	U2af1-rs1	11	M	
Ndn	7	M	+	Dlk	12	M	
Zfp127	7	M		Meg3/Gt12	12	P	+
Zfp127as	7	M		Htr2a	14	P	
Ipw	7	M	+	Igf2r	17	P	
Ube3a	7	P	+	Igf2ras/Air	17	M	
H19	7	P	+	Impact	18	M	
Igf2	7	M	+	Ins1	19	M	

Source: Moore (2001), adapted from www.mrc.mgu.ac.uk

Nutritional effects

Extremes of nutrition appear detrimental to growth and survival of embryos in several species. In gilts, although ovulation rates are increased following feeding of a high plane of nutrition prior to mating, litter size is rarely affected (Aherne and Kirkwood, 1985). This implies that increased embryo mortality has occurred under these circumstances. Similarly, in embryo transfer experiments in pigs, a higher embryonic loss was observed in gilts fed *ad libitum* prior to mating compared with the control gilts (Bazer *et al.*, 1968). In sheep, when embryos were transferred from uniformly fed ewes to recipients consuming 50 or 150% of maintenance rations, embryo mortality was lower in recipient ewes receiving the lower plane of nutrition (Bazer *et al.*, 1968). Feeding either a high or low plane of nutrition during early pregnancy is also detrimental to embryo survival. Cummings *et al.*, (1975) reported that embryo survival was maximal in ewes receiving maintenance rations, rather than 25 or 200% of maintenance. In pigs, there is evidence for a detrimental effect of feeding a high plane of nutrition in some studies with gilts (Dyck and Strain, 1983; Kirkwood and Thacker, 1988), but not in sows (Toplis *et al.*, 1983). During lactation, increased embryo mortality has been documented for sows with low feed intake (Hughes *et al.*, 1984; Kirkwood *et al.*, 1987).

An adverse relationship has been observed between plane of nutrition and circulating progesterone concentrations after mating in ewes (Rhind *et al.*, 1985; Williams and Cummings, 1982) and gilts (Dyck *et al.*, 1980; Prime *et al.*, 1988). It is believed that increased embryo mortality in well-fed animals is due to a decrease in plasma progesterone levels as the restoration of nutritionally induced reductions in plasma progesterone concentrations by administration of progesterone improved embryo survival in both ewes (Parr *et al.*, 1987) and pigs (Ashworth, 1991). Wilmut *et al.* (1986) concluded that changes in progesterone profiles after mating were critical for the establishment of pregnancy in ewes.

Asynchrony between embryos and the uterus

The establishment of pregnancy depends upon a very close synchrony between the developmental stage of the embryos and the corresponding developmental stage of the uterus. In litter-bearing species, embryos that are delayed in their development are at a disadvantage because more advanced conceptuses induce changes in the uterine environment that are detrimental to survival of developmentally retarded conceptuses (Pope *et al.,* 1990). For example, transfer of asynchronous pig embryos to an advanced uterus for only 4 to 6 hours is detrimental to their subsequent survival (Geisert *et al.,* 1991; Jarrell *et al.,* 1990), or when both small and large conceptuses are present in the same litter, and embryonic deaths are at the expense of small conceptuses (Pope, 1988).

Paternal effects

It has generally been accepted that early embryonic mortality is an important contributor to reproductive failure, and is usually considered to be due to hormonal or physiological malfunction in the female. However, there is increasing evidence suggesting that paternal factors can also play a vital role in the survival of the embryo in the uterus. Burfening *et al.* (1977) reported the levels of embryonic mortality of 19 and 11% respectively from ewes mated to rams born from dams selected for low and high prolificacy.

Maternal heat stress

Embryonic death has been reported to be influenced by maternal heat stress at different stages, during the periovulatory period (Biggers, 1987; Putney *et al.,* 1989), before d 7 of gestation (Monty and Racowsky, 1987), and during early pregnancy (Ryan *et al.,* 1992; DelaSota *et al.,* 1998). High temperatures are especially harmful when combined with elevated humidity (Ulberg and Sheenan, 1973). Monty and Racowsky (1987) reported that dairy cows subjected

to high seasonal temperatures had embryos which were less viable at the morula and blastocyst stages (days 6 to 8) than embryos obtained from cows during the cool season. Similarly, gilts subjected to high ambient temperature during the first 8 or 15 d after mating experienced reduced pregnancy rates and had a higher percentage of embryonic mortality (Edwards *et al.,* 1968; Omtvedt *et al.,* 1971). Edwards (1974) suggested that direct effects of heat on dividing cells were the primary cause of embryonic death after fertilization. Additionally, Dziuk (1992) reported that high embryo mortality rate could be due to a direct effect of a high uterine temperature on the embryo and the shunting of blood away from the uterus to the periphery in an attempt to maintain body temperature, resulting in a reduced nutrient load. The altered progesterone/estradiol ratio can also be a cause of embryonic death as Kreider *et al.,* (1978) reported that plasma concentration of progesterone increased while concentrations of estradiol decreased after gilts were exposed to an ambient temperature of 35°C for the first 8 days after mating.

Embryonic deaths in females subjected to heat may be also a result of chromosome abnormalities as it is known that chromosome abnormalities of the gametes can adversely affect embryonic development, and hence embryo survival. It is possible that heat stress adversely affects oocyte maturation and/or induces an increased frequency of chromosome abnormality as Lenz *et al.* (1983) reported that heat stress during the late stages of oocyte maturation disrupted oocyte meiotic maturation to metaphase II. These ova may be capable of being fertilized, but the resulting embryos develop abnormally and die. Putney *et al.* (1989) found that a 10-h period of heating in superovulated heifers from immediately after onset of estrus and before insemination resulted in severe retardation of development of the embryos and a marked increase in embryonic death at day 7. In cattle, Putney *et al.* (1988) showed that embryos collected from dairy heifers subjected to elevated temperature of 42°C had only

20.7% normal embryos on day 7, compared with 51.5% normal embryos in heifers housed at 20°C.

Other factors regulating embryonic mortality

Embryonic wastage in farm animals can also be affected by other factors, including genotype. In sheep, the Romanov has a lower embryonic mortality than the Finn at the same ovulation rate (Ricordeau, 1986). Among the purebred pigs, the Chinese Meishan appears to have higher rate of embryonic survival than the conventional breeds (Wilmut *et al.,* 1992). Age of animals also affects embryonic mortality. Blockey *et al.* (1975) reported that a lower embryonic loss was observed in mature ewes than in younger maiden ewes. Embryo mortality rates also depend on the number of ova ovulated. In ewes, embryo survival decreases as ovulation rate rises to three (Fahmy, 1989; White, 1981).

2.4 Heat effects on testes and male reproduction

In order to maintain normal production of spermatozoa and testicular androgens, and other activities in the testis, the temperature in the scrotal testis in most species of animals must be lower than that of the body. This physiologically low temperature is believed to be regulated by two thermoregulatory systems, the scrotum and the pampiniform plexus (Mieusset and Bujan, 1995). A rise in scrotal temperature has been reported to reduce the fertility of male animals in a number of species (Heitman *et al.,* 1984; Rathore, 1970), including humans (Comhaire, 1983; Turner, 1983). The rates of infertility depend on the degrees of the heat (mild or strong heating) and the length of time the animals have been exposed to heat.

2.4.1 Testis weight

A reduction in testis weight has been observed in heat-stressed males when either their testes were heated directly (Bowler, 1972; Bergh and Damber, 1984; Setchell *et al.,* 1998; Yin *et al.,* 1998) or their whole bodies were heated (Meistrich *et al.,* 1973; Gomes *et al.,* 1971). The decrease in testis weight was believed to be due to the degeneration of cells in the seminiferous epithelium (Chowdhury and Steinberger, 1964) as evident by the reduction in size of the seminiferous tubule diameter (Hochereau-de Reviers *et al.,* 1993).

The extent of the testis damage varies depending on the degrees of heat stress. Gomes *et al.* (1971) reported a reduction in testis weight to about 70% of control values when the entire body of rams was exposured to a hot environment for 14 days. A 50% reduction in testis weight was observed when testes were heated locally at 42°C for 30 min (Mice: Sailer *et al.,* 1997) or for 45 min (Rams: Setchell *et al.,* 1991; Hochereau-de Reviers *et al.,* 1993).

The effects of heat stress on testis weight can be detected at different days after heating depending on levels of temperature, types of animals, and age of animals in the same species. In induced-cryptorchid males, a reduction in testis weight occurred by day 2 in young rats (Shikone *et al.,* 1994), by 5-7 days in adult rats (Clegg, 1963; Fujisawa *et al.,* 1988) and by day 7 in mice (Meistrich *et al.,* 1973; Yin *et al.,* 1998). The minimum testis weight was reported between days 7 and 28 after heating in rats whose testes had been exposed to a single episode of heat at either 41°C for 60 min or 43°C for 30 min.

2.4.2 Germ cells

It is well established that an increase in testicular temperature is closely related with a reduction in testis weight (see 2.4.1). This decrease in testis weight is believed to be due to the degeneration of germ cells in seminiferous epithelium (Chowdhury and Steinberger, 1964,

1970) by means of apoptosis (Sinha Hikim *et al.,* 1995; Blanco-Rodriguez and Martinez-Garcia, 1997). It has been reported that in rats the germ cells most susceptible to heat stress are the pachytene, diakinetic and dividing spermatocytes, and the young spermatids (Chowdhury and Steinberger, 1970). However, there have been reports suggesting that some other types of germ cells can also be affected by heat treatment. Allan *et al.* (1987) reported that heating the testis locally resulted in increased apoptosis of spermatogonia. The decrease in the number of sperm head counts in mouse testes measured 28 days after heating (Gasinska and Hill, 1990) also indicates that spermatogonia are not unaffected by increased testicular temperature because according to the spermatogenic cycle at the time of heating the germ cells are at the A spermatogonia stage.

2.4.3 Endocrine secretions

There are a number of reports suggesting that heat stress can cause changes in endocrine secretions in male animals in various species. Most studies involving the whole body heating reported the reductions in blood testosterone concentrations and increases in blood cortisol concentrations providing that the heating temperature is high and the period of heat exposure is long enough. Decreased serum testosterone concentrations in males exposed to a hot environment have been found in rats (Bedrak *et al.,* 1980), bulls (Rhynes and Ewing, 1973; Minton *et al.,* 1981), rams (Gomes *et al.,* 1971) and boars (Larsson *et al.,* 1983; Wettemann and Desjardins, 1979). This decrease in testosterone may be one of the many factors that can affect semen quality of the males as very high levels of testosterone in the testes are known to be necessary for normal testicular and epididymal functions. Indeed, testosterone injections in boars inhibited the increased production of abnormal spermatozoa during the hot summer months (Nakayama *et al.,* 1991).

While low levels of blood testosterone were observed in males animals subjected to elevated ambient temperature, various levels of testosterone were found in animals whose testes had been heated locally, depending on species, age of animals and periods after heating. Scrotal insulation in bulls (Prabhakar *et al.,* 1990; Sidibe *et al.,* 1992) or heating rat testes locally in a water bath for 30 min to 43°C (Damber *et al.,* 1980; Galil and Setchell, 1988) produced little effect on serum testosterone concentrations. In rams subjected to scrotal insulation for 28 days the decrease in blood levels of testosterone was found after day 14 of treatment (Byers and Glover, 1984). Scrotal insulation suppressed the levels of testosterone in blood in mature boars but not in immature boars (Malmgren, 1990).

The production of cortisol and luteinizing hormone (LH) has also been reported to be affected by heat treatment. Blood cortisol concentrations significantly increased after boars were exposed to a hot environment of 35°C for 100h (Larsson *et al.,* 1983). Likewise, increased blood LH concentrations were found with cryptorchid rats (Gupta *et al.,* 1975) and rams (Lunstra and Schanbacher, 1988), and rams subjected to scrotal insulation (Byers and Glover, 1984).

2.4.4 Sperm production and quality

It is generally accepted that thermal stress adversely affects semen quality of animals in a number of species. A reduction in numbers of live sperm ejaculated was observed in bulls after the local heating of the scrotum for 1 or 3 days (Moule and Waites, 1963; Gerona and Sikes, 1970). Exposure of rams to high temperature leads to increased percentages of abnormal spermatozoa and a reduction in both sperm concentration and percentage of motile sperm (Howarth, 1969). Similar results, i.e. decreased sperm output and percentage of motile spermatozoa, were observed in boars (Stone, 1982), bulls (Meyerhoeffer *et al.,* 1985), men (French *et al,* 1973; Mieusset *et al.,* 1987), and rats (Blackshaw *et al.,* 1973). It has been

reported that in boars semen quality deteriorates about 2 wk after whole body exposure to increased ambient temperature (Wettemann *et al.,* 1976). The time for the sperm motility to return to normal values varies between species, for example 5 weeks after the end of heat stress in boars (Wettemann *et al.,* 1979), 8 weeks in bulls (Meyerhoeffer *et al.,* 1985), and 4 weeks in rams (Rathore and Yeates, 1967). Rathore and Yeates (1967) reported that heating of rams for two days (8 h daily) at 40.5°C resulted in the appearance of periform sperm, while in boars abnormal spermatozoa show proximal cytoplasmic droplets, abnormal heads and coiled and bent tails (Nakayama *et al.,* 1991).

The adverse effect of increased testicular temperatures on sperm characteristics has been well documented. If the rise in temperature is great enough and if it is maintained over a sufficient period of time, both of which vary for different animal species, sperm with poor quality and/or low quantity will be produced. Sperm parameters, which are normally measured for such assessment, include sperm concentration, motility rates, percent live sperm and percent normal sperm.

Of these sperm parameters, motility rate is likely to be the first character that is affected by heat stress. In stallions subjected to scrotal insulation for 1 or 2 days, a fall in sperm motility was observed within a few days but decreases in sperm concentrations and percent normal sperm were seen only after day 10 (Freidman *et al.,* 1991). Similar results were observed when the scrotal insulation technique was employed in bulls (Austin *et al.,* 1961; Ross and Entwistle, 1979; Wildeus and Entwistle, 1986; Vogler *et al.,* 1991, 1993; Karabinus *et al.,* 1997), and in rams (Braden and Mattner, 1970; Mieusset *et al.,* 1992).

Changes in sperm characteristics are also obtained when testes of rats and mice are heated by immersion in a water bath. Mice whose testes had been heated to 40°C for 60 min produced spermatozoa with increased abnormality rate (Sailer *et al.,* 1997). When the heating

temperature was increased to 42°C for a shorter period, 20 min, a lower number of sperm in the epididymis was observed (Jannes *et al.,* 1998). Increasing the heating period to 60 min, at 42°C, resulted in very few spermatozoa produced (Sailer *et al.,* 1997).

Heating the whole body of male animals including men also adversely affects sperm production. Bulls exposed to high ambient temperature have been reported to produce sperm with high numbers of abnormal and dead cells, low motility and reduced concentrations (Casady *et al.,* 1953; Skinner and Louw, 1966). Similar results were observed in rams (Dutt and Hamm, 1957; Moule and Waites, 1963; Smith, 1971).

Changes in sperm parameters due to heat treatment can be detected at different times after heating and persist for a different length of time depending on the temperatures and the durations of exposure to heat. They can be seen from the first week after heating, in rams (Moule and Waites, 1963; Smith, 1971), in bulls (Skinner and Louw, 1966) and in mice (Sailer *et al.,* 1997), and this depressing effect could last for 70 days in rats (Mauss, 1971).

2.4.5 Fertility

The harmful effects of heat stress on male reproduction have long been recognized, and the effects are known to be manifest via disrupted spermatogenesis, which then results in low sperm output and/or poor semen quality. The extent of unfavourable effects on fertility varies between individuals, and depends on the degrees of heat stress. In the field conditions where the environments are not easily controlled, low fertility during summer months ranges from poor conception rates (buffaloes: Bahga and Gangwar, 1988; cows: Ray *et al.,* 1992; pigs: Stone, 1982; Love *et al.,* 1995) to low farrowing rates (pigs: Love *et al.,* 1995). In the more controlled conditions, in the laboratory, heating the whole body of male animals has also been reported to be associated with poor reproduction. In rams exposed to an ambient temperature of 32°C and relative humidity of 65% for 4 days, reduced fertility was observed in week 2 and

sterility in week 3 (Howarth, 1969). Rathore (1970) found that fertilization rates of rams subjected to heat stress at 40.5°C for one, two, three or four days, 12h per day, were decreased to 66, 42, 23, and 6%, respectively, compared to 93% in controls. Boars exposed to whole body heat stress have been reported to produce low pregnancy rates in normal sows in both natural mating and artificial insemination (Wettemann *et al.,* 1976, 1979). In mice, reduced pregnancy rates (Garriott and Chisman, 1981) and fertilization rates (Burfening *et al.,* 1970) were observed in normal females mated to heated males.

The fact that whole body heating can adversely affect not only testis functions but also other systems related to fertility, led to a number of studies that heated only the testes/scrotum of animals to investigate if heat treatment had a direct effect on testis functions and fertility. The results of these studies showed clearly that poor fertility in male animals was caused directly by malfunctions of the testes due to heat stress. If the testes are exposed to very high temperatures (greater than core body temperature) sterility is more likely to occur. Sterility was reported in male mice whose testes had been heated at 42°C for 20 min (Setchell *et al.,* 1998), in rats at 44.3°C for 20 min (Elfving, 1950) and in rams at 40.5°C for 2h (Braden and Mattner, 1970). Reduced fertility was frequently seen when testes were heated at lower temperatures. For example, reduced fertilization rates were observed with rams whose testes were insulated for 5 weeks, 8h per day (Ekpe *et al.,* 1992, 1993).

2.4.6 Embryonic mortality

While moderate/strong heating in male animals can kill most or all of the germ cells in the testis, which subsequently affects fertilization rates in normal females, mild heating seems not to affect the production of normal appearing spermatozoa. However, there have been reports suggesting that even though the spermatozoa from mildly heat-exposed males are capable of fertilization, they are incapable of producing viable zygotes. In an experiment in ewes,

Mieusset *et al.* (1992) found a 50% decrease in the number of viable embryos in ewes mated to rams whose scrotal temperatures had been increased by only 2°C. Similar results were observed in other species, including mice (Burfening *et al.,* 1970; Bellve, 1972), rats (Setchell *et al.,* 1988), and rabbits (Burfening and Ulberg, 1968; Howarth *et al.,* 1965).

The mechanisms by which paternal heat stress influences embryonic mortality are not known. Bishop (1964) suggested that lethal factors could be carried by the spermatozoa in the form of mutant genes or structural chromosome aberrations. This proposal is supported by the data showing the association between a large proportion of embryonic mortality and the presence of known chromosome anomalies (Mcfeely, 1967). Chromosomal abnormalities may induce embryonic mortality through abnormal embryo development. Bellve (1973) suggested that following exposure of males to high ambient temperature, a major portion of the embryonic death that had occurred by d 10 of gestation was a result of a developmental retardation and/or arrest at the morula stage.

CHAPTER 3

GENERAL MATERIALS AND METHODS

CHAPTER 3. General materials and methods

3.1 Reagents

All reagents used in this study were purchased from the Sigma Chemical Co, St. Louis, MO, USA, except Pregnant Mare Serum Gonadotropin and Human Chorionic Gonadotropin which were purchased from Intervet (Australia) Pty. Ltd., Sydney, NSW, and Histolene which was purchased from from Fronine Pty. Ltd., Sydney, NSW, Australia.

3.2 Experimental animals

3.2.1 Type and strain of animals

Mice were used throughout this study. The use of these animals was approved by The University of Adelaide Animal Ethics Committee. C57BL males and CBA females were purchased from the Central Animal House, Adelaide University for use as breeders. They were kept and bred in the Small Animal House Department of Animal Science, the University of Adelaide. The F1 crossbred offspring from these 2 strains were used as experimental animals. C57BLxCBA is a common cross used in laboratory studies of reproduction and development because the F1 animals are highly prolific and breed well.

3.2.2 Animal maintenance

All breeders and experimental mice were maintained in a temperature-controlled room at 21°C, and on a 10/14 hours dark/light cycle. They were given a commercial pelleted diet (manufactured by Ridley Agriproducts Pty., Ltd., SA, Australia) ad libitum and had a free access to water. Each breeder was kept individually in a standard mouse cage. During a mating period, one male was caged with one female. They were allowed to mate until the signs of pregnancy were observed (swollen womb), after which the male was returned to its

own cage. Pups were weaned at 21 days old, then sexing was performed, and males and females were separated and kept in groups of 10-15.

3.3 Heating procedures

3.3.1 Scrotal heating

A water bath equipped with a stirrer and a thermometer was used to heat the testes of male mice. Mice were held in an empty plastic syringe barrel with a slot cut along one side and a hole in the base for the tail to pass through. Mice were restrained by tying up their tails with the post attached to the syringe. When the temperature of the water in the water bath was stable at the required level (42°C), six syringe barrels, each with a mouse inside were arranged so that only the testes, scrota and tail of the mice were immersed in the warm water.

3.3.2 Whole body heating

Before heating mice, the cabinet was pre-heated for 12 hours. When the required temperature was obtained, mice were placed into the cage inside the cabinet. Due to the limited size of the cabinet, only 16 mice were heated each time. Food and water were available ad libitum throughout the period of heating. The heating cabinet and the equipment used to monitor temperatures and humidity inside the cabinet are shown in Figure 3.1. The cabinet was made of steel and Perspex with doors from four sides, and was transparent in order that mouse activities could be monitored clearly without opening the cabinet. This heating cabinet was kept in a temperature-controlled room to ensure that the stable temperature inside was maintained throughout the heating period. The temperatures inside the cabinet were recorded regularly (every 15 min to 1 hour). To allow calibration of the unit, a series of temperature measurements were made over 2 day periods with the controller set between 34-36°C, as per table 3.1-3.3.

Figure 3.1 Photographs showing the heating cabinet used for heating mice (plate A) and the equipment used to monitor temperatures and humidity (Plate B)

Plate A The heating cabinet used to heat the whole body of mice was equipped with a heater (A) (EWT heater, Model 01S, Australia), a universal programming controller (B) (R&H Wholesale (SA) Pty. Ltd., Australia), wet and dry bulb thermometers (C), Tele-thermometer probes (D), mercury thermometers (E) and mouse cages (F). Two buckets of water (G) were placed inside the cabinet to maintain a constant humidity.

Plate B Wet-and dry-bulb thermometers (A) were hung in the middle of the cabinet to measure the relative humidity. To monitor the temperature, two mercury thermometers were placed on two locations in the cabinet: the first one (B) on the floor of the cage and the other one (C) next to the dry bulb thermometer. Additionally, five wires sensors of a Tele-thermometer system (D) were placed at 5 locations within the cabinet: 2 (E) on the floor of the cages, 2 (F) in the middle of the cages and 1 (G) in the middle of the cabinet next to the dry-bulb thermometer.


A
B
E
C
F
E
A
G
G

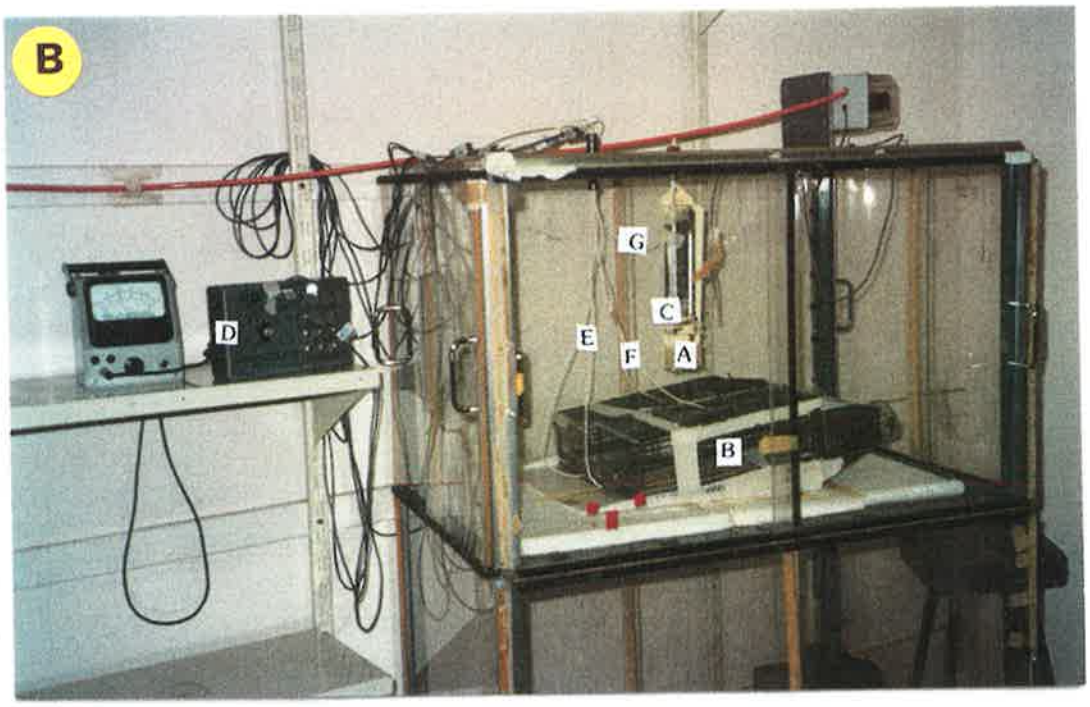

B
G
C
D
E
F
A
B

Table 3.1 Recorded temperatures set for 34°C

Time	Dry-bulb Thermometer (D) (°C)	Wet-bulb Thermometer (W) (°C)	Tele Thermo-meter (°C) *	Mercury Thermometer (°C)		D-W (°C)	Relative Humidity (%)
				Middle cabinet	On cage's floor		
Day1							
10:30	34.0	28.0	33.6	34.2	33.9	6.0	63
10:45	34.0	27.5	33.5	34.2	34.1	6.5	61
11:00	34.0	28.5	33.8	34.5	34.2	5.5	66
11:30	34.0	28.0	33.8	34.2	34.1	6.0	63
12:00	34.0	28.0	33.8	34.2	34.1	6.0	63
13:30	34.0	28.0	33.6	34.2	33.8	6.0	63
14:45	34.0	28.0	33.9	35.0	34.5	6.0	63
15:00	33.9	29.0	33.8	34.6	34.4	4.9	69
15:15	34.0	30.0	33.9	34.6	34.6	4.0	75
16:00	34.0	30.0	33.9	34.8	34.6	4.0	75
16:30	34.0	30.0	33.9	34.6	34.5	4.0	75
17:30	34.0	29.5	33.9	34.8	34.2	4.5	72
18:30	34.0	29.5	33.9	34.8	34.5	4.5	72
20:00	34.0	28.5	33.9	34.8	34.5	5.5	66
22:45	34.0	28.5	33.9	34.8	34.5	5.5	66
M ± SD	34.0±0.0	28.7±0.9	33.8±0.1	34.6±0.3	34.3±0.3	5.3±0.9	67.5±5.1
Day2							
10:00	33.8	25.0	33.8	33.9	33.8	8.8	49
10:15	33.8	26.0	33.8	34.0	33.5	7.8	54
10:30	34.0	27.0	33.9	34.4	34.5	7.0	58
11:00	34.0	28.0	33.9	34.4	33.8	6.0	63
12:00	34.0	28.5	33.9	34.4	34.5	5.5	66
13:00	34.1	29.0	33.9	34.5	34.8	5.1	69
14:00	34.1	29.0	34.0	34.5	34.8	5.1	69
15:00	34.0	29.0	34.0	34.1	34.1	5.0	69
16:00	34.0	29.0	33.8	34.1	34.1	5.0	69
17:00	34.0	29.5	33.8	34.1	34.0	4.5	72
18:00	34.0	27.5	33.6	34.1	34.0	6.5	61
M ± SD	34.0±0.1	28.0±1.4	33.9±0.1	34.2±0.2	34.2±0.4	6.0±1.4	63.5±7.3

* The values recorded in the middle of the cabinet, next to the dry bulb thermometer.

Table 3.2 Recorded temperatures set for 35°C

Time	Dry-bulb Thermometer (D) (°C)	Wet-bulb Thermometer (W) (°C)	Tele Thermo-meter (°C) *	Mercury Thermometer (°C)		D-W (°C)	Relative Humidity (%)
				Middle cabinet	On cage's floor		
Day1							
09:15	35.0	27.0	35.0	36.0	35.5	8.0	54
09:45	35.0	26.0	34.5	34.8	34.8	9.0	49
10:00	35.2	25.8	34.5	35.0	34.9	9.4	47
10:15	35.0	27.2	34.5	35.0	34.9	7.8	55
12:00	35.0	28.5	34.5	35.2	35.0	6.5	61
12:45	35.0	28.0	34.5	35.2	35.2	7.0	59
14:30	35.0	28.8	34.5	35.2	35.2	6.2	62
15:15	35.0	29.0	34.5	35.2	35.2	6.0	64
16:45	35.0	29.0	34.5	35.1	35.1	6.0	64
17:45	35.0	29.0	34.8	35.4	35.4	6.0	64
21:45	34.8	30.0	34.4	35.4	35.4	4.8	70
M ± SD	35.0±0.1	28.0±1.4	34.6±0.2	35.2±0.3	35.1±0.2	7.0±1.4	59.0±7.0
Day2							
09:30	35.0	28.5	34.0	34.9	35.2	6.5	61
10:30	35.0	29.5	34.5	35.2	35.2	5.5	67
11:00	35.0	30.0	34.5	35.4	35.8	5.0	69
13:00	35.0	29.0	34.5	35.0	35.0	6.0	64
15:00	35.0	27.5	34.5	35.2	35.5	7.5	56
17:00	35.0	28.5	34.5	34.9	35.0	6.5	61
17:30	35.0	28.5	34.5	34.9	35.1	6.5	61
21:30	35.0	28.0	34.5	34.9	35.1	7.0	59
24:00	35.0	28.0	34.5	34.8	34.8	7.0	59
M ± SD	35.0±0.0	28.6±0.8	34.4±0.2	35.0±0.2	35.2±0.3	6.4±0.8	61.9±4.1

* The values recorded in the middle of the cabinet, next to the dry bulb thermometer.

Table 3.3 Recorded temperatures set for 36°C

Time	Dry-bulb Thermometer (D) (°C)	Wet-bulb Thermometer (W) (°C)	Tele * Thermo-meter (°C)	Mercury Thermometer (°C)		D-W (°C)	Relative Humidity (%)
				Middle cabinet	On cage's floor		
Day1							
09:45	36.0	29.0	35.4	35.8	36.0	7.0	59
10:30	36.2	30.5	35.6	35.8	36.0	5.7	67
12:30	36.0	28.0	35.4	36.0	36.0	8.0	54
14:30	36.0	29.5	35.5	36.0	36.1	6.5	62
17:30	36.0	30.0	35.6	36.0	36.2	6.0	64
21:45	36.0	30.5	35.8	35.9	36.1	5.5	67
M ± SD	36.0±0.1	29.6±1.0	35.6±0.2	35.9±0.1	36.1±0.1	6.5±0.9	62.2±5.0
Day2							
09:45	36.0	29.0	35.5	36.1	36.0	7.0	59
10:15	36.0	28.5	35.4	36.0	36.2	7.5	57
12:00	36.0	30.0	35.5	36.0	35.8	6.0	64
15:00	36.0	28.0	35.2	36.0	36.0	8.0	54
17:45	36.0	29.0	35.5	36.1	36.1	7.0	59
21:45	36.0	29.5	35.5	36.1	35.9	6.5	62
M ± SD	36.0±0.0	29.0±0.7	35.4±0.1	36.1±0.1	36.0±0.1	7.0±0.7	59.2±3.5

* The values recorded in the middle of the cabinet, next to the dry bulb thermometer.

3.4 Media used

The Bicarbonate-buffered HTF and Hepes-buffered HTF (human tubule fluid) media (Quinn *et al.*, 1985) were used throughout this study, including for in vivo and in vitro fertilization trials. The media were made up of stock solutions A, B, C, D or E (Tables 3.4-3.8).

Stock solutions

Table 3.4 The compositions of Stock solution A

Reagents	Methods
1. NaCl 5.931g	1. The reagents no.1-6 were weighed and put in a glass bottle, and no. 7 was added. 2. The bottle was gently shaken, then no. 8 was added. 3. The solution was filtered in a laminar flow cabinet using a 22-μm pore syringe filter. 4. The solution was kept in a glass bottle at 4°C, and used within 3 months.
2. KCl 0.350g	
3. KH_2PO_4 0.050g	
4. $MgSO_4.7H_2O$ 0.050g	
5. Glucose 0.500g	
6. Penicillin 0.060g	
7. Milli Q water 96.30ml	
8. Sodium lactate 3.70ml	

Table 3.5 The compositions of Stock solution B

Reagents	Methods
1. $NaHCO_3$ 0.210g	1. The reagents no.1-3 were put in a glass bottle. 2. The bottle was gently shaken. 3. The solution was filtered in a laminar flow cabinet using a 22-μm syringe filter. 4. The solution was kept in a Falcon tube at 4°C and used within 2 weeks
2. Phenol Red 2/3 grains	
3. MilliQ water 10 ml	

Table 3.6 The compositions of Stock solution C

Reagents	Methods
1. Sodium pyruvate 0.051g 2. MilliQ water 10 ml	1. The reagents no.1-2 were put in a glass bottle. 2. The bottle was gently shaken. 3. The solution was filtered in a laminar flow cabinet using a 22-μm syringe filter. 1. The solution was kept in a Falcon tube at 4°C and used within 2 weeks

Table 3.7 The compositions of Stock solution D

Reagents	Methods
1. $CaCl_2.2H_2O$ 0.262 g 2. MilliQ water 10 ml	1. The reagents no.1-2 were put in a glass bottle. 2. The bottle was gently shaken. 3. The solution was filtered in a laminar flow cabinet using a 22-μm syringe filter. 4. The solution was kept in a Falcon tube at 4°C and used within 3 months.

Table 3.8 The compositions of Stock solution E

Reagents	Methods
1. HEPES 3.254g 2. Phenol red 2/3 grains 3. MilliQ water 50 ml	1. The reagents no.1-2 were put in a glass bottle. 2. MilliQ water (35 ml) was added. 3. The bottle was gently shaken. 4. The pH of the solution was adjusted to 7.5 with 1N HCl. 5. MilliQ water (15 ml) was added. 6. The solution was filtered in a laminar flow cabinet using a 22-μm syringe filter. 7. The solution was kept in a glass bottle at 4°C, and used within 3 months.

Table 3.9 Media preparations

Stock solutions	HCO_3-buffered HTF (HTF-Bicarb)	Hepes-buffered HTF (HTF-Hepes)
Stock A	10.0 ml	10.00 ml
Stock B	10.0 ml	1.60 ml
Stock C	0.71 ml	0.71 ml
Stock D	1.15 ml	2.57 ml
Stock E	0.00 ml	8.40 ml
MilliQ water	78.10 ml	78.10 ml
Total	**100 ml**	**100 ml**

Methods

1. The media were prepared 24h before use.
2. MilliQ water was put in a glass bottle.
3. All stock solutions were added, with stock D added last.
4. Osmolarity of the solutions was measured using a Vapor Pressure Osmometer, Model 5520 (Wescor, USA), and adjusted to 285 mmol/kg (mOsM). To increase osmolarity, drops of stock A were added, and to reduce osmolarity drops of milliQ water were added.
5. BSA (5 mg/ml) was added to the top of the media and allowed to slowly dissolve in the media.
6. The pH was adjusted to 7.4 with 1N HCl.
7. The media were filtered into Falcon tubes using a 22-μm syringe filter.
8. The media were equilibrated in a culture incubator (in an atmosphere of 5% CO_2 in air) overnight at 37°C before use.

3.5 Sperm Preparation

3.5.1 Sperm preparation for a histological study and analysis of the number of sperm heads

1. Male mice were killed by carbon dioxide inhalation, after which their testes and epididymides were removed.
2. Left testes were fixed in Bouin's fixative and kept in 70% Ethanol for histological studies.
3. Right testes were frozen at –20°C for subsequent analysis of the number of sperm heads.

3.5.2 Sperm preparation for analysis of sperm motility, concentration and viability, and for *in vitro* fertilization

1. Male animals were killed by cervical dislocation 1.5h prior to egg collection.
2. Their reproductive tracts were removed, vas deferentia and epididymides separated and placed in 35-mm tissue culture dishes containing 2 ml of HTF-Hepes medium.
3. Each epididymis was transferred to a 500-ul drop of HTF-Bicarb (pregassed overnight with CO_2) under oil.
4. The epididymis was punctured using a 26g needle, and slight pressure applied to release spermatozoa.
5. The spermatozoa were allowed 20 minutes to disperse, then the sperm samples were taken for the analysis of motility rate, concentration and the number of live/dead sperm.
6. The sperm suspensions were diluted in HTF-Bicarb medium to give a final motile sperm concentration of 1-2x10^6 /ml, and left in an incubator (37°C, 5% CO_2 and 95% air) for 1.5-2 h to capacitate.

3.6 Analysis of sperm characteristics

3.6.1 Sperm head counts

To assess testicular sperm production, the numbers of sperm heads (spermatid nuclei, steps 12 to 16 of development) were counted following sonication of the testis as per Meistrich and van Beek (1993). The procedures adopted were as follows:

1. The frozen testes (3.5.1) were taken out of the freezer and thawed.
2. The tunica albuginea of each testis was removed using a razor and 2 tweezers.
3. Each testis was placed in a tube containing 2 ml of deionized water.
4. The testis was sonicated using Microson Ultrasonic Cell Disruptor (Model XL 2000, output power 100 watts, output frequency 22 khz) for 60 seconds at a setting of 6.
5. An aliquot of the resultant suspension was drawn and transferred into both chambers of a haemocytometer.
6. The number of sperm heads were counted using a microscope with phase contrast optics.

3.6.2 Sperm concentration assessment

3.6.2.1 Spermicidal solution (1% formaldehyde in physiological saline)

200 mg of formaldehyde was dissolved in 20 ml of physiological saline, and the solution was stored at 4°C in a refrigerator until use.

3.6.2.2 Methods

The numbers of epididymal spermatozoa per ml and per epididymis were determined using standard methods with a Neubauer haemocytometer. The procedures were as follows:

1. Sperm suspensions (3.5.2) were diluted 10 times in the spermicidal solution (3.6.2.1).
2. The preparations were mixed well using a micropipette.
3. Both chambers of a haemocytometer were filled with the preparations.
4. Spermatozoa were allowed 20 min for to precipitate onto the haemocytometer.

5. The number of spermatozoa was counted in 5 small squares of the 1 mm middle square (4 corner squares and 1 middle square) under the microscope using a x40 objective. Both chambers of the haemocytometer were counted and an average taken.
6. Calculations:

   The 1-mm middle square (25 small squares) of the haemocytometer has a volume of $1x10^{-4}$. Hence, sperm concentration can be calculated as follow:

   A = the number of spermatozoa in 5 squares

   B = the number of spermatozoa in 25 squares (Ax5)

   C = dilution factor (=10)

   D = total volume (ml)

   E = the number of spermatozoa per ml = $BxCx10^4$

   The total number of spermatozoa per epididymis = DxE

3.6.3 Percent live sperm using Nigrosin/Eosin stain

The measurement of percent live sperm was performed using the Nigrosin/Eosin staining method as described in Section 3.6.3.2. Live spermatozoa would not take up stain. They, therefore, appeared clear or light pink while the dead spermatozoa stain dark purple.

3.6.3.1 Stain preparation

1. Nigrosin (10 g) was dissolved in 40 ml of boiled water.
2. Eosin, yellowish, Gurr (0.67 g) was then added to the solution.
3. The mixture was allowed to cool down, then water was added to 100 ml.
4. The mixture was filtered into a glass bottle using filter paper (Wattman No. 540), and kept at 4°C in the refrigerator.

3.6.3.2 Slide preparation and assessment

1. The stain (75µl) was drawn into a conical tube, and heated in a water bath at 37°C.
2. An aliquot of sperm suspension (75µl) was added to the stain.
3. The mixture was incubated for 5 min at 37°C.

4. The smears were prepared by placing a 7-µl drop of the mixture onto one end of preheated clean slide, then another slide was placed close to the stain (just touch) at 30° to the plane axis and drawn across towards the other end of the first slide.
5. The slides were allowed to air-dry, then shaken in a mixture of 1:1 5% v/v acetic acid: 0.6 N- perchloric acid for 10 to 30 sec, then washed off in running tap water.
6. They were immersed briefly in ethanol and then ether to remove the water, then were air-dried and covered using DPX as a mountant.
7. Finally, they were observed under light microscope using a x40 objective for live (clear, no stain) and dead (dark purple) spermatozoa.

3.6.4 Sperm acrosome integrity using triple staining

The triple stain technique was performed as described by Talbot and Chacon (1981). The advantages of this technique, according to Risopatron *et al.* (2001), are: 1) specimens can be preserved for a long time; 2) it can be performed in less well-equipped laboratories as it is neither expensive nor time-consuming; 3) it also allows determination of sperm morphology.

3.6.4.1 Dye preparation

1% Trypan blue in culture medium containing 12 mg/ ml BSA at 37°C

- Bovine serum albumin (BSA)-containing medium was prepared freshly by dissolving 120 mg BSA in 10 ml of HTF-Hepes medium.
- Trypan blue (50 mg) was dissolved in 5 ml of the medium.
- The solution was centrifuged at 500g for 5 min prior to use.

0.8% Rose Bengal in deionized H_2O, pH 5.3, 24°C

- Deionized H_2O was heated to 24°C.
- Rose Bengal (2 g) was mixed with 250 ml of the heated water.
- The solution was adjusted to pH 5.3 using 2N HCl.
- The solution was centrifuged at 500g for 5 min prior to use.
- The stain was kept at room temperature and used within 1 week.

0.8% Bismarck brown in deionized H_2O, pH 2.3, 40°C

- Deionized H_2O was heated to 40°C.
- Bismarck brown (2 g) was mixed with 250 ml of the heated water.
- The solution was adjusted to the pH of 2.3 using 2N HCl.
- The solution was centrifuged at 500g for 5 min prior to use.
- The stain was kept at room temperature and used within 1 week.

3.6.4.2 Fixative preparation

3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4)

- 0.1 M Cacodylate buffer was prepared by adding 1.6g sodium cacodylate to 100 ml of deionized water
- 0.1M cacodylate buffer (88 ml) was mixed with 12 ml of 25% glutaraldehyde solution.
- The solution was adjusted to pH 2.3.

3.6.4.3 Staining Methods

1. All dyes were centrifuged in a microcentrifuge prior to use.
2. Mice were killed and their sperm were collected and prepared as per the procedure described in Section 3.5.2.
3. Sperm concentration was adjusted to $5x10^5$/ml in HTF-Hepes medium.
4. 200-µL droplets of the sperm suspensions was mixed 1:1 with the 1% Trypan blue solution for 10 min under oil at 37°C.
5. The mixture was transferred to a microcentrifuge tube and diluted to 1.5-ml with culture medium.
6. The mixture was centrifuged at 500g.
7. The residual trypan blue was removed and the sperm were washed once with BSA-free culture medium.
8. The sperm were then fixed for 30 min in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4).
9. After fixation, the sperm were washed at least twice in deionized water and air-dried onto clean glass slides.
10. The slides were gently stained in 0.8% Bismarck brown for 5 min and washed with distilled water.

11. They were then stained in 0.8% Rose Bengal for 15 min, and subsequently washed in distilled water, dehydrated in ethanol, and cleared in xylene.
12. They were mounted with a cover-slip, and finally observed using oil immersion bright-field optics at x100 objective.

3.6.5 Sperm morphology using eosin staining

In order to observe sperm morphology, spermatozoa were stained with 5% aqueous eosin using the methods described in Section 3.6.5.2.

3.6.5.1 Dye preparation (5% aqueous eosin, w/v)

The stain was prepared by adding 500 mg of eosin, Gurr to 10 ml of water, and it was kept in a glass bottle and stored in a refrigerator at 4°C.

3.6.5.2 Staining methods

1. A 7-µl drop of sperm suspensions was placed on each warm clean slide, and the smears were made as the procedure described in Section 3.6.3.2.
2. The smears were air-dried, and fixed for 1h in acetic acid alcohol (a mixture of 3:1, absolute Ethanol:Glacial acetic acid).
3. The smears were stained with 5% (w/v) aqueous eosin, air-dried and covered using DPX as a mounting medium.
4. The slides were examined under an oil-immersion at x100 objective.
5. The percentages of normal and abnormal heads and tails were determined in 200 spermtozoa from each male.

3.6.6 Sperm motility using light microscopy

Sperm motility rates and motile sperm concentrations were determined using a Neubauer haemocytometer and a light microscope

1. Sperm suspersion was diluted 20 times (dilution factor = 20) by adding 10µl of sperm suspension to 190µl of warm (37°C) HTF-Hepes medium (see Table 3.9).
2. The mixture was then mixed gently.
3. Both chambers of a warm haemocytometer were filled with 10-µl aliquot of diluted sperm suspension.
4. The haemocytometer was placed on a warm stage equipped microscope.
5. The number of motile and non-motile spermatozoa was counted in all 25 small squares in the 1-mm middle square (10^{-4} ml).
6. Percent motile sperm and the concentration of motile sperm per ml were calculated as follow:

   A = the total number of spermatozoa (motile and non-motile) in 25 squares.

   B = the number of motile spermatozoa in 25 squares

   C = dilution factor

   Percent motile spermatozoa $= \frac{B \times 100}{A}$

   Motile sperm per ml $= B \times C \times 10^4$

3.6.7 Sperm motility using a computer aided sperm analysis system (CASA)

The computer aided sperm analysis (CASA) was performed using the Hamilton Thorne Sperm Motility (HTM) analyzer, HTM-2000 (Hamilton Thorn Research Inc., Denvers, USA) to analyze motility of mouse epididymal spermatozoa. This machine can possibly be used with various animal species. However, the set-up parameters of the machine must be adjusted to suit each type of animal species. Below are the parameters used for mice. They were adjusted for a number of times until the motility rate obtained was comparable to that from the conventional method (using a haemocytometer). The set-up parameters (Table 3.10) were, then subsequently used in the experiment.

3.6.7.1 Methods

1. The machine was switched on and approximately 5-10 min was allowed until the required temperature inside the machine (37.0°C) was obtained.
2. A 10-µl aliquot of epididymal sperm suspensions was drawn and placed into a Makler counting chamber (Sefi-Medical Instruments, Israel) and covered with a coverslip.
3. The chamber was placed on the heated stage and loaded into the machine
4. Six to nine fields which were free of or contained the least debris were manually selected before the machine started to analyze the samples. The motility rates were recorded in a database file on a computer attached to the HTM.
5.

Table 3.10 The set-up parameters of the HTM analyser system for determining sperm motility in mice.

Temperature	37.0°C
Chamber	Makler (10µL)
Image type	Phase contrast
Field selection	Manual selection
Calculate ALH	yes
Frames at frame rate	20, at 7/sec
Minimum contrast	4
Minimum size	5
Lo/Hi size gates	0.9, 1.5
Lo/Hi intensity gates	0.9, 1.5
Non motile head size	8
Non motile intemsity	90
Medium VAP value	50
Low VAP value	10
Slow cells motile	No
Threshold STR	60

3.6.8 Increasing motile sperm concentration by a swim-up procedure.

1. Male mice were sacrificed by cervical dislocation.
2. Epididymides were dissected from each male to a dish containing 2ml of HTF-Hepes medium (see Table 3.9).
3. Fat pad was removed and blood was drained away from the epididymides.
4. The pair of cauda epididymides were transferred into a dish containing 400 µl of HTF-Bicarb medium (see Table 3.9) under oil, and punctured with a 27G needle.
5. Sperm suspensions were drawn from the dish and slowly deposited to the bottom of the tube containing 3 ml of HTF-Bicarb medium.
6. The tube was incubated at 37°C in an atmosphere of 5% CO_2 in air to allow sperm to swim up into the medium.
7. After 20 min of incubation, the sperm-rich top fraction (1ml) was pipetted off for use in an IVF assay.

3.7 Tissue fixation

3.7.1 Testis fixation for light microscopy (LM)

In order to fix testes for a histological study, mouse testes (see Section 3.5.1) were immersion-fixed in Bouin's fixative overnight using standard procedures.

3.7.1.1 Bouin's fixative

1. Saturated Picric acid solution (21g+1000ml distilled water) 75 ml
2. 40% formaldehyde 25 ml
3. Glacial acetic acid 5 ml

All the ingredients were mixed, and the solution was kept in a plastic container and stored at room temperature.

3.7.1.2 Fixation methods

1. The whole freshly removed testis from each male was put in 2ml of Bouin's fixative in a plastic tube.
2. After 24-h fixation, the fixative was removed and the testis was washed twice with 70% ethanol.
3. The testis was left in 70% ethanol until analysis.

3.7.2 Testis fixation for transmission electron microscopy (TEM)

3.7.2.1 Phosphate buffer (0.2M, pH 7.4) for 200 ml

1.	0.2 M Na_2HPO_4	154.0 ml
2.	0.2 M NaH_2PO_4	46.0 ml

Both ingredients were mixed and the solution was adjusted to pH 7.4.

3.7.2.2 1.5% Formaldehyde / 1.5%glutaraldehyde Fixative for 500 ml

1.	0.2M NaH_2PO_4	22.5 ml
2.	0.2M Na_2HPO_4	202.5 ml
3.	25% paraformaldehyde	30.0 ml
4.	25% glutaraldehyde	30.0 ml
5.	PVP-40	12.5 g

The fixative was made by mixing all of the ingredients. The pH of the fixative was then adjusted to 7.4, and water was added to obtain the required volume.

3.7.2.3 Rinse solution for 500 ml

1.	NaCl	4.5 g
2.	PVP-40	12.5 g
3.	Procain-HCl	2.5 g

All the ingredients were dissolved in water (500 ml) and the pH was adjusted to 7.4, then 0.3 ml of Heparin was added before use.

3.7.2.4 Fixation methods

The procedure for fixing the whole body of mice were as follows:

1. Mice were anesthetized by intraperitoneal injection of 0.05ml pentobarbitone sodium.
2. Abdominal skin and tissue were cut to expose the body cavity.
3. The right side of the heart was cut.
4. A perfusion needle (20G) was inserted into the left ventricle of the heart, and the fixative was released into the heart by gravity for 15min.
5. The scrotum was dissected and the testes and epididymides were removed.
6. The testis was then cut into small cubes (approximately 1 mm^3).

3.7.3 Embryo fixation

3.7.3.1 Methods

1. The fixative (4% paraformaldehyde) was prepared on the day of use by dissolving it (4g) in 100 ml PBS at 65°C.
2. Embryos were dissected out of the decidua and extraembryonic membrane.
3. Embryos were fixed in 3 ml of the fixative in a glass vial for 4h.
4. The fixative was removed, and the embryos were washed at 4°C as follows:
    - twice with 0.83% NaCl,
    - once with 100% ethanol+0.83% NaCl (1:1), and
    - twice with 70% ethanol
5. Embryos were left in 70% ethanol for 7 days, then weighed to 3-decimal places.

3.8 Histological process for light microscopy

3.8.1 Tissue processing

Testes were processed in an automatic tissue processing machine (Shandon, Citadel 2000; A.E. Stansen and company Pty. Ltd., Victoria) using standard processing programs for a total of 22 hours as follows:

1. Bouin's-fixed testes were washed overnight in 70% ethanol to get rid of the remaining fixative.
2. The testes were cut transversely to the long axis at the equator and one-half of each was put in a micro cassette and processed in the tissue processing machine.

1	70% Ethanol	1hr 30min
2	80% Ethanol	1hr 30min
3	90% Ethanol	2 hr
4	95% Ethanol	2 hr
5	95% Ethanol	2 hr
6	Absolute Ethanol	2 hr
7	Absolute Ethanol	2 hr
8	Histoclear	2 hr
9	Histoclear	2 hr

10	Histoclear	2 hr
11	Wax (Histoplast)	1 hr 30min
12	Wax	1 hr 30min

3. At the completion of processing, tissues were kept in the last container containing clean paraffin wax until embedding.

3.8.2 Tissue embedding

The processed testes (Section 3.8.1) were embedded in paraffin wax using a Tissue-Tek (TEC, Tissue Embedding Console System, Sakura, Finetek, USA) as the following procedure.

1. Clean embedding moulds were heated by putting on a warm plate.
2. The mould was filled with clean, filtered paraffin wax.
3. The processed one-half testis was placed in the middle of the mould with the cut surface facing down.
4. The mould was then placed on the cool plate.
5. The tissue was oriented and it was firmed into the wax with warmed forceps to ensure that the correct orientation was maintained.
6. The identifying label was placed on the top of the wax.
7. The block was removed from the mould when the wax had cooled and hardened.

3.8.3 Tissue sectioning

Sectioning was performed using a rotary microtome (Leitz 1512) with standard procedures.

1. The paraffin blocks were trimmed so that a block face size of approx. 1cm x 1cm was obtained, with sides parallel, and the tissue surrounded with 2-3 mm of wax.
2. A sharp knife was installed in the microtome, and the trimmed block was fitted into the block holder.
3. The block was coarse cut at 10 μm until the full face had been trimmed.
4. The block was then fine cut at 5 μm until a ribbon of 4-5 sections was obtained.
5. The ribbon was separated from the knife edge with a moist camel-hair brush and pulled across the surface of a warm water bath (60°C).
6. Four to five floating sections were collected onto a clean slide.
7. The slides were air-dried, ready for staining.

3.8.4 Tissue staining

Sections of the testes were stained with hematoxylin and eosin using standard procedures.

3.8.4.1 Lillee Mayer's Haematoxylin

1.	5 g	Haematoxylin
2.	50 g	Aluminium Ammonium Sulphate
3.	300 ml	Glycerol
4.	700 ml	RO Water
5.	1 g	Sodium Iodate
6.	20 ml	Glacial Acetic Acid

Haematoxylin was dissolved in a few volume of ethanol. Aluminium Ammonium Sulphate was dissolved in water with gentle heating. The solutions were then combined, and the remaining ingredients were added.

3.8.4.2 Acid Ethanol (1% conc. HCl in 70% Ethanol)

5 ml of conc. HCL was mixed with 495 ml of 70% Ethanol, then the mixture was kept in a glass bottle.

3.8.4.3 Eosin

1.	50 ml	1% Aqueous Eosin Yellowish
2.	390 ml	95% Ethanol
3.	5 ml	1% Aqueous Phloxine
4.	2 ml	Glacial Acetic Acid

3.8.4.4 Staining methods

1. Air-dried slides were put in staining racks.
2. The tissues were dewaxed, cleaned and stained in a series of solutions as follow:

1 Histoclear	15 min
2 Absolute Ethanol	2 min
3 80% Ethanol	2 min
4 30% Ethanol	2 min

5	Lillee Mayer's Haematoxylin	1.5 min
6	Tap Water	20 sec
7	Acid Ethanol	10 sec
8	Running Water	10 min
9	Eosin	7-10 sec
10	80% Ethanol	10 sec
11	Absolute Ethanol	2-4 min
12	Histoclear	5 min

3. The slide was covered with a cover-slip using DPX mounting medium.
4. The stained sections were viewed with a Leitz microscope (Leitz Wetzlar, Germany) under light-field optics.

3.9 Histological process for transmission electron microscopy (TEM)

3.9.1 Tissue processing/embedding

3.9.1.1 Methods

1. Testes were cut into small cubes (approximately 1 mm^2).
2. They were fixed in formaldehyde/glutaraldehyde fixative at room temperature for 2 to 4 hours, then washed in 0.2 M phosphate buffer twice, 15 min each.
3. Tissues were then post-fixed in 1% OsO_4 in phosphate buffer for 60 min, washed in 0.2 M phosphate buffer for 15 min, and dehydrated as follows:

30% ETOH	15 min
50% ETOH	15 min
70% ETOH	15 min
75% ETOH	15 min
80% ETOH	15 min
85% ETOH	15 min
90% ETOH	15 min
95% ETOH	15 min
100% ETOH	30 min
100% $CuSO_4$	60+60+30 min

4. They were washed in Propylene Oxide twice, 30 min each, and infiltrated in:

Propylene Oxide : Resin (2:1)	overnight
Propylene Oxide : Resin (1:2)	9am-4pm
Pure resin	overnight

5. They were then embedded next morning.
6. The resin block was polymerised at 60°C for 48 hours.

3.9.2 Staining for semi-thin sections

3.9.2.1 Toluidine blue solution

The solution was made by mixing 5 ml of 0.05% toluidine blue with 5 ml of 1% borax sodium tetraborate.

3.9.2.2 Staining methods

2. Approximately 3 drops of toluidine solution was placed on a clean warm slide.
3. The semi-thin (0.5 μm) sections were collected and transferred to the droplets of the solution on the slide.
4. The slide was dried on a hot plate for 2 min, then mounted using a coverslip and a mounting medium.

3.9.3 Staining for ultrathin sections

3.9.3.1 Staining methods

1. Lead citrate was centrifuged at 3000 rpm/min for 10min before use.
2. Droplets of filtered aqueous Uranyl acetate were prepared in a Petri dish, and the Petri dish was covered with a black box.
3. Droplets of Lead citrate were made in a Petri dish, which contain sodium hydroxide pellets (to absorb carbon dioxide).
4. One grid section was placed up side down in droplets of Uranyl acetate stain for 9 minutes.
5. The grids were taken out of the stain using forceps, then they were washed once in 70% ethanol and 3 times in distilled water.
6. The back of the grid and inside of the forceps were dried using filter paper.

7. The grid was placed in droplets of lead citrate for 9 min, then washed in 4 changes of distilled water.
8. The back of the grid and inside of the forceps were dried again.
9. The grid was placed in a dry labeled Petri dish.

3.10 Fertilization

3.10.1 In vivo fertilization

3.10.1.1 Protocols

Superovulation and mating

- Day 1 1800 h PMSG injection (7.5 I.U.)
- Day 3 2130 h hCG injection (5.0 I.U.)
- Day 3 2135 h Mating
- Day 4 0600 h Plug checks, and separation of the females from the males

Egg recovery and assessment

- Day 5 0930 h Egg collection, in HTF-Hepes medium
- Day 5 0935 h Egg Examination, in HTF-Hepes medium

3.10.1.2 Methods

1. Female mice were superovulated by intraperitoneal injections of 7.5 I.U. PMSG and 5.0 I.U. hCG 48-52h later.
2. Immediately after hCG injection, each female was paired with 1 male of the control or treatment groups.
3. The animals were examined next morning for copulatory plugs to determine whether mating had occurred.
4. The mated females were killed by cervical dislocation 36h post hCG.
5. The oviducts were removed and placed in a tube containing 2ml of HTF-Hepes medium.
6. They were then transferred into a 35-mm dish containing 2ml HTF-Hepes medium supplemented with 0.1% hyaluronidase, and torn to release ova using a sharp needle under a dissecting stereomicroscope (Olympus SZ40) at 30x magnification.

7. After the cumulus cells surrounding the ova were dispersed the ova were examined using a stereomicroscope.

3.10.2 In vitro fertilization

3.10.2.1 Protocols

Superovulation

- Day 1	1800 h	PMSG injection (5.0 I.U.)
- Day 3	2130 h	hCG injection (5.0 I.U.)

IVF Procedure

- Day4	0930 h	Sperm collection in HTF-Hepes
- Day4	1100 h	Egg collection in HTF-Hepes
- Day4	1130 h	Sperm-egg coincubation, in culture incubator

Staining and assessment

- Day4	1235 h	Egg transfer and wash using HTF-Bicarb
- Day4	1245 h	Egg fixation in Phosphate-buffered formalin
- Day4	1315 h	Egg staining using Orcein/acetic acid
- Day4	1320 h	Egg assessment under light/phase microscope

3.10.2.2 Egg preparation

1. Females were induced to superovulate by intraperitoneal injections of 7.5 I.U PMSG and 5.0 I.U hCG 48-52 h later.
2. The females were killed 13.5h post-hCG injection, then their oviducts removed and placed in a tube containing 2 ml of HTF-Hepes.
3. The oviducts were transferred to a 35-mm dish containing 2 ml of HTF-Hepes medium.
4. The cumulus bulge was torn under a dissecting stereomicroscope (Olympus SZ40) at 30x magnification with a 27G needle to release cumulus masses.
5. The masses were washed twice with fresh HTF-Bicarb medium.

3.10.2.3 Sperm preparation

1. Mice were killed and their sperm collected as the procedures described in Section 3.5.2.

2. A fertilization dish was made by placing a 50-µl droplet of capacitated sperm suspension in the middle of a 35-mm dish.
3. The dish was then overlaid with 2 ml of paraffin oil and left in an incubator (37°C, 5% CO_2 and 95% air) for 1.5-2 h to capacitate

3.10.2.4 Sperm and eggs incubation

1. Immediately after washing, two cumulus masses were carefully added from each female to the preincubated sperm suspension.
2. The eggs and sperm were coincubated for 65 mins at 37°C in 5% CO_2 in air.

3.10.2.5 Fertilization rate assessment

1. After 65-min coincubation, eggs were removed and washed through fresh medium to remove bound sperm, and then left in a 100-µl drop of HTF-Bcarb medium under oil.
2. At 75 minutes after incubation, the eggs were fixed with phosphate-buffered formalin.
3. After 1-h fixation the eggs were washed twice with fresh HTF-Hepes medium, then transferred to a clean slide.
4. As much as possible of the solution on the slide was sucked out, leaving the eggs in place, and then one drop of the stain (0.75% aceto-orcein) was added to the eggs.
5. The slide was covered with a cover slip.
6. The slides were assessed immediately for the sign of fertilization using a light and phase contrast microscope.

3.11 Sperm-egg interactions

3.11.1 Solutions

H-33342 (1mg/ml)

1. H-33342 (10 mg) was dissolved in 10 ml 0.85% NaCl
2. 100-µl aliquots were made and kept in the dark at -70°C in a freezer

Hyaluronidase (3 mg/ml)

1. Hyaluronidase (30 mg) was dissolved in 10 ml of deionized H_2O.
2. 1-ml aliquots were made and frozen at -20°C in a freezer.

3.11.2 Methods

1. Superovulated female mice were killed by cervical dislocation 13.5 h post hCG and their cumulus/oocytes clumps (COC) collected.
2. The COC was treated with 0.1% hyaluronidase for 3 min, then washed in fresh medium 2 times to remove loosely attached cumulus cells.
3. Cumulus-free oocytes were transferred to 3 ml of medium in a culture dish containing H-33342 (10 μg/ml) and incubated at 37°C for 30 min.
4. Male mice were killed 1.5h prior to oocyte collection.
5. Sperm were collected using the swim-up method as described in section 3.6.8.
6. A 150-μl sperm droplet was placed in a 35-mm culture dish.
7. The droplet was covered with paraffin oil and incubated at 37°C in a humidified incubator.
8. After 30-min incubation, oocytes were washed 3 times with fresh medium to remove excess stain outside the oocytes.
9. Approximately 20 eggs were transferred to a sperm droplet.
10. Oocytes were removed after the 20-min coincubation, washed once with fresh medium and then fixed in 2.5% glutaraldehyde.
11. After 10-min fixation oocytes were washed to remove unbound sperm, and then transferred to a clean slide.
12. Silicone gel was applied in circle around the oocytes to prevent the cover slip from crushing the oocytes.
13. The slides were observed under light and fluorescent microscope and photos were taken.

CHAPTER 4

EFFECTS OF HEAT STRESS ON TESTIS AND EPIDIDYMIS OF MALE MICE

4.1 Effects of scrotal heating on body and testis weights, and spermatogenesis of mice

Introduction

Low fertility in domestic animals has been reported to be associated with heat stress during summer months, and this adverse effect has been observed in both males (Yakayama *et al.*, 1991; Pena *et al.*, 1998) and females (Putney *et al.*, 1989; Love *et al.*, 1995; Rocha *et al.*, 1998). However, effects on the male have drawn a greater deal of attention as a single male has a greater impact than any single female on the number of offspring produced per year. In the field, where the entire body of the male is exposed to heat it is difficult to draw a conclusion as to whether poor fertility in the male is a result of the direct effect of heat on the testis or from an indirect effect on the testis via changes in other systems (eg. endocrine). In order to verify if poor fertility in heat-stressed males is a result of the direct effect of heat on the testis, a number of studies involving local heating of the testes have been undertaken over the years. In those studies testes were normally exposed to heat by one of 3 methods, namely scrotal insulation, experimental cryptorchidism, or immersion of the testes in hot water baths with adverse effects reported in various species. For example, scrotal insulation has been reported to increase sperm defects in bulls (Bartg and Bowman, 1994), decrease testis weight and the number of germ cells (Hochereau-Reviers *et al.*, 1993), and increase embryonic mortality rate in the rams (Mieusset *et al.*, 1992); immersion of the scrotum/testicles in a water bath at 42°C for 20 minutes damaged seminiferous tubules and disrupted spermatogenesis (Chowdhury and Steinberger, 1970; Miraglia and Hayachi, 1993), and decreased fertilizing capacity in the rat (Miraglia and Hayachi, 1993); heating the testis locally also suppressed spermatogenesis in humans (Mieusset *et al.*, 1987). In the mouse, scrotal heating has been

shown to reduce sperm head counts, (Reid *et al.,* 1981; Gasinska and Hill, 1990), testis weight and fertility (Reid *et al.,* 1981, Setchell *et al.,* 1998).

It is obvious that heating the testes locally adversely affects spermatogenesis and subsequent reproduction. To extend these observations by investigating the effects of whole body heating on male fertility, it was considered appropriate to undertake a preliminary study employing local testis heating initially to allow the investigator to establish experimental procedures and baseline criteria for heat-induced changes in testis function, and most importantly to confirm the adverse effect of heat stress in our specific laboratory environment and with the strain of animal employed.

Materials and Methods

A total of 30 adult male C57BL/CBA F1 mice were used in this study. In the control group, six mice were kept in their original room (21°C) without being heated. In the heated group, the scrota, tail and the hind part of the mice were heated by immersion in a water bath at 42°C (see Section 3.3.1) for 5, 10, 20, or 30 min (six mice for each period). After heating, the animals were returned to their original room. They were weighed once preheating and weekly post-heating. All mice were killed by carbon dioxide inhalation 3 weeks after heating, at which time their testes and epididymides were removed and weighed. The left testes and epididymides were placed in Bouin's fixative for 24 h and then washed with 70% ethanol and kept in this agent for subsequent histological studies (Section 3.7.1). The right testes were kept at -20°C in the freezer until analysed for sperm head counts.

Sperm head counts

To analyze the number of sperm heads, assessed from the number of sonication resistant spermatid nuclei in steps 12 to 16 of development, the frozen testes were taken out of the freezer, thawed and sonicated using a sonicator as described in section 3.6.1.

Histological procedures

Bouin-fixed testes were washed overnight in 70% ethanol, cut transversely to the long axis at the equator, put in micro cassettes and subjected to routine histological processing (see Section 3.8.1) before being embedded in paraffin wax (see Section 3.8.2). Blocks were then sectioned at 5 μm using a rotary microtome (see Section 3.8.3) and the resultant sections stained with hematoxylin and eosin (see Section 3.8.4).

Results

Body weight

Mean body weights between groups at days 0, 7, 14 and 21 after heating were not significantly different (Table 4.1). The mice in all groups gained weight. The gains ranged between 2.6 and 3.6 g during the 21-day period post-heating, and they were not significantly different between treatments.

Table 4.1 Mean weekly body weights and weight changes during a period of 21 days after testicles and scrota of male mice were heated locally by immersion in a hot water bath of 42°C for either 5, 10, 20 or 30 minutes. Values are mean ± SEM.

Treatment/ heating durations	No. of mice	Body wt (g)				Wt. changes (g) (between d0 and d21)
		day 0	day 7	Day 14	day 21	
Control	6	27.3±1.1	29.1±1.0	28.7±1.0	29.6±0.9	+2.6±0.7
5 min	6	27.6±1.0	29.0±0.9	29.5±1.1	31.4±1.0	+3.8±0.4
10 min	6	26.4±0.5	28.0±0.7	27.9±0.8	29.5±0.8	+3.1±0.6
20 min	6	27.1±0.8	28.5±0.9	29.2±1.0	30.0±1.0	+2.9±0.5
30 min	6	26.2±1.0	27.6±1.0	28.5±1.0	29.8±1.0	+3.6±0.2
P-value (one-way ANOVA)		0.7876	0.7604	0.8166	0.7373	0.4587

Testis and epididymis weights

The data on testis and epididymis weights are presented in Table 4.2. These parameters were measured at day 21 postheating. Testis weights were not significantly different between the control, 5-min and 10-min heating groups. When the period of heat exposure increased to 20 or 30 minutes, a significant decrease in testis weights was observed by day 21. Mean epididymis weight was lower in the 30-min heating group, but not in the other groups when compared to that of controls. However, this difference was not significant.

Table 4.2 Mean testis and epididymis weights at day 21 after testicles and scrota of male mice were heated locally by immersion in a hot water bath of 42°C for either 5, 10, 20 or 30 minutes. Values are mean ± SEM.

Treatment/ Heating durations	No. mice	Testis weight (mg)	Epididymis weight (mg)
Control	6	109.9±2.9[a]	41.6±2.1
5 min	6	114.9±4.8[a]	40.2±0.9
10 min	6	108.9±6.0[a]	38.8±3.0
20 min	6	90.6±3.8[b]	38.8±2.1
30 min	6	50.5±3.9[c]	33.6±1.9
P-value (one-way ANOVA)		0.0001	0.1192

[a, b, c] Numbers with different superscripts within one column are significantly different.

The number of sperm heads

The number of spermatids per testis in the control animals was $20.5x10^6$, and was similar in the 5-min group (Table 4.3). This suggests that this short period of heating the testis induces minimal if any effect on spermatogenesis. However, the adverse effect of heat stress on spermatogenesis was evident when the period of heating was increased to 10 minutes, as judged by a significant reduction in the number of spermatids produced.

As expected, increasing the period of heat exposure increased the amount of damage to spermatogenesis, with a progressively larger fall in sperm head counts in males heated for 20 and 30 minutes, respectively (Table 4.3).

Table 4.3 Mean numbers of sperm heads per testis at day 21 after testicles and scrota of male mice were heated locally by immersion in a hot water bath of 42°C for either 5, 10, 20 or 30 minutes. Values are mean ± SEM.

Treatment/ heating durations	No. mice	Sperm head counts per testis ($x10^{-6}$)
Control	6	20.5 ± 1.2^a
5 min	6	20.8 ± 1.9^a
10 min	6	14.7 ± 1.0^b
20 min	6	8.3 ± 0.5^c
30 min	6	2.3 ± 0.5^d
P-value (one-way ANOVA)		0.0001

$^{a, b, c, d,}$ Numbers with different superscripts within one column are significantly different.

Histology of the testis

The photomicrographs of seminiferous tubules of the males are presented in Figures 4.1 to 4.4. In unheated (control) males all seminiferous tubules contain all types of germ cells specific for each stage of spermatogenic cycle, and spermatids are present in every tubule (Figure 4.1). Heating the testis locally for 5 or 10 min in a water bath produced little or no effect on seminiferous tubules (Figure 4.2) with the histology of the testis being similar to that of controls.

The adverse effects of heat stress on seminiferous tubules were evident when testes of mice were exposed to heat for longer periods. Heating the testes for 20 or 30 minutes caused damage to all seminiferous tubules even though the degrees of damage was variable. All tubules of these heat-stressed males were smaller in diameter when compared to controls, as clearly shown in Figure 4.3. In some tubules germ cell loss was so severe that when assessed 21 days after heating no germ cells were observed. As a consequence, only Sertoli cell and spermatogonia could be seen. In less damaged tubules even though almost all types of germ cells were present their numbers were reduced (Figure 4.4).

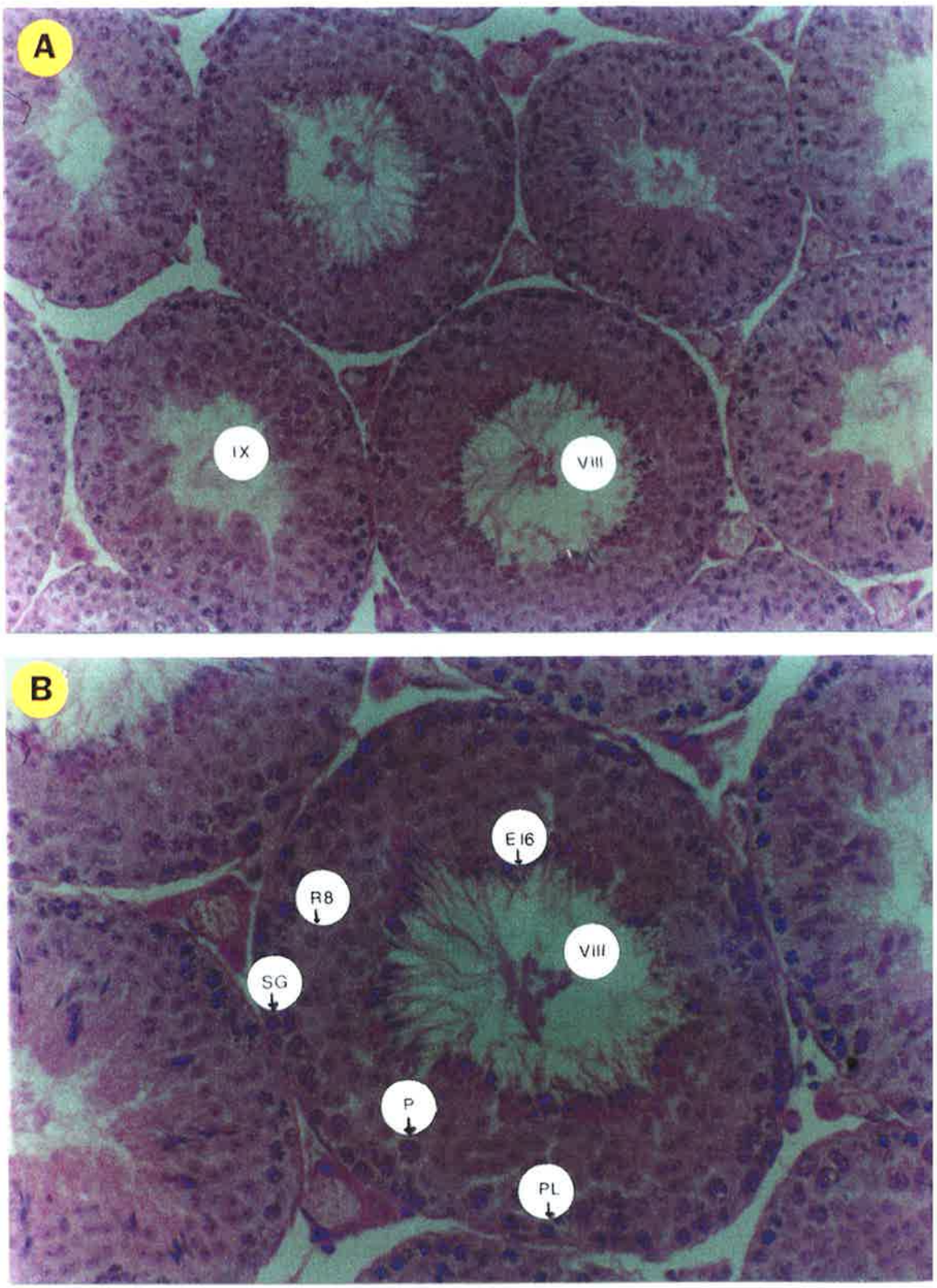

A
IX
VIII
B
E16
R8
VIII
SG
P
PL

Figure 4.1

Cross-sections of a control mouse testis at magnifications of 250x (A) and 400x (B). The sections show tubules with normal spermatogenesis. Every tubule contains spermatids and a range of other germ cells. At least 3 generations of germ cells are present in each tubule. For example, the stage-VIII tubule in the middle of plate B contains step 8 round spermatids (R8) step 16 elongated spermatids (E16), pachytene spermatocytes (P), leptotene spermatocytes (L) and spermatogonia (SG). The Roman numbers represent stages of spermatogenic cell cycle (Leblond and Clermont, 1952). The sections were stained with hematoxylin and eosin.

Figure 4.2

Cross-section of a mouse testis heated for 10 minutes at 42°C and excised 21 days later, showing the tubules with morphology similar to those of controls. These tubules have normal cell associations regarding quantity and germ cell types in each stage of the spermatogenic cell cycle. Abbreviation: IX stage 9 of spermatogenic cycle; R_9 step 9 round spermatids; ST spermatid; P pachytene; L leptotene. The sections were stained with hamatoxylin and eosin. Magnification 400x.

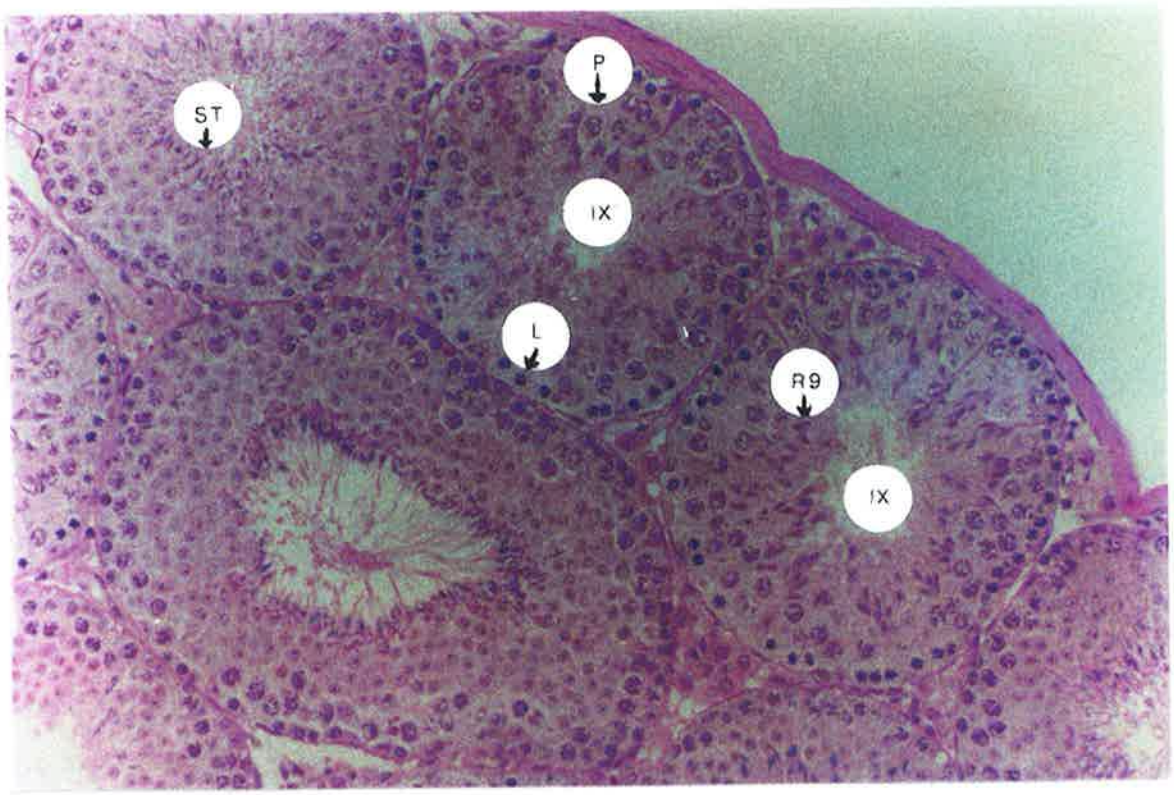

ST
P
IX
L
R9
IX

Figure 4.3

Cross-sections of testes from a control mouse (A) and from a mouse whose testes have been heated at 42°C for 30 min (B). The assessment was made at day 21 after heating. Even though size of the seminiferous tubules was not objectively measured it is obvious that the tubules in plate B have relatively smaller size in diameter than those in plate A (control). This suggests that there was an extensive loss of seminiferous epithelium in the heated testes. It is also clearly shown that the Leydig cell hyperplasia (L) occurred in the heated testis. The sections were stained with haematoxylin and eosin. Magnification 250x.

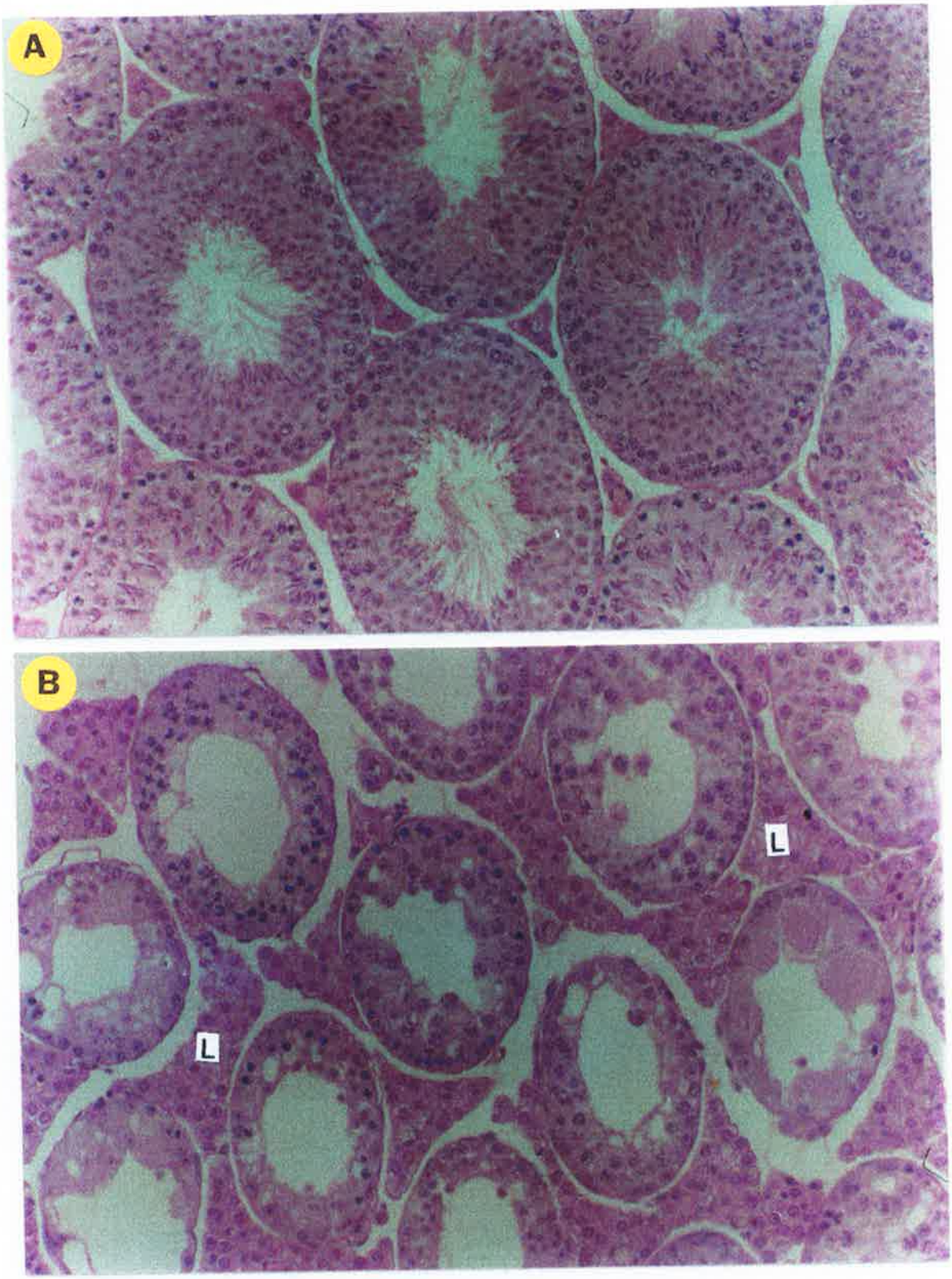
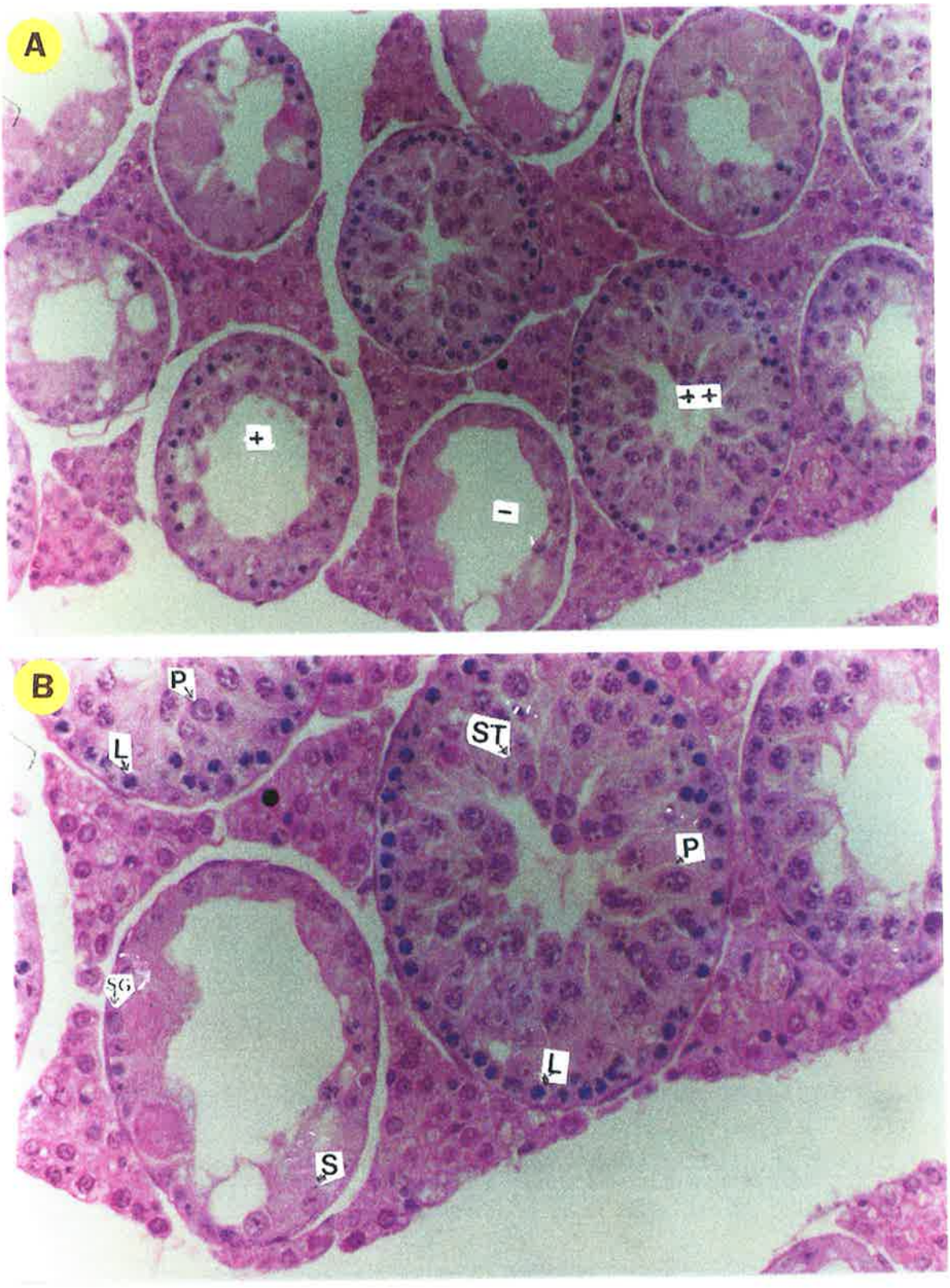

A
B
L
L

Figure 4.4

Cross-sections of mouse testes heated for 30 minutes at 42°C and excised 21 days later. Plate A shows severely damaged (-), moderately damaged (+) and slightly affected (++) tubules. In the severely damaged tubules only vacuoles, early-stage germ cells, and Sertoli cells are present, whereas in the moderately damaged tubules germ cells at more advanced stages are observed. Plate B shows types of cells in severely, moderately and slightly damaged tubules. Abreviation; SG spermatogonia, S Sertoli cell nucleus, L leptotene spermatocyte, P pachytene spermatocyte, ST spermatid. Magnifications 250x (A) and 400x (B).


A
+
+ +
-
B
P
L
ST
P
SG
L
S

Discussion

Because of its easy-to-handle characteristics and low cost of maintenance, the mouse has been a popular choice for various types of experiment. The adverse effects of localized heating of the testis on testicular functions have been reported in a number of mouse strains, including C 3 Hf/Kam (Reid *et al.*, 1981), CBA/Ht Gy f TO (Gasinska and Hill, 1990), C57BL/6JxC3H/HeJ (Sailer *et al.*, 1997), CBAxC57 (Setchell *et al.*, 1998). Throughout the present study the C57BL/CBA F1 mice were used. This preliminary study was undertaken to clarify the response of this mouse strain to heat stress. It is demonstrated from Table 4.2 that heating the testes locally at 42°C for 20-30 min resulted in a decrease in testis weight and a reduction in the number of sperm heads counted, but had no effect on body weight gain (Table 4.1). The losses of testis weight and number of sperm heads after heat treatment are believed to be due to the depletion of spermatogenic cells in seminiferous tubules, as clearly shown in figures 4.3(B) and 4.4(B). These findings are in agreement with the results of previous investigations, e.g. Gasinska and Hill, 1990; Van Zelst *et al.*, 1995; Sailer *et al.*, 1997. It is obvious that our selected mouse strain reacted to heat stress in the same manners as the strains used in previous studies.

4.2 Effects of heating the whole body of male mice at various temperatures and lengths of exposure time on the testis and spermatogenesis of mice

Introduction

The preliminary study demonstrated that heating mouse testes directly by immersing them in a water bath at 42°C for 20-30 minutes greatly reduced testis weight and sperm production. The fertility of these males was expected to be greatly affected by heat treatment, as a number of studies have reported the close relationship between scrotal heating, reduced sperm quality and/or quantity, and subsequent fertility (Setchell *et al.,* 1988, 1998; Reid *et al.,* 1981; Gasinska and Hill, 1990; Sailer *et al.,* 1997).

Similar results have been reported in a number of experiments when the entire body of the males were heated. However, most of those studies exposed males to heat at either very high temperatures (40.5°C: Rathore, 1970) or for long periods (1 to 5 days: Bellve, 1972, 1975; Burfening *et al.,* 1970; Garriott and Chrisman, 1981; Waldbieser and Chrisman, 1986).

The current experiment was designed to investigate the effect of heat in a more subtle situation i.e. repeated heating with a shorter exposure times. The whole body of mice were subjected to heat at a range of temperatures between 33°C and 36°C, at various durations from 8 to 24 hours, and at different frequencies of heating (1 to 3 times).

Materials and Methods

In an attempt to determine the minimal temperature and duration of exposure of heat that would produce clear effects on the testis, a total of 144 adult male mice (C57BLxCBA) were used. Four groups of 36 mice were allotted to 1 of 4 temperatures, namely 33°C, 34°C, 35°C or 36°C. These experimental temperatures were selected on the basis of results reported by Burfening et al. (1970) and Bellve (1972), but extended to a wider range by lowering the minimum temperature to 33°C and increasing the maximum temperature to 36°C. At each temperature level, 6 animals were randomly assigned to 1 of 6 treatments, namely control, 8h/d-1day, 8h/d-2days, 8h/d-3days, 12h/d-2days and 24h/d-1day. The whole body of each mouse was exposed to heat as per the procedure described in section 3.3.2. All mice were killed at day 21 after heating by carbon dioxide inhalation. Their testes were removed and fixed in Bouin's fixative (left testes) or kept at –20°C in a freezer (right testes). They were then processed for a histological study or for sperm head counts as per the procedures previously described in section 4.1 (Materials and Methods).

Results

Body weights and weight changes

During the course of the 21 days after heating, all animals heated at 33°C and 34°C gained weight, ranging between 4.2 to 5.2 g, and the mean weight gains are presented in Tables 4.4 and 4.5. These body weight gains were not significantly different between groups. At the higher temperature of 35°C and above, weight gains during the 21-day period were significantly lower ($p<0.005$) (Tables 4.6 and 4.7). The mean gains of between 3.3 to 4.5 g of the heated groups were significantly ($p<0.005$) lower than that of the control group (5.0 g). Among the treated animals, the (12+12) h group gained the least weight and this was significantly lower than in the other groups, but similar to that of the 24 h group. At 36°C, apart from the 12h group, the heated animals gained significantly less weight than the control group with the lowest value (+1 g) being in the (12+12) h group (Table 4.7). There was no result from the 36°C, 24h group because the animals suffered a great deal from heat stress, and the heating was terminated prematurely.

Table 4.4 Mean body weights and weight changes over the 21-d period post-heating of male mice exposed to whole body heating at 33°C in a temperature-controlled heating cabinet. Values are mean ± SEM.

Treatments/ heating durations	No. of mice	Body wt. (g)		Weight changes (g) Between d 0 and d 21
		day 0	day 21	
Control	6	27.0±0.4	31.8±0.5	+4.8±0.3
8h	6	25.7±0.5	30.8±0.3	+5.2±0.5
(8+8)h	6	26.2±0.7	30.7±1.0	+4.5±0.4
(8+8+8)h	6	27.3±0.7	31.8±0.5	+4.5±0.4
(12+12)h	6	26.3±0.7	31.3±0.8	+5.0±0.4
24h	6	28.2±0.6	33.2±0.4	+5.0±0.4
P-value (one-way ANOVA)		0.0684	0.1004	0.8052

Table 4.5 Mean body weights and weight changes over the 21-d period post-heating of male mice exposed to whole body heating at 34°C in a temperature-controlled heating cabinet. Values are mean ± SEM.

Treatments/ heating durations	No. of mice	Body wt. (g)		Weight changes (g) between d 0 and d 21
		day 0	day 21	
Control	6	27.5±0.7	31.7±1.5	+4.2±0.8
8h	6	25.8±0.6	31.0±0.6	+5.2±0.3
(8+8)h	6	29.2±0.7	34.2±0.7	+5.0±0.4
(8+8+8)h	6	27.8±0.8	32.2±0.9	+4.3±0.2
(12+12)h	6	27.5±1.0	31.7±1.1	+4.2±0.3
24h	6	28.0±0.9	32.7±1.3	+4.7±0.5
P-value (one-way ANOVA)		0.1414	0.3818	0.4931

Table 4.6 Mean body weights and weight changes over the 21-d period post-heating of male mice exposed to whole body heating at 35°C in a temperature-controlled heating cabinet. Values are mean ± SEM.

Treatments/ heating durations	No. of mice	Body wt (g)		Weight changes (g) between d 0 and d 21
		day 0	day 21	
Control	6	26.0±0.3	31.0±0.4	+5.0±0.3^{a}
8h	6	26.0±0.6	30.2±0.6	+4.2±0.2^{bc}
(8+8)h	6	26.0±0.9	30.5±0.7	+4.5±0.2^{ab}
(8+8+8)h	6	24.8±0.5	29.2±0.7	+4.3±0.3^{bc}
(12+12)h	6	25.7±0.4	29.0±0.5	+3.3±0.3^{d}
24h	6	25.2±0.5	28.8±0.7	+3.7±0.3^{dc}
P-value (one-way ANOVA)		0.5616	0.1045	0.0037

$^{a, b, c, d,}$ Numbers with different superscripts within one column are significantly different.

Table 4.7 Mean body weights and weight changes over the 21-d period post-heating of male mice exposed to whole body heating at 36°C in a temperature-controlled heating cabinet. Values are mean ± SEM.

Treatments/ heating durations	No. of mice	Body wt (g)		Weight change (g) between d 0 and d 21
		day 0	day 21	
Control	6	25.8±0.2	31.0±0.3	+5.2±0.3^{a}
8h	6	25.2±0.7	28.3±0.9	+3.3±0.3^{c}
(8+8)h	6	26.0±0.5	30.0±0.5	+4.0±0.4^{bc}
(8+8+8)h	6	25.8±0.6	30.5±0.8	+4.7±0.4^{ab}
12h	6	26.0±0.5	31.7±0.9	+5.7±0.4^{a}
(12+12)h	6	26.7±0.6	27.7±0.5	+1.0±0.4^{d}
P-value (one-way ANOVA)		0.5581	0.0017	0.0001

$^{a, b, c, d,}$ Numbers with different superscripts within one column are significantly different.

Testis weight, epididymis weight and sperm head counts

Heating at 33°C had no effect on testis and epididymis weights, and sperm head counts were also similar in the control and the heated groups (Table 4.8). At 34°C (Table 4.9), all the 8-h heating groups (heated 1, 2 or 3 times) had similar testis weight, epididymis weight, and the number of sperm heads as the controls. In the (12+12)h and 24h groups all the values measured were lower than those of the controls, even though the differences were not significant. When the temperature was raised to 35°C the effects of heat stress on the testis were clearly evident (Table 4.10). Exposure of the whole body of male mice to an elevated ambient temperature of 35°C at 8h per day for either 2 or 3 days, at 12h per day for 2 days or for 24h once, significantly reduced testis and epididymis weights and the number of sperm heads. Testis weight decreased by 21% and 32% in the (12+12)h and 24h groups, respectively. The considerable decreases in epididymis weight and sperm head counts were also observed in the (12+12) h and 24h groups. Epididymis weight and sperm head counts decreased by 24% and 67% of the control values, respectively. When the males were exposed to the ambient temperature of 36°C (Table 4.11) all heated groups exhibited significantly lower ($p<0.05$) testis weight, epididymis weight and sperm head counts than the control group, the lowest values being in the (12+12)h group. At this temperature, heating the mice even once for 8h adversely affected the testis and epididymis, with testis weight, epididymis weight and sperm head counts being at 85%, 83% and 63% of the control values, respectively. The greatest reductions in testis weight, epididymis weight and sperm head counts were found in the (12+12)h group, with the values being 59%, 69% and 22% of the controls, respectively.

Table 4.8 Testis and epididymis weights, and sperm head counts of male mice exposed to whole body heating at 33°C in a temperature-controlled heating cabinet. Values are mean ± SEM.

Treatments/ heating durations	No. of mice	Testis wt. (mg)	Epididymis wt.(mg)	No. of sperm head per testis ($x10^{-6}$)
Control	6	111.0±1.4	33.4±0.2	20.3±0.7
8h	6	108.1±2.1	32.6±0.5	19.2±1.2
(8+8)h	6	109.3±2.8	31.7±0.7	19.4±1.2
(8+8+8)h	6	107.5±1.5	32.1±0.6	19.5±0.6
(12+12)h	6	104.8±3.1	32.3±0.5	19.0±1.2
24h	6	108.3±1.8	33.2±0.6	18.6±0.4
P-value (one-way ANOVA)		0.5091	0.2405	0.8537

Table 4.9 Testis and epididymis weights, and sperm head counts of male mice exposed to whole body heating at 34°C in a temperature-controlled heating cabinet. Values are mean ± SEM.

Treatments/ heating durations	No.of mice	Testis wt.(mg)	Epididymis wt.(mg)	No. of sperm head per testis ($x10^{-6}$)
Control	6	106.2±2.1	31.7±0.6	20.8±1.1
8h	6	106.0±3.0	30.3±0.8	20.2±1.0
(8+8)h	6	107.6±3.0	33.3±0.7	17.7±1.0
(8+8+8)h	6	102.9±2.9	30.8±1.1	18.5±0.5
(12+12)h	6	96.8±4.5	28.7±1.3	16.8±1.6
24h	6	96.8±2.9	29.4±0.9	16.8±1.4
P-value (one-way ANOVA)		0.0657	0.0180	0.0805

Table 4.10 Testis and epididymis weights, and sperm head counts of male mice exposed to whole body heating at 35°C in a temperature-controlled heating cabinet. Values are mean ± SEM.

Treatments/ heating durations	No. of mice	Testis wt. (mg)	Epididymis wt.(mg)	No. of sperm head per testis ($x10^{-6}$)
Control	6	106.9 ± 1.3^{a}	32.0 ± 0.5^{a}	14.4 ± 0.2^{a}
8h	6	99.7 ± 6.0^{ab}	29.3 ± 1.4^{ab}	12.2 ± 1.0^{ab}
(8+8)h	6	92.1 ± 4.4^{bc}	27.5 ± 0.9^{bc}	10.5 ± 1.2^{c}
(8+8+8)h	6	91.1 ± 3.6^{bc}	26.2 ± 1.0^{cd}	9.4 ± 1.1^{c}
(12+12)h	6	84.7 ± 2.6^{c}	25.6 ± 0.9^{cd}	8.5 ± 0.8^{c}
24h	6	72.8 ± 2.0^{d}	24.4 ± 0.6^{d}	4.7 ± 0.7^{d}
P-value (one-way ANOVA)		0.0001	0.0001	0.0001

a, b, c, d, Numbers with different superscripts within one column are significantly different.

Table 4.11 Testis and epididymis weights, and sperm head counts of male mice exposed to whole body heating at 36°C in a temperature-controlled heating cabinet. Values are mean ± SEM.

Treatments/ heating durations	No. of mice	Testis wt. (mg)	Epididymis wt. (mg)	No. of sperm head per testis ($x10^{-6}$)
Control	6	107.7 ± 2.0^{a}	31.3 ± 0.4^{a}	16.5 ± 0.6^{a}
8h	6	91.8 ± 1.9^{b}	25.9 ± 0.5^{c}	10.4 ± 1.0^{b}
(8+8)h	6	88.8 ± 1.7^{c}	27.7 ± 0.5^{b}	8.1 ± 1.1^{bc}
(8+8+8)h	6	84.8 ± 3.0^{c}	26.3 ± 0.8^{b}	6.3 ± 1.3^{c}
12h	6	87.2 ± 1.7^{bc}	27.1 ± 0.6^{bc}	6.9 ± 0.5^{c}
(12+12)h	6	64.0 ± 2.8^{d}	21.7 ± 0.7^{d}	3.6 ± 1.1^{d}
P-value (one-way ANOVA)		0.0001	0.0001	0.0001

a, b, c, d, Numbers with different superscripts within one column are significantly different.

Histology of the testis

The histology of a testis from a control male is presented in Figure 4.5. It is clear that this testis contains normal seminiferous tubules with no sign of damage. All specific cell types for each stage of the spermatogenic cycle are present. Heating the whole body of male mice at either 33°C or 34°C for any durations from 8 to 24 hours had no obvious effect on testis histology. Figure 4.6 shows sections from the testis of male mice exposed for the longest duration (24 h) of heat to 33°C (Plate A) and 34°C (Plate B). The tubules in the testes of these mice look normal and contain all the expected germ cell types for any given stage of the spermatogenic cycle. At 35°C, after sperm head counts were performed, although a reduced number of spermatids was obvious (see Table 4.10) the changes in seminiferous tubule structure were not as easily detected by light microscopy. It is known that individual counts of germ cell types in tissue sections would be helpful in assessing subtle effects of heat on testis. The techniques are now quite advanced (Sun *et al.,* 1990; Yang *et al.,* 1990; Zhengwei *et al.,* Wreford *et al.,* 2001), but it was not possible to access the required facilities for this study. Additionally, this study was more concentrating on male fertility rather than testis anatomy. Therefore, sperm head counts were used as one quantitative measure by which to more accurately assess effects on spermatogenesis.

When the ambient temperature was raised to 36°C, the effects of heat stress on the testis were observed at all durations of heat exposure, even though the effect was less severe in the males heated once for 8h, and the majority of tubules were normal. As shown in figures 4.7 and 4.8 the obvious damages found in the tubules include vacuolization, and a lack of or reduced numbers of spermatids.

Figure 4.5

Cross-sections of untreated mouse testis at magnification of 250x (A) and 400x (B). The tubules show normal spermatogenesis, with all classes of germinal cells present in each stage of the spermatogenic cycle. Spermatogonia (SG), leptotene spermatocyte (L), pachytene spermatocyte (P), round spermatid (R) and elongated spermatid (ST). The sections were stained with hematoxylin and eosin.

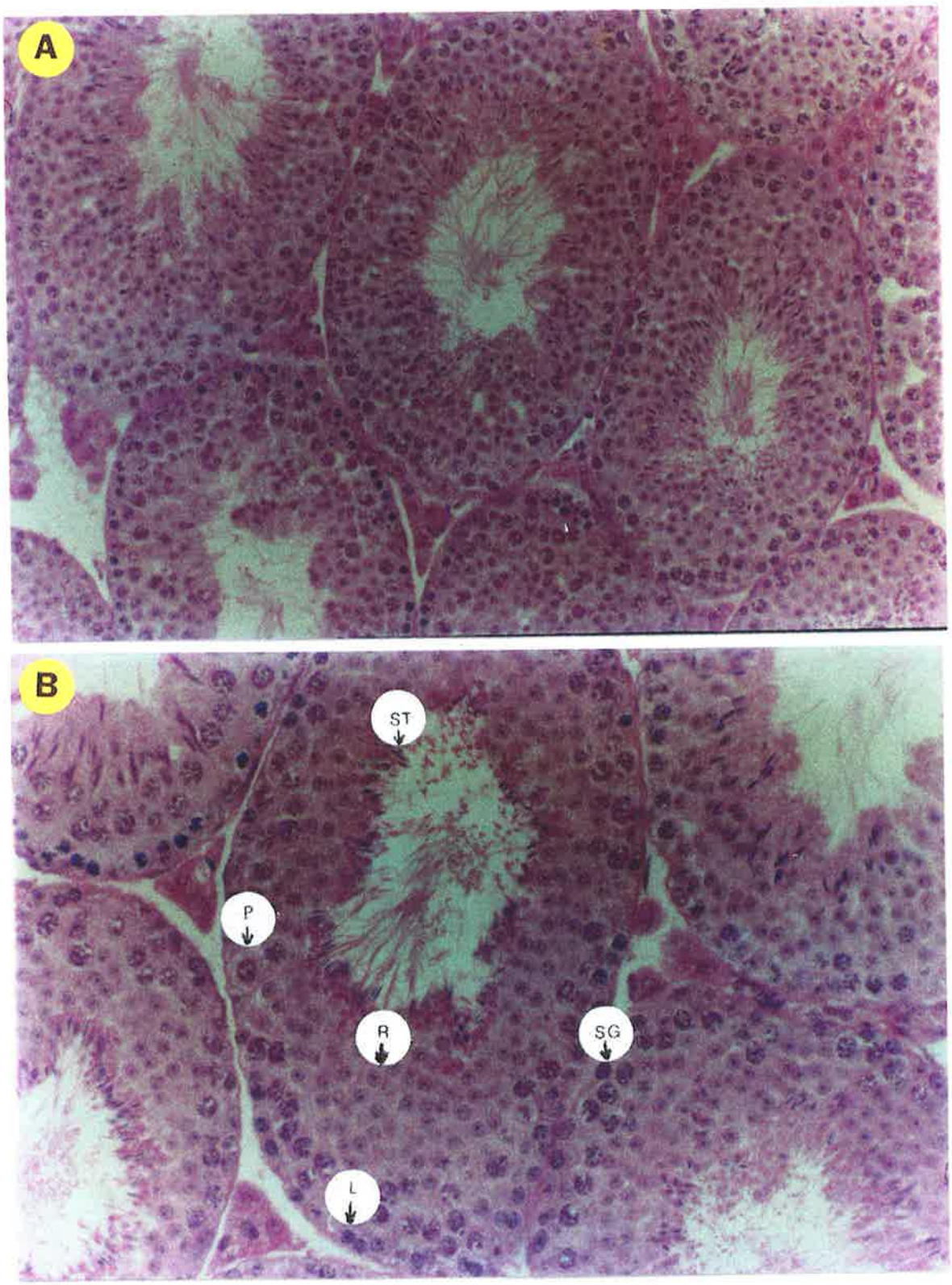

A
B
ST
P
R
SG
L

Figure 4.6

Cross-sections of testes from mice whose whole body was heated for 24h at 33°C (A) or 34°C (B), and excised 21 days later. Photomicrographs show normal appearance of the tubules. Every tubule contains spermatids at different stages and all other types of germ cells that are specific for the relevant stage of the spermatogenic cycle. Magnification 250x.

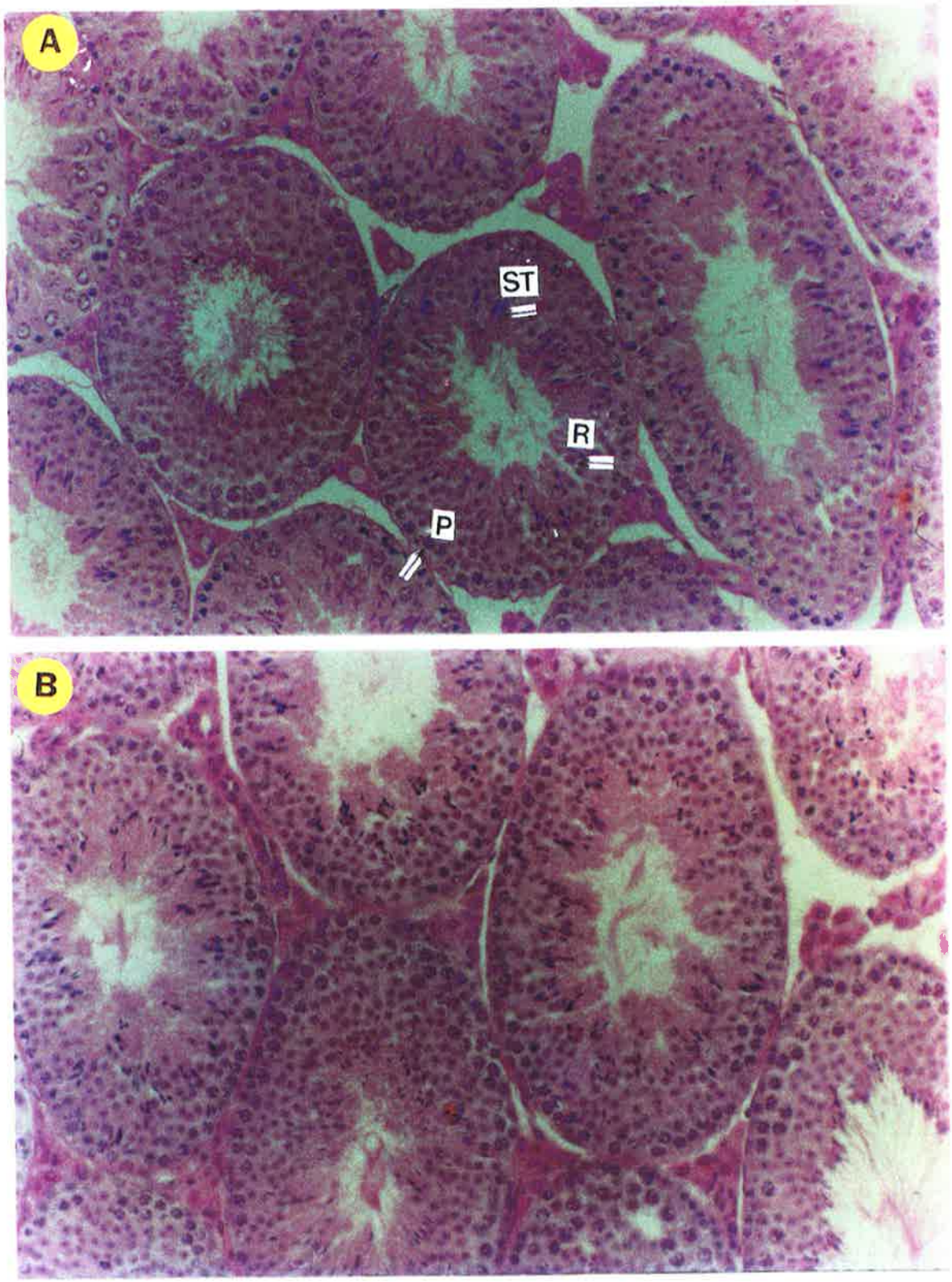

A
ST
R
P
B

Figure 4.7

Cross-sections of testes from mice whose whole body was heated at 36°C for 1 day, 8h (A) and for 2 days, 12h per day (B), and excised 21 days later. Plate A demonstrates an obviously damaged tubule (arrow) which contain vacuoles (V) and contains no spermatids, however the majority of tubules appear normal (N). Similar damage is shown in plate B where one tubule contains no spermatids, and there is some evidence of germ cell loss (*). Magnification 250x.

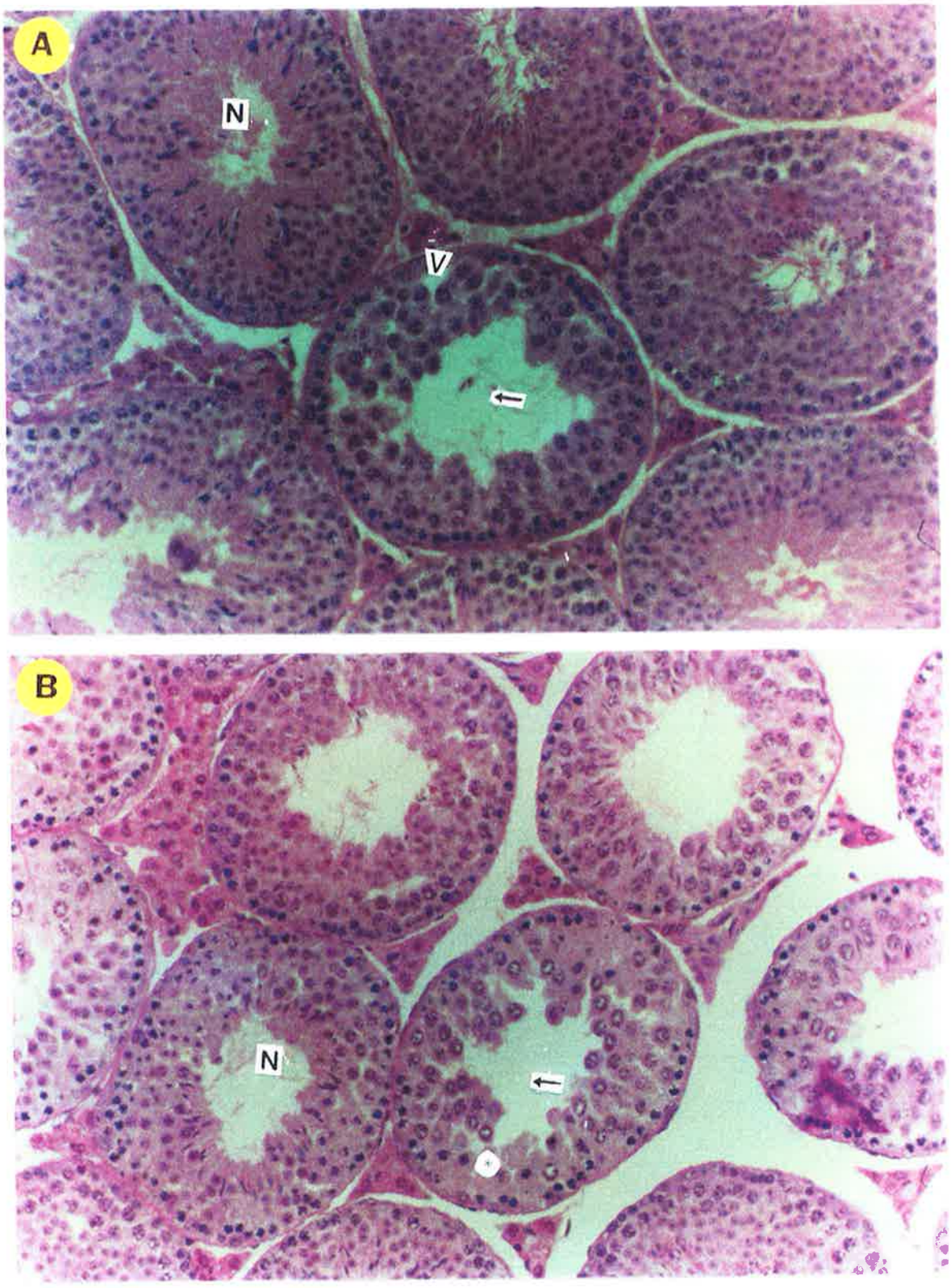
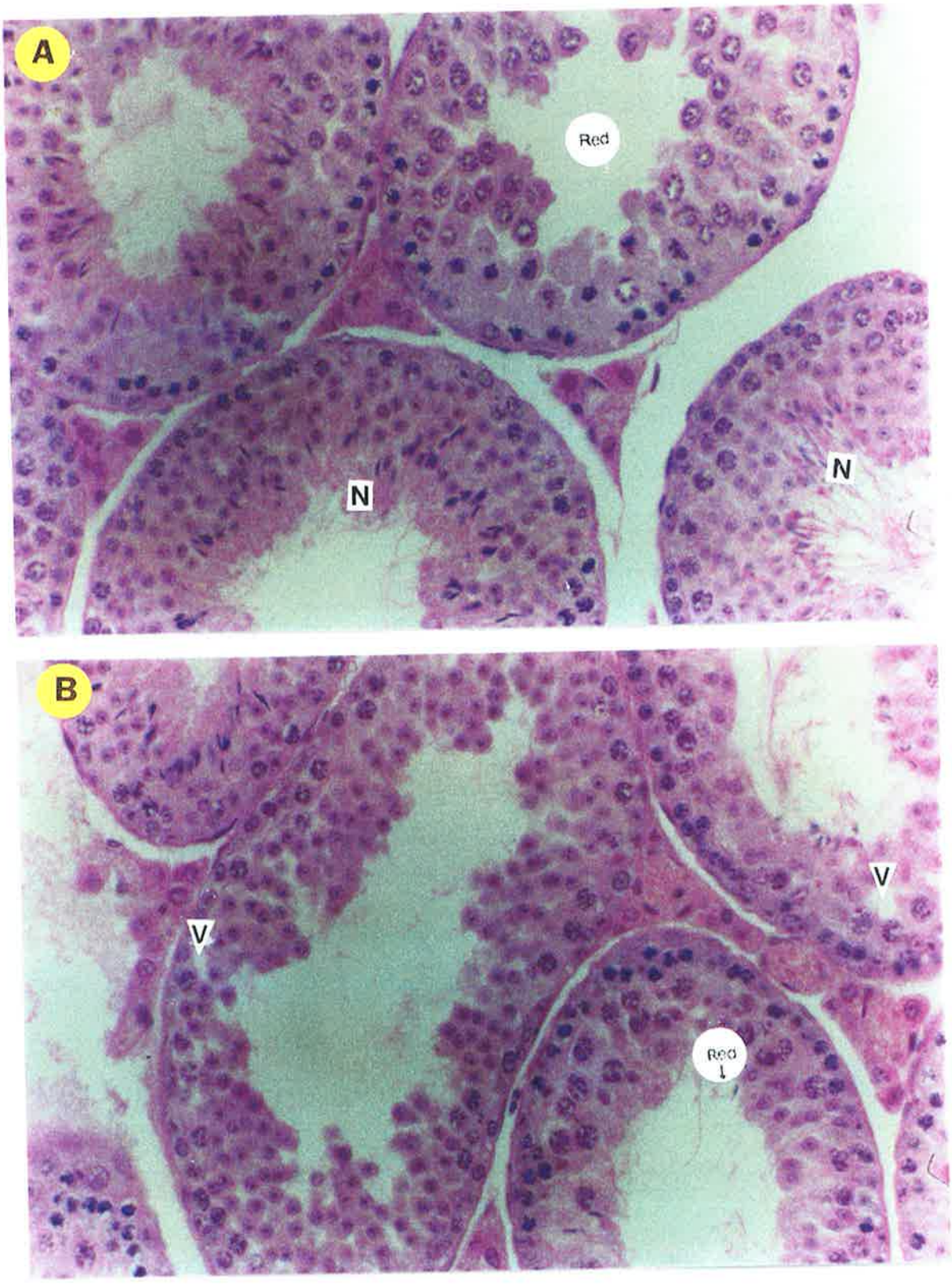

A
N
V
B
N

Figure 4.8

Cross-sections of testes from mice whose whole body was heated at 36°C for 1 day, 12h (A) and for 2 days, 12h per day (B), and excised 21 day later. Photomicrographs show obvious signs of damage to some seminiferous tubules, i. e. vacuoles (V) and reduced number of spermatids (Red) while other tubules look normal (N) with a large number of spermatids being observed. Magnification 400x.


A
Red
N
N
B
V
V
Red

Discussion

It would appear from the investigations reported here that heating the whole body of male mice at 33°C for various durations from 8 to 24 hours (Table 4.8) had no obvious effect on testis weight, epididymis weight or the number of sperm heads. Similar results were obtained when the animals were exposed to the higher temperature of 34°C. It is likely that the ambient temperatures of 33°C and 34°C are not severe enough to induce changes in testicular and epididymal functions. That body weight gains during the period of 21 days after heating at these temperatures (Tables 4.8 and 4.9) are not influenced suggests that, like the testis, other physiological systems are also not affected by this level of heat stress. However, when the level of ambient temperature was increased by only 1°C to 35°C detrimental effects of heat treatment became evident. As shown in Table 4.10, at 35°C testis weight, epididymis weight and the number of sperm heads of the males in the group most affected by heat stress (24h group) dropped significantly ($p<0.0001$) to 68%, 76.3%, 32.6% of the control values, respectively. At 36°C group, the effects of heat stress were even more pronounced (Table 4.11).

In the mouse, the duration of the spermatogenic cycle (from A1 spermatogonia to spermatozoa) is approximately 35.2 days; 7.4 days from A1 to B spermatogonia, 13.7 days from Preleptotene 1st spermatocyte to 2nd spermatocyte and 14.1 days from 2nd spermatocyte to spermatozoa (Meistrich *et al.*, 1978). From this cycle, the spermatids present at the time of assessment (21 days after heating) must have been at the stage of Zygotene or Pachytene spermatocytes at the time of heating. The finding in this study that the number of sperm heads (the nuclei of spermatids in steps 12 to 16) declines dramatically at day 21 after heating suggests that a large number of spermatocytes at the time of heating died before they could progress to the more advanced, spermatid stage. This is in agreement with Chowdhury

and Steinberger (1970) who reported that the cells in the seminiferous tubule most susceptible to heat were spermatocytes and early spermatids.

The adverse effect of heat stress on testis found at 35°C but not 34°C, i.e. only 1°C difference, would suggest that there was a threshold of heat stress that would produce a harmful effect to the testis. Such a threshold is likely to be affected also by other factors such relative humidity, animal species, behaviour, and possibly also strain.

It is obvious from these studies that if the ambient temperature is high enough, whole body heating can induce disruption of spermatogenesis. The present study also suggests that 35°C is the minimum ambient temperature that can affect testis weight and morphology.

CHAPTER 5

MALE FERTILITY

5.1 Reproductive performance of normal female mice mated to males whose whole bodies have been heated at 35°C for 24 h

Introduction

Using mice (Albino ICR) as a model, Bellvé (1972,1973) found that heating the whole body of males at 34.5°C for 24h resulted in an increase in embryonic mortality rate and a decrease in subsequent litter size in normal females mated to heated males between days 3 and 8 after heating, day 1 being the day of heating. In our previous experiment (Section 4.2) using a different strain of mice (CBA/C57, F1) we observed that testis weight, epididymis weight and sperm head counts were decreased to 68.1%, 76.3% and 32.6% of the control values, respectively when mice were exposed to whole body heat at 35°C for 24h. The next experiment extended this work to determine if these effects were also impacting on male fertility and at what time after heating.

Materials and Methods

Twelve adult male mice were heated at 35°C for 24h as previously described (see Section 3.3.2), and another 12 mice were kept in their original room without heating for use as controls. After heating, each heated male was caged with 2 females overnight. In the morning a check for copulatory plugs was performed. The females with plugs were removed to separate cages (pregnant cages) and they were assumed to be at 0.5 d of gestation. The females with no plug were separated from the males and put in "non-pregnant cages". The males were placed with females on days 1, 7, 14, 21, 28 and 35 post-heating. The pregnant females were killed at day 10.5 post-mating by carbon dioxide inhalation. They were then dissected along the ventral midline and the uterus exposed. The number of implantation sites were counted and dissected, after which trophoblasts were separated and weighed. The

number of viable and nonviable embryos were counted and fixed in freshly prepared, ice-cold 4% paraformaldehyde for 4h, washed twice at 4°C with saline, then with 1:1 ethanol:saline, and then twice with 70% ethanol. They were left in the second 70% ethanol wash for 7 days and then weighed (see Section 3.7.3).

Results

Pregnancy rates

Successfully-mated females in the control group were all pregnant at day 10.5 after mating (Table 5.1). There was no sterile mating (100% pregnancy rate) when the males were used on days 1, 7 or 35 after heating (Table 5.1, Figure 5.1). In mice mated on days 14, 21 and 28 after heating, there were a number of sterile matings; the pregnancy rates of these groups ranged between 50 and 60% (Figure 5.1).

Table 5.1 The number of sterile matings as calculated from the numbers of mice mated (measured next morning after mating by observing copulatory plugs) and pregnant on day 10.5 after mating. The females were regarded as pregnant when at least 1 embryo was present in their reproductive tracts.

Treatments	No. of mice mated	No. of mice pregnant	No. of sterile matings
Control	11	11	0
D1 post-heating	4	4	0
D7 post-heating	7	7	0
D14 post-heating	5	3	2
D21 post-heating	6	3	3
D28 post-heating	5	3	2
D35 post-heating	4	4	0

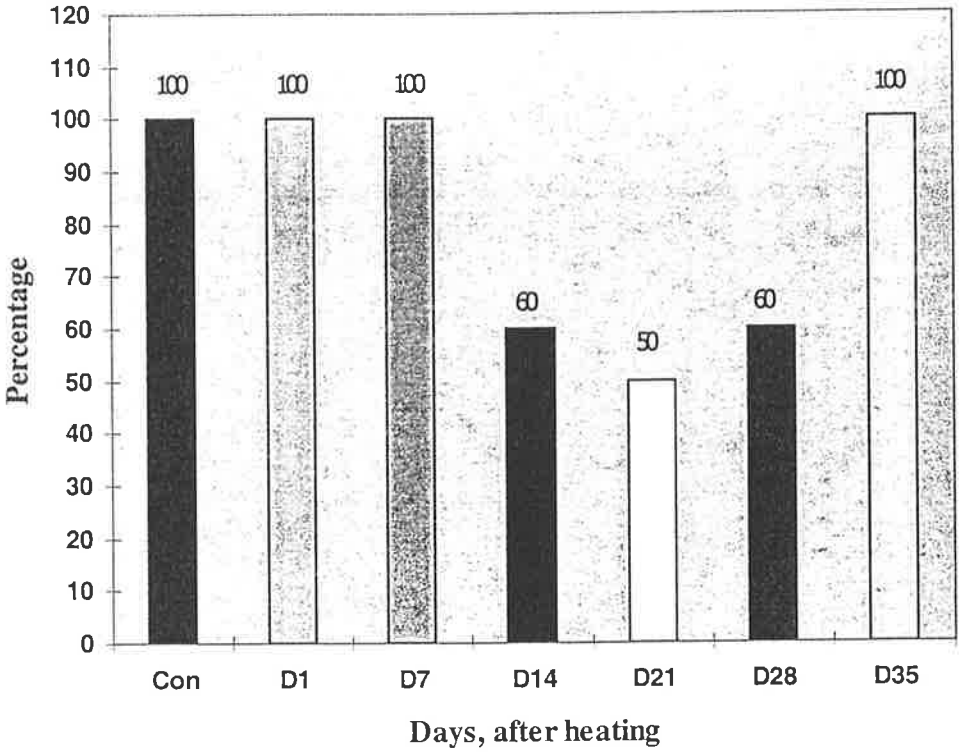


Figure 5.1 Pregnancy rates at 10.5 days after mating as calculated from the numbers of mice mated and pregnant. The females were regarded as successfully-mated when a copulatory plug was observed next morning after mating, and as pregnant when at least 1 embryo was present in their reproductive tracts.

Embryo survival rate

The data in table 5.2 demonstrate that heat-stressed males had a great influence on the number of embryos that were capable of implanting in the uterus. Significant decreases in number of implantation sites and viable embryos were observed when the males were used between days 14 and 28 after heating, the lowest values, which were less than 50% of the controls, being at day 21. At day 35 , the values were still below those of the controls but the differences were no longer significant. Heat treatment, however, had little effect on post-implantation embryonic loss (Table 5.2), and embryo survival rates ranged between 94.8 to 100% (Figure 5.2).

Table 5.2 Post-implantation embryonic loss on day 10.5 of pregnancy as calculated from the numbers of implantation sites and viable embryos. Values are mean ± standard errors.

Treatments	No. of pregnant mice	No.of implantation sites per pregnant female	No.of embryos per pregnant female	No.of embryos lost per pregnant female
Control	11	10.8±0.5	10.5±0.4	0.3
D1 post-heating	4	10.5±0.5	10.0±0.6	0.5
D7 post-heating	7	9.6±0.9	9.1±0.9	0.5
D14 post-heating	3	6.3±3.2	6.0±3.2	0.3
D21 post-heating	3	4.7±1.7	5.0±2.0*	0.0
D28 post-heating	3	5.3±2.8	5.3±2.8	0.0
D35 post-heating	4	9.3±0.5	9.0±0.4	0.3
P-value (one-way ANOVA)		0.0035	0.0069	0.0155

* The number of embryos is greater than the number of implantation sites because one implantation site has 2 embryos

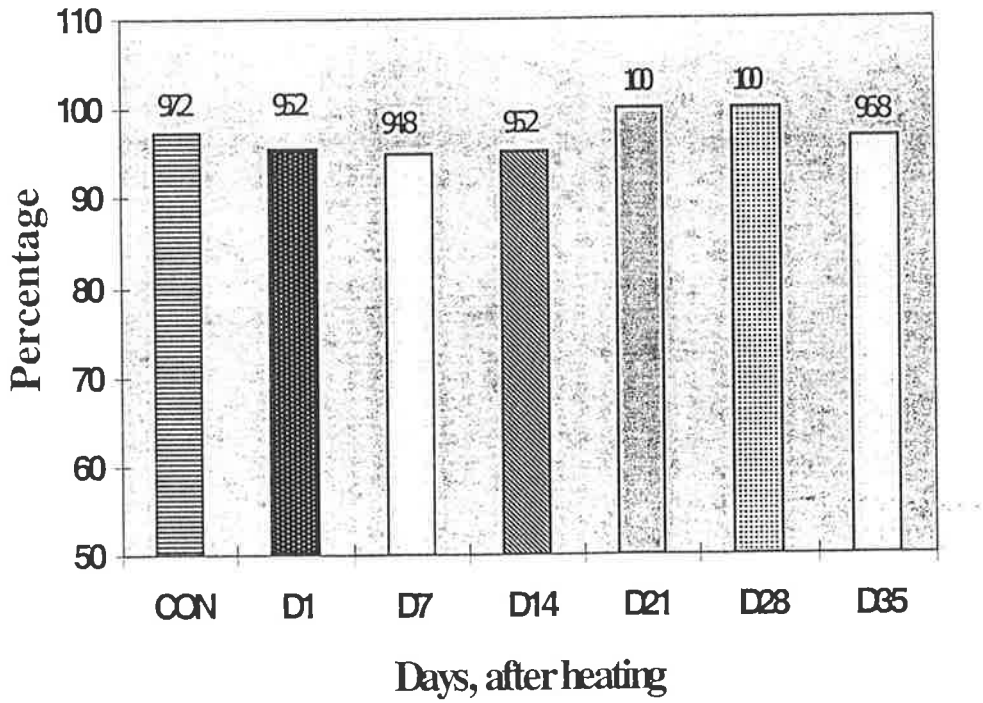


Figure 5.2 Percentages of post-implantation embryos surviving at day 10.5 of pregnancy as calculated from the numbers of implantation sites and viable embryos.

Embryonic and trophoblast weights

Embryonic weights measured on day 10.5 of pregnancy decreased ($p = 0.06$) in the females mated to the heated males between days 7 and 35 after heating, even though the differences were not significant at $p<0.05$ (Table 5.3). Trophoblast weights were not significantly different between groups.

Table 5.3 Embryonic and trophoblast weights at day 10.5 of pregnancy. Successfully-mated females were killed 10 days after mating, their reproductive tracts were removed and dissected. Trophoblasts were separated from embryos and weighed. Embryos were fixed in 4% paraformaldehyde, washed with 0.9% saline, saline+ethanol and 70% ethanol. They were kept in 70% ethanol for 7 days, then weighed.

Treatments	Embryonic weight (mg)	Trophoblast weight (mg)
Control	28.9±1.3	46.0±1.4
D1 post-heating	29.7±2.3	46.1±2.1
D7 post-heating	26.6±1.1	47.2±1.1
D14 post-heating	25.8±1.7	48.0±1.8
D21 post-heating	24.3±1.3	49.1±2.6
D28 post-heating	23.3±1.6	46.9±1.5
D35 post-heating	24.5±1.2	47.4±1.2
P-value (one-way ANOVA)	0.0609	0.9030

Discussion

The results of this experiment suggest that heating the whole body of the male mice at 35°C for 24h adversely affected the reproductive performance when they were mated to normal females. The pregnancy rates started to decrease when the females were mated to the males 14 days after heating, remained low on days 21 and 28 and were recovered by day 35. This suggests that spermatogenic cells in the testis at different stages respond differently to heat stress. Male germ cells present in the epididymis as sperm and in the testis as spermatozoa, late spermatids or spermatogonia are unlikely to be as severely affected by the heat treatment, since the results in this experiment indicate that heated males reproduced normally at days 1, 7 and 35 after heating with pregnancy rate, implantation rate and litter size being not affected. It is known that low pregnancy and implantation rates in the females can be caused by either low fertilization rate or high pre-implantation embryonic mortality rate. Bellvé (1972, 1973) reported that pre-implantation loss of embryos sired by heat-stressed males was due to the retardation of embryonic development during early stages.

Heat stress is not likely to influence post-implantation embryonic loss because, as demonstrated in Table 5.2, once implantation is completed embryonic loss is minimal and comparable in all groups (treatment and controls). It would appear from the present study that if heat-sired embryos can survive until implantation they are likely to continue to develop through gestation normally.

It is obvious that the ambient temperature of 35°C is sufficient to produce unfavorable effects on the testis if male animals are exposed to this temperature for a consecutive period of 24h. The next experiment was designed therefore, to investigate the effect of heat stress when males were heated at the same temperature but shorter exposure period.

5.2 Reproductive performance of normal female mice mated to males whose whole bodies have been heated at 35°C for 2 days, 12 h per day

Introduction

The previous study (Section 5.1) demonstrated clearly that pregnancy rate and litter size at d10.5 of pregnancy of normal female mice were reduced significantly when mated to the males 14-28 days after their whole bodies had been exposed to heat at 35°C for 24h. The present study was to investigate further if heating the males at this temperature but for a shorter period, which is more like that likely to occur naturally, would affect their fertility. The (12+12)h heating scheme was chosen for this study. This was based on the results of experiments in section 4.2 where heating the whole body of the males at 35°C for 8h per day for either 1, 2 or 3 days did not adversely affect testis morphology or spermatogenesis, while heating for 12h daily over 2 days greatly reduced testis and epididymis weights, and sperm head counts. The current experiment, therefore, aimed:

1) to investigate if fertility of normal females would be affected by males exposed to heat at 35°C for 2 consecutive days, 12h daily, and
2) to monitor the changes of testis weights of the males during the course of 35 days after heating

Materials and Methods

Male mice were exposed to heat by the procedure previously described (see Section 3.3.2), at 35°C for 2 days, 12h per day. Six control and 36 heated male mice and 44 females were used in the fertility study. An additional 42 heated males were used to monitor testis weight changes at 5-day intervals during the 35-day period after heating, and another 42 were used

as controls. For the fertility trial, the methods for mating the males and females, and collecting and assessing embryos were as described in section 5.1. For the testis weight-change study, six heated and six control males were killed by carbon dioxide inhalation every 5 days after heating, at which time their testes were removed and weighed.

Results

Pregnancy rate

Heating the males at 35°C for 2 days, 12h per day, had no effect on pregnancy rate of normal females, with all 44 mated animals being pregnant at day 10.5 of gestation (Table 5.4).

Table 5.4 Pregnancy rates of normal female mice at 10.5 days after mating as calculated from the numbers of mice mated and pregnant. The females were regarded as successfully-mated when a copulatory plug was observed next morning after mating, and as pregnant when at least 1 embryo was present in their reproductive tracts.

Treatments	No. of Mice mated	No. of Mice pregnant	Pregnancy rate (%)
Control	11	11	100
D1 post-heating	7	7	100
D7 post-heating	5	5	100
D14 post-heating	6	6	100
D21 post-heating	6	6	100
D28 post-heating	6	6	100
D35 post-heating	3	3	100

Embryo survival rates

Even though minor decreases in the number of implantation sites and viable embryos at day 10.5 of pregnancy were observed with the males used on day 7 post-heating, significant ($p<0.05$) decreases were only found with the males used on day 21 (Table 5.5). Post-implantation embryonic loss per litter was not significantly different between the control and heated groups, nor among the heated groups. The similar percentages of post-implantation embryo survival rates between groups are shown in figure 5.3.

Table 5.5 Post-implantation embryonic loss at day 10.5 of pregnancy as calculated from the numbers of implantation sites and viable embryos. Values are mean ± standard errors.

Treatments	No. of mice	No. of implantation sites per pregnant female	No. of embryos per pregnant female	No. of embryos lost per pregnant female
Control	11	10.7 ± 0.5^{a}	10.5 ± 0.4^{a}	0.2
D1 post-heating	7	10.4 ± 0.3^{a}	10.4 ± 0.3^{a}	0.0
D7 post-heating	5	10.0 ± 0.5^{a}	9.6 ± 0.7^{a}	0.4
D14 post-heating	6	9.8 ± 0.3^{a}	9.8 ± 0.3^{a}	0.0
D21 post-heating	6	6.2 ± 1.5^{b}	6.2 ± 1.5^{b}	0.0
D28 post-heating	6	9.3 ± 1.1^{a}	9.0 ± 1.0^{a}	0.3
D35 post-heating	3	9.0 ± 0.6^{a}	8.7 ± 0.9^{a}	0.3
P-value (one-way ANOVA)		0.0047	0.0048	

[a, b,] Numbers with different superscripts within one column are significantly different.

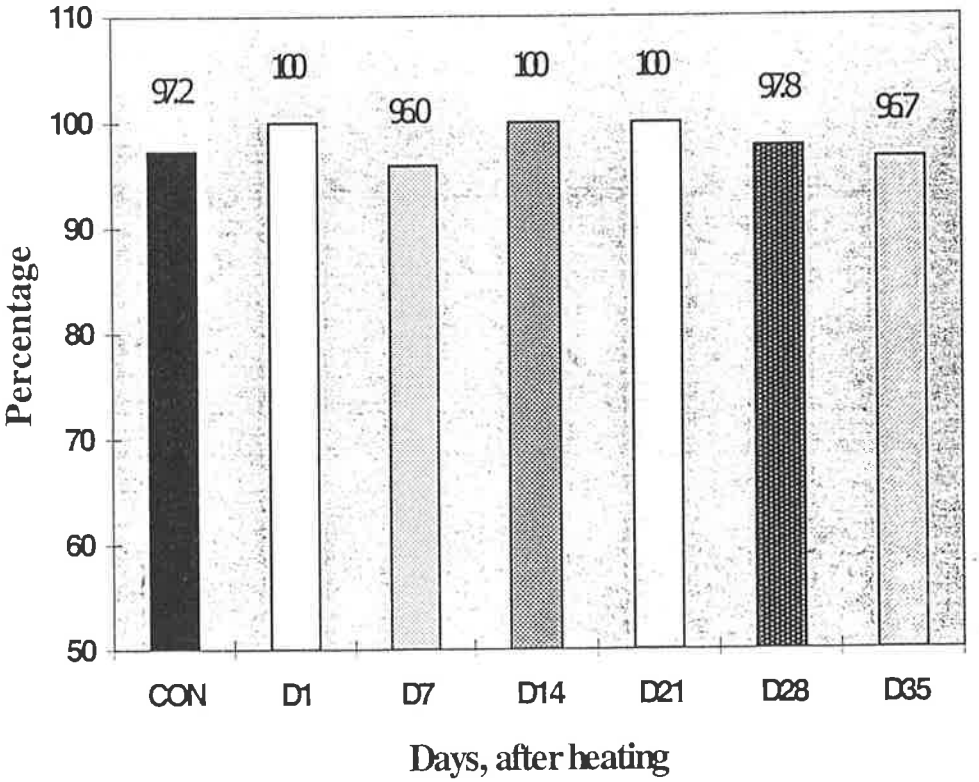


Figure 5.3 Percentages of post-implantation embryo survival rates at day 10.5 of pregnancy as calculated from the numbers of implantation sites and viable embryos.

Embryonic and trophoblast weights

Embryonic weights were not significantly different between groups although they were slightly lower when sired by males between days 7 and 35 after heating (Table 5.6). Heat treatment also had no effect on trophoblast weight.

Table 5.6 Embryonic and trophoblast weights at day 10.5 of pregnancy. Successfully-mated females were killed 10 days after mating, their reproductive tracts were removed and dissected. Trophoblasts were separated from embryos and weighed. Embryos were fixed in 4% paraformaldehyde, washed with 0.9% saline, saline+ethanol and 70% ethanol. They were kept in 70% ethanol for 7 days, then weighed. Values are means±SEM.

Treatments	Embryonic weight (mg)	Trophoblast weight (mg)
Control	28.9±1.3	46.0±1.4
D1 post-heating	27.0±1.3	44.0±1.7
D7 post-heating	25.5±1.2	48.5±1.5
D14 post-heating	25.4±1.8	49.5±1.9
D21 post-heating	24.4±1.0	49.5±1.6
D28 post-heating	26.5±1.4	49.8±1.8
D35 post-heating	23.8±1.5	47.7±2.3
P-value(one-way ANOVA)	0.1233	0.2091

Testis weight changes

The individual testis weights of males killed at 5-day intervals during the 35-day period after heating are given in table 5.7, and the mean weights are presented in figure 5.4. The mean testis weights of the heated mice at day 5 after heating were lower than those of the controls, but the difference was not significant. The reductions in testis weight were greatest ($p<0.0001$) at days 10, 15, 20 and 25. At days 30 and 35 the decreases were smaller, but the values were still significantly below ($p<0.001$) those of the controls.

Table 5.7 Individual testis weights of control and heated male mice measured every 5 days from day 5 to day 35 after heating. Values are averages of left and right testes.

	Mice	D5	D10	D15	D20	D25	D30	D35
Contr. (C)	1	130.5	119.3	123.5	116.8	124.5	125.6	113.5
	2	116.0	124.7	114.0	128.5	115.0	118.0	125.0
	3	111.5	115.4	117.5	118.2	117.0	120.4	120.0
	4	106.0	118.0	117.0	116.4	125.5	122.6	125.5
	5	120.5	120.5	111.5	122.6	126.0	117.3	129.5
	6	118.0	117.2	117.0	119.1	123.0	126.4	114.5
Mean		117.2	119.2	116.8	120.3	121.8	121.8	121.3
Heated (H)	1	111.0	89.0	80.0	99.0	100.5	123.0	113.0
	2	111.5	89.0	79.5	91.0	96.0	117.5	108.0
	3	108.5	84.5	79.5	83.5	98.0	115.5	110.0
	4	115.5	86.0	87.5	78.5	105.0	108.5	118.0
	5	111.0	73.5	81.0	97.5	95.5	118.5	109.0
	6	112.5	84.5	84.0	85.5	95.5	114.5	107.5
Mean		113.0	84.4***	81.9***	89.2***	98.4***	116.3**	110.9**

Note: ** P<0.001, *** P<0.0001, compared to control means

Discussion

It was shown in section 5.1 as well as in the work of Garriot and Chrisman (1981) that the ambient temperature of 35°C effectively suppresses fertility of the male if the duration of heat stress is long enough (>24h). The current experiment found that the degree of heat stress was likely to be less when the same duration (24h) of heat treatment was split into 2 periods, 12h per day for 2 days. It is shown in this experiment that all the successfully-mated females, irrespective of treatments, were pregnant. Except at day 21, mating the males at any other time point produced no differences in the number of implants nor post-implantation embryonic survival rate. However, this repeated-heating scheme did influence testis functions.

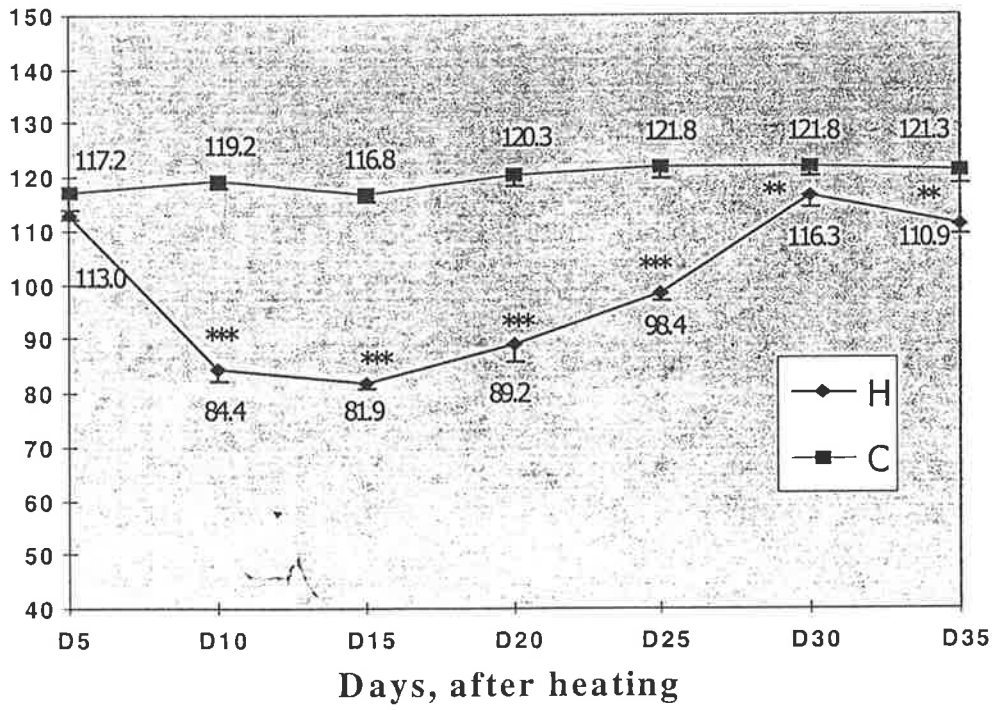


Figure 5.4 Mean testis weights of the control (C) and heated (H) mice at 5-day intervals from D5 to D35 after heating.

Note: ** P<0.001, *** P<0.0001, compared to control means

As shown in figure 5.4, significant decreases in testis weights were observed in the males killed between days 10 and 35 after heating. The lowest mean testis weight was 81.9 mg at day 15. The number of sperm head count was not measured in this study. However, it was found in the earlier study (Section 4.2, Table 4.10) that heating the whole body of male mice at 35°C for 2 consecutive days, 12h per day resulted in a reduction in the number of sperm head per testis by 41%, from 14.4×10^6 in the control group to 8.5×10^6 in the heated group. This implies that the impact on testis function seems not to be severe enough to suppress subsequent fertility. Indeed, Jones (1990) reported that to affect fertility of the heat-stressed males testis weight must be decreased to a level at least 60% of the control value. The loss of testis weight in the current experiment appears to be not great enough as the lowest weight (81.9 mg) found in the males 15 days after heating was only 70.1% of the controls (116.8 mg).

5.3 Reproductive performance of normal female mice mated to males whose whole bodies have been heated at 36°C for 2 days, 12h per day

Introduction

Given the tendency for summer heat to fluctuate in waves over successive days, and given the minimal results found at 35°C, 12+12h, it was of interest to elevate the temperature slightly to 36°C and to examine further the effect of this on reproduction.

Materials and methods

Of the 74 male mice used in this study, 26 served as controls. The remaining 48 mice were heated at 36°C, 12h per day for 2 consecutive days. Between days 3 and 20 after heating, 1 heated male was caged with 2 females overnight. In the morning a check for copulatory plugs was performed. The females with plugs were removed to pregnant cages, and they were assumed to be 0.5 d of gestation. The females with no plug were separated from the males, kept in a non-pregnant cage, and in the evening, caged with another male. Some successfully-mated males were killed on the day that a successful mating was recorded during different periods after heating, i.e. during d3-6, d7-9, d10-12 and d14-20, while the remainder were allowed at least 3 days before the next mating. After sacrifice, the males were opened on the ventral midline and the testes exposed. The testes and epididymides were separated and weighed. The testes were then put in a conical tube and kept at -20°C in a freezer for the analysis of sperm head counts. The pregnant females were killed at day 10.5 post-mating and their reproductive tracts were exposed by the procedure described in section 5.1. The number of implantation sites and embryos of each female were counted. The ovaries were also examined for the numbers of Corpora Lutea (C.L).

Results

Pregnancy rate

The pregnancy rates of females mated to the control males and to the heated males between days 3 and 6 after heating were not significantly different, the values being 94.9% and 96.1%, respectively (Table 5.8). When the heated males were used during days 7 to 9 after heating the rate of pregnancy dropped to 69.2%. The pregnancy rate continued to drop during days 10 to 12 (36.4%), and the minimum value was observed during days 14 to 20 (8.1%) (Figure 5.5).

Table 5.8 The number of sterile matings as calculated from the numbers of mice mated (measured next morning after mating by observing copulatory plugs) and pregnant on day 10.5 after mating. The females were regarded as pregnant when at least 1 embryo was present in their reproductive tracts.

Treatment	No. of mice mated	No. of mice pregnant	No. of sterile matings
Control	39	37	2
Days 3-6 post-heating	26	25	1
Days 7-9 post-heating	26	18	6
Days 10-12 post-heating	33	12	11
Days 14-20 post-heating	37	3	34

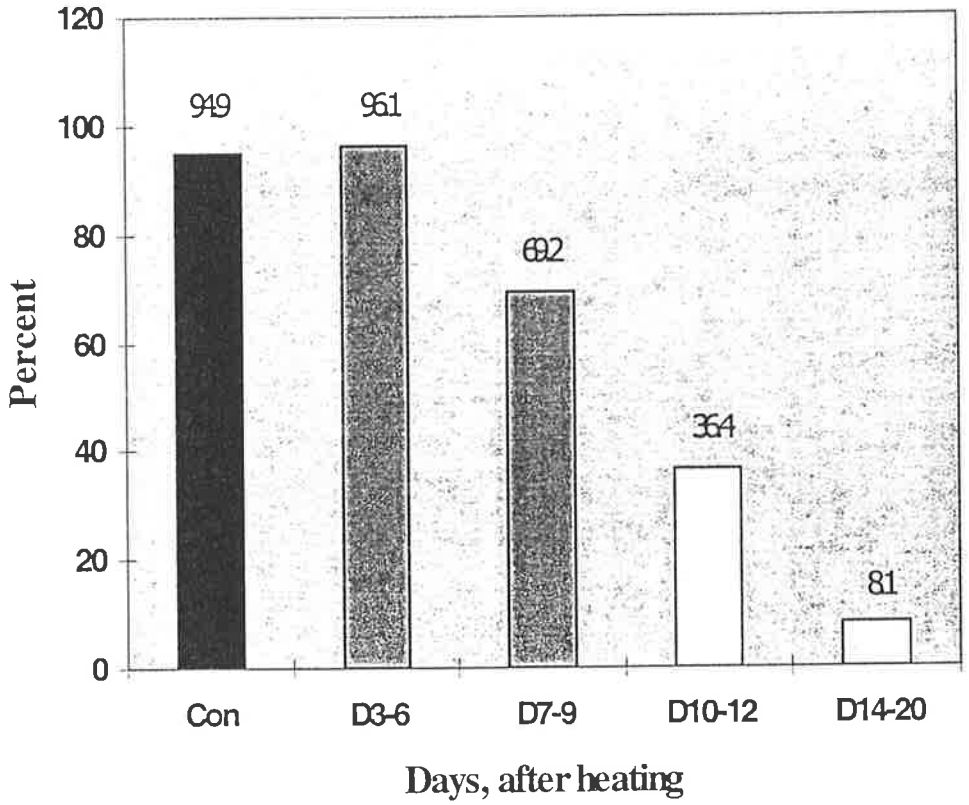


Figure 5.5 Pregnancy rates at 10.5 days after mating as calculated from the numbers of mice mated and pregnant. The females were regarded as successfully-mated when a copulatory plug was observed next morning after mating, and as pregnant when at least 1 embryo was present in their reproductive tracts.

The numbers of corpora lutea and embryos

The numbers of corpora lutea and embryos assessed at day 10.5 of pregnancy are presented in Table 5.9. While the number of corpora lutea were similar between the control and heated groups, the number of embryos per litter were significantly lower in the females mated to the males 7 to 20 days after heating.

Table 5.9 The numbers of corpora lutea and embryos at day 10.5 post-mating. Successfully-mated females were killed 10 days after mating, their reproductive tracts were removed and dissected, and the numbers of corpora lutea and embryos recorded. Values are mean ± SEM.

Treatment	No. of pregnant females	No. of C.L per pregnant female	No. of embryos per pregnant female	Differences between No. of CL and No. of embryos	
				No.	%
Control	26	9.5±0.3	9.2±0.3	0.3±0.1	3.2
Days 3-6 post-heating	14	8.9±0.3	8.4±0.3	0.5±0.3	5.6
Days 7-12 post-heating	23	9.3±0.3	6.3±0.7	3.0±0.7	32.3
Days 14-20 post-heating	3	9.0±1.0	5.3±2.0	3.7±2.2	41.1
P-value (one-way ANOVA)		0.6983	0.0004	0.0003	

Post-implantation embryonic loss

The calculation of post-implantation embryonic loss was based on the number of implantation sites and the number of embryos present. As shown in Table 5.10, the number of embryos produced declined over time during the period of 20 days after heating. The extent of post-implantation embryonic loss per litter, however, was not significantly different at any time point. This was because of the corresponding decline in the number of implantation sites and the number of embryos present, as is evident in Figure 5.6 where the 2 lines run parallel over time from days 3 to day 20 after heating. Therefore, if embryonic loss was a factor contributing to reduced fertility in normal females it would occur during preimplantation embryonic development.

Table 5.10 Post-implantation embryonic loss on day 10.5 of pregnancy as calculated from the numbers of implantation sites and viable embryos. Values are mean ± SEM.

Treatment	No. of litters	No. of implantation sites per litter	No. of embryos per litter	No. of embryo loss per litter	Embryo survival rate (%)
Control	26	9.3±0.2	9.2±0.3	0.1±0.1	98.9
Days 3-6 post-heating	14	8.5±0.3	8.4±0.3	0.1±0.1	98.8
Days 7-12 post-heating	23	6.5±0.7	6.3±0.7	0.2±0.1	96.9
Days 14-20 post-heating	3	5.7±1.8	5.3±2.0	0.4±0.3	93.0
P-value (one-way ANOVA)		0.0005	0.0004	0.7723	

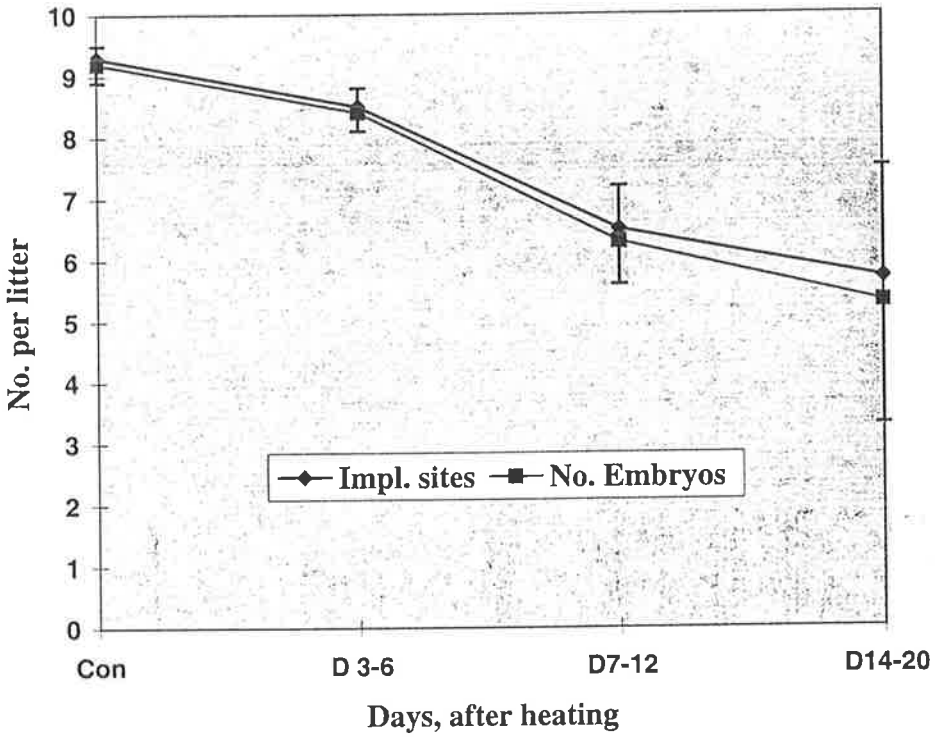


Figure 5.6 The numbers of implantation sites and embryos per litter at day 10.5 of pregnancy of normal females mated to either control males or heated males between days 3 and 20 after heating.

Male performance

The data on testis and epididymis weights and the number of sperm heads per testis of the males used to mate the females in the present study are presented in Table 5.11. Testis weight dropped dramatically ($p<0.0001$) at each time point measured during the 20-day period after heating with the lowest value (49.3 mg) being observed between days 14 and 20. Epididymis weights were not significantly different among the treated groups, but they were all significantly lower ($p<0.0001$) than those of the controls. The number of sperm heads per testis dropped accordingly with the decline in weight of the testis over the 20-day period after heating, with the minimum value ($0.4x10^6$) being also observed between days 14 and 20 after heating.

Table 5.11 Testis and epididymis weights and sperm head counts of the males used to mate normal females. After successfully mating, males were killed, their reproductive tracts removed, their testes and epididymides separated and weighed. Right testes were frozen at -20°C, and later analyzed for the number of sperm heads per testis. Values are mean ± SEM.

Treatment	No. of males	Testis weight (mg)	Epididymis weight (mg)	Sperm head counts per testis ($x10^{-6}$)
Control	23	118.4±2.1	36.1±0.7	22.5±0.6
Days 3-6 post-heating	7	66.3±3.6	26.1±0.7	14.3±2.5
Days 7-12 post-heating	24	53.6±2.0	25.8±0.4	6.0±1.0
Days 14-20 post-heating	20	49.3±1.0	27.0±0.3	0.4±0.1
P-value (one-way ANOVA)		0.0001	0.0001	0.0001

Relationship between the number of sperm heads, pregnancy rate and the number of embryos per litter

Pregnancy rate, the numbers of embryos per litter and the number of sperm heads were closely related. As demonstrated in figure 5.7, these three measurements move in the same direction, which are downward and almost parallel, over the 20-day period after heating.

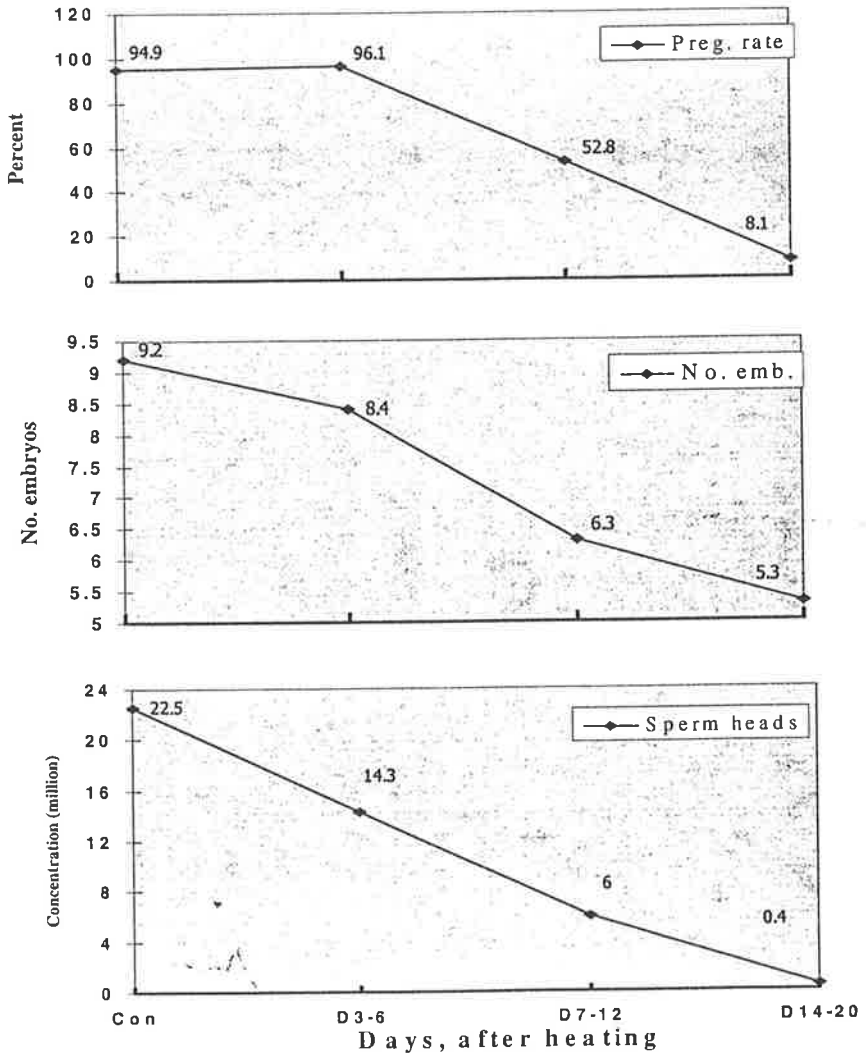


Figure 5.7 Relationships between pregnancy rates and the number of embryos per litter of normal females and the number of sperm heads per testis of males used to mate those females. Males were killed and their sperm characteristics assessed on the day of successful mating.

Discussion

It is shown in this study that a 1°C-rise in ambient temperature (from 35°C to 36°C) not only caused a more marked reduction in testis weights but also impacted on the fertility of the males. For example, the pregnancy rate of normal females mated to heat-stressed males at day 14 after heating was 8.1% in this study (36°C) compared to 100% in the previous one where the males were heated at 35°C. It would appear that in the conditions used in the current study 35°C was the maximum temperature at which the males could retain their normal reproduction, and a further increase in temperature, even by only 1°C, results in a dramatic decrease in their fertility.

It is suggested from this study that the temperature of 36°C affected spermatogenic cells as late as elongating and elongated spermatids. This statement is based on the results in table 5.11 when the decrease in testis weights was found not only with the heated males killed between days 7 and 20 after heating but also with those killed during days 3 to 6. The time taken for spermatogenic cycle in the mouse is, from A1 spermatogonia to spermatozoa, approximately 35.2 days and the elongating and elongated spermatids are normally present during the last 3 to 6 days. However, there are still a large number of spermatids that survive heat stress and become fertile spermatozoa. This is evident in the normal pregnancy rate, implantation and embryo survival rates (8.4 embryos VS 8.9 C.L) that were observed with the males used during 3 to 6 days after heating. The data in table 5.11 suggests that heat treatment severely damaged testes of the males killed between days 14 and 20 after the exposure to heat. This, based on the spermatogenic cycle, implies that the cells most susceptible to heat are those at the stages from spermatocytes to round spermatids. As a result, the males used during days 14 to 20 after heating were capable of only producing very

poor reproduction, i.e. low pregnancy rate of 8.1-36.4%, decreased litter size (5.3 embryos per litter) and implantation rate (63.3%, 5.7/9.0). This poor performance can be due to the low fertilizing ability of the sperm or because of the high mortality rate of preimplantation embryos. The next experiment was conducted to test fertilizing capacity of the sperm.

5.4 In vivo fertilization

Introduction

It was discovered in the previous experiment (Section 5.3) that pregnancy rate and the number of embryos per litter at d 10.5 of pregnancy were lower in the females mated to heat-stressed males between days 7 and 20 after heating. It is known that a reduced pregnancy rate can be a result of either the poor fertilizing capacity of the sperm, a high preimplantation embryo mortality rate, or a combination of these two factors. The current experiment was conducted to test the effect of heat stress on the fertilizing ability of the sperm. Superovulated females were mated with heated males, and 36h later their ova were recovered and examined for signs of fertilization.

Materials and Methods

As shown in section 5.3, the effect of heat stress on male reproduction was evident between days 7 to 20 after heating. Between days 14 and 20 male fertility was extremely low, i.e. 34 out of 37 mated females were not pregnant. The current experiment was, therefore, designed not to measure fertility beyond day 14 after heating. Instead, it investigated male fertility at various time points, namely days 3, 7, 10 and 14 during the first 2 weeks after heating.

Thirty three male and 47 female mice were used in this study. The treated males were heated by the procedure described in section 3.3.2. The procedure for in vivo fertilization was as described in section 3.10.1. Briefly, females were superovulated using a standard PMSG/hCG protocol (Section 3.10.1.1), mated with males immediately after hCG injection, and sacrificed by carbon dioxide inhalation 36h post-hCG. Their eggs were recovered and examined for stages of development (Section 3.10.1.2). Fertilized eggs were expected to be in the 2-cell

stage at this time (Summers *et al.*, 1995). The fertilization rates recorded were based on the ratio of cleaved ova to the number of ova recovered. The significant difference of numbers of cleaved and non-cleaved ova per litter was analyzed using the Chi-square test.

Results

The numbers of females used and ova recovered from the females 36h after hCG injection are presented in table 5.12. The ovulation rates of each superovulated females ranged between 16 and 32 per litter. The average number of ova recovered per litter in each group ranged between 24.5 and 27.7.

Table 5.12 The numbers of females used and ova recovered. Superovulated females were mated overnight with control or heated males at different days after heating. They were killed 36h post-hCG injection, and their eggs recovered and recorded.

Treatment	Total No. of females used	Total No. of ova recovered	Average No. of ova per litter
Control	10	277	27.7
D3 Post-heating	6	156	26.0
D7 Post-heating	6	147	24.5
D10 Post-heating	6	166	27.7
D14 Post-heating	6	150	25.0

The numbers of female mice with and without fertilization and the number of ova recovered from those with fertilization are shown in table 5.13. All the females mated to the control males and to heat-stressed males 3 and 7 days after heating produced cleaved ova. When females were mated to males 10 and 14 days after heating only 1 in 5 females for each group had cleaved ova. When only the females with fertilization were analyzed, fertilization rates of the females mated to control and heated males on day 3 after heating were not significantly different ($p>0.05$). Heat treatment started to produce adverse effects on day 7 after heating when fertilization rate dropped to 78.8 % from 88.1% of the controls. Between days 10 and 14 post-heating the fertilizing capacity of the sperm of heat-stressed males was severely affected, as can be seen from the low fertilization rates of 18.8% and 7.7% for day 10 and day 14 sperm, respectively (Figure 5.8).

Table 5.13 The number of cleaved ova and fertilization rates of superovulated normal female mice mated to control males or heated males at different days after heating. They were killed 36h post-hCG injection, and their eggs recovered and assessed under a dissecting stereomicroscope at 30x. Eggs were considered fertilized if they had cleaved to 2-cell stage.

Treatment	No. females with /without fertilization	No. ova per litter of females with fertilization (Mean±SD)	No. cleaved/not cleaved ova per litter	Percent fertilization
Control	10/0	27.7±4.2	24.4/3.3	88.1
D3 Post-heating	6/0	26.0±3.5	23.5/2.5	90.4
D7 Post-heating	6/0	24.5±3.6	19.3/5.2	78.8
D10 Post-heating	1/5	32.0	6.0/26.0 **	18.8
D14 Post-heating	1/5	26.0	2.0/24.0 **	7.7
Chi-Square value			χ^2 = 0.07; D3/Con χ^2 =0.82; D7/con χ^2 =28.6; D10/con χ^2 =34.7; D14/con	

Note : ** $p<0.01$

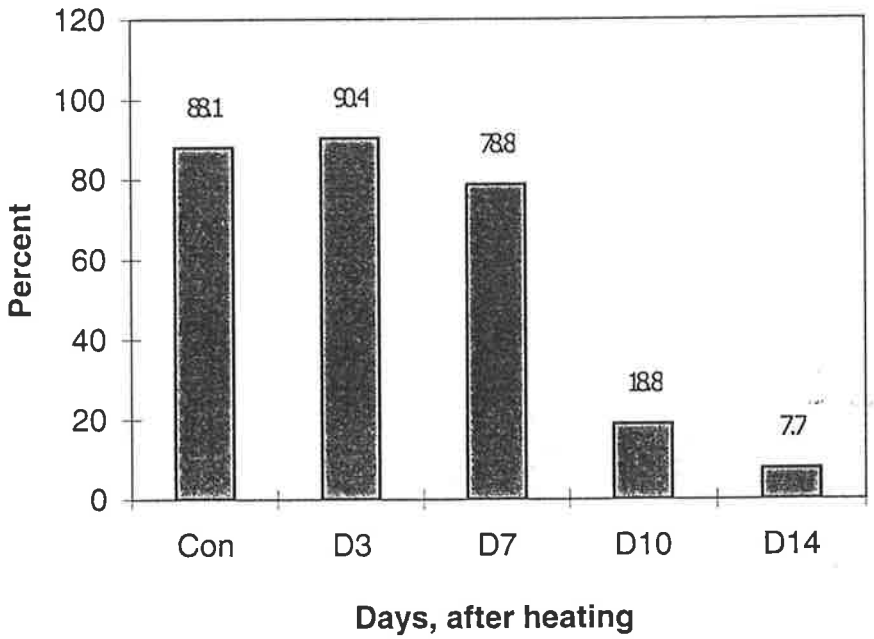


Figure 5.8 Fertilization rate of superovulated normal female mice mated to control males or heated males at different days after heating. They were killed 36h post-hCG injection, and their eggs recovered and assessed under a dissecting stereomicroscope at 30x. Eggs were considered fertilized if they have cleaved to 2-cell stage.

Discussion

Our 6 to 8-wk old female mice responded well to the injections of PMSG and hCG at the doses of 5.0 I.U. This was evident by the fact that all animals ovulated the next morning after hCG injection with a large number of eggs, ranging between 16 and 32. These levels of PMSG and hCG have been used and reported to be successful in inducing superovulation in a number of mouse species (Steven *et al.*, 1983; Summers *et al.*, 1995; Vergara *et al.*, 1997), including F1 (C57BL/CBA) (Fraser and Drury, 1975) as used in the current study. A high fertilization rate (24.4/27.7; 88.1%) was achieved when superovulated females were mated to control males. A similar result (23.5/26.0; 90.4%) was observed when the females were mated with D3-heated males, suggesting that heat treatment had no effect on fertilizing capacity of sperm which were in the epididymis at the time of heating. In other words, epididymal sperm are resistant to heat stress at the level of 36°C when heat exposure was repeated for 2 days, 12h per day. At day 7 after heating, fertilization rates of heated males were reduced slightly suggesting that elongating or elongated spermatids were essentially also resistant to heat stress of 36°C.

When the heated males were used at later times (days 10 and 14) after heating the effects of heat stress were clearly observed. After day 10 postheating fertilization rates of spermatozoa from heated males were at very low levels, i.e. only 6 in 32 (18.8%) and 2 in 26 (7.7%) eggs were fertilized in each litter when the males were used on days 10 and 14, respectively. This fits with the developing hypothesis that primary spermatocytes are the cell type most susceptible to heat stress as the sperm present between days 10 and 14 after heating would have been at the primary spermatocyte or early spermatid stage at the time of heating.

5.5 In vitro fertilization using non-swim up spermatozoa

Introduction

It has been shown from the previous experiment (Section 5.4) that fertilization rates in vivo were significantly lower with males used between days 10 and 14 postheating. The decreases in fertilization rates could be because the sperm were too weak, and as a result they could not swim along the distance from the site of ejaculation deposition to the site of fertilization. In addition, large numbers of dead or immature sperm could also impact on healthy sperm. It is, therefore, possible that there were only a small proportion of the sperm that managed to meet and fertilize eggs.

The current experiment was designed to overcome this obstacle by employing the technique of *in vitro* fertilization (IVF) to test sperm fertilizing ability at 4 time points, namely day3, day7, day10 and day14 after heating. This technique was employed to ensure that sperm reach fertilization sites regardless of their moving ability (motility rate).

Materials and Methods

Animals

Forty eight male and 43 female mice were used in this experiment. The whole body of the male was exposed to heat at 36°C for 2 days, 12h per day as the procedure previously described in section 3.3.2. The females and control males were maintained at 21°C.

Sperm preparation

The males were killed by cervical dislocation 1.5h prior to egg collection. Male reproductive tracts were removed after which vasa deferentia and epididymides were separated (see Section

3.5.2) and placed in 35-mm tissue culture dishes containing 2 ml of HTF-Hepes medium (Section 3.4). Testes were kept in the freezer for the later analysis of sperm heads. Each epididymis was transferred to a 500-ul drop of pregassed HTF-Bicarb under oil, punctured using a 26-gauge needle, and slightly pressed to release spermatozoa. The spermatozoa were allowed 20 minutes to disperse. After 20 min sperm samples were taken for the analysis of motility rate (see Section 3.6.6), concentration (see Section 3.6.2) and the number of live/dead sperm (see Section 3.6.3). The sperm were then diluted to give a final motile sperm concentration of $1\text{-}2x10^6$ /ml. A fertilization dish was made by placing a 50-µl droplet of capacitated sperm suspension in the middle of a 35-mm dish. It was then overlaid with 2 ml of paraffin oil and left in an incubator (37°C, 5% CO_2 and 95% air) for 1.5-2 h to capacitate.

Oocyte collection

The females were induced to superovulate by intraperitoneal injections of 5.0 I.U PMSG and 48-52 h later 5.0 I.U hCG. They were killed 13.5 h post-hCG injection, at which time their oviducts were removed and placed in a tube containing 2 ml of HTF-Hepes (Section 3.10.2.2). The oviducts were transferred to a 35-mm dish containing 2 ml of HTF-Hepes medium. Under a dissecting stereomicroscope at 30x, the cumulus bulge was torn with a 27G needle to release cumulus masses, after which the masses were washed 2 times with fresh HTF-Bicarb medium.

Fertilization

Immediately after washing, two cumulus masses from each females were carefully added to the droplet of preincubated sperm suspension in the fertilization dish. The eggs and sperm were then incubated for 65 mins at 37°C in 5% CO_2 in air.

Fertilization assessment

After incubation for 65 mins the eggs were removed, washed through fresh medium to remove bound sperm, and left in 100µl medium under oil. At 75 mins after incubation the eggs were fixed with phosphate-buffered formalin (Section 3.10.2.4). After ½-1 h fixation the eggs were washed twice with fresh medium, then transferred to a clean slide. The solution on the slide was drained off as much as possible, then one drop of the stain (0.75% aceto-orcein) was added to the eggs. The slide was covered with a cover slip and fertilization status immediately assessed using a light and phase contrast microscope.

Results

The number of ova recovered from superovulated mice ranged between 14 and 30, the average values being 21.0, 20.0, 20.9, 23.4 and 21.0 in the control, D3, D7, D10 and D14 groups, respectively (Table 5.14). The fertilizing capacity of the sperm is presented in Table 5.15. All the females in the control, D3 and D7 groups produced fertilized eggs in vitro, whereas in groups D10 and D14 only 4 in 7 and 1 in 8 females, respectively, gave ova with fertilization. The control and D3 sperm produced similar (90.5% and 91.5%, respectively) fertilization rates. The rates of fertilization of D7 sperm significantly decreased to 55.5%. At days 10 and 14 after heating the sperm of heat-stressed males generated very low fertilization rates, the values being 17.4% and 6.7%, respectively, although similar numbers of motile sperm were used in all groups.

Table 5.14 The number of females used and ova recovered. Superovulated females were killed 36h post-hCG injection, after which their eggs were collected and recorded.

Treatment	Total No. of females used	Total No. of ova recovered	Average No. of ova per litter
Control	22	462	21.0
D3 Post-heating	8	160	20.0
D7 Post-heating	7	146	20.9
D10 Post-heating	7	164	23.4
D14 Post-heating	8	168	21.0

Table 5.15 Fertilization rates of ova from superovulated normal female mice. The ova were fertilized in vitro by similar numbers of motile spermatozoa from either normal males or heated males at different days after heating. They were then washed with fresh medium, stained with 0.75% aceto-orcein, and assessed using a phase contrast microscope. The success of fertilization was assessed from either the presence of sperm head or tail in egg cytoplasm or egg activation as can be seen from the release of cortical granules or chromosomes at anaphase.

Treatment	No. of females with /without fertilization	Average No. of ova recovered in females with fertilization	Average No. of fertilized/ not fertilized ova	Fertilization rates (%)
Control	22/0	21.0	19.0/2.0	90.5
D3 Post-heating	8/0	20.2	18.3/1.7	91.5
D7 Post-heating	7/0	20.9	11.6/9.3 *	55.5
D10 Post-heating	4/3	25.8	4.5/21.3 **	17.4
D14 Post-heating	1/7	30.0	2.0/28.0 **	6.7
Chi-square value			χ^2 =0.4; D3:Con χ^2 =4.9; D7:Con χ^2=21.9;D10:Con χ^2=32.5;D14:Con	

* $p<0.05$ ** $p<0.01$

Testis and epididymis weight of the males used for IVF study are shown in Table 5.16. Testis weight started to drop from day 3 postheating and this trend was observed until day 14 where the average weight was at the level of less than 50% of the control value (117.5 VS 55.3 mg). An adverse effect of heat stress on the epididymis was seen from day 3 after heating. At day 3 the epididymis weight of heat-stressed males was significantly ($p<0.05$) lower (32.6 mg) compared to that of the controls (39.9 mg). Between days 3 and 14 after heating, the epididymis weights among the heated males were not significantly different.

Table 5.16 Testis and epididymis weights of male mice used for in vitro fertilization. The males were killed by cervical dislocation. Their testes and epididymides were collected, separated and weighed. Values are means ± SEM.

Treatment	No. of mice	Testis wt. (mg)	Epididymis wt. (mg)
Control	18	117.5 ± 1.9^{a}	39.9 ± 0.6^{a}
D3 Post-heating	8	82.5 ± 2.1^{b}	32.6 ± 1.2^{b}
D7 Post-heating	7	76.1 ± 2.2^{b}	34.9 ± 1.4^{b}
D10 Post-heating	7	67.0 ± 3.3^{c}	32.1 ± 0.7^{b}
D14 Post-heating	8	55.3 ± 2.5^{d}	29.1 ± 0.5^{c}
P-value (one-way ANOVA)		0.0001	0.0001

$^{a, b, c, d}$ Numbers with different superscripts within one column are significantly different.

The sperm parameters assessed prior to use for IVF are presented in table 5.17. The numbers of sperm heads per testis were not significantly different between control males and heated males used on days 3 and 7 after heating. On days 10 and 14 the number of sperm heads per testis decreased dramatically ($p<0.0001$) as compared to those of controls (see Table 5.17). Sperm concentrations per epididymis were not different among the heated groups but were significantly lower ($p<0.0001$) than those of the controls. Percent live sperm on day 3 postheating was not affected by heat stress, but was significantly reduced ($p<0.0001$) on days 7, 10 and 14. Sperm motility was also influenced ($p<0.0001$) by heat treatment during days 7 and 14, but not on day 3 after heating.

Table 5.17 Sperm parameters assessed 1 to 1.5h prior to sperm-ova incubation. Males were killed 1h before ova collection, and spermatozoa were collected from the cauda epididymides. Approximately 20 min after collection, sperm suspensions were analyzed for motility rate, concentration and number of live/dead sperm. Their testes were frozen at -20°C and the number of sperm heads per testis was assessed using a sonicator at appropriate time later. Values are means ± SEM.

Treatment	No. of mice	Sperm head counts/testis ($x10^{-6}$)	Sperm conc./ epididymis ($x10^{-6}$)	Live sperm (%) (Nigrosin/ Eosin)	Sperm motility (%)
Control	18	26.7 ± 0.6^{a}	38.9 ± 1.0^{a}	74.4 ± 1.5^{a}	73.4 ± 1.1^{a}
D3 Post-heating	8	25.4 ± 0.9^{a}	24.3 ± 0.4^{b}	74.9 ± 0.8^{a}	70.3 ± 1.4^{a}
D7 Post-heating	7	25.4 ± 0.6^{a}	21.4 ± 2.0^{b}	61.6 ± 2.6^{b}	56.8 ± 1.1^{b}
D10 Post-heating	7	8.4 ± 1.0^{b}	22.1 ± 1.2^{b}	52.1 ± 3.9^{c}	44.2 ± 2.0^{b}
D14 Post-heating	8	5.3 ± 0.9^{c}	21.3 ± 2.8^{b}	50.1 ± 2.7^{c}	30.6 ± 1.5^{b}
P-value (one-way ANOVA)		0.0001	0.0001	0.0001	0.0001

$^{a, b, c, d}$ Numbers with different superscripts within one column are significantly different.

Discussion

The fertilizing ability of the sperm in this experiment was very much the same as that of the previous in-vivo fertilization study. It was found that heat treatment had no effect on fertilization rates when males were used on day 3 after heating (see Figure 5.9). From days 7 to 14 the fertilization rates dropped dramatically ($p<0.05$), despite the fact that the same number of motile sperm were used. There was also a low number of cleaved ova obtained, with the most severe effect being observed in the D14 group.

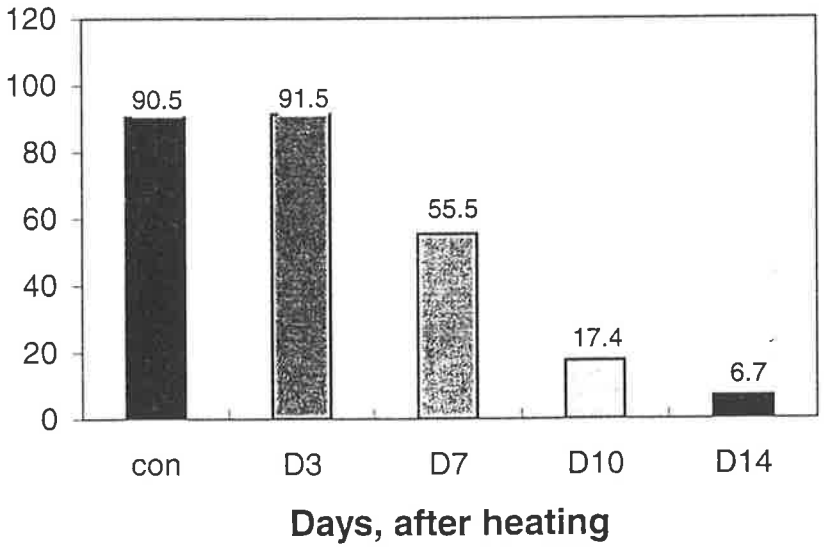


Figure 5.9 Fertilization rate of ova from superovulated normal females. The ova were fertilized in vitro by the same number of motile spermatozoa from either normal males or heated males at different days after heating. Fertilization rates were based on the proportion of fertilized ova compared with the total number of ova incubated.

Some sperm characteristics, i.e. sperm concentration, percent live sperm and sperm motility were assessed concurrently with the fertilization assay. As expected, all the sperm characteristics measured were significantly lower ($p<0.0001$) in the D10 and D14 groups compared with those of controls. The relationship between sperm characteristics and fertilization rates are presented in table 5.18 and figure 5.10. Except for epididymal sperm

concentration, other sperm characteristics together with the rates of fertilization are affected after day 3 through day 14 postheating, suggesting that these parameters are closely related. However, the more rapid fall in fertilization rate compared with the other mentioned sperm characteristics suggests that there must be other factor(s) involved.

Table 5.18 Fertilization rates and sperm characteristics of male mice at different stages (3 to 14 days) after heating

	Control	D3	D7	D10	D14
Fertilization rate (%)	90.5	91.5	55.5	17.4	6.7
Sperm conc. Per ml (x10 ^{6})	38.9	24.3	21.4	22.1	21.3
Live sperm (%)	74.4	74.9	61.6	52.1	50.1
Sperm motility (%)	73.4	70.3	56.8	44.2	30.6

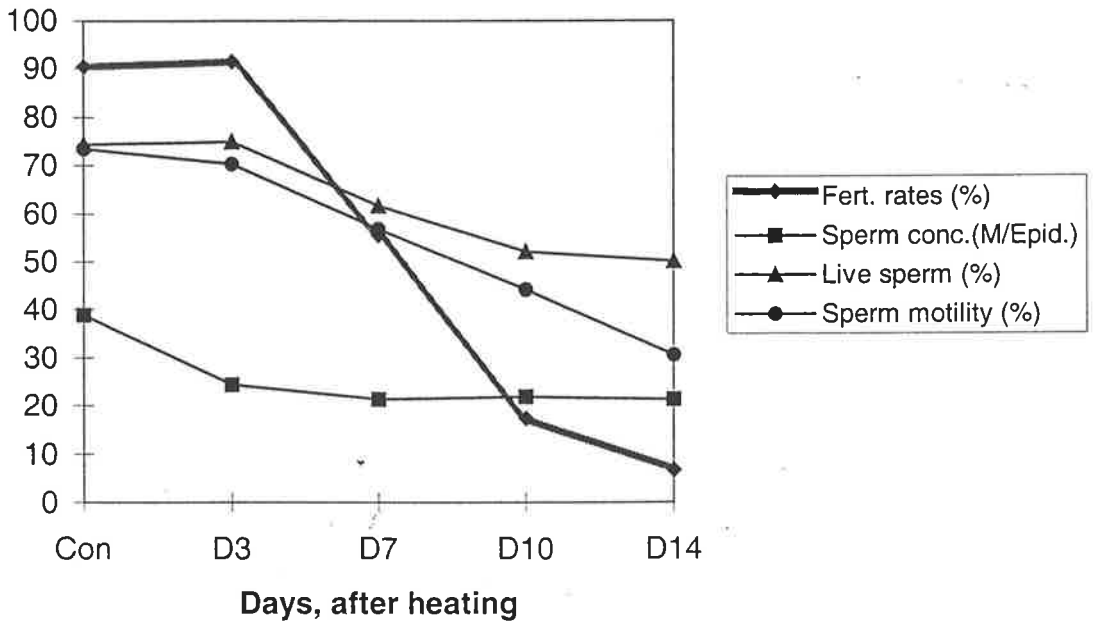


Figure 5.10 Relationships between sperm characteristics and fertilization rates of male mice at different stages (3 to 14 days) after heating

5.6 In vitro fertilization using swim-up sperm

Introduction

It was demonstrated in section 5.5 that only a small proportion of eggs were fertilized in vitro by the same number of motile sperm from heat-stressed males between days 10 and 14 after heating. In this experiment because motility rates of the sperm in each treatment group were different, it was necessary to dilute the sperm suspensions before use so that the same numbers of motile sperm were used in all groups. When the sperm suspensions of the high motility group were diluted it was inevitable that the number of immotile (presumably dead) sperm would be less accordingly. As a result, the number of dead sperm would be greater in the groups which have a lower percent live sperm. It has been reported that defective sperm can produce free radicals which can subsequently harmfully affect the fertilizing ability of live sperm in the same sample (Aitken et al, 1989b; Aitken and Krausz, 2001; Aitken and Baker, 2002). The low fertilization rates in D10 and D14 groups, thus could be due to the harmful effects caused by dead sperm. The current experiment used the swim-up technique in order to obtain as far as possible only motile sperm so that the effect of dead sperm could be eliminated.

Materials and Methods

To increase the number of motile sperm, the swim-up method (see section 3.6.8) was used. Briefly, male mice were sacrificed by cervical dislocation, their epididymides dissected, and punchured with a 27G needle. The sperm suspensions were slowly deposited to the bottom of a test tube containing 3 ml of HTF-Bicarb medium. The tubes were then incubated at 37°C for

20 min, then the sperm-rich top fraction (1ml) was pipetted off, put in a conical tube and left in the incubator.

Swim-up sperm characteristics assessment

A small volume of each sperm sample was taken for the analysis of sperm concentration (using a haemocytometer, see section 3.6.2), percent live sperm (using Nigrosin/Eosin staining, see Section 3.6.3) and sperm motility (using the Hamilton Thorne Motility Analyser, HTM, see Section 3.6.7). After the motility rates of the sperm of all groups were obtained the sperm suspensions with higher concentration of spermatozoa were diluted so that the number of motile sperm in all groups was equalised to $1x10^6$.

Preparation for in vitro fertilization dish

A 50-µl droplet of sperm suspension from each group was transferred to the middle of a 35-mm culture dish, overlayed with paraffin oil and incubated at 37°C in an atmosphere of 5% CO_2 in air for 1.5h to allow sperm to capacitate.

In vitro fertilization

Except for the sperm preparation, the other procedures involving in vitro fertilization were the same as those previously described in section 5.5.

Results

The number of ova per litter of individual superovulated mice ranged between 14 and 30 and the average values for all groups are presented in table 5.19.

Table 5.19 The number of females used and ova recovered. Superovulated females were killed 36h post-hCG injection, after which their eggs were collected and recorded.

Treatment	Total No. of females used	Total No. of ova recovered	Average No. of ova per litter
Control	6	118	19.7
D7 Post-heating	4	85	21.3
D10 Post-heating	4	68	17.0
D14 Post-heating	4	87	21.7

The numbers of females with and without fertilization, and fertilized/not fertilized ova per litter are shown in table 5.20. All the females except one in the D14 group produced fertilized eggs in vitro. The data in table 5.20 in this experiment show that the fertilizing capacity of the swum-up sperm of heated males was improved compared to those in the previous experiment (Section 5.5) where the swim-up technique was not used. However, it can be seen that the fertilization rates in D7, D10 and D14 groups, were still significantly lower ($p<0.01$) than those of controls.

Table 5.20 Fertilization rates of ova from superovulated normal female mice. The ova were fertilized in vitro by swum-up spermatozoa from either normal males or heated males at different days after heating. They were then washed with fresh medium, stained with 0.75% aceto-orcein, and assessed using a phase contrast microscope. The success of fertilization was assessed from either the presence of sperm head or tail in egg cytoplasm or egg activation as can be seen from the release of cortical granules or chromosomes at anaphase.

Treatment	No. of females with /without fertilization	Average No. of ova per litter of females with fertilization	Average No. of fertilized/ not fertilized ova per litter	Fertilization rates (%)
Control	6/0	19.7	17.7/2.0	89.8
D7 Post-heating	4/0	21.3	13.3/8.0 *	62.4
D10 Post-heating	4/0	17.0	6.5/10.5 **	38.2
D14 Post-heating	3/1	21.7	5.0/16.7 **	23.0
Chi-Square value			χ^2=7.8; D7:Con χ^2=8.7; D10:Con χ^2=16.0; D14:Con	

* $p<0.05$ ** $p<0.01$

The characteristics of swum-up sperm used in the IVF assay, which were measured prior to the commencement of the assay, are shown in Table 5.21. All the sperm parameters measured (concentration, viability and motility) were similar among the heated groups but significantly lower ($p<0.006$) than those of controls.

Table 5.21 Sperm parameters assessed 1 to 1.5h prior to sperm-ova incubation. Males were killed 1h before ova collection, and spermatozoa were collected from the cauda epididymides. Approximately 20min after collection, sperm suspensions were analyzed for motility rate, concentration and number of live/dead sperm.

Treatment	No. Mice	Sperm concentration per ml ($x10^6$) (Mean±SE)	% live sperm (N/E staining) (Mean±SE)	Sperm motility (%)
Control	6	17.8 ± 0.7^{a}	83.8 ± 2.0^{a}	83.3 ± 1.7^{a}
D7 Post-heating	4	8.8 ± 1.3^{bc}	68.5 ± 3.3^{b}	65.5 ± 2.3^{b}
D10 Post-heating	4	10.9 ± 1.6^{b}	73.4 ± 3.7^{b}	68.3 ± 2.6^{b}
D14 Post-heating	4	7.0 ± 0.8^{c}	66.6 ± 4.6^{b}	63.3 ± 1.7^{b}
P-value (one-way ANOVA)		0.0001	0.006	0.0001

Discussion

This experiment used the swim-up technique for sperm preparation for use in IVF study. It was expected that by using this technique motile and live sperm would be obtained without contamination by dead or dying sperm. This is because the motile sperm would swim up to the top fraction of the medium. Indeed, following the swim-up technique we obtained an increased sperm motility and percent live sperm (Table 5.21) when compared to the non-swim-up procedure (Table 5.17, Section 5.5). When the swim-up sperm were used for IVF it was therefore expected that their fertilization rates would also increase. The results of this experiment showed that fertilization rates of the sperm of heated males were improved at all time points after heating (Table 5.20) compared with those of the previous study (Table 5.15, Section 5.5) where the epididymal sperm were collected without using the swim-up technique. However, the magnitude of improvement in fertility was marginally small, as can be seen in figure 5.11, and the rates of fertilization by sperm from heated males were still significantly lower than those of controls.

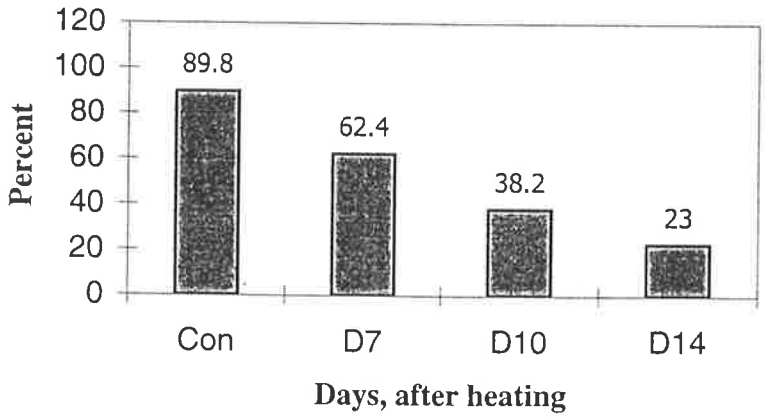


Figure 5.11 Fertilization rate of ova from superovulated normal females. The ova were fertilized in vitro by swum-up spermatozoa from either normal males or heated males at different days after heating. Fertilization rates were based on the proportion of fertilized ova over the total number of ova incubated.

Table 5.22 Relationships between sperm characteristics and fertilization rates

	Control	D7	D10	D14
Fertilization rate (%)	89.8	62.4	38.2	23.0
Sperm conc. Per ml (x10 ^{6})	17.8	8.8	10.9	7.0
Live sperm (%)	83.8	68.5	73.4	66.6
Sperm motility (%)	83.3	65.5	68.3	63.3

When sperm characteristics were considered it was found that sperm motility rates and percent live sperm in the samples from the heated males increased dramatically following the swim-up procedure. However, the values were still much lower ($p<0.01$) than those of controls. Prior to use the sperm suspensions of these heated males and those of controls were diluted with pregassed medium so that the same concentration of approximately $2x10^6$ motile sperm per ml was obtained. Even though the same numbers of motile sperm were used in all groups it was found that fertilization rates were significantly lower with samples from the heated males (Table 5.20). It is clear that sperm motility influences fertilization rates to some extent, and fertilization rates increased from 55.5, 17.4 and 6.7% on days 7, 10 and 14, respectively in the experiment without using the swim-up technique (Section 5.5) to 62.4, 38.2 and 23.0% in the present experiment.

From this small improvement in fertilization rates after the use of swim-up technique it is clear that there are some other factors apart from sperm motility that contribute to the reduced fertility of heat-stressed males. The next experiment was designed to investigate some characteristics of sperm that could contribute to their low fertilizing capacity, such as their

ability to fuse with oolemma, ability to bind to zona pellucida, sperm head shape, sperm morphology and ultrastructure and sperm acrosome integrity.

CHAPTER 6

FACTORS RESPONSIBLE FOR REDUCED FERTILIZING ABILITY OF SPERM FROM HEAT-STRESSED MALE MICE

6.1 The ability of the sperm of heat-stressed male mice to bind to the zona pellucida of normal females

Introduction

It was found in previous experiments that fertilizing ability in vivo (Section 5.4) and in vitro (Section 5.5) of spermatozoa from heat-stressed male mice was significantly reduced. Following selection by the swim-up method, the percentage of motile sperm was significantly improved. However, their fertilization rates were still appreciably lower than those of the controls (Section 5.6). There are a number of events which are involved in the successful fertilization of an egg by a spermatozoon. The spermatozoon must first be able to penetrate the cumulus mass around the egg, bind to the zona pellucida (ZP), penetrate the ZP, bind to and fuse with the oolemma and finally enter the egg cytoplasm. The disruption of any one of these events could cause failure of the entire process. From the observations in previous studies (Sections 5.5, 5.6), after 65min coincubation with spermatozoa it was found that all eggs were free of cumulus masses and moving within the fertilization medium due to the pushing force from bound spermatozoa. This suggested that spermatozoa from heated males had the ability to digest egg investments and reach the zona pellucida. However, it was not known whether those spermatozoa were able to bind to and subsequently penetrate the zona. The current study was therefore conducted to test this ability of spermatozoa to bind to the zona pellucida of the egg. Additionally, it has also been known that sperm acrosome status can greatly influence the success of sperm-zona binding. The release of acrosomal enzymes from the sperm facilitates penetration through the ZP and entry into the perivitelline space. Normally the acrosome stays intact until it is reacted when the sperm have bound to the ZP. If spermatozoa lose their acrosomes prematurely they also lose the ability to bind to the zona. Therefore, the integrity of the sperm acrosome was also investigated in this study.

Materials and methods

Sperm-zona binding assay

The capacity of sperm to bind to the zona pellucida was tested at 3 time points, i. e. days 7, 10 and 14 after heating at 36°C (12h/d for 2 days). At each time point 6 females and 4 males were used. Males were killed 1.5h prior to egg collection and their spermatozoa collected (see Section 3.5.2) and subjected to the swim-up procedure as described in section 3.6.8. Females were superovulated by the procedure described in section 3.10.2.1, killed by cervical dislocation 13.5h post hCG and their cumulus/oocyte clumps (COC) were collected by the procedure described in section 3.10.2.2. The procedure for the sperm-zona binding assay was as described in 3.11.1. Briefly, the cumulus masses were removed using 0.1% hyaluronidase. The cumulus-free oocytes were then stained with Hoechst-33342 stain (10μg/1ml culture medium) and incubated at 37°C with $2x10^6$ swum-up spermatozoa in an incubator. After 20-min incubation eggs were fixed in 2.5% glutaraldehyde, washed in fresh medium and transferred to a clean slide. Coverslips were placed onto the slides, and the preparation observed using a Leitz Othoplan microscope equipped with a 50-w mercury and bulb for epifluorescence and a filter block with an excitation filter BP 350-410, beam splitting mirror RKP 455 and suppression filter LP 470 (Leitz filter Block B2). The H-33342 dye/DNA complex was excited with 355 nm u.v. light and the epifluorescent emission (465 nm) viewed and photographed

Triple staining

Twelve male mice were used to investigate acrosome integrity of the sperm from mice killed on days 7, 10 and 14 post-heating, and an additional 4 mice were used as controls. To determine structural integrity of the acrosome spermatozoa were stained by a triple staining

method using 1% Trypan blue, 0.8% Bismarck brown and 0.8% Rose bengal, as described in section 3.6.4. By using this method the stained spermatozoa can be classified into 4 groups, namely:

1) live, acrosome-intact sperm:

(brown postacrosomal region, pink acrosome, and dark pink apical region)

2) live, acrosome-reacted sperm

(pale brown postacrosomal region, white/pale pink acrosome, clear apical region.)

3) dead, acrosome-intact sperm

(pale blue/ gray head, dark pink apical ridge)

4) dead, acrosome-reacted sperm

(grey heads, no pink staining in the acrosomal region)

Results

Sperm-egg binding

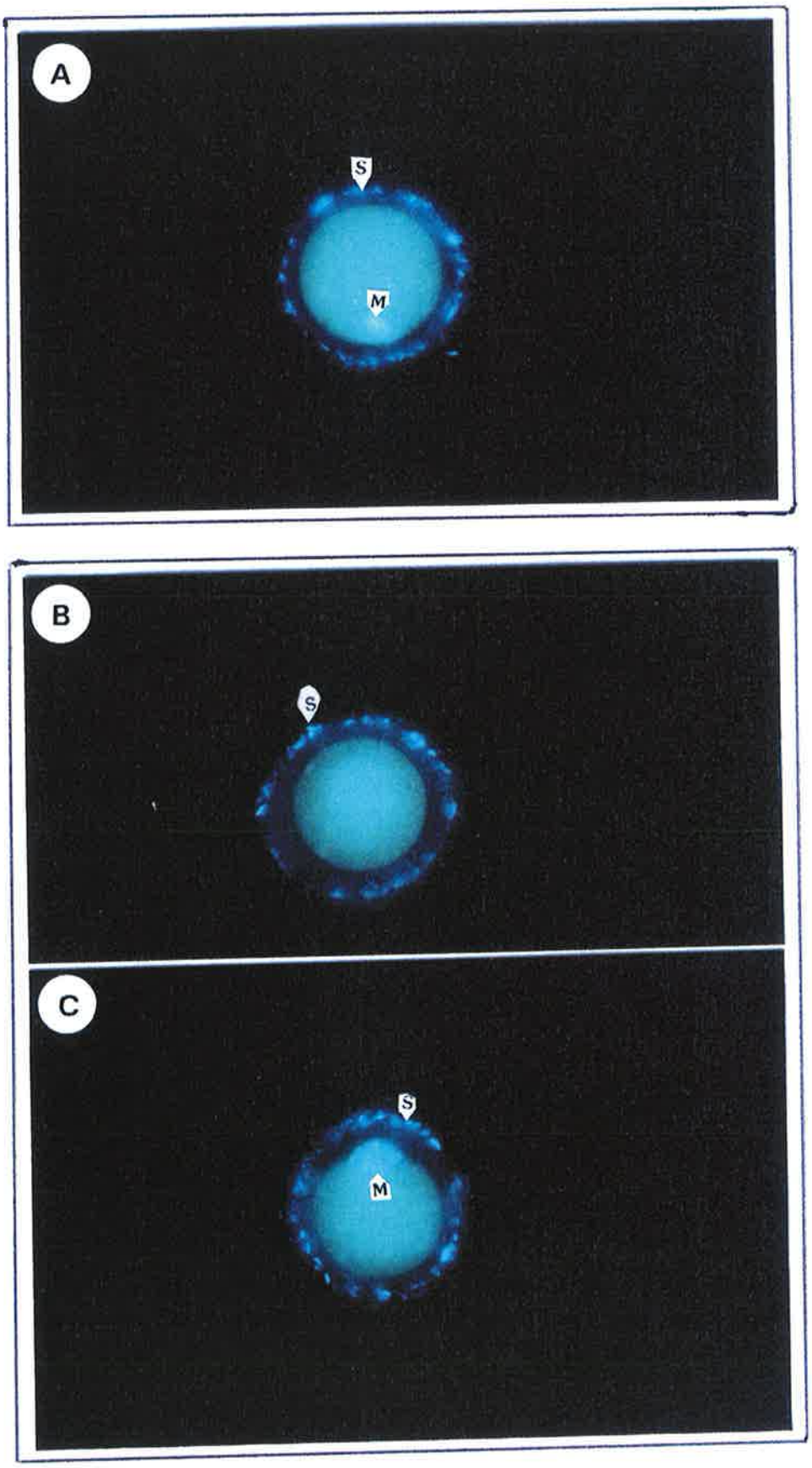
A total of 345 eggs were used in the sperm-egg binding assay. After a 20-min incubation with swum-up spermatozoa all of these eggs were successfully bound to different numbers of spermatozoa ranging from 10 to 32 in day 7 group, 7 to 32 in day 10 group and 7 to 33 in day 14 group (Table 6.1). The average numbers of bound sperm per egg were not statistically different ($p>0.05$) among male animals regardless of treatments and periods after heating.

Table 6.1 The number of spermatozoa bound to the zona pellucida of mouse cumulus-free eggs observed under fluorescence microscope.

Days, post-heating	No. of males	No. eggs incubated	No. eggs with spermatozoa	No. bound sperm per egg	
				Average	Range
Control	12	87	87	23.0±0.4 ^{NS}	10-38
Day 7	4	86	86	21.8±0.5 ^{NS}	10-32
Day 10	4	82	82	21.7±0.5 ^{NS}	7-32
Day 14	4	90	90	22.5±0.9 ^{NS}	7-33
P-value (one-way ANOVA)				0.3517	

NS = non-significance

Figure 6.1 Fluorescence micrographs of zona-intact oocytes preloaded with H-33342 (10μg/ml) and then mixed with swum-up spermatozoa ($2x10^6$/ml) from control (A) or heated males (B, C) collected on day 14 after heating. After 20 min, numerous spermatozoa have bound to the zona pellucida. S = bound spermatozoa, M = metaphase II chromosomes. 250x.


A
S
M
B
S
C
S
M

Acrosome status of spermatozoa

Swum-up spermatozoa were stained by triple staining and assessed using light microscopy. They were classified into 4 groups, live-acrosome intact, live acrosome reacted, dead acrosome intact and dead acrosome reacted. It is shown in table 6.2 that the percentages of live sperm ranged from 67.8% in mice killed on day 14 after heating to 83.1% in the control group, and most of the live sperm were acrosome intact. The percentages of live acrosome-intact spermatozoa were significantly ($p<0.05$) higher in the control group. On the contrary, most of dead spermatozoa were acrosome-reacted, and the numbers of these were significantly ($p<0.001$) higher in the heated groups. Only small proportions of dead spermatozoa had intact acrosomes and the numbers were similar in the various treatment groups.

Table 6.2 Effects of heat stress on acrosome status of live or dead spermatozoa assessed using a light microscope with an oil-immersion objective at 100x.

Days, post-heating	No. of males	Percentages of sperm			
		Live AR-intact	Live AR-reacted	Dead AR-intact	Dead AR-reacted
Control	4	74.8±3.9^{a}	8.3±0.9 ^{NS}	3.0±0.9 ^{NS}	14.0±0.4^{a}
Day 7	4	65.0±1.8^{b}	7.0±0.4 ^{NS}	3.8±0.9 ^{NS}	24.3±1.5^{b}
Day 10	4	63.5±0.9^{b}	8.0±0.9 ^{NS}	2.5±0.6 ^{NS}	26.0±1.2^{bc}
Day 14	4	60.0±1.1^{b}	7.8±1.0 ^{NS}	2.8±0.5 ^{NS}	29.5±2.0^{c}
P-value (One-way ANOVA)		0.0210	0.7434	0.6712	0.0001

NS non-significance

$^{a, b, c,}$ Numbers with different superscripts within one column are significantly different.

Discussion

It has been reported that in mice the primary binding of capacitated spermatozoa to the zona is complete within 15 minutes (Saling and Storey, 1979). The current study incubated sperm and eggs for 20 minutes to make sure that they had sufficient time to interact. Before being incubated with swum-up spermatozoa eggs were preloaded with Hoechst-33342 stain. This staining technique has been successfully used in the sea urchin (Hinkley *et al.*, 1986) and mouse (Conover and Gwatkin, 1988). The success of this technique in detecting the sperm that bind to the zona of eggs while these eggs are still viable depends on the concentration of the stain used. If the stain is too diluted the zona-bound sperm do not obtain an adequate amount of stain to be excited by ultraviolet/violet light in a fluorescence microscope. If the stain concentration is too high it may reduce viability of eggs. The concentration of 10μg/ml used in this study was based on the study of Conover and Gwatkin (1988) which reported that the concentration of 10μg/ml stained the zona-bound sperm well, and had no harmful effect on ova viability as judged by the normal development of embryos after fertilization. Preloading eggs with Hoechst-33342 stain at a concentration of 10μg/ml has proved successful in our study. As clearly shown in figure 6.1 the zona-bound spermatozoa show up clearly using 355 nm u.v. light. Because there were a large number of spermatozoa bound to the zona around the eggs, the counting of numbers of bound spermatozoa per egg were made at only one plane of focus.

The sperm-zona binding assays were performed in order to determine if whole body paternal heat stress would affect the ability of spermatozoa to bind to the zona pellucida. The spermatozoa were tested at 3 times, days 7, 10 and 14 after heating. It was discovered in this study that spermatozoa from mice subjected to whole body heat stress did not lose the ability to bind to the zona pellucida of eggs, as evident in table 6.1 with the average numbers of zona-

bound sperm per egg being not significantly different between the control and the heat-stressed groups.

It is known that in the mouse only the acrosome-intact capacitated spermatozoa will bind to the zona. In this study because the number of zona-bound spermatozoa appeared to be normal in heat-stressed mice, it was expected that these spermatozoa contained intact acrosomes. Indeed, the data in table 6.2 revealed that the numbers of live acrosome-intact spermatozoa were high (60 to 65%) in the heated groups. Such high level of "healthy" spermatozoa will certainly produce high rates of binding to the zona. The acrosome itself, however, is not involved in the process of sperm-zona binding. It has been known that during the binding process some molecules located in the sperm plasma membrane overlying the acrosome bind to the *O*-linked oligosaccharides of the ZP3 glycoproteins. The molecules of the spermatozoa reported to bind to ZP receptors of eggs include galactosyltransferase (GalTase) (Shaper *et al.*, 1988; Lopez *et al.*, 1985; Miller *et al.*, 1992; Youakim *et al.*, 1994), galactose-binding protein (sp56) (Bleil and Wassarman, 1990), spermadsin (Sanz *et al.*, 1992) and zona receptor kinase (ZRK) (Hardy and Garbers, 1995).

Thus, it is likely that heat treatment in the current study did not affect the acrosomal integrity or the molecules on the plasma membrane that are responsible for the binding to the oligosaccharides of the ZP.

6.2 Effects of heat stress in males on sperm ability to penetrate zona pellucida of eggs

Introduction

Based on the observations in the studies in sections 5.4, 5.5 and 5.6, together with the results of the previous study (Section 6.1) it is clear that the swum-up spermatozoa from heat-stressed males do not lose the ability to digest the cumulus masses around the zona pellucida (ZP) or to bind to molecules on the zona surface. Poor fertilization found in earlier studies, therefore, must be a result of a disruption of events at later stages after sperm-zona binding. The current study investigated the events immediately following sperm-zona binding, namely zona penetration.

Methods

Sperm-zona penetration assay

The precedure for the zona penetration assay was the same as that for the sperm-zona binding assay (see Section 6.1), with the exception that in the sperm-zona penetration assay oocytes were stained with a lower concentration of the Hoechst-33343 stain (1μg/ml), and the incubation period was longer (75 min) to provide adequate time for the sperm to penetrate the zona.

Results

Sperm-zona penetration

The capacity of spermatozoa from heat-stressed males to penetrate the zona pellucida was assessed at 3 times after heating (36°C, 12h/d for 2 days) on days 7, 10 and 14. The success of spermatozoa in penetrating the zona was judged by the presence of sperm either in the

perivitelline space (PVS) or in the egg cytoplasm. It is shown in tables 6.3, 6.4 and 6.5 that at each time studied after heating only about half or fewer of the eggs had a spermatozoon or spermatozoa in the perivitelline space (PVS) and the values did not differ significantly from one another ($p>0.05$). On the contrary, in the control group, most eggs had spermatozoa in the cytoplasm or the perivitelline space and the numbers of eggs with one or more spermatozoa were significantly higher in the control than the heated groups ($p<0.05$, day 7; $p<0.0001$, days 10 and 14).

Table 6.3 The numbers of eggs having spermatozoa in their perivitelline space (PVS) or cytoplasm. Cumulus-free eggs were incubated with swum-up spermatozoa ($2x10^6$/ml) collected on day 7 after heating. After 75-min coincubation they were observed under fluorescence microscope at 400x.

Days, post-heating	Male	No. eggs incubated	No. eggs with spermatozoa	
			In PVS	In cytoplasm
Day 7	C1	20	2	16
	C2	25	1	20
	C3	18	0	15
	C4	26	3	18
Control means		22.3	1.5	17.3
	H1	23	1	10
	H2	22	0	9
	H3	19	0	12
	H4	25	1	7
Treatment means		22.3	0.5^{NS}	9.5*
Chi-square value			$\chi^2 = 0.52$	$\chi^2 = 5.69$

NS = Non significant , * = $p<0.05$, compared with controls

Table 6.4 The numbers of eggs having spermatozoa in their perivitelline space (PVS) or cytoplasm. Cumulus-free eggs were incubated with swum-up spermatozoa ($2x10^6$/ml) collected on day 10 after heating. After 75-min coincubation they were observed under fluorescence microscope.

Days, post-heating	Male	No. eggs incubated	No. eggs with spermatozoa	
			In PVS	In cytoplasm
Day 10	C1	23	2	17
	C2	20	1	15
	C3	24	3	18
	C4	26	3	19
Control means		23.3	2.3	17.3
	H1	19	0	4
	H2	21	1	2
	H3	25	0	3
	H4	22	1	1
Treated means		21.8	0.5^{NS}	2.5**
Chi-square value			$\chi^2 = 1.11$	$\chi^2 = 18.02$

NS Non significant ** $p<0.0001$, compared with controls

Table 6.5 The numbers of eggs having spermatozoa in their perivitelline space (PVS) or cytoplasm. Cumulus-free eggs were incubated with swum-up spermatozoa ($2x10^6$/ml) collected on day 14 after heating. After 75-min coincubation they were observed under fluorescence microscope.

Days, post-heating	Male	No. eggs incubated	No. eggs with spermatozoa	
			In PVS	In cytoplasm
Day 14	C1	20	5	14
	C2	26	3	20
	C3	24	2	18
	C4	16	1	14
Control means		21.5	2.8	16.5
	H1	18	0	3
	H2	25	2	2
	H3	28	1	0
	H4	22	0	1
Treated means		23.3	0.8^{NS}	1.5 **
Chi-square value			$\chi^2 = 1.39$	$\chi^2 = 23.0$

NS Non significant ** $p<0.0001$, compared with controls

Figure 6.2 Phase contrast (A) and fluorescence (B) micrographs of the same zona-intact mouse oocyte preloaded with H-33342 (1μg/ml) and then mixed with swum-up spermatozoa ($2x10^6$/ml) from a heated male 14 days after heating. At 75 min after insemination numerous spermatozoa (sp) were bound to the zona pellucida, but none of them had fused with the oolemma or been incorporated into the egg cytoplasm. Thus, the egg was not activated, and only the metaphase II chromosomes (M) fluoresce. 400x.

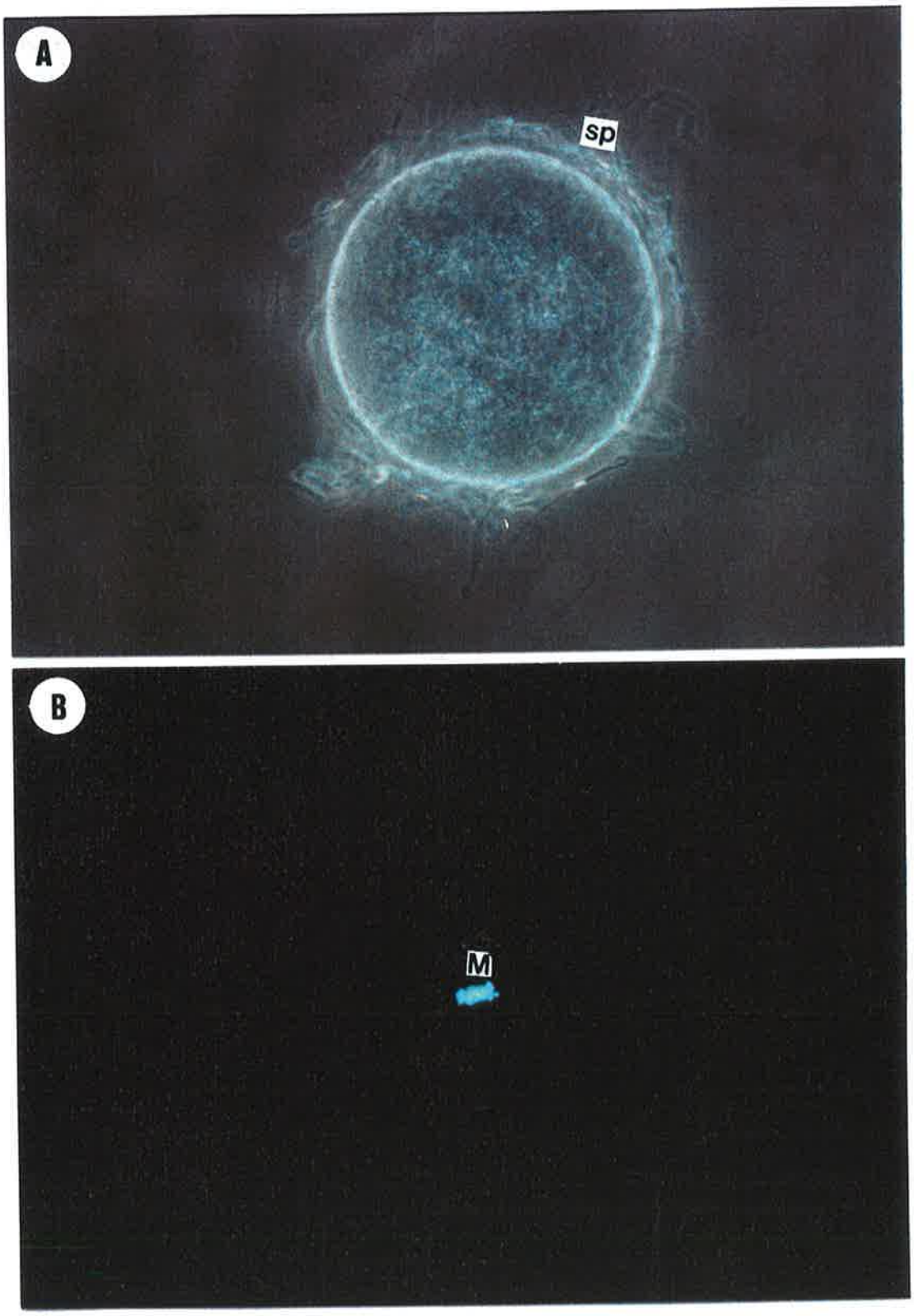
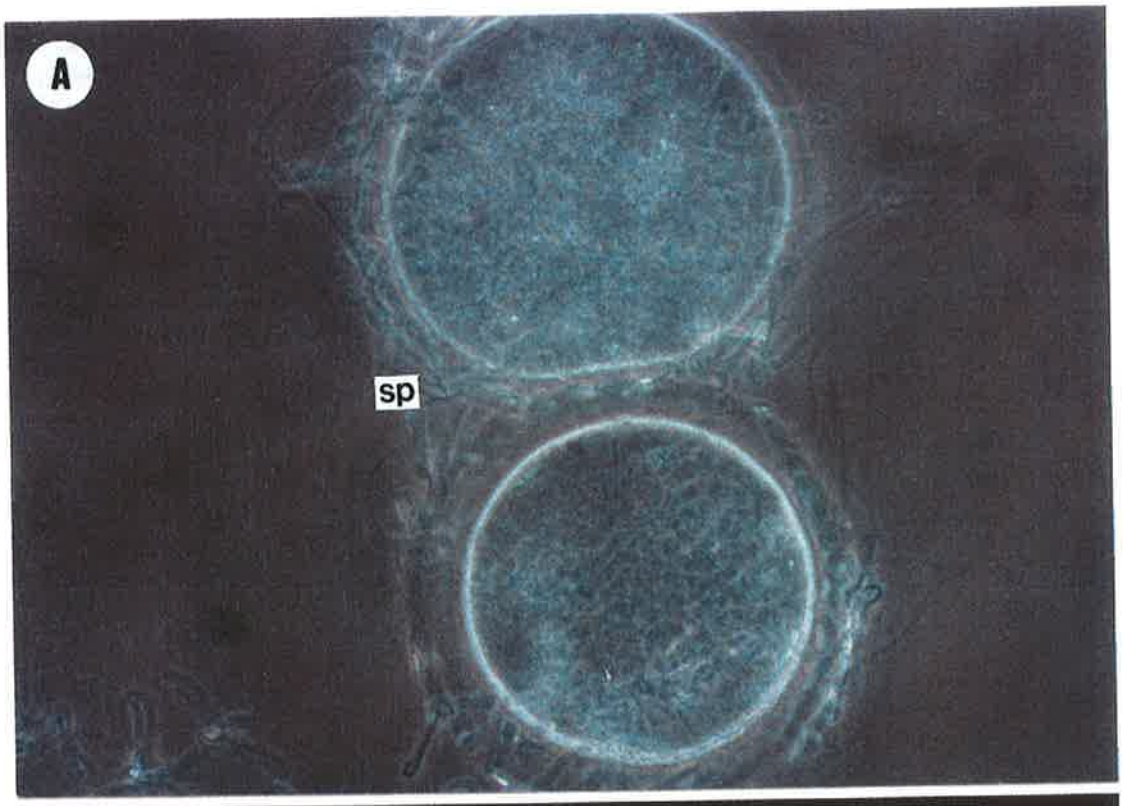

A
sp
B
M

Figure 6.3 Phase contrast (A) and fluorescence (B) micrographs of the same two zona-intact mouse oocytes preloaded with H-33342 (1μg/ml) and then mixed with swum-up spermatozoa ($2x10^6$/ml) from a heated male 14 days after heating. At 75 min after insemination numerous spermatozoa (sp) were bound to the zona pellucida, but none of them had fused with the oolemma or been incorporated into the egg cytoplasm. Thus, the egg was not activated, and only the metaphase II chromosomes (M) fluoresce. 400x.


A
sp

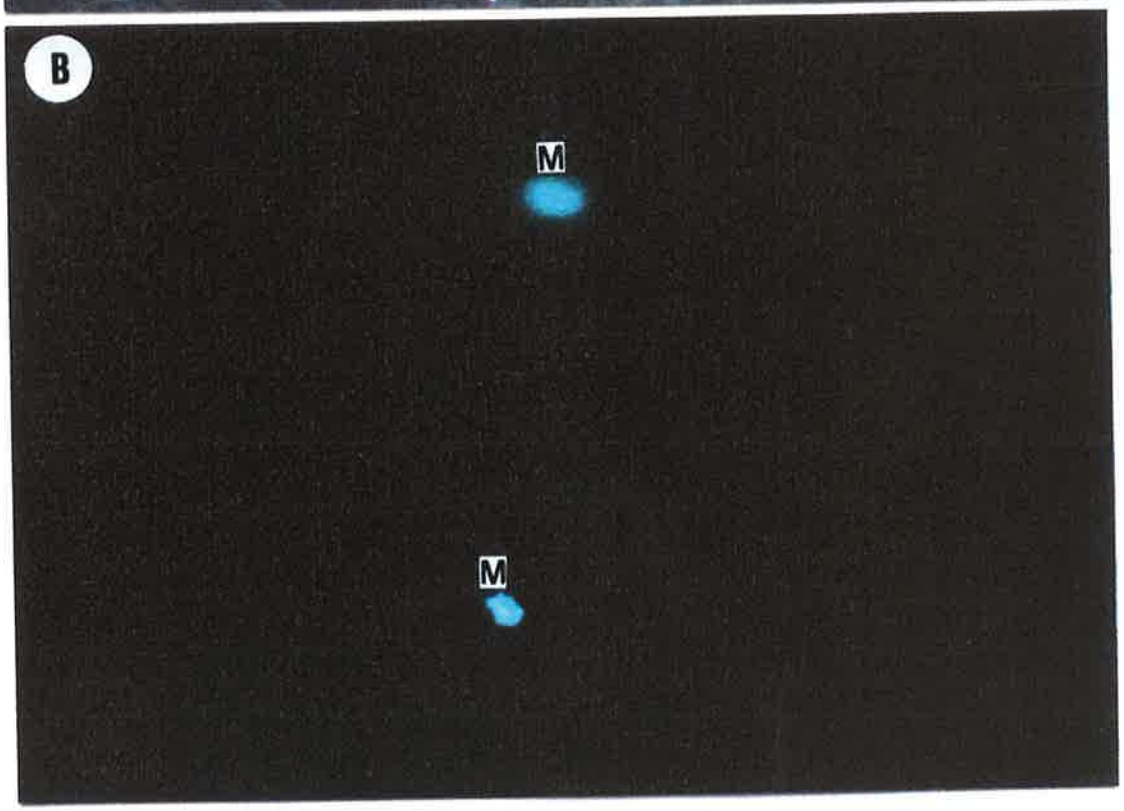
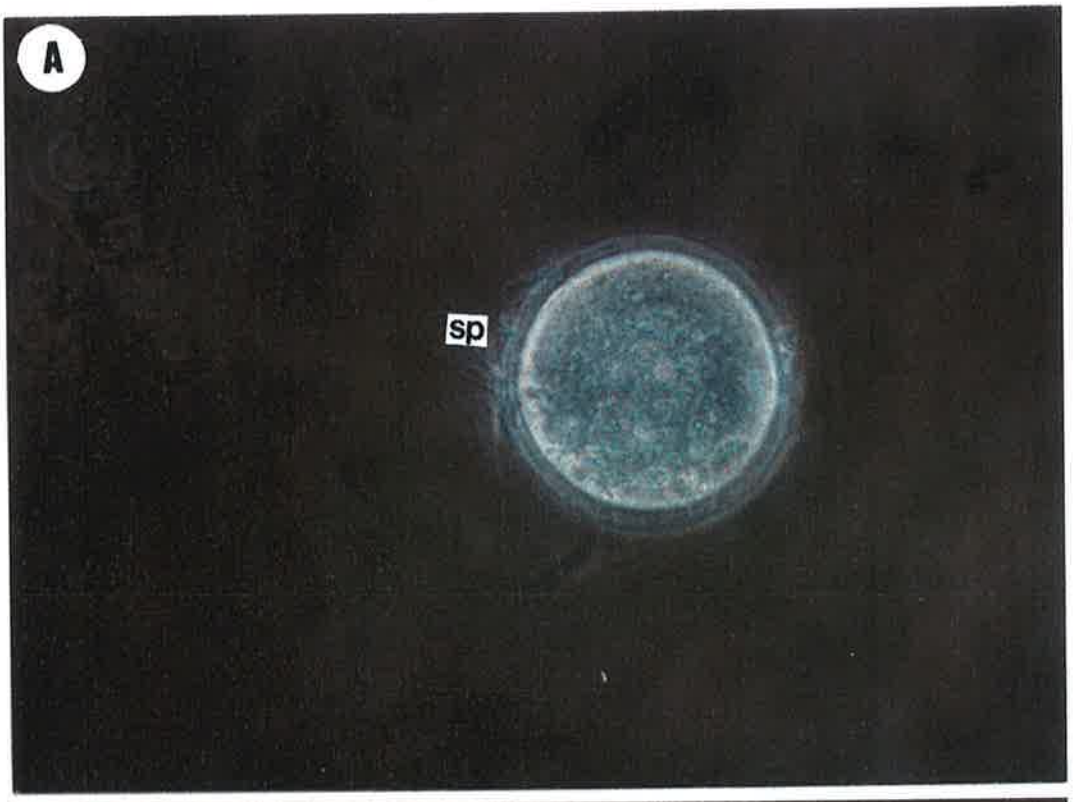

B
M
M

Figure 6.4 Phase contrast (A) and fluorescence (B) micrographs of the same zona-intact mouse oocyte preloaded with H-33342 (1μg/ml) and then inseminated in vitro with swum-up spermatozoa ($2x10^6$/ml) from a control male. At 75 min after insemination there were still a few spermatozoa (sp) binding to the zona pellucida and only one of them had entered the oocyte. Only the fertilizing spermatozoon (S) is beginning to decondense, and the maternal (metaphase II) chromosomes (M) show fluorescense. 400x.


A
sp

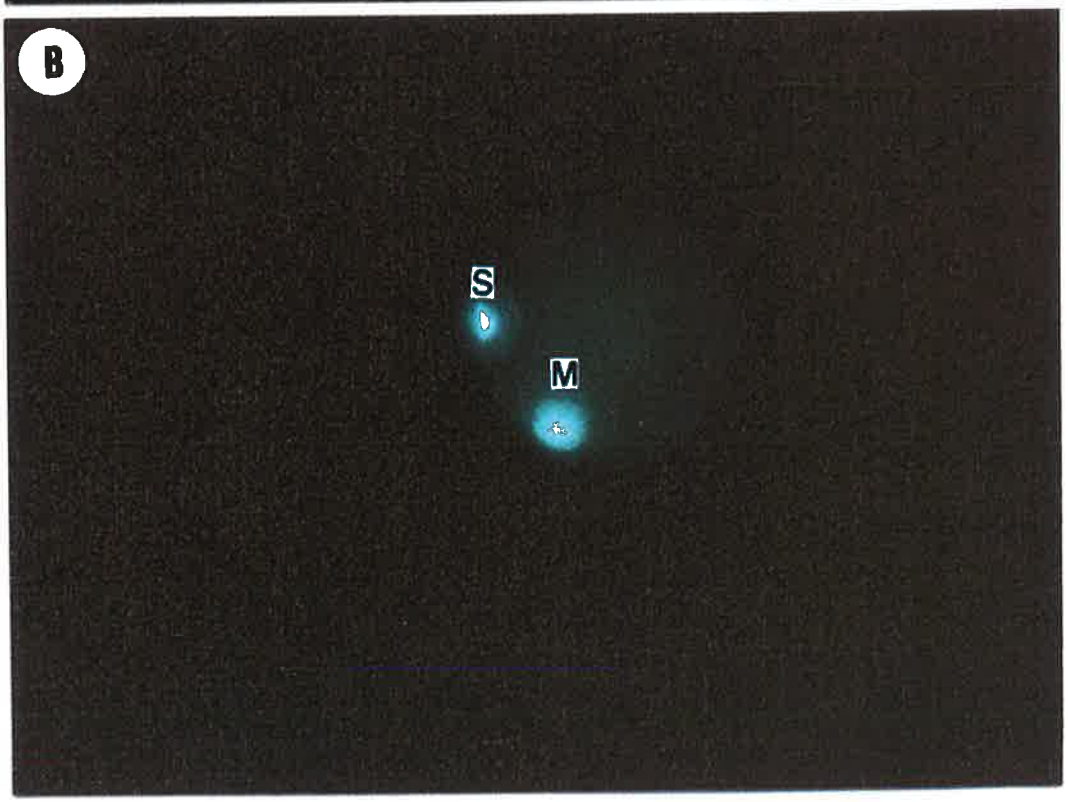
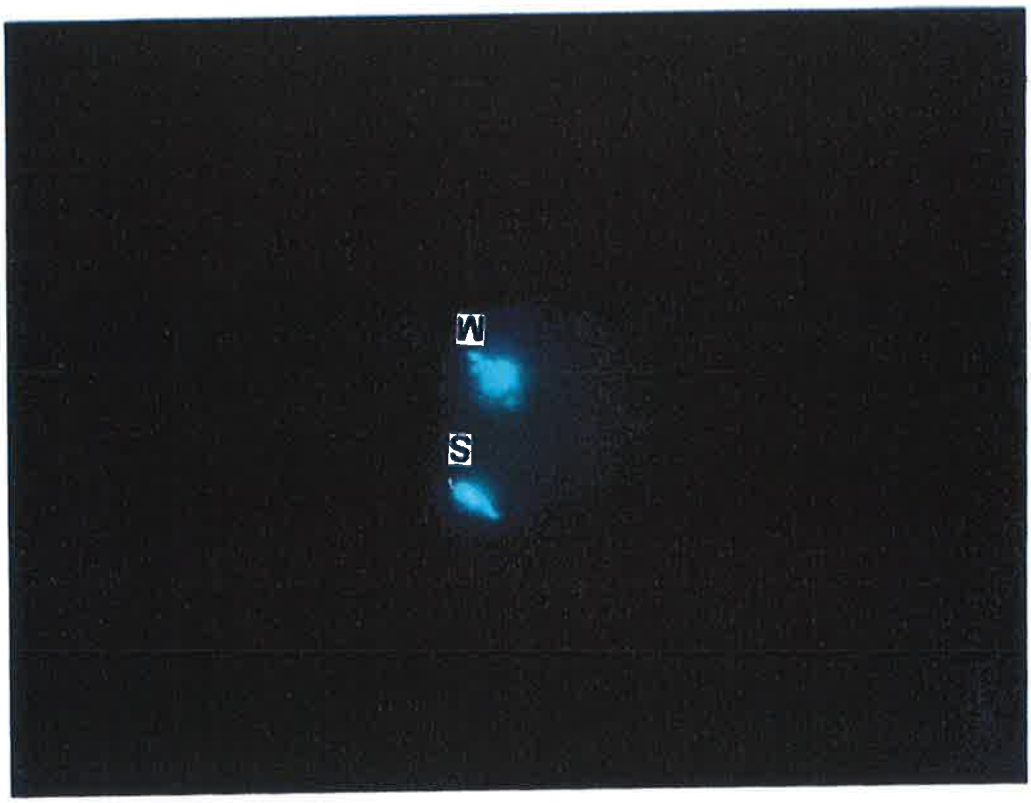

B
S
M

Figure 6.5 A fluorescence micrograph of a zona-intact mouse oocyte preloaded with H-33342 (1μg/ml), then inseminated in vitro with swum-up ($2x10^6$/ml) spermatozoa from a control male and examined at 75 min after insemination. Note the decondensing spermatozoon (S), and the maternal (metaphase II) chromosomes (M). 250x.


W
S

Discussion

The sperm-zona penetration assay was conducted using eggs preloaded with Hoechst-33342 stain, and designed to detect only the sperm that has successfully passed through the zona pellucida. The assay will be successful only if there is no excess stain outside the egg cytoplasm so that only the sperm that has fused with the egg plasma membrane or has entered the egg cytoplasm, but not the sperm at the surface of the zona, are stained. To get the eggs that have the stain only in their cytoplasm before use, two important steps must be carefully carried out. Firstly, eggs must be incubated in a medium containing a low concentration of the stain, otherwise the leakage of the stain from the egg cytoplasm can occur. The concentration of the 1μg/ml has been reported by Conover and Gwatkin (1988) to give satisfactory results. Secondly, H-33342 preloaded eggs must be washed thoroughly (3 to 4 times) in fresh medium before incubation with the spermatozoa.

In the current study, eggs were preloaded with Hoechst-33342 stain at a concentration of 1μg/ml and yielded very good results. As shown in figures 6.2 and 6.3 there are a large number of spermatozoa on the surface of the zona, however, they are not stained, and therefore do not show up under a fluorescence microscope. In figures 6.4 and 6.5, there was one spermatozoon that had entered the cytoplasm of each egg, hence only the sperm head and egg chromosomes showed fluorescence.

The numbers of eggs that were successfully penetrated by a spermatozoon/spermatozoa show clearly that the whole body heat stress at 36°C for 2 days, 12h per day greatly reduced the capacity of spermatozoa to penetrate the zona pellucida. The degrees of damage to sperm functions depended on the time when the sperm were used. It is likely that the spermatozoa collected on day 14 after heating were more damaged than those collected at earlier times, as judged by their low penetration rate (only the average of 2.3 in 23.3 eggs were penetrated,

suggesting that testicular germ cells at different stages responded differently to heat stress. The most severe effects found on day 14 after heating suggested that early spermatids were more susceptible to heat than late spermatids, or sperm in the epididymis.

It was found in the previous experiment (Section 6.1) that in both control and heated mice, a large proportion of live spermatozoa were acrosome-intact, and they had a similar capacity to bind to the zona pellucida. Before zona-bound spermatozoa can pass through the zona they must undergo the acrosome reaction to release hydrolytic enzymes that are necessary for zona digestion. In other words, sperm-zona penetration will not occur if the acrosome reaction is prevented. The initiation of an acrosome reaction depends on the signal released from the zona, and it is possibly an increased level of Ca^{++}. However, in many cases the rise in levels of Ca^{++} do not always culminate in the acrosome reaction of spermatozoa. The failure of zona-bound spermatozoa to respond to Ca^{++} influx is believed to be due to biochemical and physiological alterations of the sperm plasma membrane.

6.3 Effects of heat stress in males on sperm morphology

Introduction

After sperm-zona binding, zona penetration will not occur unless the sperm undergo the acrosome reaction. It has been reported that the process of sperm acrosome reaction can be inhibited by reactive oxygen species (ROS) (Aitken, 1994; Aitken and Fisher, 1994; Griveau *et al.,* 1995), and a significant level of ROS can be produced by morphologically abnormal, defective and immature sperm (Aitken and Clarkson, 1988; Aitken *et al.*,1989; Aitken and West, 1990; Sharma and Agarwal, 1996). It is possible therefore, that the failure of zona-bound spermatozoa to penetrate the zona in the previous experiment (Section 6.2) was due to the inhibition of acrosome reaction by ROS produced by morphologically abnormal or defective spermatozoa. The next study was designed to investigate the effect of heat treatment on sperm morphology, at both light microscopic and electron microscopic levels.

Methods

Sperm morphology by light microscopy

Eosin staining was used to examine epididymal sperm morphology at days 7, 10 and 14 after heating (36°C, 12h/d for 2 days). Six male mice were used at each time point and another six were used as controls. They were killed by cervical dislocation, and their spermatozoa collected as the procedure described in section 3.5.2. The spermatozoa were then stained with 5% (w/v) aqueous eosin as the procedure described in section 3.6.5. Sperm morphology was determined under light microscope with an oil-immersion objective at 100x. The percentages of head and tail abnormalities were determined in a total number of 400 sperm from each male.

Sperm morphology by electron microscopy

Ultrastructure of maturing germ cells and spermatids within the testis and epididymal spermatozoa was determined by a transmission electron microscopy (TEM). Heated male mice were killed on days 8 and 12 after heating, 2 mice at each period, with another 2 mice used as controls. The whole body of each mouse was perfused with 1.5% formaldehyde/1.5% glutaraldehyde fixative made up in 0.2 M phosphate buffer pH 7.4 as described in section 3.7.2. The testes and epididymides were dissected out of the scrotum, cut into small cubes (1 mm^3), and fixed in 3% formaldehyde / 3% glutaraldehyde fixative in 0.2 M phosphate buffer pH 7.4 at room temperature for 2-4h. They were then processed and embedded in Taab embedding resin and polymerized at 60°C for 48h (see Section 3.9.1). The tissue blocks were trimmed and cut at 0.5μm thickness (semi-thin) using glass knives. The sections were stained in toluidine blue solution (see Section 3.9.2), and observed under light microscopy to select the suitable areas for electron microscopy. The tissue blocks were trimmed to centralize the desired features, then ultra-thin sections cut at a thickness of approximately 0.1μm using a diamond knife. The ultrathin sections were collected onto specimen grids, stained with uranyl acetate and lead citrate (see Section 3.9.3) and observed with a Philips 100S transmission electron microscope (TEM).

Results

Sperm morphology by light microscopy

Spermatozoa from males killed on days 7, 10 and 14 were examined. The morphology of the sperm was classified into 4 categories, namely normal head and tail, abnormal head but normal tail, normal head but abnormal tail, and abnormal head and tail. Figures 6.6, 6.7 and 6.8 show photographs of spermatozoa in the 4 categories used. The numbers of normal sperm (with a normal head and tail) were significantly lower and sperm with a normal head but an abnormal tail were significantly higher in heated animals at every time point after heating, compared with controls (Table 6.6). The numbers of misshaped sperm with a normal tail were not significantly different between the control and day 7 groups but significantly higher at day 10 and day 14. A small number of spermatozoa with head and tail abnormalities compared with other categories were found at every time after heating, and the numbers were significantly higher in days 10 and 14 groups compared with controls.

Table 6.6 Effects of whole body heat stress on abnormalities of sperm heads and tails of epididymal spermatozoa. Values given are mean percentages (±SEM) derived by counting 400 sperm per each male.

Days, post-heating	Male	Percentages of sperm			
		Normal head and tail	Abnormal head normal tail	Normal head abnormal tail	Abnormal head and tail
Control	6	72.1±2.0^{a}	2.1±0.3^{a}	25.0±2.4^{a}	0.8±0.3^{a}
Day 7	6	59.9±1.0^{b}	3.9±0.4^{a}	34.8±0.9^{b}	1.5±o.2^{ab}
Day 10	6	52.3±2.1^{b}	7.5±1.4^{b}	35.9±2.9^{b}	4.4±0.7^{bc}
Day 14	6	43.0±4.0^{c}	9.4±1.0^{b}	40.3±3.4^{b}	7.4±2.0^{c}
P-value (one-way ANOVA)		0.0001	0.0005	0.0091	0.0043

$^{a, b, c}$ Numbers with different superscripts within one column are significantly different.

Figure 6.6 Photographs showing spermatozoa with normal and abnormal head shapes. Epididymal spermatozoa were stained with 5% aqueous eosin, then observed under a light microscope using an oil-immersion objective at 100x. The normal sperm heads are shown in plates A and B, and the various forms of abnormal heads are shown in plates C to H.

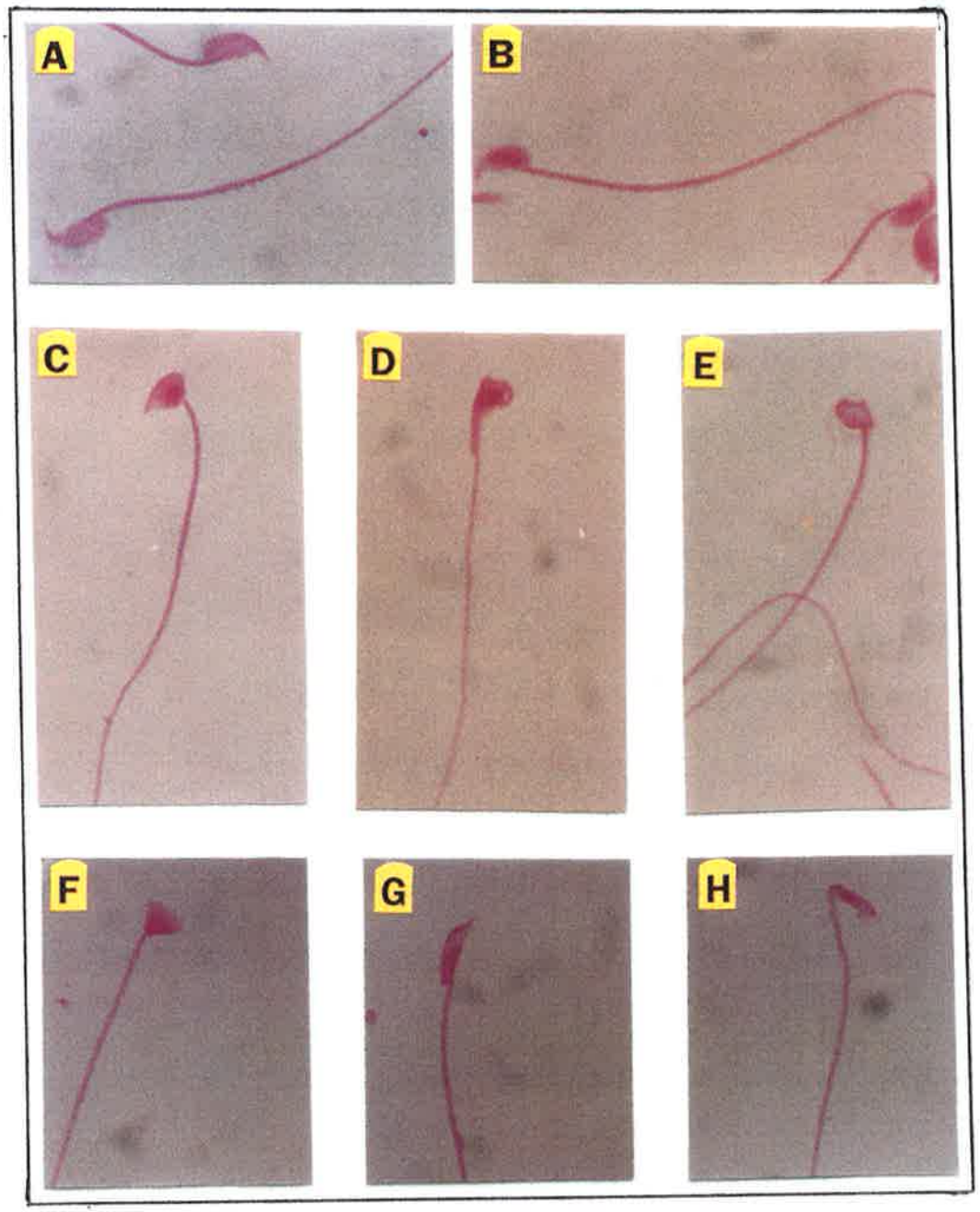

A
B
C
D
E
F
G
H

Figure 6.7 Photographs showing epididymal spermatozoa with normal heads, but abnormal tails. Note that the flagella of the spermatozoa are bent at different locations; neck (A), midpiece (B and C), midpiece and principal piece (D and E) and tail (F). A cytoplasmic droplet is seen attached in C. 1000x.

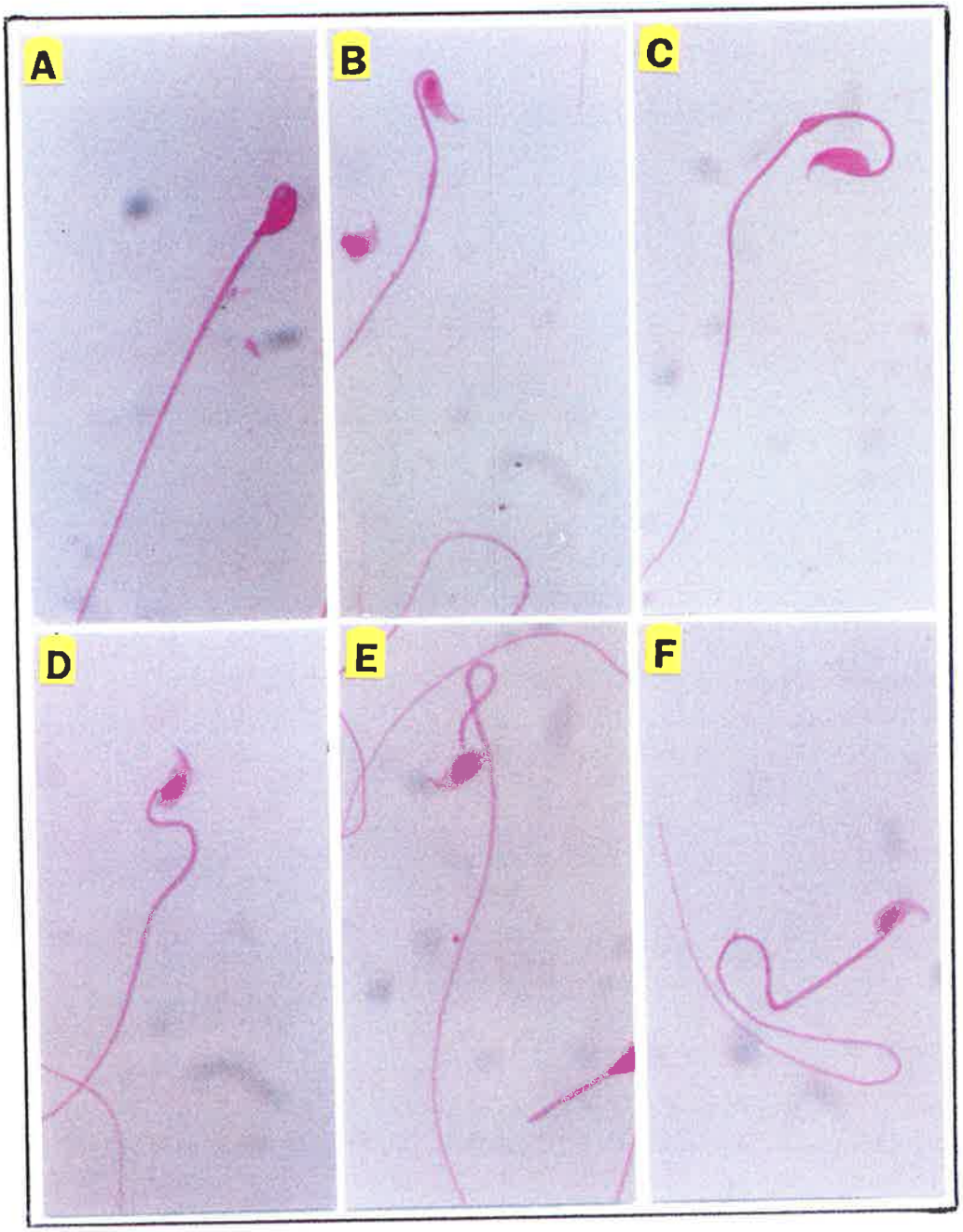

A
B
C
D
E
F

Figure 6.8 Photographs showing epididymal spermatozoa with abnormal heads and flagella; misshaped head + missing mitochondria (A), misshaped head + looped midpiece (B), misshaped head + bent tail (C), misshaped head + cytoplasmic droplet + abnormal principal piece (D), misshaped head + bent principal piece (E) and tiny head + missing mitochondria + cytoplasmic droplet (F). 1000x.

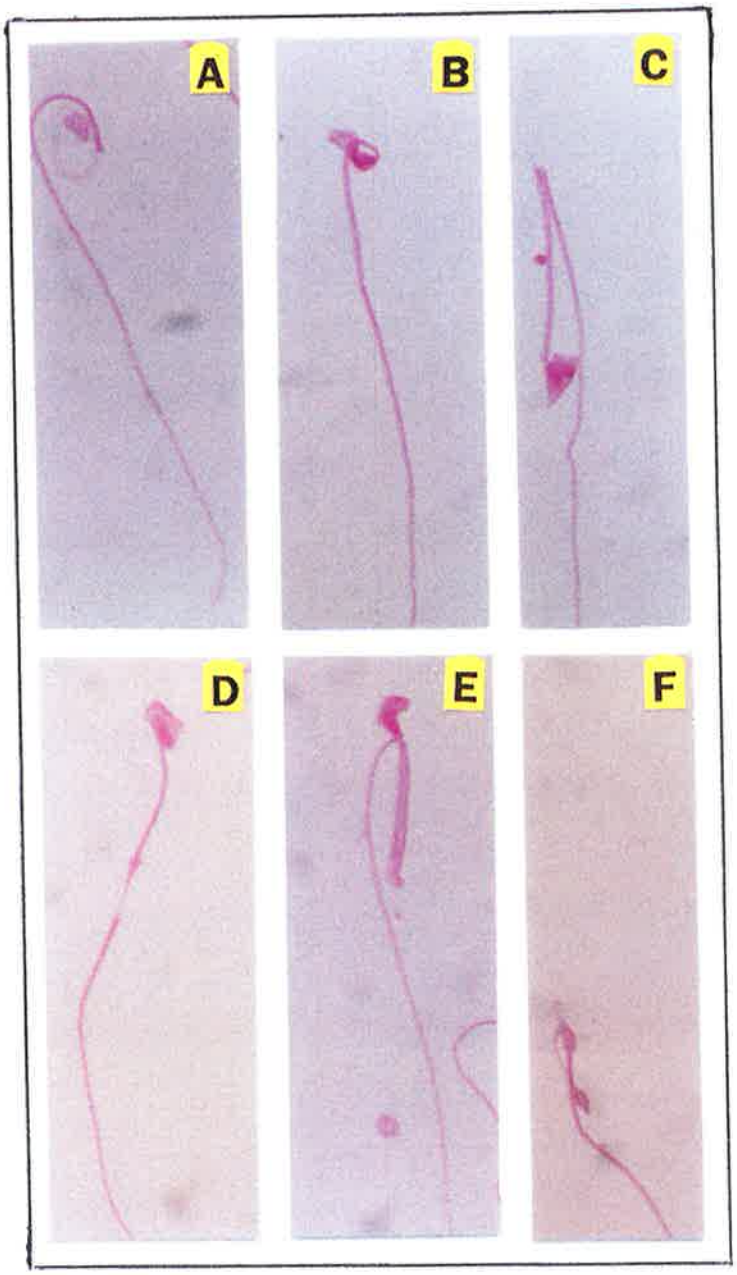

A
B
C
D
E
F

Figure 6.9 A photograph of epididymal spermatozoa from control males. All spermatozoa have normal heads while some of them have abnormal tails, bent (B) and coiled (C). 1000x.

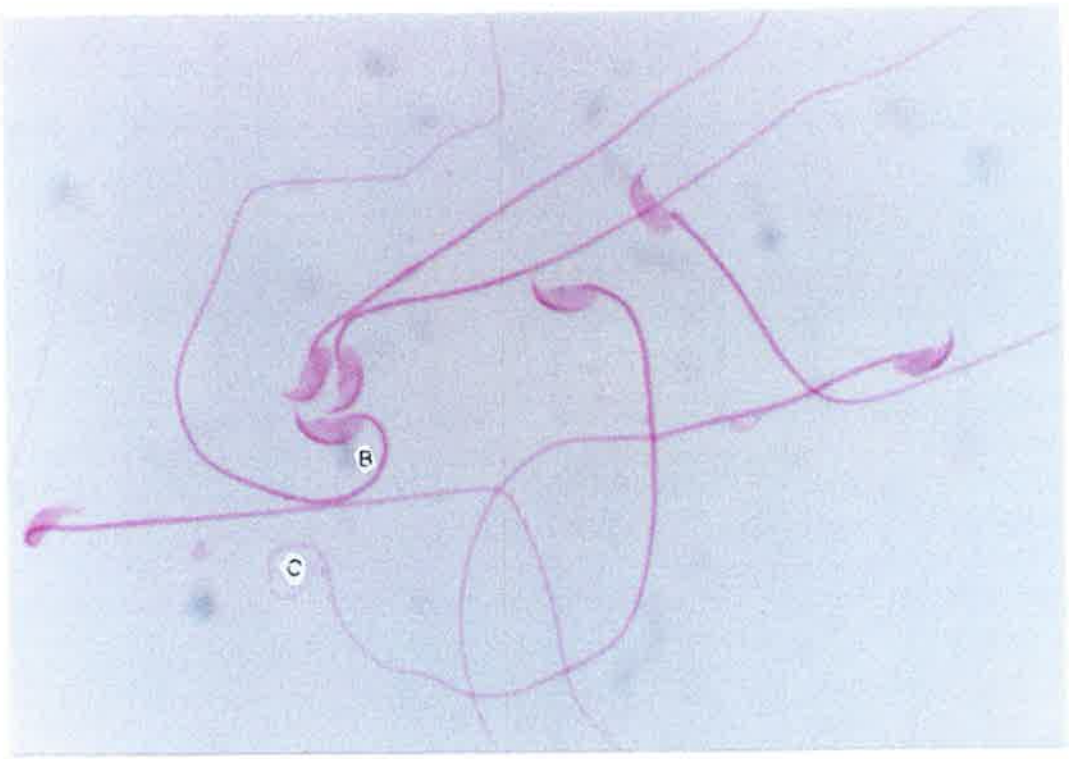
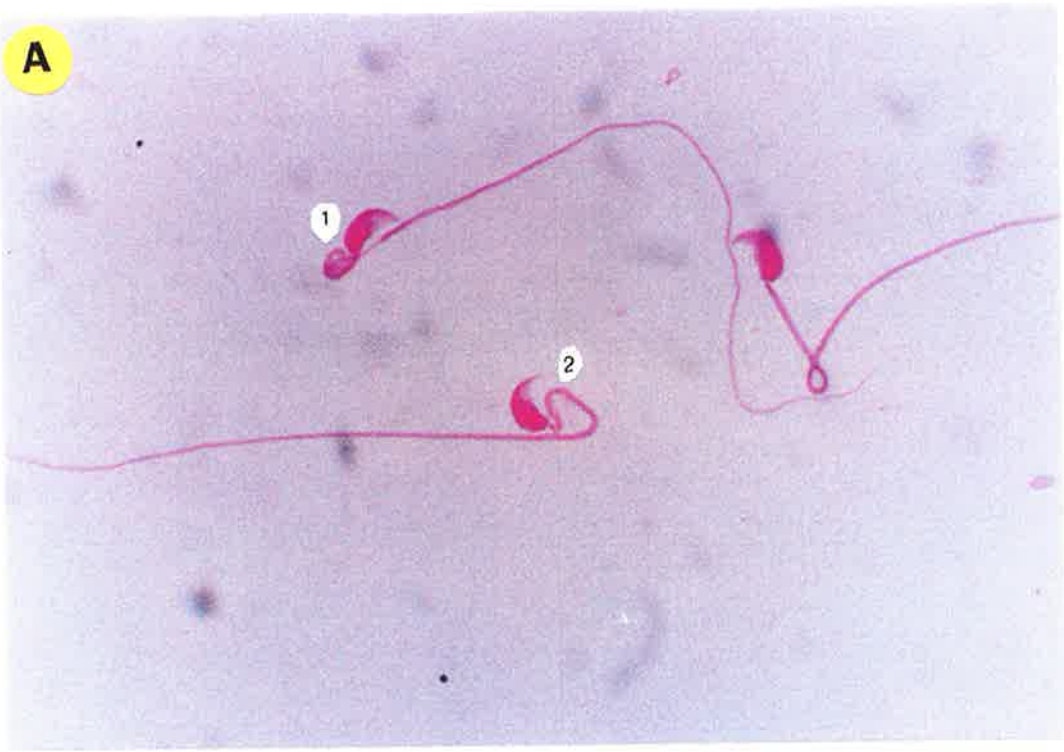

B
C

Figure 6.10 Photographs of epididymal spermtozoa from heat-stressed males, showing different forms of tail abnormalities; coiled and thin midpiece (1), bent midpiece (2) and looped midpiece (3). 1000x.


A
1
2

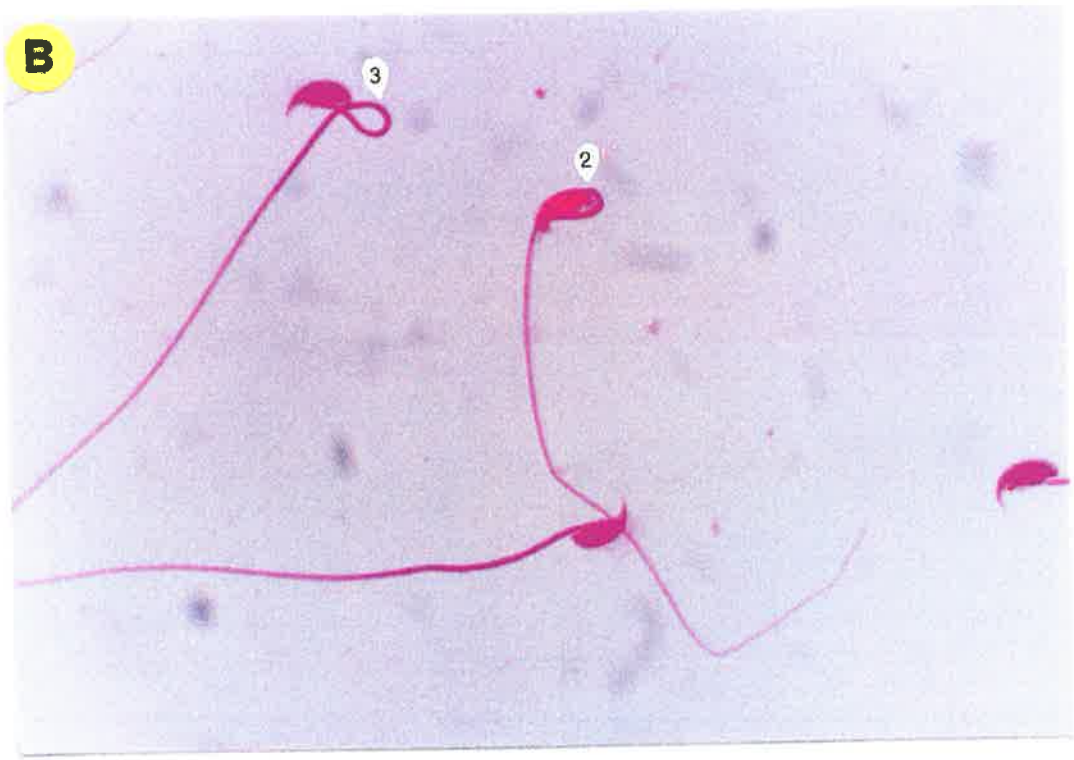
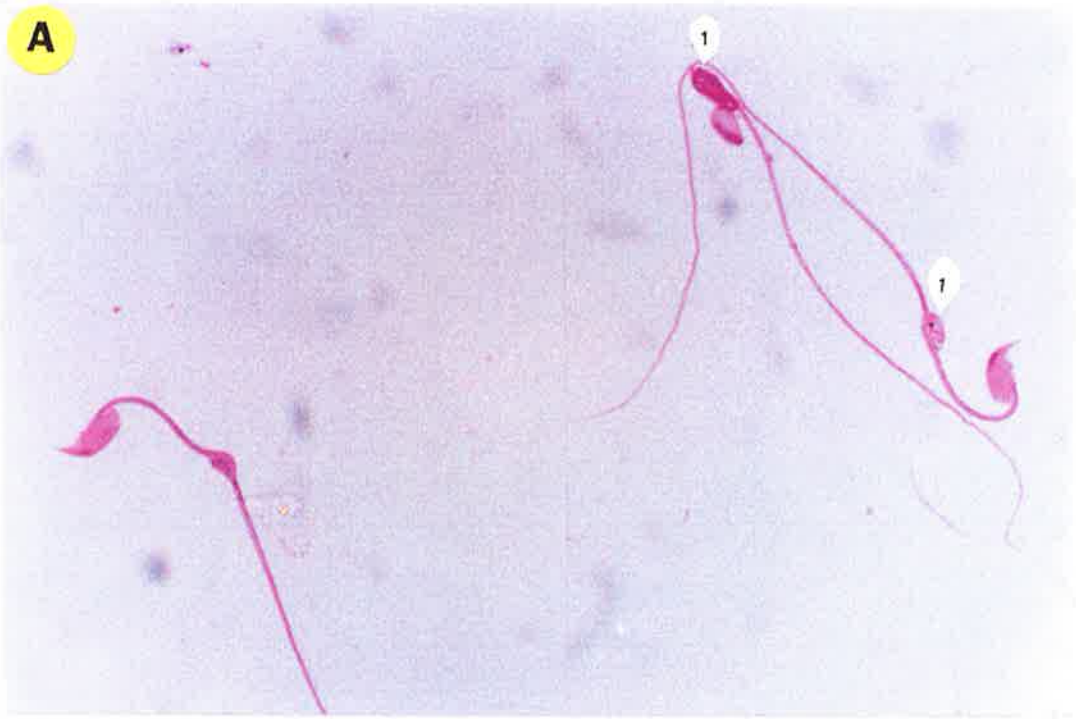

B
3
2

Figure 6.11 Photographs of epididymal spermatozoa from heat-stressed males, showing different forms of abnormalities; cytoplasmic retention (1), bent flagellum (2), coiled midpiece (3), thin midpiece (4) and deheaded (5). 1000x.


A
1
1

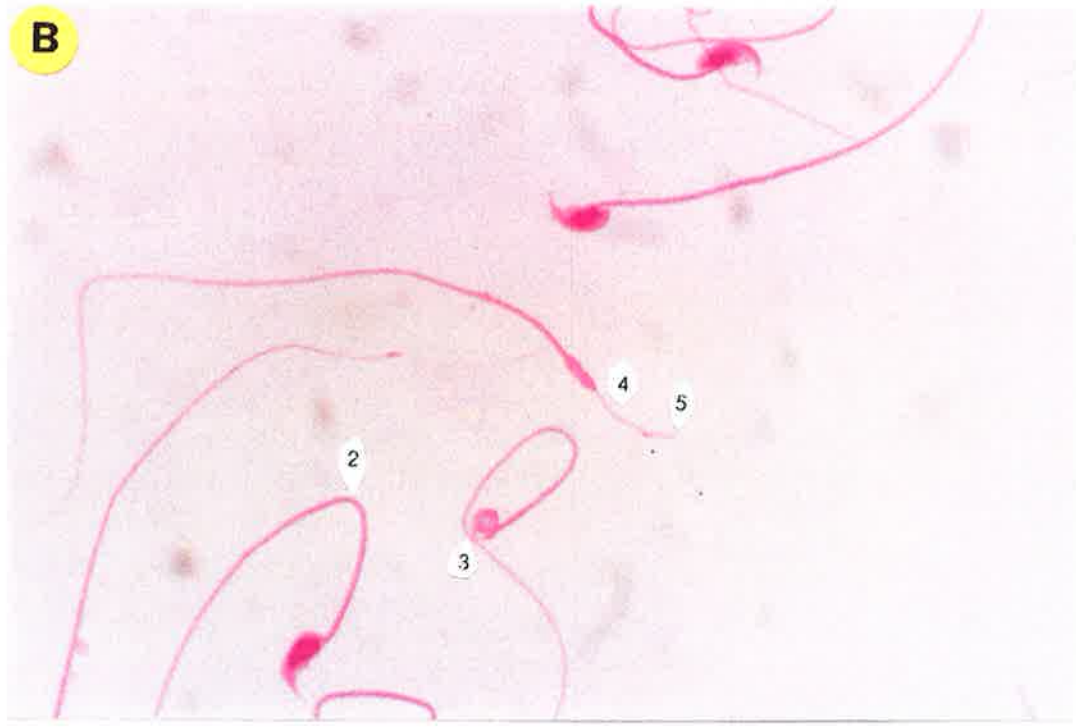
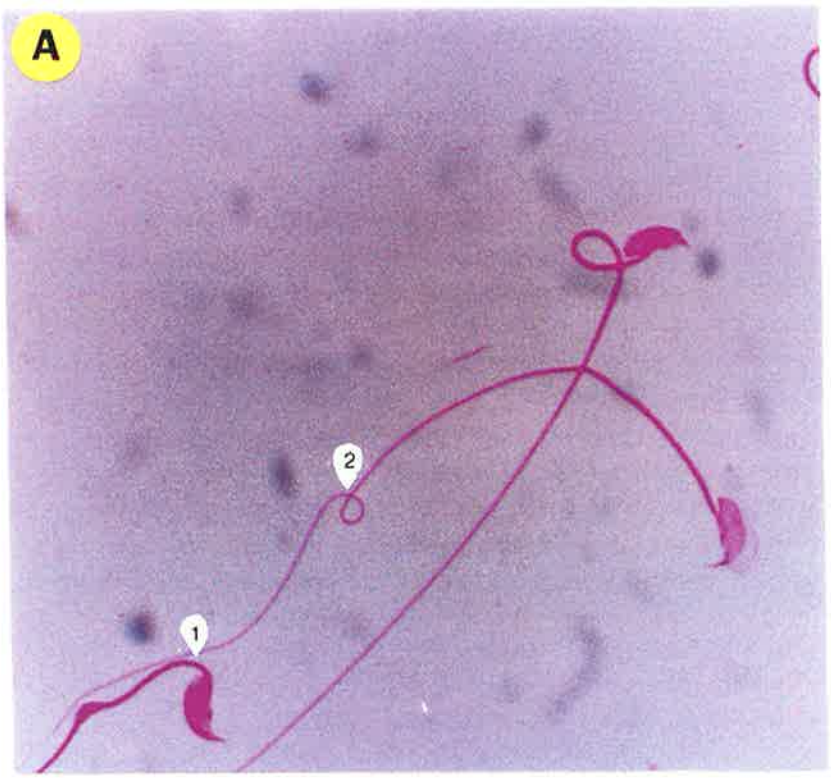
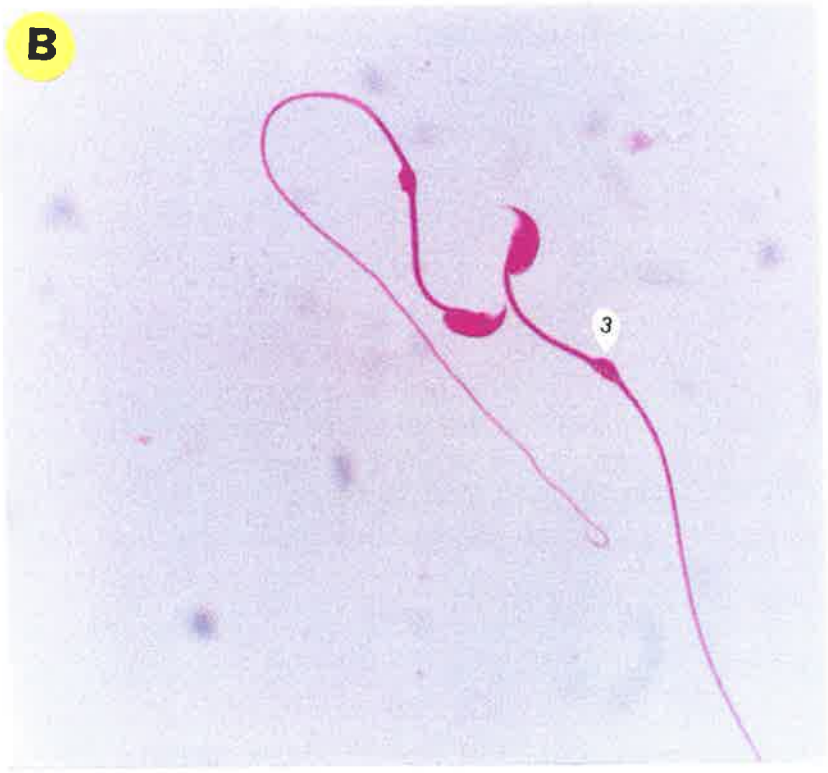

B
4
5
2
3

Figure 6.12 Photographs of epididymal spermatozoa from heat-stressed males showing bent midpiece (1) and coiled tail (2) (Plate A), and cytoplasmic retention (3) (Plate B). 1000x.


A
1
2


B
3

Ultrastructure of spermatids/spermatozoa by transmission electron microscopy

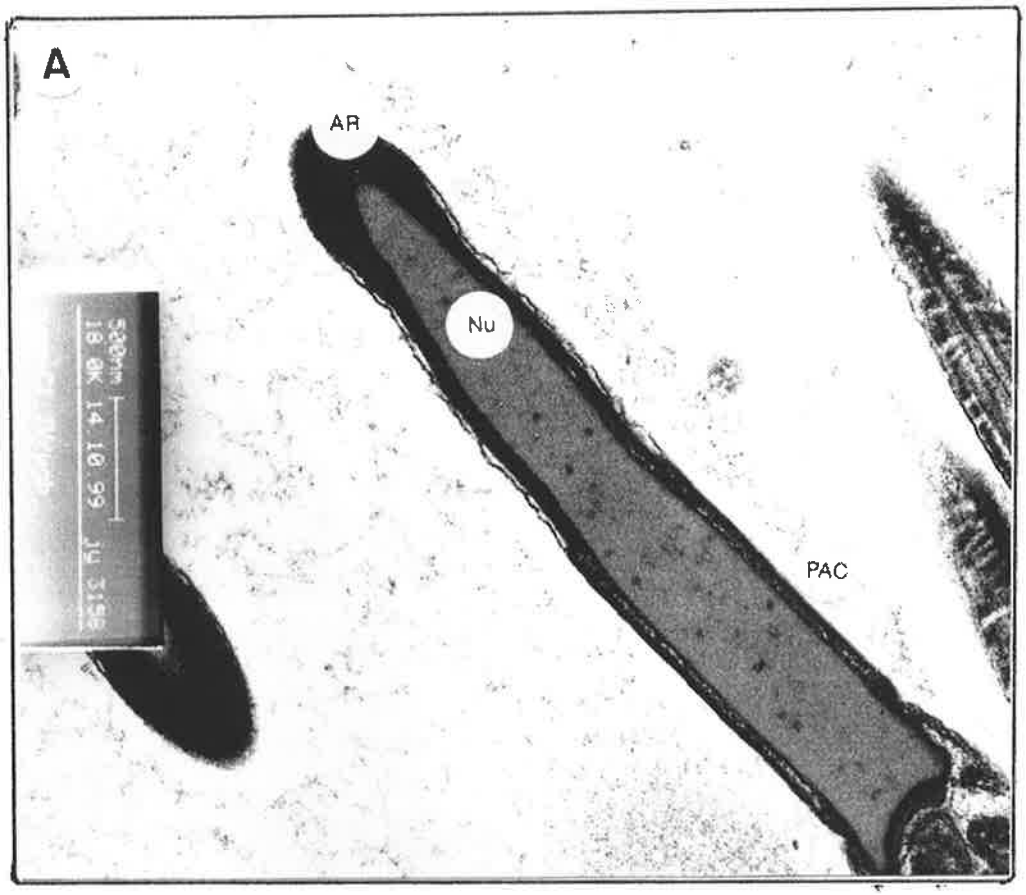
Swim-up procedure employed in the earlier fertility trials would screen out many gross morphological dysfunctions such as bent tails, deformed flagellar etc. However, given the poor zona penetration results it was of interest to examine the ultrastructure of the developing spermatozoa after heat treatment. This study aimed to examine heat-stress effects on germ cell (spermatid) ultrastructure in the testis and sperm morphology in both testis and epididymis. However, no attempt was made to quantitate the number of abnormal cells because at present there is no simple method for performing this task. Therefore, the results of this study were based on qualitative observations only. The morphology of testicular spermatids/spermatozoa is shown in figures 6.13-6.21, and that of epididymal spermatozoa is shown in figures 6.22-6.26.

Under electron microscope the morphology of particular spermatids/spermatozoa differs due to the plane of section. In the control animals the testicular spermatids/spermatozoa had intact acrosomes, small subacrosomal space, and a straight flagellum (see Figures 6.13 and 6.14). In the heated animals killed either on day 8 or day 12 after heating a number of abnormalities, which were not found in the control group, were observed. The abnormalities found in testicular tissue were dead germ cells (Figure 6.15), abnormal acrosomes during the cap phase (Figure 6.16), abnormal nuclear shapes, perhaps due to abnormal manchette development during nuclear condensation (Figures 6.17 and 6.18), excess retention of material in the subacrosomal space (Figure 6.19), abnormal acrosome (Figure 6.20), cytoplasmic droplet around the neck of spermatozoa (Figure 6.21).

In the controls the morphology of epididymal spermatozoa (Figure 6.22) was similar to that of spermatids/spermatozoa from the testis. The abnormalities found in the epididymal spermatozoa of heated testes included, bent flagellum (Figure 6.23), broken or partly lost

acrosomes (Figure 6.24), and obvious retention of cytoplasmic contents in the subacrosomal space (Figure 6.25) so that big bleb was formed in the space between the plasma membrane and the outer acrosomal membrane (Figure 6.26).

Figure 6.13 A transmission electron micrograph (**A**) of normal testicular sperm from a control mouse, and a corresponding drawing (**B**) to show the plane of section. Note that in (**A**) the acrosome contents (AR) appear compact, as does the nuclear material (Nu), and the post-acrosomal region (PAC) is well defined.


A
AR
Nu
PAC

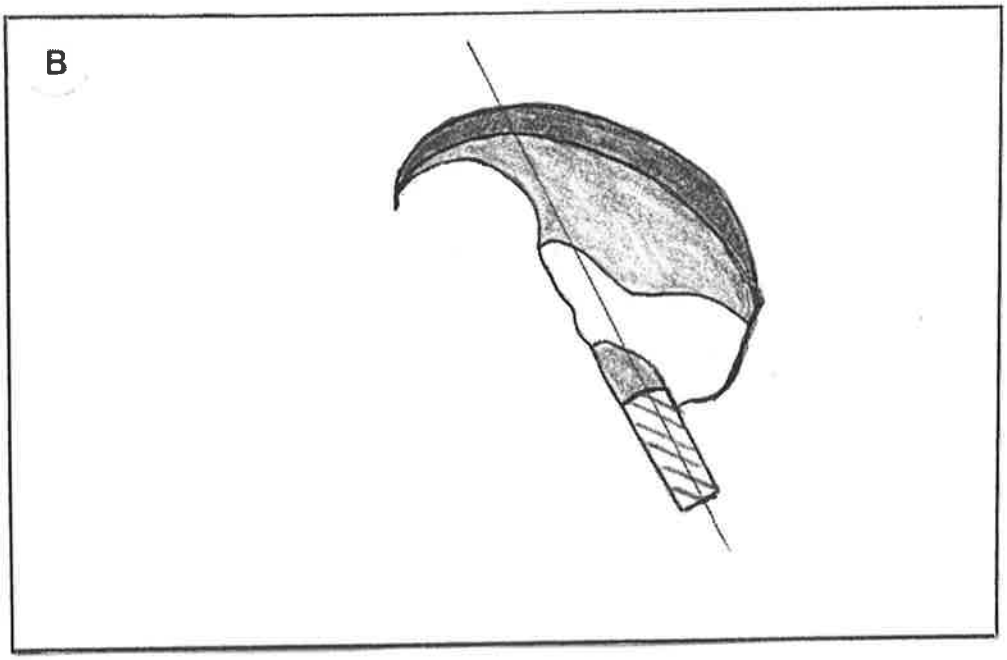
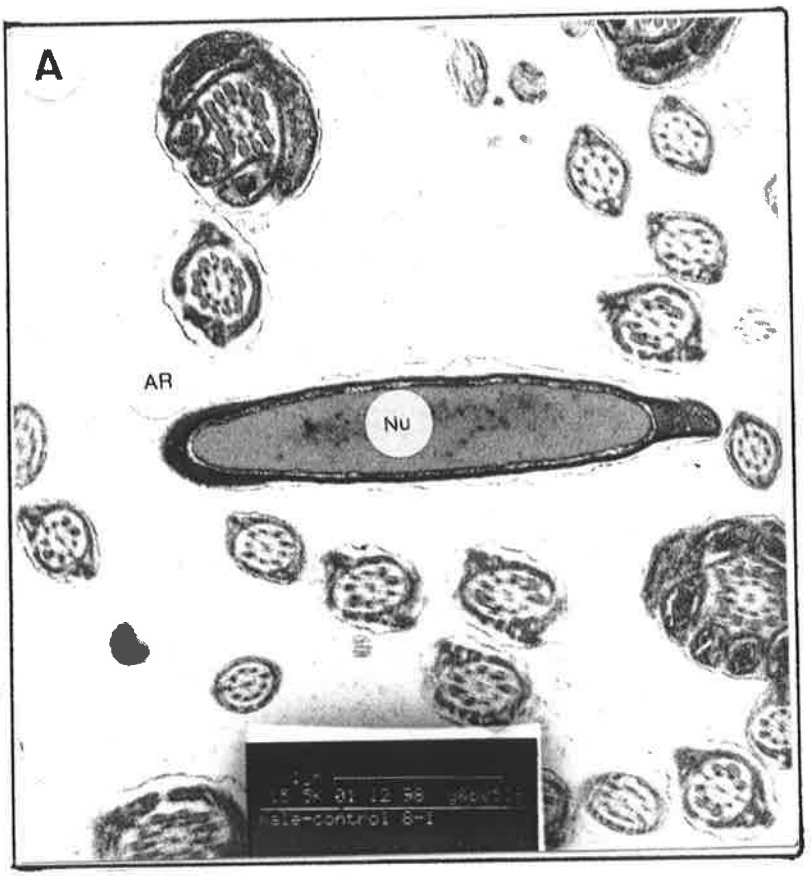

B

Figure 6.14 A transmission electron micrograph (**A**) of normal testicular sperm from a control mouse, and a corresponding drawing (**B**) which shows the orientation of the section. Note that in (**A**) the acrosome (AR) is dense and compact, as is the nuclear material (Nu).


A
AR
Nu

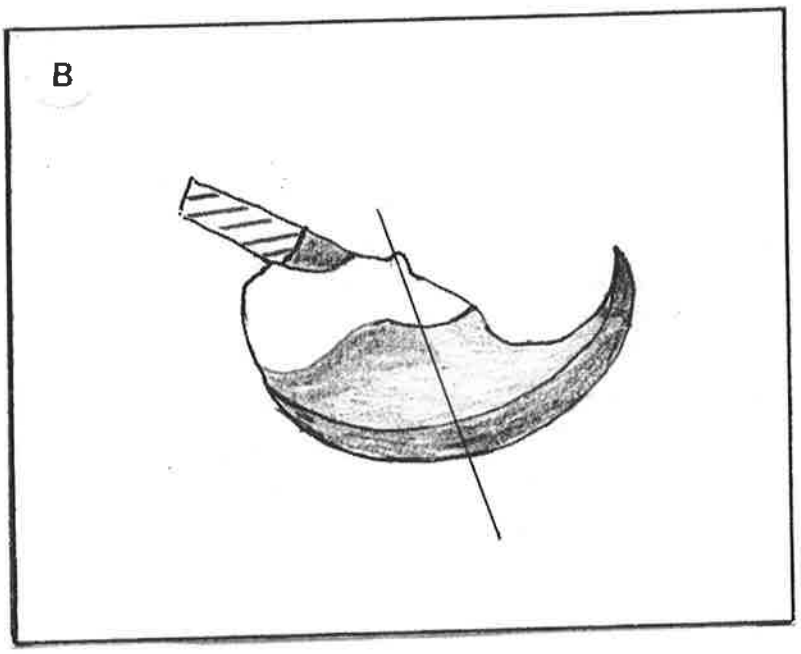

B

Figure 6.15 A transmission electron micrograph, showing testicular germ cells (D) with dark color and vacuoles, presumed dead.

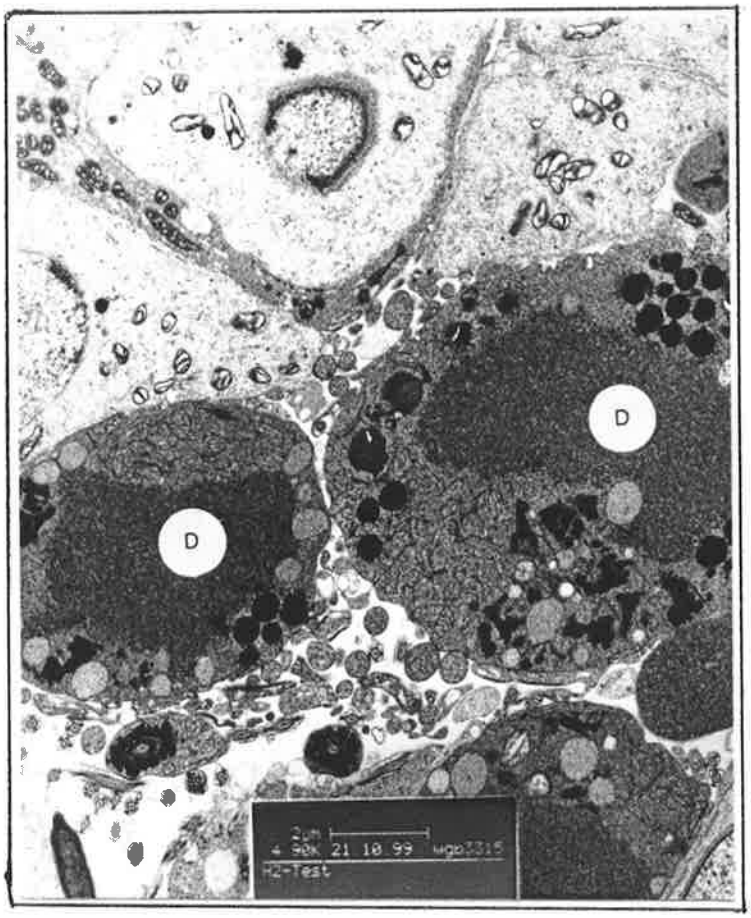

D
D
2µm
4 90K 21 10 99 wgo2315
H2-Test

Figure 6.16 A transmission electron micrograph, showing an abnormal curled-shape acrosome (AR) of an early-stage spermatid from the testis of a heated mouse killed on day 12 after heating (36°C, 12h/d for 2 days).

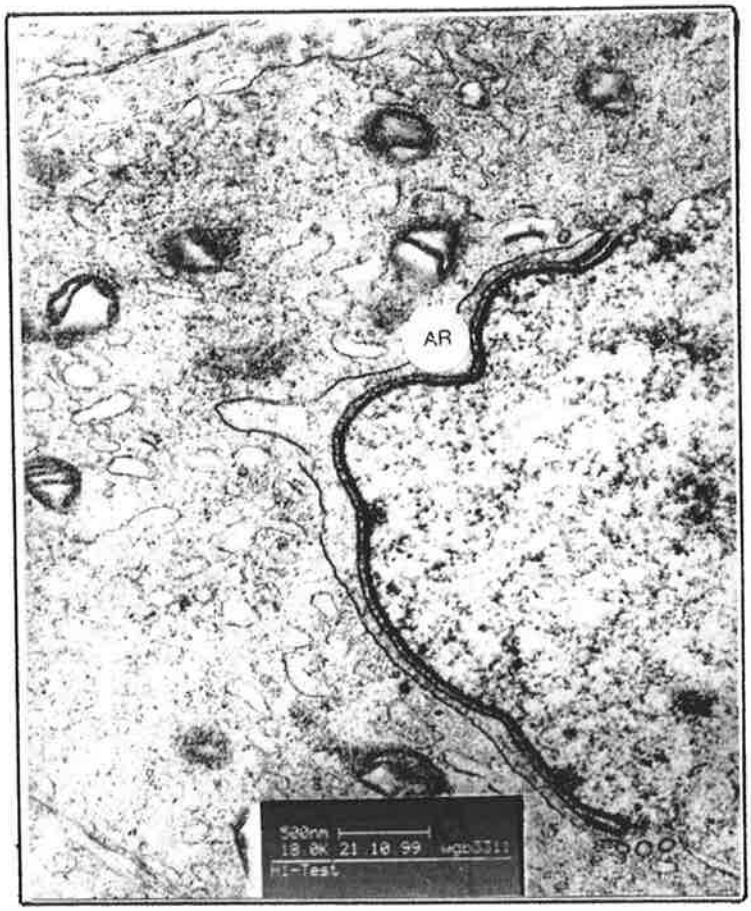
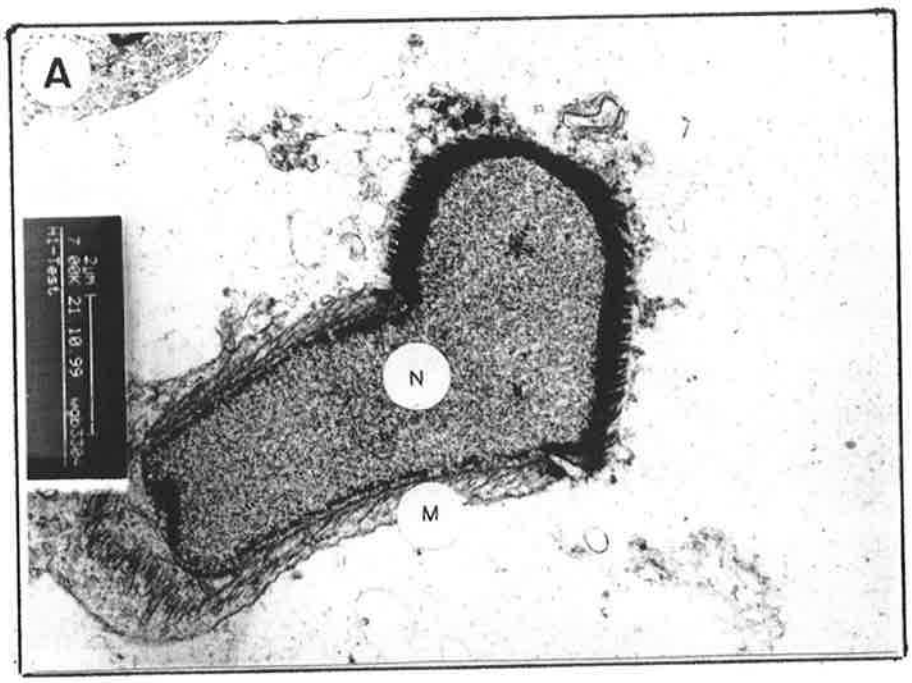

AR
500nm
18.0K 21 10 99 wgb3311
HI-Test

Figure 6.17 Transmission electron micrographs showing spermatids of heated mice killed on day 12 after heating (36°C, 12h/d for 2 days). Note abnormal manchette (M) development and a deformed nucleus (N).


A
N
M

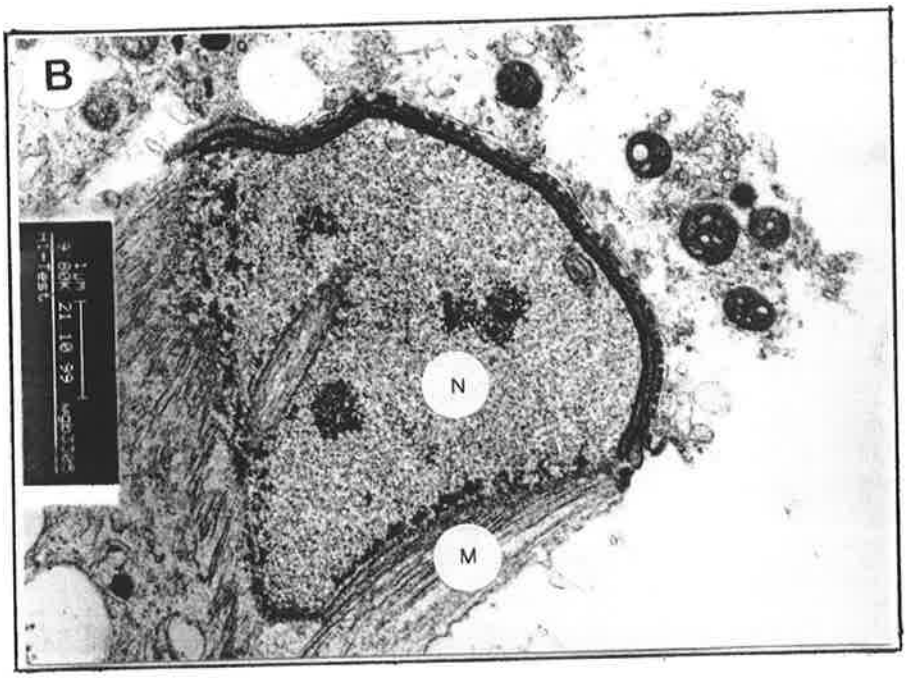
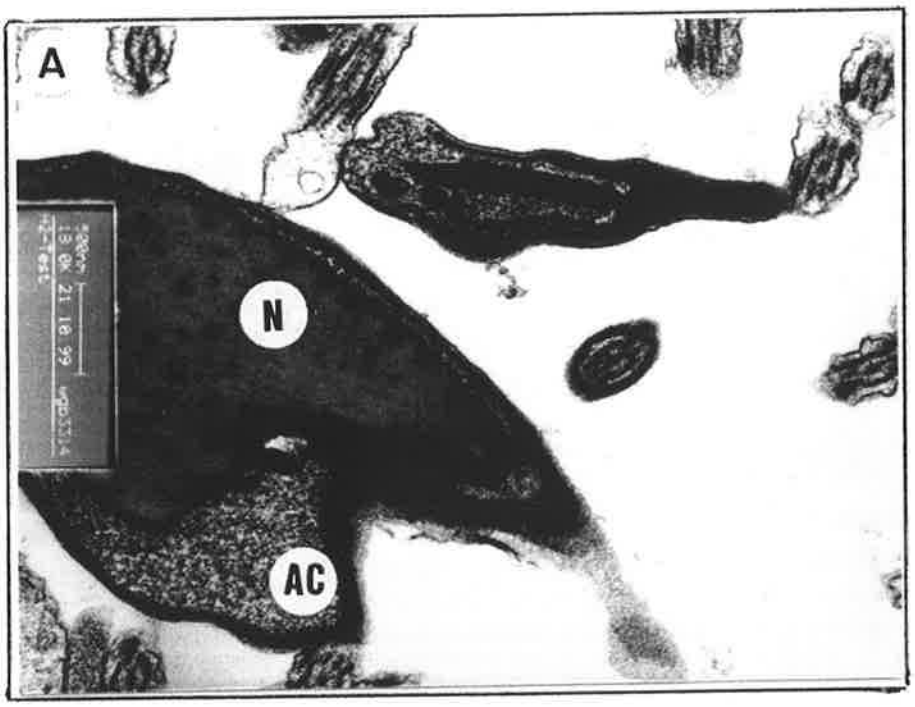

B
N
M

Figure 6.18 Transmission electron micrographs showing abnormal spermatids of heated mice killed on day 12 after heating (36°C, 12h/d for 2 days). Plates **A** and **B** show a deformed nucleus (N) and an expanded subacrosomal space (AC).


A
N
AC

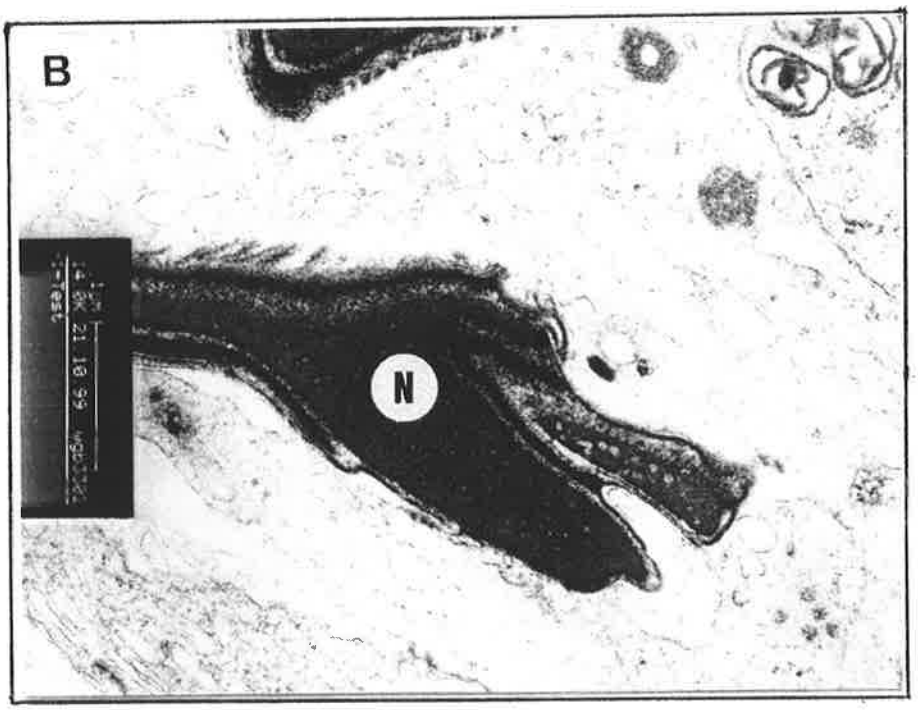

B
N

Figure 6.19 A transmission electron micrograph of spermatids of a heated mouse killed on day 12 after heating (36°C, 12h/d for 2 days). All spermatids in this micrograph have normal ultrastructures, except the one in the middle which has an expanded subacrosomal space which contains electron-dense material (arrows) within the subacrosomal space. The space between the post acrosomal dense lamina and the nuclear envelope is also expanded

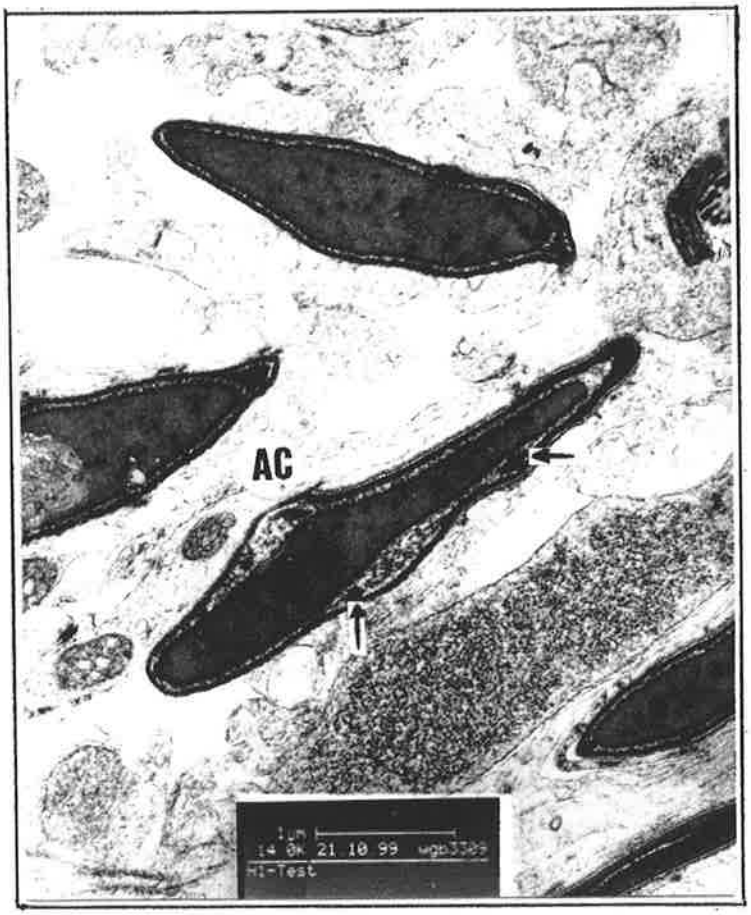
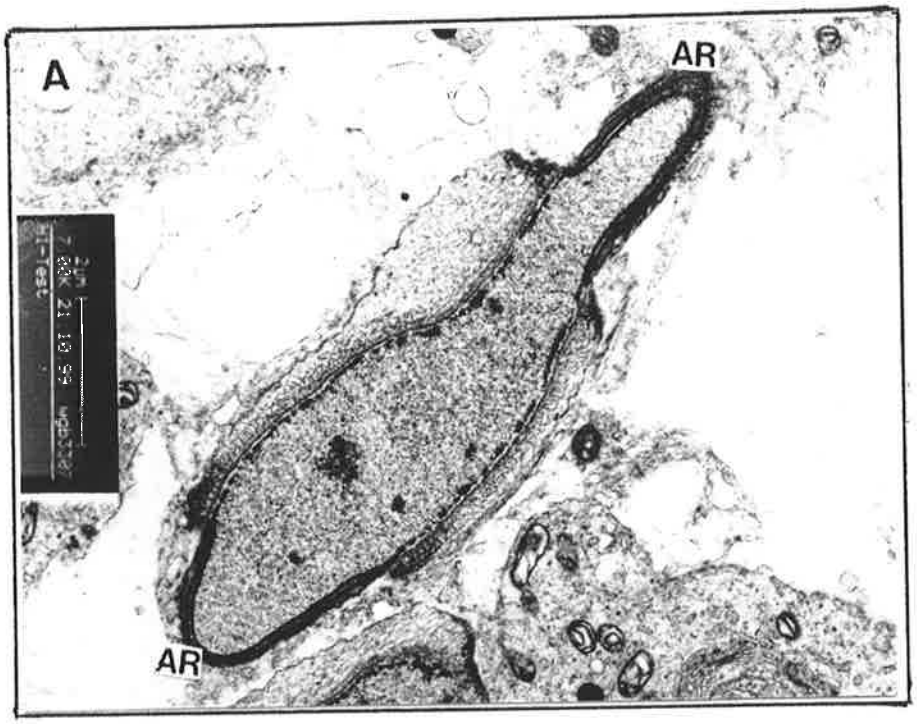

AC
1µm
14 0K 21 10 99
Hi-Test

Figure 6.20 Transmission electron micrographs showing abnormalities of acrosome in spermatids of heated mice killed on day 12 after heating (36°C, 12h/d for 2 days). Plate **A** shows a spermatid which appears to have two acrosomes (AR). Plate **B** shows a wavy inner acrosomal membrane and the electron-dense material within the subacrosomal space (arrows).


A
AR
AR

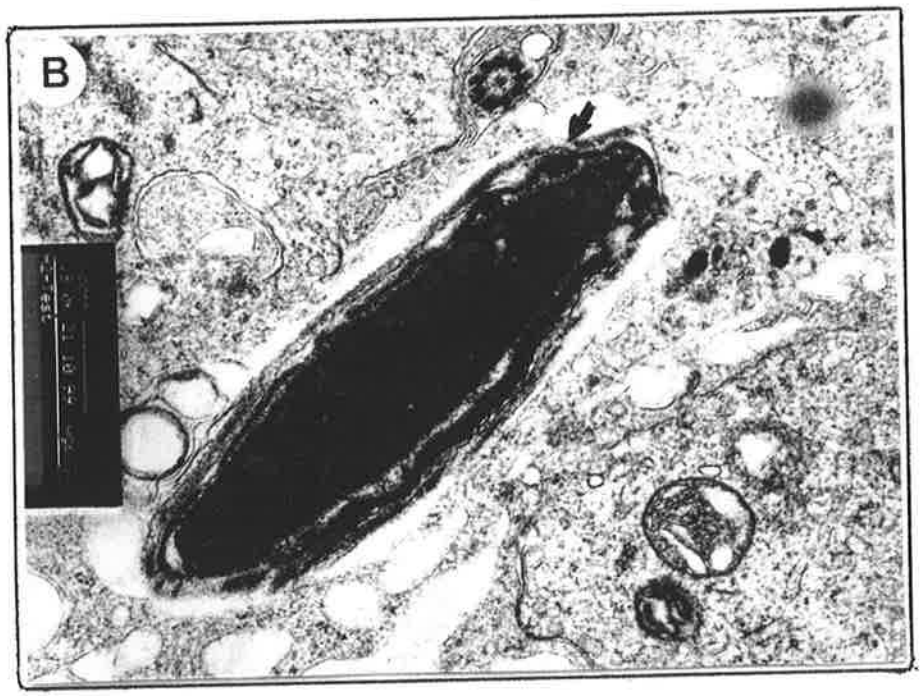

B

Figure 6.21 A transmission electron micrograph of spermatids of a mouse killed on day 12 after heating (36°C, 12h/d for 2 days). Note the spermatid in the middle of the micrograph has a normal nucleus (Nu) but appears to have a bent mid-piece flagellum (M) and a proximal cytoplasmic droplet (Cy) at the neck region of the spermatozoon.

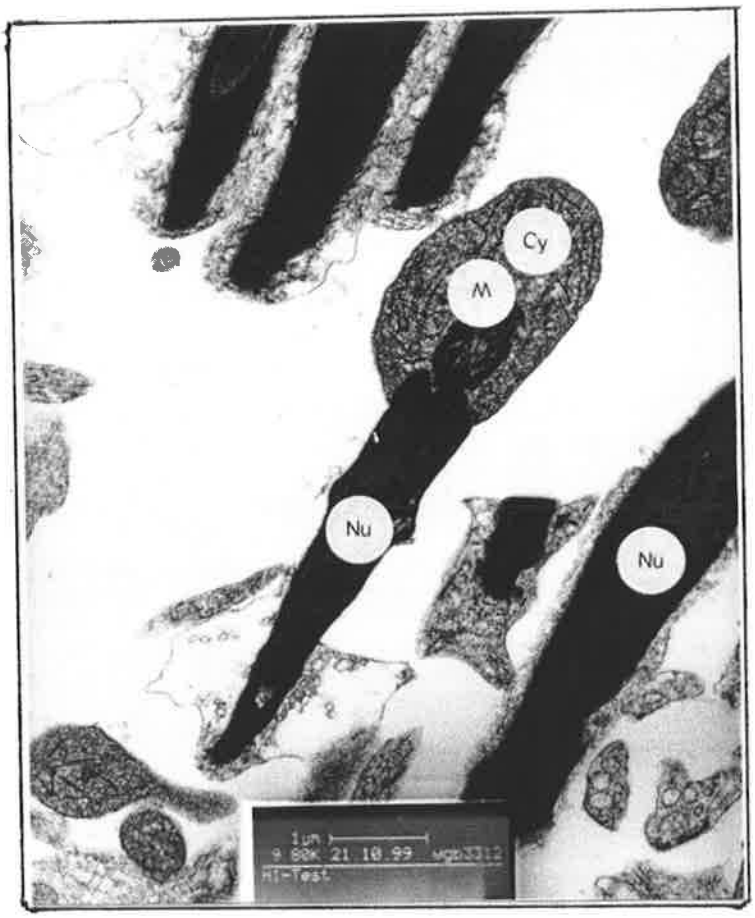
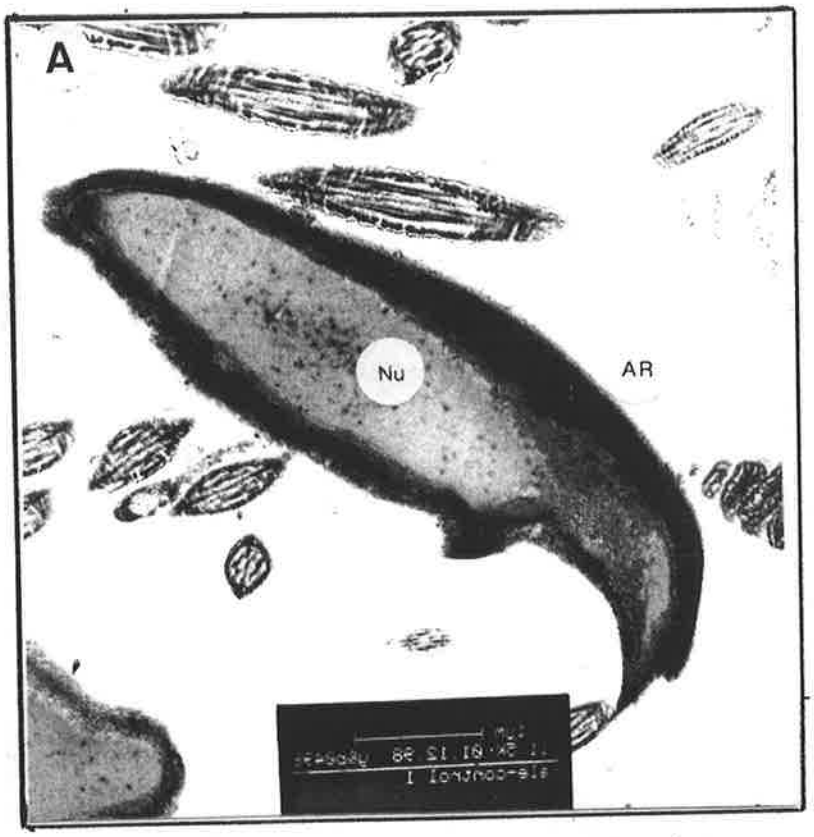

Cy
M
Nu
Nu
1um
9.88K 21.10.99
HT-Test

Figure 6.22 A transmission electron micrograph (A) of a normal epididymal sperm from a control mouse, and a corresponding drawing (B) which shows the orientation of the section. Note that in A the acrosome (AR) is dense and compact, as is the nuclear material (Nu).


A
Nu
AR

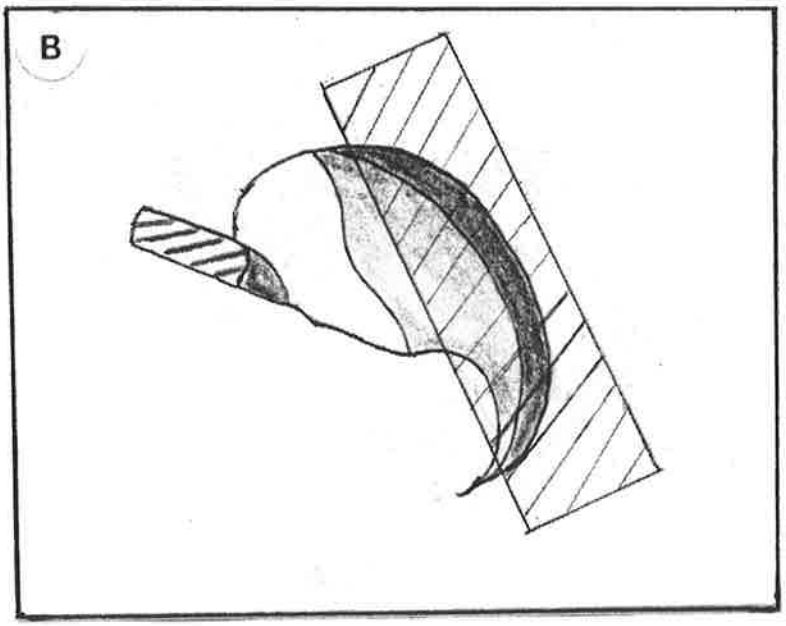

B

Figure 6.23 A transmission electron micrograph of epididymal spermatozoa of a heated mouse killed on day 12 after heating (36°C, 12h/d for 2 days), showing an abnormal spermatozoon with a bent midpiece (Mid) and an expanded subacrosomal space (*).

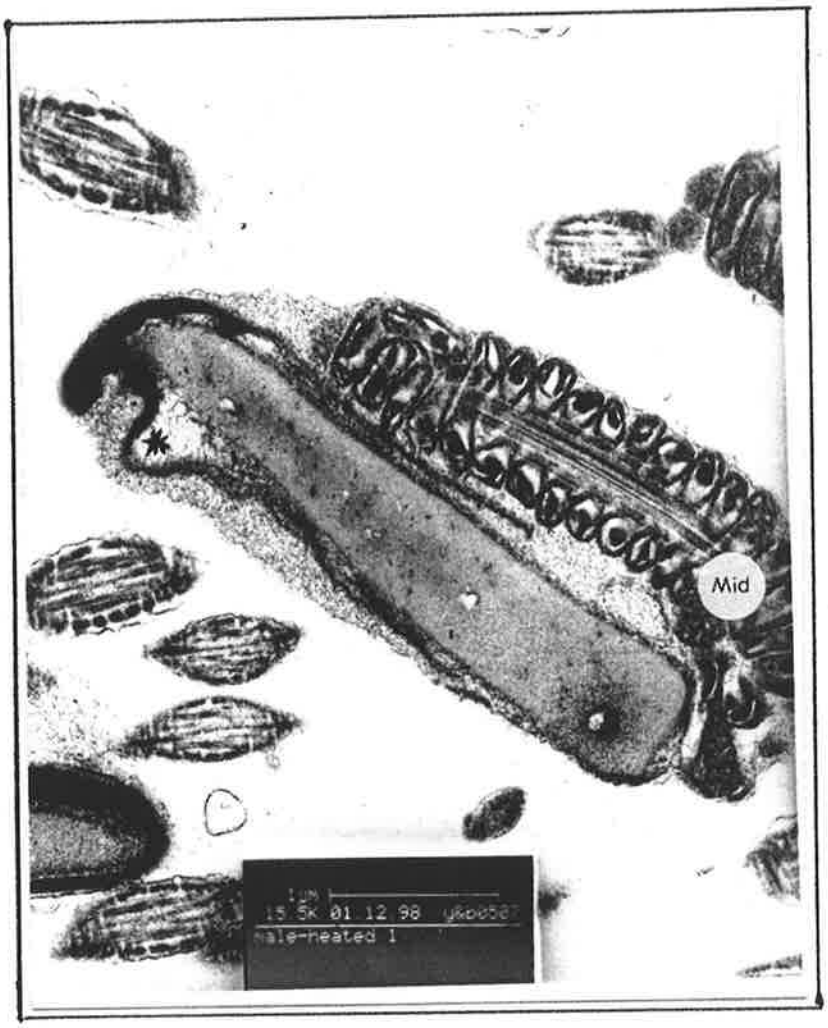

Mid
1 µm
15 5k 01 12 98
ale-heated 1

Figure 6.24 Transmission electron micrographs of epididymal spermatozoa of heated mice killed on day 8 (A, B) and day 12 (C) after heating (36°C, 12h/d for 2 days), showing the detached acrosomes and an expanded subacrosomal space.

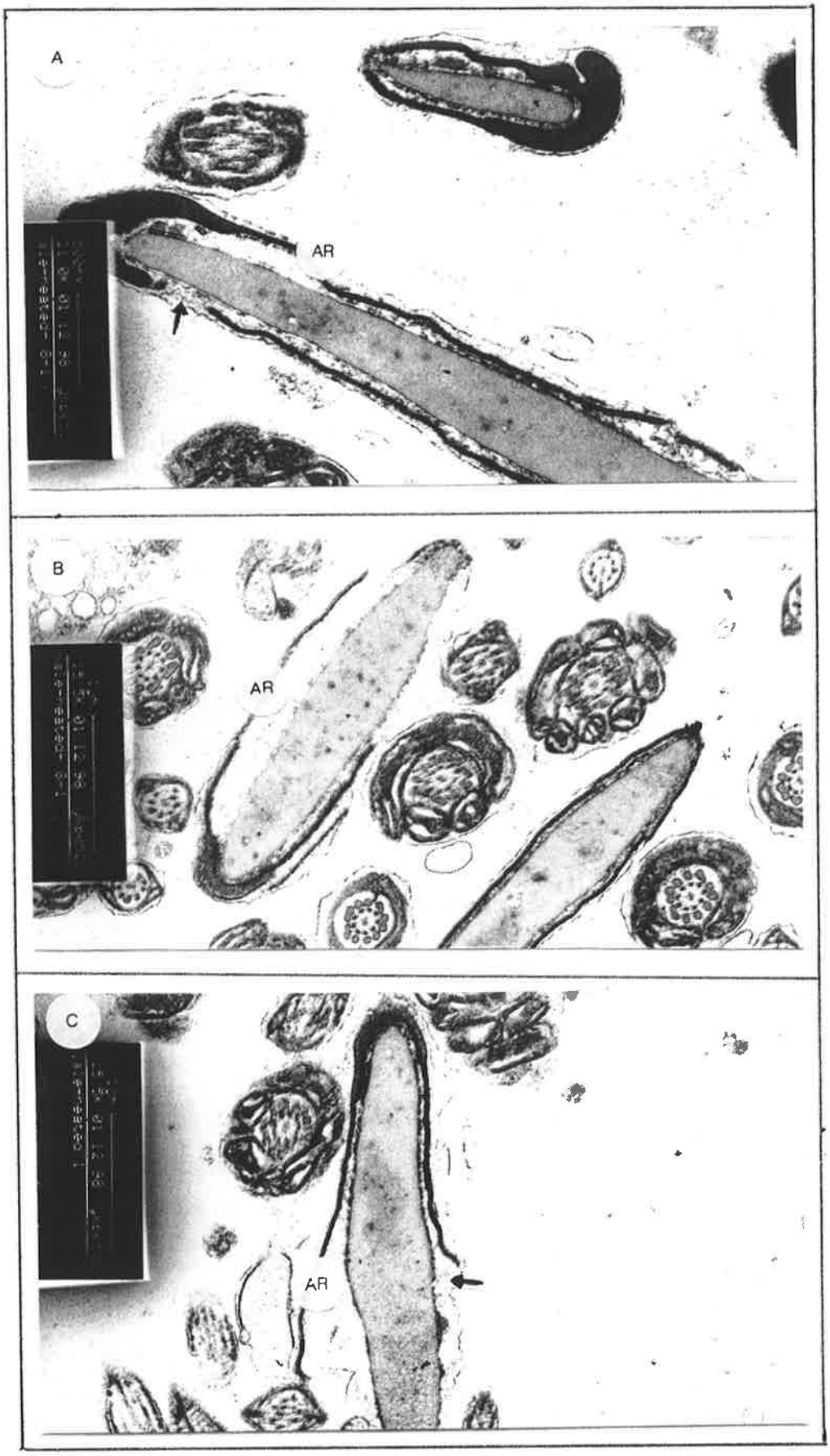

A
AR
B
AR
C
AR

Figure 6.25 Transmission electron micrographs of spermatozoa of heated mice killed on day 12 after heating (36°C, 12h/d for 2 days), showing large blebs (*), due to expansion of subacrosomal space and the appearance of cytoplasmic contents (arrows) within the space.

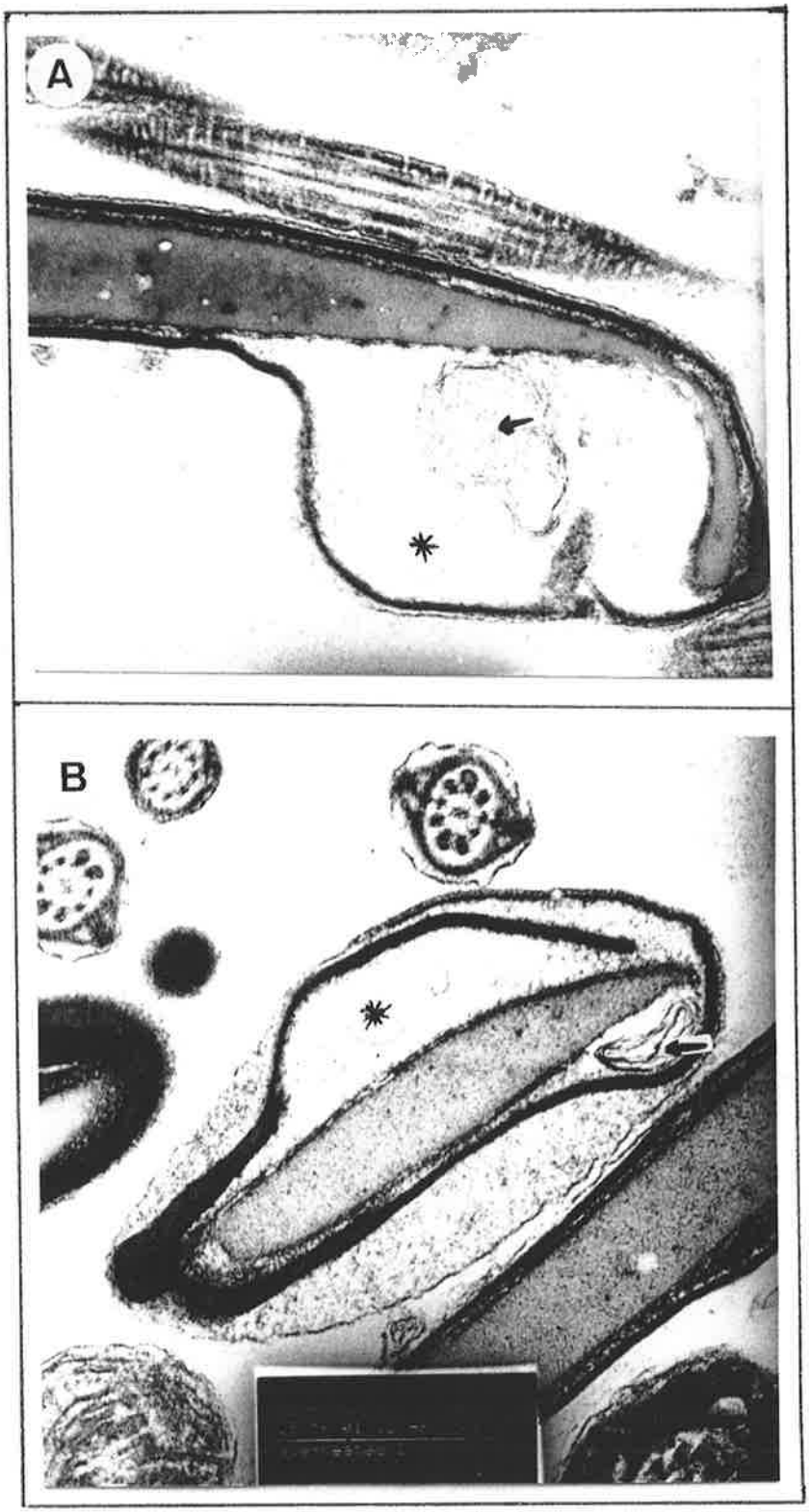
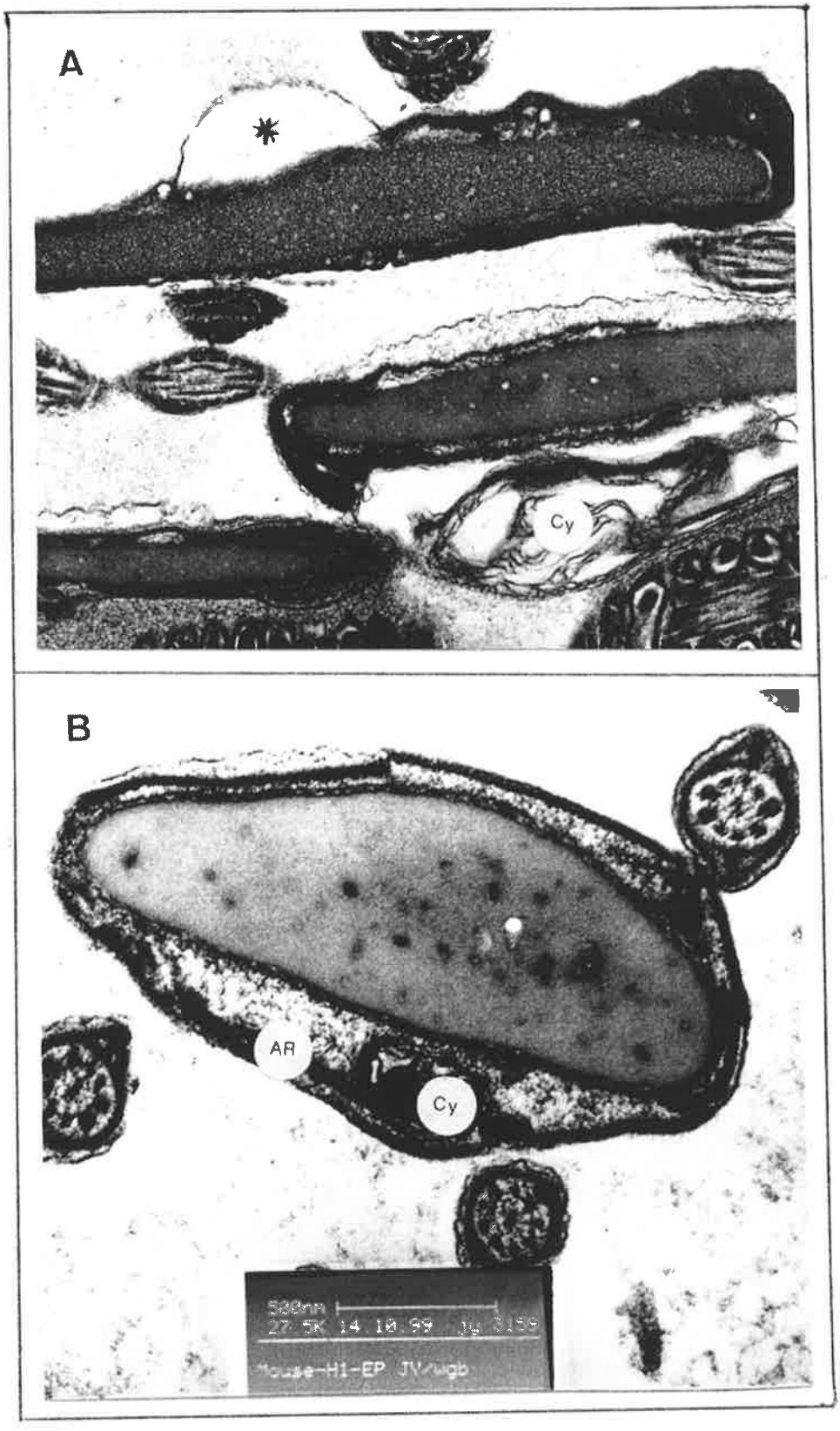

A
B

Figure 6.26 Transmission electron micrographs of spermatozoa of heated mice killed on day 12 after heating (36°C, 12h/d for 2 days), showing cytoplasmic contents within the subacrosomal spaces (Cy), a large bleb between plasma membrane and outer acrosomal membrane (*) and separation of acrosomes (AR).


A
Cy
B
AR
Cy
500nm
27.5K 14.10.99
mouse-H1-EP JV/wgb

Discussion

In the current study sperm morphology was assessed at both light and electron microscopic levels. It was found that under the light microscope higher percentages of abnormal sperm were observed in heat-stressed males (see Table 6.6). The obvious abnormalities were deformed sperm heads (see Figure 6.6), and bent and coiled flagella at different parts (see Figures 6.7, 6.10, 6.12). Some sperm were found to be abnormal in both head and tail (see Figure 6.8). Another common characteristic found in spermatozoa from heated mice was the retention of cytoplasmic droplets (see Figures 6.11, 6.12), which suggests the immaturity or premature release of spermatozoa.

Abnormal sperm ultrastructure was also found in the electron microscope study. Both spermatids in the testis and spermatozoa in the testis and epididymis were found to be adversely affected by heat treatment. The obvious abnormalities observed are shown in figures 6.18 to 6.26. The most frequently seen characteristics were deformed nucleus of spermatids, expansion of the subacrosomal space, bent midpiece of the flagellum and retention of cytoplasmic droplets. Apparently, all of these abnormalities are products of disturbed/disrupted spermatogenesis.

Taking all the information obtained from this study together, it is likely that heat stress disrupted spermatogenesis of male mice, as reflected by the production of morphologically abnormal spermatids and spermatozoa. Spermatozoa with altered plasma membranes then, although they still retained the ability to bind to the zona, did not respond well or did not respond at all to the stimulation from the zona such as the increased level of Ca^{++}. As a consequence, zona-bound spermatozoa failed to undergo acrosome reaction and penetrate the zona. These defective spermatozoa in turn probably produced ROS. If the amount of ROS was sufficiently great it could induce lipid peroxidation in the sperm plasma membrane, which would result in changes in the molecular composition or the structure of the membrane.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Summer infertility is a serious problem with the potential to undermine profits in farm animal operations in tropical or subtropical countries around the world (Armstrong *et al.*, 1986; Clark *et al.*, 1986; Bahga and Gangwar, 1988; Ray *et al.*, 1992; Love *et al.*, 1995; Alnimer *et al.*, 2002). Infertility or low fertility in these animals is known to be contributed to, and possibly caused by heat stress. Although a large number of experiments concerning heat stress have been conducted during the last few decades, the precise mechanisms by which mild heat stress in males reduces fertility in normal females is not clear.

The present study was undertaken to address this particular issue, and with the fertility problem in farm animals in mind, was designed to be conducted in an environment which was similar to that seen otherwise in the field. The design of this study had at least 2 points that are commonly found in the natural situation; the animals were exposed to heat repeatedly, and the levels of heat were not too extreme, i. e. lower than core body temperature.

The results have clearly demonstrated that heat stress caused by repeated whole body heating has a significant influence on male fertility in mice, with reductions in litter sizes and pregnancy rates commonly recorded in normal female mice mated to heated males.

In the natural situation, heat is normally seen coming in waves during the summer months. Once it comes it usually stays for a few days or weeks during which time it produces high temperatures during the daytime and cooled temperatures during the night. Because of this pattern, animals are likely to suffer heat stress intermittently, but repeatedly. The present study has demonstrated that repeated heating at less than 2 times for a period of 12h produces sufficient heat stress to adversely affect fertility of male mice, providing that the temperature is sufficiently high (36°C).

It has been previously reported that reduced fertility was observed in male mice whose whole body had been exposed continuously for 24h to heat at 34.5°C (Bellve *et al.*, 1972). In the

current study, because animals were exposed to heat for less than 24h each day it was expected that the temperature that would produce the same results must be higher. This is because the levels of heat stress are a product of the combination of temperature and duration of heat exposure. Indeed, it was discovered in this study that heating the whole body of male mice at temperatures less than 34.5°C, i. e. at 33°C or 34°C at any frequencies; 8h/d for 1 or 2 or 3 consecutive days, or 12h/d for 1 or 2 consecutive days, had little or no effect on either testis morphology or male fertility. When the temperature was raised to 35°C the clear effect of heat stress on male fertility was observed only with the males whose whole body had been heated for 24h continuously, but not with the ones that had been heated repeatedly for 2 days, 12h per day. Surprisingly, when the ambient temperature was increased as little as 1°C, i. e. to 36°C, a significant effect of intermittent heat stress on fertility was demonstrated. That is, heated males produced significantly lower litter size and pregnancy rates in normal females, compared to controls. This treatment was also shown to disrupt spermatogenesis and reduce subsequent fertility.

These results seem particularly relevant to the natural situation in which male animals might suffer heat stress and reproduce poorly during the summer months in many parts of the world. This is because the environmental conditions are often much more severe than in this study, i.e. ambient temperatures of above 40°C are not unusual and heat waves commonly last for more than 2 days.

Given this, it was of interest to further understand "what cause (s) reduced fertility in heat-stressed males".

In this study two aspects of reduced fertility found in females mated to heat-stressed males were low pregnancy rate and small litter size (Section 5.1). It is well known that low pregnancy rates and small litter sizes can be influenced by two factors, namely low

fertilization rates or high embryonic mortality rates. In the fertility trial (Section 5.1), however, it was found that reduced litter size at day 10.5 of pregnancy of normal females mated to heated males was a result of reduced implantation sites, not post-implantation embryonic loss, as similar numbers of embryos and implantation sites were found. For example, in the D14 group the number of embryos and implantation sites per litter were 6.0±3.2 and 6.3±3.2, respectively (Section 5.1), while the number of corpora lutea (CL) were similar among the females, ranging from 8.9 to 9.5 (Section 5.3). Therefore, either high pre-implantation embryonic loss or reduced fertilizing ability of sperm or both were the main causes for low litter size or pregnancy rates in normal females mated to heated males.

Several fertilization trials, both in vivo (Section 5.5) and in vitro (Sections 5.5 and 5.6) were carried out to test fertilizing ability of spermatozoa. It was discovered in these studies that spermatozoa from heat-stressed males started to lose their capacity to fertilize eggs on day 7 after heating. By day 10 their fertilization rates were extremely low, and these low levels continued until day 14. In *in vivo* and *in vitro* studies, only 2 in 26 eggs (7.7%) and 2 in 30 eggs (6.7%), respectively were fertilized by males used on day 14 after heating while in the control group the fertilization rates were between 88 and 91%. Based on these results it is likely that low fertilization rates are a major factor contributing to reduced pregnancy rates and litter sizes. The adverse effects of heat stress on fertilization, regardless of the methods of heating used, have been reported in a number of animal species; mice (Burfening *et al.*, 1970; Jannes *et al.*, 1998), rats (Setchell *et al.*, 1988) and rams (Rathore, 1970; Ekpe *et al.*, 1992, 1993).

Fertilization of an egg by a spermatozoon is a complex process comprising a number of steps, including cumulus cell digestion, zona binding, the acrosome reaction, zona penetration, oolemma binding and fusion, egg incorporation, egg activation and resumption of meiosis II. Disruption of any of these steps will inevitably cause the whole process to fail. It was found in

this study (Sections 6.1, 6.2) that the first 2 steps, namely cumulus penetration and sperm-zona binding, were not affected by heat treatment. Eggs used in both IVF studies, using swum-up and non swum-up spermatozoa, were all found to be free of cumulus cells within 10 minutes after coincubation between eggs and sperm. Because a large number of sperm were bound to the zona's surface and their tails were continuously beating, the eggs were seen moving like balls around the culture dish. This suggests that the plasma membrane of spermatozoa from heated males still retained enzymes or molecules that are responsible for the digestion of cumulus masses around the eggs. Additionally, the sperm receptors for binding with the ligands in the zona pellucida appeared to be functioning normally, as judged by the similar number of bound spermatozoa found in the control and heated animals, i.e. 23.0 vs 22.5 in the control and the D14 heated group.

In the mouse, only acrosome-intact spermatozoa are capable of binding to the zona pellucida. This is supported by our results (Section 6.1) which show that most of the live sperm in both the control and heated groups were acrosome-intact (90.0% in the control and 88.5% in the day-14 heated groups).

The step following the sperm-zona binding event, the zona penetration (Section 6.2), was investigated next, and it was found that most of the bound spermatozoa from the heated animals failed to penetrate the zona. To traverse the zona pellucida the zona-bound spermatozoa must have undergone the acrosome reaction so that the acrosomal contents are released to digest the zona. So, in the present study where the zona-bound spermatozoa were unable to pass the zona it is possible that those sperm either did not undergo the acrosome reaction and hence there was no hydrolytic enzyme for the zona digestion, or they lacked critical functional enzymes within the acrosomal contents.

The acrosome reaction, which involves a few steps including the binding of capacitated spermatozoa with the zona pellucida, the release of zona factors to stimulate sperm plasma membranes and finally the fusion of the outer acrosomal and sperm plasma membranes and release of the acrosomal contents, occurs only with spermatozoa that have already been capacitated (Fraser *et al., 1990;* DasGupta *et al.,* 1994; Adeoya-Osiguwa and Fraser, 1996). Unfortunately, in the present study it was not possible to investigate the state of capacitation in spermatozoa used for the IVF studies. However, morphological studies of spermatozoa using both light and electron microscopy (Section 6.3) were performed. These studies have provided some information that is helpful in explaining the incidence of low zona-penetration by spermatozoa from heated males.

Light microscopic techniques in the present study have shown high levels of spermatozoa with abnormal head and/or tail morphology, as well as immature spermatozoa as judged by their retention of cytoplasmic droplets. These defects are believed to be caused by the disruption of spermatogenesis, as indicated by the results of the electron microscopic study, which revealed the abnormal development of acrosomes and nuclei of young spermatids, the loss or separation of acrosomes of late spermatids or epididymal spermatozoa, the excessive retention of cytoplasmic contents in the midpiece of the flagellum, and bent midpieces.

The plasma membranes of spermatozoa contain high levels of unsaturated fatty acids, and because of this condition they are known to be easily attacked by reactive oxygen species (ROS), especially by hydrogen peroxide (H_2O_2) or peroxide anions (O_2^-) (Jones *et al.,* 1979). In the semen of male animals ROS are produced by two sources, leucocytes (Aitken *et al.,* 1994) and immature and defective spermatozoa (Sharma and Agarwal, 1996). ROS are believed to be associated with low fertility of male animals including humans, as a high level of ROS and a great number of immature and defective spermatozoa have been found in infertile men (Iwasaki and Gagnon, 1992).

If the population of abnormal spermatozoa is large enough it is likely that high levels of ROS will be produced, inducing damage to the plasma membrane of the spermatozoa in the same population. That is, the elevated levels of ROS will induce lipid peroxidation in the sperm plasma membrane and cause changes in the composition and organization of the membrane. Aitken and Clarkson, (1987) reported that because of this altered plasma membrane, sperm may lose their capacity to respond to the calcium signal from the egg that normally initiates the acrosome reaction.

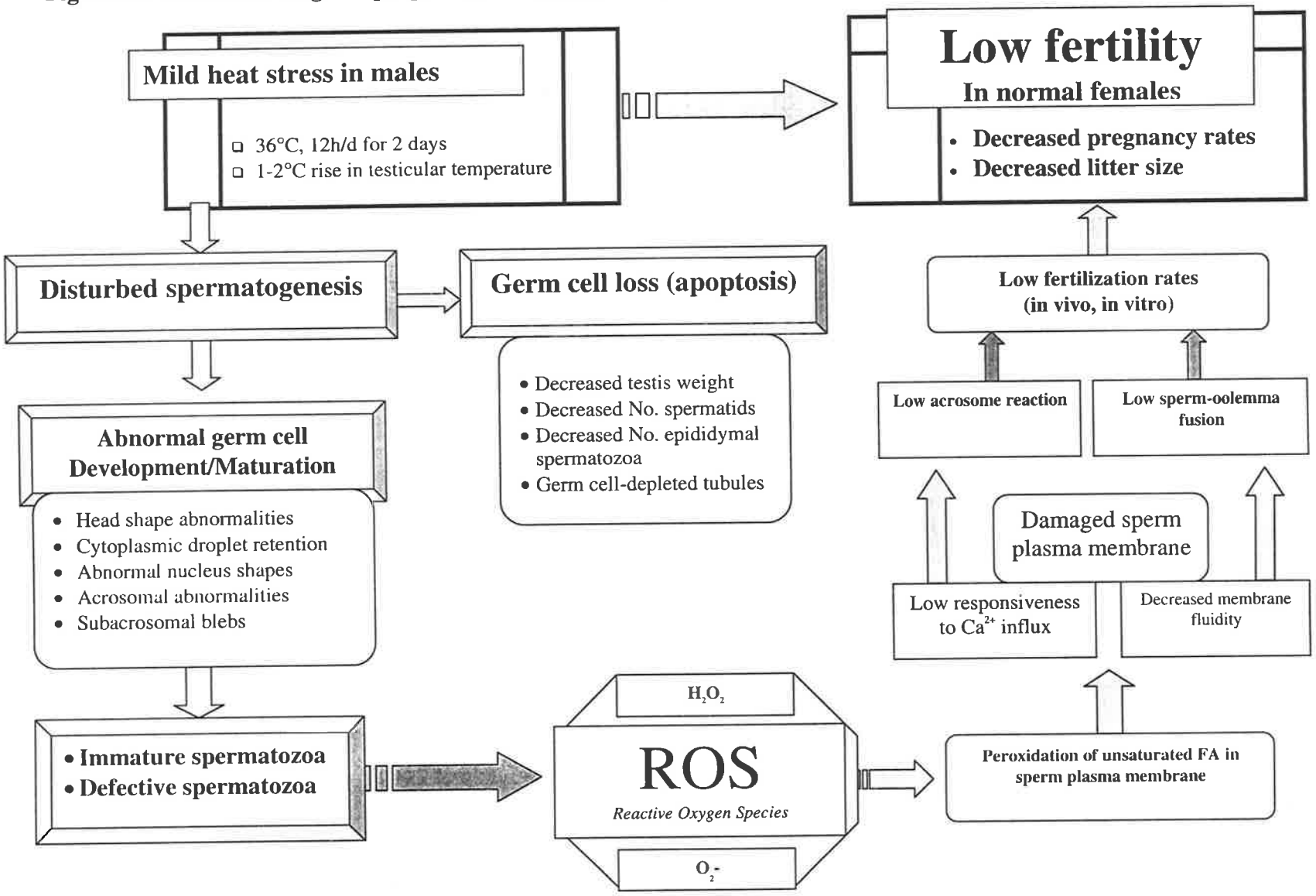
This may explain the incidence of low zona penetration rates in the present study. It could be that, due to structural or biochemical changes, bound spermatozoa failed to respond to the signals released from the zona, and did not undergo the acrosome reaction. As a consequence, the acrosome contents were not released and the zona were not digested, and fertilization could not occur. These issues would warrant further investigation.

A schematic diagram summarizing the effects of heat stress on male fertility recorded in this study and some possible mechanisms involved is provided in Figure 8.1.

In summary, the present study has demonstrated clearly that whole body heat stress has the potential to cause disruption of spermatogenesis in male mice, providing the heat load produced by the heat treatment is sufficiently high. As a result, semen with a large number of abnormal and immature spermatozoa can be produced. Associated with this, plasma membrane changes are induced in other sperm making them less responsive to factors from the zona pellucida at the time of fertilization. As a result, the acrosome reaction can not occur, sperm fail to pass through the zona and fertilization can not occur. Exactly what triggers these changes in the plasma membrane remains to be determined. However, the likely importance of lipid peroxidation in such processes, and the presence of large numbers of abnormal

spermatozoa leads us to speculate that reactive oxygen species could be a significant trigger, and are worthy of further investigation in this respect.

Figure 8.1 Schematic diagram proposing a mechanism may be responsible for low fertility of heat-stressed male mice



CHAPTER 8.

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