Direct and indirect effects of whole body heat exposure on germ cells and spermatozoa.

A thesis submitted to the University of Adelaide in total fulfilment of the requirements for the degree of Doctor of Philosophy

by

HARSHA WECHALEKAR

School of Medical Sciences

The University of Adelaide

Adelaide, South Australia

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This thesis is dedicated to *my family*

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DECLARATION

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Principal Author

Name of Principal Author (Candidate)	Harsha Wechalekar		
Contribution to the Paper	Involved in the experimental design, conducted the animal experiments, recorded,analyzed the data, and prepared the manuscript for submission.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

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Name of Co-Author	Prof. Brian Setchell		
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Please cut and paste additional co-author panels here as required.

Name of Co-Author	Dr. Eleanor Peirce
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Contribution to the Paper	Conducted the animal experiments, recorded and analyzed the data, and prepared the manuscript for submission.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

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Name of Co-Author	Prof. Brian Setchell		
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Name of Co-Author	Prof. William G. Breed		
Contribution to the Paper	Assisted in the general plan of the study, data interpretation and editing of the manuscript.		
Signature		Date	August 4th, 2015

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Ms. K.R. Pilkington
Contribution to the Paper	Assisted in interpretation and preparation of flow cytometry data in the manuscript including the flow graphs.
Signature	Date 1/9/2015

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ABSTRACT

Exposure to extreme temperature conditions such as occurs in certain occupations is known to induce male infertility. In humans and most of the eutherian mammals, it has been shown that whole body heat stress decreases fertility and produces defective embryos. However, the mechanisms producing fertility defects post exposure to whole body heat are not yet fully determined. Hence, the present study aimed at gaining some insight into the mechanisms producing fertility defects after whole body heat stress.

Laboratory mice were exposed to constant temperatures higher than their core body temperature of 37-38^{o}C for 8h on three consecutive days. A decrease in testis weight occurred as early as 7 days post exposure to heat with weights comparable to controls by 21 days post heat exposure. Histology of the testicular tissue showed germ cell apoptosis affecting the pachytene spermatocytes and the spermatids. Germ cell apoptosis was investigated by TUNEL 16h, 7, 14 and 21 days following the heat treatment. Apoptosis was found to be stage-specific affecting early and late stages of the seminiferous epithelium cycle on day 7 and 14 post exposure to heat stress with involvement of all stages of seminiferous epithelium 16h following heat stress. Reduction in germ cell apoptosis was evident 21 days post exposure. To determine the mechanism of germ cell apoptosis, caspase-3 proteins were detected in apoptotic germ cells following heat stress which showed strong positivity. Hence, we concluded that whole body heat stress results in caspase-3 mediated germ cell apoptosis. Furthermore, changes in cauda epididymal spermatozoa were investigated to determine sperm apoptosis following heat stress. Sperm apoptosis was detected by the exteriorization of phosphotidylserine (PS) on the outer layer of the plasma membrane. The results showed an early and late apoptosis in caudal epididymal spermatozoa 16hr following heat treatment. A second experiment was conducted to determine the time of appearance of changes in spermatozoa following whole body heat stress for which the duration of exposure to heat of

37-38ºC was reduced to one-day period. This showed more spermatozoa in the early phase of apoptosis with fewer dead spermatozoa, which is suggestive of a time and temperature dependant pattern of sperm apoptosis.

Arid-adapted *Notomys alexis* (hopping mice) were also investigated to determine whether differences in response to whole body heat have evolved in extreme climate conditions. Therefore hopping mice were exposed to similar temperatures as laboratory mice to investigate heat influences on germ cells and spermatozoa. Like the laboratory mice, germ cells and spermatozoa of hopping mice also showed apoptosis. However, unlike in laboratory mice, stage specificity in apoptosis could not be determined in hopping mice because of the presence of more than one cell association within the cross sections of the seminiferous tubules. Similar to laboratory mice, germ cell apoptosis in hopping mice was caspase-3 mediated. The vasculature of hopping mice was also determined to look for any variations in the cooling mechanisms, which could have resulted in germ cell apoptosis. We found the absence of a coiled testicular artery suggestive of a lack of such a cooling mechanism in hopping mice that could have resulted in germ cell and sperm apoptosis. We also investigated changes in germ cells following experimental cryptorchidism, which showed tubular degeneration with low numbers of germ cells lining the seminiferous epithelium. Thus the findings indicated that whole body heat had a detrimental effect on developing germ cells and spermatozoa.

It is also known that heat stress changes other bodily functions to maintain body homeostasis. Heat stress is associated with an activation of the hypothalamic-pituitary axis, which is followed by an increase in blood cortisol levels in the circulation as a result of increased synthesis of corticosteroids from the adrenocortical cells. Hence the current study also determined changes, if any, in the adrenal glands, especially the adrenocortical cells of laboratory and hopping mice following whole body heat exposure.

Heat stress resulted in the formation of vacuoles, dilated capillaries and interstitial fibrosis in cortical areas of the adrenal glands in both the species. In addition, large syncytial bodies were evident in hopping mice adrenal cortical cells. Thus it is evident from the study that germ cell and sperm apoptosis are a result of either a direct effect of heat on germ cells and/ or due to changes in the body hormonal milieu following whole body heat stress.

Thus this study showed an activation of caspases in producing germ cell apoptosis and externalization of PS in inducing sperm apoptosis following whole body heat stress with changes in the adrenocortical cells following heat stress. This study demonstrated that an arid-adapted species *Notomys alexis*, although evolved in extreme environmental conditions, also is affected by high temperatures. Hence, this study gives some insight in the reasons for reduced fertility following whole body heat stress.

CHAPTER 1

INTRODUCTION

CHAPTER 1. INTRODUCTION

This section gives a general overview of various testicular positions in mammalian species and their role in maintaining an ambient temperature for normal spermatogenesis to occur. Animal models *Mus* m*usculus* (laboratory mice) and *Notomys alexis* (hopping mice) were chosen to investigate degenerative and apoptotic changes in the seminiferous epithelium therefore an introduction to the cellular organization of testes, spermatogenesis and cycle of seminiferous epithelium of these rodents has been included in this section before describing the effects of heat stress. The cycle of the seminiferous epithelium of humans has also been presented in order to potentially extrapolate our results to those of the human species.

1.1 Descent of testis

The testes of most mammals descend from their embryonic position in the abdomen into the scrotum where a lower temperature occurs; this descent however is neither complete nor is the final destination fixed, with considerable inter-species variability apparent in testes location. Three anatomical testicular positions have been described; these are testicondy (testicles are situated close to the kidneys with an absent scrotal sac), descended ascrotal (testicles have descended but are retained either intra-abodminal or extra-abominal i.e. subcutaneous testicles) and descended scrotal (testicles have descended into a well-developed extraabdominal scrotal sac) (Werdelin and Nilsonne, 1999). Testicondy is present in Monotremata and in all of the Afrotherian mammals, descended ascrotal testes exist in some eutherian placental mammals, while descended scrotal testes exist in most other eutherian placental mammals as well as marsupials (Kleisner et al., 2010). Even though testicular descent in marsupials and eutherians appears to be similar, the marsupial scrotum is prepenial while that of eutherians is postpenial (Kleisner et al., 2010). Numerous hypotheses have been proposed to explain the diversity of testicular positions in mammals. These include the need for a lower temperature for normal spermatogenesis (Moore, 1926), or to reduce mutation rates in germ cells (Short, 1997), and for maturation and prolonged sperm storage in the

epididymides (Bedford, 1978). The most accepted hypothesis is the requirement of an ambient temperature for normal spermatogenesis to occur. This cooler scrotal environment is achieved by counter-current heat exchange between the blood in the descending testicular artery and the ascending blood in the pampiniform plexus of veins (Waites and Moule, 1961) as well as by the presence of abundant sweat glands in the scrotal skin (Waites and Voglmayr, 1963). Contraction and relaxation of the dartos muscle in the scrotum regulates the surface area of the scrotal skin with contraction of the dartos, in conjunction with the cremasteric muscle, elevating the scrotum closer to the lower abdominal wall thereby controlling the amount of heat loss (Morgentaler et al., 1999). In addition, absence of subcutaneous fat in the scrotal skin helps dissipate heat and thermoregulation in the intra-scrotal temperature (Glad Sorensen et al., 1991). However, in mammals with subcutaneous testicles, cooling is achieved by coiled testicular arteries (Bedford, 1978) while in descended ascrotal testes cooling is carried out through specialized arterio-venous heat exchange between lumbar venous and spermatic arterial plexus (Rommel et al., 1992, Rommel et al., 1995). In testicondid mammals, body temperatures have been suggested as being lower than the average body temperatures of mammals with scrotal testes, although this is not supported (Wislocki, 1933). Thus irrespective of the location of the testes, the testicular temperatures are maintained lower than the core body temperature and this is necessary for normal spermatogenesis to occur.

1.2 Organization of the testis

1.2.1 Cytoarchitecture of the testis

Mammalian testes consist of two major interrelated compartments: the interstitial/intertubular and the seminiferous tubular compartment. The former contains blood and lymphatic vessels, testosterone secreting Leydig cells, macrophages, and various other somatic cells. Multiple layers of developing germ cells and Sertoli cells line seminiferous tubules.

Sertoli cells provide structural support to the developing germ cells (Griswold, 1995, Vogl et al., 2000), secrete tubular fluid to nourish germ cells (Setchell, 1978, Griswold, 1988),

function as macrophages (Kerr and de Kretser, 1974), secrete inhibin and androgen binding proteins (Bardin et al., 1988, Steinberger and Steinberger, 1977), facilitate germ cell movement and release (Russell et al., 1988) as well as maintain the blood-testis barrier (BTB) (Waites and Gladwell, 1982). Inhibin modulates the secretion of FSH while androgen binding protein is a carrier protein which regulates spermatogenesis (Collins et al., 2003). The BTB is formed between adjacent Sertoli cells by junctional complexes namely desmosomes, gap and tight junctions (Dym and Fawcett, 1970, Russell and Peterson, 1985, Gilula et al., 1976, Russell, 1977). BTB is situated at the basal third of the seminiferous epithelium dividing the tubule into basal, intermediate and adluminal compartments. The basal compartment contains spermatogonia, preleptotene and leptotene spermatocytes, the intermediate zone transits germ cells between the basal compartment and tubular lumen whereas the adluminal compartment contains spermatocytes, round spermatids and elongated spermatids (Russell, 1978). BTB acts as an immunogenic barrier to protect germ cells as well as limiting the diffusion of substances and/or fluid into the tubular lumen (Dym and Fawcett, 1970, Weber et al., 1988). Any breach in the integrity of this barrier leads to increased permeability of fluid into the tubular lumen with dilations of intercellular spaces between the Sertoli cells.

1.2.2 Spermatogenesis

Spermatogenesis is a process that results in highly differentiated and specialized cells developing from spermatogonia: the spermatozoa. This process is divided into three phases (Russell et al., 1990):

1. Proliferative phase
2. Meiotic phase
3. Differentiation or spermiogenic phase

Proliferative phase: This phase involves successive divisions of spermatogonia. There are two groups of spermatogonial cells: undifferentiated A type (Huckins, 1971) and differentiated, Intermediate and type B spermatogonia (Huckins, 1978). Undifferentiated spermatogonia are of two types: stem cell A_{stem} (A_s) (Gallardo Bolanos et al., 2012) and proliferative spermatogonia A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}) (Abreu and David-Ferreira, 1982)]; these cells rest on the basement membrane of the seminiferous epithelium. As spermatogonia undergo frequent mitotic divisions to give rise to A_{pr} which divide to produce A_{al} which then give rise to chains of A_{al} 4 to 16 cells (A_{al-4}, A_{al-8} and/or A_{al-16}) (Chiarini-Garcia and Russell, 2001). A_s cells have been shown to have the potential to survive a toxic or environmental insult, however, high mitotic rates of proliferative and differentiating spermatogonia result in them being vulnerable to damage (Noller et al., 1977). Subsequent divisions of A_{al} give rise to A1, A_2, A_3, A_4, Intermediate and type B spermatogonia (Abreu and David-Ferreira, 1982). These spermatogonia are connected by intercellular bridges with the consequence that cytoplasmic continuity between germ cells is notable (Weber and Russell, 1987). These communications help to synchronize various cellular activities (Lee et al., 1995, Braun et al., 1989). Type B spermatogonia finally enter the meiotic phase (Fig. 1.2. 2. a). To summarize, the steps of the proliferative phase of spermatogonia are as follows:

$$\mathbf{A_s \rightarrow A_{pr} \rightarrow A_{al\text{-}4} \rightarrow A_{al8} \rightarrow A_{al\text{-}16} \rightarrow A_1 \rightarrow A_2 \rightarrow A_3 \rightarrow A_4 \rightarrow In \rightarrow B}$$

Meiotic phase - Type B spermatogonia divide mitotically to produce preleptotene spermatocytes that enter an exceptionally long lasting prophase of the first meiotic division wherein preleptotene spermatocytes sequentially develop into leptotene, zygotene, pachytene and diplotene spermatocytes. In the leptotene stage chromosomes condense with the result that condensed chromosomes form homologous pairs in the zygotene stage, crossing over and exchange of genetic material then occurs in the pachytene stage and separation of homologous chromosomes in the diplotene phase is the last stage of the long prophase. This is rapidly followed by metaphase I, anaphase I and telophase I of the first meiotic division and hence separation of chromosomes into haploid cells, the secondary spermatocytes. These secondary

spermatocytes then undergo a second meiotic division to give rise to a pair of spermatids that then enter the spermiogenic phase.

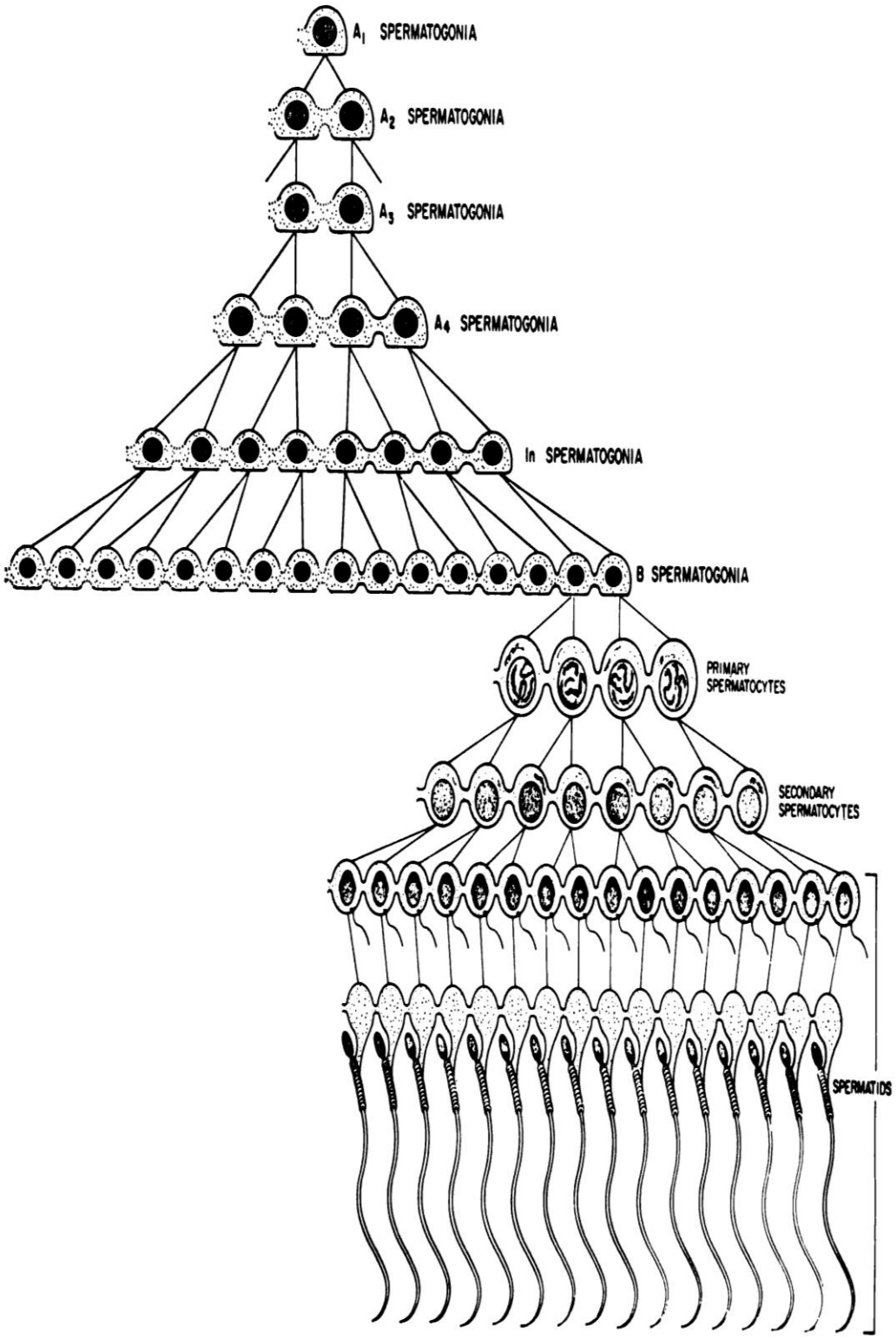

Fig.1.2.2a Schematic diagram showing the spermatogonial cells undergoing mitotic and meiotic divisions to give rise to spermatids. Note the intercellular bridges connecting the spermatogonia. (Dym and Fawcett, 1971)

Differentiation or spermiogenic phase – There is an absence of cell division in this phase, although spermatids undergo dramatic morphological changes to form highly differentiated spermatozoa. These changes include development of an acrosome, formation of a flagellum, nuclear reshaping and condensation and elimination of excess cytoplasm (Leblond and Clermont, 1952). Here the various stages that occur during genesis of mature spermatozoa are discussed.

Development of the acrosome -The steps involved in the formation of the acrosome form the basis for staging the cycle of the seminiferous epithelium. The main steps involved are similar in all species albeit there are subtle detailed microscopic differences between species. This process commences with the perinuclear appearance of the Golgi apparatus; this organelle gives rise to proacrosomal vesicles, which fuse together to form a single granule or vesicle, referred to as an acrosomal vesicle (step 1-3 spermatids). The acrosomal vesicle makes contact with the nuclear envelope and spreads over its surface (steps 4-7 spermatids). The angle subtended by the acrosomal complex (Fig.1.2.2b) increases at this time. In step 4 spermatids the angle is 40^{o}; in step 5 spermatids this ranges between 40-95^{o}; in step 6 spermatids it is 95 to 120^{o} and in step 7 spermatids the angle is >120^{o}. Step 8 spermatids are identified by the opposition of the acrosomal complex to the inner plasma membrane. This demarcates the head, or the nuclear region, from the tail or flagellum of the spermatid.

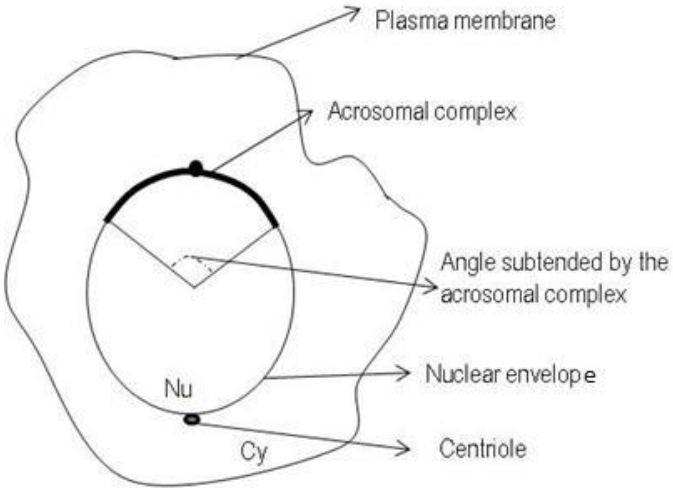

Fig. 1.2.2b Schematic diagram showing the nucleus (Nu) and the cytoplasm (Cy) (Bedard et al., 2005) of an early spermatid. The acrosomal complex spreads over the nuclear envelope to form an angle with the center of the nucleus, which increases in step 2-7 spermatids.

Development of the flagellum - The centriole at the base of the nucleus forms the flagellar axoneme which extends from the nuclear membrane to form the implantation fossa on the opposite pole to that of the acrosome of differentiating spermatids (Fawcett and Phillips, 1969). The accessory structures of the flagellum, the middle, principle and end pieces develop towards the later phases of spermiogenesis (Fawcett, 1975).

Nuclear shaping and condensation - The shape of the sperm nucleus differs between different species of mammals. In the mouse, the nucleus is falciform in shape with the change in nuclear shape during spermatogenesis being attributed to the occurrence of the manchette (Wolosewick and Bryan, 1977) and nuclear chromatin condensation (Fawcett et al., 1971).

Elimination of extra cytoplasm - The volume of the spermatid is reduced to 25% by elimination of water plus expulsion of the residual body (Sprando and Russell, 1987) before the spermatozoon is released into the tubular lumen.

1.3 Stages of the seminiferous epithelium

The structural organization of germ cells within the seminiferous epithelium has been studied in a number of rodent species including laboratory rats (Clermont and Perey, 1957, Leblond

and Clermont, 1952), laboratory mice (Oakberg, 1956) and hopping mice (Peirce and Breed, 1987) . A cell association or stage is defined as a grouping of germ cell developmental stages at particular phases of development in a tubular cross section (Russell et al., 1990). These developmental events occur in a well-timed sequential manner that results in precise and distinct germ cellular associations. A cycle of seminiferous epithelium is defined as the sequence of events occurring from the disappearance of a given cellular association to its reappearance (Clermont, 1972).

In mammals, two different methodologies have been developed for staging the cycle of seminiferous epithelium. These are (1) the tubular morphology system and (2) the acrosomal system. The tubular morphology system is based upon the number of meiotic events, shape of the spermatid nucleus and the relative position of the spermatids within the seminiferous epithelium (Roosen-Runge and Giesel, 1950, Grocock and Clarke, 1975). In the acrosomal system, the morphology of the developing spermatids and the shape of the acrosome define the various stages (Leblond and Clermont, 1952)

1.3.1 Spermatogenic cycle in the laboratory mouse

In 1956, Oakberg proposed the first classification of the mouse seminiferous epithelium. His classification, which was mainly based on the acrosomal system, allocated the mouse seminiferous epithelium into 12 different stages (Fig.1.3.1 a)

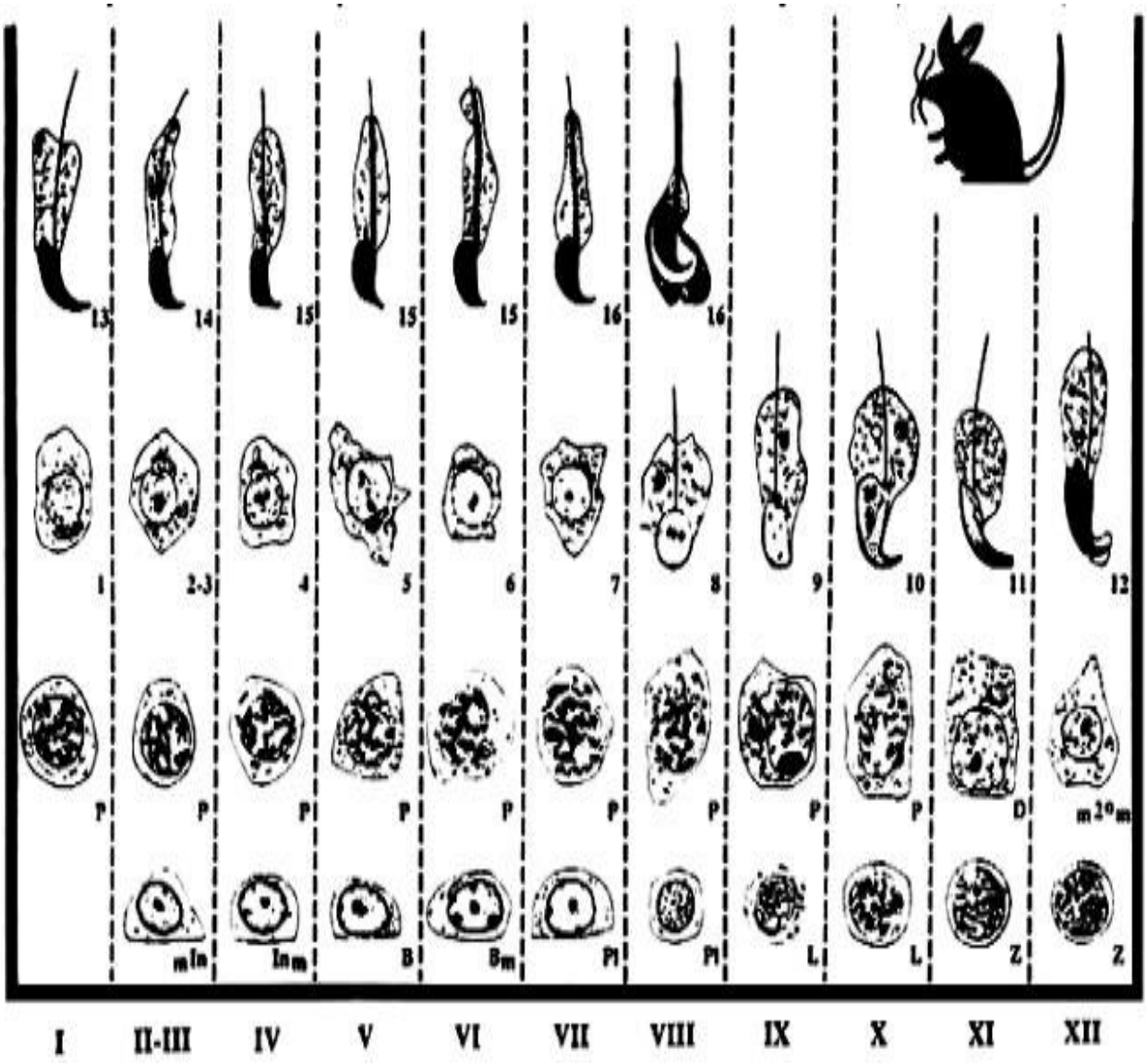

Fig. 1.3.1.a Diagram showing the 12 different stages in the cycle of seminiferous epithelium of mouse ((Russell et al., 1990)

The criteria used to determine the 12 stages within the seminiferous epithelium of the laboratory mouse were subsequently refined by Russell et al. (1990) as follows:

Stage	**Criteria**
I	No acrosome is seen in spermatids by light microscopy; step 1 spermatids are smaller than secondary spermatocytes.
II-III	An early proacrosomal granule and /or an acrosomal granule are seen. The acrosomal vesicle remains rounded and lies close to the nuclear surface.
IV	The stage begins as the acrosomal vesicle flattens on the nuclear surface and ends before the angle subtended by the acrosome becomes 40^0
V	The angle subtended by the acrosome extends from 40^0 to a maximum of 95^0
VI	The angle subtended by the acrosome extends along the plasma membrane 95^0 to a maximum of 120^0; elongate spermatids remain within crypts of the Sertoli cell.
VII	Elongate spermatids move to the luminal aspect of the seminiferous epithelium; the angle subtended by the acrosome is greater than 120^0; round spermatid nuclei have not yet made contact with the cell surface.
VIII	Nuclei make contact with the plasma membrane; the caudal end of the nucleus is slightly tapered.
IX	Spermatid nucleus is deformed; dorsal and ventral surfaces appear.
X	A ventral angle is formed.
XI	The spermatid shows a dorsal angle; diplotene cells have not yet progressed to metaphase
XII	The presence of meiotic metaphase, anaphase or telophase of meiosis I, secondary spermatocytes, or any of the phases of meiosis II define this stage.

1.3.2 Spermatogenic cycle in hopping mice

In hopping mice, 8 stages of the cycle of seminiferous epithelium based on the tubular morphology classification system have been described by Peirce and Breed (1987) . They are:

Stage I Presence of AI and A2 spermatogonia, leptotene primary spermatocytes and rounded spermatids surrounding the lumen of the seminiferous tubule.

Stage II Presence of A3 spermatogonia, pachytene and leptotene spermatocytes with elongating spermatids.

Stage III Intermediate spermatogonia, zygotene spermatocytes and darkly stained spermatids

Stage IV Intermediate spermatogonia predominantly with pale type Al spermatogonia, diplotene spermatocytes and darkly stained spermatids.

Stage V. Anaphase and metaphase spermatocytes, secondary spermatocytes with a nucleus containing a prominent heterochromatin.

Stage IV. Elongated spermatids in the recesses of the Sertoli cells within the seminiferous epithelium.

Stage VI Spermatogonia with bundles of elongated spermatids lying between the rounded spermatids.

Stage VII Abundant Type B spermatogonia, early pachytene spermatocytes, and 3-4 layers of round spermatids in the adluminal compartment.

Stage VIII Spermatozoa lining the tubular lumen with leptotene and pachytene spermatocytes and round spermatids.

1.3.3 Spermatogenic cycle in humans

In humans, 6 stages have been described for the cycle of seminiferous epithelium with stages based on changes in the developing spermatids. Six types of spermatids have been described. They are S_a, S_{b1}, S_{b2} S_c, S_{d1} and S_{d2} based on their nuclear shape, size, staining and changes in the cytoplasm (Clermont, 1963). The seminiferous epithelium of humans shows more than one cellular association in a cross-section of the tubule, unlike the seminiferous epithelium of

laboratory mice (Clermont, 1963, Heller and Clermont, 1964, Johnson et al., 1996). These cellular associations are:

Stage I with two generations of spermatids (S_a and S_d) with maturing spermatids (S_d) in bundles within the Sertoli cell membrane, early pachytene primary spermatocytes and spermatogonia (type A and B).

Stage II mature spermatids lining the lumen of the tubule, pachytene spermatocytes and spermatogonia (Ap, Ad, B).

Stage III containing one generation of spermatids (S_b) and two generations of spermatocytes the pachytene primary spermatocytes and type A spermatogonia.

Stage IV contains spermatids showing signs of elongation of nuclei (S_b), pachytene and leptotene primary spermatocytes together with numerous basal spermatogonia.

Stage V has spermatids with elongated nuclei (S_c), late pachytene, leptotene and zygotene primary spermatocytes with basal spermatogonia (A_p, A_d).

Stage VI consists of maturing spermatids, a generation of zygotene primary spermatocytes, and secondary spermatocytes.

1.4 Apoptosis

In 1972, the term apoptosis was coined to describe the morphological changes occurring in a dying cell (Kerr et al., 1972). It was suggested that apoptosis is a mode of cell death, essential for removing an excess of cells from the body during development so that homeostasis is maintained. Apoptosis occurs in most tissues during development, in mesenchymal tissue (Zuzarte-Luis and Hurle, 2002), brain (Hutchins and Barger, 1998) and developing reproductive organs (Levy and Seifer-Aknin, 2001). Another pathway of cellular death is necrosis characterised by leaky and swollen cells with signs of inflammation (Van Cruchten and Van Den Broeck, 2002) as opposed to apoptosis which involves cell shrinkage, chromatin

condensation and formation of apoptotic bodies without signs of inflammation (Searle et al., 1982).

1.4.1 Mechanisms inducing apoptosis

1.4.1.1 Proteins involved in apoptosis

Apoptosis involves activation of several cytosolic, intra-nuclear and intra-membranous molecules. The most studied are the Bcl-2 family of proteins that play a pivotal role in regulating the process of apoptosis. Bcl-2 is a membrane protein localized on the outer mitochondrial membrane, endoplasmic reticulum and nuclear membrane (Krajewski et al., 1993). The NH_2 terminals of Bcl-2 protein project into the cytosol while the hydrophobic –COOH end (TM) attaches to the membrane of the organelle (Nguyen et al., 1993).These proteins have four domains, BH1-4, and a transmembrane protein (TM) at the hydrophobic end. Depending upon the domains, they are classified into either pro-survival or pro-apoptotic proteins (Fig. 5.4.1.1 a). The pro-survival Bcl-2 proteins (Bcl-2, Bcl-X_L, Bcl-W, Mcl-1, A1) contain domains BH1 to 4 and TM while pro-apoptotic proteins contain either domain BH1-3 and TM (Bax,Bok,Bak) or BH3 and TM (Blk, BNIP) and/ or BH3 alone (Bad,Bid,Bim,EGL-1) (Adams and Cory, 1998). A balance exists between pro-apoptotic and pro-survival proteins, with any change in the relative fractions of these proteins deciding the susceptibility of the cells to death signals (Oltvai et al., 1993).

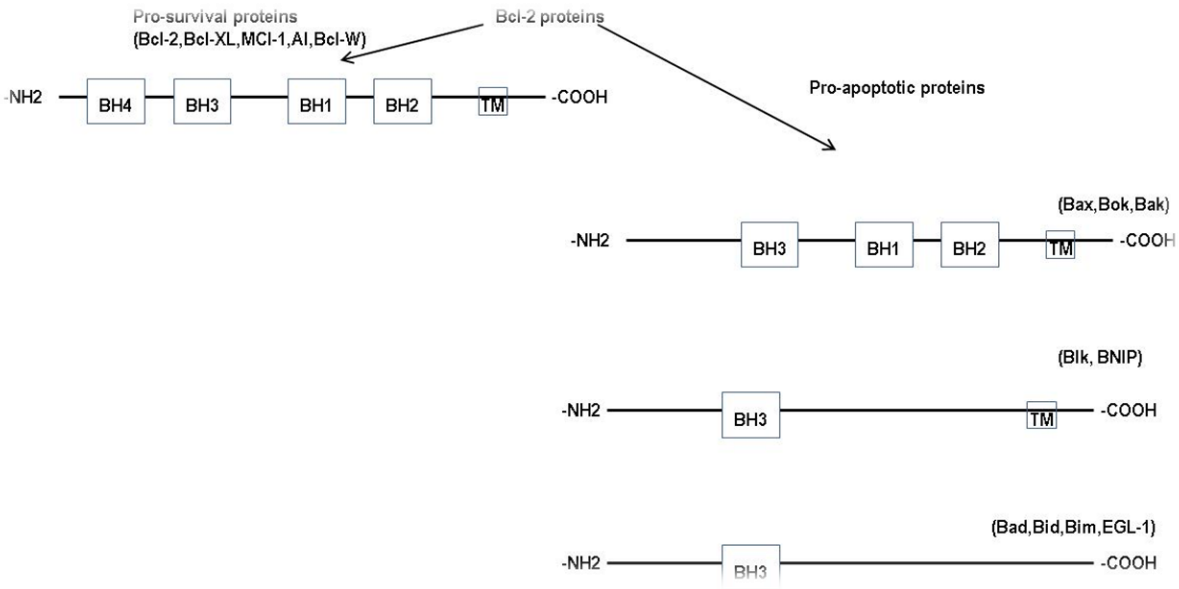

Fig 1.4.1.1 a Schematic diagram showing the Bcl-2 family of proteins.

The principle mechanism by which the Bcl-2 group of proteins regulates apoptosis is by modulating the release of a set of proteins from the mitochondria like cytochrome *c* (Kluck et al., 1997) which is a water soluble protein present in the mitochondrial intermembranous space (Hatefi, 1985).This protein functions in the respiratory chain to transport electrons from one substrate to another (Branden et al., 2001). However, it has been shown that in cells undergoing apoptosis, cytochrome *c* is translocated into the cytosol from the mitochondrial intermembranous space (Wang et al., 2010). The translocated cytochrome *c* in apoptotic cells binds with yet another protein called apoptosis protease-activating factor-1 (Apaf-1) in the cytosol to initiate apoptosis (Liu et al., 1996). Apaf- 1 is a cytosolic protein which remains dormant in the cytosol of the cell until it binds to cytochrome *c* and forms an apoptosome which activates a cascade of cysteine proteins called the caspases (Caroppi et al., 2009) (Fig. 1.4.1.1b). Caspases are a family of cysteine-dependent aspartate-specific acid proteases that regulate and execute apoptosis in a cell (Lavrik et al., 2005). These proteins are strongly expressed in dying cells (Yuan et al., 1993). Caspases are present in the cytosol as latent proteins made up of three domains, the N-terminal prodomain, a large domain (p20, 20kDa) and a small domain (p10, 10kDa) (Thornberry and Lazebnik, 1998) (Fig.1.4.1.1b). Depending upon the N-terminal prodomains, caspases are divided into three categories (Lavrik et al.,

2005): caspases with larger prodomains (>90 amino acids) are inflammatory caspases, those with relatively smaller prodomains are the initiator caspases, and prodomains of 20-30 amino acids are the effector caspases. Inflammatory caspases are 1, 4, 5, 11, 12, 13 and 14, initiator caspases are 2, 8, 9 and 10, and effector caspases are 3, 6 and 7. The prodomains of caspases are a family of death proteins namely death effector domain (DED) and a caspase recruitment domain (CARD) (Martinon et al., 2001). Caspases-1, 4, 5, 11, 12, 13, 14 possess CARD prodomain (Fuentes-Prior and Salvesen, 2004) while caspases-8 and 10 contain two sets of DED (Lavrik et al., 2005, Sprick et al., 2002). Inflammatory caspases activate a series of cytokines which initiate an inflammatory response (Martinon and Tschopp, 2004) while initiator caspases induce apoptosis by activating the effector caspases later in the cascade. These effector caspases degrade cellular proteins and produce morphological changes evident in a dying cell (Nagata et al., 2003, Coleman et al., 2001).

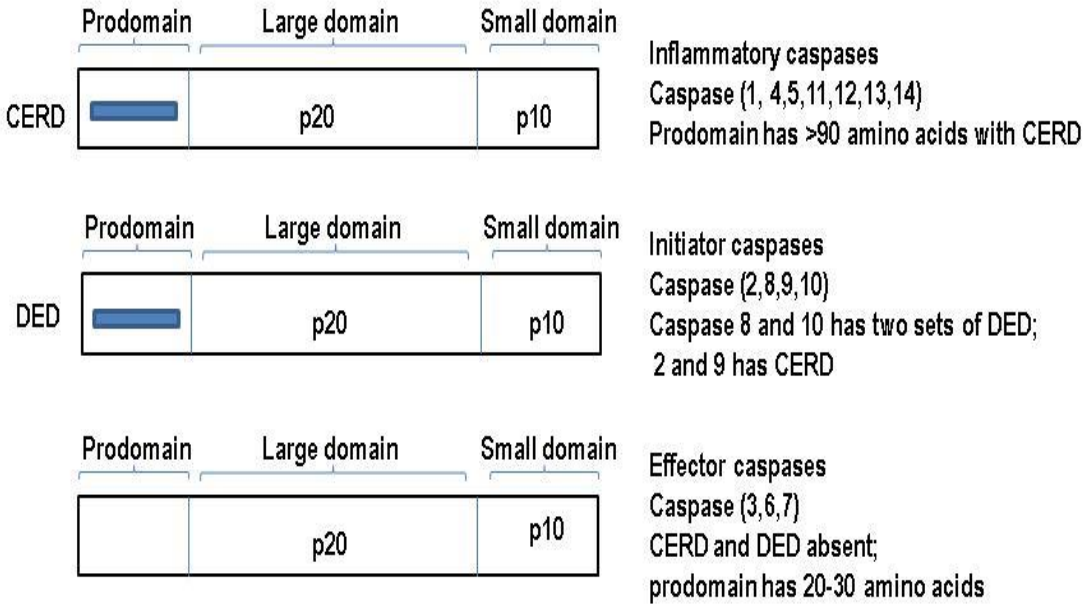

Fig. 1.4.1.1b Schematic diagram showing the different types of caspases and their structure.

1.4.1.2 Caspase-3 and its role in apoptosis

Of the effector caspases, caspase-3 has been shown to have an important role in producing morphological changes within a cell undergoing apoptosis (Porter and Janicke, 1999).

Caspase-3 is also known as the CPP32 (32kDa cysteine protease), Yama or apopain executioner protein (Cohen, 1997). It has been shown in caspase-3 knockout mice that the dying cells do not show chromatin condensation and DNA fragmentation, which are the hallmark features of an apoptotic cell (Woo et al., 1998). DNA fragmentation is brought about by the activation of a 45 kDa subunit of DFF 45 (DNA fragmentation factor 45) protein by caspase-3; this activated subunit can in itself produce further DNA fragmentation without the requirement of caspase-3 (Liu et al., 1997, Janicke et al., 1998, Enari et al., 1998). Furthermore, caspase-3 is required for cleavage of α-fodrin protein, which initiates membrane-associated morphological changes within an apoptotic cell, which is characterised by formation of membrane blebs and cytoplasmic shrinkage (Cryns et al., 1996). α-Fodrin is a trans-membrane protein believed to provide dynamic and structural stability to a cell by binding to its cytoskeletal elements (Bennett, 1990). There are other structural proteins like the actin regulatory protein, gelosin (Kothakota et al., 1997, Mashima et al., 1997) and p21-activated kinase 2 (PAK-2) (Rudel and Bokoch, 1997) which are cleaved by caspase-3 to produce cytoskeletal disintegration in an apoptotic cell. In addition, one of the suggested actions of caspase-3 is to activate the upstream proteins of the apoptotic pathways; in particular, it can influence the release of cytochrome c from the mitochondria to the cytosol and convert Bcl-2 protein into a Bax-like death effector protein (Cheng et al., 1997). Thus caspase-3, the downstream protein in all apoptotic pathways has an indispensable role in mediating cytoskeletal and nuclear apoptosis within a cell.

1.4.1.3 Apoptotic pathways (Refer to Fig. 1.4.1.3a)

Apoptotic pathways are triggered by a variety of stimuli (apoptotic or death signals) such as cytotoxic drugs, heat stress, and oxidative stress; however, the pathways which are activated vary with the stimulus and the type of tissues and cells which are involved in the apoptotic process. Two major pathways have been documented, namely the intrinsic and extrinsic apoptotic pathways.

Intrinsic pathway (mitochondrial-mediated apoptotic pathway)- Cytochrome *c* is the primary mitochondrial protein that activates caspases in this pathway. Apoptotic or death signals stimulate the cytosolic pro-apoptotic BH3-only domain proteins (Bid, Bad) which combine with other pro-apoptotic proteins from the Bax family of proteins (Bax, BaK, Bok) in the pores of the outer mitochondrial membrane (Gross et al., 1999). These proteins undergo conformational changes in the outer mitochondrial membrane which alter the mitochondrial membrane potential and permeability thereby permitting the leakage of cytochrome *c* into the cytosol of a dying cell through permeability transition pores (PTP) or voltage-dependent anion channels (VDAC) (Green and Reed, 1998, Tsujimoto and Shimizu, 2002). The released cytochrome *c* then binds with the Apaf-1 protein in the cytosol to form a protein complex called 'apoptosome' that activates procaspase 9 converting it to caspase 9. Caspase 9 inturn activates effector caspases (3, 6 and 7) that produce morphological changes seen in a dying cell (Coleman et al., 2001). This pathway is regulated by the anti-apoptotic Bcl-2 family of proteins which inhibits cytochrome *c* release from the intermembranous space by the mitochondrial membrane potential and maintains its permeability at a permissible level (Reed, 1998).

Extrinsic pathway (death receptor-mediated apoptotic pathway)- This pathway involves the tumor necrosis factor (TNF) family of transmembrane proteins located on the plasma membrane of the cell (Locksley et al., 2001). The transmembrane proteins are death domains with an extracellular cysteine-rich domain called the 'death ligand' or TRAIL (tumor necrosis related apoptosis-inducing ligand) and an intracellular domain made up of about 80 amino acids referred to as the 'death receptor' (Ashkenazi and Dixit, 1998). Different death ligands and corresponding receptors have been identified, some of the important ones are FasL/FasR, TNF-/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Chicheportiche et al., 1997, Peter and Krammer, 1998, Ashkenazi and Dixit, 1998, Suliman et al., 2001). Death signals are transmitted from the cell surface to the intracellular region. In the cytosol, there are adapter

proteins which bind with the death receptor to form death-inducing signal complex (DISC) (Kischkel et al., 1995). The adapter proteins binding to the death receptor vary with the type of death domains activated on the cell membrane by the death signal. TRADD (TNF-receptor associated protein with a death domain) and FADD (Fas-associated death domain) are the adapter proteins for TNF-/TNFR1 and FasL/FasR (Hsu et al., 1995, Wajant, 2002). The DISC complex thus formed converts procaspase 8 to its active counterpart caspase 8 (Kischkel et al., 1995). Caspase 8 either directly activates effector caspases (3, 6, 7) to induce cell death or indirectly activate proteins of the mitochondrial apoptotic pathway like Bid, Bim, Bax proapoptic proteins in the cytosol to induce cell death (Igney and Krammer, 2002, Zha et al., 1996).

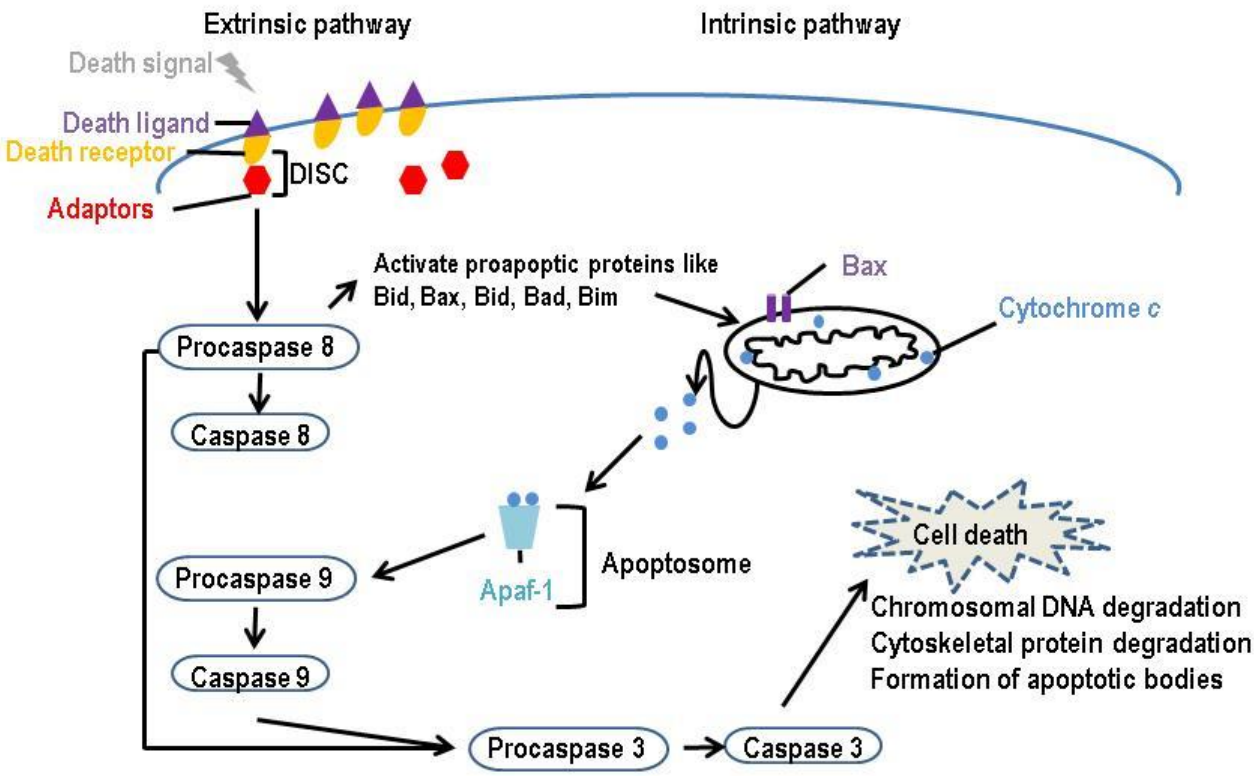

Fig. 1.4.1.3a Schematic diagram showing the extrinsic and the intrinsic apoptotic pathways.

1.4.2 Germ cell apoptosis

Spontaneous germ cell apoptosis occurs during spermatogenesis; it is a mechanism by which unwanted or aberrant germ cells are eliminated in order to maintain germ cell number within

the testes (Jacobson et al., 1997). When testes are differentiating, the process of apoptosis removes excess premeiotic spermatogonia to maintain an appropriate ratio between Sertoli cells and germ cells (Aitken et al., 2011). However, there are other factors, including hormones (GnRH, testosterone) (Hikim et al., 1995, Blanco-Rodriguez and Martinez-Garcia, 1998), drugs (Cai et al., 1997, Alam et al., 2010, Wang et al., 2007), toxins (Lee et al., 1999, Xu et al., 1999, Alam et al., 2010), metabolic diseases e.g diabetes (Tsounapi et al., 2012) and also heat stress (Lue et al., 1999, Lue et al., 2000, Kanter et al., 2013), which can trigger apoptosis in testicular germ cells. Furthermore, deletion of genes like Gonadotropin-regulated testicular helicase (DDX25) (Gutti et al., 2008), Jmjd1a demethylase (Liu et al., 2010), crem (Kosir et al., 2012) or over-expression of androgen-binding protein (Jeyaraj et al., 2003) have all been shown to increase germ cell apoptosis. Moreover, it has been shown that overexpression of Bcl 2/BclxL survival proteins reduces apoptosis in germ cells thereby altering the ratio of germ cells and Sertoli cells which may result in male infertility (Rodriguez et al., 1997). Thus spontaneous apoptosis during germ cell development is essential to maintain germ cell number and male fertility.

1.4.3 Apoptosis in spermatozoa

Apoptotic spermatozoa show well-defined signs of cell damage (Gorczyca et al., 1993, Baccetti et al., 1996, Sun et al., 1997, Barroso et al., 2000, Lachaud et al., 2004), characterised by sperm membrane changes, decreased mitochondrial membrane potential, caspase activation and DNA fragmentation (Glander and Schaller, 1999, Weng et al., 2002). One of the earliest features to appear is in the plasma membrane of the spermatozoa. The plasma membrane of mature spermatozoa consists of an outer and an inner leaflet, aminophospholipids phosphatidylserine (PS) and phosphotidylehanolamine (PE) are concentrated in the inner leaflet while neutral phospholipids sphingomyelin (SM) and phosphotidylcholine (Withers et al., 1979) are present on the outer leaflet (Muller et al., 1994, Hinkovska et al., 1986, Hinkovska-Galcheva and Srivastava, 1993, Nolan et al., 1995, Rana et

al., 1993). As spermatozoa undergo maturation the concentrations of PS and PE increase within the inner leaflets of the plasma membrane (Muller et al., 1997). In apoptotic spermatozoa, PS translocates to the outer surface of the plasma membrane which is considered to be the earliest feature to appear in a dying cell (Zhang et al., 2008a, Paasch et al., 2003, Glander and Schaller, 1999, Martin et al., 2005, Pena et al., 2003, Oosterhuis and Vermes, 2004, Marti et al., 2008, de Vantery Arrighi et al., 2009, Kadirvel et al., 2012) which is then followed by cytoplasmic condensation, nuclear fragmentation, and blebbing of the cell into apoptotic bodies (Anzar et al., 2002). Various techniques have been adapted to identify membrane changes. Annexin V, a calcium-dependent phospholipid-binding protein, has an affinity for PS and has been shown to bind to cells with externalised PS (Koopman et al., 1994, Vermes et al., 1995). Annexin V conjugated with fluorescein isothiocyanate (FITC) is widely used for flow cytometric detection of apoptotic spermatozoa in combination with propidium iodide that binds to the nuclear DNA of cells with a leaky membrane. However, use of TUNEL (TdT-mediated deoxyuridine triphosphate nick end-labeling) in conjunction with Annexin V in flow cytometry allows detection of spermatozoa, not only with early membrane damage, but also with DNA breaks (Barroso et al., 2000).

Externalization of PS onto the outer surface of the plasma membrane is not only seen in apoptotic spermatozoa but also in spermatozoa undergoing capacitation (Gadella and Harrison, 2002, Muratori et al., 2004, de Vries et al., 2003). The presence of PS on the acrosomal plasma membrane shows that aminophospholipids are an essential and integral part of capacitation/ acrosomal reaction (Kurz et al., 2005). Externalization of these proteins has also been shown to increase the affinity of spermatozoa to bind with the zona pellucida (Flesch et al., 2001). Spermatozoa subjected to capacitating agents like bicarbonate /CO_2 underwent PE externalization, indicating that PE externalization was necessary for capacitation/ acrosomal reaction. However, these spermatozoa did not show any nuclear changes which suggests that this is not a part of apoptotic events (de Vantery Arrighi et al.,

2009, Gadella and Harrison, 2002). Studies have also shown that proteins like albumin can also induce capacitation/ acrosomal like reaction (Fraser, 1985). Capacitation like changes produced by these agents are reversible unlike apoptotic events, which are irreversible (Harrison et al., 1996). Sperm capacitation involves loss of cholesterol from the plasma membrane which increases membrane fluidity, thereby facilitating the binding of spermatozoa to the zona pellucida (Bailey, 2010). Studies show that efflux of decapacitation factors like oxysterols, formed as oxidative derivatives of cholesterol in sperm plasma membranes facilitate capacitation by inhibiting tyrosine phosphatise and producing cAMP (Brouwers et al., 2011). Increase in tyrosine phosphorylation is also essential for capacitation and this increase is noticeable on the sperm tail (Bailey, 2010). ROS (reactive oxygen species) generated during capacitation bring about these changes when produced at optimal levels by spermatozoa (Aitken et al., 1998b, Aitken and Curry, 2011). ROS superoxide (O_2^-*) and nitric oxide (oxidative metabolites) combine to form peroxynitrite ($ONOO^-$) which stimulates the tyrosine phophorylation cascade essential for capacitation (Lewis and Aitken, 2001). However, failure to fertilize the ovum leads to continued release of ROS which initiates the process of apoptosis in spermatozoa by release of Cytochrome c, caspase activation, PS externalization and DNA fragmentation (Aitken and Baker, 2013).

An alteration in the mitochondrial membrane potential (MMP) is yet another early apoptotic change seen in sperm undergoing cell death changes (Barroso et al., 2006, Barroso Villa et al., 2002, Kadirvel et al., 2012). PS translocation is associated with lowering of MMP and generation of reactive oxygen species, which induce sperm DNA fragmentation (Marchetti et al., 2002, Wang et al., 2003a, Wang et al., 2003b, Kadirvel et al., 2012). These early apoptotic changes are also associated with an increase in activated caspases, which bring about degradation of the cytoskeletal elements within the spermatozoa and also DNA damage (Paasch et al., 2003, Wang et al., 2003b, Sakkas et al., 2002, Weng et al., 2002, Paasch et al., 2004, Zribi et al., 2012, Kotwicka et al., 2008). These caspases are normally found in

spermatozoa and are region specific: they are expressed in the implantation fossa (Pichardo et al., 2010) and also in the mid-piece of the spermatozoa (Garcia Vazquez et al., 2012). However, their role in normal functioning of spermatozoa has not yet been determined; but it is now thought that activated caspases result in cell death. Thus it is evident that intrinsic apoptotic pathways are involved in mediating sperm apoptosis. The role of the extrinsic pathway involving activation of Fas receptor is negligible in inducing apoptosis as this process does not enhance the expression of caspase-3 which is thought to be an essential component to execute the final stages of apoptosis (Grunewald et al., 2005). In the literature some studies have shown absence of Fas receptors on spermatozoa (Perticarari et al., 2008) while other have shown the presence of these receptors (Sakkas et al., 1999, Grunewald et al., 2001). Thus there are no confirmatory results available to show involvement of the extrinsic pathway of sperm apoptosis.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2: REVIEW OF LITERATURE

The effects of heat on the male reproductive tract due to high scrotal temperatures have been studied extensively in the past. Various techniques have been employed for studying the effects of heat on male reproduction. The following techniques have been used by various groups to study the effects of heat stress on developing male germ cells and spermatozoa:

a. Whole body heating (Ray et al., 1968, Magal et al., 1981, Yaeram et al., 2006).
b. Local heating of the testes by scrotal insulation (Mieusset et al., 1991, Karabinus et al., 1997) or immersion of the testes in a water bath (Setchell et al., 1998, Gasinska and Hill, 1990)
c. Heating by microwave radiation (Kesari and Behari, 2010, Yu et al., 2004).
d. Experimental cryptorchidism (Fujisawa et al., 1988a, Xu et al., 2000).

Of all these techniques, the most commonly used methods are scrotal insulation, immersion of testes into a water bath and induction of experimental cryptorchidism.

The effects of heat on male reproduction have been described with respect to five different aspects of the reproductive process (Setchell, 1998). Each of these aspects has been described separately.

1. Effects of heat on the testes in relation to its weight, structural organization of the seminiferous tubules and changes in germ cells in the seminiferous epithelium.
2. Effects of heat on the number, structure and motility of the spermatozoa in rete testis fluid.
3. Effects of heat on the number, structure and motility of the spermatozoa in semen.
4. Effects on the fertilizing ability of the heat effected spermatozoa.
5. Capability of the heat effected spermatozoa to form a normal embryo after fertilization.

Here the effects of heat on testes and epididymal spermatozoa are discussed.

2.1 Effects of heat on testes

2.1.1 Effects on testicular weight after local heat stress

Reduction in testis weight is the most apparent change evident after application of localized heat to the scrotum. In rats a decrease of 40% in testis weight compared to controls was evident as early as 7 days post exposure to scrotal heat of either 43ºC for 30min or 41ºC for 60min with the changes continuing to persist until 28 days post exposure. Furthermore testes weights remained below the control values for at least 63 days post exposure (Setchell and Waites, 1972, Fridd et al., 1975, Main et al., 1978, Galil and Setchell, 1988, McLaren et al., 1993). Moreover, chronic exposure to scrotal heat of 43.5ºC for 20min seven times a week every 6 weeks results in 40% decrease in rat testis weight, this decrease in testes weight persists for 24 weeks post exposure (Bowler, 1972). A similar 40% decrease in rat testes weight was observed 35 days post exposure to scrotal heat stress of 43ºC for 30min (Kanter et al., 2013).Similarly in mice, scrotal heat stress of 43ºC for 15 and/or 30min resulted in 50 to 65% decrease in testes weights as early as 14 days post exposure (Gasinska and Hill, 1990, Miura et al., 2002). By reducing the temperature to 42ºC with a longer duration of exposure of 45 min, a 50% decrease in ram testes weight was seen 21 days post exposure (Setchell et al., 1991, Hochereau-de Reviers et al., 1993). A similar reduction in testes weight was seen in rats exposed to the same temperature for 30min with no change in testes weight on day 1 and a 50% decrease in testes weight as early as 3 days post exposure (Sailer et al., 1997). In mice exposed to local heat of 42ºC for 30 min there was 40 to 60% reduction in testes weight 7 and 14 days respectively post exposure (Paul et al., 2008). Thus it is evident that exposing the scrotum to a range of temperatures between 41-43ºC for a varying time period of 15 to 60 min results in decreased testis weights of 40% to 60% starting from day 3 post-exposure, with testes weights continuing to be below normal until 35-63 days post exposure. This reduction in testes weight after high temperature is suggested by some as a result of an increase in germ cell loss (Li et al., 2013) and /or a reduction in testicular blood flow (Galil and Setchell, 1988,

Ewing and VanDemark, 1963) following heat stress. In contrast, a few studies also suggest that heat stress increases blood flow to the testes (Glover, 1966, Waites et al., 1973). Thus the possible reasons for a reduction in testes weights following heat stress are not yet determined**.**

2.1.2 Effects on testicular weight post-experimental cryptorchidism

Experimental cryptorchidism also reduces testes weight. Studies on cryptorchid rat testes have shown that testes weight remains unchanged after 24 h of exposure to abdominal temperature (Bergh and Damber, 1984) with a 17% decrease in rat testes weight 2 days post cryptorchidism (Shikone et al., 1994b) and 25% to 49% decrease in rat testes weight 7 days post cryptorchidism (Meistrich et al., 1973, Yin et al., 1998a, Fujisawa et al., 1988a) . Similarly a 40% reduction in mice testes weights was seen 7 days post cryptorchidism (Socher et al., 1997).

2.1.3 Effects on testicular weight post whole-body heat stress

Whole body heat exposure of rams to a temperature of 32^{o}C for 14 days produced a 70% decrease in testes weight post exposure (Gomes et al., 1971).By contrast, studies on rats exposed to whole body heat of 35^{o}C for 30 days did not produce any change in testes weight post-exposure (Magal et al., 1981). A recent study has shown a 60% decrease in mice testes weight 21 days post-exposure to whole body heat of 36^{o}C for 8 h for three consecutive days (Yaeram et al., 2006).

From the above studies, it is clear that exposing the scrotum to high temperatures can produce a significant reduction in testicular weight. This reduction depends on the duration of heat exposure and the techniques employed to produce heat stress. However, the outcomes of the whole body heating on testis weight are less clear. The varied responses observed in studies may be due to changes in the physiological and hormonal status of the body in response to whole body heat which may indirectly affect the testis (Setchell, 1998).

2.2 Effects of heat on structural organization of the seminiferous tubules

2.2.1 Germ cells affected by heat

Effects of heat on the structural organization of the seminiferous epithelium and the developing germ cells in heat-treated and cryptorchid testes have been known since the early 1900s. Studies in the 1960's and 70's have shown changes to pachytene spermatocytes in the rat testis, 1h post exposure to a temperature of 43ºC for 15mins without any effects on the spermatogonia (Chowdhury and Steinberger, 1964, Chowdhury and Steinberger, 1970). Exposure of rat testes to a similar temperature for a longer duration of 30min produced strong dye chromophilia with eosin and/or alcian blue in the pachytene spermatocytes within 1h of local heating and by 2h these cells had large aggregates of acid phosphatase and aminopeptidase enzymes (Blackshaw and Hamilton, 1970). Heat stress has also been shown to affect other developing germ cells: in pigs exposed to a hot environment for 90 days exposure brought about changes in the spermatids without any effects on pachytene spermatocytes (Wettemann et al., 1979), however, scrotal insulation for 100h revealed a reduction in numbers of both pachytene spermatocytes and early spermatids post-insulation (Malmgren and Larsson, 1989). A 33% reduction in the number of late spermatids was evident in mice testes 14days post exposure to heat of 43ºC for 30min (Gasinska and Hill, 1990). A decrease in the number of round and elongated spermatids was evident in homogenates of mouse testis 3 days post-exposure to 43ºC for 60min (Sailer et al., 1997). Similar findings were evident in rat testes with the involvement of pachytene spermatocytes by day 9 after a heat stress of 43ºC for 15min (Lue et al., 1999) and also in mice testes as early as 16 h post exposure to a scrotal temperature of 43ºC for 20min (Rockett et al., 2001). An increase in germ cell apoptosis has been reported in rat testes heated to a temperature of 43ºC for 30min with an increase in incidence of apoptosis at day 1 and 14 and a decrease in incidence of apoptosis at day 35 post exposure (Kanter et al., 2013). Male germ cells isolated from rat testis maintained at a temperature of 43ºC for 1h showed DNA fragmentation (Ikeda

et al., 1999) while human testes fragments heated for a longer duration of 22h at a lower temperature of 37ºC had reduced numbers of spermatids (Nakamura et al., 1987). There is also evidence of changes in the spermatogonial cells after local heating of the testis (Allan et al., 1987, Chowdhury and Steinberger, 1964, Davis and Firlit, 1966). These findings have been supported by recent studies that have used apoptotic markers, and showed apoptotic changes in the spermatogonial cells of rat testis 1 to 3 months after experimental cryptorchidism (Dundar et al., 2005). Furthermore, in experimental cryptorchidism pachytene spermatocytes and spermatids are the first germ cells to be affected in both rat (Henriksen et al., 1995a, Blackshaw and Massey, 1978, Parvinen, 1973, Xu et al., 2000) and mouse testes (Yin et al., 1997b). Experimental cryptorchidism also induces DNA fragmentation in pachytene spermatocytes day 7 post surgery in rat testes (Shikone et al., 1994a) and as early as day 1 in adult mouse testes (Ohta et al., 1996). Changes in spermatocytes after heat stress have also been confirmed by ultrastructural studies which showed apoptotic changes in pachytene spermatocytes as early as the 3rd and 7th day post exposure to a temperature of 43ºC for 15 min on 6 consecutive days in monkey testes (Lue et al., 2002). Extreme temperatures have been shown to alter calcium homeostasis within rat pachytene spermatocytes and round spermatids with an associated decrease in intracellular pH (Herrera et al., 2001). These ionic changes within germ cells may induce apoptosis (Lizama et al., 2007). In a recent study using TUNEL assay, a 38-fold increase in spermatocytes with DNA strand breaks was shown in mice exposed to a scrotal heat stress of 42ºC for 30min (Paul et al., 2008). Recently researchers have shown the existence of DAZL (RNA- binding protein, 'deleted in azoospermia') containing stress granules (SG's) within the spermatogonia and spermatocytes which protect these germ cells from stress, however an alteration in these granules can induce apoptosis (Kim et al., 2012).

Thus it is clear that primary spermatocytes and early spermatids are cells that are sensitive to heat during spermatogenesis (Yin et al., 1997a, Setchell, 1998, Yamamoto et al., 2000). Furthermore, differentiating spermatogonia are also heat sensitive (Gasinska and Hill, 1990,

Sailer et al., 1997, De Vita et al., 1990) with both Type B (Hadziselimovic and Herzog, 2001) and Type A spermatogonia being affected by experimental cryptorchidism (de Rooij et al., 1999). By contrast, recent studies have shown that with increasing temperatures the elongated spermatids (Cataldo et al., 1997), and the round spermatids are heat resistant when compared with the pachytene spermatocytes. This resistance to high temperatures observed in rounded spermatids was attributed to the high levels of antioxidants in their cytosol and low mitochondrial membrane potential following heat stress when compared to other germ cells (Pino et al., 2013). Thus the above studies suggest that most of the germ cells are susceptible to heat; however, the sensitivity to heat varies with duration, temperature and mode of heat stress.

Apoptosis in germ cells may be time-temperature dependent (Morgentaler et al., 1999). In experimental cryptorchidism, germ cell apoptosis is evident 6-7 days post-surgery (Yin et al., 1997a) while local scrotal heat stress of 43^{o}C for 20min induces germ cell apoptosis as early as 8h post-exposure however, lower temperatures of 39-40^{o}C had a negligible effect on germ cell apoptosis (Rockett et al., 2001). Furthermore, rat testes exposed to a temperature of 43^{o}C for 10min did not show any signs of germ cell apoptosis, albeit increasing the exposure time to 15min and 30min induced apoptosis within a few days post exposure (Collins and Lacy, 1969). In addition, intermittent exposures of the scrotum to a heat of 39.9-41.1^{o}C for 30min, 3 days a week for 4 weeks have been shown to significantly affect spermatogenesis (Loughlin et al., 1991). The overall conclusion from these observations is that germ cell apoptosis is time and temperature dependent.

2.2.2 Effects of heat on Sertoli cells (SCs) and Leydig cells

Heat stress also affects Sertoli cells (SCs) within the seminiferous epithelium. Studies have shown that cryptorchid testes induce formation of vacuoles and lipid inclusions within the cytosol of SCs, dilated endoplasmic reticulum and induced changes in the nuclear membrane as well as expansion of the intercellular spaces between the adjoining SCs (Rune et al., 1992,

Kerr et al., 1979). Changes in cytoskeletal proteins of the SCs were also apparent in the form of altered expression of vimentin, cytokeratin-18 and desmin in monkey testes as early as 5 days post-experimental cryptorchidism (Zhang et al., 2004). Cytokeratin-18 is a marker for Sertoli cell differentiation (Miettinen et al., 1985, Moll et al., 1982). This protein is expressed in immature Sertoli cells and is absent from adult testes (Steger et al., 1996). Increased expression of Cytokeratin-18 after experimental cryptorchidism (Zhang et al., 2006b) and scrotal hyperthermia (Zhang et al., 2006c) resulted in dedifferentiation of SCs into immature cells (Maymon et al., 2002). An increase in expression of vimentin around the perinuclear region of the SCs in cryptorchid rat testes has been shown to interrupt contact mediated communication between Sertoli cells and germ cells resulting in germ cell apoptosis (Wang et al., 2002). In addition, ultrastructural studies have shown defragmentation of the Sertoli cell cytoskeletal protein actin as early as 2 days post scrotal heat stress of 43^{o}C for 30 min (Cai et al., 2011). Similarly other molecules like liver receptor homolog-I (LRH-1) normally absent from Sertoli cells were found in Sertoli cells after scrotal hyperthermia (Guo et al., 2007). Heat stress also alters the tight junction (TJ) proteins between Sertoli cells (Chen et al., 2008, Gladwell, 1977). These TJ proteins are occludins, zonula occludens and claudins, and form an integral part of blood-testis barrier (BTB) (Chen et al., 2008) which regulates the movement of molecules from the testicular lymph in the interstitial tissue to the adluminal compartment of the seminiferous epithelium (Meng et al., 2005). Scrotal hyperthermia of 43^{o}C for 30 min alters the TJs as early as 24-48h post heat stress resulting in disruption of BTB and also formation of fluid-filled cavities between the intercellular spaces of SCs (Cai et al., 2011). Other changes evident in the Sertoli cells are accumulation of lipid droplets in the cytosol after experimental cryptorchidism (Nistal et al., 2005), irradiation (Abreu and David-Ferreira, 1982) and scrotal hyperthermia (Furland et al., 2011, Liu et al., 2012). The lipid droplets are present in Sertoli cells, with some being present in the residual body of the elongated spermatids (Paniagua et al., 1987). In stress, it has been shown that lipid droplets within the Sertoli cells accumulate as a result of phagocytosis of apoptotic germ cells (Wang et al., 2006,

Xiong et al., 2009). The changes within the SCs also affect their secretory capacity thereby reducing the production of androgen binding protein (ABP) (Kerr et al., 1979, Karpe et al., 1982, Hagenas and Ritzen, 1975). Thus molecular changes within the Sertoli cells may control or induce germ cell apoptosis (Griswold, 1995).

Similar to Sertoli cells, Leydig cells are also vulnerable to heat stress. Leydig cells secrete testosterone for germ cell and sperm maturation (Sharpe et al., 1992). It has been shown that heat stress transiently affects the production of testosterone by Leydig cells (Liu and Stocco, 1997, Murphy et al., 2001). Decreasing levels of circulating testosterone induce germ cell apoptosis (Troiano et al., 1994, Henriksen et al., 1995b). The only morphological changes reported in Leydig cells after heat stress were accumulation of lipid droplets in the cytosol (Aktas and Kanter, 2009, Damber et al., 1980). Thus Sertoli and Leydig cells appear to be vulnerable to heat stress like the developing germ cells and spermatozoa.

2.2.3 Heat induces stage-specific apoptosis

Earlier studies using rats reported stage specific degeneration of pachytene spermatocytes in stages IX-XII of the spermatogenic cycle, spermatocytes in XIII-XIV and young spermatids in stage I within 1h of heat stress of 43^{o}C for 15min (Chowdhury and Steinberger, 1970, Chowdhury and Steinberger, 1964). Increase in heat exposure time to 4h induced changes, additionally, in the pachytene spermatocytes of stages VII-VIII. In accordance with this study, stage-specific apoptosis was also apparent in early (I-IV) and late (XII-XIV) stages of rat seminiferous epithelium exposed to a temperature of 43^{o}C for 15 mins, however, there were minimal effects on stages VII-VIII (Lue et al., 1999). Similar findings were observed in cryptorchid testes of both mice and rats (Shikone et al., 1994a, Henriksen et al., 1995a). The difference in the expression of apoptosis was further explored by introducing GnRH antagonist along with heat stress, which showed comparable numbers of apoptotic cells in stages (VII-VIII) and stages (I-IV and XII-XIV). In addition, this study reported a marked reduction in intra-testicular testosterone levels within 2 days of heat treatment. Therefore, it

was evident that stages (VII-VIII) were hormone-dependant stages within the seminiferous epithelium. In yet another study, there were reports of a protective role of intra-testicular testosterone and FSH (follicle stimulating hormone) on the testicular germ cells (Shetty et al., 1996).On further investigations preleptotene and pachytene spermatocytes, and round and elongated spermatids of stages VII-VIII were found to be affected by GnRH antagonist and by reduced levels of intra-testicular testosterone, which further confirmed that stages VII-VIII were hormone-dependant (Sinha Hikim and Swerdloff, 1999, Sinha Hikim et al., 1997, Billig et al., 1995). However, heat induced stage-specific apoptotic changes mainly affect the early (I-IV) and late (XII-XIV) stages within the rat seminiferous epithelium as early as 6hrs post exposure to a temperature of 43^{o}C for 15 mins (Yamamoto et al., 2000, Hikim et al., 2003). Thus apoptosis within the seminiferous epithelium appears to be stage specific, with heat-induced apoptosis affecting the early (I-VI) and late stages (XII-XIV) while hormone-dependent apoptosis targeted the stages VII-VIII within the seminiferous epithelium.

2.2.4 Mechanisms producing heat induced apoptosis in germ cells

Germ cell apoptosis is initiated by activation of either the intrinsic, extrinsic or p53 mediated pathways. There is substantial evidence in the literature suggesting the involvement of mitochondrial–dependent pathways in heat-induced germ cell apoptosis. In the mitochondrial-dependant pathway, the Bax family of proteins initiates the process of apoptosis (Adams and Cory, 1998). Redistribution of Bax proteins from the cytosol to the para-nuclear regions was apparent in late spermatocytes and spermatids 2 hours post exposure to a heat of 43^{o}C for 15 min in mouse testis (Vera et al., 2004). Similar translocations of Bax proteins around the para-nuclear area were reported in the rat testis exposed to a temperature of 43^{o}C for 15 mins (Hikim AP, 2003, Vera et al., 2004, Sinha Hikim et al., 2003, Yamamoto et al., 2000). Furthermore, experimental cryptorchidism in mice also augments the expression of Bax proteins within 6-15 days post-surgery (Xu et al., 2000). In experimental cryptorchidism redistribution of Bax to the peri-nuclear region was seen in spermatogonia, spermatocytes and

spermatids of rhesus monkey testis (Zhang et al., 2003). In yet another study, spermatogonia and spermatocytes of rat testis showed increased expression of Bax proteins in their cytoplasm after one month of experimental cryptorchidism (Dundar et al., 2005). Elevations in Bax mRNA levels were also evident in cryptorchid mouse testis within 6hrs of surgery (Absalan et al., 2010). In a recent study, reduced expression of Bax protein with elevated levels of Bcl-2 proteins was observed in mouse testis 2 months post treatment of cryptorchid testis (Absalan et al., 2012) which indicates that the levels of these proteins regulate the recovery process. Redistribution of Bax proteins activates the mitochondrial release of cytochrome c into the cytosol of the germ cells, which in turn activates caspases 3, 6 and 7 as early as 2 h post exposure to a heat of 43^{o}C for 15min (Sinha Hikim et al., 2003). A 9 fold or 27 fold increase in expression of caspase-3 was evident in mice testes 6h and 24h respectively, post exposure to a heat of 42^{o}C for 30 min (Paul et al., 2009). These executioner caspases cause breakdown of PARP, laminin, actin and other cytoskeletal elements within the germ cell (Hikim et al., 2003). Furthermore use of caspase and cytochrome c inhibitors has been shown to reduce germ cell apoptosis after heat stress which indicates that these proteins play a vital role in inducing germ cell apoptosis (Vera et al., 2005, Matsuki et al., 2003). Various other upstream proteins have been identified in the mitochondrial dependant pathway which induces heat mediated germ cell apoptosis, e.g mitogen-activated protein kinase (MAPK 14) (Jia et al., 2009) inactivates Bcl 2, nitric oxide synthetase (Lue et al., 2003, DeFoor et al., 2004, Ishikawa et al., 2005) and caspase 2 (Johnson et al., 2008). However, there are studies which show reduced levels of Bcl-2 proteins without any significant change in the expression of Bax family proteins after a heat stress of 43^{o}C for 15 mins (Miura et al., 2002). This suggests the possible roles of other pathways in producing heat- induced apoptosis. Involvement of the p53 mediated apoptotic pathway was demonstrated in heat treated mouse testis (Socher et al., 1997, Absalan et al., 2010) post cryptorchidism and also in the cynomologus monkey heat-treated testis (Zhang et al., 2006a). This pathway induces apoptosis in spermatogonia and spermatocytes without affecting the

spermatids (Ohta et al., 2003). In p53 knockout cryptorchid mice, germ cell death was delayed suggesting that apoptosis is mediated in the germ cells by p53 dependant and independent pathways (Yin et al., 1998b). However, it was shown that p53 alone cannot initiate and complete the process of germ cell apoptosis, therefore, there is always an associated activation of other apoptotic pathways especially the Fas signalling pathway (Yin et al., 2002) to induce testicular germ cell apoptosis. Investigations on estimating the levels of Fas expression in the mouse testis 6-12 hours postexposure to a temperature of 44^{o}C ±0.5 for 15min, showed a significant increase in the expression of these proteins (Lee et al., 1999). A similar increase in expression of Fas was demonstrated in the spermatogonia, spermatocytes and spermatids of mouse testis 4 days after experimental cryptorchidism (Ogi et al., 1998). However, studies have shown that mice with non-functional Fas L and Fas receptors, when exposed to a scrotal temperature of 43^{o}C for 15min, show apoptotic changes in the diplotene spermatocytes, suggesting that the Fas signalling system (extrinsic pathway) is not essential for inducing germ cell apoptosis (Vera et al., 2004, Sinha Hikim et al., 2003).Thus heat stress induced apoptosis in germ cells is likely to be brought about by intrinsic, or p53 mediated apoptotic pathways.

Recently, the process of autophagy has been proposed as a probable mechanism for producing cell death changes in heat stress (Oberley et al., 2008). Autophagy is referred to as type II programmed cell death which is distinct from type 1 programmed cell death (apoptosis), as it does not involve caspases or DNA breaks which are classical features of apoptosis (Levine and Yuan, 2005). The molecular basis of autophagy is not known although this process is suggested as being regulated by autophagy genes (Atg genes) (Boya et al., 2013). A recent study on mouse germ cell death after local scrotal heat stress of 42^{o}C for 15 min showed not only apoptotic changes in the germ cells as early as 0.5 h to 12 h post exposure, but also an up-regulation of Afg 7 genes 6h post exposure. This suggests both apoptosis and autophagy are essential for inducing germ cell death after heat stress (Zhang et al., 2012).

2.2.5 Heat shock proteins (HSP) and male germ cell apoptosis

HSP are molecular chaperones, which assemble and modify proteins. These proteins are induced by various stressful conditions like raised temperatures, oxidative stress and ischemia (Lindquist, 1986, Welch, 1992). Different families of HSP have been identified out of which HSP 60 and 70 are essential for reproduction (Neuer et al., 2000). HSP 60 is found in the mitochondria (Jindal et al., 1989) while Hsp70 is in the cytosol and nuclei of cells (Hunt and Morimoto, 1985). HSPs are expressed in a different stage of spermatogenesis (Dix, 1997, Meinhardt et al., 1999). Hsp70 is up-regulated during mouse and rat spermatogenesis (Allen et al., 1988). Hsp70-2 is a heat non-inducible protein however Hsp70-1 and Hsp70-3 may be heat inducible. In Hsp70-2 knockout mice, germ cell apoptosis and infertility were evident with spermatocytes arrested in meiosis with an associated decrease in testes size (Dix et al., 1996). Reduced expression of Hsp60 is evident in infertile men which correlates well with reduced spermatogenic function (Werner et al., 1997).By contrast, studies have shown that Hsp70-1 in spermatocytes does not protect germ cells from apoptosis (Vydra et al., 2006, Widlak et al., 2007). HSPs are activated by heat shock transcription factors (HSFs). Four different types of HSFs have been described namely HSFs 1, 2, 3, and 4 (Wu, 1995, Morimoto, 1998, Fujimoto et al., 2010) with HSF 1 and 4 being important for spermatogenesis and fertility (Wang et al., 2004). Activation of Hsf-1 has been shown to block spermatogenesis at pachytene and induce apoptosis in spermatocytes (Nakai et al., 2000). Phlda 1 may be the target gene of Hsf1 to initiate apoptosis (Hayashida et al., 2006). Increased expression of these proteins Hsf1 and Phlda1 was evident in cryptorchid rat testes 7 days post-surgery (Liu et al., 2011). However, there are genes like uncoupling protein 2 and cyclooxgenease 2 which are suggested to be up-regulated with heat stress to protect germ cells from apoptosis (Zhang et al., 2007, Kubota et al., 2011). By contrast, genes like DNA polymerase beta/gamma and poly (ADRP) polymerase, which are essential for DNA repair (Fujisawa et al., 1988b, Tramontano et al., 2000), and genes necessary for cell division like

Cdc2, Cyclin B1 are down-regulated post heat stress inducing germ cell apoptosis (Kong et al., 2000, Zhang et al., 2008b). However, how these genes induce or protect germ cells from apoptosis is still unclear.

2.3 Effect of heat on epididymal spermatozoa

2.3.1 Effect of heat on the caudal epididymal environment

The epididymis in all eutherian mammals stores and transports sperm, and provides a suitable environment for the spermatozoa to mature and maintain their viability (Bedford, 1994, Bedford et al., 1973, Brooks, 1983). The proximal segment or caput epididymidis is the site for sperm maturation (Temple-Smith et al., 1998) whereas the distal/ caudal segments store spermatozoa (Finlayson, 1941). Like the testes, the epididymis also requires an ambient temperature of 2-4^{o}C lower than the core body temperature for normal functioning. Any change in temperature produces considerable effects on the epididymal physiology with consequent effects on the stored spermatozoa (Foldesy and Bedford, 1982, Bedford, 1978). Morphological changes include a reduction in the diameter and length of the cryptepididymis and the vas deferens (Bedford, 1991) and apoptosis in proximal segments of the cauda affecting mainly the principal cells of the epithelium of cryptepididymal mice, as early as 24h post-surgery (Jara et al., 2002). A 5- fold decrease in the storage capacity of the cauda with a reduction of 25% of the sperm numbers was seen in epididymides maintained at abdominal temperature for 3 months compared with the scrotal cauda (Foldesy and Bedford, 1982). Physiological changes involve alteration in the ionic composition of the caudal fluid due to defects in the ion and water transport across the principal cells of the cauda epididymides of the rat when the epididymides are exposed to abdominal temperature (Rasweiler and Bedford, 1982). An increase in the Na^{+} and Cl^{-} ions and decrease in K^{+} ions was seen in caudal epidydimal fluid of rats, 2 and 7 days post-surgery (Wong et al., 1982). Furthermore, heat stress reduces the capacity of the caudal epithelium to synthesize and secrete proteins which are essential for maintaining the viability of the spermatozoa (Esponda and Bedford, 1986).

There are heat shock proteins, especially Hsp70-1, in the lining epithelium of the epididymidis that protect the spermatozoa from the detrimental effects of raised temperature. An increase in expression of Hsp 70-1 was documented in the basal cells of the epididymal epithelium of the cauda epididymides exposed to abdominal temperature (Legare et al., 2004). This suggests that like the testes, the epididymis, which houses spermatozoa, is also a heat-sensitive organ and any change in this organ can produce adverse effects on the stored spermatozoa. Below is discussed the changes seen in spermatozoa as a result of heat stress.

2.3.2 Effect of heat on epididymal and ejaculated sperm numbers and motility

A reduction in sperm counts and motility as a result of heat stress has been known since the 1940s (see review by (Setchell, 1998). Local heating of the bull testes by insulation resulted in a decrease in sperm numbers and motility as early as 1 to 2 weeks post insulation (Karabinus et al., 1997). Similar effects were evident as late as 21 to 31 days after insulating the scrotum for 4 days in bulls (Brito et al., 2003) with a reduction in sperm numbers evident even after insulating only the scrotal neck of the bull (Kastelic et al., 1996). However, no effect on sperm numbers was evident in pigs after insulating the scrotum for 100h, although a decrease in sperm motility was evident between 2-4 weeks post insulation (Malmgren and Larsson, 1989). Similar effects on sperm numbers and motility were also reported in rams after scrotal insulation (Mieusset et al., 1992). In stallions, a decrease in motility was evident a few days after scrotal insulation for 24 to 48h with a reduction in sperm numbers after 10 days but motility and sperm numbers returned to normal levels by day 70 post insulation (Freidman et al., 1991). Mice exposed to local heat of 42^{o}C for 60min had fewer spermatozoa in the epididymis compared to controls (Sailer et al., 1997). Furthermore, reduction of exposure time to 20min reduced sperm numbers and motility 28 days after heat stress (Jannes

et al., 1998). Exposure of mice to a temperature of 42ºC for 30min reduced sperm numbers as early as 7-21 days post exposure (Banks et al., 2005).

A similar decrease in sperm motility and numbers was observed after whole body heat stress. Whole body heating of bulls reduced sperm numbers within 1 week of heat stress with effects persisting for up to 2 months (Skinner and Louw, 1966). In rams, a reduction in sperm numbers was followed by their return to normal within 1 to 1½ months after heat exposure (Moule, 1963). In boars exposed to whole body heat, the number of spermatozoa was not affected but there was a decrease in motility 1-6 weeks after heat stress (Larsson and Einarsson, 1984, Malmgren and Larsson, 1984). Laboratory mice exposed to a whole body heat of 36ºC for 12 h periods for two consecutive days showed decrease sperm numbers 21 days post exposure to heat (Yaeram et al., 2006).

Recently, bilateral cryptorchidism of adult male rats has been shown to reduce sperm motility as early as day 1, 3 and 5 post surgery (Ren et al., 2006).

Thus the above studies on effects of heat stress on sperm numbers and motility using different heating techniques indicate that sperm numbers and motility are temperature dependent and that increasing the time of exposure and temperature can produce a proportional decrease in sperm motility and also sperm numbers with motility changes appearing first followed by the reduction in sperm numbers.

2.3.3 Effect of heat on sperm DNA

Various techniques have been employed in the past to study the effects of heat on sperm DNA, commonly used are the COMET and Sperm Chromatin Structure (SCSA) assays. COMET assay measures single and double stranded breaks in the sperm DNA (Haines et al., 1998, Irvine et al., 2000, Singh et al., 1988) whereas SCSA measures the susceptibility of sperm chromatin to acid denaturation (Evenson et al., 1999), DNA strand breaks and defective chromatin packaging (Larson et al., 2000, Evenson et al., 2002). Recent literature suggests that TUNEL may be a more efficient, simple and objective assay for detecting DNA

damage in spermatozoa (Sharma et al., 2013). Previously SCSA studies on mice exposed to a scrotal heat stress of 40^{o}C for 60min showed 48% abnormal sperm heads by day 14 and defective sperm chromatin acid denaturation as early as day 11 post exposure (Sailer et al., 1997). Exposure of mice to a higher temperature of 42^{o}C for 30min induced DNA damage as shown by COMET assay, within 1h following heat treatment with abnormal spermatozoa with defective chromatin remaining in the cauda until day 7. Furthermore, SCSA analysis of the spermatozoa from the same group showed DNA strand breaks as early as 1h following heat treatment with damaged spermatozoa persisting in the cauda for up to 14 to 32 days post exposure (Banks et al., 2005). TUNEL assays on mouse spermatozoa exposed to similar temperature conditions of 42^{o}C for 30 min also indicate DNA strand breaks from day 7 post exposure until 28 days post heat treatment with the greatest number of spermatozoa with DNA breaks seen on day 14 (Perez-Crespo et al., 2008) and spermatozoa numbers returning to normal by day 60.The above studies show that DNA damaged spermatozoa found on day 7 post exposure to heat would have arisen from maturing spermatids within the testis, or sperm that must have entered the epididymis at the time of heat stress. It has been suggested that it takes around 3-7 days for the maturing spermatozoa to enter the cauda epididymis (Meistrich et al., 1975). These studies have also shown that the changes in the spermatozoa persisted for 28 days post exposure to heat. This indicates that the damaged spermatozoa developed from the immature cells within the testes at the time of heat stress. In addition, this process appears to be reversible as normal spermatozoa were found in the epididymis after 60 days post treatment, which would have arisen from the germ cells present in the subsequent spermatogenic cycle. These studies suggest that sperm DNA damage is inevitable after heat stress; however, studies showing an association between sperm DNA damage and fertility outcomes may give a better insight about the relationship between the two parameters.

2.4 Effects of heat stress on fertilization

The effects of heat on male fertility have been known since the early 1920s. Local heating of guinea pig testes has been shown to produce temporary sterility lasting for 7 to 44 days post heat whereas rat testes treated with infra-red or hot air, induced sterility between 16 and 82 days and 34 and 85 days respectively post exposure and rat testes treated at a temperature of 44.3^{o}C for 20min became sterile between 10 to 20 days post heat (see review by (Setchell, 1998).

Whole body heating of adult mice to a temperature of 32.7^{o}C or 36.1^{o}C for 2h showed 100% sterility in animals exposed to the higher temperature and 43% sterility in animals exposed to the lower temperature (Pennycuik, 1967). Mice exposed to a lower whole body temperature of 32^{o}C for 24h, showed a 79% decrease in fertility on day 1 to 5 and 11% reduction after 16-20 days post exposure (Burfening et al., 1970). Similarly, boars exposed to a temperature of 34.5^{o}C for 8h and 31^{o}C for 16h for 6 weeks when allowed to mate for the next 5 weeks post exposure, exhibited a 59% decrease in fertility compared with 80% fertility in controls (Wettemann et al., 1976, Wettemann et al., 1979). Mice exposed to whole body heat of 35^{o}C for 24h showed no effect after 24h but there was a significant reduction in fertility rates 48, 96 and 120h post exposure (Garriott and Chrisman, 1981). Exposure of mice to a higher temperature of 36^{o}C for 12h for two successive days produced 53% and 8% decrease in fertility compared to 95% fertility rates in controls 7 and 14 days post-exposure to heat (Yaeram et al., 2006) .

In later experiments local heating of rat testes to a temperature of 43^{o}C for 30 min showed reduced fertility rates of 76% 16 days post exposure to 17% after 20 days exposure (Setchell et al., 1988). In a recent study, female mice mated with male mice exposed to a local scrotal heat stress of 42^{o}C for 30min, showed a seven-fold decrease in fertility rate compared with controls (Paul et al., 2009).

It has been shown that there is not only an effect of heat stress on fertility rates but also on the development of embryos. Artificial insemination of ewes with semen collected from rams 4

days post scrotal insulation showed loss of embryos as early as day 17 and day 65 of pregnancy with more losses seen after insemination with semen collected 11 or 18 days post scrotal insulation (Mieusset et al., 1992). Normal female mice mated with male mice exposed to a scrotal heat of 42^{o}C for 20min produced 20% smaller embryos compared with controls (Setchell et al., 1998). *In vitro* fertilization rates of mouse oocytes fertilized with spermatozoa from males exposed to local scrotal heat stress of 42^{o}C for 20min were significantly lower compared with controls (Jannes et al., 1998). A similar reduction was seen *in vitro* fertilization rates of ova with spermatozoa obtained from mice exposed to whole-body heat of 36^{o}C for 12h for two consecutive days (Yaeram et al., 2006).*In vitro* culture of zygotes obtained from female mice mated with mice exposed to an ambient temperature of 36^{o}C ± 1^{o}C for 24h showed defective four cell stage embryos, morulae and blastocysts at 14, 21, 28, 35 days post exposure to heat (Zhu et al., 2004). *In vitro* fertilization with abnormal spermatozoa obtained after scrotal insulation for 48h also affected the time of cleavage during blastocyst formation (Walters et al., 2005). Further studies have shown that failure in pronuclear formation as a result of defective decondensation of the sperm nucleus occurs before it affects the time of cleavage (Walters et al., 2006). Formation of defective 4-cell stage embryos and blastocysts was observed i*n vitro* fertilization with spermatozoa obtained 16h and 23 days post-exposure to a scrotal heat of 42^{o}C for 30 min (Paul et al., 2009).There are also reports on changes in protein profiles of 2 -cell staged embryos 21 days post-exposure to paternal heat stress of 36^{o}C for 24 h (Zhu and Maddocks, 2005). In addition, studies have shown that heat stress has mutagenic potential. Mice exposed to a temperature of 35^{o}C for 5 days showed increased rates of X-Y univalent at metaphase I of the spermatogenic cycle (Garriott and Chrisman, 1980, Waldbieser and Chrisman, 1986). Similar reports were seen in rat testis heated to a temperature of 42^{o}C, 6 and 12 days post exposure (van Zelst et al., 1995). Thus it is evident from the above studies that heat stress induces meiotic abnormalities in the germ cells that develop into abnormal spermatozoa and when these

morphologically defective spermatozoa fertilize normal ova chromosomally defective zygotes result.

2.5 Role of reactive oxygen species in germ cell apoptosis

Cellular changes and cell death observed in germ cells after heat stress can be attributed to oxidative stress (Buttke et al., 1994) which produces reactive oxygen radicals/species (ROS) mainly superoxide radicals (O_2^{-*}), hydroxyl radicals ($^*OH$), hydrogen peroxide (H_2O_2), nitric oxide (NO*) hypochlorite ion ($HCIO3^{-*}$) and peroxynitrite ($ONOO^-$). These radicals are highly reactive molecules with unpaired electrons (Papa and Skulachev, 1997). All living cells generate ROS within normal range as a by- product of various biochemical reactions and the rate of generation of these free radicals in the testes and spermatozoa is thought to be temperature-dependant (Yu, 1994). However, an abnormal accumulation of ROS results in cellular and apoptotic changes within the germ cells (Ikeda et al., 1999, (Ahotupa and Huhtaniemi, 1992, Paul et al., 2009, Shiraishi et al., 2010) and spermatozoa (Aitken et al., 1998a, Aitken and Krausz, 2001, Aitken, 2006).Testicular germ cells and spermatozoa normally generate ROS although there is a balance between their generation and antioxidative mechanisms in the male reproductive tract (Griveau et al., 1997).

Germ cell plasma membranes and mitochondria may be the major sites for ROS production (Shiraishi, 2012). In testicular germ cells, the plasma membrane is rich in polyunsaturated fatty acids which are susceptible to oxidation by ROS (Peltola et al., 1995). Stable ROS products like NO and MDA are used as markers to detect oxidative stress within the testes. High levels of NO induce germ cell apoptosis (Shiraishi et al., 2001, Turner and Lysiak, 2008, Shiraishi and Naito, 2007). An increase in expression of NO has been shown in cynomologus monkeys 3-8 days post exposure to a scrotal temperature of 43^{o}C for 30 min for 2 consecutive days (Guo et al., 2009). Overexpression of nitric oxide synthase increases germ cell apoptosis in mice with induced experimental cryptorchidism (Ishikawa et al., 2005). Studies have shown that oxidative stress is the chief cause of heat-induced germ cell apoptosis (Perez-

Crespo et al., 2008, Paul et al., 2008, Paul et al., 2009, Turner and Lysiak, 2008, Li et al., 2006). ROS have been shown in the testicular tissue of infertile subjects (Koksal et al., 2003). These studies thus show that heat stress induces production of ROS, thereby producing germ cell apoptosis.

Germ cells are protected by antioxidants and testicular antioxidants are broadly categorized into enzymatic and nonenzymatic. Enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidise (GPX), glutathione S- transferase (GST), and heme oxygenase while non-enzymatic antioxidants are Vitamins E, A, C, and glutathione (Shiraishi, 2012). In the enzymatic group, SOD is a vital antioxidant for testicular germ cells (Fujii et al., 2003, Mruk et al., 2002). In addition to an increase in ROS post heat stress there are also reports of an increase in expression of antioxidant proteins like superoxide dismutase, glutathione peroxidise and glutathione S-transferase, which protect the germ cells from oxidative stress (Bauche et al., 1994, Gu and Hecht, 1996). However, antioxidants like glutathione reductase and aldo-keto reductase are thought to be lower in germ cells compared with Sertoli and Leydig cells (Iuchi et al., 2004). Xanthine oxidase inhibitors have been shown to inhibit germ cell apoptosis induced by experimental cryptorchidism in rats (Kumagai et al., 2002).

Optimum levels of reactive oxygen radicals are also suggested to be essential for normal functioning of the spermatozoa (de Lamirande et al., 1997). Disruption in the balance between antioxidants and free radicals or excessive production of ROS can lead to oxidative stress (Lewis et al., 1995). ROS induces lipid peroxidation of polyunsaturated fatty acids of the head and the middle piece of spermatozoa (Aitken et al., 1989). In cultured mouse spermatozoa, elevation of lipid peroxidation has been shown to increase with temperature (Alvarez and Storey, 1985). This increases the cell permeability, producing DNA damage and cell death (Aitken et al., 1995). An increase in production of ROS induces lipid peroxidation which reduces sperm motility (Alvarez et al., 1987, Aitken and Clarkson, 1987, Aitken and Fisher, 1994, Sharma and Agarwal, 1996, Aitken, 1999, Aitken, 2004). Reduction in sperm motility

is directly related to the level of lipid peroxidation (Gomez et al., 1998, Aitken et al., 1989, Suleiman et al., 1996). Hydrogen peroxide has been identified as the main radical responsible for producing sperm motility changes by inducing lipid peroxidation (Aitken et al., 1993a). Lipid peroxidation not only affects sperm motility but also affects sperm-oocyte fusion and acrosomal exocytosis (Aitken et al., 1993c, Aitken et al., 1993b, Jones et al., 1978). It releases the compound malondialdehyde (MDA) as a by-product and it is the levels of MDA, which have been used as an indirect measure of oxidative stress (Hellstrom et al., 1992). Oxidative stress also produces sperm mitochondrial and nuclear DNA damage(Sawyer et al., 2003). However, nuclear DNA is more resistant to damage compared to mitochondrial DNA (Aitken and Baker, 2006). There is substantial evidence that sperm DNA damage is induced by oxidative stress (Kodama et al., 1997, Barroso et al., 2000, Kemal Duru et al., 2000, Twigg et al., 1998). Thus ROS initiates the process of apoptosis in spermatozoa by release of cytochrome *c*, caspase activation, PS externalization and DNA fragmentation (Aitken and Baker, 2013).

2.6 Effects of heat stress on human male infertility

Exposure to high heat in humans in their occupations has been a long-standing concern. However mechanisms involved in this process are not yet clearly demonstrated. Occupational heat exposure in welders, workers in aluminium, plastic, and ceramic factories is known to influence semen quality and fertility. Welders, exposed to a temperature of 31-41^{o}C for 5h daily for 6 weeks showed a raised scrotal temperature of 1.4^{o}C with a decrease in sperm motility and abnormal spermatozoa (Bonde, 1992). There are also reports of conception defects and reduced semen quality in welders (Rachootin and Olsen, 1983, Mortensen, 1988). Furthermore, reduced birth rates have been reported in workers of aluminium and ceramic factories (Mur et al., 1998, Figa-Talamanca et al., 1992). Studies on the relationship between occupational heat exposure and the time required to achieve a pregnancy (TTP) in professional drivers (3h/day heat exposure), welders and bakers have shown a significant

increase in TTP compared with controls, suggestive of occupational heat exposure being a potential hazard for inducing male infertility (Thonneau et al., 1997). Abnormal spermatozoa were significantly higher in workers from plastic factories compared with controls without any difference in sperm numbers and motility. These reports suggest that exposure to high environmental temperatures has a negative impact on male fertility however apart from high heat exposure these individuals were also exposed to harmful chemical substances which may have contributed to the reduction. Finally, a recent study reports that acclimatization to high temperatures may occur without producing any major impact on fertility (Momen et al., 2010). More definitive data is thus required to reach a conclusion on the effect of heat stress on human male infertility.

2.7 General Aims and Objectives

This project aims to investigate the effects of whole body heat stress on developing germ cells and spermatozoa in mice. The following questions have been addressed:

1. Is whole body heat exposure known to reduce fertility rates due to
    a. Germ cell apoptosis?
    b. If so, is it mediated by molecules involved in apoptotic pathways?
    c. Is germ cell apoptosis stage-specific?
    d. Do spermatozoa, like testicular germ cells, undergo apoptosis following whole body heat exposure, and if so, what changes occur in the spermatozoa?
2. Do similar changes to those in laboratory mice occur in arid-adapted rodents when exposed to high temperatures? For this the spinifex hopping mouse, *Notomys alexis* was used. In this species
    a. Are the germ cell changes following exposure to high temperatures similar to those of the laboratory mouse?
    b. Are there any anatomical differences in the testicular vasculature in this species compared to that of the laboratory mouse?

3. Since adrenal glands are known to respond to stress, are there any changes in the adrenal gland morphology in laboratory mice and hopping mice following exposure to whole body heat?

Hypotheses-

1. Whole body heat exposure results in activation of caspase-3 that induces stage-specific germ cell apoptosis and membrane changes in spermatozoa of laboratory mice.

2. In the arid-adapted hopping mouse, *Notomys alexis* similar heat related changes in germ cells and spermatozoa may not occur due to its adaptation to extreme environmental conditions.

3. The changes in germ cells and spermatozoa are either a direct effect of heat and/or an indirect effect resulting from structural changes within the adrenal glands of these species.

CHAPTER 3

GENERAL MATERIALS AND METHODS

CHAPTER 3: GENERAL MATERIALS AND METHODS

3.1 Preliminary study

This study was carried out to identify the morphological characteristics of different germ cells and stages of the spermatogenic cycle in the laboratory mouse before commencing the experiments (Fig.3.1a). The identifying features of the germ cells were adapted from descriptions provided in '*Histological and Histopathological Evaluation of the Testis'* (Russell et al., 1990) .

Cell Descriptions:

Intermediate spermatogonia (Fig. 3.1a a) - Nuclei are ovoid with a rim of heterochromatin on the inner surface of the nuclear membrane. Occasionally, a few strands of heterochromatin occur centrally.

B spermatogonia (Fig.3.1a b) – Morphology is similar to intermediate spermatogonia but more heterochromatin is present than in intermediate spermatogonia.

Leptotene spermatocytes (Fig.3.1a c) - Nuclei contain fine threads of chromatin giving a 'speckled' appearance.

Zygotene spermatocytes (Fig3.1a d) – Nuclear appearance is similar to leptotene spermatocytes, but chromatin threads are thicker, and their staining is intense. These cells are characterised by the presence of the synaptonemal complex (→).

Pachytene spermatocytes (Fig.3.1a e) - Large chromatin cords are spread throughout the nucleus that has a sex vesicle (►) and synaptonemal complex (→). Sex vesicle contains nuclear material and stains intensely compared to nucleoplasm. These cells become progressively larger with advancement in the stages of the cycle of seminiferous epithelium.

Diplotene spermatocytes (Fig.3.1a f) – These are large cells with no heavily stained nuclear chromatin; by contrast the nucleolus associated material stains densely with a clear central area (→).

Secondary spermatocytes- These are very short lived cells that develop after completion of meiosis I. Their short life span without any clear morphological features makes identification difficult. These cells have indistinct heterochromatin and are similar in appearance to newly formed spermatids.

Early spermatids (Fig.3.1a g to k)- Step 1 (g) These cells have an additional prominent juxtanuclear golgi apparatus (→) and a densely stained nucleolus. Step 2 (h) begins with the formation of proacrosomal granules (→), which fuse to form a large proacrosomal granule at the end of step 3 (i). In steps 4 and 5 (j), the proacrosomal granule spreads over the nuclear membrane. Depending upon the extent of the spreading of the acrosome over the nuclear membrane step 6 and 7 spermatids are defined (not shown). In step 8 (k) spermatids, the nuclear membrane makes contact with the plasma membrane and becomes ovoid.

Late spermatids (Fig.3.1a l)-Late spermatids are more apico-caudally elongated. Step 9 spermatids are characterised by an apex, ventral flat and dorsal curved surface. The dorsal surface and the apex are covered by the acrosome. Step 10 spermatids have a prominent apex with an expanding acrosome over the dorsal surface. Step 11 spermatids elongate further with continuous protrusion of the apex. Step 12 shows the most elongated nucleus of any stage of spermiogenesis; with a distinct bend at the junction of the caudal and middle third. Step 13 spermatids show an acrosomal granule in the middle of the dorsal curvature and a ventral fin; step 14 spermatids decrease in length by 30% as compared with the spermatid in step 13. These cells also develop a dorsal fin. Step 15 is characterised by alignment of mitochondria along the flagellum to form the prominent middle piece. In step 16, spermatids have a prominent, definitive hook with the cytoplasmic remnant forming a hood over the sperm head.

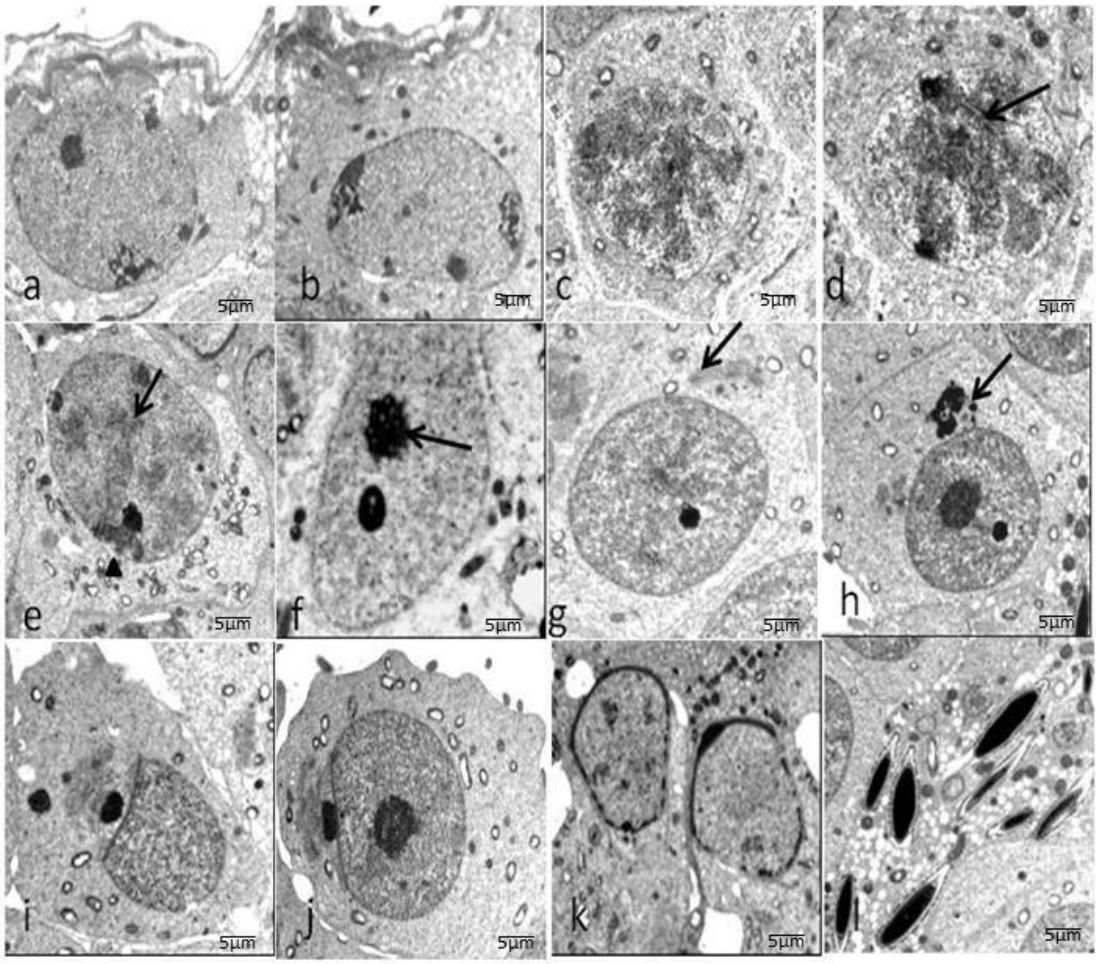

Fig. 3.1a TEM of germ cells within the seminiferous epithelium of the laboratory mouse. Spermatogonia (a,b) with ovoid nucleus with heterochromatin rim close to the nuclear membrane; leptotene spermatocyte (c) has speckled appearance of nuclear chromatin; Zygotene spermatocyte (d) is characterised by presence of synaptonemal complex (→); pachytene spermatocyte (e) shows sex vesicle (►) and synaptonemal complex (→) ; diplotene spermatocyte (f) has nucleolus associated material with a clear centre (→); and early spermatids (g-k) in different stages of development; late spermatids (I)- elongated in apicocaudal direction. (Scale bar- 5µm)

3.1.1 Staging of the mouse seminiferous epithelium

A preliminary study on identifying the stages of the spermatogenic cycle in laboratory mice was carried out before the start of the experiments. For the purpose of this study, stages were grouped into 6 separate categories that correspond to the temperature and hormone dependant apoptotic cycle stages as described in previous studies of laboratory mouse seminiferous epithelium. These catagories are illustrated and described in Figure 3.1b.

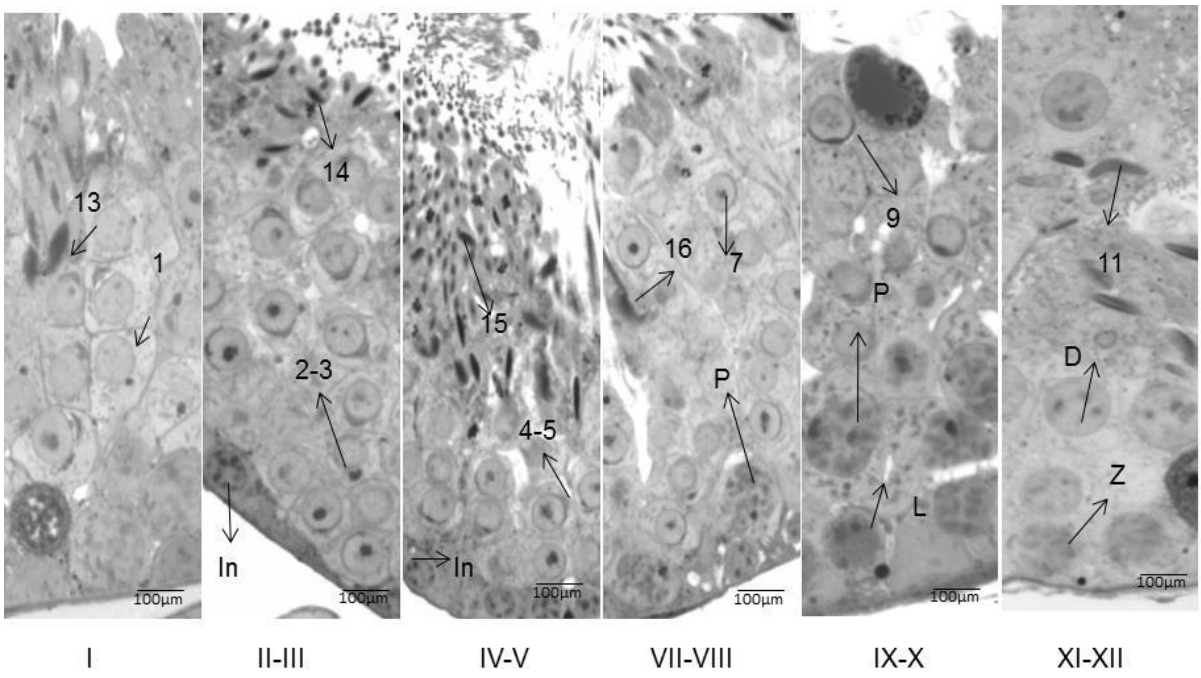

Fig. 3.1b Light micrographs showing the different stages of the seminiferous epithelium of laboratory mouse testis. Numbers indicate the spermatids in different stages of the cycle of seminiferous epithelium. The first stage (I) of the seminiferous epithelium was identified by thepresence of early spermatids with a proacrosomal granule (step 1) and late spermatids (step 13). Stage II-III was characterised by the presence of intermediate spermatogonia, step 2-3spermatids and elongating spermatids (14). Stage IV-V was outlined by intermediate spermatogonia, step 4/5 spermatids and step 15 spermatids. Stage VII-VIII was characterized by the pachytene spermatocytes (P), step 7 spermatids and hook shaped step 16 spermatids. Stage IX-X showed spermatocytes in pachytene (P) and leptotene (L) phase with step 9 spermatids. Stage XI-XII showed diplotene (D) and zygotene (Z) spermatocytes with step 11 spermatids.

3.2 Experimental animals

3.2.1 Strain of animals

Two species of animals were used for this study. They were inbred laboratory mice C57 BL6 (Harlan, Indinapolis, USA) and spinifex hopping mice (*Notomys alexis*). The use of these animals was approved by the Animal Ethics committee at the University of Adelaide (M/39/07). C57BL6 males were purchased from laboratory animal services, located at the Adelaide Medical School, University of Adelaide whereas the hopping mice were purchased from the Nature Education Centre, Norwood, Adelaide.

3.2.2 Animal maintenance

All the experimental animals were kept for a week at the laboratory animal services facility at a temperature maintained at 23-24^{o}C, and on a 12-hour light and dark photoperiod cycle, before the start of the experiment.

Laboratory mice were provided with a commercial pellet diet and water at all times whereas hopping mice were given a similar diet to that they received at Nature Education, which consisted of a mixture of seed and pellet with greens such as carrot and apple slices. A sipper bottle was used to provide water at all times.

3.2.3 Heating procedures

3.2.3.1 Whole body heating

Heating cabinet - The heating cabinet used for all the experimental procedures was a translucent chamber made up of steel and Perspex (Figure 3.2.3.1a). The cabinet had an upper and lower compartment. The upper compartment was utilised to house the experimental animals whereas the lower compartment contained the heater (EWT heater, model 01S, Australia). The temperature inside the cabinet was automatically controlled to between 37^{o}C and 38^{o}C with the aid of a programming controller (R&H, wholesale (SA) Pty. Ltd.,

Australia). Mercury thermometers were installed in order to monitor the accuracy of the temperature controller. In addition, a digital thermometer with humidity monitor was also placed inside the chamber. A glass container filled with water was placed in the lower compartment of the chamber to maintain humidity of 60-70%.

Standardization of the heating chamber- The heating chamber was kept inside a temperature controlled room with a temperature of 23-24^{o}C so that a relatively stable temperature inside the heating cabinet was maintained. In order to calibrate the temperature and humidity inside the chamber, a serial recording of the temperature and humidity was carried out every hour for 8h a day for three consecutive days. The temperature programming control was set at a temperature of 38^{o}C. The temperatures varied between 37-38^{o}C and the humidity between 66-70%.

Figure 3.2.3.1.a The heating chamber used for the heating experiments. This chamber was equipped with a heater (EWT heater, Model 01S, Australia) (a), a universal programming controller (b) (R&H Wholesale (SA) Pty. Ltd., Australia), mercury thermometer (c), digital thermometer (d & e), animals cages (f) and glass container containing water to maintain optimum humidity (g).

Experimental animals - The animals were kept in cages in the upper compartment of the heating cabinet. Food and water were supplied at all times throughout the experiment. The warm water in the sipper bottles was replaced with cold tap water frequently during the experiment. The animals were exposed to a temperature of 37-38^{o}C for 8h for three consecutive days. If signs of heat stress namely restlessness, drowsiness, inactivity and rapid respiratory rates were continuously noticed, the animals were removed from the heating chamber.

Control animals - All the control animals were kept at a room temperature maintained at 23-24^{o}C with a 12-hour light and dark photoperiod cycle.

3.2.3.2 Cryptorchidism

In experiments on cryptorchidism, hopping mice were anaethesized with fluothane, the midline abdominal wall opened and one testis was attached to the body wall, with the use of a single 6/0 silk suture placed through the epididymal capsule. The body wall and abdominal skin were subsequently closed with a 4/0 silk suture. The animals were sacrificed, either 7 or 21 days later.

3.2.4. Collection of tissue specimens

Animals were anesthetized with pentobarbitone sodium (Nembutal, Bomac laboratories, Asquith, NSW) at a dose of 60mg/10gm of body weight injected intraperitoneally.

Vascular fixation was achieved through the left ventricle of the heart. The vascular system was first rinsed with heparinised phosphate buffer followed by infusion with 4% paraformaldehyde and 3% glutaraldehyde made up in 0.1 M phosphate buffer, pH 7.4 for ~ 5mins. The testes and/or epididymides were removed and fixed overnight in 4% paraformaldehyde and 3% glutaraldehyde made up in 0.1 M phosphate buffer for routine histology and electron microscopy and/or in Bouin’s fixative overnight for

immunohistochemistry. In addition, adrenal glands were removed from the laboratory and hopping mice and fixed overnight in Bouin's fixative for routine histology.

In experiments where caudal epididymal spermatozoa were used for flow cytometry studies, mice were euthanized by an intraperitoneal injection of pentobarbitone. Cauda epididymides were removed from animals, blotted on filter paper and placed in 500µL of prewarmed modified Biggers, Whitten, and Whittingham (BWW) medium (Biggers et al., 1971). Spermatozoa were extruded by retrograde perfusion of BWW into the vas deferens after making a small incision in the cauda epididymidis (Walsh et al., 2008). They were allowed to disperse into the medium in an incubator, maintained at 37ºC with 5% CO_2 in air, for 10mins and then were collected for immediate use.

In experiments on cryptorchidism, the cryptorchid and the contralateral control testes were removed and fixed by immersion for 24 h in Bouin's solution.

3.2.5 Histology for light microscopy

Tissue processing- Testes and adrenal glands from the laboratory and hopping mice were processed overnight in an automatic machine (Shandon, Citadel 2000; A.E. Stansen and company Pty. Ltd., Victoria) using the standard protocol as follows:

Testes and adrenal glands fixed in 4% paraformaldehyde and 3% glutaraldehyde made up in 0.1 M phosphate buffer, were washed with 70% ethanol overnight.

Tissues were cut transversely to the long axis and each half placed in a micro cassette for processing in the automatic machine.

a. 70% ethanol, 1 hour 30 mins
b. 80% ethanol, 1 hour 30 mins
c. 90% ethanol, 2 hours
d. 95% ethanol, 2 hours

e. Absolute ethanol, 2 hours-two changes
f. Histoclear, 2 hours- three changes
g. Wax (Histoplast) for 1 hour 30 mins.

Tissue embedding and sectioning- Tissues were embedded in paraffin wax using a Tissue – Tek (TEC, Tissue Embedding Console System, Sakura, Finetek, USA). Tissues were sectioned at 7µm thick using a microtome with standard procedures. Serial sections were cut for staining and analysis and transferred to glass slides that were air-dried.

3.2.5.1 Haemotoxylin and eosin staining of sections of testes and adrenal glands

Air dried slides were stained using the following protocol:

Histoclear, three change 5 mins s each, agitating the slides periodically

a. Absolute alcohol, 3 mins
b. 80% ethanol, 3 mins
c. 70% ethanol, 3 mins
d. 30% ethanol, 3 mins
e. Distilled water, 3 mins
f. Lillee Mayer's Haematoxylin, 2mins (See Appendix)
g. Running tap water, wash
h. Ammonia, 1 min wash
i. Running tap water, wash
j. Eosin, 1min (See Appendix)
k. 80% ethanol, 3 mins
l. 85% ethanol, 3 mins
m. Absolute ethanol, 3 mins
n. Histoclear, 5mins two changes

Then the slides were mounted with a cover-slip using DPX mounting medium and viewed under Olympus BX 51 microscope coupled with a CCD camera (Olympus, Tokyo, Japan).

3.2.5.2 Masson's trichrome staining of sections of adrenal glands

Air dried slides were stained using the following protocol:

Weigert's iron hematoxylin for 10min (See Appendix)

a. Rinse in running tap water, 10min
b. Distilled water, 1min
c. Biebrich scarlet-acid fuschin, 10-15min (See Appendix)
d. Distilled water, 1min
e. Differentiate in 5% phophomolybdinic acid, 10 mins
f. Aniline blue solution, 10mins (See Appendix)
g. Distilled water
h. Dehydrate in 95% alcohol and 100% alcohol
i. Mount with DPX

Note: The collagen fibres stain blue, nuclei bluish-black and cytoplasm red.

3.2.5.3 TUNEL (Terminal Deoxynucleotidyl Transferase-mediated Deoxy-UTP nick end Labelling)

DNA strand breaks in germ cells were identified in paraffin-embedded testicular sections (7µm) by TUNEL, using an *in situ* cell death detection kit, AP (Roche).

Sections were-

Adhered to slides coated with 2% 3-aminopropyl triethoxysilane (Sigma- Aldrich, St. Louis, MO) in 100% ethanol.

Deparaffinized by heating for 30mins at 60^{o}C followed by treatment with xylene and hydration in 100%, 95%, 90%, 80%, 70% alcohol and distilled water.

Incubated with 0.1%Triton- X in 0.1% sodium carbonate buffer for 8 mins.

Treated with TDT buffer (See appendix) for 10mins at room temperature.

Incubated for 3 hours at 37^{o}C with TUNEL reaction mixture that contains enzyme and label solution (See Appendix).

Washed with PBS and DNA break sites were labeled with an anti-fluorescein antibody conjugated with the enzyme alkaline phosphatase (Converter AP) for 1 hour at 37^{o}C.

Washed to remove unbound enzyme conjugate.

Visualized by a substrate reaction with fast red and counter stained with haemotoxylin.

3.2.5.4 Immunolocalization of Cleaved Caspase -3

Caspase 3 activation in germ cell apoptosis was identified in 7µm thick testicular sections using cleaved caspase-3 (Asp 175) antibody conjugated with Alexa fluor® 488 (Cell Signaling Technology).

For this sections were -

a. Deparaffinized and rehydrated followed by antigen masking by boiling sections in 10mM sodium citrate buffer at 95-99^{o}C for 10mins.
b. Washed with PBS and incubated with blocking buffer for 60mins at room temperature.
c. Treated with primary antibody conjugated with Alexa fluor® fluorochrome at a dilution of 1:10 overnight at 4^{o}C.
d. Rinsed with high salt PBS to decrease background staining treated with antifade reagent and examined under a fluorescent microscope (Olympus BX 51).

3.2.5.5. Transmission electron microscopy of testes and adrenal glands

Tissues were cut into ~1mm^3 cubes, and processed as follows:

1. Overnight fixation in 4% paraformaldehyde and 3% glutaraldehyde made up in 0.1 M phosphate buffer, pH 7.4.
2. Washed twice for 15 mins each in 0.2M phosphate buffer.
3. Post-fixed in 1% osmium tetraoxide (OsO_4) in phosphate buffer for 60mins followed by 0.2M phosphate buffer wash and dehydration.

4. Dehydration by passing through:
    a. 30% ethanol, 15mins
    b. 50% ethanol, 15 mins
    c. 70% ethanol, 15mins
    d. 75% ethanol, 15 mins
    e. 80% ethanol, 15 mins
    f. 85% ethanol, 15 mins
    g. 90% ethanol, 15mins
    h. 95% ethanol, 15 mins
    i. 100% ethanol, 30 mins
    j. 100% ethanol ($CuSO_4$), 60 mins three changes
5. Dehydration was followed by two washes, 30 mins each, in propylene oxide.
6. Tissues were infiltrated with
        a. Propylene oxide: Resin (2:1), overnight
        b. Propylene oxide: Resin (1:2), 7-hours
        c. Placed in pure resin, overnight
7. Embedded next morning.
8. Polymerisation carried out by incubating at 60^{o}C for 24 hours.

Sectioning and Staining **-**Tissues were cut using a Reichert-Jung Ultracut ultramicrotome. Semi-thin sections (0.5-1.0µm) were cut with a glass knife and stained with toluidine blue to select the area of interest. Blocks were trimmed and ultrathin sections (70-90µm) of silver to gold interference colours cut using a diamond knife (Diatome Ltd., Bienne, Switzerland). Thick plastic sections were collected on a clean glass slide with 2 drops of toluidine blue solution (See Appendix) and dried on a hot plate for a few mins and then mounted with DPX before viewing under Olympus BX 51 microscope coupled with CCD camera (Olympus, Tokyo, Japan).The ultrathin sections were collected onto copper/palladium grids (200 mesh).

Sections were stained with 2% uranyl acetate followed by lead citrate. These stained sections were then taken for viewing with a Philips CM 100 transmission electron microscope.

3.2.5.6. Fluorescence microscopy for spermatozoa stained with Annexin V-PE and 7-AAD

Sperm suspensions from control and heat-treated groups were stained with Annexin V-PE and 7-AAD. To remove excess stain, sperm suspensions were washed twice with phosphate buffered saline, smeared on slides and examined under a fluorescent microscope. Images were captured at X 100 magnification using an Olympus BX 51 microscope coupled with CCD camera (Olympus, Tokoyo, Japan).

3.3 Flow Cytometry

Flow cytometry is a laser-based technology, which measures the physical characteristics of a single cell in a suspension.

3.3.1 Principles of Flow Cytometry

(Jaroszeski and Radcliff, 1999, Radcliff and Jaroszeski, 1998)

Flow cytometry consists of four interrelated systems; these are the fluid, illumination, optical and data analysis and storage systems. The fluid system has two sources of fluid, an outer 'sheath fluid' layer of PBS (Phosphate-buffered saline) with a central core of fluid (PBS) containing the particles under analysis, spermatozoa being the cells analysed in this study. The pressure of the sheath fluid aligns individual particles in suspension through the central core for optical analysis. The illumination system contains a laser as the light source. Light emitted from the laser beam strikes the particle under analysis within the central core of the fluid system. The most commonly used beam is an argon ion laser, which emits a beam with a

wavelength of 488nm. When the laser beam strikes the particle the light is scattered in all directions. The optical system collects the scattered light to quantify it. The light scattered in the direction of the laser beam is referred to as forward angled scatter scan (FSC) and light scattered at 90^{o} to the laser beam is called the side angled scatter scan (SSC). FSC assesses the basic morphological characteristics of the particle while SSC assesses granularity within the particle. In addition to these morphological details, use of fluorochromes helps to identify other characteristics of the cells such as membrane changes after heat stress in the current study. The fluorescent emission is detected by a series of dichroic mirrors, optics, beam splitters and photomultiplier tubes (PMT). Green fluorescence of FITC (fluorescein isothiocynate) is detected by a FL1 filter. Orange-red fluorescence emitted by PE and PI is detected by a FL2 filter while red fluorescence from dyes like red-670 tricolor is detected by a FL 3 and 7-AAD by a FL4 filter. In the current study, dual flow cytometry was performed using both Annexin V-PE and 7-AAD flurochromes. The emissions from the flurochromes Annexin V-PE and 7-AAD were detected with a 576/26nm band pass filter behind a 600 nm long pass dichroic mirror and a 695/40nm band pass filter behind a 645-nm long pass dichroic mirror respectively. In Dual flow cytometry, the emission spectra can overlap producing false positive results. This is normally adjusted to compensate using software to avoid false positive results. The next step involves conversion of electrical signals into digital data. The information from a particle/cell is called a single event. Before processing the sample, standardization of flow cytometry is essential using negative and positive controls to minimise false positive results. Negative controls containing unstained cells were used to obtain a baseline reference point to set up FSC and SSC (Fig 3.3.1). A gate was drawn to demarcate a single population of cells. The sperm population was identified on the basis of FSC and SSC scatter properties, respectively; the sperm population showed a typical L-shaped distribution (Fig. 3.3.1 a-d). This population was gated to exclude debris.

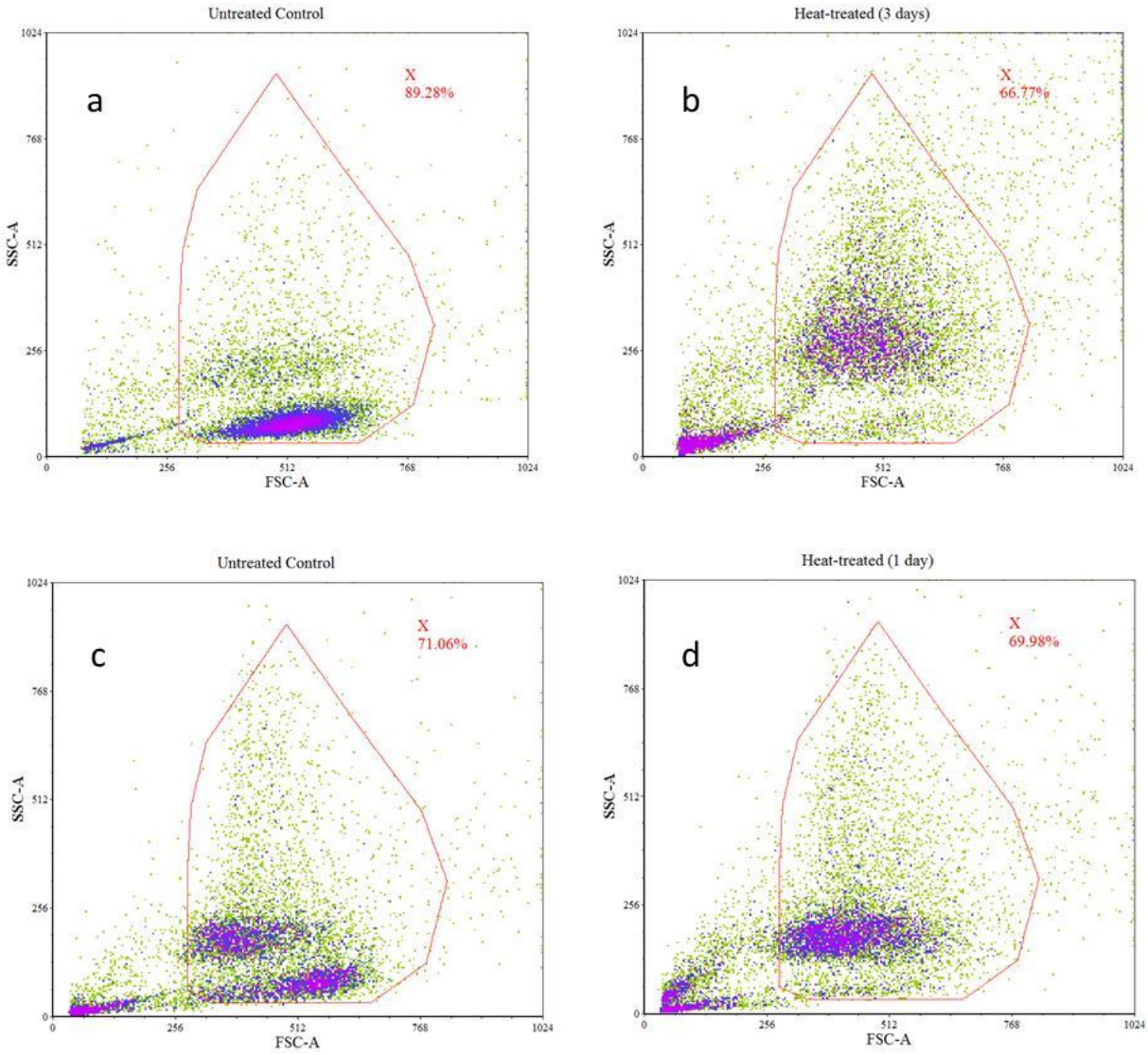

Fig. 3.3.1 (a-d) Shows the negative controls. Gate "X' represents the L-shaped distribution of the sperm population. The sperm population in gate 'X' was analysed in control (a,c) and experimental samples (3 days (b) and 1 day (d) post exposure) to minimise false positive results from debris.

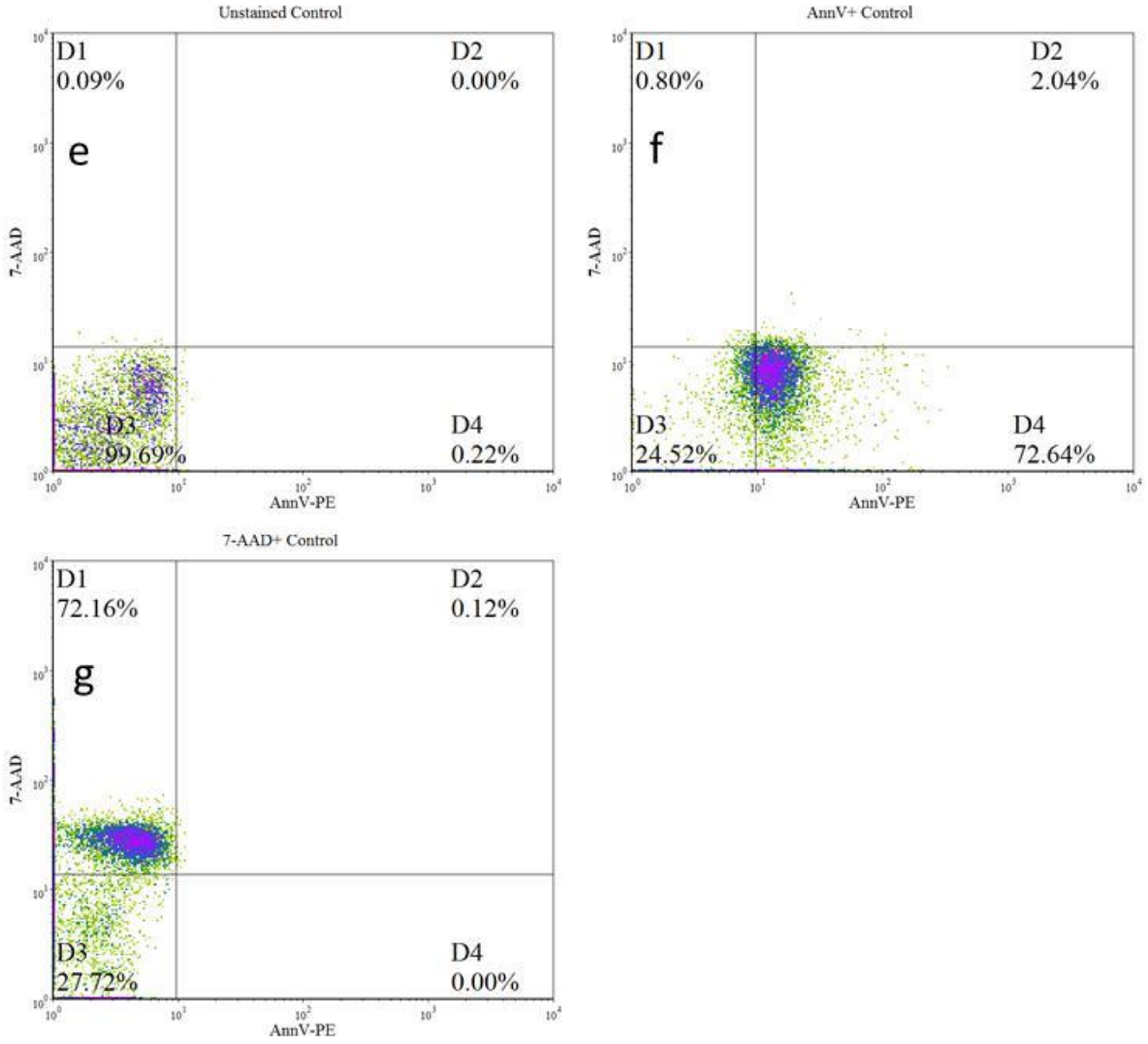

Fig. 3.3.2 (e-g) Shows setting of quadrant limits with unstained (e), Annexin V (annV)–phycoerythrin (PE)- positive (f) and 7-aminoactinomycin D (7-AAD)- positive (g) controls.

Detector voltages were set using an unstained sample as a negative control (Fig. 3.3.2e). Positive controls were used to reduce the overlap of spectral emission when two fluorochromes are used as in the current study. Two positive controls one stained with Annexin V-PE and the other with 7-AAD were used to set up quadrant limits before running the samples stained with both the dyes in control and experimental groups. (Fig. 3.3.2 f,g). A combination of FMO controls (Fluorescence minus one) and internal experimental positive controls was used to determine the correct position of the gate fluorescent parameters. The gates to define AnnV-PE+ staining were set using samples treated with Me2SO (dimethyl

sulphate) as a positive control (Fig. 3.3.2 f) and a PE FMO was checked to ensure the PE+ boundary was accurate (Roederer, 2001). Gates for 7-AAD+ staining were set with samples treated with ethanol (Fig. 3.3.2 g) and a 7-AAD FMO was checked to ensure the 7-AAD$^+$ boundary was accurate. There are different ways to represent flow data. Data are depicted in the form of histograms, overlay plots and dot plots. The current results were represented by dot plots in which each event is depicted by a dot and the position of the dot within the plots represents the intensities of the two parameters FL2/FL4.

3.3.2 Standardization of flow cytometry technique for spermatozoa

Flow cytometric analyses were carried out using an EPICS-PROFILE II XL flow cytometer (Beckman Coulter) with a 15-mW 488-nm air–cooled argon ion laser for excitation and non-standard optics configuration for collection of emitted light. Integral (area) signals were recorded for all parameters. Data were analysed using FSC Express (Version 4;DeNovo Software, Los Angeles, CA, USA). For each sample a minimum of 10,000 spermatozoa was assessed. The sperm population was first identified on the basis of forward and side scatter properties (FSC and SSC, respectively); the sperm population showed a typical L-shaped distribution (Fig. 3.3.1 a to d). This population was gated (X) to eliminate the majority of debris. As described by Petrunikina *et al* (2010), this gate is insufficient to assess accurately the genuine number of spermatozoa because these samples are likely to contain non DNA-containing particles such as cytoplasmic droplets or cellular debris that commonly show similar FSC and SSC properties to those of spermatozoa and, as such, cannot be eliminated on the basis of light scatter properties alone (Petrunkina et al., 2010). The true percentage of non-DNA-containing particles per events in the D3 quadrant (Fig. 3.3.1 e) was estimated using the following formula (Petrunkina and Harrison, 2010):

% Non-stained events in D3= [% total events in D3 – (%DNA-negative events)] / [%total events- (% DNA-negative events)] x 100

where the % DNA-negative events is the percentage of events in the D3 + D4 quadrants in the 7-AAD- positive control sample and the % of total events is the percentage of events in the D1 + D2 + D3 + D4 quadrants. The percentage of non-stained events in quadrant D3 should be < 3% to eliminate any misinterpretation in the analysis. In the current study the true percentage of non- DNA events in the D3 quadrant was estimated to be 0.125, which indicates negligible misestimation in the analysis. The percentage of spermatozoa in each quadrant was estimated in the control (Fig.3.3.3 h,j) and heat treated group (Fig.3.3.3 i,k). Propidium iodide (PI) has been used in most of the earlier studies (Glander and Schaller, 1999), (Pena et al., 2003), however in the current study this was replaced with 7-AAD because of its higher DNA binding affinity, and emission maxima of higher wavelength in the red region where emission maximum is 610nm to 670nm. Therefore the emission spectrum of 7-AAD and PE have minimal overlap (Schmid et al., 1992).

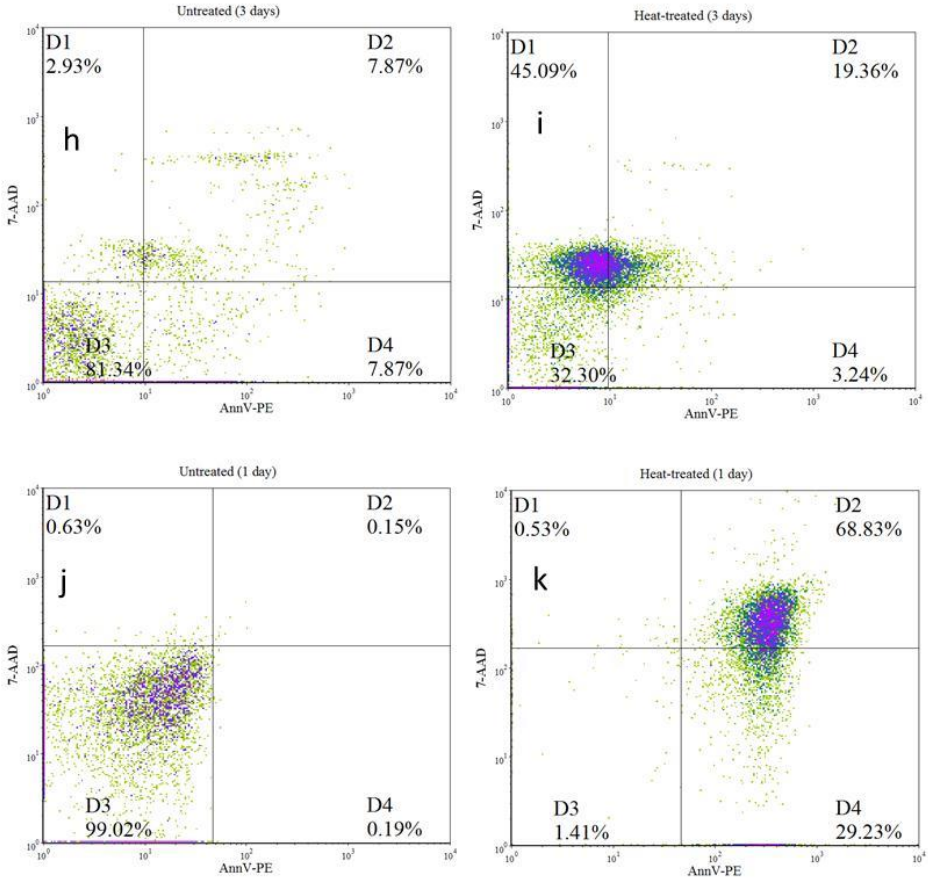

Fig. 3.3.3 The X and Y-axes represent percentages of 7-AAD and Ann V-PE positive spermatozoa respectively. The gates were used to divide spermatozoa into four

subpopulations in four different quadrants (D1-D4) in the controls (h,j) and heat-treated groups exposed to 1 day (k) and 3 days (i) of heat.

3.3.3 Different sub populations of spermatozoa

Thus four different subpopulations of spermatozoa were defined in control (Fig.4.3.3 h,j) and heat-treated (Fig.4.3.3 i,k) groups: the D1 quadrant with AnnV-PE$^-$/7-AAD$^+$ spermatozoa (dead spermatozoa); the D2 quadrant with Ann V-PE$^+$/7-AAD$^+$ spermatozoa (late apoptotic); the D3 quadrant with unstained (live spermatozoa or viable spermatozoa); and the D4 quadrant with Ann V-PE$^+$/7-AAD$^-$ spermatozoa (early apoptotic spermatozoa) based on the classification by Pena et al. (2003)

3.4 Vascular cast studies

Animals were perfused with a resin that was prepared according to the manufacturer's instructions (Boston No. 17, Plastic Replica and corrosion kit, Polysciences, Inc.). For the preparation of the stock solution, 20mls of base solution A, 2mls of red and blue pigment (2%) and 4mls of methyl methacrylates were mixed vigorously and then divided into two equal parts. 5.5 ml of catalyst was added to one half and 3 drops of promoter were added to the other half. These two portions of the solutions were mixed together immediately before use. The vessels were first flushed with heparinised PBS. Then resin was pumped into the circulation through a needle placed in the left ventricle. The specimens were then placed in an ice bath and the resin was allowed to set overnight. Subsequently, the specimens were placed in a solution of potassium hydroxide for several days to obtain the cast.

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Original Article

Whole-body heat exposure induces membrane changes in spermatozoa from the cauda epididymidis of laboratory mice

Harsha Wechalekar[1,2], Brian P. Setchell[1], Eleanor J. Peirce[1], Mario Ricci[1], Chris Leigh[1], William G. Breed[1]

[1]*Discipline of Anatomy and Pathology, School of Medical Sciences, Faculty of Health Sciences, University of Adelaide, Adelaide, South Australia 5005, Australia*

[2]*Division of Health Sciences, University of South Australia, City East Campus, Adelaide, South Australia 5000, Australia*

Abstract

This study was carried out to determine if exposure to hot environmental temperatures had a direct, detrimental effect on sperm quality. For this the effect of whole-body heat exposure on epididymal spermatozoa of laboratory mice was investigated. C57BL/6 mice (n = 7) were housed in a microclimate chamber at 37°C–38°C for 8 h per day for three consecutive days, while control mice (n = 7) were kept at 23°C–24°C. Cauda epididymal spermatozoa were obtained 16 h after the last heat treatment. The results showed that sperm numbers were similar in the two groups (P = 0.23), but after heat treatment, a significant reduction in the percentage of motile sperm was present ($P < 0.0001$). Membrane changes of the spermatozoa were investigated by staining with phycoerythrin (PE)-conjugated Annexin V, which detects exteriorization of phosphotidylserine from the inner to the outer leaflet of the sperm plasma membrane, and 7-aminoactinomycin D (7-AAD), which binds to the sperm nucleus when the plasma membrane is damaged. The percentage of spermatozoa showing positive staining with Annexin V–PE or 7-AAD or both, was significantly higher ($P < 0.05$) in heat-exposed mice compared with controls. These results show that whole-body heat exposure to 37°C–38°C induces membrane changes in the epididymal spermatozoa of mice, which may lead to apoptosis.

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Keywords: laboratory mice, sperm membrane damage, whole-body heat

1 Introduction

In most mammals, testes occur in a scrotum, which has a temperature that is several degrees below that of core body temperature. This lower temperature is brought about by a counter current heat exchange between descending blood in the testicular artery and ascending venous blood in the pampiniform plexus, with the somewhat lower temperature being essential for male germ cell maturation [1]. The epididymidis, similar to the testis, is also maintained at a lower temperature than that of core body temperature, and it has been suggested that cooling the epididymidis is the prime mover in the evolution of the scrotum [2]. Numerous studies have shown that local heating of the scrotum results in the disruption of spermatogenesis [3, 4]. Scrotal heat temperature of 42°C for 30 min also resulted in reduction of epididymal sperm number, viability and motility [5], and an increase in scrotal temperature by 2°C for 16 h a day resulted in fewer embryos in inseminated ewes as early as 4 days of heat treatment [6]. The possible effects of whole-body heating on spermatozoa are less clear. There is some

Correspondence to: Dr William G. Breed, Discipline of Anatomy and Pathology, School of Medical Sciences, Faculty of Health Sciences, University of Adelaide, Adelaide, South Australia 5005, Australia.
Fax: +61-8-8303-3788 E-mail: bill.breed@adelaide.edu.au
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suggestive evidence that paternal heat stress of 36°C for 24 h a day may result in abnormalities in early embryo development [7, 8], but these studies did not show any observable changes to the spermatozoa that could have produced such defects.

In ejaculated spermatozoa, studies have shown that temperature changes during freezing and thawing may result in sperm plasma membrane damage [9, 10], whereas heating of spermatozoa to 58°C for 30 min denatures sperm proteins, with an exposure to 100°C resulting in sperm DNA fragmentation [11]. Sperm plasmalemma defects, mitochondrial damage and/or DNA fragmentation are all characteristic features of apoptosis [12, 13], but whether whole-body heat exposure produces such adverse effects on the sperm population residing in the epididymis is not known.

The sperm plasmalemma contains phosphatidylserine (PS) and phosphatidylethanolamine in the inner leaflet, whereas sphingomyelin and phosphatidylcholine are present on the outer leaflet [14]. Translocation of PS from the inner to the outer leaflet takes place in early apoptosis [15] as well as during sperm capacitation [16] and after incubation with bicarbonate [17]. Damaged membranes owing to temperature effects have been shown to bring about impaired sperm motility [18], but whether *in vivo* heat exposure induces changes in the membrane phospholipid organization that results in subsequent reduced sperm viability appears to be unknown.

The hypothesis that we tested in the current study is that whole-body heating results in changes in membrane phospholipid organization of cauda epididymal spermatozoa. For this study, we exposed mice to a temperature of 37°C–38°C for 8 h per day for 3 consecutive days. This temperature was selected because body temperature of laboratory mice varies between 35°C and 36°C, and to simulate naturally occurring hot environmental conditions, we selected a temperature of 1°C–2°C higher than that of the core body temperature of mice. We chose to expose mice to these high temperatures for 8-h time periods to simulate the approximate period of time the mice would be exposed to high temperature during the day. There are also reports that exposure of mice to 36°C for a 24-h period increases the body temperature by ~1°C–2°C within 8 h, with an increase in testicular temperature by 3°C [7]. Thus in the present study, to ascertain whether short-term increase in body temperature can result in sperm membrane damage, we exposed mice to an elevated temperature of 37°C–38°C for 8 h for 3 consecutive days.

2 Materials and methods

2.1 Animals

For this study, 2- to 3-month-old, C57BL/6 male mice (n = 14) were used. Experimental animals (n = 7) were placed in a microclimate chamber maintained at 37°C–38°C for 8 h per day for 3 consecutive days, while controls (n = 7) were left at 23°C–24°C. Animals had access to food and water at all time periods, and the procedures were approved by the Animal Ethics Committee of the University of Adelaide, Australia (M/39/07).

2.2 Collection of spermatozoa

Mice were killed by an intraperitoneal injection of pentobarbitone, 16 h after the end of the last heat exposure. One cauda epididymidis was removed from each animal from control and heat-treated groups, then blotted on filter paper and placed in equal volume of 500 μL prewarmed modified Biggers, Whitten and Whittingham (BWW) medium [19]. Spermatozoa were extruded by retrograde perfusion of BWW into the vas deferens after making a small incision in the cauda epididymidis [20]. They were allowed to disperse into the medium in an incubator, maintained at 37°C with 5% CO_2 in air, for 10 min.

2.3 Assessment of sperm count and motility

Number of sperm extruded from the cauda epididymidis was determined using a Neubauer hemocytometer by the formula:

Sperm number = (dilution factor) × (sperm count in five squares) × 0.05 × 10^6

All spermatozoa with noticeable tail movements were recorded as motile and the percentage of motile spermatozoa was calculated as

Number of motile spermatozoa in 25 squares × 100/total number of spermatozoa in 25 squares.

2.4 Annexin V-phycoerythrin (PE) and 7-aminoactinomycin D (7-AAD) staining

Dual fluorescent staining of spermatozoa with Annexin V–phycoerythrin (PE) and 7-AAD (ApoScreen Annexin V Apoptosis Kit-PE, Beckman Coulter, Fullerton, CA, USA) for determination of changes in cell membrane PS was carried out as follows: spermatozoa were diluted in warm modified BWW medium to a concentration of ~10 × 10^6 sperm per mL. A volume of 50 μL of diluted sperm suspension was stained with

10 μL of Annexin V–PE and/or 7-AAD. The samples were incubated in darkness at room temperature (RT) for 15 min and then 500 μL of modified BWW was added before carrying out flow cytometry. Annexin V-positive controls were prepared by treating spermatozoa with dimethyl sulfoxide (Me_2SO, 1:1 v/v) for 10 min at RT. The 7-AAD-positive controls were prepared by mixing sperm suspensions with 100% ethanol (1:1 v/v) for 10 min on ice to induce DNA and membrane damage. Both Me_2SO- and ethanol-treated sperm were washed twice and resuspended in 50 μL of BWW. Unstained cells were used as negative controls.

2.5 Flow cytometry

Dual color flow cytometry was carried out with an EPICS-PROFILE II XL (Coulter, Miami, FL, USA), and data were analyzed using CXP software (version 2.2; Applied Cytometry System, Sheffield, UK). For each sample from control (Figure1F) and heat treated (Figure 1G) mice, a minimum of 10 000 spermatozoa were assessed. Gates were set for forward and side scatter with unstained sperm, to exclude debris from the analysis. The intensity of staining with Annexin V–PE and 7-AAD was determined for spermatozoa in gate 'A', control (Figure 1A), and heat-treated (Figure 1B) mice. Membrane changes were assessed by staining with Annexin V–PE and 7-AAD. The fluorochromes were excited with an argon ion laser emission at 488 nm and fluorescent detection was carried out with FL2 (576/26 nm) and FL4 (695/40 nm) filters, respectively. Unstained spermatozoa were first run to set negative controls (Figure 1C), following which positive controls, treated with Me_2SO and stained with Annexin V–PE, were analyzed to set the gates for Annexin V–PE staining (Figure 1D). To avoid Annexin V–PE spill over into FL4, compensation was set. Subsequently, positive controls treated with 100% ethanol and stained with 7-AAD were analyzed to set gates for 7-AAD staining (Figure 1E). Thus, four different subpopulations of spermatozoa were defined in control (Figure 1F) and heat treated mice (Figure 1G), on the basis of the classification by Pena *et al.* [21]. The D3 quadrant (Annexin $V^-/7\text{-}AAD^-$) contained live spermatozoa that did not show fluorescence, whereas the D4 quadrant contained spermatozoa stained with Annexin V–PE but not with 7-AAD (Annexin $V^+/7\text{-}AAD^-$), representing early apoptotic spermatozoa in which the DNA did not stain but exteriorization of the PS on the plasma

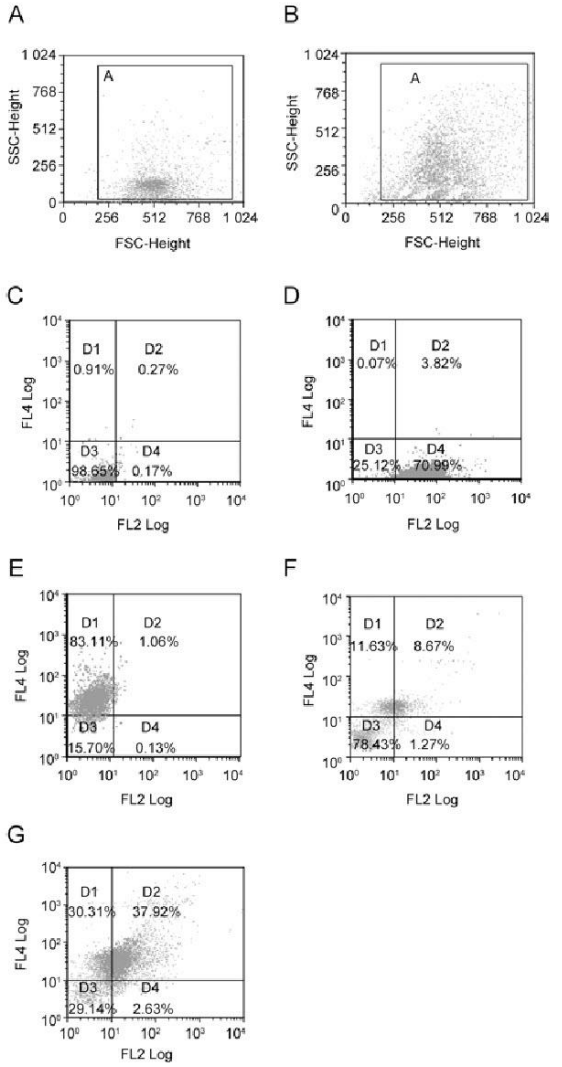

Figure 1. Representative forward (FSC) and side scatter (SSC) dot plots of unstained spermatozoa from control (A) and heat-treated (B) mice. Gate 'A' in dot plots was selected to exclude debris before flow cytometric analysis. In control (A), spermatozoa were mostly concentrated in the lower region of gate 'A', whereas in heat-treated group (B), an upward shift is apparent. Panels C–E show the quadrant limits set with unstained (C), Annexin V–PE-positive (D) and 7-AAD-positive (E) controls. Panels F and G show four different subpopulations of stained spermatozoa in four different quadrants (D1–D4) in control (F) and heat-treated (G) groups. D1, Annexin $V^-/7\text{-}AAD^+$ (dead spermatozoa); D2, Annexin $V^+/7\text{-}AAD^+$ (late apoptotic spermatozoa); D3, Annexin $V^-/7\text{-}AAD^-$ (live spermatozoa); and D4, Annexin $V^+/7\text{-}AAD^-$ (early apoptotic spermatozoa). The percentages shown in the quadrants represent values from a single control and heat-treated mouse.

membrane had taken place. Spermatozoa in the D2 quadrant (Annexin V^+/7-AAD^+) had both exteriorized PS and DNA staining, which may reflect late stages of apoptosis. Those in D1 quadrant (Annexin V^-/7-AAD^+) had DNA staining with no detectable PS exteriorization and were assumed to be dead.

2.6 Mean intensity of fluorescence (MIF) of Annexin V–PE and 7-AAD staining

A shift in the MIF of spermatozoa stained with Annexin V–PE and 7-AAD from heat-treated (n = 7) and control (n = 7) groups was also evaluated with the aid of overlay plots in CXP software (version 2.2; Applied Cytometry System). Unstained spermatozoa were analyzed to set peaks for negative controls (black peak, Figure 2), following which the shift in MIF of Annexin V–PE-positive spermatozoa from control (red line, Figure 2A) and heat-treated (blue peak, Figure 2A) groups was evaluated. Similar shifts in MIF of 7-AAD-stained spermatozoa from control (green line, Figure 2B) and heat-treated (red peak, Figure 2B) groups were also analyzed. The values of MIF were obtained from the CXP software.

2.7 Fluorescence microscopy

The sperm suspensions stained with Annexin V–PE and 7-AAD from control and heat-treated groups were used for fluorescence microscopy. For this, sperm were washed twice with phosphate-buffered saline, smeared on slides and examined by fluorescent microscopy with images captured at × 100 magnification using Olympus BX 51 microscope coupled with a CCD camera (Olympus, Tokyo, Japan).

2.8 Ultrastructure of Annexin V–PE and 7-AAD-stained spermatozoa

Spermatozoa from control and heat-treated groups were separated by a cell sorter (BDFACS Aria, San Jose, CA, USA) into two subpopulations, Annexin $V–PE^+$/7-AAD^+ and Annexin $V–PE^-$/7-AAD^-. Sperm pellets (~4 000 spermatozoa) from each subpopulation from heat-treated and control animals were fixed in 4% paraformaldehyde and 3% glutaraldehyde, made up in 0.1 mol L^{-1} phosphate buffer (pH 7.4) and processed for transmission electron microscopy (TEM) for morphological studies.

2.9 Statistical analysis

Data from control and heat-treated groups were compared by unpaired t-test using Graph Pad Prism software (version 5.01; Aberystwyth, Wales, UK) and presented as mean ± SEM, with level of statistical significance set at $P < 0.05$.

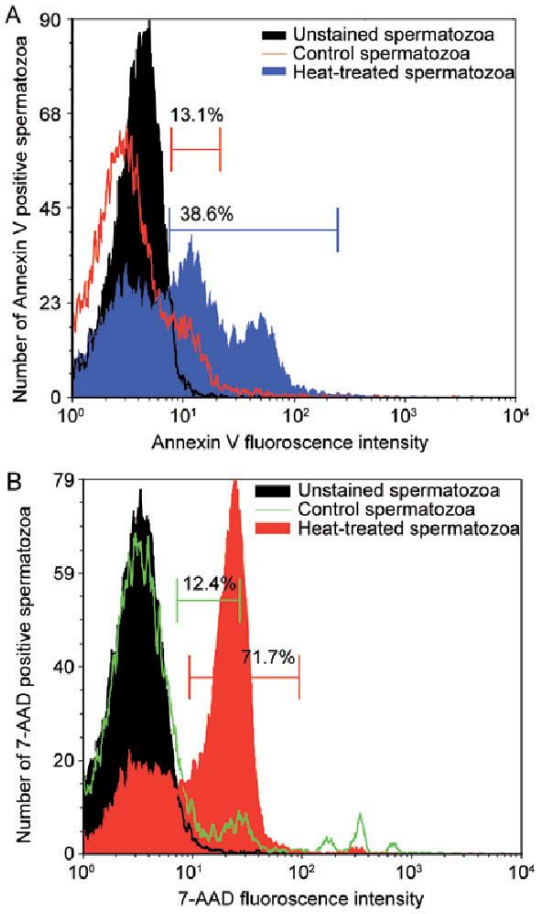

Figure 2. Representative overlay plots showing changes in mean intensity of fluorescence (MIF) in control and heat-treated groups after staining with Annexin V–PE and 7-AAD. In panels A and B, negative controls are shown in black. Panel A shows a positive shift in the MIF of Annexin V–PE staining in heat-treated (blue) compared with control (red line) mice. Similarly, panel B shows a positive shift in the MIF of 7-AAD staining in heat-treated (red) compared with control (green line) mice. The percentages shown in the overlay plots represent values from a single animal from control and heat-treated groups.

3 Results

3.1 Sperm numbers and motility

There were no significant differences in numbers

of caudal epididymal sperm between control and experimental animals (mean ± SEM, $29.0 \times 10^6 \pm 4.3 \times 10^6$ *vs.* $24.8 \times 10^6 \pm 3.1 \times 10^6$; $P = 0.23$). The percentage of motile spermatozoa, however, was significantly reduced in heat-treated compared with the control group (mean ± SEM, 85.0% ± 2.6% *vs.* 42.5% ± 1.5%; $P < 0.0001$).

3.2 Subpopulations of spermatozoa

Flow cytometry showed that exposure to heat significantly increased the percentage of spermatozoa stained with either Annexin V–PE ($P = 0.015$) or 7-AAD alone ($P < 0.001$) or both Annexin V–PE and 7-AAD ($P < 0.001$) (D4, D1 and D2, respectively, Figure 3).

A noticeable positive shift in the MIF of spermatozoa stained with Annexin V–PE and 7-AAD from heat-treated compared with control groups was estimated with the overlay plots. In Annexin V–PE-stained spermatozoa from heat-treated groups, the MIF was significantly higher compared with control mice (mean ± SEM, 1.50% ± 0.15% *vs.* 0.57% ± 0.07%; $P = 0.0001$). Similarly, in 7-AAD-stained spermatozoa from heat-treated group the MIF was significantly higher in heat-treated compared with control groups (mean ± SEM, 2.12% ± 0.11% *vs.* 0.86% ± 0.20%; $P = 0.0002$).

The percentage of spermatozoa showing staining with Annexin V–PE was significantly higher in heat-exposed mice compared with controls (mean ± SEM, 37.7% ± 4.3% *vs.* 15.4% ± 1.2%; $P = 0.002$). In addition, the percentage of 7-AAD-positive spermatozoa was significantly higher in heat-exposed mice compared with controls (mean ± SEM, 70.5% ± 2.4% *vs.* 14.8% ± 1.5%; $P = 0.0001$).

3.3 Structure of the Annexin V–PE and 7-AAD-stained spermatozoa

Spermatozoa stained with Annexin V–PE and 7-AAD did not appear to differ in their ultrastructural morphology, as observed by TEM, compared with the unstained controls. The sperm nuclei invariably had condensed chromatin; they also had an intact acrosome, subacrosomal space, plasma membrane and sperm tail. Flourescence microscopy of stained sperm from control and heat-treated groups showed speckled staining of PS by Annexin V–PE on the plasma membrane of head and tail regions, whereas 7-AAD stained the sperm nuclei (Figure 4A) indicating a dead population of

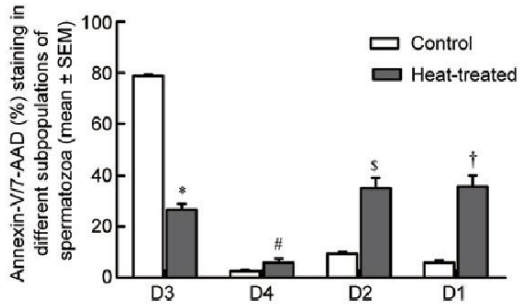

Figure 3. Histograms showing percentage of Annexin V–PE and 7-AAD-stained cells in different subpopulations of spermatozoa in control ($n = 7$) and heat-treated ($n = 7$) mice. There were significantly reduced percentages of live spermatozoa (D3) in heat-treated mice compared with controls (mean ± SEM, 26.6% ± 2.2% *vs.* 78.8% ± 0.6%; $^*P < 0.0001$) and a significant increase in percentage of Annexin V–PE-positive (5.8% ± 1.6% *vs.* 2.6% ± 0.4%; $^\#P = 0.015$; D4), Annexin V–PE and 7-AAD-positive (35.0% ± 4.1% *vs.* 9.4% ± 0.8%; $^\$P < 0.001$; D2) and Annexin V–PE negative and 7-AAD-positive spermatozoa (35.7% ± 4.4% *vs.* 5.9% ± 0.9%; $^\dagger P < 0.001$; D1).

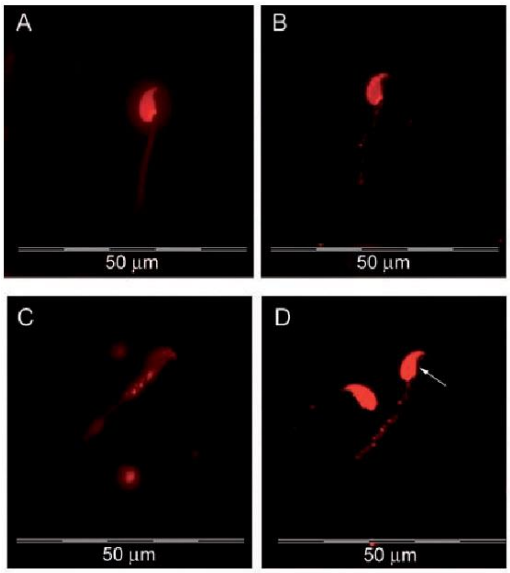

Figure 4. Fluorescent images of laboratory mouse spermatozoa stained with Annexin V–PE and 7-AAD. (A): Nucleus stained with 7-AAD represents a dead spermatozoon; (B): speckled staining of phosphatidylserine on the plasma membrane of sperm head and tail (C) in an early apoptotic spermatozoon; (D): staining with both Annexin V–PE (speckled staining in tail region) and 7-AAD (→ stained sperm nucleus) represents a late apoptotic spermatozoon.

spermatozoa. Speckled staining of PS on the plasma membrane of sperm head and tail suggests early apoptosis (Figures 4B and C), whereas sperm stained with both Annexin V–PE and 7-AAD were probably in late apoptosis (Figure 4D). In the figure, Annexin V–PE staining is only evident on the sperm tail. Staining of PS on the head region is not clear because of similar color emissions from Annexin V–PE and 7-AAD.

4 Discussion

The results of this study indicate that, when male mice are exposed to 37°C–38°C for 8 h per day for 3 consecutive days and examined 16 h after the last heat exposure, there is impaired sperm motility and an increased number of spermatozoa with plasma membrane changes within the cauda epididymidis.

As it takes, in laboratory mice, about 7 days for the sperm to migrate from caput to cauda epididymidis [22], the population of spermatozoa affected by heat exposure would have been already present within either the corpus or cauda epididymidis at the time of heat exposure, as the first exposure to heat took place only 3 days before the retrieval of sperm from the cauda.

In an earlier study, 3 days after exposure to 36°C for two 12-h periods, no changes in sperm motility was found to occur, whereas changes were observed 14 days after heat exposure [23]. This suggested that testicular germ cells are likely to have been the source of deleterious effects of heat on sperm. In contrast, in the present study, sperm with impaired motility and apoptotic-like changes were found 96 h after the start and 16 h after end of the heat treatment, suggesting direct affects of heating on the extratesticular sperm population. This decrease in motility could have been due to either the high temperature having a direct effect on the spermatozoa and/or an adverse effect of altered epididymal environment in which the sperm reside. This could have come about by either an influx of free radicals producing cellular damage [24] and/or the sperm mitochondria producing reactive oxygen species (ROS), with an increase in ROS reducing the percentage of motile sperm [25]. It is also possible that deep body temperature could have altered the ionic and protein composition of caudal epididymal fluid as a result of changes in the secretory and absorptive behavior of the surrounding epithelium [26, 27]. Future studies are required to tease out these various options.

Membrane changes, as evident from PS translocation from inner to outer leaflet of plasma membrane, DNA fragmentation [28], defects in mitochondria [29] and activation of caspases [30], are all characteristic features of apoptosis. In the present study, in the sperm of heat-treated mice there was an increase in Annexin V–PE-positive spermatozoa (Annexin V^+/7-AAD^-), suggestive of a temperature-dependent exteriorization of the PS. These findings are similar to those of several other studies in which temperature effects on PS translocation in the sperm plasma membrane have been found to occur [31, 32], but the previous studies were mostly related to sperm plasma membrane damage caused during freezing and thawing.

The population of spermatozoa stained with Annexin V^+/7-AAD^- may either be in early apoptosis [15, 33] or have undergone premature capacitation and/or the acrosome reaction [16]. Heat-induced premature capacitation has been found to occur in cryptepididymal spermatozoa [34], but, in the current study, the possibility of capacitation and/or acrosomal reaction bringing about these changes is unlikely, as fluorescence microscopy showed the presence of PS exteriorization over both the sperm head and tail plasma membrane and not just over the apical head plasma membrane as occurs during capacitation [17, 35]. The exteriorization of PS in this sperm population is thus highly suggestive of early apoptotic membrane damage due to heating.

A previous study has shown DNA fragmentation in apoptotic mouse spermatozoa after local heat treatment to the scrotum [5]. In addition, apoptotic nonviable human sperm subpopulations have been shown to have DNA fragmentation by TUNEL assay [36]. However, positivity with TUNEL does not differentiate apoptosis from other forms of cellular damage such as necrosis and autolytic cell death [37]. It has previously been suggested that ultrastructural studies need to be performed for documentation of apoptosis [38], although TEM studies have shown that TUNEL-positive spermatozoa may not have DNA fragmentation [39]. Similarly, in the current study, we found that the mouse spermatozoa stained positively with Annexin V–PE and 7-AAD had normal nuclear morphology under TEM. This supports the view that sperm DNA fragmentation, determined by apoptotic markers, may not be reflected in chromatin ultrastructure. Clearly, further study needs to be performed to conclusively show that the results are indicative of apoptosis.

PUBLICATION 2

Wechalekar, H., Setchell, B.P., Peirce, E.J., Leigh, C. and Breed, W.G. (2011). Are male germ cells of the arid-zone hopping mice (*Notomys alexis*) sensitive to high environmental temperatures?
Australian Journal of Zoology, v. 59 (4), pp. 249-256

PUBLICATION 3

Wechalekar, H., Setchell, B.P., Pilkington, K.R., Leigh, C., Breed, W.G. & Peirce, E. (2014). Effects of whole-body heat on male germ cell development and sperm motility in the laboratory mouse.
Reproduction, Fertility and Development, v. 28 (5), pp. 545-555

CHAPTER 7 Effects of heat stress on the adrenal glands of hopping mice and laboratory mice.

(IN PAPER FORMAT)

Effects of heat stress on the adrenal glands of hopping mice and laboratory mice.

H.Wechalekar, B.P.Setchell , E.Peirce and W.G.Breed

Abstract

The present study compares the normal morphological and histological differences in the adrenal glands of laboratory mice and arid-adapted hopping mice and the changes, if any, that occur after exposure to whole-body heat of 37-38ºC for 8h for three consecutive days. Adrenal glands are known to respond to stressful stimuli. Hence results from adrenal glands were used to confirm that the temperature regimens selected in our previous studies induced stress in these two species that may have subsequently resulted in germ cell apoptosis.

At ambient temperature (23-24ºC), adrenal glands of hopping mice are much smaller than those of laboratory mice; however the ratio of thickness of the medulla to the cortex was greater in hopping mice. Histologically, adrenal glands from both species showed two distinct layers, the cortex and medulla with the cortex consisting of three indistinct zones, the zona glomerulosa (ZG), zona fasiculata (ZF) and zona reticularis (ZR).

Heat stress produced vacuolization of the cells in the zona fasciculata and reticularis in both the species with formation of distinct, large, syncytial-like structures at the corticomedullary interface of the hopping mice adrenal glands at 16h post heat exposure. Dilated sinusoidal spaces were also present within the adrenocortical layers in both species. An increase in interstitial fibrosis was evident with fibrosis limited to the zona fasiculata and reticularis of the adrenal cortex.

In conclusion, heat stress induced vacuolization of the adrenocortical cells with dilatation of the sinusoidal spaces and interstitial fibrosis in the adrenal glands across both the species.

Introduction

Previous studies on effects of heat on developing germ cells and spermatozoa of laboratory and hopping mice have shown that exposure to a temperature of 37-38ºC for 8 hrs for 1 and

/or 3 days resulted in germ cell apoptosis and membrane defects in spermatozoa (Wechalekar et al., 2010, Wechalekar et al., 2014). Furthermore, in the hopping mouse an absence of a pamipiniform plexus of veins was evident (Wechalekar et al., 2011), which in most eutherian mammals is proposed to be essential for thermoregulation of the scrotum and for normal spermatogenesis to occur. These findings indicate that heat has a detrimental effect on developing germ cells and spermatozoa in these species. Heat stress is also known to change other bodily functions to maintain body homeostasis (Michel et al., 2007). Heat stress is associated with activation of the hypothalamic-pituitary axis followed by an increase in blood cortisol levels by activation of adrenocortical cells (Judelson et al., 2008). Hence the current study was undertaken to determine changes, if any, in the adrenal glands, especially the adrenocortical cells of laboratory and hopping mouse following whole body heat exposure. Arid-adapted hopping mice were included in this study to determine any difference in response of adrenocortical cells to whole body heat exposure because of this species adaptation to extreme environmental conditions.

Adrenal glands are the paired glands situated close to the upper poles of the kidneys. They are composed of two distinct regions, an outer cortex and inner medulla. The mouse adrenal cortex is composed of three indistinct cellular layers the zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) however, thickness of these cortical layers varies in different strains of mice especially the ZR zone (Tanaka et al., 1995). Another distinct layer called the 'X zone' occurs between the ZF/ZR and medulla, the cells of this layer are arranged in a less ordered manner (Howard- Miller, 1927). The 'X' zone in the mouse is evident 10-14 days after birth with enlargement of cells evident in this zone until 3 weeks with regression in puberty as a result of apoptosis (Zubair et al., 2008). The function of the 'X' zone is not completely determined but it is proposed to have steroidogenic potential (Zubair et al., 2006) . This region is vacuolated in some rodent species and non- vacuolated in others with a complete absence of this zone in male C5 BL6 mice at puberty (Tanaka and Matsuzawa, 1995). In laboratory mice, the zona glomerulosa (ZG) contains basophilic cells arranged in

clusters; the zona fasciculata (ZF), has parallel and radial columns of eosinophilic cells filled with lipid droplets and separated by sinusoids, and the zona reticularis (Zribi et al., 2012) has an irregular network of compact cells with lipid droplets (Cater and Lever, 1954, Vinson, 2003). Functionally, the ZG zone secretes mineralocorticoids especially aldosterone into the circulation and its secretion is controlled by the renin-angiotensin system (Bassett et al., 2004), the ZF layer in rodents secretes corticosterone (Nussdorfer, 1986) and ZR cells secrete androgens. The inner medulla secretes catecholamines, adrenalin and norepinephrins (Ehrhart-Bornstein et al., 1998).

All hormones in the adrenal gland are synthesized from a common precursor, cholesterol. Heat stress results in an increase in circulating ACTH released by the pituitary following activation of the HPA axis. ACTH stimulates transfer of cholesterol into the inner mitochondrial compartment of adrenocortical cell layers. Cholesterol in ZF cells is converted by various isoezymes into progesterone and deoxycortisol (Lavoie and King, 2009). In the final stages deoxycortisol is converted into corticosterone in the mouse by the action of 11 β-hydroxylase (Williams, 2005)

The normal morphology and cytoarchitecture of the adrenal glands of the laboratory mouse is evident in the literature (Tanaka et al., 1995) however morphology of adrenals of arid-adapted hopping mice has not yet been investigated. Hence in the current study adrenal glands of hopping mice were examined and compared with those of the laboratory mouse to compare the cytoarchitecture. In addition, since hopping mice are an arid-adapted species the study also aimed to determine the differences, if any, in responses of the adrenal gland between the two species following heat treatment.

Material and methods

Animals

Adrenal glands of C57BL6 male laboratory mice (n=5) and hopping mice (n=5) were obtained from the animals that were used to investigate the effects of heat on germ cells. The

experimental animals were placed in a microclimate chamber maintained at a temperature of 37-38ºC for 8h/day for three consecutive days, while controls (n=5) were left at 23-24ºC.

Collection of sample

16 h after the last heat treatment animals were perfused through the heart with 4% paraformaldehyde and 3% glutaraldehyde made up in 0.1 M phosphate buffer, pH-7.4. The adrenal glands were removed and embedded in paraffin wax and 7µm thick sections were cut for light microscopy.

Staining

Adrenal gland sections were stained with haematoxylin and eosin to determine the effects of heat stress on the cytoarchitecture of the adrenal gland and with Masson trichrome stain to see the effects of heat on the adrenal gland interstitial tissue. Masson trichrome imparts green colour to collagen fibers.

Results

On gross inspection, the control group adrenal glands of laboratory mice appeared to be much larger than those of the hopping mice from the control group (Fig 1A). A transverse section through the middle of the gland showed a larger medulla: cortex ratio in the hopping mouse compared with laboratory mice (Fig 1B &C). H&E stained sections showed a cortex and medulla with three cortical layers namely zona glomerulosa, zona fasciculata and the zona reticularis in both laboratory and hopping mice (Fig. 2a &b). Large vacuoles were evident in the adrenocortical cells of the zona fasciculata and the zona reticularis (Fig. 3a) in the heat-treated groups of both laboratory mice and hopping mice. In addition large syncytial structures were seen at the corticomedullary junction of heat-treated hopping mice (Fig. 3b) without any evidence of such structures in the adrenal glands of the laboratory mice. Staining with Masson's trichrome showed interstitial fibrosis in cortical areas of the adrenal glands of the heat stressed animals sparing the medullary zone. Interstitial fibrosis was more evident in the zona reticularis layer of the cortex in both the animal groups (Fig. 4b,d).

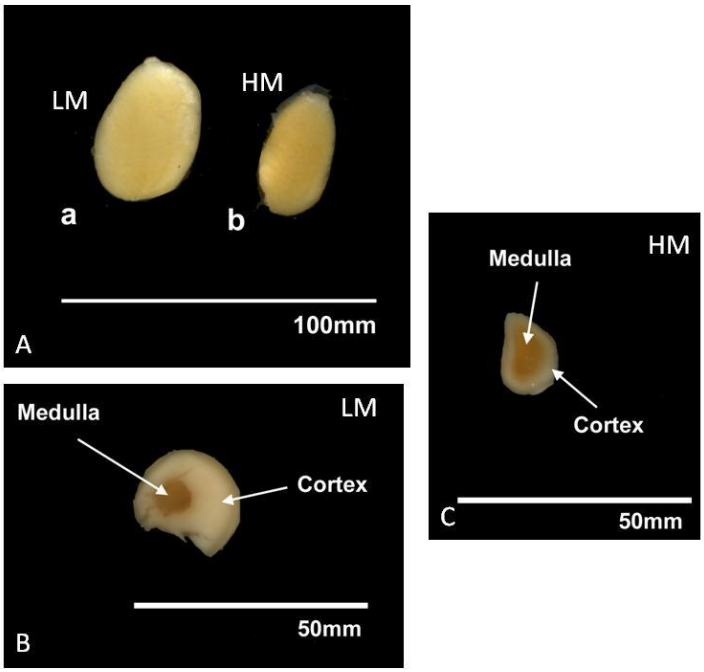

Fig 1. Adrenal glands from laboratory mouse (LM) and hopping mouse (HM) of control group (A) under a dissecting microscope. Transverse section through the adrenal gland of laboratory mouse (B) and hopping mouse (C) shows that the adrenal medulla:cortex ration is much larger in HM compared to LM.

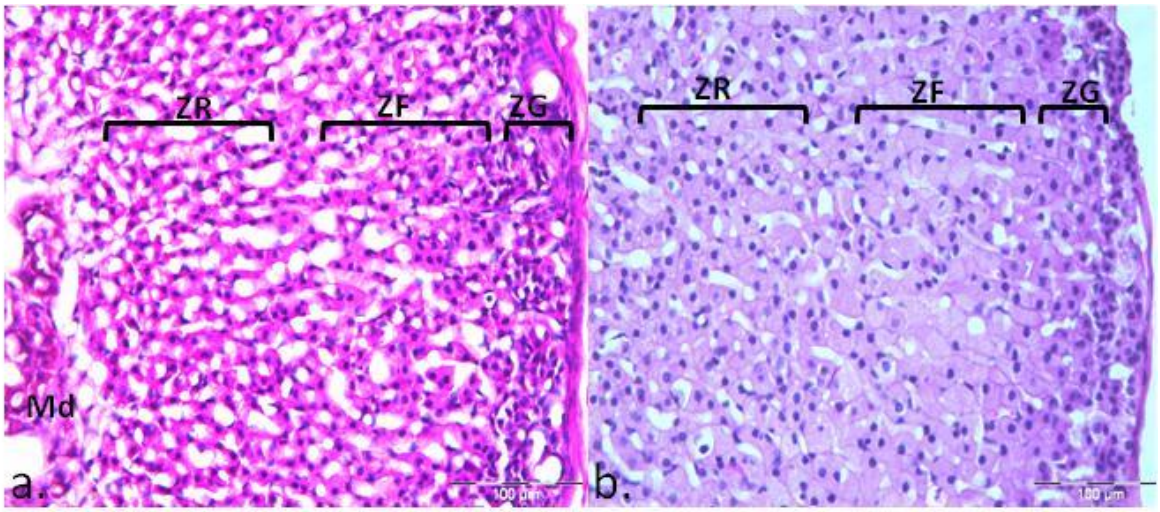

Fig 2. Haematoxylin and eosin stained adrenal gland sections from control laboratory mouse (a) and control hopping mouse (b) showing the layers of the adrenal gland. ZG (zona Glomerulosa), ZF (Zona Fasiculata), ZR (Zona Reticularis) and adrenal medulla. (Scale bar-100µm)

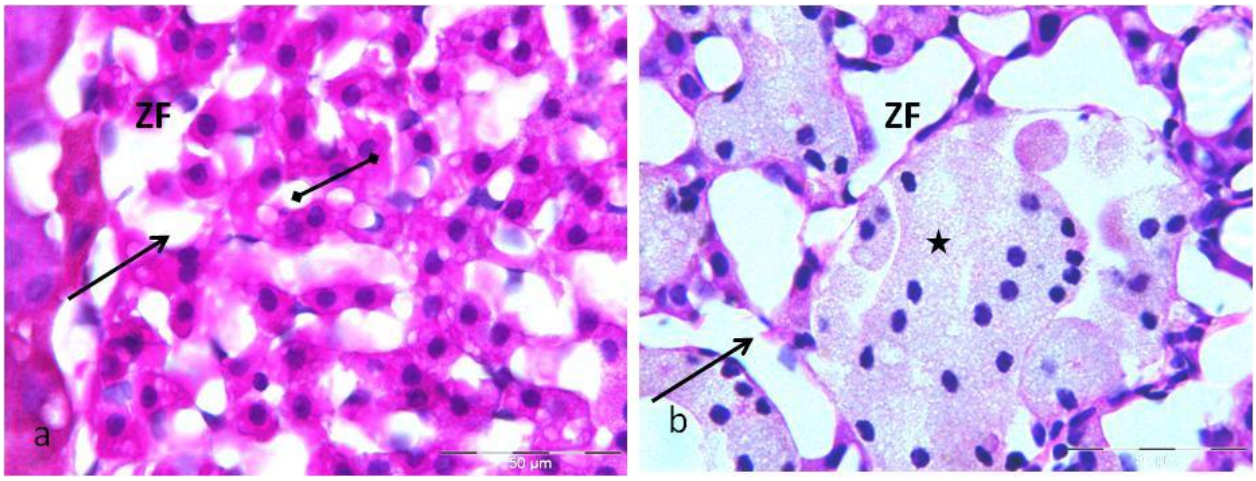

Fig. 3. H&E stained adrenal glands from heat-treated laboratory mouse (a) showing vacuoles (⟷) within the adrenocortical cells, and large syncytial structures (★) at the adrenomedullary junction of hopping mouse (b) adrenals. Dilated capillaries (→) were present in cortical layers of both the laboratory mouse (a) and hopping mouse (b). (Scale bar- 50µm)

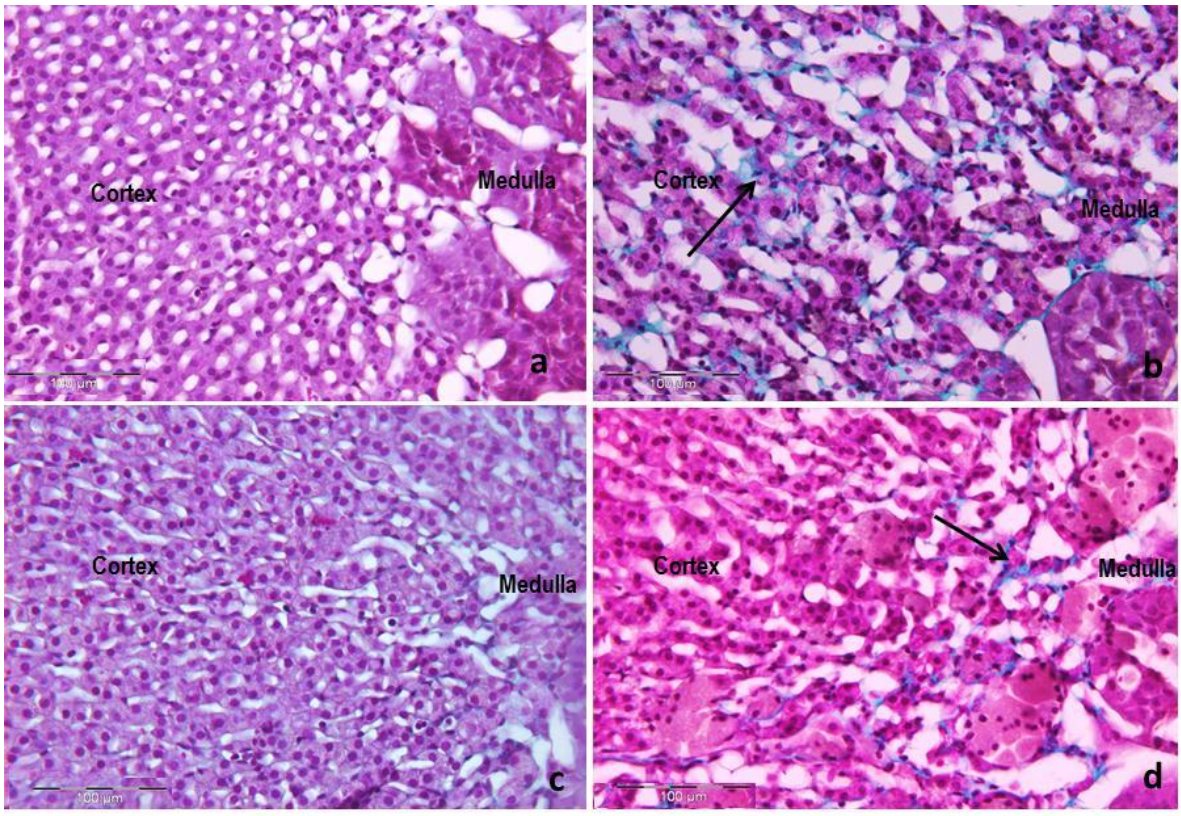

Fig.4. Masson's trichrome stained adrenal gland sections from the control laboratory mouse (a) and hopping mouse (c). Heat-treated groups showed increased interstitial fibrosis (→) between the zona reticularis of the laboratory mouse (b) and the hopping mouse (d).

Discussion

Heat stress of 37-38ºC for 8h/day for three consecutive days produced changes in the adrenal glands of laboratory and hopping mice. In both the groups whole body heat stress enlarges the adrenocortical cells, and causes dilatation of the sinusoids and interstitial fibrosis within the cortical layers of the adrenal gland.

The adrenal medulla, which is a part of the sympathetic nervous system, secretes adrenaline and noradrenaline in response to heat stress (Edens and Siegel, 1975, Kemppainen and Behrend, 1997). Morphologically, the smaller size of the adrenal cortex and the larger medullary region in hopping mice adrenals compared to those of the laboratory mice in the control groups may result in more secretion of stress hormones from the medullary region as an adaptation to extreme environmental conditions. However, histologically there were no differences in the cortical layers of the adrenal gland of hopping mice compared with those of the laboratory mice. This study reports the normal morphology and histology of the adrenal glands of hopping mouse and also the changes in adrenal gland cytoarchitecture post heat exposure.

Stress is known to increase the levels of circulating ACTH in response to activation of the hypothalamic-pituitary-adrenal axis (HPA). This however depends upon the type and duration of the stressful stimulus, which also influences the response of the adrenal glands to stress (Mazzocchi et al., 1986, Robba et al., 1985). Increased levels of circulating ACTH have been shown to induce adrenocortical cell hypertrophy with an increase in steroid hormone synthesis (Nussdorfer and Mazzocchi, 1983). More specifically, stress induces hyperplasia in the outer layers of the ZF zone and hypertrophy of the inner layers of ZF with an associated hypertrophy of the adrenal medulla (Ulrich-Lai et al., 2006). Not only the cells of ZF but also ZG cells undergo similar changes to those of the ZF zone in response to stress (Mazzocchi et al., 1986). An associated depletion of lipid droplets has also been observed in the ZF region (Koko et al., 2004) with hyperplasia of the ZR cells. Swelling in the adrenocortical cells with an associated hyperaemia has also been reported in male rats exposed to a heat stress of 39ºC

for 30 min (Mete et al., 2012). Furthermore, a recent study reports stress induced apoptosis and vacuolization within the adrenal cortical layers (Burkhardt et al., 2011). Heat stress has also been shown to induce interstitial capillary dilatations within the ZG and ZF cortical layers (Pugachev, 1977, Pugachev, 1983) with extravasations of blood in the ZR and the medullary region of the adrenal gland of the rat (Vlad et al., 2010). Thus in the current study the changes seen within the ZF and ZR zones of the adrenal glands could be attributed to the elevated levels of circulating ACTH as result of heat stress, however; measurements of circulating levels of ACTH post heat stress need to be determined to confirm this.

Heat stress also induces malignant transformation of collagen- binding glucoprotein, a heat shock protein (Hsp 47) essential for collagen synthesis under normal conditions; however alterations in these proteins as a result of heat stress can cause excessive collagen deposition within the interstitium of an organ (Nagata et al., 1986). Heat stress has been shown to up regulate the expression of these heat shock proteins within the adrenal glands of rats exposed to heat of 42^{o}C for 20 min (Inaguma et al., 1995). These proteins are normally expressed in the adrenocortical layers sparing the medulla and exposure to heat has been shown to increase the expression of these proteins in the cortical layers (Shimizu et al., 1997). In the current study an increase in the collagen within the interstitium of the cortical mantle and its absence from the medullary zone points towards the possibility of heat induced activation of heat shock proteins with the subsequent deposition of collagen within the interstitium of the adrenal cortex of both the species. This is the first report to show the changes in adrenocortical layers and collagen deposits in the adrenal gland of arid adapted hopping mice after heat stress. However, quantitative analysis of the expression of heat shock proteins and collagen fibres across both species is required to determine whether there are differences in the response pattern of adrenal glands in the two species.

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CHAPTER 8

GENERAL DISCUSSION AND CONCLUSION

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSION

This project studied the effects of heat stress on developing germ cells and spermatozoa of the laboratory and hopping mice. The study was designed to extrapolate findings to humans with reduced fertility when exposed to extreme temperature conditions in their occupations. Two species of rodents were used *Mus musculus* (laboratory mouse) and *Notomys alexis* (hopping mouse). Heat stress was induced by whole body heat of 37-38^{o}C (8h for three consecutive days and/or for 1 day) as well as by cryptorchidism.

The core body temperature of laboratory mice has been shown to range between 35-36^{o}C therefore the temperature selected for the experiments was a few degrees higher than the body's core temperature. Furthermore, temperatures of 18-33^{o}C are ambient for laboratory mice (Barnett, 1956) therefore controls were maintained at 23-24^{o}C which falls within the ambient temperature range for these species. The time frame of 8h was chosen to simulate the situation to which humans are exposed during the day whilst at work.

It has been shown in the past that increasing temperatures to a few degrees above core body temperature produce a proportional increase in scrotal temperature. For instance, rats, rams and mice exposed to a temperature of 35^{o}C (Sod-Moriah et al., 1974), 40^{o}C and 42^{o}C respectively, showed an increase in scrotal temperatures close to core body temperature. *Notomys alexis* was selected for study as this species occurs in the arid zone of Australia which is hot during the day for most days of the year. The hopping mouse was included to determine whether responses to whole body heat stress differ in an arid-adapted species when compared with the laboratory mouse. Furthermore, the cycle of seminiferous epithelium of hopping mice is similar to humans with slight differences in cell associations in tubular cross-sections during the cycle of seminiferous epithelium. This allows extrapolating the results to humans who are exposed to whole body heat stress in various circumstances.

Hopping mice inhabit the sand dunes of Central and Western Australia and are well-adapted behaviourally and physiologically to survive in extreme climate conditions (MacMillen and Lee, 1969). Their adaptations include the presence of large ears to facilitate

heat dissipation, long back feet for rapid locomotion, excretion of highly concentrated urine to minimise water loss as well as confinement to their burrows during the day (MacMillen and Lee, 1969, Breed and Ford, 2007, Dickman et al., 2010). In order to determine whether adaptations to an arid environment protect developing germ cells and spermatozoa from high temperatures, animals were exposed to whole body heat and induced cryptorchidism. Little is known about reproductive adaptations of this species to extreme temperature conditions although reproduction can take place any time of the year (Breed, 1990) with fertility rates increasing after prolonged periods of rain as suggested by increase in population (Finlayson, 1941, Newsome and Corbett, 1975, Letnic et al., 2004, Dickman et al., 2010). In addition, in sexually mature hopping mice, the testes vary from scrotal to inguinal or even lower abdominal in position, which is related to their small size relative to the inguinal canal (Breed, 1981). Furthermore, in hopping mice extratesticular sperm storage occurs in the urethral segment of the vas deferens that lies in the lower abdominal region, as well as in the cauda epididymides (Breed, 1997, Peirce et al., 2003). Thus we speculated that periodic exposure of germ cells and spermatozoa to temperatures similar to the body's core might have resulted in resistance to the damaging effects of heat. However, it was surprising to find that the testes of hopping mice even though adapted to extreme climate conditions appear to be more heat sensitive compared to the laboratory mice. This could be due to the fact that the animals are crepuscular and nocturnally active on the surface and confined to their burrows during the day, which are situated up to 1m below the surface and are hence maintained at a lower temperature (Lee et al., 1984).Thus hopping mice may limit to their exposure to high environmental temperatures (above 30^{o}C) albeit that they inhabit an arid environment. Furthermore, our findings indicate that in *Notomys alexis* there is absence of a coiled testicular artery to facilitate counter-current heat exchange with blood in the testicular veins. It is known that a coiled testicular artery is essential for counter-current heat exchange with the pampiniform plexus of veins in most mammal species in order to maintain scrotal temperature below the body's core for normal spermatogenesis (Ivell, 2007). Thus it is clear

from the current study of hopping mice that exposure of germ cells to high temperatures results in degeneration of the seminiferous epithelium and germ cell apoptosis.

In laboratory mice, whole body heat induced stage-specific apoptosis with early and late stages being more vulnerable to heat stress compared to the intermediate stages. In hopping mice, whole body heat induced germ cell apoptosis although the cycle of seminiferous epithelium could not be specifically defined due to tubule cross-sections showing multiple cell associations (Peirce and Breed, 1987). In laboratory mice by day 21 post whole body heat stress seminiferous epithelium appeared similar to that of controls whereas in cryptorchid hopping mice testes little effect was seen after seven days, but by day 21 of cryptorchidism degenerative changes were evident in at least a few of the seminiferous tubules. On the contrary, in laboratory mice degenerative changes have been shown to appear as early as 24h and/or 6-7 days post cryptorchidism (Bergh and Damber, 1984, Blackshaw and Massey, 1978). The absence of marked degenerative effects on hopping mice germ cells seven days post cryptorchidism could be due to the fact that the testes are much smaller relative to their body mass and also due to the fact that testes are exposed periodically to abdominal temperatures. In the present study, both laboratory and hopping mouse germ cells were affected by high temperatures irrespective of the mode of heat treatment. However, degenerative changes in cryptorchid testes appeared much later, as well as after longer duration of exposure to body temperature, when compared with whole body heat. In both the species pachytene spermatocytes and spermatids were affected after heat stress. The mechanisms which make pachytene spermatocytes and early spermatids vulnerable to heat stress compared with other germ cells are still unknown. Recent studies have shown that high temperatures increase cytosolic Ca^{2+} ions and lower pH in pachytene spermatocytes and rounded spermatids (Herrera et al., 2001) which then alters the cell signaling pathways leading to apoptosis (Lizama et al., 2007) . Furthermore, pachytene spermatocytes may be more heat sensitive compared with round spermatids because the latter contain more antioxidant properties (Pino et al., 2013). Stage-specific apoptosis after whole body heat

stress in laboratory mice affected all stages (I-XII) 16h, 7 and 14 days after the last heat treatment with apoptosis in intermediate stages comparable to controls at 14 and 21 days post exposure. Unlike whole body heat exposure, scrotal hyperthermia induces stage-specific apoptosis in early (I-IV) and late (XII-XIV) stages without affecting the intermediate stages (VII-VIII) which are protected by gonadotropic hormones (Lue et al., 1999) and the presence of androgen receptors (Bremner et al., 1994). However, we found that whole body heat exposure induces apoptosis in all stages, which suggests some changes in circulating hormones in response to heat stress. It has been shown that whole body heat exposure causes an acute decrease in gonadotropin hormones as well as raised cortisol and testosterone levels (Li et al., 2013). Moreover, a decrease in gonadotropins (Lue et al., 1999) and an increase in cortisol and testosterone levels induces germ cell apoptosis (Shiraishi et al., 2010). Unlike whole body heat stress, scrotal hyperthermia does not elevate blood gonadotropins therefore germ cells in the intermediate stages may be protected however, gonadotropin hormone antagonist has been shown to induce germ cell death in intermediate stages as early as 5-7 days post heating (Sinha Hikim et al., 1997). In addition, use of gonadotropin antagonist and scrotal hyperthermia at the same time induces apoptosis in all the stages of the seminiferous epithelium (Lue et al., 1999). In the current study, there is no clear indication as to whether changes in the hormonal milieu after heat stress is directly responsible for germ cell apoptosis or is just another contributing factor to germ cell apoptosis. Future studies determining the hormone levels may better establish a relationship between hormonal changes, apoptosis and effects on stage specificity.

Heat stress also brings about changes in the spermatozoa of laboratory and hopping mice. The affected population in both the species appears to be extratesticular spermatozoa since it takes 7 days for the spermatozoa to migrate from caput to cauda epididymidis (Banks et al., 2005) therefore samples were withdrawn either at 16h after whole body heat and 7 days post cryptorchidism. However, the abnormal spermatozoa obtained 21 days post cryptorchidism are likely to be the ones, which developed from the heat affected germ cells. Sperm motility

was reduced as early as 16 h after whole body heat exposure for 3 and /or 1day in laboratory mice while motility changes appeared much later in hopping mice after 7 and 21 days post cryptorchidism. Thus an ambient temperature is required for maintaining sperm motility within the cauda epididymidis and ductus deferens. Reduction in sperm motility resulted either from direct effect of heat and/or alteration in the epididymal environment due to heat. Heat stress is known to generate reactive oxygen radicals (ROS) (Ikeda et al., 1999). ROS thus produced inhibit the enzymes of glycolytic pathways and/or oxidative phosphorylation depleting mitochondrial ATP and also these radicals fail to induce phosphorylation of axonemal components thereby impairing sperm motility (de Lamirande and Gagnon, 1992). In addition, ROS is known to oxidise polyunsaturated fatty acids (PUFAs) associated with the membranes of various cell organelles with plasma membranes of spermatozoa being rich in polyunsaturated fatty acids (Jones et al., 1979). Thus increased oxidative stress leads to lipid peroxidation of the sperm plasma membrane that may reduce sperm motility (Burruel et al., 2013). More specifically, ROS induces peroxidative damage of the sperm tail and motility apparatus producing motility defects (Koppers et al., 2008). Thus in the current study production of ROS would have resulted in loss of sperm motility, however further studies on quantitative analysis of ROS post heat stress needs to be undertaken to give a definitive answer.

Even if the motility is decreased, sperm numbers remained similar after whole body heat exposure although cryptorchidism did reduce the sperm numbers in the cauda and ductus in most of the animals.

Whole body heat exposure is known to reduce sperm numbers, but only after 1-6 weeks following heat stress (Moule, 1963, Skinner and Louw, 1966, Larsson and Einarsson, 1984, Malmgren and Larsson, 1984, Yaeram et al., 2006). In addition, scrotal hyperthermia also reduces sperm numbers 1-4 weeks post heat stress (Freidman et al., 1991, Kastelic et al., 1996, Karabinus et al., 1997, Jannes et al., 1998, Banks et al., 2005, Brito et al., 2003). Similarly in the present study reduced sperm counts were evident 7 and 21 days post

cryptorchidism. However, our results did not show any change in sperm counts 16h after whole body heat. This could be due to the fact that in earlier studies sperm counts were investigated 1-6 weeks post heat stress, whereas in the present study counts were carried out 16h post heat stress which could have been too early to show changes, if any, in sperm counts. Moreover, studies have shown that it takes days to weeks for the nonviable spermatozoa to undergo degradation within the cauda epididymidis (Jones, 2004) which could be another reason for finding normal sperm count shortly after heating.

Heat stress also alters the epidydimal environment by changing the ionic and protein composition of epididymal fluid (Rasweiler and Bedford, 1982, Esponda and Bedford, 1986, Bedford, 1991), by decreasing the storage capacity of epididymides (Jara et al., 2002) and also by reducing the ability of the epididymal epithelium to secrete various ions and water essential for sperm maturation (Wong et al., 1982).

The changes evident in the spermatozoa after whole body heat stress confirmed the occurrence of apoptosis. The characteristic features of sperm apoptosis are PS translocation, DNA fragmentation, defective mitochondria and caspase activation. In the present study, temperature-dependant exteriorization of PS in spermatozoa was evident which indicates early apoptosis. These observations were very similar to the findings from other studies showing temperature dependent exteriorization of PS albeit these changes were observed after freezing and thawing of spermatozoa (Woelders, 1997, Sion et al., 2004). Exteriorization of PS is also evident in spermatozoa undergoing capacitation or the acrosomal reaction (Kurz et al., 2005). In capacitation, changes are induced by endogenous production of ROS, mainly in $ONOO^-$ (peroxynitrite) which yields oxysterol essential to remove cholesterol from the plasma membrane and to regulate tyrosine phosphorylation and cAMP production vital for capacitation (Ecroyd et al., 2003, Rivlin et al., 2004, Aitken and Baker, 2013). However, in capacitation, PS exteriorization is limited to the apical region of the sperm head (Gadella and Harrison, 2002, de Vries et al., 2003). In the present study exteriorization of PS was not limited to the sperm head but also extended to the plasma membrane enveloping the sperm

tail indicating sperm apoptosis. In the present study, membrane changes in the form of PS exteriorization were evident as early as 8h post exposure to heat of 37-38^{o}C and increasing the time of exposure to 8h for three consecutive days resulted in cell death. This suggests that apoptotic changes in spermatozoa are temperature and time dependant with membrane changes appearing first followed by an irreversible damage to the spermatozoa. Sperm DNA fragmentation is yet another feature of spermatozoa undergoing apoptosis, which was not a feature in the sperm population we studied after whole body heat stress. However, sperm DNA fragmentation is known to occur after scrotal hyperthermia and is also reported in semen samples from infertile men. These studies have used TUNEL to detect DNA defects in apoptotic spermatozoa but TUNEL does not differentiate the different cell death types like necrosis and autolysis. Ultrastructural studies are required to confirm sperm apoptosis although spermatozoa showing TUNEL positivity do not always show fragmented DNA. This supports our findings of normal nuclear morphology in late apoptotic spermatozoa. It has also been suggested that compartmentalization within the spermatozoa separates the mitochondria in the middle piece from the apically situated nucleus therefore apoptotic enzymes or free radicals released from the mitochondria as a result of stress remain within the middle piece without extending to the nucleus (Koppers et al., 2011). This could result in a normal nuclear morphology.

In order to establish that whole body heat exposure produces heat stress in laboratory mice and the arid adapted hopping mice we investigated adrenal gland morphology. In addition, whether there is any difference in responses to whole body heating in arid–adapted hopping mice when compared to the laboratory mice was also investigated. Morphologically, there appeared to be a larger medullary to cortex ratio when compared with the laboratory mice, which suggests the need for these animals to secrete stress hormones to adapt to extreme environmental conditions. Histology of the glands showed similar cytoarchitecture in both the species. This study reports on the normal morphology and histology of the adrenal glands of hopping mice. Our findings showed vacuolation in the adrenocortical cells, interstitial fibrosis

and capillary dilatations in the cortical layers and abnormal synctial structures at the corticomedullary junction. The adrenal cortex is known to secrete corticosteroids in response to stress by activating the hypothalamic-pituitary-adrenal axis (HPA), however this depends on the type and duration of stressful stimulus (Mazzocchi et al., 1986, Robba et al., 1985). Raised ACTH levels induce adrenocortical cell hypertrophy in order to synthesize and release more steroids into the circulation in response to stress (Nussdorfer and Mazzocchi, 1983). Hypertrophy is limited to the inner layers of zona fasiculata (ZF) and medulla while the outer layers of ZF zone undergo hyperplasia (Ulrich-Lai et al., 2006). There is also evidence of depletion of lipid droplets from the zona fasiculata cells (Koko et al., 2004). Similar changes were seen in zona glomerulosa following heat stress (Mazzocchi et al., 1986). Furthermore, heat stress produces interstitial capillary dilatations within ZG and ZF (Pugachev, 1977, Pugachev, 1983) with extravasation of blood in the ZR and adrenal medulla (Vlad et al., 2010). Thus vacuolated cells in ZF and ZR in the current experiment appear to be due to interstitial capillary dilatations following whole body heat. Recent reports suggest 30 min exposure to whole body heat of 3^{o}C is sufficient to induce hypertrophy in adrenocortical cells (Mete et al., 2012) and elevated levels of circulating ACTH can increase the risk of apoptosis (Burkhardt et al., 2011). Furthermore, in heat-treated hopping mice testes, formations of syncytial masses were evident at the corticomedullary junction, which could be due to the need to secrete and synthesize excess stress hormones, corticosteroid, to thrive in high temperatures. Thus in the current study changes evident in the ZF and ZR of adrenal glands of hopping mice and laboratory mice appear to be due to changes in the levels of ACTH following whole body heat stress, although measurements of ACTH and corticosteroid levels after heat stress need to be defined to give us a definitive answer. The changes in the cytoarchitecture of the adrenals following heat stress in both species support observations that the regimen of temperature exposures selected for this study is sufficient to induce germ cell and sperm apoptosis following whole body heating, either via direct or indirect heat affects, possibly involving hormone mediated events. Heat stress is known to induce changes in the

collagen-binding heat shock protein, Hsp 47 thereby increasing the deposition of collagen within the interstitium (Nagata et al., 1986). In adrenal glands, heat shock proteins are normally expressed in cortical layers (Shimizu et al., 1997) with increase in the expression of these proteins in adrenal glands of animals exposed to heat stress (Inaguma et al., 1995, Shimizu et al., 1997). Hence our findings of increase in interstitial collagen in the cortical layers after whole body heat stress and cryptorchidism indicates heat stress in animals. This is the first report to show the changes in adrenocortical layers and collagen deposits in the adrenal gland of arid adapted hopping mice after heat stress. However, the quantitative analysis of expression of heat shock proteins and collagen fibres across both the species are required to be investigated in future to determine the difference in response pattern of adrenal glands in species adapted to different environmental conditions.

9.1 Future directions

Findings from this study have extended data on the proteins involved in germ cell apoptosis, stage-specificity of apoptosis and sperm membrane protein defects after whole body heat exposure however whether the changes are a result of the direct effect of heat and/or are hormone induced is not known. Hence future studies on estimation of blood GnRH and androgens need to be performed. Furthermore, determining the role, if any, of sperm mitochondria in producing sperm membrane defects may provide an explanation for sperm apoptosis following whole body heating. In addition, further experiments using a combination of TUNEL, Annexin V/and 7-AAD may provide definitive answers on sperm apoptosis. Moreover, reduction in fertility rates post exposure to whole body heat could be explained better by undertaking fertility studies i.e. fertilization rates, resultant embryo quality and pregnancy outcomes.

9.2 Implications

The results obtained from the present study could possibly be extrapolated to humans since a few individuals are exposed to extreme conditions in their occupations. Human studies have

shown that welders exposed to temperatures of 31-45ºC for 5h daily over 6 weeks in their occupation have an increase in scrotal temperature of 1.4ºC with a decrease in sperm motility and abnormal spermatozoa. In addition, conception defects and a reduction in semen quality were observed in subjects involved in this occupation (Rachootin and Olsen, 1983, Mortensen, 1988). Reduced birth rates have been found to occur in workers from the aluminium industry and ceramic factories exposed to high environmental temperatures (Figa-Talamanca et al., 1992, Mur et al., 1998). In addition, subjects working in plastic factories also showed abnormal spermatozoa in semen samples (Jelnes, 1988). Thus occupational heat exposure may have effects on semen quality and fertility. Finally, changing environmental temperatures as a result of global warming could possibly have an impact on human fertility. Therefore further insight into the mechanisms contributing to heat-induced male infertility may enable identification of ways in which to reduce its impact with increasing environmental temperatures.

Lastly, the comparative study with *Notomys alexis* showed that whole body heat and/or experimental cryptorchidism is detrimental to the developing germ cells and extratesticular sperm cells. However, fertility retention in these animals in extreme conditions is likely to be due to the fact that during the day animals live in deep burrows without being exposed to high environmental temperatures. Thus the changing environmental conditions could have a major impact on survival of this species.

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APPENDIX A

List of laboratory chemicals used for this project:

Buffers and solutions		
BWW Stock	**91.5 mM**	**NaCl**
	4.6mM	**KCl**
	1.2mM	**KH_2PO_4**
	1.2mM	**$MgSO_4.7H_2O$**
	1.7mM	**$CaCl_2.2H_2O$**
BWW (Working solution)	**25mM**	**$NaHCO_3$**
	5.6mM	**D-glucose**
	0.27mM	**Sodium Pyruvate**
	44mM	**Sodium Lactate**
	5U/ml	**Pencillin**
	5µg/ml	**Streptomycin**
	20mM	**HEPES buffer**
Eosin	**1%**	**Aqueous eosin**
	95%	**Ethanol**
	1%	**Aqueous phloxine**
	2ml	**Glacial acetic acid**
Lille Mayer's Haemotoxylin	**5gm**	**Haemotoxylin**
	50gm	**Aluminium ammonium sulphate**
	300ml	**glycerol**
	700ml	**RO water**
	1gm	**Sodium iodate**
	20ml	**Glacial acetic acid**

Weigert's iron hematoxylin (Stock solution)		
Stock A	**1gm**	**Hematoxylin**
	95%	**Alcohol**
Stock B	**29%**	**Ferric chloride in water**
	95 ml	**Distilled water**
	1ml	**Hydrochloric acid**
Working solution		**Equal parts of Stock A and B**
Aniline blue solution	**2.5g**	**Aniline blue solution**
	2ml	**Glacial acetic acid**
Biebrich Scarlet-acid Fuschin	**1%**	**Biedrich aqueous solution**
	1%	**Aqueous acid fuschin**
	1ml	**Glacial acetic acid**
Phosphate buffers		
Stock solutions **1. 0.2M Na_2HPO_4** **2. 0.2M NaH_2PO_4**		
0.2M Na_2HPO_4	**35.6gms**	**Na_2HPO_4 $2H_20$**
	53.65gms	**Na_2HPO_4 $7H_20$**
	71.64gms	**Na_2HPO_4 $12H_20$**
	28.4gms	**Na_2HPO_4 anhydrous**
	1000ml	**Distilled water**
0.2M NaH_2PO_4	**27.6gms**	**NaH_2PO_4 H_20**
	31.21gms	**NaH_2PO_4 $2H_20$**

	1000ml	**Distilled water**
0.2 M Phosphate buffer	**154ml**	**Na_2HPO_4**
	46ml	**NaH_2PO_4**
Toludine blue solution	**0.025%**	**Toludine blue**
	0.5%	**Sodium tetraborate**
TDT buffer	**30mM**	**Trizma base**
	140mM	**Sodium cacodylate**
	1mM	**Cobalt chloride**
	0.1%	**Bovine serum albumin**
TUNEL reaction mixture		
Enzyme solution		**Terminal deoxynucleotidyl transferase from calf thymus, recombinant in *E.coli(E.C 2.7.7.31)*, in storage buffer 10xconc, 5x5µl**
Label solution		**Nucleotide mixture in reaction buffer, 1xconc, 5x550µl**

TABLE OF FIGURES