The role of exogenous HSPA8, a conserved oviductal protein in sperm physiology

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Abstract

Sperm storage in the female oviduct is an evolutionary solution for overcoming the temporal dissociation between mating and ovulation in vertebrates. The female sperm reservoir provides a secure and efficient setting for spermatozoa to remain viable until ovulation by producing secretory fluid into the oviduct lumen. Recently, the production of a novel group of proteins known as extracellular heat shock proteins (HSPs) by the oviductal epithelial cells in response to the presence of spermatozoa in the oviduct has gained considerable attention. Proteomics has identified the presence of a constitutively expressed 70kDa heat shock protein (HSPA8) in the porcine apical epithelial membrane which enhances sperm viability *in vitro*. However, the exact mechanisms that are involved in the preservation of sperm survival remain largely unknown. This study hypothesised that, as an oviductal protein, HSPA8 plays an active role in regulating sperm physiology in favour of a successful fertilisation.

The *in vitro* studies described in this thesis sought to characterise the effect of a recombinant exogenous HSPA8 on aspects of sperm physiology and function using a variety of techniques. This study suggests that the female oviductal sperm reservoir uses HSPA8 to regulate sperm viability (membrane integrity), mitochondrial activity, binding to oviductal epithelial cells and *in vitro* fertilisation performance. This study reports a novel aspect of extracellular HSPA8 as a 'rapid response' extracellular cytoprotector and modifier of cell function which rapidly restores cell membrane integrity by influencing membrane microviscosity via a mechanism which is dependent on membrane cholesterol.

Publications

Moein-Vaziri N., Philips I., Smith S., Almiñana C., Maside C., Gil M.A., Roca J., Martinez E.A., Holt W.V., Pockley A.G., Fazeli A. **Exogenous heat shock protein A8 rapidly repairs and restores sperm membrane integrity by increasing plasma membrane fluidity via a cholesterol dependent process.** Submitted.

Abstracts and Presentations

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Abbreviations

AC	Adenylyl cyclase
APC	Antigen presenting cell
CC	Ciliated cells
CLC	Cholesterol loaded cyclodextrine
CTC	Chlortetracycline
COCs	Cumulus-oocyte comples
cAMP	Cyclic AMP
ELIZA	Enzyme-linked immunosorbent assay
FSH	Follicular stimulating hormone
FRAP	Fluorescence recovery after photobleaching
GnRH	Gonadotropin releasing hormone
HSP	Heat shock protein
HRP	Horse-radish peroxide
IAM	Inner acrosomal membrane
IVC	*In vitro* culture
IVF	I*n vitro* fertilisation
LH	Luteinising hormone
MβCD	Methyl-beta-cyclodextrin
NCM	Non-capacitating medium
NBD	N-terminal nucleotide binding domain
NC	Non-ciliated

NKC	Natural killer cell
OAM	Outer acrosomal membrabe
OEC	Oviductal epithelial cell
PKA	Protein kinase A
sAPM	Soluble oviductal apical plasma membrane
SBP	Substrate binding site
TLR	Toll-like receptor

Chapter 1

General Introduction

1.1 Overview

Internal sexual reproduction requires the fusion of male and female gametes to occur inside the female reproductive tract (Aviles, Gutierrez-Adan et al. 2010). However, this strategy does not necessarily lead to fertilisation, as mating and ovulation do not always coincide for many species and sperm insemination in the female reproductive tract is not immediately followed by egg deposition. This temporal dissociation has led to development of the female ability to store sperm in specialised sperm storage organs. This sperm reservoir allows the female to remain fertile for prolonged periods in the absence of potential mates and ensures successful fertilisation and embryo development after mating has occurred (Holt and Fazeli 2010; Holt and Lloyd 2010; Roy and Krishna 2011; Schnakenberg, Siegal et al. 2012).

The oviductal tube is the specialised principal site for important reproductive events beneficiary to fertilisation and embryo formation in mammals. The events include female and male gamete transport and final maturation, male gamete storage, fertilisation and eventually early embryonic cleavage (Hunter and Rodriguez-Martinez 2004). A successful pregnancy depends on each individual event to be precisely timed and regulated. The oviduct provides a supportive environment by producing a host of factors that are required by the gametes and for the maturation and development of the embryo/s.

Until very recently, it was believed that the oviductal environment and the composition of oviductal secretions were primarily regulated by systemic hormones. However, studies have now provided strong evidence for the presence of a local regulatory system within the oviduct which involves active transcriptional changes in response to interactions between reproductive members (Georgiou, Sostaric et al. 2005). Reproduction would never be satisfactorily fulfilled without successful, active interactions between gametes, embryo/s and the oviduct.

This reciprocal interaction is known as maternal communication and was first proposed following the observations of protein production as a result of the presence of sperm and their attachment to the oviductal epithelial cell (OEC) monolayer (Ellington, Ignotz et al. 1993). The definition was later extended to embryo-oviduct interactions (Alminana, Heath *et al.* 2012). The communication defines series of signals which are exchanged between the

members and are initiated when the oocyte is released at ovulation or when spermatozoa are deposited in the female reproductive tract at coitus. Signals generated from any of the reproductive components not only affect their own properties and transport, but also alter gene expression and physiology of the oviduct (Holt and Fazeli 2010; Holt and Lloyd 2010).

As part of gamete-oviduct communication, in many species the inseminated spermatozoa ascend to the oviduct and bind tightly to OECs. Such cell to cell binding favours the formation of a sperm reservoir for holding spermatozoa that are competent for fertilisation (Rodriguez-Martinez 2007). Several sperm properties that are essential for successful reproduction have been shown to be maintained, regulated or improved in the oviduct. Sperm maturation, known as capacitation, is promptly induced and the speed of the process is finely modulated in the oviduct (Hunter and Rodriguez-Martinez 2004; Rodriguez-Martinez 2007).

Sperm motility is finely modulated in favour of formation of sperm reservoir and oocyte penetration at ovulation (Hunter and Wilmut 1984). Moreover, the sperm-OEC interaction maintains sperm viability during the storage period in order to ensure an appropriate number of viable sperm are available at the time of ovulation (Smith and Yanagimachi 1990; Pollard, Plante et al. 1991). This early aspect of reproduction has attracted major scientific attention due to the significance of interactions for successful fertilisation and the long-term well-being of off-spring. As a step towards understanding the underlying mechanism of this sort of communication, genomic and proteomic approaches have shed light on the protein factors that are involved *in vitro* and *in vivo.* Studies have identified a number of seemingly relevant heat shock proteins: HSPA1A (HSP70) as a component of the oviductal secretory fluid (Georgiou, Sostaric et al. 2005) and HSPD (HSP60) and HSPA8 (HSC70) on the surface of the epithelial cells (Boilard, Reyes-Moreno et al. 2004; Elliott, Lloyd *et al*. 2009). The role of extracellular heat shock proteins (HSPs) in mammalian reproduction remains unclear.

As chaperone proteins, HSPs perform their protective role by protecting gametes and embryos from environmental stressful conditions within the reproductive tract (Neuer, Spandorfer et al. 2000). They also take part in other reproductive processes such as enhancing sperm viability (Elliott, Lloyd *et al*. 2009), increasing the fertilisation rate and accelerating embryonic development (Neuer, Mele et al. 1998; Neuer, Spandorfer *et al*. 2000). Nevertheless, minimal

knowledge currently exists regarding the heat shock protein-gamete and particularly HSPA8-spermatozoa association. Even much less is known about the receptors which are activated and pathways which are triggered during this type of cell-protein interaction.

This chapter will provide a brief overview of the biology and physiology of maternal interactions with gametes and embryos and will focus on the final maturation of gametes, fertilisation and early embryonic development. It will describe the environment in which these events take place. It will also introduce heat shock protein family, classes, general functions and finally consider HSPs and their role in maternal communication with gametes and the embryo.

1.2 Characters of mammalian reproduction

1.2.1 Female reproductive tract

The general structure of the female reproductive tract is similar in most mammals and is composed of a number of tubes with different anatomies and functions: the vagina, cervix, uterus, a pair of oviducts (called fallopian tubes in primates and human) and ovaries (Figure 1.1A). However, differences exist in forms and anatomical details among species. For example, marsupials have a pair of vaginas each of which lead to a separate uterine horn, while in pigs the vagina unites to form a uterus with two diverging uterine horns (Figure 1.1B). In primates and humans, the uteri are combined to form a single uterus with no horns. The general anatomy of the female reproductive tract in humans is illustrated in Figure 1.1 and the anatomy and physiology of each part is briefly described below:

The **Vagina** acts as a muscular tube which serves as a passage for spermatozoa to ascend the tract after copulation and for the delivery of the foetus during labour. The vaginal wall consists of an inner mucosal layer lined with stratified squamous non-keratinised epithelium, a middle muscular layer and an outer fibrous sheath.

The **uterus** is a hollow thick-walled muscular organ which is situated deeply in the pelvic cavity, opening to the fallopian tubes on the upper part and to the vagina on the lower part. The uterus measures about 6-7 cm in length and is divided hypothetically into cervix, uterine body and the fundus. The cervix is the lower constricted segment which connects the uterine body

to the vagina and acts as a barrier between external and internal environment of the female reproductive tract. The uterine body is the main cavity for embryo implantation and development. Finally the most upper part is the convex-shaped fundus which narrows to form the isthmus on each side. The uterine wall is composed of three distinct layers; the external serous perimetrium, the middle muscular myometrium, and the internal mucosal endometrium. The endometrium is divided into the basalis layer facing the myometrial junction and the thicker functional layer facing the uterine cavity. Menstrual shedding and renewal occurs in the functional layer with each cycle.

The **Fallopian tubes,** also called oviducts or uterine tubes, are a pair of open-ended tubes that connect the uterus to the ovaries on each side. The anatomy and function of the oviduct were first described by Gabriel Fallopius in 1561 and Reigner De Graaf, respectively (Ankum, Houtzager *et al.* 1996) and the early view was that the oviduct was a simple passive conduit for gametes and embryo transport. However, recent observations demonstrate that the oviduct has a more complex dynamic role in regulating gamete maturation and fertilisation, as well as being involved in early embryonic development.
As described above, the focus of this thesis is to investigate the role that the oviduct plays as sperm reservoir and particularly on the interactions between oviductal epithelium and spermatozoa in the pig model. Hence, a relatively detailed anatomy, and histology of the pig oviduct are described here.

The porcine oviduct is approximately 25 cm in length. For descriptive purposes, each oviduct is anatomically divided into three main segments based on their anatomical and functional characteristics. The proximal segment is the isthmus that originates from the uterus through the utero-tubal junction and comprises one third of the total length of the tube. The isthmus is the principal site for sperm reservoir formation. The ampulla is the middle section which takes about two thirds of the oviduct length. Ampulla hosts the major events including fertilisation and early embryonic development and hence, the mucosal epithelium in this region is more developed compared with the other oviductal segments. The third segment is the funnel-shaped infundibulum which surrounds the ovary with its fimbria and this is instrumental in ovum-pick up at the time of ovulation (Crow, Amso et al. 1994). Connecting areas including terminal

section or ostium, the ampulla-isthmic junction and utero-tubal junction are also distinguished as transitions between segments.

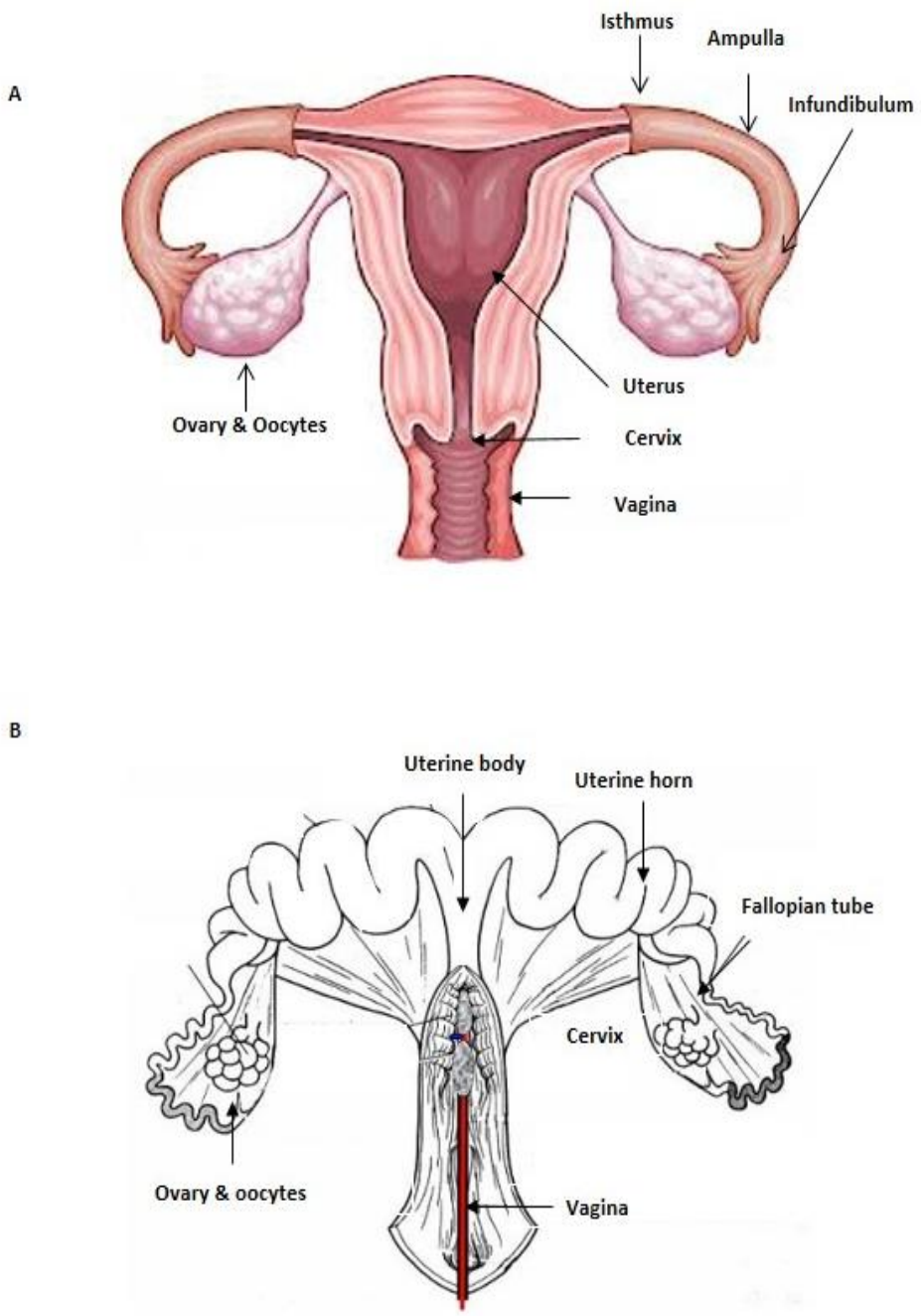

Figure 1.1 Gross anatomy of the female reproductive tract in; (**A**) human and (**B**) pig. (Picture kindly provided by Dr. Carmen Almañana, University of Sheffield, United Kingdom).

The histo-architecture of the oviduct displays three distinct layers in the oviduct wall (Figure 1.2). The outer connective tissue known as the tunica serosa that coats the outer surface of the tube. The middle tunica muscularis layer which is composed of double-layered longitudinal and circular smooth muscle fibres. Thickness of the tunica muscularis layer decreases towards the ostium leaving the isthmus the thickest and the infundibulum the widest segments of the tube. The inner mucosal coating (tunica mucosa) that faces the interior lumen consists of two layers of lamina propria and lamina epithelialis. Lamina propria is a thin gland-free connective tissue which lies between the epithelium and the muscle layer (Yaniz, Lopez-Gatius et al. 2006).

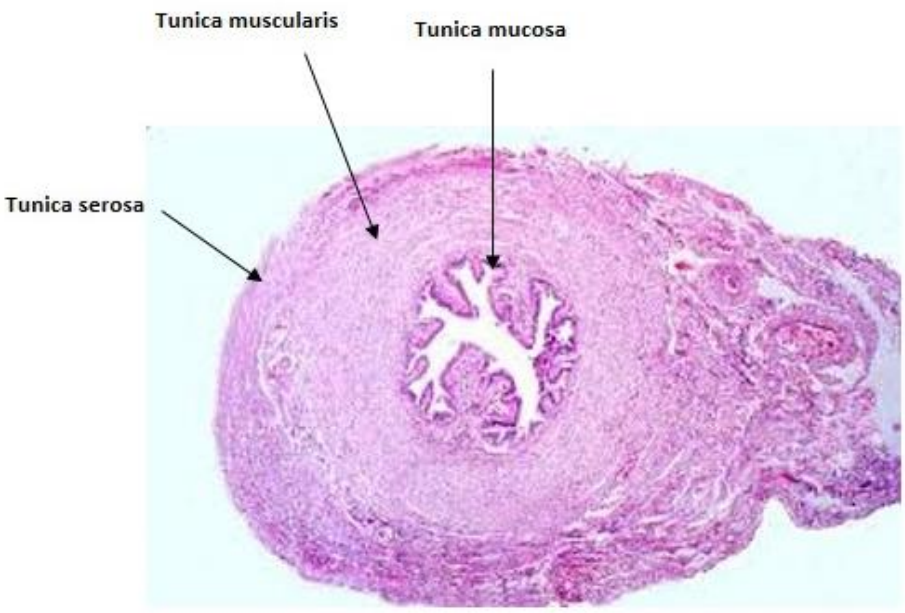

Figure 1.2 Histo-architecture of oviduvt; illustrating the three layers of tunica serosa, tunica muscularis and tunica mucosa, derived from http://www.netterimages.com.

Light microscopy shows the lamina epithelialis as a single layer of columnar or cuboidal epithelium with some degree of ciliation at the luminal surface. A high proportion of cells exhibit oval or elongated nuclei with open chromatin pattern typical of epithelial cells and a few mononuclear cells scatter in the basal epithelium. The underlying stroma shows hyperchromatic dense nuclei characteristic of lymphoid cells.

Ultrastructural analysis shows that the oviductal epithelium consists of four morphologically distinct cell types; ciliated cells (CC), non-ciliated or secretory cells (NCC), peg cells (intercalated) and basal cells. Although CCs and NCCs cover the entire length of the oviductal lumen, there is a progressive increase in the proportion of CCs from the isthmus outwards the highest numbers in fimbria. The higher number of CCs in the infundibulum indicates the role of this segment in egg transportation. Cilia measure about 10 µm long and 0.25 µm wide and project to the oviduct lumen. CCs have oval centrally located nuclei with a large number of mitochondria at the peri-nuclear region (Crow, Amso et al. 1994).

NCCs also known as secretory cells, exhibit apical dome-shaped protrusions of variable heights compared to the flat luminal surface in the CCs. The nuclei are irregular in shape and located in the basal third of the cell. NCCs contain larger mitochondria scattered sparsely in the cytoplasm. NCCs contain dense granules which vary in size and cytoplasmic location during the menstrual cycle. Granules are usually released into the tube lumen along with surface protrusions as form of apocrine or decapitation secretory process which leads to the reduction in the cell height. NCCs with surface domes and dense cytoplasmic granules appear most prominently in the ampulla and are less developed in other tubal sections (Crow, Amso et al. 1994).

Both main cell type, CCs and NCCs have numerous microvilli at their luminal surfaces which appear between the cilia of the CCs and on the surface of domes in the NCCs. Peg or intercalated cells are identified as slender rod-like cells with little cytoplasm which are squeezed in between the other epithelial cells and appear shorter than the other cells in the plane of the section. This cell type is believed to be the NCC residual cells after granular secretion.

Basal cells with small round dark nuclei comprise 1% of the total epithelial cell population and were initially thought to be undifferentiated cells with the potential to differentiate and replicate into CC and NCC cells, since epithelial cell loss into the oviduct lumen occurs and there is a need for replication and replacement of the lost cells. However, some immunohistochemical studies have categorised basal cells as cytotoxic T lymphocytes (Crow, Amso et al. 1994).

The **Ovaries** are the female reproductive glands that are responsible for oogenesis and storage of the oocytes. They are usually found in pairs as part of the vertebrate reproductive system and are the equals for testes in males, in that they are both gonads and endocrine glands which produce the reproductive hormones that are essential for reproduction. The ovaries on each side are attached to the outer layer of the uterus via the ovarian ligaments but have no attachment to the oviduct. Every month one ovary takes turn to release eggs, unless one ovary is absent or dysfunctional. In that case, the functional ovary would be the source of oocytes in each cycle. The porcine ovulatory activity is an exception, in that ovulation occurs bilaterally in each cycle (Brussow, Ratky et al. 2008).

1.2.2 Menstrual cycle

The mammalian female reproductive years are characterised by rhythmical alterations in the secretion of a group of hormones and related physical changes in the female reproductive tract, most notably the ovaries, oviducts and the uterine endometrium. This cyclical pattern is called the menstrual cycle in human and oestrus in other mammals (Guyton 2000).

The rhythmic property of the female sexual cycle is dependent on a complex hierarchy of hormones secreted from the hypothalamus (gonadotropin releasing hormone, GnRH) which regulates secretion of follicular stimulating hormone (FSH) and luteinising hormone (LH) or sex hormones from the anterior pituitary gland (Guyton 2000). The latter two steroid hormones control the production of oestrogen and progesterone by the ovaries, both of which exert direct effects on the female reproductive tract. The secretion of the hormones is not in a constant rate and fluctuates throughout the cycle. This fluctuation is responsible for the the different patterns in the female reproductive tracts during the cycle (Guyton 2000).

The female menstrual cycle has two major outcomes: Firstly, the oocyte/s is/are released from

the ovaries in the middle of each cycle, so that if fertilisation happens, foetus will be produced. Secondly, the uterine endometrium becomes prepared for implantation of the fertilised ovum at the appropriate time of the cycle.

The mammalian female cycle is divided into two distinct phases by ovulation, and the events which take place in each phase are specific to each organ. During the first half, termed as follicular phase in the ovaries, there is an accelerated growth of a few number of primary follicles under the effect of moderately increased FSH. After several days one of the follicles begins to outgrow all of the other stimulated follicles. The outgrown follicle reaches its mature size immediately before ovulation. In the middle of the ovarian follicular phase, as the follicles continue to grow larger, oestrogen is secreted from the granulose cells surrounding the follicles and reaches its highest level before ovulation (Guyton 2000; Johnson 2000).

The first phase of the cycle in the endometrium is known as the proliferative or oestrogen phase and contains two distinct parts. Most of the endometrium becomes desquamated and the cells start shedding during the initial days of the phase. Covert bleeding happens only in human and close species like chimpanzees and is known as menstrual bleeding. In other female mammals, it is called estrous and shedding is totally absorbed. Shedding continues to the extent that only a thin layer of deeper epithelial cells remains. Desquamation stops as the ovarian oestrogen level rises to a certain level. The endometrial epithelial cells proliferate very quickly and increase the endometrial thickness until ovulation occurs (Guyton 2000).

Ovulation occurs in the middle of the menstrual cycle, along with a surge in LH level in blood. The follicular out-layer of the mature oocyte ruptures and the ovum is released to the oviduct. Here the ovarian luteal phase begins. The remaining follicular cells named corpus luteum after the ovum expulsion is a secretory organ and secretes large amounts of progesterone and oestrogen (Guyton 2000; Johnson 2000).

These hormones induce strong inhibitory feedback on FSH and LH and their levels in the blood decrease. They also cause additional cellular proliferation and the swelling and secretory development of the endometrium. The latter phase of the cycle is consequently known as the secretory phase in the uterus. The main purpose of the endometrial change during the secretory

period is to produce a highly secretory endometrium containing large amounts of nutrients which provide an appropriate condition for the fertilised ovum to implant in the uterine wall (Guyton 2000). If the ovum is not fertilised, the corpus luteum in the ovaries degenerate towards the end of the cycle and the ovarian hormones, oestrogen and progesterone decrease to low levels in the blood (Guyton 2000; Johnson 2000). Menstruation follows and a new cycle begins.

The oviductal epithelium expresses varying patterns of receptors for oestrogen and progesterone according to the stages of the ovarian cycle and therefore undergoes morphological and functional changes during the female cycle (Lyons, Saridogan et al. 2006). Oestrogen stimulates hypertrophy, secretion and ciliogenesis in epithelial cells, whereas progesterone causes atrophy and deciliation. Accordingly, during the luteal phase the epithelial cell height, secretion and ciliation is at its lowest and increase gradually to the maximum level in the peri-ovulatory phase when ciliated and secretory cells reach approximately equal sizes. Secretory cells display peak activity around ovulation, release their contents into the oviduct lumen and shrink in height compared to ciliated cells. The prominence of ciliated cells at this stage facilitates efficient transportation of particulates or fluids along the tube (Lyons, Saridogan et al. 2006).

Mammals share the fundamental aspects of reproduction system including the regulatory hypothalamic, pituitary and ovarian hierarchy. However, species vary significantly in their detailed functioning. For instance, animals with oestrus cycles reabsorb the endometrium if conception does not occur, whereas species with menstrual cycles (primates), shed the endometrium through the menstruation. Moreover, females with oestrus cycles are only sexually active during the oestrus phase (referred to as being in heat), but females with menstrual cycles are sexually active at any time during their cycle regardless of the ovulation time.

The basic mechanism of the oestrus cycle in pig is very similar to human, characterised by the development of follicles and release of the oocytes at the best time for fertilisation, preparation of the uterus for embryo accommodation and resumption of further follicular development for a new cycle and new ovulation. During pig oestrus cycle like menstrual cycle, the follicular

phase (5 days) characterised by FSH/oestrogen surge and the luteal phase (16 days) characterised by LH/progesterone surge can be distinguished.

1.3 Gametes and gametogenesis

1.3.1 Oogenesis

The oocyte or egg is produced in the female gonad, the ovary, via the process of oogenesis during early fetal life. In mammals, the initial step of oogenesis starts in the germinal epithelium by the development of ovarian follicles which are the functional units of the ovary. The female ovarian primordial germ cells undergo mitosis to form oogonia. Oogonia transform to primary oocytes as they go under the first meiotic division. Perinatally, primordial follicles formed as the primary oocytes get arrested in the prophase of the first meiotic division and are covered by a layer of squamous somatic granulosa cells. Secondary and tertiary follicles appear as the granulosa cells proliferate and differentiate into multiple layers of cuboidal cells surrounding the oocyte (cumulus cells) and fluid-filled cavities form within the follicle (Uyar, Torrealday et al. 2013).

With the commencement of puberty, the follicles grow into pre-ovulatory (antral) follicles under the influence of the pituitary follicle stimulating hormone (FSH). At this stage, a layer of specialised granulosa cells known as cumulus cells surround the oocyte inside the follicle. With the leutinising hormone (LH) surge, the oocyte resumes the maturation process by undergoing the second meiotic division. Now the cumulus-oocyte complex (COC) containing an oocyte arrested in the metaphase of the second meiotic division is ready for ovulation and fertilisation, should sperm be present nearby (Uyar, Torrealday et al. 2013). Figure 1.3A illustrates the different stages of oogenesis in the ovary.

Each ovary contains a defined number of follicles and each follicle contains one egg. At each female cycle usually only one oocyte is released and fertilised by sperm in the ampulla, should sperm be present in the near vicinity (Guyton 2000). However, in polyovular species like pigs each follicle contains more than one oocyte and therefore more then 15-20 eggs are usually ovulated during each oestrus cycle (Yaniz, Lopez-Gatius et al. 2006).

1.3.2 Oocyte

While released from the ovary into the fallopian tube, the mammalian oocyte is arrested in the metaphase of the second meiotic division, ready to be fertilized. The secondary oocyte is encased with multicellular cumulus oophorus and the glycoprotein egg coat named zona pellucida and contains the first polar body along with the metaphase plate produced as result of the first meiotic division (Huang and Wells 2010). Unlike spermatozoa, oocytes are rich in cytoplasm which contains the nucleus, ribosomes and mitochondria all derived from the maternal cells. A picture of mature follicle at ovulation stage is depicted in Figure 1.3B

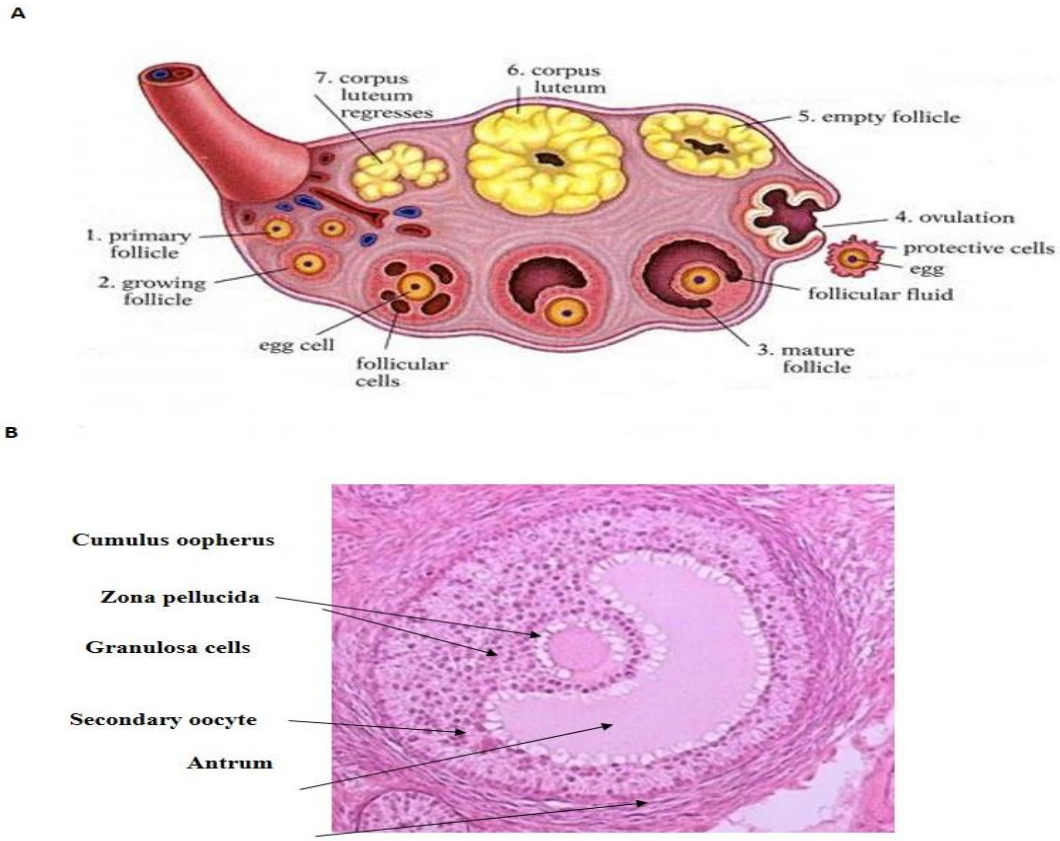

Figure 1.3 Oogenesis and oocyte. (**A**) Different stages of oogenesis redrawn from www.studyblue.com, (**B**) different segments of a mature follicle derived from http://anatomytopics.wordpress.com.

1.3.3 Spermatogenesis

Spermatogenesis is the male gametogenesis and its purpose is to generate mature male gametes, known as spermatozoa, with the ability to fertilise its female counterpart, the oocyte to produce the offspring. In mammals, it occurs in the male reproductive gonads, testes in a stepwise pattern. Unlike oogenesis, it starts at puberty and lasts uninterrupted through the entire life. The whole process can be divided into several stages, each producing a distinct cell type (Johnson 2000).

Briefly, in the basal section of seminiferous tubules, spermatogonial stem cells multiply mitotically into diploid primary spermatocytes. On moving towards the tubal lumen, each primary spermatocyte gives rise to four haploid spermatids by undergoing a series of meiotic divisions. Upon release into the lumen of seminiferous tubules, spermatids undergo an extensive morphological remodelling and develop into fully shaped spermatozoa by nuclear condensation, acrosomal cap formation and development of a tail. However, at this stage, sperm cells do not possess motility and fertilising ability. Peristaltic contractions of the seminiferous muscular wall transport the cells to the epididymis which serves as a reservoir for spermatozoa to gain motility and maturation. Mature spermatozoa are stored in the epididymis tube until ejaculation. At the time of ejaculation, spermatozoa are pushed out of the epididymis towards the ejaculatory ducts and finally to the urethra in the penis through the muscular tube of vas deferens. Seminal vesicles and prostate are male sex glands located along the way whose function is to secrete their content into the tube upon ejaculation. The glandular secretions contain a nutrient mixture of proteins, enzymes, sugar, mucus, vitamins, phosphorylcholine and prostaglandins and constitute 50 to 75% of the semen volume (Johnson 2000). Figure 1.4A illustrates the different stages of spermatogenesis.

1.3.4 Spermatozoa

Mammalian spermatozoa are highly differentiated cells with specific features and organelles which carry half of the nuclear genetic material of the diploid offspring. Male gametes vary in length from 28 µm to 394 µm in different species (Gage 1998). A normal human sperm measures about 50 to 60 µm and a pig sperm is about 45 µm in length. As displayed in Figure 1.4B, a mammalian spermatozoon generally consists of three major parts; the head, midpiece

and principle piece, all of which are surrounded by sperm plasma membrane or plasmalemma (Gage 1998).

1. The **head** varies in shape among different species. For example, rodents' sperm have hooked-shape head, whereas primates and human have spatula-shaped heads. Progressing from inwards out, the head contains the nucleus, cytoplasm, the acrosome and plasmalemma. The nucleus is covered by the nuclear envelope and occupies the most part of the head. This leaves space for only a minimal cytoplasm. The sperm nucleus is haploid and has the most densely packed DNA compared to the somatic cells. Nucleus hypercondensation in sperm is made possible by specific DNA proteins named as protamines during spermiogenesis. This highly compact nucleus facilitates sperm motility and penetration through the egg vestments (Eddy 1988). The nucleus is covered by the acrosome at its apical part. The acrosome comprises a vesicle which originates from Golgi complex during spermiogenesis and contains hydrolytic enzymes necessary for egg penetration during the fertilisation process (Knobil 1994). The acrosome is wrapped by the inner acrosomal membrane (IAM) fronting the top of the nucleus and the outer acrosomal membrane (OAM) facing the plasma membrane. The area where the acrosome cap is located is named anterior acrosome and the area overlying the nucleus posterior to the acrosome is the posterior acrosome (Eddy 1988). The acrosome and postacrosome domains are separated by an equatorial segment which delineates the IAM and OAM.

2. The **Midpiece** is connected to head by the connecting piece and is covered by mitochondrial sheath. It holds a large helical complex of 75-100 sperm mitochondria and provides energy for sperm motility.

3. The **principle piece**, also called tail or flagellum, is composed of a unique symmetric arrangement of nine peripheral microtubule doublets connected to a pair of microtubules in the centre (Eddy 1988). The tail is responsible for sperm motility and its whip-like motion generates the force which is required for moving the sperm towards the egg inside the female reproductive tract (Knobil 1994).

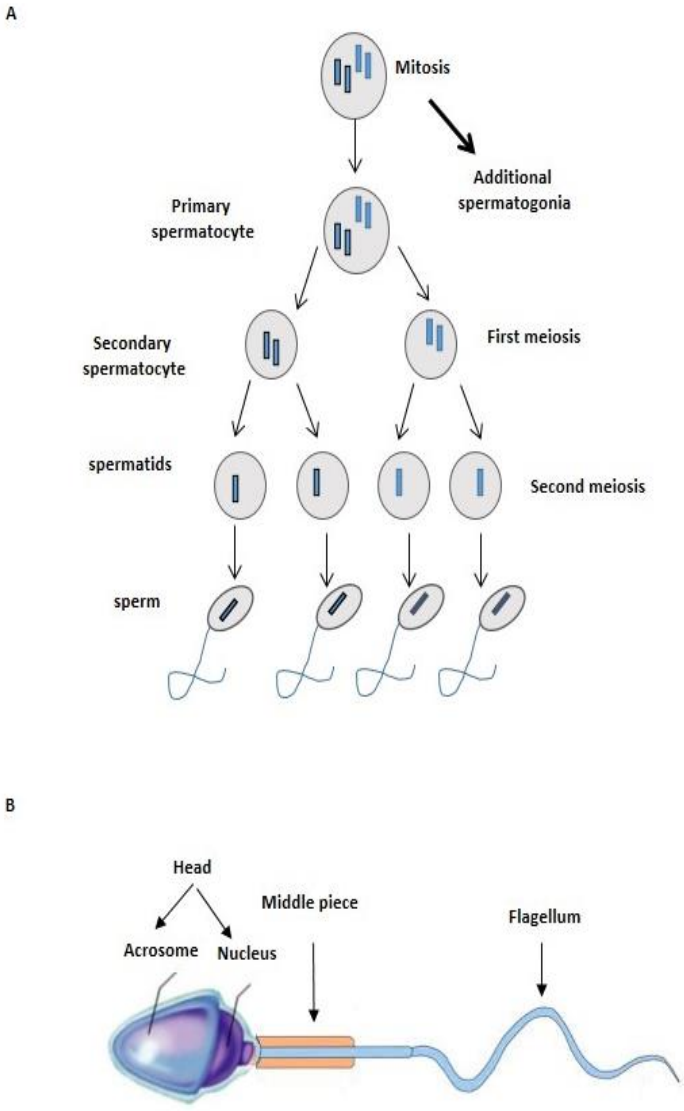

Figure 1.4 Spermatogenesis and sperm. (**A**) Different stages of spermatogenesis, (**B**) Different segments of the mammalian spermatozoon.

1.3.5 Embryogenesis

Embryogenesis is the process via which a new organism is created, and it starts with the fertilisation of the ovum by sperm which gives rise to the zygote. As a consequence, the zygote is a diploid single cell which possesses half the DNA of each of its parents. Immediately after fertilisation, the created zygote undergoes sequential mitotic divisions (known as cleavage) with no significant change in the initial size or cellular differentiation. This leads to the development of a multicellular organism. Meanwhile, it is transported to the uterus and becomes implanted in the uterine wall (Carlson 1999). At implantation, it adheres to the uterine wall to receive oxygen and nutrients from the mother to be able to further grow into a complete organism. For descriptive purposes the pre-implantation embryonic development is separated into early and late embryonic developmental stages that correspond to the time which the embryo spends in the oviduct and uterus, respectively. During this stage, a minimum of four cell divisions occur inside the oviduct in order to produce a dense ball of a 16-cell morula. The morula continues to divide and moves to the uterine cavity in which it develops into the blastocyst. The blastocyst is a fluid-filled cavity consisting of an inner cell mass which forms into the future embryo and the outer cell mass. Implantation occurs at blastocyst stage (Carlson 1999). The pre-implantation embryonic development is schematically displayed in Figure 1.5.

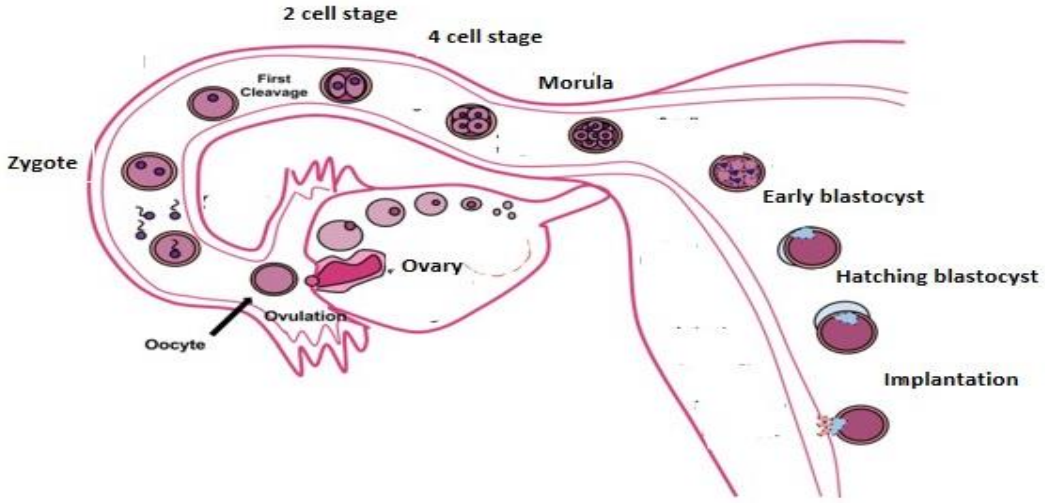

Figure 1.5 A schematic picture of the pre-implantation process; consisting of fertilisation, zygotic division and embryo implantation in the female reproductive tract.

1.4 Physiology of reproduction: from sperm and oocyte to embryo

The spermatozoa formed in the male testes are deposited inside the female reproductive tract via coitus. Although morphologically perfect, the sperm requires further maturation in order for it to be able to fertilise the oocyte. To acquire this full functional maturity, the sperm ascends up to the proximal oviduct, in which motile spermatozoa establish a strong transient bond with the oviductal epithelium to form a reservoir (Hunter, Flechon et al. 1987; Suarez 2002; Rodriguez-Martinez 2007). Here, the competent spermatozoa transiently reduce their motility and wait for the release of oocyte at ovulation.

Subsequent to ovulation, the inter sperm-oviduct bonds disappear, and the spermatozoa become hyperactivated (capacitated) and are guided towards the fertilisation site in the ampulla. To accomplish the fertilisation mission, the spermatozoon has to overcome two final hurdles - the multi-cellular cumulus oophorus and the zona pellucida layers around the oocyte (Sutovsky 2009). By penetrating these layers, the spermatozoon acquires access and fusibility to the oocyte plasma membrane (oolemma). At this stage, exocytosis of the acrosomal hydrolytic enzymes (acrosome reaction) helps the spermatozoon to penetrate the outer oocyte layers and fuse with the oolemma (Kaji and Kudo 2004). Sperm-oocyte fusion occurs on the highly localised area on the sperm surface membrane overlying the equatorial segment (Kaji and Kudo 2004). Cell to cell fusion triggers membrane ion transportation and intracellular pathways in the oocyte which lead into release of sperm content into the oocyte and completion of oocyte second meiotic division, collectively known as fertilisation. After fertilisation, the resulting zygote multiplies and migrates to the uterus, into which it becomes implanted and then grows into a fully grown embryo until the end of pregnancy.

Since the principle theme of this thesis deals with the interaction between the sperm and the female oviduct, the following discussion will closely focus on sperm-epithelial binding, the formation of the sperm reservoir in the oviduct and the subsequent interactions that occur preceding ovulation.

1.4.1. Sperm in the female reproductive tract

Of the many millions of spermatozoa that are deposited in the lower female reproductive tract at coitus, only a few thousand can get past the uterus, and typically only one will fertilise the egg (Petrunkina AM 2001).This gradual reduction in sperm number is due to the 'sieving' action of the lower parts in the female reproductive tract (Smith, Koyanagi et al. 1987). Sperm can spend from hours to days in the mammalian female genital tract (Rodriguez-Martinez 2007). The female reproductive tract and particularly the caudal segment of the isthmus have a special responsibility for regulating a protective pre-ovulatory sperm reservoir prior to its interaction with oocyte.

The isthmus has been located as sperm storage site during female estrous (pre- ovulatory) period in several mammalian species including mice, guinea pigs (Yanagimachi R 1976), hamsters (Smith, Koyanagi et al. 1987), rats (Shalgi and Kraicer 1978), rabbits (Overstreet and Cooper 1978), pigs (Hunter 1981), sheep (Hunter and Nichol 1983) and cows (Hunter and Wilmut 1984).

1.4.2. Role of isthmic sperm reservoir in reproduction

The isthmic sperm reservoir provides a conduit for a secure number of spermatozoa to pass through to the oocyte (Suarez and Pacey 2006; Suarez 2008) and it has been shown that the resection of the isthmus increases the incidence of polyspermia (entrance of more than one spermatozoon into the ovum (Hunter and Leglise 1971). The isthmic sperm reservoir also functions as an environment in which to maintain sperm viability and motility (Suarez 2002). The motility and fertility of bovine sperm are maintained longer *in vitro* when incubated with oviductal epithelium compared to other types of epithelial cells or in medium alone (Pollard, Plante et al. 1991). Thirdly, The isthmic sperm reservoir regulates the final step of sperm maturation (capacitation) in order to ensure successful fertilisation (Smith 1998).

1.4.3. Mechanism of sperm reservoir formation

Anatomically, the proximal isthmus has a very narrow lumen and thicker tunica muscularis as compared with the ampulla (Suarez, Redfern et al. 1991). The lining mucosa is arranged in folds in order to create blind channels (Suarez 2002). In addition, this narrow lumen is filled with thick mucus (Suarez 2002). All the above factors, combined with oedema of the isthmus wall (Boyle, Cran et al. 1987) and a decrease in sperm motility (Overstreet and Cooper 1978; Suarez 1987), are known to play a part in the formation of a sperm reservoir. Furthermore, spermatozoa form a strong transient bond with apical oviductal epithelial cells when they come to close contact with the oviduct epithelium. This tight attachment is known to be a significant contributing factor to the formation of the oviductal sperm reservoir (Hunter 1981; Hunter and Nichol 1983; Suarez 2002).

1.4.4. Direct sperm-oviductal epithelial cell interaction

Using scanning electron microscopy, Hunter and colleagues demonstrated a direct association between the somatic oviductal epithelial cells and sperm in mated pigs and cows *in vivo* (Hunter, Flechon et al. 1987; Hunter, Flechon et al. 1991). Similar associations between sperm and oviductal epithelial explants (Pollard, Plante et al. 1991; Suarez, Redfern et al. 1991) and cell monolayers (Ellington, Padilla et al. 1991; Ellington, Ignotz et al. 1993) have been observed *in vitro*. Contact between the sperm and the epithelium takes place between the intact acrosomal region on the sperm head and apical segment of oviductal epithelial cells (Pollard, Plante et al. 1991; Suarez, Redfern et al. 1991). The establishment of such an intimate contact is due to the presence of ligands (lipopolysaccharides) on epithelial cells and complementary receptors (lectins) on the sperm head (Topfer-Petersen, Wagner et al. 2002).

The treatment of sperm-epithelial cell co-cultures with competitive inhibitors that cross- react with receptors reduces the number of sperm that are bound to OECs (Suarez 2001; Suarez 2002). It appears that different species possess unique epithelial cell sugar ligands and complementary sperm protein receptors - for example, terminal sialic acid in hamster; galactosein horse and fucose in cattle oviductal epithelium (Suarez 2001; Suarez 2002). Figure 1.6 illustrates scanning electron micrograph of spermatozoa bound to the oviductal epithelium.

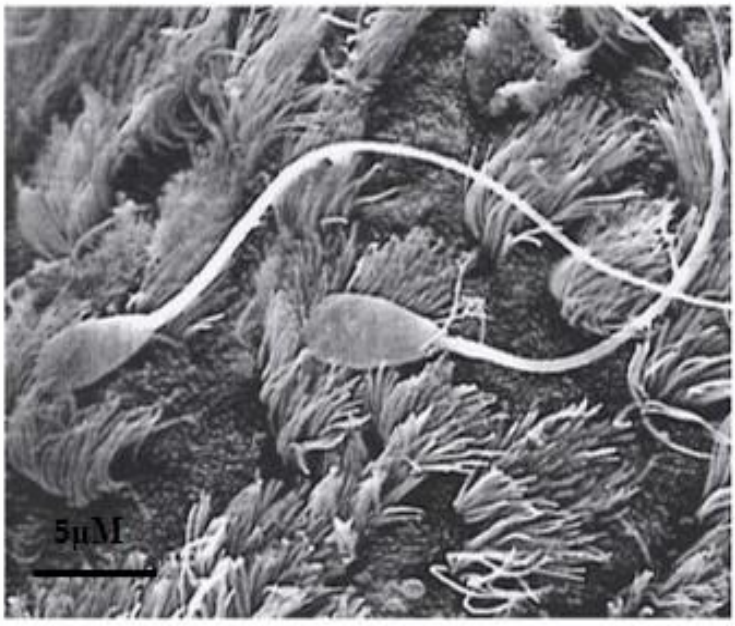

Figure 1.6 Scanning electron micrograph of spermatozoa bound to the oviductal epithelium. Photograph kindly provided by Dr. Edita Sostaric, University of Utrecht, Netherlands.

1.4.5. The effect of sperm-OEC binding on sperm characteristics

The discovery that spermatozoa come into direct contact with the oviductal epithelium posed questions concerning the physiologic impacts of this intimate interaction. Reports from a large number of experiments indicated that oviductal-sperm interactions are of physiologic significance rather than a mere physical attachment. Figure 1.7 presents a model of sperm-oviductal epithelial cell interaction and consequent alterations in sperm characteristics.

Effect of sperm- OEC binding on sperm maturation (capacitation)

Spermatozoa are formed via the process of spermatogenesis in the male genital tract (testes). Initially, spermatozoa do not possess the characteristics that are required for fertilisation such as motility, fertilising ability or morphological differentiation. The gradual passage of sperm in the male genital tract exposes the immature sperm to local and hormonal secretions that are partially beneficial to sperm maturation. Although ejaculated testicular sperm is morphologically complete, it is not of sufficient functional maturity for immediate egg fertilisation (Rodriguez-Martinez, Saravia et al. 2005).

About 50 years ago, Austin reported that ejaculated spermatozoa are incapable of penetrating the oocyte if inseminated directly at the time and site of ovulation. Spermatozoa must spend some time in the female reproductive tract in order to acquire the ability of successful egg penetration. Therefore, the term 'capacitation' was coined in the field of reproductive biology to illustrate a process involving biochemical, biophysical and metabolic modifications of all sperm domains (Rodriguez-Martinez 2007) for the attainment of full maturity in the female reproductive tract. Capacitated spermatozoa are principally able to fertilise the oocyte.

Sperm capacitation happens gradually *in vivo* during sequential exposure of spermatozoa to the different compartments of the female reproductive tract and is completed in the oviduct (Rodriguez-Martinez 2007). The female reproductive tract induces sperm capacitation by removal of seminal plasma and epididymal proteins that coat the sperm membranes (Rodriguez-Martinez 2007).

The oviductal isthmus effectively takes part in modulating the capacitation process in spermatozoa (Hunter, Huang et al. 1998; Smith 1998). Co-incubation of sperm with isthmic

cells delays capacitation (Dobrinski, Smith et al. 1997) and sperm- epithelial cell adhesion terminates as the capacitation status in attached spermatozoa completes (Lefebvre and Suarez 1996). Since capacitated sperm die very quickly (an undesirable event in fertilisation process) (Rodriguez-Martinez 2007), the physiological delay prolongs the viability of sperm until ovulation in order to synchronise sperm function with the ovulation time (Rodriguez-Martinez 2007). As ovulation occurs and the oocyte is released in the oviduct, a chemotactic mechanism draws the spermatozoa to the egg (Eisenbach 1999). The sperm-OEC bond disappears at this time and viable capacitated sperm move towards the egg. On the other hand, exposure of sperm to isthmic fluid leads to rapid sperm capacitation (Hunter and Rodriguez-Martinez 2004).

Although sperm capacitation can be mimicked by particular capacitating media *in vitro*, the major difference between the laboratory and natural conditions is the rate of sperm capacitation. This occurs immediately in media, but only very gradually inside the female reproductive tract in response to ovulation. Upon ovulation, the gradual reduction in the number of spermatozoa along a gradient from the caudal isthmus outwards indicates that there is a constant release, but not a massive one, associated with ovulation. These observations highlight the regulatory and synchronising role of the oviduct in the sperm capacitation process in order to ensure the presence of an appropriate number of competent sperm at the site of ovulation in order to reduce the chance of polyspermy (Rodriguez-Martinez, Saravia et al. 2005).

Effect of sperm-OEC binding on sperm motility

Although motility is important to the transportation of spermatozoa inside the female reproductive tract, sperm are temporarily immotile in the isthmic oviduct. This is assumed to be a contributing factor to the formation of the sperm reservoir. The first *in situ* observation of sperm motility within the oviduct confirmed the immotile phase for the spermatozoa which were held within mouse isthmus (Suarez 1987). Hunter and Wilmut reported that sperm were in a quiescent state in the oviduct (Hunter and Wilmut 1984) for up to 20 hours in cows or 10 hours in rabbits (Hunter and Wilmut 1984; Smith and Nothnick 1997) during the pre-ovulatory period.

Spermatozoa bathe in isthmic secretions and are in contact with oviduct-specific proteins, enzymes, glycol- and lipoproteins (Rodriguez-Martinez 2007). The transient depression of motility in stored sperm in the isthmus can be due to the presence of an inhibitory component or the lack of a stimulatory factors in the region (Suarez 1987). Experiments in rabbits have shown that most of the spermatozoa that are recovered from the isthmus are immotile, and that motility is acquired once again when incubated with ampullar fluid (Overstreet, Katz et al. 1980).

Grippo and colleagues evaluated the effect of fluids collected from two oviductal regions at luteal and non-luteal phases on sperm motility and demonstrated the capacity of isthmic secretions to inhibit sperm motility (Grippo, Way et al. 1995). Following these observations, the presence of a molecular motility inhibitor in the rabbit oviduct was reported (Overstreet, Katz et al. 1980). It is also sensible to conclude that sperm motility is restricted when they form strong bonds with OECs. Furthermore, around ovulation time, quiescent spermatozoa which are bound to isthmic epithelium exhibit a hyperactivated state in response to chemotactic mechanisms from the oocyte (Eisenbach 1999), detach from the epithelium and swim up towards the egg. The low number of spermatozoa recovered from the ampulla during their travel to the fertilisation site (Overstreet and Cooper 1978; Smith and Nothnick 1997) explains the gradually controlled release of highly motile sperm from the reservoir.

All reported results suggest that the isthmus has a major role in synchronising sperm transport and ovulation by keeping the spermatozoa quiescent prior to ovulation and sustaining the release of spermatozoa in response to ovulation. This way it prevents accumulation of a large number of sperm at ovulation site and consequent undesirable polyspermy.

Effect of sperm-OEC binding on the maintenance of sperm viability

Mating in mammals usually occurs either around the time of, or at ovulation. The period between sperm insemination in the female reproductive tract and ovulation can be a few hours to a few days. There must therefore be a mechanism to preserve the fertile life span of the

stored spermatozoa inside the female reproductive tract and particularly the isthmus (the site where accommodates the sperm for the majority of its life in the female reproductive tract). *In vivo* and *in vitro* investigations in hamsters (Smith and Yanagimachi 1990) and cows (Pollard, Plante et al. 1991) have illustrated that the adherence of sperm to epithelial cells in isthmus has a beneficial effect on sperm viability.

The mechanism might involve the presence of epithelial secreted products at the site of interaction, or the direct interaction(s) of sperm with the apical plasma membrane of the epithelial cells. In this regard, a 95 kDa glycoprotein component of bull oviduct secretion has been shown to enhance the viability of homologous sperm *in vitro* (Abe, Sendai et al. 1995). However, studies in which sperm cells were brought into direct contact with vesicles isolated from apical plasma membranes of rabbit and equine oviduct (Dobrinski, Suarez et al. 1996; Smith and Nothnick 1997) supported the idea that co-incubation of sperm along with secretory products of isthmic epithelium is not sufficient to enhance cell viability (Smith and Nothnick 1997), and that direct interaction of epithelium with spermatozoa is essential (Suarez 2008). A similar study in pigs has shown a dose-dependent improvement of sperm viability after co-incubation of spermatozoa with apical plasma membranes (sAPM) isolated from pig oviductal epithelial cells for 24 hours (Fazeli, Elliott et al. 2003). The effects on viability were abolished when sAPM proteins were denatured, thereby suggesting proteins as effectors (Fazeli, Elliott et al. 2003).

Proteomics has since identified a 60kDa protein and 70kDa (HSPA8) heat shock protein in bovine and porcine sAPM which are reported to be responsible for enhancing sperm viability *in vitro* (Boilard, Reyes-Moreno et al. 2004; Elliott RM 2009). In line with those reports and the potential significance which oviductal components have for *in vivo* sperm preservation, we were prompted to further investigate the role of extracellular heat shock protein HSPA8 on the properties and function of sperm. The following section provides a detailed introduction to heat shock proteins; their discovery, function and the existing knowledge on their role in reproduction.

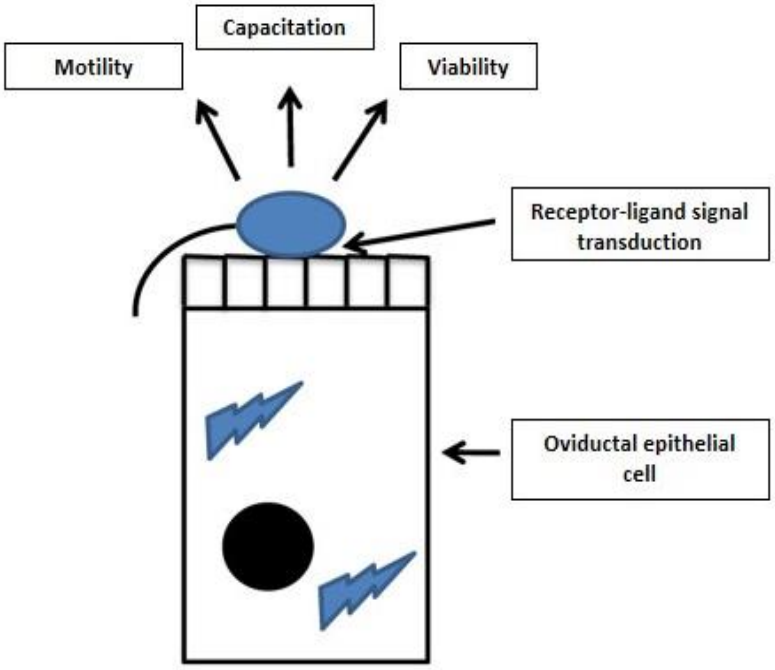

Figure 1.7 Model of sperm-oviductal epithelial cell interaction and consequent alterations in sperm characteristics. Oviductal-sperm interactions in the female reproductive tract are of physiologic significance rather than a mere physical attachment. The oviductal epithelium effectively takes part in modulating sperm fertilising capacity through synchronising sperm viability, motility and capacitation prior to ovulation and sustaining the timely release of fertilising competent spermatozoa in response to ovulation.

1.5 Introduction to Heat Shock Proteins

Living organisms are in a constant dynamic interaction with their surrounding environment. Amongst all environmental factors they face, many of them can exert deleterious impacts. In order to survive stressful conditions, organisms are equipped with evolutionary defence machinery which includes a set of complex proteins with general and specific properties termed as 'Stress' or 'Heat Shock Proteins'. Heat Shock Proteins (HSPs) are vital to the growth, development and survival of all organisms.

1.5.1 History of heat shock proteins

The discovery of heat shock proteins dates back in 1962 when Ferruccio Ritossa observed a new puffing pattern on the fruit fly's salivary gland chromosomes (Ritossa 1962) after one of his technicians accidentally adjusted the temperature of an incubator to a non-physiological level. He repeated the experiment with normal temperature controls and observed similar puffing pattern along with new RNA synthesis. The rapidity of the new RNA synthesis within 2-3 minutes was impressive (Ritossa 1964). He concluded that the increase in temperature was responsible for immediate changes in chromosome pattern and gene expression and he called the phenomenon a heat shock response.

1.5.2 Heat shock response

The heat shock response is a remarkably conserved natural adaptive response (Lindquist 1986). Organisms acquire biologic tolerance to lethal temperatures (thermotolerance) after brief exposure to sub-lethal temperatures via a rapid, but transient reprogramming of cellular activities. This rapid heat shock response takes place in bacteria, plants and animals. It protects crucial cellular components and ensures survival so that stressed cells can carry on their normal function over the recovery phase.

1.5.3 Characterisation of heat shock response

After Rittossa's report on alteration of gene activity induced by heat shock in Drosophila (Ritossa 1962), scientists started to elucidate the "heat shock response" phenomenon. Over the

following 10 years the response was studied principally at the cellular level, and several important observations were made:

1. Induction of puffs by several other stress factors (Ritossa 1962; Leenders and Berendes 1972).
2. Puffs were produced within a few minutes of stress treatment (Berendes 1968).
3. Appearance of puffs was associated with new RNA synthesis (Ritossa 1962; Leenders and Berendes 1972).
4. Puffs were produced in other types of fruit fly and many other tissues (Ritossa 1964; Berendes 1965).
5. It took sometime before it was appreciated that the response is mediated via a transient activation of a number of genes. The genes previously silent or active at low levels were activated by stress and transcribed into a number of specific proteins (Burdon 1986; Lindquist and Craig 1988). In 1974 for the first time, Tissières & Mitchell characterised a small number of proteins which were coincidentally expressed with chromosome puffs in Drosophila (Tissieres, Mitchell et al. 1974). Since the expression of the proteins was originally discovered as a consequence of exposure to thermal (heat) stress, these products of the genes were collectively termed 'Heat shock proteins'.
6. For a while, the induction of the heat shock response was thought to be restricted to Drosophila and certain tissues. In 1978, Schlesinger discovered an analogous response to heat shock in cultured avian and mammalian cells (Schlesinger 1986; Schlesinger 1990). Studies in *E.coli* (Yamamori, Ito et al. 1978) and Tetrahymena (Fink and Zeuthen 1980) revealed similar results. Following these observations, heat shock proteins were recognised as being present in almost every cell and tissue type, in explanted tissues and cultured cells and in all organisms from bacteria to human (Lindquist 1986).

1.5.4 Heat shock proteins

Heat shock/ stress proteins (HSPs) are a family of functionally-related proteins. They were initially found to be expressed intracellularly in cells subjected to stressful conditions. Their expression was transcriptionally regulated and they were believed to be part of a stress response which has evolved to protect stressed cells from adverse insults.

Not long after their discovery, several of the major HSPs were also shown to exist in cells under natural conditions and to be essential for normal physiological cell function, growth and development (Schlesinger 1990). For example HSP60, HSP90 and HSC70 are constitutively expressed under normal conditions in mammalian cells, whereas, HSP70 and HSP27 are induced by various stressful stimuli (Garrido, Gurbuxani et al. 2001). Most of the heat shock proteins have also been found to be induced by other stressful agents. Although the particular effects vary among organisms, anoxia, ethanol, certain heavy metals and chemicals and free oxygen radicals induce the expression of the proteins in almost all cells (Lindquist and Craig 1988).

The significant common feature of HSPs is that they are highly conserved during evolution among divergent organisms (Schlesinger 1990) and there have been very few changes to the amino acid sequence and structure of HSPs during evolution (Neuer, Spandorfer et al. 2000). For example, the major HSP70 protein has about 50% of its genomic sequence conserved between *E.coli* and human or 73% between human and Drosophila (Schlesinger 1990).

1.5.5 Heat shock proteins, extra or intracellular proteins

Until relatively recently, the general perception was that mammalian HSPs merely existed in intracellular compartments and the only circumstance they were found in extracellular space was in pathological conditions such as necrotic cell death (Hightower and Guidon 1989). The reason behind the old notion was, in part at least, due to the fact that HSPs do not express the typical N-terminal signal peptide sequences that are typically required for protein secretion and therefore cannot, theoretically, be released under non-pathological conditions (Horvath, Multhoff et al. 2008). However, observing detectable amounts of extracellular HSPs in normal physiological conditions circumvents the classical dogma.

A number of HSP isoforms such as HSP60 and HSP70 have been found in the peripheral circulation (Pockley, Shepherd et al. 1998; Pockley, Bulmer et al. 1999), HSP70 has been found in extracellular fluids (Georgiou, Sostaric et al. 2005) and bound to cell membranes (Schmitt, Gehrmann et al. 2007) in normal individuals. A range of cells from various types

possess the capability to secrete heat shock proteins into the extracellular environment in varying amounts, which is assumed to be due to difference in cell type capacities but the mechanism/s how HSPs are released from viable cells are not fully understood.

Investigators trying to identify heat shock protein secretory mechanisms were not successful at blocking the release of these components into the extracellular environment via inhibition of conventional secretory pathways. Hence, it was suggested that release of HSPs from intact cells occurs via selective active pathways rather than common secretory ones (Hightower and Guidon 1989). Following that, secretion via a number of other alternate non-classical secretory pathways has been suggested for HSPs including co-transport with other proteins which contain the trans membrane peptide (Multhoff 2007; Schmitt, Gehrmann et al. 2007), binding of HSPs to phosphatidylserine on the inner membrane and transport to the outer membrane by a flip flop mechanism (Arispe, Doh et al. 2004; Schilling, Gehrmann et al. 2009), transport via exosome-mediated mechanisms (Lancaster and Febbraio 2005) and finally interaction of HSPs with membrane lipid rafts (Arispe, Doh et al. 2002).

Exosomes are tiny vesicles formed within the membranes and their fusion with the cell membrane leads to the release of exosomal contents such as HSP70, HSC70 and HSP90 in tumour cells into the extracellular space (Thery, Zitvogel et al. 2002; Chaput, Taieb et al. 2004) or HSP70 in reticulocytes (Mathew, Bell et al. 1995). Specialised membrane domains which function in membrane sorting and signal transduction ('lipid rafts') might also be involved in the transport HSP70 to the cell membrane and eventually its secretion into the environment (Broquet, Thomas et al. 2003).

1.5.6 Heat shock protein families

The HSPs are classified into families on the basis of their estimated molecular weights in kilodaltons (Lindquist and Craig 1988; Garrido, Gurbuxani et al. 2001). The most comprehensively studied and functionally significant families of molecules are:

1. **HSP90:** This family includes two major isoforms of HSP90, α and β, which are integral to cell survival. They exist constitutively in high amounts and represent about 1-2% of

the cellular protein content (Lanneau, Brunet et al. 2008). A significant function of HSP90s is their selective interaction with protein kinase transcription factors and, most notably steroid receptors such as those for oestrogen and progesterone. Through binding, HSP90 keeps the receptors in their inactive state and prevents further transcriptional activity until the appropriate hormone signals interfere (Lindquist and Craig 1988).

2. **HSP70:** This is the most highly conserved family of HSPs and contains proteins ranging from 66-78 kDa. Some are primarily localised in the cytosol, such as the inducible HSP70 or constitutive HSC70, whereas others are located in mitochondria and others in the endoplasmic reticulum (Lanneau, Brunet et al. 2008). Members of this family need to bind with ATP molecules in order to fulfil their protective role in cells (Lindquist and Craig 1988).

3. **HSP60:** Also called chaperonins, members of this family are primarily located in mitochondria. Although principally expressed constitutively, slight elevations of HSP60 members can be seen under stressful conditions, particularly heat. Their ATP-dependant function is regulated by binding to HSP10 (Lanneau, Brunet et al. 2008).

4. **Small HSPs:** Members of this group vary in size between 15 to 30 kDa and have sequence homologies and biochemical properties in common. The most studied member is HSP27, an ATP-independent molecule whose function is to prevent proteins from aggregating. Its expression occurs very late after exposure to several stresses. It is expressed in very high amounts in cancer cells and is associated with cellular resistance to anti-cancer therapies (Lanneau, Brunet et al. 2008).

Table 1.1 shows different families of heat shock proteins, their related properties and main functions.

Table 1.1 Families of heat shock proteins, their related properties and main functions

Categories	Main Members	Co-chaperones	Location	Type of Expression	Main Function
HSP90	HSP90α	p50/Cdc37, P23, Aha1	Cytoplasm	Constitutive	Regulation of tyrosine kinase & steroid hormone transcriptional activities, Protein folding
	HSP90β		Cytoplasm	Constitutive	
HSP70	HSP70	HSP40,110	Cytosol/Nucleus	Stress Induced	Protein transport, repair& assembly, Anti-apoptosis
	HSC70	HSP40,110	Cytosol/Nucleus	Constitutive	
	GRP75(Mortalin)	DnaJ	Mitochondria	Constitutive	
	Bip(GRP78)	DnaJ	ER		
HSP60	HSP60(Chaperonin)	HSP10	Mitochondria	Constiutive	Prevention of protein aggregation& misfolding, Immunogenic property
	TCP1	HSP10	Mitochondria	Induced	
sHSP	HSP27		Cytosol / Membrane / Nucleus	Constitutive & Induced	Anti-apoptosis , Prevention of protein aggregation
	αCrystaline				
	βCrystaline				

1.5.7 New nomenclature of heat shock proteins

The expanding number of heat shock protein members and the inconsistencies in their nomenclature led to introduction of new guidelines for nomenclature of this protein superfamily - **HSPC** (HSP90), **HSPA** (HSP70), **HSPD** (HSP60) and **HSPB** (small HSPs).The nomenclature is largely based on the nomenclature assigned by HUGO Gene Nomenclature Committee and used in the National Centre of Biotechnology Information Entrez Gene Database for the heat shock genes (Kampinga, Hageman et al. 2009). In order to follow the up to date guidelines and avoid further confusion, the new HSP nomenclature will be used throughout this thesis.

1.6 General structure of heat shock proteins

The basic protein structures for only some of the HSP family members, notably HSPC and HSPA have been determined based on structural and proteolytic studies. HSPC consists of three structural domains: an N-terminal nucleotide binding domain (NBD) that binds to HSPC peptide inhibitors, a middle segment that interacts with client proteins and is also responsible for catalysing the ATP hydrolysis, and the C terminus, which is involved in homodimerisation (Javid, MacAry et al. 2007).

In contrast, HSPA contains two domains: an NBD and a substrate binding domain (SBD). The 44-kDa NBD (N terminus) has ATPase activity and associates with the HSPA co-chaperone DnaJ. The 27-kDa SBD (C terminus) comprises the main substrate binding domain which is composed of two four anti-parallel strands, four connecting loops that make contact with the bound substrate and a helical lid section (Javid, MacAry et al. 2007). Both HSPC and HSPA molecules have a conserved linker or lid region which mediates the connection between major subdomains and is suggested to be critical for inter-domain communication (Javid, MacAry et al. 2007).

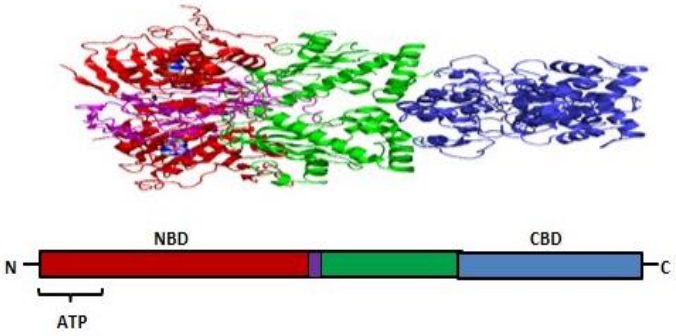

Figure 1.8 Schematic image of *E.coli* HSPA1A as determined by structural and limited proteolysis studies. HSPA1A has two major domains: a 44-kDa N-terminal NBD (red) and a 27-kDa C-terminal substrate binding domain (green and blue). This region comprises the main substrate binding domain (green; 18 kDa) that consists of two times four anti-parallel strands and four connecting loops and makes contact with the bound substrate, as well as an -helical lid region (blue; 10kDa). The molecule has a linker region (purple) that is thought to mediate communication between the major domains. The image was redrwan from http://atlasgeneticsoncology.org/Deep/HSPinCancerTreatment.html

1.7 General functions of heat shock proteins

Based on the context of their discovery, HSPs were initially renowned for the protective role they play in cells against stressful stimuli – their chaperone function inside the cells. According to a traditional definition, chaperones are "a family of unrelated classes of proteins that mediate the correct assembly of other poly peptides, but are not themselves components of the final functional structure" (Ellis and van der Vies 1991). In other words, chaperones are intracellular proteins that bind to and stabilise other unstable proteins. Stabilisation refers to folding, the oligomeric assembly of newly synthesised poly-peptides, their transport to particular sub-cellular compartments, switching between active and inactive conformations and preventing deleterious aggregation of target proteins (Hendrick and Hartl 1993).

HSP chaperoning activity was initially thought to be mainly limited to stressful conditions. However, abundant expression of different types of functional HSPs in normal unstressed cells led to the present concept that *in vivo* homeostatic action of HSPs covers both stressful and normal cellular situations. It is now apparent that HSPs are essential for maintaining normal cellular homeostasis.

1.8 Specific functions of heat shock proteins

1.8.1 Immunogenicity

In addition to their homeostatic and protective properties when in the intracellular environment, HSPs can elicit either innate or adaptive immune responses when located in the extracellular space or on the plasma membrane (Schmitt, Gehrmann et al. 2007). Members of HSPA and HSPC families are the principal regulators of the host immune system (Schmitt, Gehrmann et al. 2007), and this capacity is manifested via three different pathways:

1. HSPs have been reported to be important in the process of cross-presentation of tumour-derived antigenic peptides on antigen presenting cells (APCs) to appropriately-specific responding $CD4^+$ and $CD8^+$ -T cell populations. Exposure of the antigen initiates an antigen specific cellular immune response. This is named as peptide carrier function (Wells and Malkovsky 2000).

2. Tumour cells have been identified as being natural sources for extracellular HSPs and particularly HSPA1A (former HSP70). HSPs initiate the secretion of pro-inflammatory cytokines including TNF-α, IL-1, 2, 6 (Asea, Kraeft et al. 2000) via interactions with Toll-like receptors (TLRs) and CD14 on antigen presenting cells (APCs). This is the cytokine inducing effect of HSPs (Schmitt, Gehrmann et al. 2007).

3. Tumour cells have been found to have the capacity to selectively express HSPA1A, a member of HSPA family, on their surface (Multhoff, Botzler et al. 1995). HSPA1A selectively expressed on the membrane of tumour cells acts as a target recognition structure for activated natural killer (NK) cells. The interaction of membrane HSPA1A positive tumour cells with activated NK cells result in a perforin independent, granzyme B-mediated killing of the former by the latter (Multhoff, Botzler et al. 1995; Schmitt, Gehrmann et al. 2007).

1.8.2 Regulation of cell apoptosis

Apoptosis is a natural process of cell death aimed at removing old or unhealthy cells and results from a series of genetically programmed events. Two pathways lead to apoptosis: the intrinsic pathway in which the mitochondria act as a coordinators of the catabolic reactions leading to apoptosis, and the extrinsic pathway in which plasma membrane death receptors initiate the process via interaction with intracytoplasmic apoptotic effectors (Garrido, Gurbuxani et al. 2001).

Some HSP members regulate the apoptosis pathways at multiple stages in the cell. HSPB1 (formerly known as HSP27) exerts its anti-apoptotic effect by neutralising the toxic effects of oxidised proteins (Rogalla, Ehrnsperger et al. 1999) and cooperating with other intrinsic anti-apoptotic members (Garrido, Bruey et al. 1999). It has also been shown to block death receptor pathway by deactivating the associated receptors (Garrido, Gurbuxani et al. 2001).

On the other hand, HSPA1A increases the survival of cells by protecting them from energy deprivation related to cell death (Wong, Menendez et al. 1998), or by reducing mitochondrial

damage, nuclear fragmentation (Buzzard, Giaccia et al. 1998) and blocking the apoptotic activator factors in intrinsic pathway. Extracellular HSPA1A promotes the killing of tumour cells by interacting with lipid complexes on their membrane (Schilling, Gehrmann et al. 2009). In contrast, HSPD1 (former HSP60) and HSPC1 (former HSP90) at some circumstances appear to act as pro-apoptotic effectors in cells along with their original cytoprotective role. The interactions of different HSP members with various stages of intrinsic and extrinsic apoptotic pathways are illustrated in Figures 1.8 and 1.9, respectively.

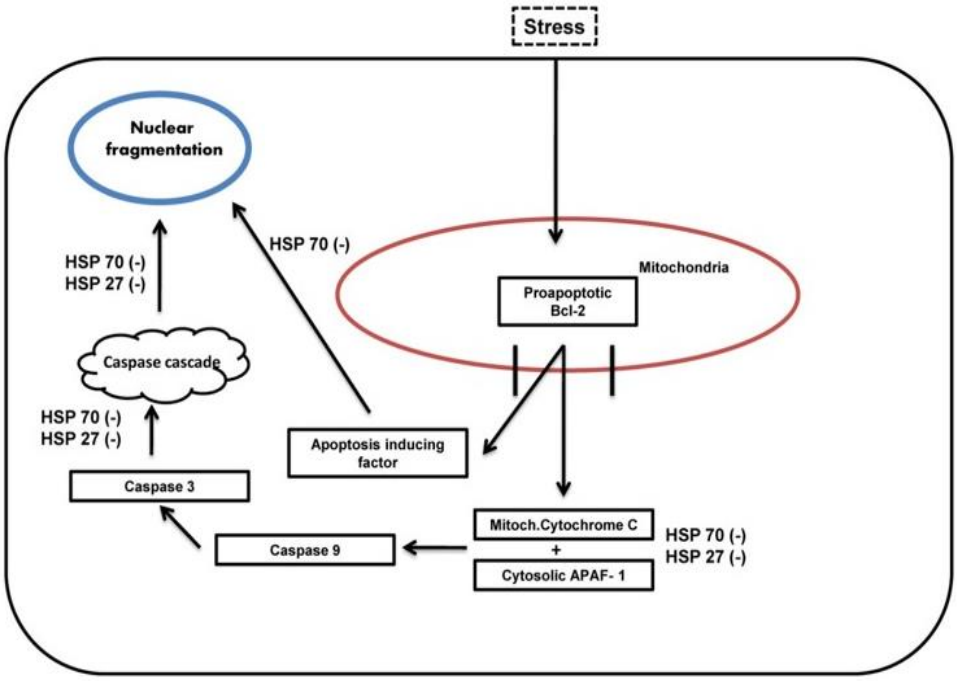

Figure 1.9 Schematic model of intrinsic cell apoptosis; Mitochondrial outer membrane permeabilisation in response to apoptotic signals from varying origins results in the release of molecules which are normally captured within the mitochondria such as cytochrome C. Cytosolic cytochrome C interacts with apoptotic protease activating factor 1 (Apaf-1) which in turn binds to caspase-9. Activated caspase-9 triggers the apoptotic caspase pathway. Apoptotic inducing factor (AIF) also as a apoptogenic molecule released from mitochondria is directly transported to nucleus to cause nucleus alterations independent of caspase cascade. HSPA1A and HSPB1 interact with apoptotic molecules at multiple stages to prevent the progression of the pathway.

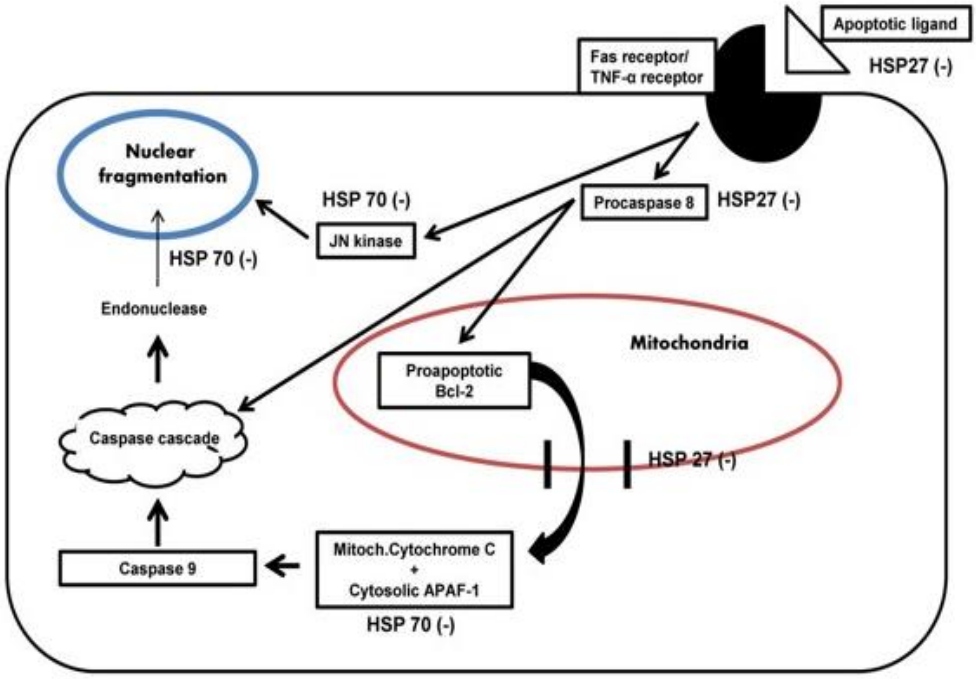

Figure 1.10 Schematic model of extrinsic cell apoptosis; Activation of plasma membrane death receptors, provokes caspase 8 which in turn leads into either direct activation of caspase cascade or induction of mitochondrial apoptotic pathway. HSPA1A and HSPB1 exert anti-apoptotic functions by blocking this pathway at various stages.

1.9 Extracellular heat shock protein function; inflammatory, anti-inflammatory, cytoprotective effects

The discovery of exogenous HSPs prompted a re-evaluation of the classical concept of HSPs as being exclusively intracellular maintainers of homeostasis and introduced a new perspective in the functioning domain of these proteins in various systems. Investigating and understanding the physiological efficacy of heat shock proteins as extracellular components continues to be an emerging field. Heat shock proteins are highly versatile molecules which take different and even opposing roles in different systems and circumstances.

In a number of human organ diseases, various exogenous heat shock protein isoforms have been associated with the inflammatory components of the disorder such as rise in serum HSPD1 in early stages of atherosclerosis (Pockley, Wu et al. 2000), elevated levels of HSPA1A complexed with IgG and albumin in plasma of type 1 and 2 diabetic individuals (Finotti and Pagetta 2004; Hunter-Lavin, Hudson et al. 2004) and high serum concentrations of HSPA1A along with inflammatory markers TNF-α and IL-6 in acute infection (Njemini, Lambert et al. 2003). These findings led to the general perception that extracellular heat shock proteins act as inflammatory mediators.

However, in other inflammatory situations such as organ transplantation, anti-inflammatory properties of HSPs dominate and they appear to be involved in down-regulating the adverse consequences of the inflammatory process. This in turn, will lead into delay in graft rejection (Birk, Gur et al. 1999). Exogenous heat shock proteins achieve this clinically significant matter by diverting the pro-inflammatory process towards an immune-regulatory pathway.

In addition to the immunological activities of circulating heat shock proteins which has attracted immense scientific attention, a number of investigations have also considered the non-immunological influence which heat shock proteins have on a range of systems and cells. Cytoprotection, among all the non-immunological functions is an important aspect to consider, since such properties provide a potential basis for novel preventative and therapeutic strategies.

For years it was known that intracellular heat shock proteins protect cells from environmental insults. As the authentic function of the heat shock protein family, this seems applicable to extracellular isoforms as well. One of the early reports on HSP release documented the transfer of HSPA1A and HSPA8 (formerly known as HSC70) to squid axon released by the adjacent glial cells (Tytell, Greenberg et al. 1986). This discovery led to assumption that neuronal cells which cannot respond to physical and metabolic insults acquire protection via receiving HSPs released by neighbouring glial cells. Another report suggesting cytoprotective roles for circulating heat shock proteins was the observation that extracellular members of the HSPA family could prevent the death of spinal sensory neurons induced by axotomy (Houenou, Li et al. 1996), or the death of cultured aortic smooth muscle cells subjected to heat stress *in vitro* (Johnson, Berberian et al. 1990).

In the reproductive system, extracellular HSPA8 has been identified in the isolated fraction derived from the apical segment of the oviductal epithelial cells and been shown to extend the survival of sperm from a number of species *in vitro* (Smith and Nothnick 1997; Fazeli, Elliott et al. 2003; Boilard, Reyes-Moreno et al. 2004; Elliott, Lloyd et al. 2009). All of these observations support the validity of the concept that HSPs perform survival enhancing roles in nature.

Rationally speculating, the cytoprotective effects of HSPs must be achieved by target cell membrane interactions which include or exclude internalisation. Preliminary studies have provided useful insights by revealing that the delayed death of stressed cells induced by extracellular HSPA1A is a result of increases in intracellular levels of HSPA1A (Berberian, Myers et al. 1990). It has also been shown that cellular protection by exogenous HSPs does not necessarily involve internalisation (Berberian, Myers et al. 1990).

The HSP-membrane relationship has been reported elsewhere in studies on mammalian spermatozoa in which scientists observed that a brief co-incubation with surface membrane extracts of oviductal epithelial cells enhanced sperm viability and motility (Boilard, Reyes-Moreno et al. 2004; Elliott RM 2009). The search for the effectors discovered detectable amounts of HSPD1 tightly bound to the surface of bovine spermatozoa after exposure to extracts of the apical oviductal epithelium (Boilard, Reyes-Moreno et al. 2004). Since it is

known that HSPs positively affect cellular functions by correcting protein folding and influencing transport across cellular membranes, it was concluded that the exogenous forms of this family of proteins in cellular membrane extracts protect the integrity of sperm plasma membranes via direct or mediated association, and that these effects are likely accomplished by modulating membrane protein transport and conformation. Consequently, various signalling pathways in favour of cell longevity are triggered or blocked as a result of these interactions (Boilard, Reyes-Moreno et al. 2004).

Having noted all these, the cytoprotective functions of HSPs to a great extent is attributed to their anti-apoptotic properties. Of note, HSPA1A and HSPB1 have been shown to directly interact with particular key proteins at multiple stages of the apoptosis machinery and thereby block the downstream events which lead into cell death (Garrido, Gurbuxani et al. 2001). On the other hand, HSPs act as cellular chaperones to enable cells to cope with insults by refolding damaged and misfolded proteins to their native structure, reversing harmful protein aggregation and assisting in the degradation of irreversibly damaged proteins.

Moreover, the ability of HSPA1A and HSPD1 isoforms in some species to induce ion-conducting channels within cell membrane bilayer (Alder, Austen et al. 1990) might explain, at least in part, the ability of these proteins to enhance the viability and promote stress reducing pathways inside cells.

1.10 Heat shock proteins and reproduction

1.10.1 Effect of heat shock proteins on spermatozoa

Although somatic cells are capable of producing HSPs in response to stress, spermatozoa lose their protein translational machinery during spermatogenesis and cannot therefore synthesise new proteins. However, high resolution microscopic and immunolocalisation methods have revealed the existence of HSPD1, constitutive HSPA8, inducible HSPA1A and HSPC1 and constant dynamic changes in their localisation over consequent maturational stages in a number of mammalian spermatozoa (Huang, Kuo et al. 1999; Kamaruddin, Kroetsch et al. 2004; Spinaci, Volpe et al. 2005; Lachance, Fortier et al. 2009). Therefore, HSPs in mature

sperm are produced during spermatogenesis (rather than newly, stress-induced) (Spinaci, Volpe et al. 2005), or are absorbed from their local environment such as seminal plasma (Kamaruddin, Kroetsch et al. 2004) or the female reproductive tract.

Low levels of intracellular HSPD1 expression in spermatogonia (primary spermatozoa) is associated with a high level of apoptosis (Neuer, Spandorfer et al. 2000). Furthermore, sperm HSPA1A appears to be involved in reducing the deleterious effects of high temperature on quality and development, sperm-oocyte interactions (Kamaruddin, Kroetsch et al. 2004) and the preservation of sperm membrane integrity via direct interactions with lipids and protein components of sperm membrane (Spinaci, Volpe et al. 2005). Intracellular HSPC1 protects sperm against oxidative stress and maintains its motility by enhancing ATP metabolism (Huang, Kuo et al. 1999).

HSPD1, HSPA1A and HSPC1 have also been identified in other regions of the reproductive system such as the mammalian endometrium (Tabibzadeh and Broome 1999), oviductal epithelium (Boilard, Reyes-Moreno et al. 2004; Elliott RM 2009) and the seminal plasma (in the case of HSPC1). They are all associated with maintaining sperm membrane integrity and signalling pathways (Boilard, Reyes-Moreno et al. 2004), viability (Boilard, Reyes-Moreno et al. 2004; Elliott RM 2009) and motility (Huang, Kuo et al. 2000; Boilard, Reyes-Moreno et al. 2004).

1.10.2 Effect of heat shock proteins on oocyte

Similar to spermatogenesis, oogenesis in mammals is also accompanied by HSP expression, although this area is little studied. Fully developed oocytes are unable to synthesise inducible HSPs and therefore are very sensitive to high temperatures (Neuer, Spandorfer et al. 2000). This reveals the important function of HSPs in the female gamete. However, HSPs, particularly HSPA8, are expressed in high amounts during oocyte growth and early stages of development, the synthesis of which disappears completely as the gamete approaches the end of its maturation process. Hence, HSPs seem to play roles in the preservation of oocyte metabolic activity, survival, and also the ovulation process (Neuer, Spandorfer et al. 2000).

1.10.3 Effect of heat shock proteins on fertilisation and embryo development

Fertilisation begins at the point of sperm-oocyte recognition, their attachment and zygote formation. HSPs are among the first proteins that are synthesised in the growing mammalian embryo (Neuer, Spandorfer et al. 2000). Constitutive HSPA8 is expressed as soon as zygotic cleavage begins, whereas expression of the inducible form is delayed until the blastocyst (16 cell) stage.

A dynamic re-localisation and distribution of HSPA1A from acrosomal to equatorial region on bovine spermatozoa indicates its role in gamete interaction (Matwee, Kamaruddin et al. 2001). The supporting evidence is that exposure to HSPA1A monoclonal antibodies disrupts gamete interaction, fusion and fertilisation (Matwee, Kamaruddin et al. 2001). It is also very likely that HSPA1A exerts significant effects on early embryonic development, since antibodies to HSPA1A in the 3 to 9 day bovine embryo increases apoptosis and hinders the embryo reaching different developmental stages with higher number of cells (Matwee, Kamaruddin et al. 2001). Another group has determined the effect of three anti-HSPD1, HSPA1A and HSPC1 antibodies on 2-day mouse embryos and observed that each antibody inhibits the progress of unique embryonic developmental stages. The anti-HSPD1 antibody exerts the most detrimental effect on the third day, anti-HSPA1A on the fifth day and anti-HSPC1 at later times (Neuer, Mele et al. 1998). Collectively, inhibiting cellular HSP function during early stages of embryogenesis leads into embryonic growth retardation or arrest.

Experiments exposing early embryos to high temperatures, have revealed lower proportions of apoptotic cells in them (Matwee, Kamaruddin et al. 2001). As it is clear, heat induces HSP synthesis through heat shock response and the newly-induced protein protects the embryo.

1.11 Concluding remarks

Heat shock proteins are essential components of life. They guard living cells from harmful stimuli, regulate protein structure and location and prevent cell death when necessary. Like all other organs, mammalian reproduction relies on the beneficial action of HSPs in various stages. HSPs terminate stressful effects to gametes and maintain their viability and motility.

Fertilisation and embryo development also heavily depend on the presence of these proteins. Taken together, growth, development, reproduction and life are not possible without HSPs. However, most of the studies so far have investigated the effect of intracellular HSP isoforms on reproduction and very few studies have considered the role of extracellular HSPs in this field. Further insight into this area is required given the evidence that exogenous HSPs appear to have a number of protective and therapeutic properties which might be highly relevant to the development of novel reproductive technologies.

1.12 Aims and objectives of the thesis

Previous investigations have documented the expression of extracellular isoforms of HSPA in response to the presence of gametes within the mammalian oviduct (Georgiou, Sostaric et al. 2005) and also interactions between gametes and exogenous HSPs that are released from oviductal cells into the environment (Boilard, Reyes-Moreno et al. 2004). Furthermore, it has been demonstrated that the regulation of essential gamete properties that are favourable to successful fertilisation is not achieved unless there is an intimacy between the induced oviductal products and gametes. An example would be the effect of HSPA8 which resides on the oviductal epithelial cell surface has on the maintenance of sperm survival (Elliott, Lloyd et al. 2009). All of these effects constitute a local regulatory system which is created by reciprocal associations that result in the maintenance of homeostasis and reproductive potential. Nevertheless, minimal knowledge currently exists regarding the exogenous heat shock protein-gamete and particularly HSPA8-spermatozoa associations. Even much less is known about the receptors which are activated and pathways which are triggered during this type of cell-protein interaction.

This project hypothesised that extracellular HSPA8 is an active component of the oviductal sperm storage system, positively affects sperm properties and function in favour of successful fertilisation. Hence the overall aim of this study was to characterise the effect of extracellular HSPA8 on sperm physiology and function and to identify the biological significance that this oviductal protein might have on the establishment of fertilisation.

In search for the answer, uterine bovine recombinant HSPA8 was generated in an *E. coli* over expression system in our laboratory to be applied in *in vitro* studies on pig sperm. The initial objective was to determine the influence of this extracellular protein on sperm characteristics such as viability, capacitation, mitochondrial activity and their capacity to bind oviductal epithelial cells (Chapter 2). With the effect of HSPA8 on sperm physiology characterised, a well-established pig *in vitro* fertilisation (IVF) system was employed to investigate the effect of this protein on sperm fertilisation performance (Chapter 3). The major part of this thesis was dedicated to understanding the mechanisms associated with the influence of HSPA8 on maintenance of sperm cell viability.

The work presented in Chapter 4 was pursued following observations regarding the beneficial enhancing effect of HSPA8 on sperm viability on the basis of membrane integrity viability assays. Work evaluated the consequences of HSPA8-membrane associations using 'fluorescence recovery after photobleaching' (FRAP).

Given the observations from HSPA8-induced alterations in sperm membrane lipid dynamics and the insight to the significant role of cholesterol in mediating membrane fluidity, the aim of work in Chapter 5 was to decipher HSPA8-cholesterol interactions and the possible role which membrane cholesterol plays as the mediator of HSPA8 effects on the sperm membrane. These studies were undertaken by modifying membrane cholesterol using established cholesterol acceptors and donors.

Membrane lipid alterations in response to HSPA8 treatment led to the development of the work presented in Chapter 6 which used confocal imaging technology to determine the localisation of HSPA8 in sperm. Protein fluorescent labelling and flow cytometry techniques were used to elucidate physiological characteristics of spermatozoa which associated with HSPA8.

The work presented in Chapter 7 investigated the intracellular pathways in sperm that are activated downstream to HSPA8-membrane stimulation.

Finally in chapter 8, all the results obtained from each individual chapter and their biological, clinical and commercial implications are discussed and potential future prospects for following this work are presented.

Chapter 2

Effect of extracellular HSPA8 on boar sperm characteristics

2.1 Introduction

Despite great achievements made in assisted reproductive technologies in human and animal reproduction, there are still practical obstacles in the artificial reproductive methods (O'Hara, Hanrahan et al. 2010). The *in vitro* maintenance of sperm viability is one major constraint (O'Hara, Hanrahan et al. 2010). Many factors including osmotic stress, oxidation, temperature fluctuations as well as extender composition, decrease sperm survival by causing damage to spermatozoa during handling, storage and preparation procedures (Tapia, Macias-Garcia et al. 2012; Jimenez, Perez-Marin et al. 2013). The optimisation of conservation protocols and extenders is therefore an important element for enhancing *in vitro* semen storage methods and maximising fertilisation potential.

The ability of the female reproductive tract to prolong sperm survival has been an inspiration for scientists to decipher and employ the naturally existing *in vivo* factors and mechanisms to preserve sperm viability for long-term *in vitro* storage. The maintenance of sperm viability over prolonged periods inside the female reproductive tract is highly developed in those species with gaps of hours to days and even months between mating and ovulation (Topfer-Petersen, Wagner et al. 2002; Holt and Fazeli 2010; Roy and Krishna 2011). These species such as snakes, turtles, salamanders as well as birds and mammals (Elliott, Lloyd et al. 2009) have adopted sperm storage which is predominantly formed in the oviduct (Fazeli, Duncan et al. 1999; Green, Bredl et al. 2001). Oviductal sperm storage has been extensively documented in many vertebrates (Elliott, Lloyd et al. 2009) and the evolutionary significance of this phenomenon has been reported (Birkhead 1993).

It has been suggested that female sperm reservoir provides a secure and efficient setting for spermatozoa to remain viable until ovulation via two different local systems. One is by producing secretory fluid which contains various types of molecules into the oviduct lumen (Abe, Sendai et al. 1995; Aviles, Gutierrez-Adan et al. 2010) and the other is the mucosal epithelium (Smith and Yanagimachi 1990; Smith and Nothnick 1997; Elliott, Lloyd et al. 2009).

Upon arrival in the oviductal region, spermatozoa closely associate with both systems by bathing in the secreted fluid and establishing tight temporary bonds with the apical epithelial membrane. Each of the systems contains a number of proteomic factors that have been reported to positively influence sperm properties which are beneficiary to fertilisation (Elliott, Lloyd et al. 2009; Aviles, Gutierrez-Adan et al. 2010). Recently, the production of a novel group of proteins known as extracellular heat shock proteins (HSPs) by the oviductal epithelial cells in response to the presence of spermatozoa in the oviduct has gained considerable attention (Boilard, Reyes-Moreno et al. 2004; Georgiou, Sostaric et al. 2005; Elliott, Lloyd et al. 2009).

The term, extracellular HSPs, has not been around for very long since heat shock proteins were perceived to be strictly intracellular proteins in normal physiologic conditions. This misconception persisted for a considerable length of time among scientists simply because HSPs do not possess the signalling sequence which was originally thought to be necessary for proteins' translocation outside the cell (Multhoff 2007; Schmitt, Gehrmann et al. 2007). Therefore, a number of other alternate non-classical secretory pathways have been suggested for HSPs including co-transport with other proteins which contain the trans membrane peptide (Multhoff 2007; Schmitt, Gehrmann et al. 2007), binding of HSPs to phosphatidylserine on the inner membrane and transport to the outer membrane by a flip flop mechanism (Arispe, Doh et al. 2004; Schilling, Gehrmann et al. 2009), transport via exosome-mediated mechanisms (Lancaster and Febbraio 2005) and finally interaction of HSPs with membrane lipid rafts (Arispe, Doh et al. 2002).

In terms of mammalian reproduction, the major focus of studies so far has been largely on the expression of intracellular HSPs in male and female reproductive tract (Renoir, Radanyi et al. 1990; Tabibzadeh, Kong et al. 1996; Neuer, Spandorfer et al. 2000) and their role in different stages of reproductive process such as during early oogenesis (Dix 1997), throughout initial mitotic stages of spermatogenesis (Curci, Bevilacqua et al. 1987; Curci, Bevilacqua et al. 1991) and finally in zygote and embryo in their pre-implantation phase (Bensaude, Babinet et al. 1983; Morange, Favet et al. 1998). However, the discovery of extracellular HSPs in the female tract has kindled interest in the role of these proteins in different stages of reproduction.

HSPD (formerly known as HSP60) on the surface of bovine oviductal epithelium has been shown to maintain sperm viability and acrosome integrity following *in vitro* co-incubation (Boilard, Reyes-Moreno et al. 2004). Furthermore, HSPA8, a 70kDa heat shock protein purified from the apical segment of oviductal epithelial cells, prolongs sperm viability *in vitro* in a number of species (Elliott, Lloyd et al. 2009; Lloyd, Elliott et al. 2009).

The existence of HSPA8 (HSC70) in the extracellular environment of the oviduct and its ability to maintain sperm survival prompted us to establish studies that were aimed at providing insight into the effects that exogenous HSPA8 might have on important characteristics of spermatozoa. For this, boar spermatozoa were incubated with varying concentrations of a recombinant HSPA8 for 24 and 48 hrs *in vitro* and its effects on viability determined. The influence of short (15 min) exposure time on sperm viability and mitochondrial activity have also been studied. In addition, the effect of sperm capacitation on HSPA8-mediated effects on sperm viability after brief exposure (15 min) was also evaluated. The aim of these studies was to unravel the potential differential effects of HSPA8 on sperm in uncapacitated and capacitated states that naturally exist in the oviductal tract.

Capacitation is induced *in vivo* upon residence in the female reproductive tract and is considered to be an essential physiological milestone, as only capacitated sperm can fertilise the egg. However, capacitated spermatozoa die quickly soon due to capacitation-induced physiological changes (Hunter and Rodriguez-Martinez 2004; Rodriguez-Martinez 2007). Hence, we investigated whether HSPA8 can prolong the life span of *in vitro* capacitated sperm.

The specificity of the effect of HSPA8 on sperm viability was determined by evaluating the effect of HSPA1A (HSP70) and α-Tubulin as control proteins. We hypothesised that due to structural resemblance, HSPA1A and HSPA8 isoforms have similar functions and might therefore exert similar influence on sperm viability. Moreover, the effect of mixtures of HSPA1A/HSPA8 on sperm viability was tested based on the implications of the reports that exogenous HSPA1A/HSPA8 in combination, interact with a number of different cell types and influence their function such as modifications on membrane ion conductance or accelerating

early embryo cleavage (Brown, Martin et al. 1993; Negulyaev, Vedernikova et al. 1996; Browne, Swan et al. 2007). Additionally, studies have documented the presence of stress inducible HSPA1A and constitutive HSPA8 in extracellular forms in the oviductal secretions or in the epithelial membrane surface (Georgiou, Sostaric et al. 2005; Sostaric, Georgiou et al. 2006; Georgiou, Snijders et al. 2007; Lloyd, Elliott et al. 2009).

2.2 Materials and methods

All chemicals used for preparation of Tyrode's medium were purchased from BDH Laboratory Supplies, Poole, UK. Other chemicals were from Sigma-Aldrich company Ltd, Dorset, UK, unless otherwise stated.

2.2.1 Semen Preparation

Boar semen diluted and stored for 24 hours in Beltsville Thawing Solution (BTS) (Pursel and Johnson 1975) was obtained from a commercial artificial insemination station (JSR Healthbred Limited, Thorpe Whillougby, Yorkshire,UK). On the day of experiment, diluted boar semen was washed using Percoll gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Briefly, aliquots of 2-4 ml of diluted semen were layered over a Percoll density gradient. The iso-metric Percoll gradient consisted of 2 ml 70% (v/v) Percoll overlaid with 2 ml 35% (v/v) Percoll. The gradient was centrifuged at 200 g for 15 min followed by 15 min centrifugation at 900 g (Holt and Harrison 2002). The supernatant was removed and the pellet was resuspended in Tyrode's medium consisting of 3.1 mM KCL, 0.4 mM $MgCl_2.6H_2O$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, 0.3 mM $NaH_2PO_4.2H_2O$ (Elliott, Lloyd et al. 2009). Thereafter, semen samples were centrifuged at 900 g for 15 min and resuspended in Tyrode's medium. Sperm concentration was measured in duplicate using an improved Neubauer chamber (Marienfield, Germany) and adjusted to (5×10^6 / ml) in TALP which consisted of Tyrode's medium supplemented with 12 mg/ml bovine serum albumin, 21.6 mM sodium lactate and 1 mM sodium pyruvate, unless otherwise stated.

2.2.2 Evaluation of sperm viability

Sperm viability was evaluated using two fluorescent viability assays, Ethidium homodimer / Calcein-AM assay (Live / Dead Sperm Viability Kit, Molecular Probes, Eugene, Oregon, USA) and Propidium iodide / SYBR-14 assay (Molecular Probes). Live cells contain cytoplasmic esterase activity which can be used for enzymatic conversion of the nonfluorescent cell-permeant Calcein-AM to the intensely fluorescent Calcein. The polyanionic dye Calcein can easily pass through intact live cells' membranes and by the esterase activity of the enzyme produces a high intensity uniform green fluorescence (ex/em ~495 nm/~515 nm). Ethidium is excluded by the intact plasma membrane of live cells and is merely able to enter cells with damaged membranes. Inside the cell it binds to nucleic acid and goes under a 40-fold enhancement of red fluorescence. Hence membrane damaged or dead cells exhibit a bright red fluorescence (ex/em ~495 nm/~635 nm) under the epifluorescence microscope.

The fluorescence-based viability assay, Propidium iodide and SYBR-14, is produced specifically for analysing the viability of sperm. This combination of dyes label intracellular DNA. Live sperm cells with intact cell membranes fluoresce bright green (SYBR-14) and cells with damaged cell membranes fluoresce red (Propidium iodide). The fact that both dyes stain cellular nucleic acid prevents the vagueness of labelling different cellular components (Garner, Johnson et al. 1994; Garner and Johnson 1995).

Dyes were added to 100 µl of semen aliquots with 5 x 10^6 spermatozoa / ml to final concentrations of 0.4 µM and 0.08 µM for Ethidium homodimer / Calcein-AM, and 12 µM and 4 nM for Propidium iodide / SYBR-14, respectively. Samples were mixed and incubated for 30 min at 39°C in 5% v/v CO_2 for Ethidium homodimer & Calcein-AM assay and 15 min for Propidium iodide & SYBR-14 assay. A 10 µl aliquot of each preparation assay was placed on a slide and evaluated by using epi-fluorescence microscope (Olympus BH2, Olympus, London, UK) equipped with a dual rhodamine-FITC filter at 400x magnification. Two hundred spermatozoa were counted per slide (3 replicates for each sample) and classified as live spermatozoa (green) and dead spermatozoa (red). Spermatozoa with intact membrane (live spermatozoa) showed green fluorescence due to Calcein-AM or SYBR-14 staining, depending

on the assay. Spermatozoa with disrupted membranes (dead spermatozoa) showed red fluorescence due to Ethidium homodimer or Propidium iodide (Figure 2.1A).

2.2.3 Assessment of sperm mitochondrial activity

Mitochondrial activity in live cells based on electrochemical gradient across the mitochondrial inner membrane was evaluated by using a cationic, lipophilic dye, JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide). JC-1 is a fluorescent dye which emits red (~ 590 nm) or green (~ 525 nm) depending on normal or collapsed mitochondrial potential, respectively. Therefore, it is useful for determination of high or low mitochondrial activity in cells (Cossarizza, Baccarani-Contri et al. 1993; Gravance, Garner et al. 2000).

Due to the intact electrochemical potential gradient in normal cells, the dye forms red fluorescent aggregates (J-aggregates) in the mitochondrial matrix. Any insult that disrupts the mitochondrial membrane potential inhibits the JC-1 aggregation in the mitochondria and hence, the dye is distributed throughout the cell cytoplasm resulting in a shift from red (J-aggregates) to green fluorescence (JC-1 monomers) (Reers, Smith et al. 1991; Gravance, Garner et al. 2000). In sperm, the red JC-1 aggregates are mostly concentrated in midpiece due to the high density of mitochondria in the midpiece and to a less extent in the head (Figure 2.1B).

JC-1 kit was used according to manufacturer's instructions. Briefly, stock solutions of 200x JC-1 (1 mg/ml) were prepared in DMSO and stored in 25 µl aliquots at -20°C. On the day of experiment, 25 µl aliquot of the 200x JC-1 stock solution was thawed and vortex mixed in 4 ml of ultrapure water. The suspension was incubated for 1–2 minutes at room temperature to ensure that JC-1 is completely dissolved. Then, 1 ml of the JC-1 staining buffer 5x (supplied in the kit) was added to the suspension and mixed by inversion (staining solution). Semen (200 µl of 5 x 10^6 / ml concentration) were added to 200 µl of staining solution containing 12 µM Propidium iodide, samples were mixed and incubated for 20 minutes at 37°C in a humidified atmosphere containing 5% v/v CO_2. Then, 1x JC-1 Staining Buffer was prepared by diluting 1 volume of the JC-1 Staining Buffer 5x with 4 volumes of water and kept on ice. The semen-staining suspension was centrifuged at 600 *g* for 3–4 minutes at 2–8°C. The supernatant was removed and the pellet was placed on ice. Then, the pellet was washed with 200 µl of the ice-

cold 1x JC-1 Staining Buffer and resuspended in 400 µl of the same solution. The sample was kept on ice and analysed within 1 hour after staining.

The mitochondrial staining assay was combined with Propidium iodide to analyse mitochondrial activity in the live sperm population (Garner, Thomas et al. 1997). Spermatozoa that showed red fluorescence due to Propidium iodide were excluded. Two hundred live spermatozoa per slide (3 replicates for each sample) were evaluated using an epifluorescence microscope (Olympus BH2, Olympus, London, UK) at 400x magnification.

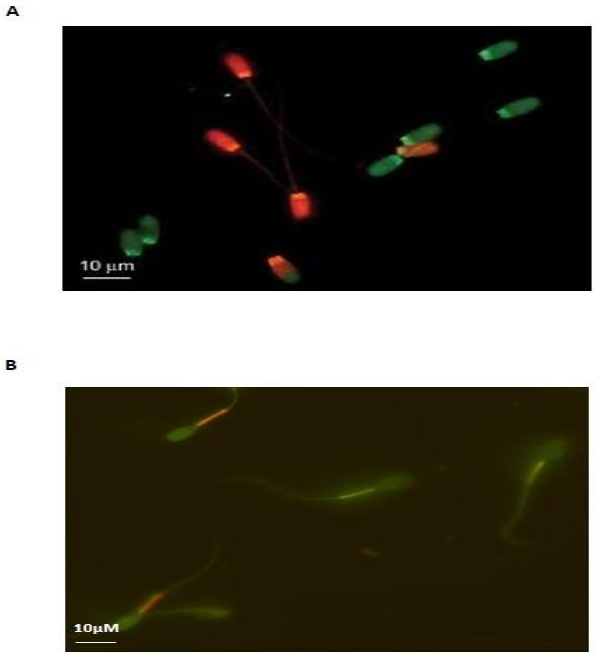

Figure 2.1 Epifluorescence microscopy of spermatozoa stained with different fluorescent probes; (**A**) SYBR-14 (membrane intact, green) & Propidium iodide (membrane disrupted, red), (**B**) mitochondrial stain Jc-1; spermatozoa with high mitochondrial activity show orange on midpeice and those with low mitochondrial activity show green.

2.2.4 Assessment of sperm capacitation

The Chlortetracycline Hydrochloride (CTC) staining method was used to assess the capacitation status of boar sperm as described by Fazeli *et al.* (Fazeli, Duncan et al. 1999) (Fazeli, Duncan et al. 1999) (Fazeli, Duncan et al. 1999) (Fazeli, Duncan et al. 1999). Briefly, a buffer containing 130 mM NaCl and 20 mM Tris was prepared. The CTC staining solution was prepared by adding 750 µM CTC and 5 mM D,L-cystein to the buffer solution. Then, the solution was filtered using a 0.22 µM filter and the pH was adjusted to 7.8. The CTC assay was performed by mixing equal volumes of semen diluted in TALP at 5 x 10^6 / ml concentration and CTC staining solution. After 30 seconds, an equal volume of 2% w/v paraformaldehyde in PBS was added to the sample. Finally, 2 drops of an antifade reagent Citifluor (Citifluor Ltd., London, UK) was added to each sample in order to preserve the fluorescence. Two hundred spermatozoa per slide (3 replicates for each sample) were evaluated immediately using an epifluorescence microscope at 400x magnification. CTC assay showed 3 patterns of uncapacitated (uniform distribution of fluorescence over the sperm head) capacitated (acrosomal fluorescence) and acrosome reacted spermatozoa (postacrosomal fluorescence) (Figure 2.2) as described by Wang et al. (Wang, Abeydeera et al. 1995).

To analyse capacitation in the live sperm population, CTC staining assay was combined with Propidium iodide staining. Spermatozoa that showed red fluorescence due to Propidium iodide were excluded from counting.

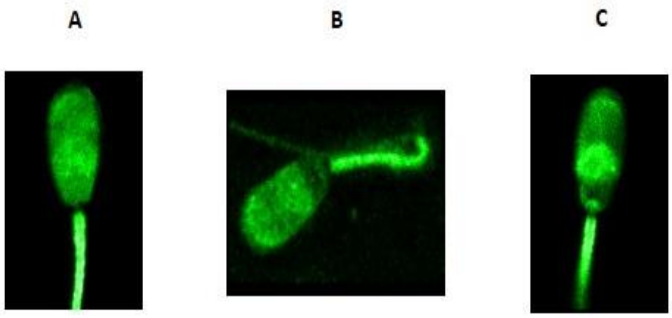

Figure 2.2 Illustration of; (**A**) non-capacitated, (**B**) capacitated and (**C**) acrosome reacted spermatozoa. Pictures kindly provided by Dr. Alireza Fazeli, University of Sheffield, United Kingdom.

2.3 Experimental design

2.3.1 Effect of different HSPA8 concentrations and HSPA8 co-incubation intervals with boar sperm on boar sperm viability

In order to characterise the effect of the recombinant bovine HSPA8 on boar sperm viability, different concentrations and different co-incubation intervals were tested. Aliquots of semen from 10 different boars diluted in TALP (5×10^6 spermatozoa / ml) were incubated with 0 (Control), 0.1, 0.5 and 1 µg/ml of HSPA8 at 39°C, 5% v/v CO_2 for 24 and 48 hours and for 15 min at room temperature. Samples were stained with SYBR-14 and Propidium iodide.

After initial experiment mentioned above, to investigate further if a short incubation time (15 min) is sufficient to extend sperm viability by HSPA8, diluted semen samples (5×10^6 spermatozoa / ml) of 3 boars were incubated with and without 0.5 µg/ml of HSPA8 at room temperature for 15 min and then, sperm viability was assessed by staining with Ethidium homodimer / Calcein-AM and Propidium iodide / SYBR-14 dyes separately for confirmation of the results.

2.3.2 Effect of varying concentrations of stress-induced HSPA1A and HSPA1A-HSPA8 combination on boar sperm viability

To identify the specificity of the recombinant bovine HSPA8 effect on enhancing sperm survival, the influence of individual HSPA1A (Recombinant human stress-induced HSPA1A, StressMarq,Victoria, BC, Canada), α-Tubulin (Abcam, Cambridge, UK) and HSPA8 proteins on sperm viability were tested. In addition, to figure out whether *in vitro* combinations of HSPA1A-HSPA8 had combinatory influence on prolonging sperm viability, Percoll-washed spermatozoa from 7 boars were incubated in TALP containing varying concentrations of HSPA1A (0.1, 0.5 and 1 µg/ml) and combinations of 0.1-0.1, 0.5-0.1, 0.5-0.5 and 1-0.5 µg/ml HSPA1A-HSPA8, respectively. A control sample with no protein as well as a sample with HSPA8 0.5 µg/ml were included for final comparison.

2.3.3 Neutralisation of the viability enhancing effect of HSPA8 by anti-HSPA8 antibody

The specificity of the observed effects were further evaluated by determining the capacity of a specific anti-HSPA8 antibody to block the effect of HSPA8 on sperm viability. For these experiments, Percoll-washed semen samples from 7 boars were pre-incubated with different concentrations (0, 0.1, 0.5, 1 µg/ml) of anti-HSPA8 antibody (polyclonal rabbit IgG antibody; Abcam, Cambridge, UK) for 30 min at room temperature. Thereafter, HSPA8 (0, 0.5 µg/ml) was incubated with pre-treated sperm samples for 15 min at room temperature, after which sperm viability was assessed.

2.3.4 Effect of short exposure time of HSPA8 on mitochondrial activity of boar sperm

To assess sperm mitochondrial activity under the immediate effect of HAPA8, the JC-1 staining was performed on the sperm samples from 9 boars and incubated with HSPA8 (0 and 0.5 µg/ml) and α-Tubulin (0.5 µg/ml), as control protein for 15 min at room temperature and assessed by fluorescence microscope.

2.3.5 Effect of sperm capacitation induction on the viability enhancing effect of HSPA8

We aimed to characterise the effect of sperm pre-incubation in capacitating conditions on HSPA8 ability to expand sperm viability during a 15 min exposure period. A total of 6 boar semen samples were incubated in TALP at 39ºC with 5% v/v CO_2 (capacitating condition) at different intervals: 0, 1, 2, 4 and 6 hrs. At each time-interval, incubated sperm samples were mixed with and without 0.5 µg/ml of HSPA8 for 15 min. Then, viability and capacitation status of each sample was determined separately.

2.3.6 Statistical analysis

Data were analysed by analysis of variance (ANOVA). When the results were significant in ANOVA, means were further tested with post-hoc Fisher's least significant difference (LSD) test to examine the effect of treatments within experimental designs by Statistica software, version 7 (WWW.StatSoft.com, 1984-2004). Data were expressed as mean ± s.e.m. A probability of $P \leq 0.05$ was considered to be statistically significant.

2.4 Result

2.4.1 HSPA8 enhanced sperm viability

Both long-term (24 and 48h) and short-term (15 min) sperm-HSPA8 co-incubation showed significant overall viability enhancement ($P \leq 0.0001$) regardless of the HSPA8 concentration. However, this positive survival enhancing effect appeared to be highest at 0.5 µg/ml at 15min and 24hrs and 1 µg/ml at 48hrs (Figure 2.3 & Table 2.1).

The outcome from brief sperm-HSPA8 exposure (15 min) was pursued further by repeating the bioassays with 0.5 µg/ml HSPA8. Sperm viability was evaluated using Propidium iodide / SYBR-14 along with another viability assay, Ethidium homodimer / Calcein-AM for comparison and validation.

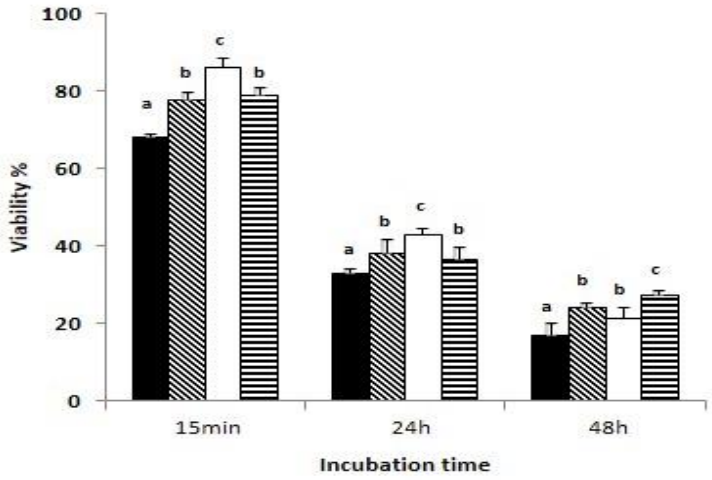

Figure 2.3 Effects of HSPA8 on boar sperm viability. Viability % (determined by SYBR-14 / Propidium iodide staining) of boar spermatozoa incubated for 15 min at room temperature, 24 and 48 hrs at 39°C in the presence of 0 (control) ■ , 0.1▨ , 0.5 □ and 1▤ µg/ml HSPA8. Significant beneficial effects of protein treatments on sperm viability was observed compared to controls at specific time intervals. Different letters denote significant ($P < 0.05$) differences within specific time points. n= 10, mean ± s.e.m.

Table 2.1 Summary of analysis of variance for the effect of HSPA8 on boar sperm viability.

Effect	SS	Degree of Freedom	MS	F	P
Intercept	672315.3	1	672315.3	8551.375	0.000000
HSPA8	7979.6	3	2659.9	33.832	0.000000
Time	155382.6	2	77691.3	988.178	0.000000
HSPA8*Time	3222.3	6	537.0	6.831	0.000001
Error	24529.7	312	78.6		

The beneficial effects of HSPA8 on sperm viability over a 15 min exposure to spermatozoa were evident as assessed by both viability assays, Propidium iodide / SYBR-14 and Ethidium homodimer / Calcein-AM ($P = 0.002$) (Figure 2.4).

2.4.2 HSPA8 enhanced sperm viability in a specific manner

The exposure of spermatozoa to α-Tubulin or varying concentrations of HSPA1A for 15 minutes had no effect on sperm survival, whereas exposure to HSPA8 significantly increased sperm viability (Figure 2.5A & Table 2.2). Combinations of HSPA1A and HSPA8 at varying concentrations appeared to have no influence on the survival of boar sperm, contrary to the positive effect of HSPA8 alone (Figure 2.5B). This suggested that addition of HSPA1A to spermatozoa may prevent HSPA8 from affecting sperm viability. The anti-HSPA8 antibody blocked the viability enhancing effect of HSPA8 in a concentration- dependent manner (Figure 2.5C & Table 2.3).

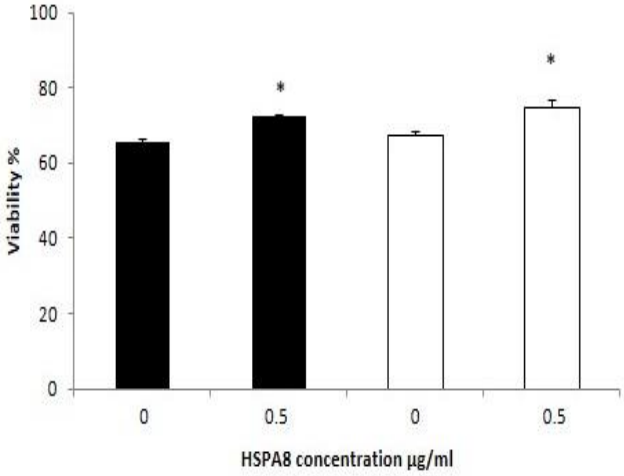

Figure 2.4 Comparing the effect of HSPA8 on sperm viability (membrane integrity) by two different dye exclusion viability assays, Ethidium homodimer / Calcein-AM ■ and Propidium iodide / SYBR-14 □. Sperm viability % of boar spermatozoa after 15 min exposure to TALP only (control) or HSPA8 0.5 µg/ml .* < 0.05, n = 3, mean ± s.e.m.

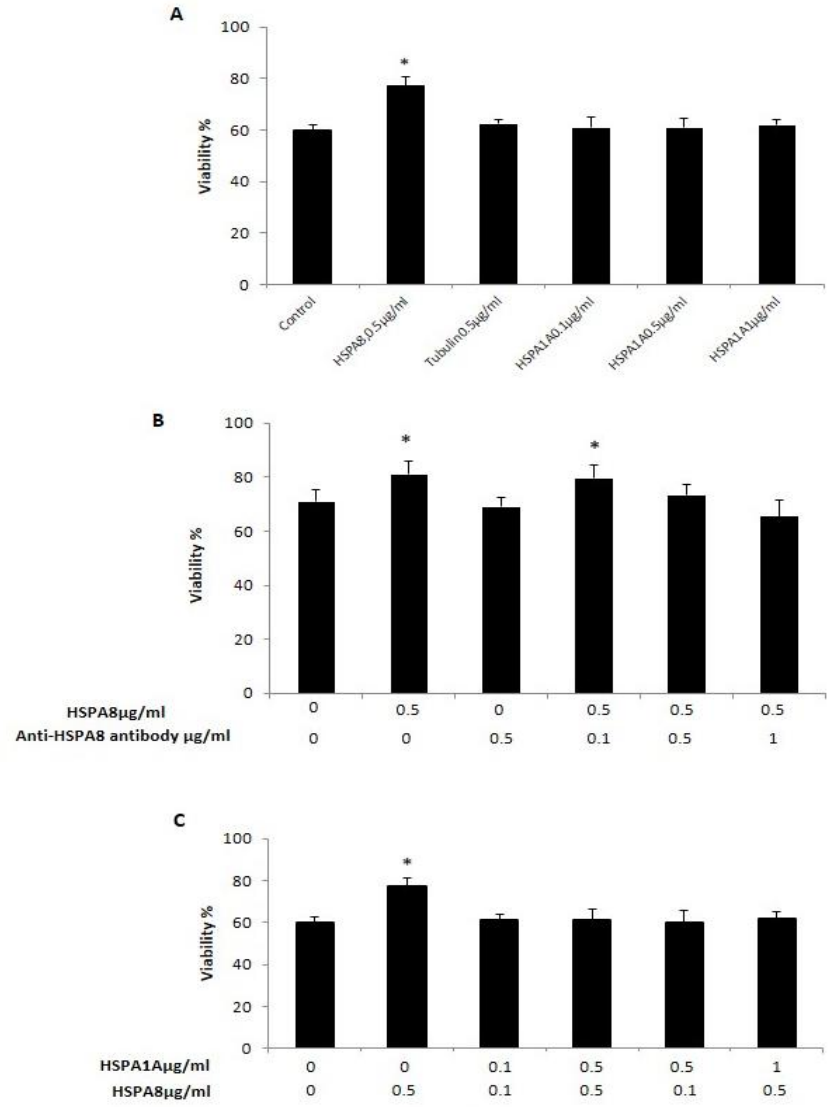

Figure 2.5 HSPA8 specificity and its combinatory effect with HSPA1A on sperm viability. (**A**) Sperm viability % (determined by Propidium iodide / SYBR-14 staining) of boar spermatozoa after 15 min exposure to 0 (control) , 0.5 µg/ml HSPA8, 0.5 µg/ml α-Tubulin and 0.1, 0.5, 1 µg/m HSPA1A, (**B**) Sperm viability % of boar spermatozoa after 15 min exposure to 0 (control), 0.5 µg/ml HSPA8, 0.5 µg/ml anti-HSPA8 antibody and combination of 0.5 µg/ml HSPA8 with 0.1,0.5 and 1 µg/ml anti-HSPA8 antibody, (**C**) Sperm viability % of boar spermatozoa after 15 min exposure to 0 (control), 0.5 µg/ml HSPA8 and combinations of 0.1-0.1, 0.5-0.5, 0.5-0.1 and 1-0.5 µg/ml HSPA1A-HSPA8, * $P < 0.05$, n = 7, mean ± s.e.m.

Table 2.2 Summary of analysis of variance for; sperm viability % of boar spermatozoa after 15 min exposure to 0 (control), 0.5 µg/ml HSPA8, 0.5 µg/ml anti-HSPA8 antibody and combinations of 0.5 µg/ml HSPA8 with 0.1, 0.5 and 1 µg/ml anti-HSPA8 antibody.

Effect	SS	Degree of Freedom	MS	F	P
Intercept	597198.0	1	597198.0	53632.89	0.000000
HSPA8	4178.0	6	696.3	62.54	0.000000
Anti-HSPA8 antibody	1147.1	5	229.4	20.60	0.000000
HSPA8*Antibody	153.3	30	5.1	0.46	0.990873
Error	935.3	84	11.1		

2.4.3 Short exposure to HSPA8 reduced sperm mitochondrial activity

A total of 9 boar semen samples were briefly (15 min) exposed to 0.5 µg/ml recombinant HSPA8 before being evaluated by Jc-1 staining for mitochondrial activity assessment. In contrast to control treatments containing no protein or 0.5 µg/ml α-Tubulin which had no effect on sperm mitochondrial activity, exposure of spermatozoa to HSPA8 did alter sperm mitochondrial trans-membrane potential and reduced mitochondrial activity (Figure 2.6).

2.4.4 Sperm capacitation induction negated the enhancing effect of HSPA8 on sperm viability

As depicted in figures 2.7A, B and Tables 2.4 A, B 15 min exposure to HSPA8 did not affect the capacitation status of spermatozoa which were pre-incubated in capacitating conditions. However, the beneficial effect of HSPA8 on maintaining sperm survival decreased along with the increase in pre-incubation time and rise in the number of capacitated spermatozoa. The ability of HSPA8 to enhance sperm viability over 15 min exposure time diminished completely after 4 hours of sperm pre-incubation in capacitating conditions. It was concluded that capacitated sperm is not responsive to the positive HSPA8 effects in respect to survival enhancement.

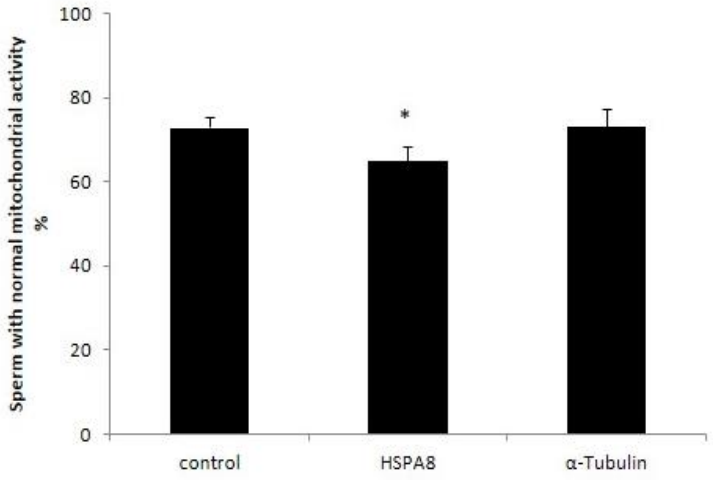

Figure 2.6 Effect of short exposure to HSPA8 on boar sperm mitochondrial activity. Percentage of spermatozoa with normal mitochondrial activity following incubation for 15 min at room temperature in the presence of TALP only (control) or HSPA8 (0.5 µg/ml) or α-Tubulin (0.5 µg/ml). * $P < 0.05$, n = 9, mean ± s.e.m.

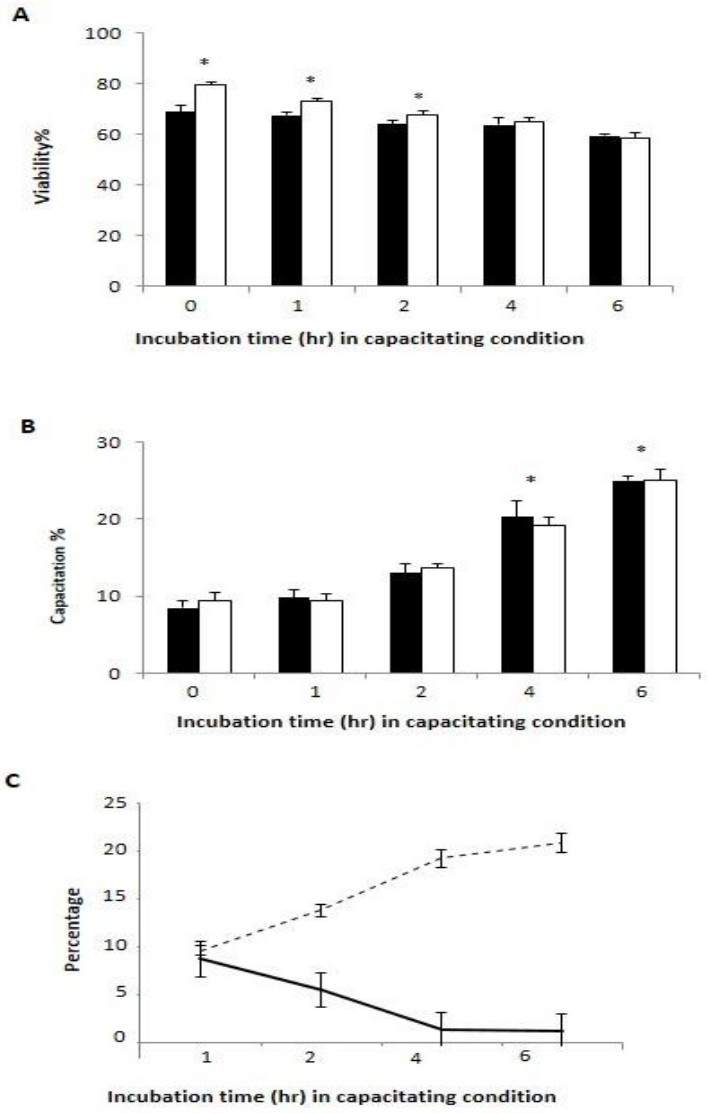

Figure 2.7 Effect of capacitation induction on the ability of HSPA8 to enhance boar sperm viability. (**A**) Viability% (determined by Propidium iodide /SYBR-14 staining), (**B**) Capaitation% (assessed by chlortetracycline assay of boar spermatozoa) after incubation at 0, 1, 2, 4 and 6 hrs at 39°C in capacitation conditions followed by 15 min exposure to 0 ■ and 0.5 □ µg/ml HSPA8 at room temperature. (**A**) * denotes significant ($P < 0.05$) differences within specific time points, (**B**) * denotes significant ($P < 0.05$) differences between different incubation time points and control (0 hr). (**C**) Percentage change in viability ——— and capacitation status – – – of boar spermatozoa after incubation at 1, 2, 4 and 6 hrs at 39°C in capacitation condition followed by15 min exposure to 0.5 µg/ml HSPA8 at room temperature. n= 6, mean ± s.e.m, r= 0.8, $P < 0.05$.

Table 2.3 Summary of analysis of variance for (**A**) viability% and (**B**) capacitation% after incubation at 0, 1, 2, 4 and 6 hrs at 39°C in capacitation conditions followed by 15 min exposure to 0 and 0.5 µg/ml HSPA8 at room temperature.

A

Effect	SS	Degree of Freedom	MS	F	P
Intercept	801667.5	1	801667.5	22342.32	0.000000
HSPA8	4900.0	1	1225.0	34.14	0.000000
Time	746.2	4	746.2	20.8	0.000010
HSPA8*Time	676.7	4	169.2	4.27	0.001240
Error	6099.8	170	35.9		

B

Effect	SS	Degree of Freedom	MS	F	P
Intercept	4657533	1	4657533	11774.48	0.000000
HSPA8	1072	1	1072	2.71	0.101555
Time	927843	4	231961	586.41	0.000000
HSPA8*Time	3465	4	866	2.19	0.072210
Error	66850	169	396		

2.5 Discussion

Herein, we have demonstrated HSPA8 has an immediate effect on sperm viability which could be observed in spermatozoa after only 15 min exposure to HSPA8. This observation is distinct to previous studies that have reported HSPA8 to prolong bull, boar and ram spermatozoa sperm survival after 24 or 48 hr incubation with HSPA8 (Elliott, Lloyd et al. 2009; Lloyd, Elliott et al. 2009). It is also distinct to other studies that have reported HSPD, HSPA1A and combination of HSPA1A/HSPA8 to protect post axotomy mice sensory neuronal cells (Houenou, Li et al. 1996) and heat-stressed macaque vascular endothelial cells (Johnson, Berberian et al. 1990) after extended co-incubation periods. The fact that there was a short interval between the addition of HSPA8 to the reinstatement of viability in spermatozoa indicated that the HSPA8-mediated enhancement of spermatozoa viability was not reliant on gene expression, protein transcription or production. This encouraged us to find out the mechanisms that were mediated by immediate exposure of spermatozoa to HSPA8.

In this study, two dye exclusion assays were used to assess sperm viability after HSPA8 treatment, both of which are based on the assumption that live (membrane intact) cells exclude impermeant dyes like Propidium iodide or Ethidium homodimer, whereas dead (membrane disrupted) cells would be stained by the dye. On the other hand, permeant dyes like SYBR-14 or Calcein-AM can easily gain access through intact membrane of live cells. The test therefore measures the plasma membrane integrity as an attribute to the viability status of the cell (Pegg 1989) and primarily determines cell membrane status. Intracellular metabolic function always depends on an intact enzyme system, but only sometimes on the structural integrity (Pegg 1989). Minor structural injuries to the cell membrane does not necessarily lead into total cellular impairment or death. The use of the general term ‘viability’ throughout this study therefore specifically refers to membrane integrity status of the cell.

A number of studies have reported on the ability of different HSPs to prolong viability via interactions with cellular membranes in different cell types (Johnson, Berberian et al. 1990; Johnson and Tytell 1993; Boilard, Reyes-Moreno et al. 2004; Elliott, Lloyd et al. 2009). There is plenty of evidence indicating that HSPs regulate membrane stability through association with plasma membrane lipid structure (Chen, Bawa et al. 2005; Horvath, Multhoff et al. 2008).

The interaction of HSP17 and bacterial GroEL with *Synechocystis* and *E. coli* membrane lipids, respectively, has been shown to stabilise membrane integrity in favour of cell survival under stressful conditions (Torok, Horvath et al. 1997; Horvath, Glatz et al. 1998). Furthermore, HSPs are also involved in stabilising organellar membranes in stressful conditions (Carratu, Franceschelli et al. 1996; Vigh, Maresca et al. 1998; Vigh, Horvath et al. 2007). HSPA1A prevents cancer cell death by inhibiting lysosomal membrane permeabilisation (Gyrd-Hansen, Nylandsted et al. 2004; Nylandsted, Gyrd-Hansen et al. 2004; Kirkegaard, Roth et al. 2010). All the available information suggest that association of HSPs with cell membrane counteracts stress induced disruptions in membrane domains by preserving functional and structural integrity of the biomembranes (Horvath, Glatz et al. 1998; Nylandsted, Gyrd-Hansen et al. 2004; Horvath, Multhoff et al. 2008).

The ability of HSPA8 to enhance the viability of spermatozoa is also consistent with the perceived functions of stress proteins, although comparatively little is known about the mechanisms underlying the cytoprotective effects of extracellular stress proteins. Certainly, heat shock proteins and particularly HSPA family members are known for their cell protecting function against various stressful stimuli through their anti-apoptotic activity (Garrido, Bruey et al. 1999; Schmitt, Gehrmann et al. 2007; Joly, Wettstein et al. 2010). Heat shock proteins function at multiple points in the apoptotic signalling pathways and via interactions with molecules that are involved in extrinsic or intrinsic apoptotic cascades (Garrido, Gurbuxani et al. 2001; Arya, Mallik et al. 2007). Apoptotic mitochondrial damage and nuclear fragmentation is inhibited in cells that have been transfected with HSPA1A (Li and Darzynkiewicz 2000). Elevated levels of HSPs in certain types of tumour cells contribute to the progress of malignancy and confer resistance against various anti-cancer treatments (Jaattela 1995; Multhoff, Botzler et al. 1995; Shin, Wang et al. 2003). The tumorigenicity of HSPs has been linked to their anti-apoptotic properties (Jaattela 1995).

Our observations revealed a consistent and significant increase of 10% to 15% in the proportion of live (membrane intact) spermatozoa after brief exposure to HSPA8. *In vivo*, the oviduct selectively associates with a small proportion of spermatozoa of superior quality in terms of viability (Smith and Yanagimachi 1990; Mburu, Einarsson et al. 1996; Tienthai,

Johannisson et al. 2004), acrosome intactness (Gualtieri and Talevi 2000) and uncapacitated status (Lefebvre and Suarez 1996; Fazeli, Duncan et al. 1999). *In vitro* investigations have also revealed that only a small number of live spermatozoa bind to a soluble fraction of oviductal apical plasma membrane (sPAM) (Elliott, Lloyd et al. 2009).

Together, these findings indicate that the oviductal epithelium associates with a small, but high quality subpopulation of sperm in order to optimise fertilisation. Interpreting our *in vitro* observations in the context of real oviductal events suggests it to be likely that HSPA8 is an oviductal component which has the ability to reverse potentially deleterious cellular alterations by selectively associating with the minority population of spermatozoa which are of the highest quality and therefore have the greatest fertilisation potential. The other interpretation would be that HSPA8 recognises and guards the cells which are at risk of stress and protects them from damage which is induced by environmental stressors and therefore increases the high quality sperm population.

Alternatively, it could be stipulated that exogenous HSPA8 binds to all cells, but that it is only competent to save those with reversible minor damage which are destined to stay alive. Visualisation of HSPA8-sperm binding pattern using imaging technologies would provide a definite answer as to which of the above stipulations are more precise.

The specificity of HSPA8-induced effects was confirmed using HSPA1A as a control protein. HSPA1A is an inducible isoform of 70kDa HSP. HSPA proteins are highly conserved and hold the highest level of sequence homology among other HSP families (Hendrick and Hartl 1993; Fagan and Weissman 1996). There is more than 84% homology between HSPA1A and HSPA8 in mammalian species (Arispe, Doh et al. 2004; Multhoff 2007). Both proteins function as cellular chaperones in folding, assembly, scavenging and transport of protein complexes in favour of cell survival (Beckmann, Mizzen et al. 1990; Shi and Thomas 1992). The constitutive and inducible isoforms are assumed to play synergistic functions, based on the report that *in vivo* combinations of these two proteins may affect functional properties of a number of cell types (Brown, Martin et al. 1993; Browne, Swan et al. 2007). However, in contrast to HSPA8, human recombinant HSPA1A had no effect on sperm viability in our investigation. Furthermore, none of the HSPA1A and HSPA8 combinations had any effect on viability,

suggesting that HSPA1A negated the positive effect which is observed with HSPA8 alone. This observation is consistent with recent reports of differential and antagonising effects of HSPA1A against HSPA8 in cellular systems. In *Xenopus* oocytes, the induction of surface expression of epithelial sodium channels by individual HSPA1A is antagonised by co-injection of HSPA1A and HSPA8 cRNA to the cells in a concentration-dependant manner (Goldfarb, Kashlan et al. 2006). Furthermore, these two proteins have been shown to counteract the stabilisation of a potassium channel subunit in human cardiomyocytes (Li, Ninomiya et al. 2011).

The female reproductive tract holds a unique responsibility in maintaining sperm viability and fertilising capacity which is most vital in species in which there is a time span between sperm deposition in the female reproductive tract and ovulation (Topfer-Petersen, Wagner et al. 2002; Holt and Fazeli 2010; Roy and Krishna 2011). Therefore, perpetuating reproduction necessitates persistent expression and consistent function of factors necessary for protection and maintenance of sperm survival in the female reproductive organ. In contrast to other body organs which express HSPA1A as a survival prolonging factor to delay cell death (Schmitt, Gehrmann et al. 2007; Kirkegaard, Roth et al. 2010), the female reproductive tract seems to use HSPA8 as a survival factor. HSPA1A is the stress-induced heat shock protein whose expression and cell protecting functions are restricted to times of stress (Schlesinger 1990; Craig, Gambill et al. 1993), whereas HSPA8 is a constitutive HSP and is present in the cells at all times (Schlesinger 1990; Craig, Gambill et al. 1993). Replacement of HSPA1A by HSPA8 as a viability protecting agent in female reproductive system might be an evolutionary solution which ensures the constant presence of regulatory factors in the sperm reservoir. This, in part, can explain the inefficiency of HSPA1A in enhancing sperm viability in our system as opposed to other biologic systems. Furthermore, the ability to block the effects of HSPA8 on sperm viability using anti-HSPA8 antibody confirmed the unique and specific effect of short exposure of sperm to HSPA8 in restoring sperm viability.

Further investigations showed that the enhancing effect of HSPA8 on sperm survival diminished gradually through the process of sperm capacitation. In other words, capacitated sperm were not any longer responsive to viability enhancing effect of HSPA8. Capacitation is

the final maturation process in sperm that takes place gradually *in vivo* inside the oviduct (Rodriguez-Martinez 2007). During capacitation, sperm membrane undergoes major structural and biochemical alterations which are integral to fulfilment of the process (Rodriguez-Martinez 2007). Hence, based on the hypothesis that HSPA8 targets sperm membrane to exert its positive effects, one can speculate that the capacitation-induced changes in sperm membrane occur in a direction which is not consistent with optimal settings required for HSPA8 to induce any survival maintaining/enhancing effects. Understanding changes occurring as a result of capacitation in spermatozoa can help us to understand the HSPA8 mechanism of action. HSPAs associate with membrane lipid domains via interactions with cholesterol (Mamelak and Lingwood 2001; Mamelak, Mylvaganam et al. 2001; Chen, Bawa et al. 2005). One of the major alterations which occur during the process of sperm capacitation is the substantial reduction in membrane cholesterol (Osheroff, Visconti et al. 1999; Buffone, Verstraeten et al. 2009). It is therefore highly probable that the capacitation-induced reduction/reorganisation in sperm membrane cholesterol/lipid structure compromises the interaction sites that are required for HSPA8 to assert its effects. Altering the cholesterol content of sperm membranes and determining the immediated effects of HSPA8 on the sperm membrane in such spermatozoa could prove the validity of the above hypothesis. In addition, spermatozoa undergoing capacitation are prone to cell death (Hunter and Rodriguez-Martinez 2004; Rodriguez-Martinez 2007), therefore it is sensible to conclude that actions aiming to expand cell survival must interfere before capacitation stage.

It is unclear if exogenous HSPA8 gets into the cell and functions internally or induces cellular alterations from outside via mediators. There are a number of studies regarding exogenous HSP-cell association, all of which have tracked protein location after they obtained positive effects from addition of the protein to their particular cell type of interest. Association of HSPA1A with arterial smooth muscle cells occurred via protein-membrane binding rather than protein internalisation (Johnson and Tytell 1993). Similarly, incubation of spermatozoa with HSPD led into significant cell membrane-protein association (Boilard, Reyes-Moreno et al. 2004). Considering the results obtained and speculations made so far, the interaction of HSPA8 with sperm membrane seems very likely. However, other possibilities, including HSPA8 internalisation or external action through mediator molecules should be considered too. Hence,

the next step is to pinpoint the location at which exogenous HSPA8 associates with boar sperm. Mitochondrial transmembrane potential is an indicator of the level of mitochondrial activity and energy production (Garner, Thomas et al. 1997) and in this brief exposure, HSPA8 reduced mitochondrial activity, as assessed on the basis of JC-1 mitochondrial staining which differentially reacts to high or low mitochondrial electrical activities. A transient decrease in sperm motility occurs in the vicinity of oviductal epithelial cells (Suarez 1987; Lefebvre, Chenoweth et al. 1995) or oviductal secretions (Overstreet, Katz et al. 1980; Grippo, Way et al. 1995; Coy, Lloyd et al. 2010) and reduced mitochondrial activity appears to be responsible for the formation of the oviductal sperm reservoir (Overstreet and Cooper 1978; Suarez 1987). Furthermore, the treatment of bull spermatozoa with isthmic non-luteal oviductal fluid significantly suppressed motility (Grippo, Way et al. 1995), although the factors involved have not been identified. These observations indicate the importance of motility inhibiting factors in the oviduct, and it is likely that HSPA8 is an important oviductal component which contributes to sperm storage formation in the oviduct by reducing mitochondrial activity and motility.

Instantaneous alterations in mitochondrial activity under the effect of HSPA8 poses questions on the pathways that might be involved. Suppression of sperm motility by HSPA8 might be due to its modulatory effects on mitochondrial respiratory pathways or direct interactions with mitochondrial membrane, ion channel induction and alterations in mitochondrial ion concentration. There are reports on the interaction of HSPs with cellular as well as intracellular membranes (Chen, Bawa et al. 2005; Horvath, Multhoff et al. 2008; Horvath and Vigh 2010; Kirkegaard, Roth et al. 2010), ion channel induction and ion transportation (Smith 1995). One other hypothesis would be that HSPA8 is converted to an inhibitory metabolite of metabolic pathways responsible for energy generation for sperm motility, similar to the effect of some male anti-fertility compounds which exert their motility suppressing effect within 10 to 15 min time (Cooney and Jones 1988; Jones 1998). Although a descriptive qualitative assay, our data partially shed light on a possible physiological effector modulating sperm motility in the oviductal reservoir, further investigations are required. Identifying correlations between visible sperm motility and mitochondrial activity in response to HSPA8 using computer-assisted or any other reliable sperm motility assays would complement the existing results.

The novel results presented in this study demonstrate that brief exposure of boar spermatozoa to extracellular bovine recombinant HSPA8 significantly increases sperm viability and decreases mitochondrial activity. HSPA8 instantly restores membrane integrity via a rapid activation of repair mechanisms in cells with membranes inefficient of preventing impermeant dyes from entering the cells. Furthermore, reducing effect of HSPA8 on sperm mitochondrial activity was suggested to be attributed to the interaction of HSPs with organellar membranes. HSP-cellular organelle interaction could be either through direct association upon internalisation in the cell or via intracellular signalling effectors activated by specific membrane receptors from the extracellular environment. Although this was an *in vitro* model, the effects created by HSPA8 on sperm seem to be similar to events happening in the oviductal sperm reservoir.

Semen storage is extremely important in preservation of genetic diversity and efficient breeding. Despite considerable advances in semen preservation techniques, no definite solution has been found to minimise the damage to sperm plasma membrane and reduce significant decrease in sperm viability over the course of semen storage (Tapia, Macias-Garcia et al. 2012). Failed insemination trials also indicate that semen fertilising performance significantly drops with the duration of semen preservation, even in the best existing extenders (Waberski, Weitze et al. 1994; Johnson, Weitze et al. 2000). Adding exogenous HSPA8 as a survival prolonging agent which is involved in membrane repair mechanism to the existing semen preserving media could be one step forward towards augmenting the efficiency of semen preservation industry.

However, further investigations on the effect of HSPA8 on sperm functional properties such as fertilisation performance would considerably expand our knowledge and the potential applicability of this protein in semen preservation and reproduction techniques. Moreover, the current novel findings necessitate an in depth investigation of the mechanism of HSPA8 action on sperm membrane. An integrative understanding of the membrane attributes that are affected by HSPA8 would complement the existing knowledge on HSP-mediated repair systems.

Chapter 3

Effect of extracellular HSPA8 on sperm-oviductal epithelial cell binding and *in vitro* fertilisation potential

3.1 Introduction

Mammals have adopted internal fertilisation strategies which involve the whole process of fertilisation taking place inside the female reproductive tract and for which the maternal interaction with gametes and embryos plays a critical role in the success of pregnancy (Topfer-Petersen, Wagner et al. 2002; Aviles, Gutierrez-Adan et al. 2010). Among various compartments in the maternal tract, the oviduct hosts some of the key reproductive events and exhibits a high level of interaction with gametes and embryos. Upon arrival in the oviduct, spermatozoa establish an intimate association with the epithelial cells in the caudal segment of the oviduct. The ability of spermatozoa to bind to the oviductal epithelium is believed to be an essential sperm function and a definitive prerequisite for the sperm-egg interaction (Pollard, Plante et al. 1991). Sperm binding to oviductal epithelial cells (OEC) initiates a complex reciprocal dialogue in the maternal tract which would sequentially lead into maintaining essential sperm fertilising competence prior to ovulation, synchronising punctual maturation of both gametes and their transport towards each other for fertilisation and finally providing a conduit for the early embryos to develop and transfer to its implantation site after fertilisation (Killian 2004; Waberski, Magnus et al. 2006; Georgiou, Snijders et al. 2007).

Oviductal epithelial cells (OECs) respond to presence of each of the gametes or embryo differently by *de novo* synthesis of new proteins (Ellington, Ignotz et al. 1993; Georgiou, Sostaric et al. 2005; Holt and Lloyd 2010). Proteins constitute a major component of the oviductal fluid and the list of oviductal proteins is constantly growing, some of which are reported to regulate different processes that occur in the oviduct (Buhi, Alvarez et al. 2000; Killian 2004; Georgiou, Snijders et al. 2007; Elliott, Lloyd et al. 2009). The release of a number of heat shock protein (HSP) members by oviductal epithelium in response to sperm has been reported in bovine, porcine and human (Georgiou, Sostaric et al. 2005; Elliott, Lloyd et al. 2009; Marin-Briggiler, Gonzalez-Echeverria et al. 2010). Constitutive 70kDa heat shock protein, HSPA8 (HSC70), secreted from porcine oviductal epithelium preserves sperm viability (Elliott, Lloyd et al. 2009; Lloyd, Elliott et al. 2009). Bovine oviductal GRP78 and HSPD (HSP60) maintain motility and prolong the viability of spermatozoa (Boilard, Reyes-Moreno et al. 2004). Heat shock proteins are also involved in the process of fertilisation and development of early embryos. HSPA8 purified from the extracellular vitelline envelope of the

amphibian oocyte exhibits a high affinity for sperm plasma membrane and it has been suggested to enhance oocyte-sperm membrane interactions (Marin-Briggiler, Gonzalez-Echeverria et al. 2010). In a study on the fertilising functions of HSPs in the sea urchin, a combination of inducible HSPA1A with constitutive HSPA8 has proven to be highly effective in accelerating the nuclear envelope breakdown in the fertilised eggs (Browne, Swan et al. 2007). Brief treatment of spermatozoa with exogenous HSPA8 has been shown to significantly improve the rate of monospermy and the speed of early embryonic development in a porcine *in vitro* fertilisation (IVF) assay (Satake N 2007; Elliott, Lloyd et al. 2009).

Previous work in our laboratory has focused on characterising the effect of exogenous HSPA8 on sperm properties. We have provided evidence that a short (15 min) exposure to extracellular HSPA8 enhances sperm viability (measured as membrane integrity) and reduces sperm mitochondrial activity (Chapter 2). Observing the alterations in the basic sperm characteristics after such brief exposure to HSPA8 prompted us to investigate potential changes that could meanwhile happen in functional properties of sperm such as their capacity to bind oviductal epithelial cells and fertilisation performance using porcine sperm-OEC binding and *in vitro* fertilisation (IVF) bioassays, respectively. Accordingly, it was hypothesised that brief (15 min) treatment of spermatozoa with exogenous HSPA8 increased sperm-OEC binding, improved fertilisation performance and early embryonic development as specific endpoints of the reproductive process.

3.2 Materials and Methods

All chemicals used for preparation of Tyrode's medium were purchased from BDH Laboratory Supplies, Poole, UK. Chemicals used in the sperm-OEC binding and IVF assays were purchased from Sigma-Aldrich Company Ltd, Dorset, UK and Sigma-Aldrich Company Ltd, Alcobendas, Madrid, Spain, respectively unless otherwise stated.

3.2.1 Sperm preparation

For the sperm-OEC binding assay, boar semen diluted and stored for 24 hours in Beltsville Thawing Solution (BTS) (Pursel and Johnson 1975) was obtained from a commercial artificial insemination station (JSR Healthbred Limited, Thorpe Whillougby, Yorkshire,UK). On the day of experiment, diluted boar semen was washed using Percoll gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Briefly, aliquots of 2-4 ml of diluted semen were layered over a Percoll density gradient. The iso-metric Percoll gradient consisted of 2 ml 70% (v/v) Percoll overlaid with 2 ml 35% (v/v) Percoll. The gradient was centrifuged at 200 g for 15 min followed by 15 min centrifugation at 1000 g (Holt and Harrison 2002). The supernatant was removed and the pellet was resuspended in Tyrode's medium consisting of 3.1 mM KCL, 0.4 mM $MgCl_2.6H_2O$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, 0.3 mM $NaH_2PO_4.2H_2O$ (Elliott, Lloyd et al. 2009). Thereafter, semen samples were centrifuged at 900 g for 15 min and resuspended in Tyrode's medium. Sperm concentration was measured in duplicate using an improved Neubauer chamber (Marienfield, Germany) and adjusted to the required concentration in TALP which consisted of Tyrode's medium supplemented with 12 mg/ml bovine serum albumin, 21.6 mM sodium lactate and 1 mM sodium pyruvate.

For the IVF experiments, spermatozoa from 3 different boars of proven fertility and motility were obtained on the day prior to each experimental replicate. Semen samples were pooled and adjusted to 10^7 sperm / ml in mTBM medium and kept at room temperature (18-22°C) till further use.

3.2.2 Oviductal epithelial cells isolation and culture

Reproductive tracts of gilts were obtained from a local abattoir on the day of slaughter and transported to the laboratory in Dulbecco's Phosphate Buffered Saline DPBS with Ca^{2+} and Mg^{2+}, supplemented with 2% v/v penicillin-streptomycin-amphotericin mix (Sigma-Aldrich, Dorset, UK) at room temperature. Oviducts with no gross pathology were dissected away from the ovaries and surrounding tissue and rinsed three times in DPBS supplemented with antibiotics. Each oviduct was sealed tightly on one end approximately 5 mm above the cutting edge by a strong cotton thread. Using a sterile syringe, the sealed oviduct was filled with 1ml of 0.25% w/v filter-sterilised collagenase type 1A (Sigma) in Ca^{2+}/Mg^{2+}-free Hanks' Balanced Salt solution (HSBB; Sigma), pre-warmed in a water bath at 39°C. The oviduct was then immediately closed tightly at the other end using a strong cotton and incubated at 37°C and 5% (v/v) CO_2 for 90 min.

The cotton thread at one end of the oviduct was then removed, the lumen content was milked manually into a centrifuge tube and washed three times by centrifugation at 300 g for 5 min in 40 ml M199 culture medium (Sigma) at room temperature. The supernatant was discarded and the pellet was mixed and vigorously shaken with 1 ml red cell lysis buffer (Sigma) for 1 min. The treated pellet was then centrifuged at 300 g for 5 min in 20 ml M199 culture medium for three times and resuspended in fresh culture medium. The concentration of the oviductal epithelial cells was determined using a haemocytometer and cellular integrity was microscopically assessed by staining the cells with 4% w/v trypan blue (Sigma) dissolved in PBS. Oviductal cells were transferred into 75 cm^2 tissue culture flasks at $1x10^6$ viable cells/ml and incubated at 39°C with 100% humidity and 5% v/v CO_2. The culture medium was refreshed every 48 hours. Oviductal cells reached confluence between 7 to 14 days.

On the day of the experiment, confluent cells were rinsed three times with Ca^{2+}/Mg^{2+}-free PBS (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM$Na_2HPO_4.12H_2O$ and 1.5 mM KH_2PO_4) (Harayama, Okada et al. 2003) and detached from the flask wall by incubation with 3 ml Trypsin-EDTA solution for 10 min at 39°C. Detached cells were rinsed twice with 2 ml Ca^{2+}/Mg^{2+}-free PBS in order to dissociate the cells. Then, 2 ml of culture medium containing 10% v/v foetal calf serum was immediately added to inhibit trypsin and prevent any potentially damaging effects.

Oviductal epithelial cells were centrifuged at 300 g for 4 min. The supernatant was discarded and the cells were resuspended in 1ml of M199 culture medium. Cells were counted using a haemocytometer and adjusted to 2 x 10^6 cell / ml in M199 medium.

3.2.3 Sperm-oviductal epithelial cell binding assay

Percoll-washed boar sperm samples were diluted and adjusted to a final concentration of 1 x 10^8 spermatozoa / ml in TALP. The sperm-oviductal epithelial cell binding assay was performed according to the method described by Green et al. (Green, Bredl et al. 2001). Briefly, 200 µl of boar sperm samples and 200 µl of oviduct epithelial cells were transferred to the same tube. The tubes were incubated at 39°C, 5% v/v CO_2 for 30 min on a rotator. To remove unbound or loosely attached spermatozoa, 400 µl of sperm-OEC suspension was layered over a two–step Percoll gradient containing 1 ml of 35% v/v Percoll overlaid on the top of 1 ml of 70% v/v Percoll in a 15 ml polypropylene tube.

Following centrifugation at 200 g for 3 min, 3 layers of cells appeared. Non-motile, unbound spermatozoa and unattached oviductal epithelial cells located at the interface between the media and Percoll 35% v/v (Layer 1). Sperm-oviductal epithelial cell complexes located at the interface of the two Percoll layers (Layer 2), and unattached highly motile spermatozoa sedimented at the bottom of the tube (Layer 3). Cells from layers 1 and 2 were removed carefully using a pipette. Then, cells from the 2 layers were combined and diluted in 15 ml of PBS. Unattached spermatozoa were removed by centrifugation at 200 g for 5 min. The supernatant was discarded, sperm-oviductal epithelial cells were resuspended in 500 µl of PBS and fixed with 1% w/v formaldehyde in PBS. In order to count the number of spermatozoa bound to epithelial cells, 10 µl of the fixed cell suspension was placed on a microscope slide and covered with a cover slip. In each sample, the number of spermatozoa attached to 100 oviductal epithelial cells were counted by light microscopy in three replicates.

3.2.4 Oocyte recovery and culture

Ovaries from prepubertal gilts were transferred from the local slaughterhouse to the laboratory in 0.9% v/v NaCl containing 70 µg/ml kanamycin, at 34-37°C within 1 hr of collection. Ovaries were gently washed three times in pre-warmed (37°C) NaCl solution and follicles of 3 to 6 mm in diameter were collected by making gentle cuts on the ovarian wall. Released oocytes with compact cumulus mass and dark evenly granulated cytoplasm were washed three times in oocyte maturation medium which was the protein-free tissue culture medium (PF-TCM) 199 (Gibco, Life technologies, Barcelona, Spain) supplemented with 0.57 mM cysteine, 0.1% (w/v) polyvinylalcohol (PVA), 10 ng/ml epidermal growth factor (EGF), 75 µg/ml potassium penicillin G, 50 µg/ml streptomycin sulphate, 0.5 µg/ml luteinising hormone (LH) and 0.5 µg/ml follicular stimulating hormone (FSH). Next, 70-80 oocytes in 500 µl of maturation medium supplemented with 10 IU/ml equine chorionic hormone (eCG, Folligon, Intervet International B.V., Boxxmeer, The Netherlands) and 10 IU/ml human chorionic hormone (hCG, Veterin Corion, Divasa Farmavic, S.A., Barcelona, Spain) were transferred into each well of a 4-well plate. These were incubated at 39°C, 5% v/v CO_2 under mineral oil for 22 hrs and then for another 22 hrs in maturation medium without hormones for *in vitro* maturation (IVM).

3.2.5 *In vitro* fertilisation

Upon completion of IVM, cumulus oocyte complexes were stripped from cumulus cells by briefly vortexing with 0.1% w/v hyaluronidase in maturation medium at 1660 revolutions / min. Denuded oocytes were then washed twice in maturation medium and three times in pre-equilibrated fertilisation medium (modified Tris-buffered medium, mTBM) described by Abeydeera and Day (Abeydeera and Day 1997) which consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2.2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 0.2% w/v BSA (fraction V; Sigma) and 0.5 mM caffeine with no antibiotics. Groups of 50 denuded oocytes were then placed in 50 µl droplets of fertilisation medium in 4-well plate dishes under mineral oil at 39°C in 5% v/v CO_2 in air.

Fresh spermatozoa were pre-incubated with HSPA8 (0, 0.5, 1, 10, 20 µg/ml) or 0.5 µg/ml HSPA1A (positive control) for 15 min at room temperature. Thirty minutes after oocyte preparation, 50 µl aliquots of semen samples diluted in mTBM medium (10^7 spermatozoa / ml) were added to oocytes in IVF droplets to give a final concentration of 25,000 spermatozoa (sperm : oocyte ratio , 500 : 1).

3.2.6 Embryo culture

Five hours post-insemination, presumptive zygotes were vigorously washed three times in pre-equilibrated embryo culture medium **(**North Carolina State University [NCSU-23]: 108.73 mM NaCl, 4.78 mM KCl, 1.19 mM K_2PO_4, 1.19 mM $MgSO_4.7H_2O$, 25.07 mM $NaHCO_3$, 1 mM L-glutamine, 7 mM Taurin, 5 mM Hipotaurin, penicillin and streptomycin (Petters and Wells 1993) to detach loosely bound spermatozoa from oocytes. At this stage, groups of 50 washed zygotes were transferred to 4-well dish containing 500 µl of culture medium under mineral oil. Zygotes were cultured in NCSU-23 supplemented with 0.4% w/v BSA (fraction V) for first two days and with NCSU-23 containing 5.55 mM D-glucose for the rest of the culture period.

Meanwhile, 16 hr after culture, 50% of the zygotes were fixed on slides in acetic acid: ethanol solution at 1:3 (v/v) ratio for 48-72 hr at room temperature for the assessment of fertilisation parameters. Fixed oocytes were then stained with 1% v/v lacmoid in 45% v/v acetic acid and examined under a phase contrast microscope (Eclipse E400, Nikon, Japan) at 200x and 400x magnification. The rest of the oocytes were continued in culture until 48 hr, at which time they were transferred to fresh culture medium for the rest 5 days of embryo culture period. A summary of *in vitro* fertilisation and embryo culture process is illustrated in Figure 3.1.

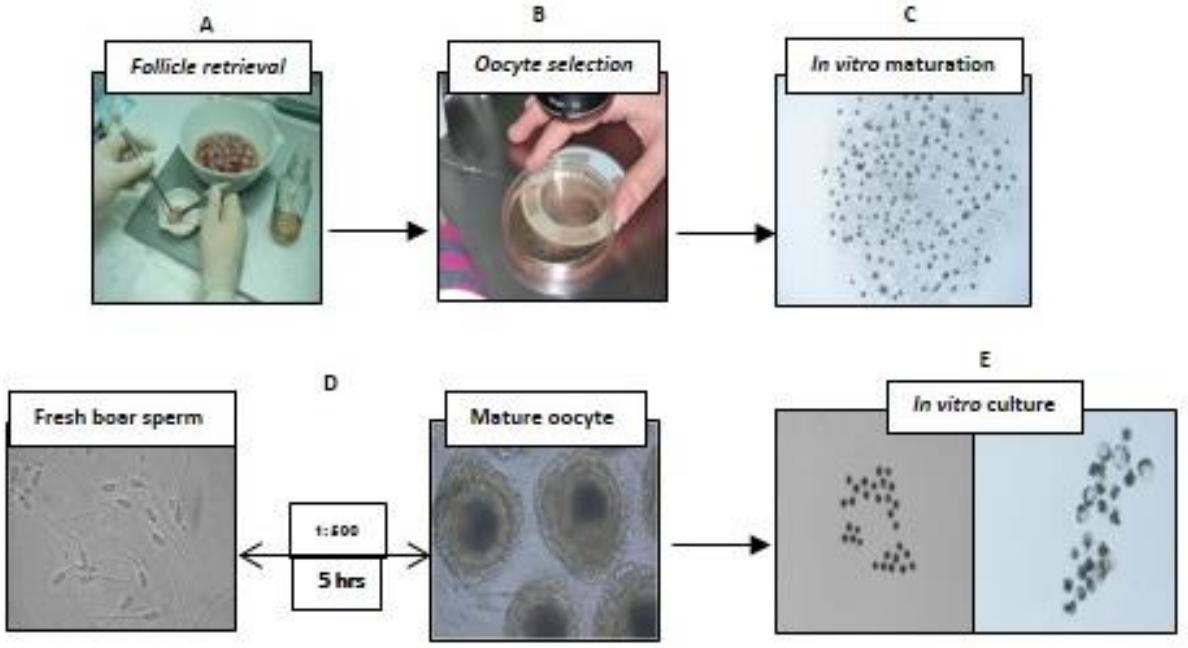

Figure 3.1 Summary of *in vitro* fertilisation and embryo culture process. (**A**) Ovarian follicles of 3 to 6 mm in diameter were collected by making gentle cuts on the wall of the ovaries from prepubertal gilts, (**B**) Released oocytes with compact cumulus mass and dark evenly granulated cytoplasm were washed and incubated in oocyte maturation medium (protein-free tissue culture medium 199) for 48 hrs, (**C**) upon completion of *in vitro* maturation, groups of 50 denuded oocytes were then placed in 50 µl droplets of fertilisation medium (modified Tris-buffered medium) under mineral oil at 39°C in 5% v/v CO_2 in air, (**D**) 30 min later, prepared oocytes droplets were inseminated with 50 µl aliquots of fresh spermatozoa diluted in mTBM medium (10^7 spermatozoa / ml) and treated with (0, 0.5, 1, 10, 20 µg/ml) or 0.5 µg/ml HSPA1A to give a final sperm : oocyte ratio of 500 : 1, (**E**) five hours post insemination, presumptive zygotes were washed and transferred to 4-well dish containing 500 µl of culture medium (North Carolina State University [NCSU-23]) under mineral oil. *In vitro* culture was carried out for 16 hrs, 2 and 7 days 39°C in 5% v/v CO_2 for assessment of fertilisation, cleavage and blastocyst formation parameters, respectively.

3.2.7 Assessment of *in vitro* fertilisation parameters

Degenerated oocytes were not assessed. Oocytes with one or more sperm heads and / or male pronuclei and sperm tails were counted as penetrated. Three fertilisation parameters were assessed:

1. penetration percentage was calculated as percentage of penetrated oocytes / total inseminated oocyte
2. monospermy was calculated as percentage of monospermic oocytes / total penetrated oocyte
3. efficiency of fertilisation was calculated as percentage of monospermic oocytes / total inseminated oocyte.

Figure 3.2 depicts microscopic images of penetrated and monospermic penetrated oocytes.

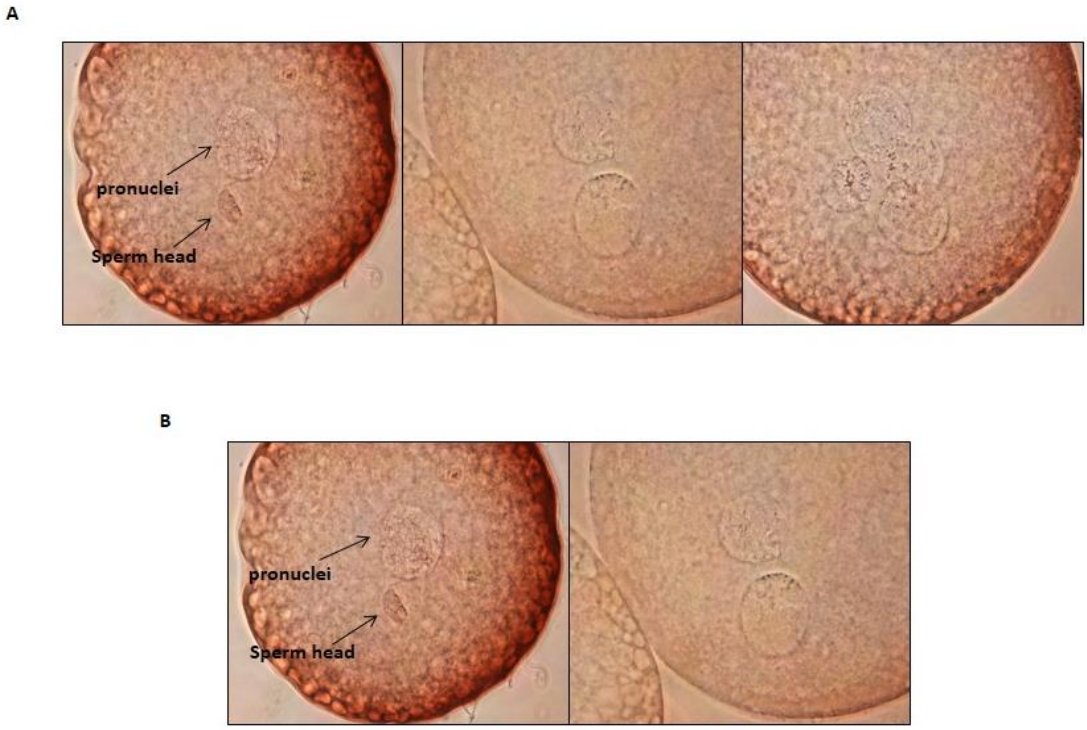

Figure 3.2 Assessment of *in vitro* fertilisation parameters. Twenty hours post insemination oocytes from each group were mounted on slide, fixed for 48-72 hrs with 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% lacmoid in 45% acetic acid and examined under phase contrast microscope, (**A**) oocytes were considered penetrated when contained one or more sperm heads / male pronuclei with corresponding sperm tails and two polar bodies, (**B**) oocytes with one sperm head / male pronuclei were considered as monospermic penetrated oocytes.

3.2.8 Assessment of *in vitro* embryo development

Two embryo culture parameters including cleavage and blastocyst formation rates were evaluated on the second and seventh days after *in vitro* fertilisation, respectively. Cleavage rate was calculated as the percentage of embryos at 2 or 4 cells / total cultivated zygotes. Blastocyst formation rate represented the percentage of blastocysts / total cultivated inseminated oocytes. Cells per blastocysts were counted after fixing and staining with fluorescent Hoechst-33342 nuclear stain. Figure 3.3 demonstrates microscopic images of cleaved and blastocyst embryos as well as a Hoechst-stained blastocyst for determination of its cell number.

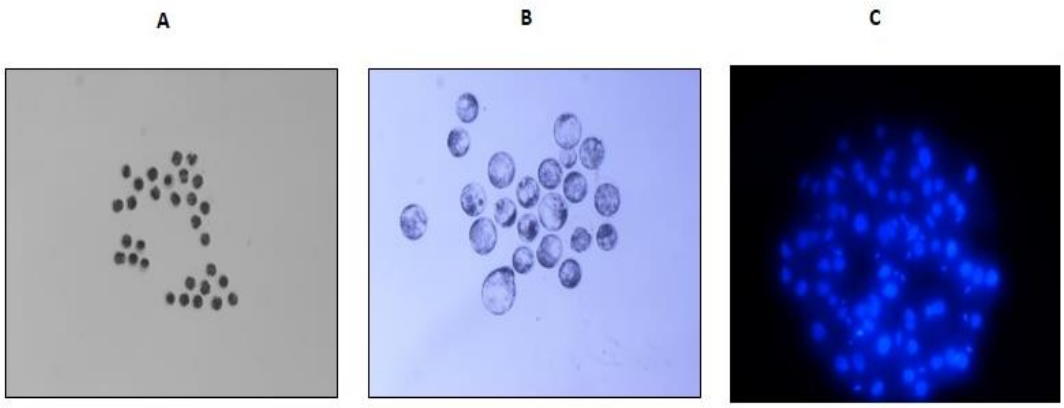

Figure 3.3 Assessment of *in vitro* embryo development. Cultured embryos from each group were evaluated under estereomicroscope (**A**) at 48 hrs (2 days) post insemination for percentage of the embryos divided into 2 and 4 cell stages (cleavage atage) and (**B**) at 168 hrs (7 days) post insemination for blastocyst formation and (**C**) number of cells per each blastocyst determined by Hoechst staining.

3.3 Experimental design

3.3.1 Effect of brief exposure to HSPA8 on sperm-oviductal epithelial cell binding

To investigate the effect of HSPA8 on boar sperm capacity to bind oviductal epithelial cells, sperm samples from 7 boars were adjusted to 1 x 10^8 sperm / ml concentration in TALP and incubated for 15 min at room temperature with 0 or 0.5 µg/ml of HSPA8 or 0.5 µg/ml α-Tubulin. The samples were then washed briefly by centrifugation (200 g for 10 min) and the pellet was resuspended in TALP. Then, 200 µl of boar sperm samples treated with or without HSPA8 and 200 µl of oviduct epithelial cells were transferred to the same tube. The tubes were incubated at 39°C, 5% v/v CO_2 for 30 min on a rotator. The rest of the experiment was performed as described in the Materials and Methods section.

3.3.2 Effect of brief exposure to HSPA8 on sperm fertilising capacity and early embryonic development

Aliquots of fresh pooled semen samples from boars with proven fertility and high motility which had been diluted in mTBM medium (10^7 spermatozoa / ml) were pre-incubated with HSPA8 (0, 0.5, 1, 10, 20 µg/ml) or 0.5 µg/ml HSPA1A (positive control) for 15 min at room temperature. After 30 min of oocyte preparation, 50 µl of treated and diluted spermatozoa were added to oocytes in IVF droplets to give a final concentration of 25,000 spermatozoa (sperm:oocyte ratio, 500:1) and the rest of the IVF experiment was carried out as explained above. It should be noted that in our IVF assays spermatozoa were treated with a range of HSPA8 concentrations because first the concentration of spermatozoa used in the IVF assays were much higher compared to sperm concentration tested in previous experiments (Chapter 2) and the second was to investigate the effect of increasing protein concentrations in IVF assay.

3.3.3 Statistical analysis

IVF experiments were repeated in 6 replicates on 6 different days. Pooled data from individual replicates for oocyte penetration, monospermy, cleavage and blastocyst formation rate as wells

as the number of cells / blastocyst were analysed by analysis of variance (ANOVA) for determination of the significant differences among the groups. When ANOVA showed significant differences, values were compared by Fisher's least significance difference (Fischer's LSD) test using Statistica software, version 7 (WWW.StatSoft.com, 1984-2004)].

Data obtained from sperm-OEC binding assay were analysed by Student t-test to examine the effect of treatments between treated and untreated samples. The results were expressed as mean ± s.e.m and differences with P values ≤ 0.05 were considered as significant.

3.4 Results

3.4.1 Brief exposure to HSPA8 enhanced the capacity of sperm to bind oviductal epithelial cells

Brief exposure (15 min) of spermatozoa to HSPA8 increased the number of spermatozoa attached to single epithelial cells (TALP control versus 0.5 µg/ml HSPA8; $P = 0.003$) (Figure 3.4).

3.4.2 HSPA8 improved *in vitro* fertilisation performance of sperm but had no effect on early embryonic development

To determine whether brief exposure to extracellular HSPA8 had any influence on sperm fertilising performance and early embryonic development, a total of 4027 oocytes in 6 replicates were inseminated, from which 1982 oocytes were assessed for *in vitro* fertilisation parameters and the rest (2045) for *in vitro* culture parameters. As indicated in Figure 3.5A, a 15-min pre-treatment of semen with 1 µg/ml HSPA8 significantly increased penetration rate ($P < 0.004$). None of the other concentrations tested had any effect. In addition, monospermy increased significantly when spermatozoa were pre-treated with 0.5 µg/ml ($P < 0.0001$) and 1 µg/ml, ($P < 0.0001$) of HSPA8 (Figure 3.5B). The efficiency rate was accordingly higher after incubating sperm with 0.5 and 1 µg/ml HSPA8 (Figure 3.5C & Table 3.1).

There were no significant differences in cleavage rate or blastocyst formation rate between groups treated with varying HSPA8 concentrations. HSPA8 had no apparent effect on the final embryo quality, as evaluated by the mean number of nuclei / blastocyst. HSPA1A had no effect on any of the measured parameters (Figure 3.6A, B, C & Table 3.2).

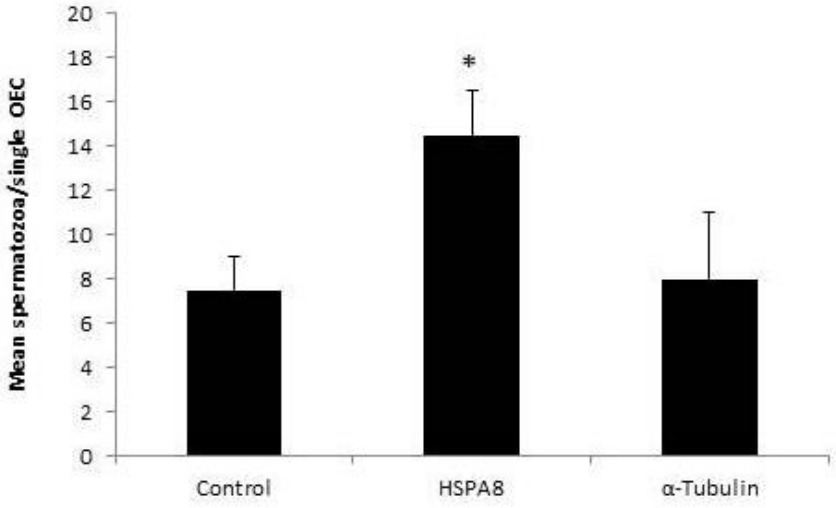

Figure 3.4 Effect of HSPA8 on sperm capacity to bind oviductal epithelial cells. Average number of sperm bound to single oviductal epithelial cells after 15 min exposure to 0 (control) and 0.5 µg/ml HSPA8. * < 0.05, n = 7, mean ± s.e.m.

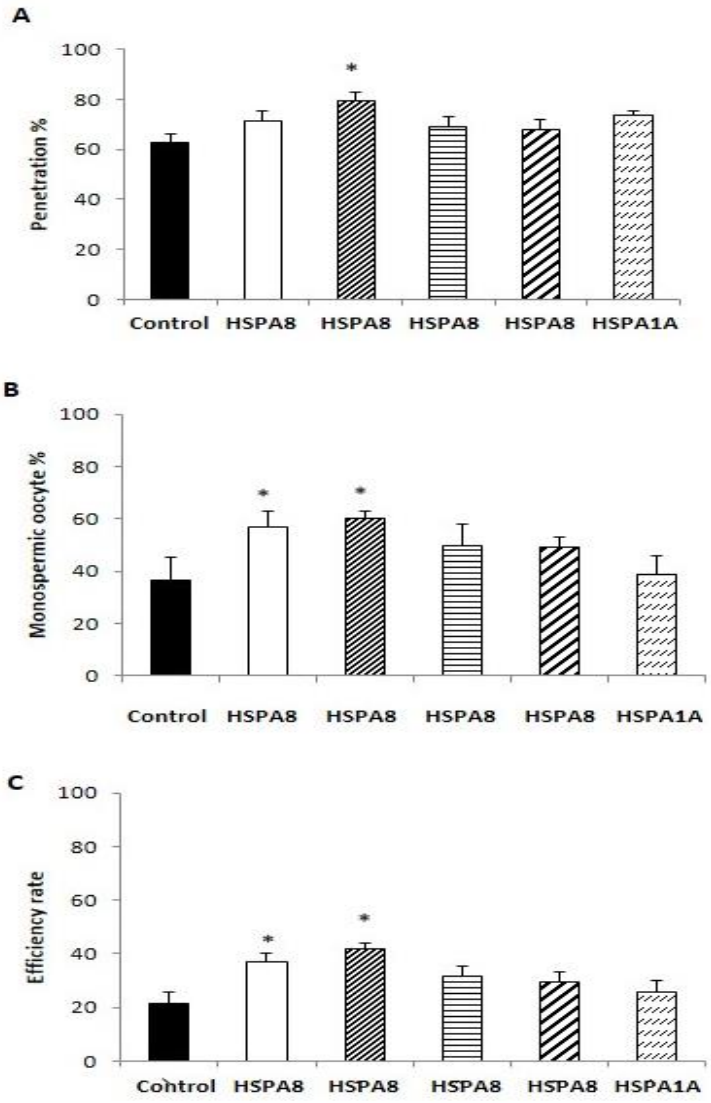

Figure 3.5 Effect of short pre-treatment with HSPA8 on sperm *in vitro* fertilisation performance. Matured oocytes were inseminated with fresh boar spermatozoa pre-treated with no protein (control) ■ , 0.5 □ , 1 ▨ ,10 ▤ and 20 ▨ µg/ml HSPA8 or 0.5 ▨ µg/ml HSPA1A for 15 min at room temperature. (**A**) penetration rate, (**B**) monosperm rate and (**C**) efficiency rate in each group were evaluated 21 hrs post insemination.* < 0.05, data were obtained from 6 replicates on 6 different days, n= maximum 350 oocytes in each group, mean ± s.e.m.

Table 3.1 *In vitro* fertilisation parameters (penetration, monospermy and efficiency rate) for oocytes inseminated with spermatozoa pre-treated with HSPA8 or HSPA1A.

Experimental Group	Total	Nº matured oocytes	Penetrated (%)	Monospermy (%)	Efficiency (%)
Control	317	287	195(68.3± 3.9 %)	68(36.4 ± 5.5%)	68(21.5 ± 2.8%)
HSPA8-0.5µg/ml	319	286	213(74.9 ± 3.9%)	119(56.8 ± 5.4%)*	119(37.2 ± 2.8%)*
HSPA8-1µg/ml	346	301	243(81.4 ± 3.9%)*	146(60.2 ± 5.3%)*	146(42.1 ± 2.7%)*
HSPA8-10µg/ml	335	297	220(74.5 ± 3.9%)	106(49.1 ± 5.4%)	106(31.6 ± 2.7%)
HSPA8-20µg/ml	333	295	202(69.8 ± 3.9%)	98(49.0 ± 5.5%)	98(29.4 ± 2.7%)
HSPAA1A-0.5µg/ml	332	301	219(73.2 ± 3.9%)	86(39.2 ± 5.4%)	86(25.9 ± 2.7%)
Total	1982	1767			

Boar spermatozoa were treated with and without HSPA8 (0.1, 0.5, 1, 10 and 20µg/ml) or HSPA1A (0.5 µg/ml), 15 min prior to insemination. * represents significant difference with control in each column, $p < 0.05$, n=6 replicates.

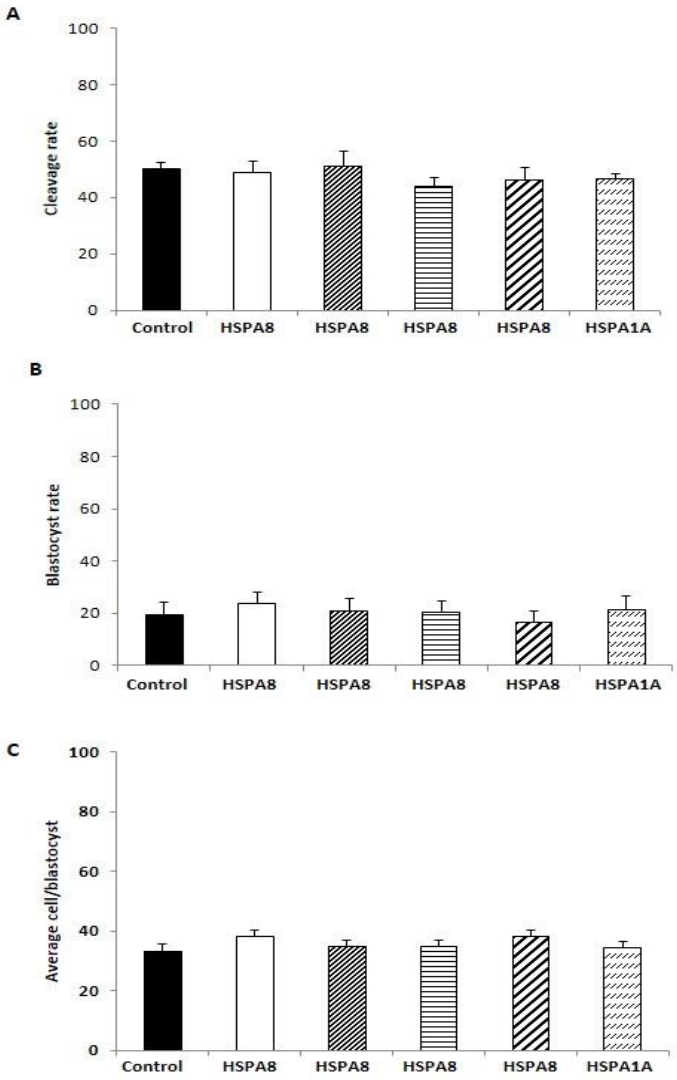

Figure 3.6 Effect of short pre-treatment of spermatozoa with HSPA8 on *in vitro* early embryonic development. Matured oocytes were inseminated with fresh boar spermatozoa pre-treated with no protein (control) ■ , 0.5 □ , 1 ▨ ,10 ▤ and 20 ◪ µg/ml HSPA8 or 0.5 ▨ µg/ml HSPA1A for 15 min at room temperature. (**A**) cleavage rate at 48 hrs post insemination, (**B**) blastocyst rate and (**C**) average cell / blastocyst at 168 hrs post insemination. Data were obtained from 6 replicates on 6 different days. n= maximum 350 oocytes in each group, mean ± s.e.m.

Table 3.2 *In vitro* embryonic development parameters (cleavage, blastocyst formation rate and average cell /blastocyst) for oocytes inseminated with spermatozoa pre-treated with HSPA8 or HSPA1A.

Experimental Group	Total	Cleaved (%)	Blastocyst (%)	Mean number of cell/blastocyst
HSPA8-0µg/ml	326	163(49.9 ± 3.5%)	(19.5 ± 4.5%)	33.19 ±2.3
HSPA8-0.5µg/ml	351	174(49.3 ± 3.4%)	(23.7 ± 4.4%)	38.03 ± 2.1
HSPA8-1µg/ml	345	178(51.4 ± 3.5%)	(21.0 ± 4.5%)	34.66 ± 2.2
HSPA8-10µg/ml	349	154(43.9 ± 3.5%)	(20.3 ± 4.4%)	34.67 ± 2.2
HSPA8-20µg/ml	341	158(46.1 ± 3.5%)	(16.5 ± 4.5%)	37.98 ± 2.4
HSPAA1A-0.5µg/ml	333	156(46.6 ± 3.5%)	(21.4 ± 4.5%)	34.37 ± 2.2
Total	2045			

Boar spermatozoa were treated with and without HSPA8 (0.1, 0.5, 1, 10 and 20µg/ml) or HSPA1A (0.5 µg/ml), 15 min prior to insemination, n=6 replicates.

3.5 Discussion

The current investigation was established on the presumption that the general effects of HSPA8 on basic sperm properties and particularly HSPA8-induced enhancement of sperm viability (observed in chapter 2) extended to sperm functional performance. Hence, investigating the effect of exogenous HSPA8 on the functional aspects of sperm physiology seemed very much reasonable.

One important aspect of sperm function is the ability to bind to the oviductal epithelial cells and form a functional sperm reservoir in the oviduct (Waberski, Magnus et al. 2006). Establishment of direct sperm-OEC attachment significantly facilitates maternal gamete interaction and contributes to formation of the sperm reservoir (Smith and Yanagimachi 1990; Pollard, Plante et al. 1991; Boilard, Reyes-Moreno et al. 2004). Sperm reservoir formation is a selective process for viable and uncapacitated spermatozoa to sustain their fertilising competence till the time of fertilisation (Hunter, Flechon et al. 1987; Thomas, Ball et al. 1994; Lefebvre and Suarez 1996; Ellington, Evenson et al. 1999; Fazeli, Duncan et al. 1999). Evidence indicates that use of aged semen samples or those obtained from individual animals with lower semen quality leads to debilitated sperm reservoir due to high degree of sperm membrane instability. Lack of membrane stability shortens sperm life span and non-viable spermatozoa are incapable of interacting with the oviduct (Saacke 1994; Waberski, Magnus et al. 2006).

Our earlier results (Chapter 2) demonstrated that exogenous HSPA8 enhanced the viability of uncapacitated spermatozoa, presumably via effects on the membranes of a selected subpopulation of sperm. Hence, the influence of HSPA8 on the capacity of boar sperm to bind oviductal epithelial cells was assessed based on the assumption that the effect is brought to sperm membrane to enable higher binding capacity. This assumption was further supported by the fact that extracellular HSPs have a tendency to associate with membrane lipid rafts (Chen, Bawa et al. 2005; Gehrmann, Doss et al. 2011). Inferring our *in vitro* results to the *in vivo* context indicates that HSPA8 plays a role in sperm reservoir formation by increasing the number of membrane-stable viable spermatozoa with high capacity to bind OECs. Whether HSPA8 directly mediates the sperm-OEC attachment or is involved in more complex pathways

remains unanswered, HSPA8 seems to function as a physiologic facilitator of the inter-cellular communication to improve efficacy of the oviductal sperm reservoir in maintaining sperm viability.

Viability is defined as the ability of a cell or tissue to exhibit a specific function or functions (Pegg 1989). The ultimate test of viability in a system is its ability to function normally in its physiologic milieu and in case of sperm, its the ability to fertilise and develop into a living organism. This is usually measured by estimating the *in vitro* fertilisation and early embryonic development rates of a given semen sample (Pegg 1989).

In our initial studies investigating the effect of HSPA8 on sperm characteristics, we demonstrated that exogenous HSPA8 had no effect on the sperm capacitation process, but reduced sperm mitochondrial activity after a short treatment period of 15 min (Chapter2). Although the observed inability of HSPA8 to promote capacitation, combined with its capacity to reduce mitochondrial activity might be a prediction of poor sperm fertilising function, the monospermic fertilisation rate of boar spermatozoa was significantly improved after a brief pre-treatment with HSPA8. Our observations are on the one hand consistent with a previous study in porcine system in which spermatozoa were briefly (10 min) pre-treated with apical segment of oviductal epithelial cells (sAPM) prior to fertilisation (Satake N 2007; Elliott RM 2009; Coy, Lloyd et al. 2010), but on the other hand contradict two other studies, again in pig, which used pre-ovulatory oviductal fluid or HSPA8 alone (Elliott RM 2009; Coy, Lloyd et al. 2010) for short treatment of spermatozoa before insemination. The former study revealed enhanced overall enhancement in penetration rate with reduced monospermy percentage, while in the latter two, monospermic penetration was enhanced with no change in overall penetration rate.

The disparity between these studies could probably be due to different fertilisation media, IVF protocols and most noticeably the agents with which spermatozoa were pre-treated. HSPA8 as an oviductal component might possess influential effects on its own, but act differently when used in conjunction with other naturally co-existing components. Even variability in the

source, method and diluents for HSPA8 production can account for differing results in similar studies.

Dynamic redistribution of endogenous HSPA1A in boar and bull sperm membranes during capacitation and acrosome reaction (Kamaruddin, Kroetsch et al. 2004; Spinaci, Volpe et al. 2005), identification of a 70 kDa heat shock protein in the purified plasma membrane from amphibian oocytes which exhibited a high affinity to sperm (Coux and Cabada 2006) and finally significant dose-dependent reduction in fertility rates after exposure to specific monoclonal anti-HSPA1A antibodies in bovine and porcine IVF assays (Matwee, Kamaruddin et al. 2001; Spinaci, Volpe et al. 2005) supported our speculation on the potential involvement of heat shock proteins in the process of oocyte-sperm interaction and fertilisation. However, none of the observations cast light on the precise mechanism which underpins HSPA8-mediated enhancement of fertilisation.

That fertilisation is, in principle, an orchestrated step-wise process, suggests that HSPA8 as an effector could influence individual or a combination of stages including sperm membrane fusion with the oocyte plasma membrane, establishment of the membrane block to polyspermy (Wortzman-Show, Kurokawa et al. 2007), sperm acrosomal exocytosis to expose the protein content of the inner acrosome to facilitate penetration (Sutovsky 2009) or the incorporation of sperm cytoplasmic and nucleus contents into the fertilised egg (Wortzman-Show, Kurokawa et al. 2007).

Generally, egg-sperm interaction at the plasma membrane level holds an essential role in fertilisation (Foltz, Partin et al. 1993; Coux and Cabada 2006). In our IVF experiment, pre-treated sperm samples were highly diluted to negligible amounts of HSPA8 in IVF medium, introducing the possibility that the observed effects could be attributed to HSPA8 localisation in sperm or its long lasting impacts on sperm and particularly on sperm plasma membrane. On this account, one speculation would be that since mature differentiated spermatozoa have lost protein synthesis machinery and do benefit from boosting effects of exogenous proteins (Pena, Rodriguez Martinez et al. 2009), exogenous HSPA8 might act directly as a synergistic effector with endogenous membrane-bound /cytoplasmic factors to enhance membrane fusogenicity.

Consequently, it is highly possible that HSPA8 mediates its positive effects upon release with sperm cytoplasmic / nuclear / membranous contents following exocytosis into the egg (Wortzman-Show, Kurokawa et al. 2007).

Alternatively, that HSPA8 elicits indirect contribution to the enhancement of fertilisation via interactions with sperm membrane lipid rafts and alterations in sperm membrane lipid fluidity is a very likely possibility, as it is a well-known fact that sperm-oocyte membrane fusogenicity depends on sperm membrane lipid hyperfluidity induced during the process of capacitation (Sutovsky 2009). All of the above assumptions reflect the need for investigating the exogenous localisation of exogenous HSPA8 in sperm which would provide helpful supplementary information. From a practical point of view, it is also very valuable to determine the precise nature of HSPA8 interactions by devising experiments which focus on different aspects of the fertilisation process that are influenced by HSPA8.

The simultaneous improvement in fertilisation and reduction of polyspermy using fresh spermatozoa pre-treated with low concentrations of HSPA8 was a surprising observation, as it generally expected that increased monospermy is accompanied by decline in penetration rate *in vitro* (Abeydeera and Day 1997; Gil, Ruiz et al. 2004). The coincidence of enhanced penetration and monospermy rates indicates that HSPA8 plays a dual physiological influence on the fertilisation capacity of sperm, rendering them able to fertilise the oocytes in favour of the highest number of embryo production. Sperm-egg membrane fusion contributes to establishment of the membrane block to polyspermy (Wortzman-Show, Kurokawa et al. 2007), however the membrane factor / factors responsible are not well elucidated. Our observation that treatment of sperm with HSPA8 improves monospermy rate indicates that HSPA8 can be one of the potential factors involved in the process.

The fact that the observed improvement in fertilisation did not result in a higher production of embryos or an increase in the number of cells in individual embryos contradicted previous results that have reported a higher proportion of embryos at different stages of early embryonic development (Satake N 2007; Elliott, Lloyd et al. 2009). Although the pace of transition in embryo development from one stage to another was not assessed in the current study, our

assessment of embryonic development *in vitro* was based on standard criteria which encompass the number of embryos and cells per individual embryos at developmental stages. However, this does not necessarily prove that HSPA8 improves embryonic development. Considering the fact that early embryos are inefficient at upregulating stress proteins and therefore are extremely stress vulnerable, the number of live cells within each embryo and the embryo's ability to resist and survive the stressful conditions could be better indicators of good quality embryos (O'Neill 2008; Ganeshan, Li et al. 2010). It has been demonstrated that treatment of sea urchin eggs with a combination of exogenous HSPA1A/HSPA8 prior to fertilisation prevents degeneration of produced embryos after exposure to high non physiologic temperatures (Browne, Swan et al. 2007). A useful step towards investigating the role of exogenous HSPA8 in pre-implantation stage of embryonic development would be to include HSPA8 in IVF/IVC media for long-term co-incubation periods. Evaluating the effect of HSPA8 pre-treatment on the survival ability of stressed embryos at different stages and also on the implantation rate would provide additional insight.

The specificity of HSPA8-mediated effects in IVF assay by using HSPA1A as a control protein, once again provided evidence on the fact that due to its constitutive expression, HSPA8 has gained an essential role in mammalian reproduction. Even in hibernating species, reproduction machinery is in a constant stand-by situation and requires persistent presence of reproductive effectors to preserve the reproducibility. HSPA8 is naturally expressed in normal physiological conditions and therefore is perfectly suitable for the task. However, blocking HSPA8-mediated effects on fertilisation by using specific anti-HSPA8 antibodies would be a further complementary approach for determining the specificity of HSPA8 in fertilisation performance of sperm in IVF systems.

In conclusion, the results presented in this study suggest that brief exposure of boar spermatozoa to exogenous HSPA8 significantly enhanced the capacity of sperm to bind OECs and their *in vitro* fertilisation performance. The available data suggest that exogenous HSPA8 not only influences the fertilisation process, presumably at multiple levels, but also takes part in the priming events prior to fertilisation by augmenting the interaction between sperm and the female oviductal epithelium.

A pioneering step towards clarifying the role of exogenous HSPA8 in reproduction and particularly on sperm fertilising function would be to perform bioassays using HSPA8 treated-spermatozoa in natural *in vivo* contexts such as intra uterine insemination. Understanding the effects of different oviductal components like HSPA8 on gamete biology, fertilisation and embryo development in animal models provides valuable information for developing more efficient IVF and embryo culture media as well as improving assisted reproduction techniques strategies in different mammalian species including the human.

Chapter 4

Extracellular HSPA8 enhances sperm membrane integrity via effects on membrane fluidity

4.1 Introduction:

In species with internal fertilisation, the timely interaction of two viable male and female gametes is of greatest importance. However, generally speaking, mating and ovulation do not necessarily coincide (Schnakenberg, Siegal et al. 2012). For instance, hibernating species exhibit clear dissociation between their male and female gametogenic cycles and this yields a substantial time span between sperm deposition in the female tract and ovulation (Roy and Krishna 2011). In humans, ovulation takes place once per each reproductive cycle, whereas sperm deposition might happen randomly at any time during the cycle. In nearly all other mammals, the female allows mating to take place at the time of oestrous (standing heat). Although standing heat is close to ovulation, spermatozoa may reside in the female reproductive tract from hours to days until ovulation takes place. To overcome this temporal separation and maximise the chance of conception, females have evolved successful means to efficiently store spermatozoa inside their reproductive tract until ovulation when eggs are available (Topfer-Petersen, Wagner et al. 2002; Holt and Fazeli 2010; Schnakenberg, Siegal et al. 2012). Storage of spermatozoa in the female reproductive tract is documented in a range of vertebrates and varies between months to years in hibernating species to a few days in mammals (Elliott, Lloyd et al. 2009). In many species sperm storage site is predominantly located in the oviduct (Fazeli, Duncan et al. 1999; Green, Bredl et al. 2001). Female sperm storage not only ensures the presence of adequate amount of spermatozoa at the ovulation time but also preserves the survival of stored spermatozoa (Lefebvre, Chenoweth et al. 1995; Schnakenberg, Siegal et al. 2012).

Although the exact mechanisms involved in preservation of sperm survival in the female tract remain largely elusive, reports suggest that direct contact between sperm and apical oviductal epithelial membranes plays a key role in this process (Smith and Yanagimachi 1990; Smith and Nothnick 1997). Both *in vivo* and *in vitro* studies have demonstrated that membrane to membrane contact initiates a reciprocal dialogue between the sperm and oviductal epithelium which ultimately stimulates gene transcription signals and production of protein factors in the oviductal epithelial cells (Ellington, Ignotz et al. 1993; Georgiou, Sostaric et al. 2005). Some oviductal membrane protein factors pertaining to heat shock protein family (HSPs) such as HSPD (HSP60) and HSPA8 (HSC70) have been proven to exhibit positive effects on viability

of spermatozoa from a number of species (Boilard, Reyes-Moreno et al. 2004; Elliott, Lloyd et al. 2009; Lloyd, Elliott et al. 2009), and it has been argued that their effect on sperm viability maintenance / enhancement is regulated via plasma membrane-mediated signalling mechanisms (Boilard, Reyes-Moreno et al. 2004; Elliott, Lloyd et al. 2009).

In preliminary experiments described in Chapter 2, we evaluated the effect that a bovine recombinant HSPA8 exerted on sperm viability. Viability was determined by dye exclusion or, in other words, membrane integrity-based viability assays which assess the intactness of membranes. These viability assays are composed of two dyes with distinct fluorescent spectra; the impermeant dye component which can only enter the cells with disrupted membranes and label them as dead, and the permeant component which gets into the cells with intact membranes and mark them as live cells (Pegg 1989).

Our data revealed that a brief (15 min) treatment period with extracellular HSPA8 was sufficient to significantly enhance the proportion of viable or membrane intact spermatozoa in a given boar semen sample. It was concluded that HSPA8 immediately restored membrane integrity in a subpopulation of spermatozoa which were postulatred to have undergone reversible membranous injuries. In other words, HSPA8 reversed non-fatal sperm membrane damage which was incurred during storage or processing and restored cell membrane integrity via interactions with, and alterations in membrane properties. However, the positive effect of HSPA8 on sperm viability was abolished by sperm capacitation, suggesting that capacitation-related modifications in sperm membrane structure counteracted the restorative properties of HSPA8.

This is a novel discovery which extends current knowledge concerning the functional aspects of HSPs in the reproductive system. There is very little evidence describing the potential mechanisms responsible for immediate HSPA8 membrane repairing effects in spermatozoa in the literature. However, reports demonstrating that certain HSP family members maintain cell membrane stability via modulating lipid membrane fluidity and interactions with membrane

lipid components exist in other systems (Boilard, Reyes-Moreno et al. 2004; Vigh, Nakamoto et al. 2007; Horvath, Multhoff et al. 2008).

In our investigations, detecting the restorative effect of HSPA8 on sperm membrane over a brief exposure time span indicated that there was a rapid mechanism involved in the membrane repair process. Moreover, it is known for a fact that lipid fluidity shifts happen very quickly over the membrane lipid plane (Ladha, James et al. 1997; Wolfe, James et al. 1998; Balogh, Horvath et al. 2005). Hence, we hypothesised that fast recovery of sperm membrane intactness under HSPA8 effect could result from HSPA8-mediated alterations in membrane lipid lateral fluidity. Accordingly, we evaluated the sperm membrane lipid diffusion patterns before and after exposure to HSPA8 in both fresh and capacitated spermatozoa as an approach to provide insight into the mechanism via which HSPA8 mediates an immediate enhancement of sperm viability / membrane integrity.

For this, an optical method named Fluorescence Recovery After Photobleaching (FRAP) was used to measure rapid two dimensional lateral diffusion of lipids within a defined area in sperm membrane after a brief exposure to HSPA8 (Ladha, James et al. 1997; Wolfe, James et al. 1998). FRAP is a useful method for investigating cell membrane lipid and protein diffusion and fluidity.

The basic principles contributing to FRAP lie in simple biological and physical facts. The biologic fact dates back to 1972 when the original fluid mosaic model of membrane structure was proposed (Singer and Nicolson 1972). The model described the membrane lipids and proteins as freely diffusing components within the membrane bilayer arrangement and Brownian motion was proposed as a contributing factor to membrane fluidity. The Brownian diffusion leads to the constant movement of molecules from highly concentrated regions to those with lower density levels and this will reduces heterogeneity of membrane lipid phase (Marguet, Lenne et al. 2006).

From the physics point of view, the photobleaching phenomenon also referred to as fluorescence fading, occurs when fluorescent molecules irreversibly lose their ability to fluoresce or in other words emit photons after being exposed to intense excitation light (Marguet, Lenne et al. 2006). Intense light causes photochemical destruction of the fluorophore and compromises the observation of fluorescent molecules during microscopy. Although undesirable in many microscopic techniques, fluorescence photobleaching is usefully exploited to study the motion / diffusion of molecules through FRAP technique.

For FRAP analysis, membrane lipid molecules were loaded with lipophilic fluorescent dye ODAF (5-(N-octa-decanoyl aminofluorescin) to produce a quantifiable signal after excitation with the appropriate wavelength (Wolfe, James et al. 1998). ODAF was used as the reporter dye for FRAP analysis of lipid diffusion in membrane surface because as a fluorescent-conjugated single fatty acid, it gets incorporated in between plasma membrane lipids on the outer leaflet of lipid bilayer and behaves similarly to membrane lipid components, so its diffusion characteristics are true reflections of real-time lipid dynamics in the membrane. Another advantage of using ODAF for FRAP analysis involving sperm samples is that it intercalates with plasma membranes of all spermatozoa and creates distinct fluorescent patterns in live and dead sperm cells (Ladha, James et al. 1997; Wolfe, James et al. 1998). The distinction between live and dead cells was crucial, as live spermatozoa were the target of our study (Figure 4.1).

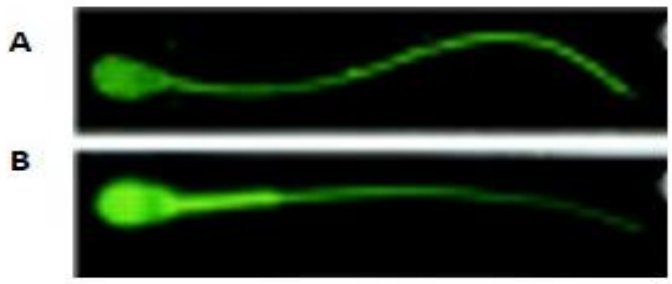

Figure 4.1 Fluorescence staining of live and dead cell boar spermatozoa with 5-(N-octa-decanoyl aminofluorescin (ODAF). (**A**) Typical uniform incorporation of ODAF into viable sperm membranes, (**B**) the classic regionalised incorporation of ODAF into dead / permeabilised cell showing higher fluorescence intensities over acrosome and midpiece domains. Pictures kindly provided by Professor Roy Jones, The Babraham Institute, Cambridge, United Kingdom.

In this section we focused on understanding the molecular mechanisms responsible for mediating the capacity of HSPA8 to repair sperm membranes. Membrane lipid dynamics of fresh and capacitated spermatozoa after exposure to HSPA8 were compared as a measure to elucidate the reason behind different responses to HSPA8 in each physiological condition. Moreover, the specificity of the effect on sperm membrane lipid fluidity was evaluated by comparing the influence of HSPA1A (HSP70) as the control protein due to their high sequential homology (Arispe, Doh et al. 2004; Multhoff 2007) and also by attempting to block effects using a specific anti-HSPA8 antibody. To best of our knowledge, this is the first study which has elucidated the molecular mechanism(s) that are responsible for the restorative functions of extracellular HSPA8 on sperm plasma membrane.

4.2 Materials and methods

All chemicals used for preparation of Tyrode's medium were purchased from BDH Laboratory Supplies, Poole, UK. Other chemicals were from Sigma-Aldrich company Ltd, Dorset, UK unless otherwise stated.

4.2.1 Semen Preparation

Boar semen diluted and stored for 24 hours in Beltsville Thawing Solution (BTS) (Pursel and Johnson 1975) was obtained from a commercial artificial insemination station (JSR Healthbred Limited, Thorpe Whillougby, Yorkshire,UK). On the day of experiment, diluted boar semen was washed using Percoll gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Briefly, aliquots of 2-4 ml of diluted semen were layered over a Percoll density gradient. The iso-metric Percoll gradient consisted of 2 ml 70% (v/v) Percoll overlaid with 2 ml 35% (v/v) Percoll. The gradient was centrifuged at 200 g for 15 min followed by 15 min centrifugation at 1000 g (Holt and Harrison 2002). The supernatant was removed and the pellet was resuspended in Tyrode's medium consisting of 3.1 mM KCL, 0.4 mM $MgCl_2.6H_2O$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, 0.3 mM $NaH_2PO_4.2H_2O$ (Elliott, Lloyd et al. 2009). Thereafter, semen samples were centrifuged at 900 g for 15 min and resuspended in Tyrode's medium. Sperm concentration was measured in duplicate using an improved Neubauer chamber (Marienfield, Germany) and adjusted to the required concentration in Tyrode's or TALP (Tyrode's medium supplemented with 12 mg/ml bovine serum albumin, 21.6 mM sodium lactate and 1 mM sodium pyruvate), based on experimental conditions.

4.2.2 Evaluation of Sperm viability

Sperm viability was evaluated by fluorescent viability assay composed of the combination of Propidium iodide & SYBR-14 (Live / Dead Sperm Viability Kit, Molecular Probes, Eugene, Oregon, USA). Propidium iodide & SYBR-14 at the final concentrations of 12 µM and 4 nM, respectively were added to 100 µl of washed semen aliquots (5×10^6 / ml). Samples were mixed and incubated for 30 min at 39°C in 5% v/v CO_2 for each assay. A 10 µl aliquot of each preparation assay was placed on a slide and evaluated by epi-fluorescence microscope (Olympus BH2, Olympus, London, UK), using a dual rhodamine-FITC filter. Three slides

were prepared for each sample and minimum of 200 spermatozoa were counted per slide. Spermatozoa were classified as live spermatozoa (green) and dead (red).

4.2.3 Assessment of sperm capacitation

The Chlortetracycline Hydrochloride (CTC) staining method was used to assess the capacitation status of boar sperm as described by Fazeli *et al.* (Fazeli, Duncan et al. 1999). The CTC staining solution was prepared by adding 750 µM CTC and 5mM D,L-cystein to the buffer solution containing 130 mM NaCl and 20 mM Tris. The solution was filtered using a 0.22 µM filter and pH was adjusted to 7.8. The CTC assay was performed by mixing equal volumes of Percoll-washed spermatozoa (5 x 10^6 / ml), previously diluted and incubated in TALP containing 12 µM Propodium iodide for 15 min at 39°C, 5% (v/v) CO_2) and CTC staining solution. After 30 seconds, an equal volume of 2% w/v paraformaldehyde in PBS was added to the sample. Finally, 2 drops of an antifade reagent Citifluor (Citifluor Ltd., London) was added to each sample in order to preserve the fluorescence. Two hundred spermatozoa per slide (3 slides for each sample) were evaluated immediately using an epi-fluorescence microscope (Olympus BH2, Olympus, London, UK). CTC assay showed 3 patterns of uniform fluorescence over the whole head (uncapacitated), fluorescence-free band in the postacrosomal region (capacitated) and no fluorescent over the whole head except for a thin band of fluorescence in the equatorial segment (acrosome reacted) described by Wang *et al*. (Wang, Abeydeera et al. 1995). Images of different sperm patterns are presented in chapter 2, Figure 2.2.

4.2.4 Assessment of sperm membrane fluidity

Lateral fluidity of lipid molecules in sperm plasma membrane was measured by FRAP analysis. For the labelling, equal volumes of Percoll-washed boar semen in Tyrode's (5 x 10^6 / ml) and 12.5 µM ODAF (5-N-octa-decanoyl aminofluorescin, Invitrogen, Eugene, Oregon, USA) in 2% v/v ethanol were incubated for 15 min at room temperature, after which they were washed twice in Tyrode's by centrifugation at 400 *g* for 10 min (Wolfe, James et al. 1998). Due to its cholesterol-accepting property and capacitating effects, bovine serum albumin was excluded from the media to prevent any secondary effects on sperm plasma membrane during FRAP studies.

ODAF uptake was monitored by a Zeiss epifluorescence microscope (Carl Zeiss Ltd, London, UK) fitted with a 100x objective lens.

In the FRAP method;

1. Laser beam with low intensity is focused on the area of interest in membrane and the initial fluorescent intensity (prebleach intensity) is recorded (Figure 4.2A).
2. The laser intensity is increased to 1000 folds for an extremely brief period (mseconds) to irriversibly bleach fluorophores located inside the laser beam area (Figure 4.2B).
3. Subsequently the fluorescent signal recovers with time over the bleached spot due to random diffusion of the surrounding unbleached fluorescent molecules from outside the bleached area (Wolfe, James et al. 1998; Marguet, Lenne et al. 2006). Their movement is recorded at low laser power and kinetic parameters are determined as:

a) Rate of diffusion (D-value) of the fluorescently labelled molecules within the membrane bilayer showing how fast the fluorescent molecules migrate back into the photobleached area which is calculated from: $W^2⁄4$ (tD), where W is the beam radius and tD is the diffusion time. Typical diffusion coefficients for membrane lipids are high and between $10^{-7}cm^2$ / sec to $10^{-9}cm^2$ / sec.
b) Rate of recovery (R%) shows the amount of light that returns to the photobleached spot relative to the amount of light that was there before the photobleaching and represents the proportion of the freely diffusing fluorescent molecules which cover the photobleached area (Figure 4.2C) (Ladha, James et al. 1997; Wolfe, James et al. 1998).

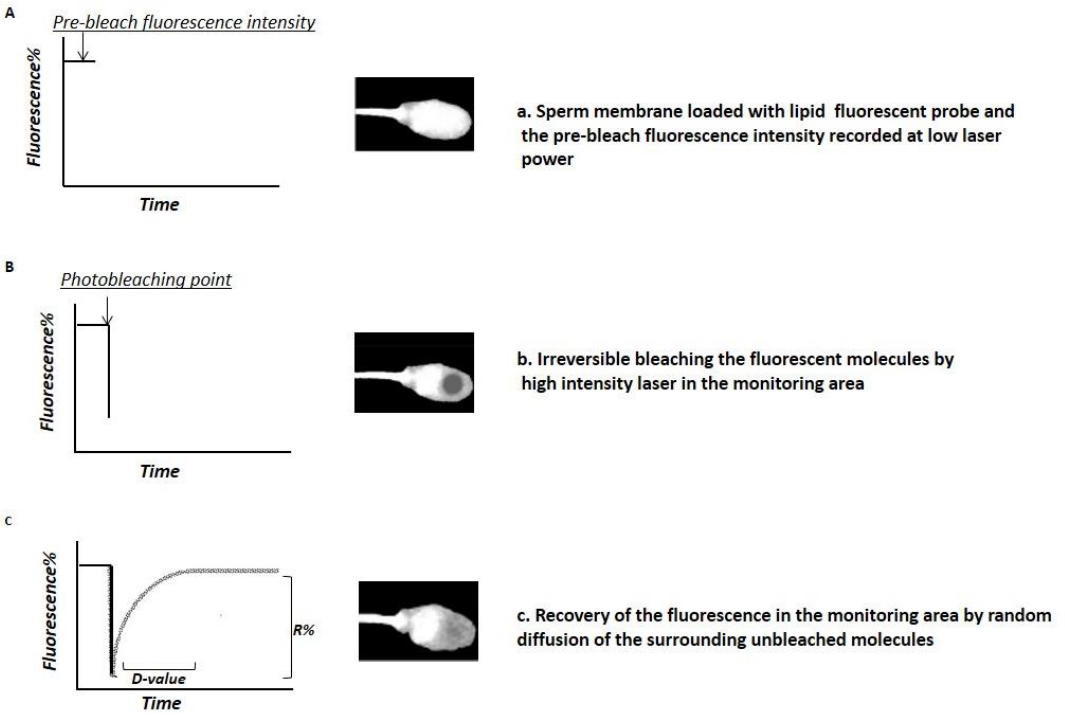

Figure 4.2 Schematic outline of the principle of fluorescence recovery after photobleaching.

The incident ~ 2.5 µm laser beam was generated by a water-cooled argon ion laser and bleaching time was 5 milliseconds at 0.2 mW. FRAP analysis was performed using a COOLSNAPHQ / ICX285 camera (Photometrics, Maidenhead, UK) using 406 nm excitation and 530 nm emission filters. The kinetics of fluorescence recovery including the rate of diffusion (D-value) and level of recovery (R %) of the diffusing reporter molecules within the membrane plane were recorded at room temperature (Wolfe, James et al. 1998). Figure 4.3 demonstrates a typical recovery curve for ODAF-loaded boar spermatozoa.

Live and dead staining patterns are markedly different in samples stained with ODAF; as depicted in figure 4.1, live spermatozoa are always stained weakly whereas the dead and membrane disrupted ones absorb more of ODAF and appear with stronger fluorescence, particularly over the acrosome and mid piece domains (Wolfe, James et al. 1998). The distinction between live and dead cells enabled us to examine the effect of HSPA8 on membrane fluidity of live spermatozoa only by excluding the dead ones. However in this investigation, live pattern spermatozoa which exhibited D-values and R% lower than viable range were also disregarded from the analysis as a further precaution step to restrict the analysis to viable cells only.

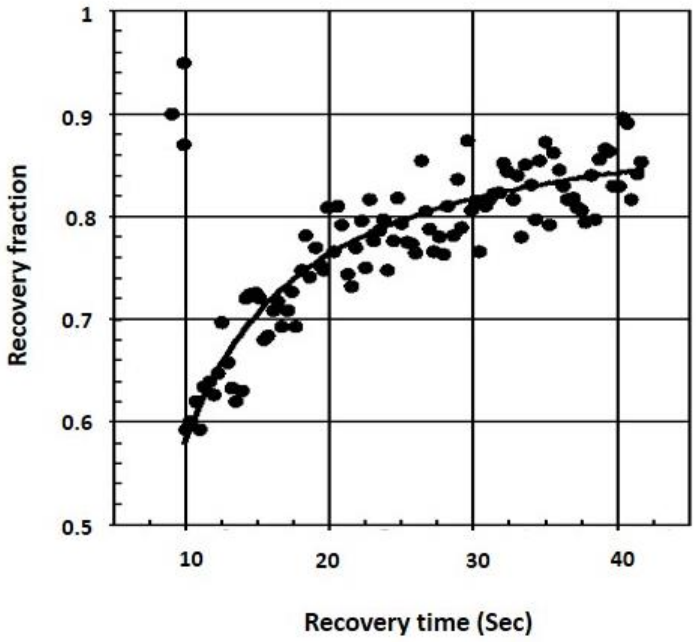

Figure 4.3 Typical recovery curve for ODAF-loaded boar spermatozoa

4.3 Experimental design

4.3.1 Effect of lipid reporter probe, ODAF on sperm viability

We suspected that as a fluorescent reporter, ODAF might have unknown secondary effects on sperm membrane integrity and cause unpredictable interference with the final results. Therefore a preliminary experiment compared the viability of spermatozoa stained with SYBR-14 / Propidium iodide and ODAF. For this, Percoll-washed semen samples from 3 different boars diluted in Tyrode's solution (5 x 10^6 spermatozoa / ml) were stained with SYBR-14 / Propidium iodide or ODAF according to their respective protocols and proportion of viable spermatozoa in each sample was determined microscopically.

4.3.2 Effect of HSPA8 on sperm membrane fluidity

For main experiments, fresh Percoll-washed semen samples (n = 4 boars) were diluted in Tyrode's solution (5 x 10^6 spermatozoa / ml) and split into two aliquots for simultaneous assessment of HSPA8 effect on sperm viability and membrane fluidity. Viability was determined microscopically after spermatozoa were incubated with HSPA8 (0 and 0.5 µg/ml) for 15 min at room temperature. For FRAP analysis, spermatozoa were loaded with ODAF and briefly (15 min) incubated with HSPA8 (0, 0.5 µg/ml) or HSPA1A (0.5 µg/ml) (recombinant human stress-induced HSPA1A, StressMarq, Victoria, BC, Canada) at room temperature. FRAP analysis on acrosomal and postacrosomal domains of live stained spermatozoa was performed at room temperature and a minimum number of 30 spermatozoa were examined in each sample.

4.3.3 Specificity of HSPA8 effect on sperm membrane lipid fluidity

To validate specificity of the observed effects on membrane fluidity, an anti-HSPA8 antibody (polyclonal rabbit IgG anti-HSPA8 antibody; Abcam, Cambridge, UK) was used to neutralise HSPA8.

Fresh Percoll-washed semen samples (n = 4 different boars) at 5 x 10^6 spermatozoa / ml, were split into two aliquots. One aliquot was used to determine sperm viability after spermatozoa were first pre-incubated with different concentrations (0, 0.5, 1 µg/ml) of anti-HSPA8 antibody for 30 min at room temperature and then treated with HSPA8 (0, 0.5 µg/ml) for 15 min at room temperature.

In the other aliquot, which was designated for FRAP analysis, ODAF pre-loaded spermatozoa were first treated with anti-HSPA8 antibody (0, 0.5, 1 µg/ml) for 30 min at room temperature and then briefly (15 min) incubated with HSPA8 (0, 0.5 µg/ml) at room temperature. FRAP analysis on acrosomal and postacrosomal domains of live stained spermatozoa was performed at room temperature and a minimum number of 30 spermatozoa were examined in each sample. For both viability and FRAP assessments, a control sample with no protein, one with 0.5 µg/ml HSPA8 only as well as a sample with 0.5 µg/ml anti-HSPA8 antibody only were included for final comparison.

4.3.4 Effect of sperm capacitation induction on HSPA8-mediated enhancement of sperm membrane lipid fluidity

Semen samples from 4 different boars (5x10^6 spermatozoa / ml) were pre-incubated in TALP at 39ºC with 5% v/v CO_2 (capacitating condition) for 0 or 6 hr. At each time point, sperm samples were divided into three aliquots for concomitant assessment of viability, capacitation status and membrane fluidity. Viability was determined microscopically after spermatozoa were treated with HSPA8 (0 and 0.5 µg/ml) for 15 min at room temperature. Capacitation status of spermatozoa in each sample was determined by CTC assay. For FRAP studies ODAF-loaded spermatozoa were treated with HSPA8 (0 and 0.5 µg/ml) for 15 min, after which analysis of a minimum of 30 spermatozoa was performed on acrosome and postacrosome regions at room temperature.

4.3.5 Statistical Analysis

Data were analysed by factorial analysis of variance (ANOVA). When the results were significant in ANOVA, means were further tested with post-hoc Fisher's least significant difference (LSD) test to examine the effect of treatments within experimental designs by Statistica software, version 7 (WWW.StatSoft.com, 1984-2004). Data were expressed as mean ± s.e.m. A probability of $P \leq 0.05$ was considered to be statistically significant.

4.4 Results

4.4.1 HSPA8 increased sperm membrane fluidity in a specific manner

The number of spermatozoa displaying live-pattern in samples stained with ODAF correlated with the viable proportions in SYBR / Propidium iodide-stained samples (70.5% ± 4 vs 72.5% ± 7.5 for SYBR14 / Propidium iodide and ODAF, respectively), indicating that ODAF was not a membrane disturbing agent and could be used safely in FRAP analysis.

For the main experiment, sperm viability was significantly enhanced from 72.35% ± 3 to 84.18 ± 4.5% after short (15 min) co-incubation with 0.5 µg/ml HSPA8 ($P < 0.005$). FRAP analysis of live-pattern spermatozoa in control samples showed higher D-values and R% rates on acrosome than on postacrosome regions, which was consistent with previous reports (Ladha, James et al. 1997; Wolfe, James et al. 1998). D-value and R% values were within viable range and indicative of membrane intactness (Ladha, James et al. 1997; Wolfe, James et al. 1998). Incubation of sperm with HSPA8 significantly increased acrosomal and postacrosomal D-values and R% of sperm plasma membrane lipids. Addition of HSPA1A to sperm samples did not alter any of the sperm membrane fluidity parameters in the two sperm regions (Figure 4.4).

The anti-HSPA8 antibody on its own had no effect on either sperm viability or membrane fluidity, however blocked the enhancing effect of HSPA8 on sperm viability and membrane fluidity in both acrosome and postacrosome regions. The blocking effect showed a concentration dependent pattern and the highest blocking effect in both analyses was observed with 1 µg/ml anti-HSPA8 antibody (Figure 4.5 and 4.6).

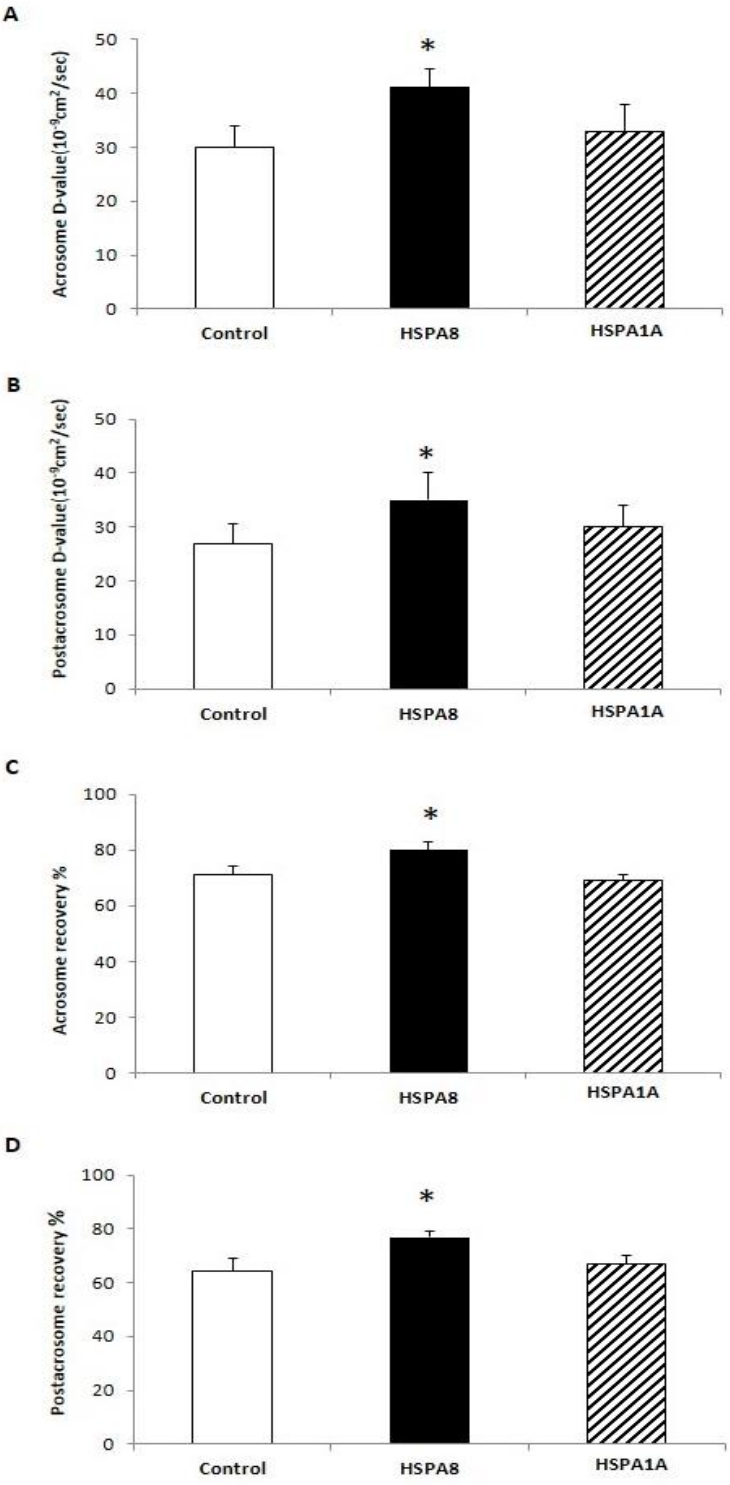

Figure 4.4 Effect of HSPA8 and its specificity on sperm membrane fluidity. (**A**) Acrosome D-values (10^{-9}cm^2 / sec), (**B**) postacrosome D-value (10^{-9}cm^2 / sec), (**C**) acrosome recovery% and (**D**) postacrosome recovery% of boar spermatozoa (30 spermatozoa / sample) after 15 min exposure to HSPA8, HSPA1A, 0 and 0.5 µg/ml. n = 4, * $P \leq 0.05$, mean ± s.e.m.

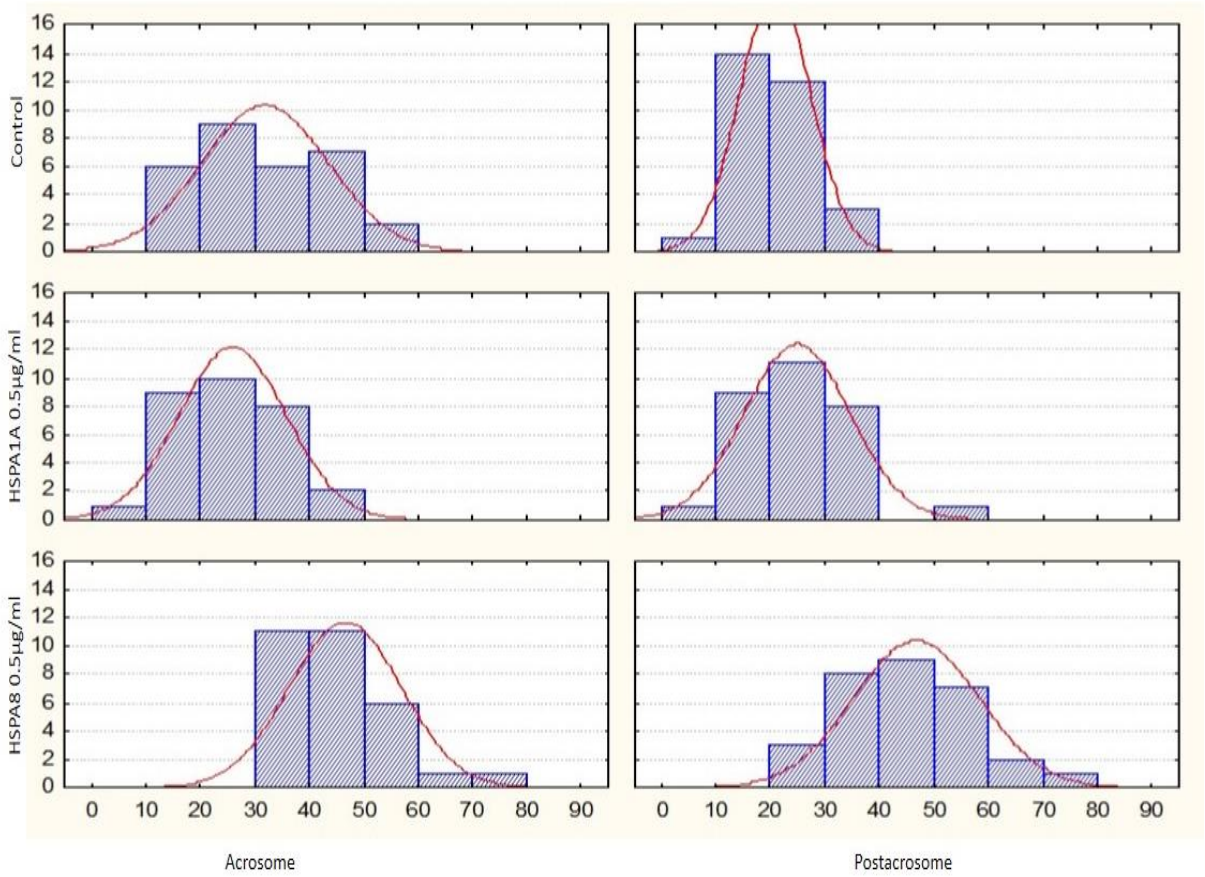

Figure 4.5 Distribution of sperm acrosome and postacrosome D-values ($10^{-9}cm^2$ / sec). D-values of boar spermatozoa after 15min exposure to HSPA8 or HSPA1A, 0 and 0.5 µg/ml, (n =30 spermatozoa).

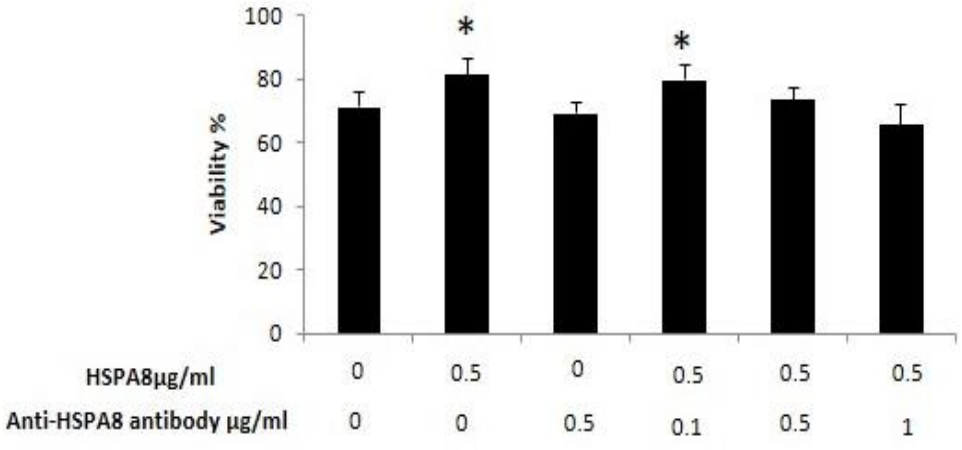

Figure 4.6 Neutralising effect of anti-HSPA8 antibody on HSPA8 effect on sperm viability (membrane integrity). Viability% (membrane integrity) of boar spermatozoa in response to HSPA8 (0.5 µg/ml) after pre-treatment with anti-HSPA8 antibody 0.1, 0.5 and 1 µg/ml, n = 4, * $P \leq 0.05$, mean ± s.e.m.

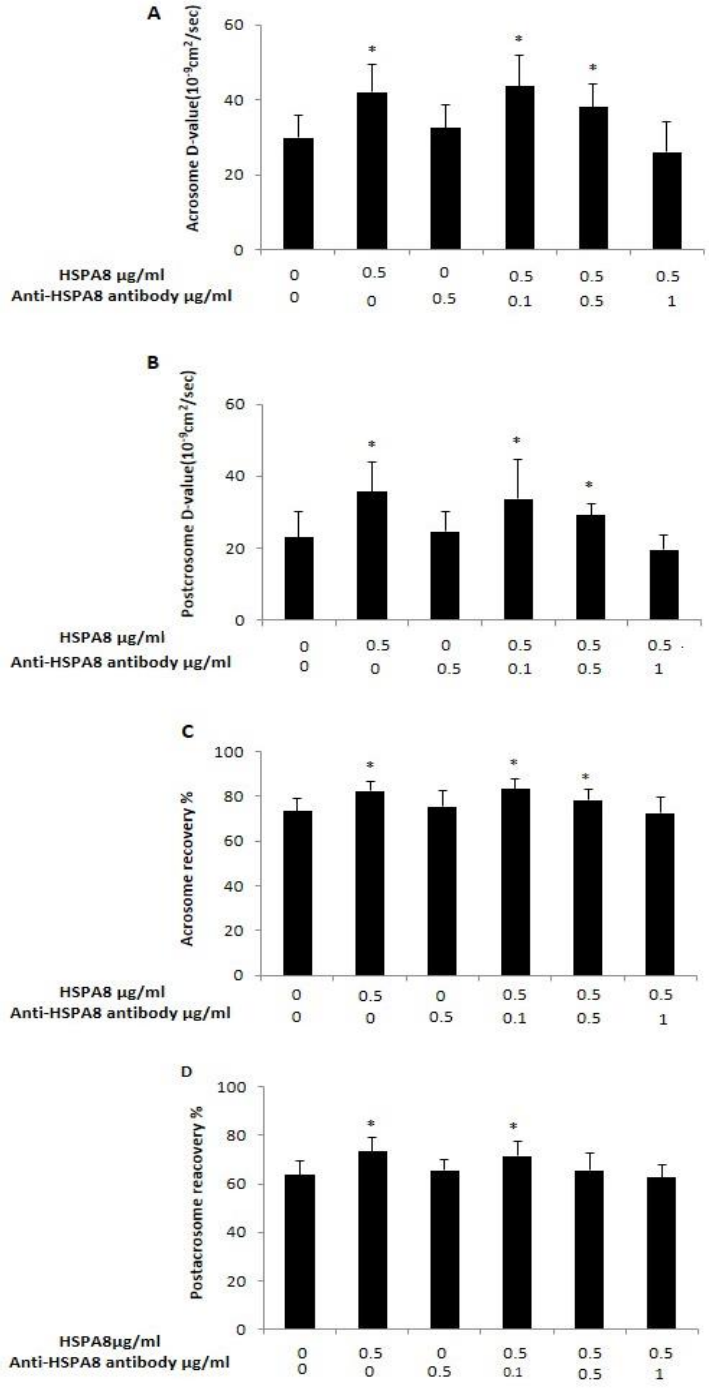

Figure 4.7 Neutralising effect of anti-HSPA8 antibody on HSPA8 effect on sperm membrane fluidity. (**A**) Acrosome D-values ($10^{-9}cm^2$ /sec), (**B**), postacrosome D-values ($10^{-9}cm^2$ /sec), (**C**), acrosome recovery%, (**D**) postacrosome recovery% of boar spermatozoa (30 spermatozoa / sample) in response to HSPA8 (0.5 µg/ml) after pre-treatment with anti-HSPA8 antibody 0.1, 0.5 and 1 µg/ml, n = 4, * $P \leq 0.05$, mean ± s.e.m.

4.4.2 Sperm capacitation negated the membrane fluidity enhancing effect of HSPA8

After 6 hours of incubation in capacitating conditions, the proportion of capacitated spermatozoa increased from 7 ± 2.5% to 67 ± 5.5% , concomitant with a significant decrease in the number of viable spermatozoa from 62 ± 5.5% to 50 ± 7 % ($P < 0.01$). Meanwhile, the enhancing effect of HSPA8 on sperm viability decreased as the proportion of capacitated spermatozoa increased (Figure 4.7A).

At 0 hr, fresh sperm exposed to HSPA8 (0.5 µg/ml) for 15 min showed significantly higher D-values and R% on acrosome and postacrosome domains compared to control samples which indicated higher lateral mobility of sperm membrane lipids in response to HSPA8. After 6 hr pre-incubation in capacitating conditions, there was an overall trend for D-values of ODAF to increase significantly and R% to decrease over the acrosome and post-acrosome compared to non-capacitated (0 hr) spermatozoa as the result of capacitation. However, brief exposure to HSPA8 had no additional effect on any of the measured parameters in the sperm head regions (Figure 4.7B, C, D and E).

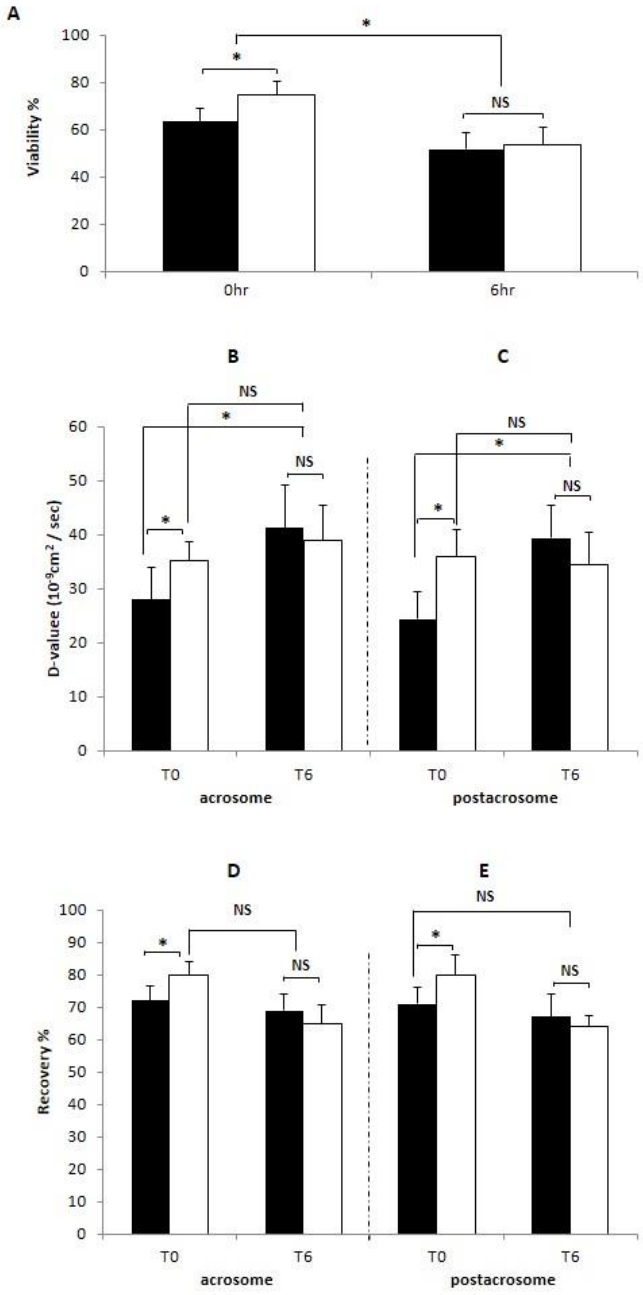

Figure 4.8 Effect of sperm capacitation on HSPPA8-mediated enhancement of sperm viability (membrane integrity) and membrane fluidity. (**A**) Viability % (membrane integrity), (**B**) arosome D-values ($10^{-9}cm^2/sec$), (**C**) postacrosome D-values ($10^{-9}cm^2/sec$), (**D**) acrosome Recovery% (**E**) and postacrsome recovery % of boar spermatozoa (30 spermatozoa / sample) at 0 and 6hr, after 15 min exposure to HSPA8, 0 ■ and 0.5 □ µg/ml. n = 4 , * $P \leq 0.05$, mean ± s.e.m, NS: not significant.

4.5 Discussion:

One of the mechanisms mediating the prolonging effects of intracellular HSPs on survival has been suggested to involve their ability to stabilise membranes by influencing lipid phase dynamics (Vigh, Maresca et al. 1998; Vigh, Horvath et al. 2007; Vigh, Nakamoto et al. 2007; Horvath, Multhoff et al. 2008). In this investigation, we used exogenous HSPA8 to induce an improvement in sperm membrane integrity and examined the downstream effect on the lipid fluidity on sperm head surface by FRAP analysis.

Membrane lipid fluidity measured by FRAP refers to the two-dimensional lateral fluidity and motion of lipid molecules in the membrane lipid bilayer. Fluidity is a characteristic feature of membranes. In spermatozoa, like any other cell, cellular viability and physiologic function are dependent on maintenance of membrane fluidity within physiologic ranges (Horvath, Glatz et al. 1998; Horvath, Multhoff et al. 2008; Tai, Yang et al. 2010). Membrane fluidity is the function of membrane lipid composition and environmental factors (Moce, Purdy et al. 2010; Tai, Yang et al. 2010). Extreme temperatures and free O_2 radicals are amongst the most common environmental factors which adversely affect membrane fluidity equilibrium, while length of membrane fatty acid, degree of fatty acid saturation and membrane cholesterol content are the defence factors used by cells to balance membrane fluidity (Moce, Purdy et al. 2010; Tai, Yang et al. 2010). For instance, high temperatures increase membrane fluidity because the membrane lipids tend to form irregular gel-like structures (Wolfe, James et al. 1998) while oxidation and cold favour more rigid crystalline structures (Wolfe, James et al. 1998; Christova, James et al. 2002; Vigh, Nakamoto et al. 2007).

In contrast, membranes containing shorter fatty acids, unsaturated fatty acids or a lower cholesterol content exhibit higher fluidity within certain temperature ranges (Moce, Purdy et al. 2010). Excessive alterations in physical state of membrane lipid structure cause loss of membrane integrity. Membrane permeabilisation in turn causes sudden transition from a fluid phase to a rigid immobile phase and spermatozoa which exhibit lipid fluidity parameters lower than certain levels are considered as dead cells (Wolfe, James et al. 1998).

In the current study, FRAP analysis revealed that along with enhanced viability (measured as membrane integrity), lipid fluidity in the acrosome and postacrosome domains of sperm membrane was increased after short exposure to HSPA8. A concomitant increase in membrane integrity and fluidity under the influence of HSPA8 suggested that there was an association between the two responses. Moreover, coincident inhibition of membrane fluidity and viability enhancements after blocking the HSPA8 effect with specific antibody further supported the concept that HSPA8-mediated enhancement of sperm membrane integrity and membrane fluidity were inter-related.

Furthermore, the unresponsiveness of spermatozoa pre-incubated in capacitating conditions to viability and membrane fluidity enhancing effects of HSPA8 further suggested a link between HSPA8-mediated increase in membrane integrity and fluidity. As the experimental conditions, including HSPA8 concentration, was maintained relatively identical at all pre-incubation time intervals and the mere difference was in the sperm capacitation status, we stipulated that the capacitation induced changes, particularly in the sperm membrane, suppressed the effects of HSPA8. It was speculated that physiological and biochemical alterations induced by capacitation rendered spermatozoa unresponsive to HSPA8, particularly in the membrane region. There seemed to be a distinct line between the membrane hyperfluidisation which was caused by capacitation in pre-incubated spermatozoa and fluidisation induced by HSPA8 in fresh spermatozoa. There was a limit to rise in membrane fluidity by HSPA8, whereas capacitation-induced membrane fluidity was far higher. Conclusively, it was speculated that capacitation-induced membrane disorganisation / hyperfluidity was far beyond the physiologic capacity of HSPA8 to further enhance membrane fluidity. An alternative possibility is that the capacitation process led to a substantial reduction in HSPA8 binding sites.

Among numerous changes during the sperm capacitation process, there is a substantial reduction in membrane cholesterol, and this results in a remarkable remodelling of lipid architecture and hyperfluidity in the sperm plasma membrane (Osheroff, Visconti et al. 1999; Buffone, Verstraeten et al. 2009; Tapia, Macias-Garcia et al. 2012). Reports on an association of HSPA family members with cholesterol within membrane lipid bilayer (Chen, Bawa et al. 2005) and particularly an ability of HSPA8 to bind membrane lipid domains (Mamelak and

Lingwood 2001; Mamelak, Mylvaganam et al. 2001) raises the prospect that capacitation-induced compositional and / or organisational changes in sperm membrane cholesterol / lipid structure compromises the interaction sites for HSPA8 in spermatozoa.

Shifts in membrane lipid fluidity state in favour of membrane stability under the effect of HSPA8 is in full accordance with existing reports on HSP-membrane interactions. Heat shock proteins regulate membrane integrity, activity and signalling by regulating the membrane lipid phase (Chen, Bawa et al. 2005; Horvath, Multhoff et al. 2008), and there is plenty of evidence that HSPs are involved in stabilising cellular and organellar membranes in stressful conditions (Carratu, Franceschelli et al. 1996; Vigh, Maresca et al. 1998; Vigh, Horvath et al. 2007). The interaction of HSP17 with membrane lipids of *Synechocystis* positively influence membrane integrity and fluidity in favour of cell survival under stressful conditions (Horvath, Glatz et al. 1998). An *E. coli* heat shock member, GroEL, has also been shown to associate with and stabilise membrane during stress (Torok, Horvath et al. 1997). HSPA1A prevents the death of cancer cells by inhibiting lysosomal membrane permeabilisation (Gyrd-Hansen, Nylandsted et al. 2004; Nylandsted, Gyrd-Hansen et al. 2004; Kirkegaard, Roth et al. 2010).

Although different observations have reported the membrane responses to HSPs to involve modifications in membrane fluidity, the direction of membrane fluidity in response to HSP stimulation is a matter of debate. It is generally accepted that association of HSPs with cell membranes serves to antagonise stress-induced hyperfluidisation of membrane domains and preserve functional and structural integrity of the biomembranes (Horvath, Glatz et al. 1998; Vigh, Escriba et al. 2005; Horvath, Multhoff et al. 2008). Our results have demonstrated that HSPA8-mediated increases in membrane fluidity contribute to the restoration of membrane integrity.

This contradiction might be explained by considering variations in species, biologic and cellular systems and environmental circumstances, as well as the different methods that have been applied in the assessment of membrane fluidity. Moreover, membrane interactions and the resulting responses strongly rely on membrane raft organisation, lipid composition and level of lipid saturation (Vigh, Escriba et al. 2005; Vigh 2010). For instance, the mammalian

sperm membrane is highly susceptible to oxidation due to higher polyunsaturated fatty acids, as well as higher cholesterol to phospholipid molar ratios compared to more resistant spermatozoa from other species (Christova, James et al. 2002; Tapia, Macias-Garcia et al. 2012). Reactive oxygen species and lipid peroxidation are common factors that cause lipid rigidification and disruption in sperm membrane integrity during *in vitro* sperm storage (Tapia, Macias-Garcia et al. 2012). Therefore, in our case it is very possible that the population of spermatozoa that reacted to exogenous HSPA8 by increasing membrane fluidity and incorporating live staining are probably those cells that have a reduced fluidity due to oxidative or other physical damages which cause adverse membrane rigidity (Aboagla and Terada 2003; Christova, James et al. 2004; Buffone, Verstraeten et al. 2009). It has been demonstrated that increases in membrane fluidity render sperm membrane flexible and more able to endure environmental damages and recover its integrity (Luvoni 2002; Aboagla and Terada 2003; Tapia, Macias-Garcia et al. 2012).

It could also be argued that the contrast between the directions of membrane lipid lateral diffusion in response to HSPs is a strong indication of different functional aspects of HSPs. At the time of stress, HSPs can quickly interfere to avert the crisis before the stress can cause fatal defects in membrane (Balogh, Horvath et al. 2005; Vigh, Horvath et al. 2007; Vigh, Nakamoto et al. 2007). However, as observed in our studies, HSPA8 seemed to repair membrane defects already caused by stress. Therefore, although both scenarios are generally regarded as membrane protecting mechanisms for HSPs, there appears to be a substantial difference between the nature and timing of the HSP effects. The first response is aimed primarily at protection and the latter at restoration of the cell membrane integrity. Investigating the effect of HSPA8 on sperm membrane lipid fluidity under known membrane fluidisers or rigidifying agents enhances our understanding of the reason behind differences found in the response to HSP-membrane interplay in different situations.

In our experiments, the recovery of membrane integrity in HSPA8-treated samples was detected in a subpopulation of spermatozoa, suggesting that there was a selective response in a group of spermatozoa with potentially specific affinity to HSPA8 treatment. The fact that there was a selective manner for HSPA8 damage restorative property on sperm suggests that

HSPA8 recognises and saves potentially functional spermatozoa with reversible membrane defects whose cost of survival outweigh their death in the fertilisation process via membrane signalling mechanisms.

In the current study, HSPA1A was used as control protein in order to provide insight into the specificity of the observed effects. The biological relevance of this comparison was that HSPA1A is the stress-induced isoform of the constitutive HSPA8 which shares more than 80% homology with HSPA8 (Arispe, Doh et al. 2004; Multhoff 2007) and therefore, is assumed to play identical functions in the cell (Goldfarb, Kashlan et al. 2006; Li, Ninomiya et al. 2011). HSPA1A did not influence sperm membrane fluidity which can be attributed to distinctive responses the spermatozoa have to variety of HSPs or more importantly, to the different functions HSPs play based on their nature and expression conditions. Moreover, the inability of HSPA8 to enhance boar sperm membrane fluidity after addition to spermatozoa pre-treated with anti-HSPA8 antibody further supported the specificity of HSPA8 effect on sperm membrane fluidity.

It is a well-established dogma that in biologic systems HSPs are expressed intracellularly in response to various injuries caused by stressors. For instance, alterations in the physical states of cell membranes caused by heat or any other membrane disturbing agents can trigger HSP gene expression and hence start the stress response to protect cell membrane equilibrium and integrity (Horvath, Multhoff et al. 2008).

However, our study revealed that extracellular HSPA8 was also able to reverse cell membrane damage by influencing membrane lipid lateral diffusion. Spermatozoa are terminally differentiated cells which are devoid of protein synthesis apparatus, incapable of producing new HSPs under any circumstances or turning over membrane components (Ladha, James et al. 1997; Hecht 1998; Mouguelar and Coux 2012). This property renders spermatozoa dependent on extracellular agents present either in their local environment or as a sperm component pre-synthesised in the spermatogenesis phase (Allen, O'Brien et al. 1988; Miller, Brough et al. 1992; Kamaruddin, Kroetsch et al. 2004). Our data clearly demonstrated that exogenous HSPA8 provided significant improvement in sperm preservation, strongly implying

that production of HSPA8 in the female reproductive tract is an evolutionary solution to facilitate regulation of boar sperm function.

The maintenance of sperm integrity *in vitro* is an immense challenge in sperm preservation technology (Tapia, Macias-Garcia et al. 2012). Our data demonstrating the role of exogenous HSPA8 in restoring sperm membrane integrity *in vitro* implies that HSPA8 could be a useful and important component in semen preservation technology.

In the current study it was revealed that a relatively brief (15 min) exposure of fresh boar spermatozoa to exogenous bovine recombinant HSPA8 was sufficient to induce alterations in sperm membrane fluidity. Simultaneously, sperm viability was significantly enhanced as the result of improvement in membrane integrity while none of the effects were reproducible in capacitated spermatozoa. Moreover, HSPA1A as a highly identical protein to HSPA8 in terms of sequence and function was not capable of inducing any of the observed effect in sperm.

Our data strongly suggest that the molecular mechanism that mediates HSPA8 repairing effect on sperm membrane occurs through alterations in dynamics of membrane lipids, the effect is specific and only detectable in fresh spermatozoa before the commencement of any capacitation-induced changes in sperm membrane lipid structure. However, since FRAP only measures the lateral lipid diffusion over several square microns, the possibility that HSPA8 also affects membrane lipid translocation between inner and outer lipid bilayer, membrane protein components or raft organisations in nanometer scales cannot be ruled out. The application of other biophysical techniques with larger target areas which measure rotational membrane lipid diffusion in conjunction with FRAP can yield a broader perspective to the effect of HSPA8 on membrane lipid dynamics. Furthermore, using techniques such as scanning electron microscopy or atomic force microscopy would provide three dimensional submicron images of sperm surface membrane to investigate whether HSPA8-mediated membrane fluidity is accompanied by any detectable morphologic / morphometric changes in sperm membrane.

It remains unclear if improvements in membrane integrity are the direct consequence of increased membrane lipid diffusibility or shifts in membrane fluidity, or whether HSPA8 initiates a complex subcellular pathway which ultimately ends in membrane repair. Investigating potential intracellular signalling pathways related with membrane re-organisation under HSPA8 effect could provide clues for deciphering the targets and detailed mechanisms behind HSPA8-mediated membrane alterations.

Chapter 5

Cholesterol mediates the restorative effect of extracellular HSPA8 on boar sperm membrane

5.1 Introduction

The literature provides evidence that some oviductal membrane protein factors pertaining to heat shock protein family (HSPs) such as HSPD (HSP60) and HSPA8 (HSC70) exhibit positive effects on viability of spermatozoa from a number of species (Boilard, Reyes-Moreno et al. 2004; Elliott, Lloyd et al. 2009; Lloyd, Elliott et al. 2009), and it has been argued that their effect on sperm viability maintenance is regulated via plasma membrane-mediated signalling mechanisms (Boilard, Reyes-Moreno et al. 2004; Elliott, Lloyd et al. 2009).

Consistent with that, results obtained during the early stages of this research (Chapters 2 and 3) suggested a novel functional aspect for HSPA8, as a brief (15 min) treatment period with HSPA8 was enough to significantly enhance the proportion of viable or membrane intact spermatozoa in a given boar semen sample. The conclusion that extracellular HSPA8 immediately restored membrane integrity in a subpopulation of spermatozoa was an attractive notion, as it extended the physiologic role of HSPs from stress protection to restoration. Furthermore, it also suggests that exogenous HSPA8 reverses non-fatal sperm membrane damage which is incurred during storage or processing and restores cell membrane integrity via interactions with, and alterations in membrane properties.

Extracellular heat shock proteins have been reported to protect and maintain cell membrane homeostasis against stressful challenges (Broquet, Thomas et al. 2003; Lancaster and Febbraio 2005; Horvath, Multhoff et al. 2008). Through specific associations with membrane lipid rafts, HSPs are shown to regulate major attributes of membrane lipid bilayer such as permeability and fluidity (Broquet, Thomas et al. 2003). In line with this property of HSPs, they also actively participate in repairing mild membrane defects caused by environmental stressors (Vigh, Maresca et al. 1998; Balogh, Horvath et al. 2005; Vigh, Nakamoto et al. 2007). Little is known about the mechanism of HSP-raft association, however there is convincing evidence that membrane-bound HSPs associate with cholesterol in the lipid raft.

Lipid rafts or ‘cholesterol-rich domains’ are dynamic liquid assemblies that are enriched in sphingolipids and cholesterol and float in the membrane bilayer (Brown and London 1998; Simons and Toomre 2000; Simons and Ehehalt 2002; Simons and Sampaio 2011). Lipid rafts

are highly organised structures significantly due to their high cholesterol content. Cholesterol plays a key regulatory role in stability, permeability and fluidity of membrane rafts (Crockett 1998) and its removal results in raft dissociation, functional disruption and dramatic alterations in membrane fluidity.

Examining the association of extracellular heat shock proteins with cell membrane cholesterol has been made possible by use of methyl-beta cyclodextrine (MβCD). MβCD is a poly-carbohydrate molecule with high affinity to cholesterol which readily removes membrane cholesterol without inserting or binding to the membrane (Klein, Gimpl et al. 1995; Simons and Toomre 2000). MβCD-mediated cholesterol depletion resulted in substantial decreases in membrane-bound HSPA (HSP70) isoforms in neuronal cells from rat brain (Chen, Bawa et al. 2005), as well as in tumour cells (Gehrmann, Liebisch et al. 2008).

In earlier chapters, we have obtained consistent findings that HSPA8 enhances the integrity and fluidity of boar sperm membrane. Membrane fluidity and cholesterol are two tightly related entities. Cholesterol molecules act as natural 'glue' which packs phospholipid molecules tightly against each other and increases membrane stiffness by limiting lateral diffusion of lipid molecules (Buffone, Verstraeten et al. 2009; Simons and Sampaio 2011). Substantial reductions in membrane cholesterol either through artificial manipulations or due to cryopreservation causes increases in the lateral diffusion of lipids (Cross 1998). On the other hand, treating sperm with cholesterol minimises the lateral fluidity of the membrane lipids (Ladbrooke, Williams et al. 1968; Moce, Blanch et al. 2010). Considering the relation between cholesterol and membrane fluidity along with HSPA8-enhancing effects on membrane fluidity, we hypothesised that cholesterol mediated the effects of HSPA8 on sperm plasma membrane.

Accordingly, inducing modifications in membrane cholesterol content and investigating the impact on HSPA8-mediated effects on membrane fluidity would provide insight into the interactions between HSP and membrane cholesterol, and their consequences. Use of Methyl-β-cyclodextrin (MβCD) and cholesterol-MβCD compound (CLC) as cholesterol depleting and cholesterol donor agents, respectively facilitated cholesterol modifications in sperm membrane. Fluorescence recovery after photobleaching allowed us to assess potential changes

in membrane lipid fluidity subsequent to cholesterol modifications. The factors studied were the ability of HSPA8 to enhance the integrity and fluidity of sperm membranes exhibiting a modified membrane cholesterol profile. Although reproduction is the central theme of this study, our results would provide additional information on the mechanisms underlying the ability of HSPs to repair membranes in other biological systems.

5.2 Materials and Methods

All chemicals used for preparation of Tyrode's medium were purchased from BDH Laboratory Supplies, Poole, UK. Other chemicals were from Sigma-Aldrich company Ltd, Dorset, UK, unless otherwise stated.

5.2.1 Semen Preparation

Boar semen diluted and stored for 24 hours in Beltsville Thawing Solution (BTS) (Pursel and Johnson 1975) was obtained from a commercial artificial insemination station (JSR Healthbred Limited, Thorpe Whillougby, Yorkshire,UK). On the day of experiment, diluted boar semen was washed using Percoll gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Briefly, aliquots of 2-4 ml of diluted semen were layered over a Percoll density gradient. The iso-metric Percoll gradient consisted of 2 ml 70% (v/v) Percoll overlaid with 2 ml 35% (v/v) Percoll. The gradient was centrifuged at 200 g for 15 min followed by 15 min centrifugation at 900 g (Holt and Harrison 2002). The supernatant was removed and the pellet was resuspended in Tyrode's medium consisting of 3.1 mM KCL, 0.4 mM $MgCl_2.6H_2O$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, 0.3 mM $NaH_2PO_4.2H_2O$ (Elliott, Lloyd et al. 2009). Thereafter, semen samples were centrifuged at 900 g for 15 min and resuspended in Tyrode's medium. Sperm concentration was measured in duplicate using an improved Neubauer chamber (Marienfield, Germany) and adjusted to the required concentration in Tyrode's or TALP (Tyrode's medium supplemented with 12 mg/ml bovine serum albumin, 21.6 mM sodium lactate and 1 mM sodium pyruvate), based on the experimental conditions.

5.2.2 Evaluation of sperm viability

Sperm viability was evaluated using Propidium iodide / SYBR-14 (Live / Dead Sperm Viability Kit, Molecular Probes, Eugene, Oregon, USA), as escribed previously in Chapter 2.

5.2.3 Assessment of sperm capacitation status

The capacitation status of boar sperm was assessed using the Chlortetracycline Hydrochloride (CTC) staining method, as described by Fazeli *et al.* (Fazeli, Duncan et al. 1999), as described previously in Chapter 2.

5.2.4 Assessment of sperm membrane fluidity by fluorescence recovery after photobleaching (FRAP)

Sperm membrane fluidity was assessed by FRAP, as described previously in Chapter 4.

5.2.5 Membrane cholesterol quantification

Sperm membrane cholesterol content was determined using the Cholesterol Liquicolor Enzymatic assay (Stanbio, Boerne, Texas, USA). For this, pellets of fresh Percoll-washed or membrane cholesterol modified spermatozoa were resuspended and washed (600 g, 10 min) in phosphate-buffered saline (PBS:136.9 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4.12H_2O$ and 1.5 mM KH_2PO_4) (Harayama, Okada et al. 2003). After a second wash, the pellet was resuspended in 1 ml PBS and sperm concentration was determined. The samples were stored at -20°C until cholesterol analysis was performed.

After thawing, samples were diluted 1/1 v/v with lysate buffer (0.4% v/v Triton x-100 in PBS) for 1 hr to solubilise the plasma membranes. Same samples were further diluted 1/5 with the reagent provided in the kit and incubated for 25 min at 37°C. Samples were then washed and the supernatant was analysed for cholesterol by spectrophotometer (Cecil Instruments, Cambridge, UK) at a wavelength of 500 nM. Cholesterol concentrations were determined by comparison of the readings with those obtained for a standard curve which was generated using defined cholesterol concentrations.

5.2.6 Depletion of membrane cholesterol

Sperm membrane cholesterol was depleted by Methyl-β-cyclodextrin (MβCD). On the day of experiment, MβCD working solution was made at 16 mM by complete dissolving of 2 mg MβCD in 1 ml Tyrode's solution in a glass tube. MβCD solutions should be freshly made on the day of usage and not be prepared ahead or stored. Fresh Percoll-washed spermatozoa were diluted in Tyrode's and treated with 0, 1, 2, 4 and 8 mM MβCD / 10^8 spermatozoa for 30 min at 20-22°C on a gentle shaker to allow even distribution of MβCD and homogeneous cholesterol removal. The mixture was washed using a 45% v/v Percoll gradient at 600 g for 20 min, the pellet was washed once more in PBS (600 g, 10 min) and resupended in Tyrode's. The remaining amount of cholesterol in the spermatozoa was measured as described above and compared to control samples for final determination of the membrane cholesterol removal.

5.2.7 Repletion of membrane cholesterol

Spermatozoa were loaded with cholesterol according to the protocol described previously (Purdy and Graham 2004; Moore, Squires et al. 2005). Cholesterol was added to sperm membrane using cholesterol-loaded Methyl-β-cyclodextrin complex (CLC). CLC was prepared by dissolving cholesterol 200 mg in 1ml of chloroform in a glass tube (solution A). MβCD 1 g was dissolved in 2 ml methanol in a separate glass tube (solution B). A 450 µl aliquot of the solution A was added to the solution B and stirred until clear. The the mixture was poured into a glass petri dish and dried in air over 48 to 72 hr to form crystals. The resulting dry crystals were stored in a glass container at room temperature for future use.

On the day of experiment, a working solution of 16 mg / ml CLC was prepared in Tyrode's by vigorous vortexing at 37°C. CLC solutions should be freshly made on the day of usage and not be prepared ahead or stored. Spermatozoa were treated with 0, 1, 2, 4 and 8 mg CLC / 10^8 spermatozoa for 30 min at 20-22°C using a gentle shaker in order to allow the CLC to distribute and affect the cells homogeneously. After removing CLC through 45% v/v Percoll gradient (600 g for 20 min), the amount of cholesterol in the sperm samples was measured as described above and compared to control samples for final determination of the total membrane

cholesterol.

5.3 Experimental design

5.3.1 Effect of HSPA8 on membrane cholesterol level

To find out whether exogenous HSPA8 altered the amount of membrane cholesterol in a brief exposure time, fresh Percoll-washed boar spermatozoa (5 x 10^6 / ml) from 5 different boars were incubated in Tyrode's medium containing 0.5 µg / ml HSPA8 for 15 min at room temperature. The samples were briefly centrifuged (400 g for 10 min) and then, the amount of membrane cholesterol was determined as described above. Bovine serum albumin, as a cholesterol acceptor was excluded from the incubation medium in order to avoid interference.

5.3.2 Effect of membrane cholesterol depletion on HSPA8-mediated enhancement of sperm membrane integrity and fluidity

To investigate whether reduction of membrane cholesterol affected HSPA8-mediated effects on sperm membrane integrity and fluidity, membrane cholesterol of semen samples from 5 different boars was depleted according to protocol described above. Thereafter, each sample was split into 6 aliquots of 5 x 10^6 / ml in Tyrode's. Viability, CTC fluorescence pattern and membrane fluidity were determined after 15 min exposure to HSPA8 (0 and 0.5 µg / ml) at room temperature.

Both MβCD and bovine serum albumin (BSA) remove membrane cholesterol. Therefore, in this experiment BSA was excluded from sperm media to avoid interference and focus specifically on MβCD cholesterol depleting effects on sperm membrane.

5.3.3 Effect of repletion of membrane cholesterol on HSPA8-mediated enhancement of sperm membrane integrity and fluidity

To confirm whether the HSPA8 effect on sperm membrane integrity and fluidity on cholesterol-depleted spermatozoa could be restored by exogenous cholesterol repletion, fresh Percoll-washed semen samples of 5 boars were first treated with MβCD (0, 2, 4 mM / 10^8).

MβCD was removed through 45% Percoll gradient, the pellets were resuspended in Tyrode's and sperm concentration determined. Then, MβCD-treated samples were exposed to 2 mg CLC / 10^8 spermatozoa for 30 min at 20-22°C.

Viability, CTC fluorescence pattern and membrane fluidity were determined after removing CLC through 45% Percoll gradient (600g for 20 min) and exposure to HSPA8 (0 and 0.5 µg / ml) for 15 min at room temperature.

5.3.4 Statistical analysis

Analysis of variance (ANOVA) was used and when the results were significant in ANOVA, means were further tested with post-hoc Fisher's least significant difference (LSD) test to examine the effect of treatments within experimental designs by Statistica software, version 7 (WWW.StatSoft.com, 1984-2004). Data were expressed as mean ± s.e.m. A probability of $P \leq 0.05$ was considered to be statistically significant.

Relationships between concentrations of cholesterol modifiers and membrane cholesterol content were analysed by regression analysis to determine the fit of the regression line and significance levels of the estimated relationship. The results were expressed as mean ± s.e.m and differences with P values ≤ 0.05 were considered as significant.

5.4 Results

5.4.1 HSPA8 had no effect on membrane cholesterol level

Brief incubation of spermatozoa with HSPA8 caused no changes to the sperm membrane cholesterol level, indicating that HSPA8 had no effect on membrane cholesterol quantity (Figure 5.1).

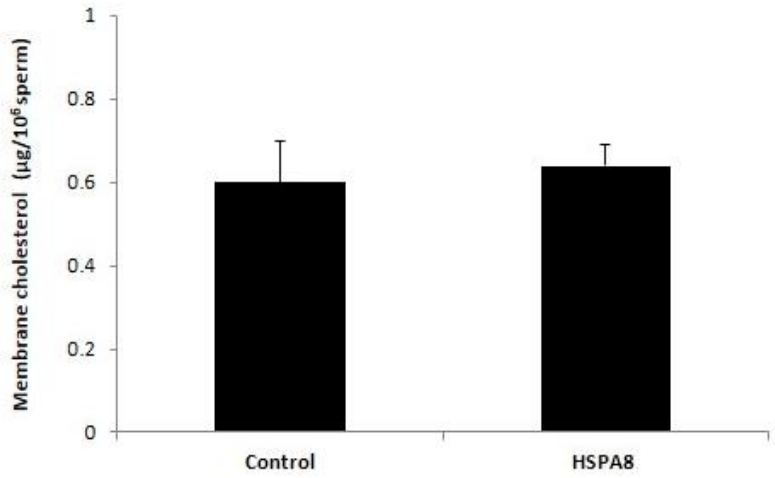

Figure 5.1 Effect of HSPA8 on sperm membrane cholesterol content. Cholesterol content (µg / 10^6 sperm) of boar sperm membrane was measured after brief (15 min) treatment with 0 and 0.5µg/ml HSPA8 at room temperature, n = 5, mean ± s.e.m.

5.4.2 Methyl-β-cyclodextrin efficiently depleted sperm membrane cholesterol content

Incubation of fresh sperm suspensions for 30 min with 0 – 8 mM MβCD / 10^8 spermatozoa reduced their cholesterol content progressively with a strong inverse linear correlation between them (r^2 = -0.99, mean ± s.e.m, n = 5, *P* = 0.0003). With 4 mM MβCD, cholesterol levels were estimated at 0.36 µg ± 0.16 / 10^6 cells relative to 0.48 µg ± 0.11 / 10^6 cells in control samples. In the presence of 8 mM MβCD cholesterol was further reduced to 0.22 µg ± 0.11 / 10^6 cells, i.e. 56% of controls (Figure 5.2A).

5.4.3 Cholesterol-loaded methyl-β-cyclodextrin efficiently repleted sperm membrane cholesterol

Incubation of fresh sperm suspension for 30 min with 0 – 8 mg CLC / 10^8 spermatozoa enhanced membrane cholesterol with a positive correlation between them (r^2 = 0.68, n = 5), however the relation did not follow a linear correlation (*P* = 0.03). The amount of membrane cholesterol following treatment with 0 and 1 mg CLC / 10^8 cells was 0.28 ± 0.04 and 0.32 µg ± 0.072 / 10^6, but showed a sharp rise at 4 mg CLC / 10^8 cells (1.22 µg ± 0.7) and followed a smooth trend 1.44 µg ± 0.6 / 10^6 and 1.57 µg ± 0.68 / 10^6 at 4 and 8 mg CLC / 10^8, respectively (Figure 5.2B). No change in sperm viability was detected between 1 - 4 mg CLC but at 8 mg CLC viability significantly decreased from 69% ± 4 to 52% ± 3 (control vs MβCD treatment, mean ± s.e.m, n = 5, *P* = 0.007).

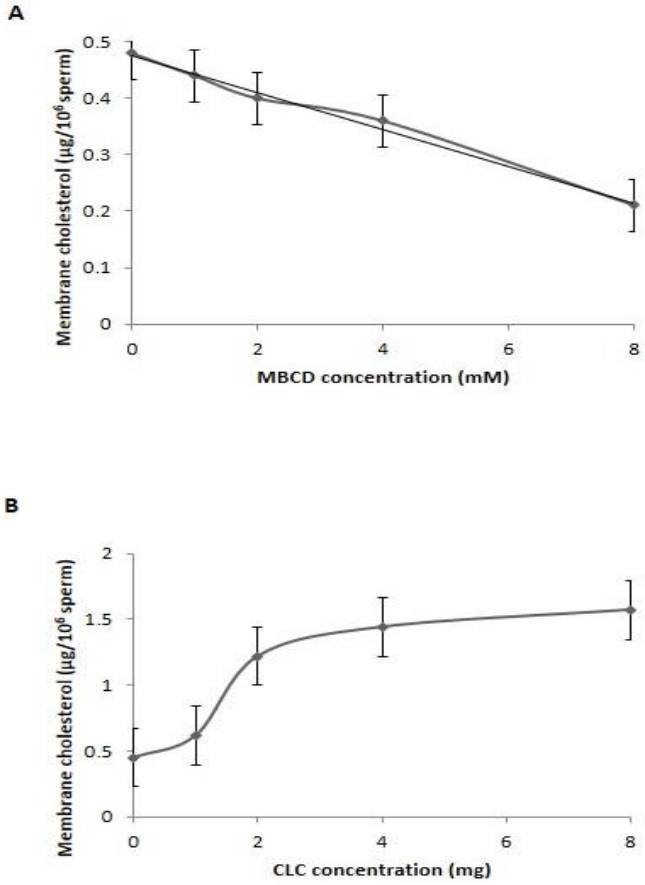

Figure 5.2 Cholesterol content of boar sperm membrane; (**A**) after 30 min treatment with methyl-beta cyclodextrine (MβCD) at 0, 1, 2, 4 and 8 mM / 10^8 spermatozoa) at room temperature, r^2 = - 0.99, *P* = 0.0003, (**B**) after 30 min treatment with cholesterol-loaded methyl-β-cyclodextrin complex (CLC) at 0, 1, 2, 4 and 8 mg CLC / 10^8 spermatozoa at room temperature, r^2 = 0.68, p = 0.03, analysed by spectrophotometry , n = 5, mean ± s.e.m.

5.4.4 Reducing sperm membrane cholesterol attenuated HSPA8 enhancing effect on sperm viability and membrane fluidity

No change in sperm viability was detected between 1 - 4 mM MβCD but at 8 mM MβCD viability decreased from 68% ± 4.5 to 53% ± 3 (control vs MβCD treatment, mean ± s.e.m, n = 5, $P = 0.003$). Thus, 4 mM MβCD lowered sperm cholesterol by 25% without adversely affecting their viability under the conditions employed (Figure 5.3A). In the presence of 1 - 4 mM MBCD diffusion of ODAF on the sperm acrosome increased progressively from 31 to 47 x $10^{-9}cm^2$ /sec ($P < 0.005$) but fell sharply to 15 x $10^{-9}cm^2$ /sec in the presence of 8 mM MBCD. Subsequent exposure of these sperm to 0.5 µg/ml of HSPA8 attenuated the increase in ODAF diffusion with the effect much reduced at 8 mM MBCD (Figure 5.3B). On the postacrosome, ODAF diffusion increased only at the lower concentrations of MBCD (1 – 2 mM), decreasing substantially at 4 – 8 mM MBCD. As observed on the acrosome, the addition of 0.5 µM HSPA8 increased ODAF diffusion only at the lower concentrations of MBCD (Figure 5.3C).

5.4.5 Repletion of membrane cholesterol restored HSPA8 function on sperm membrane integrity and fluidity

This experiment was carried out to confirm the mediating role of membrane cholesterol in HSPA8 effect on sperm membrane. First spermatozoa were treated by 4 mM MβCD. This treatment removed a significant amount of membrane cholesterol with minimal disturbing effect on membrane integrity and most importantly, negated the effect of HSPA8 on membrane integrity and fluidity (Figure 5.3). However, repletion of membrane cholesterol restored the membrane responsiveness to HSPA8-mediated effects on both membrane integrity and fluidity in the sperm acrosome and postacrosome (Figure 5.4A, B, C).

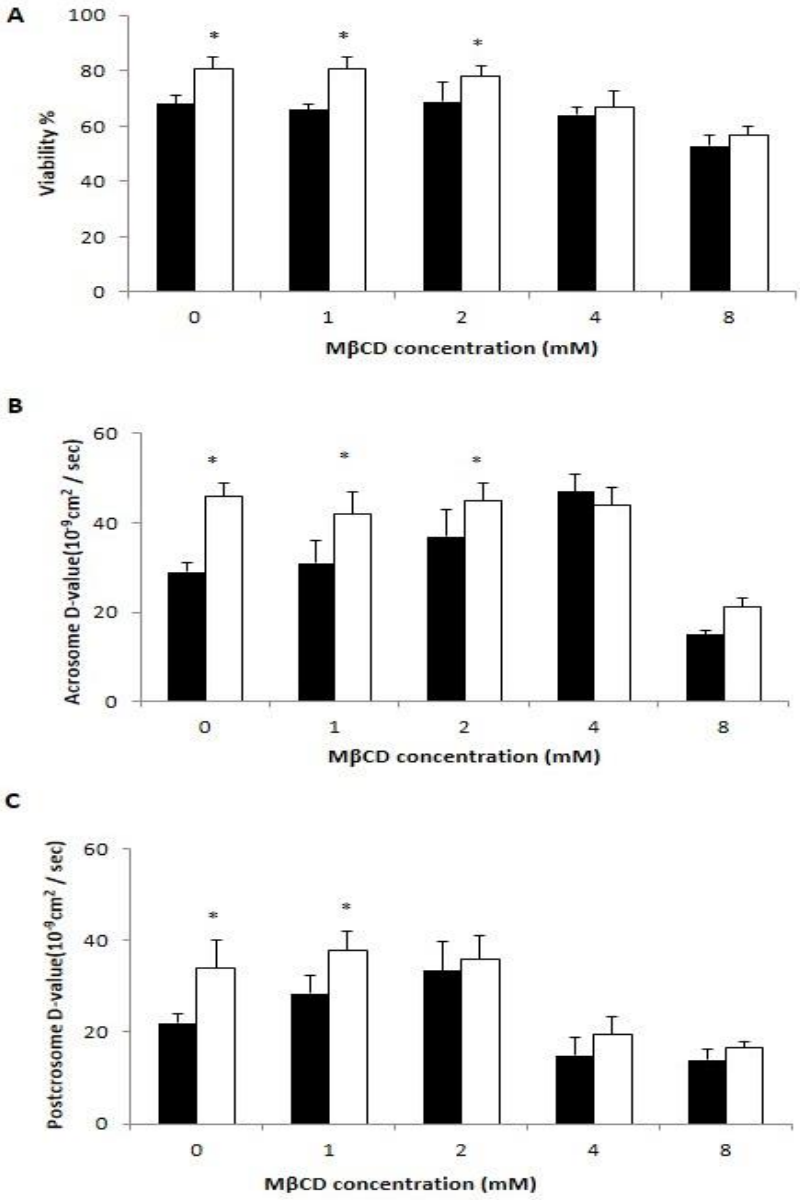

Figure 5.3 Effect of membrane cholesterol depletion on HSPA8-mediated enhancement of sperm membrane integrity and fluidity. (**A**) Viability (membrane integrity) %, (**B**) acrosome D-values ($10^{-9}cm^2$/sec) and (**C**) postacrosome D-values ($10^{-9}cm^2$/sec) of boar spermatozoa (30 spermatozoa) after treatment by MβCD (0, 1, 2, 4 and 8 mM / 10^8 spermatozoa) and 15 min exposure to 0 ■ and 0.5□ μg/ml HSPA8. * represents significant differences among individual groups, $P < 0.05$, n = 5, mean ± s.e.m.

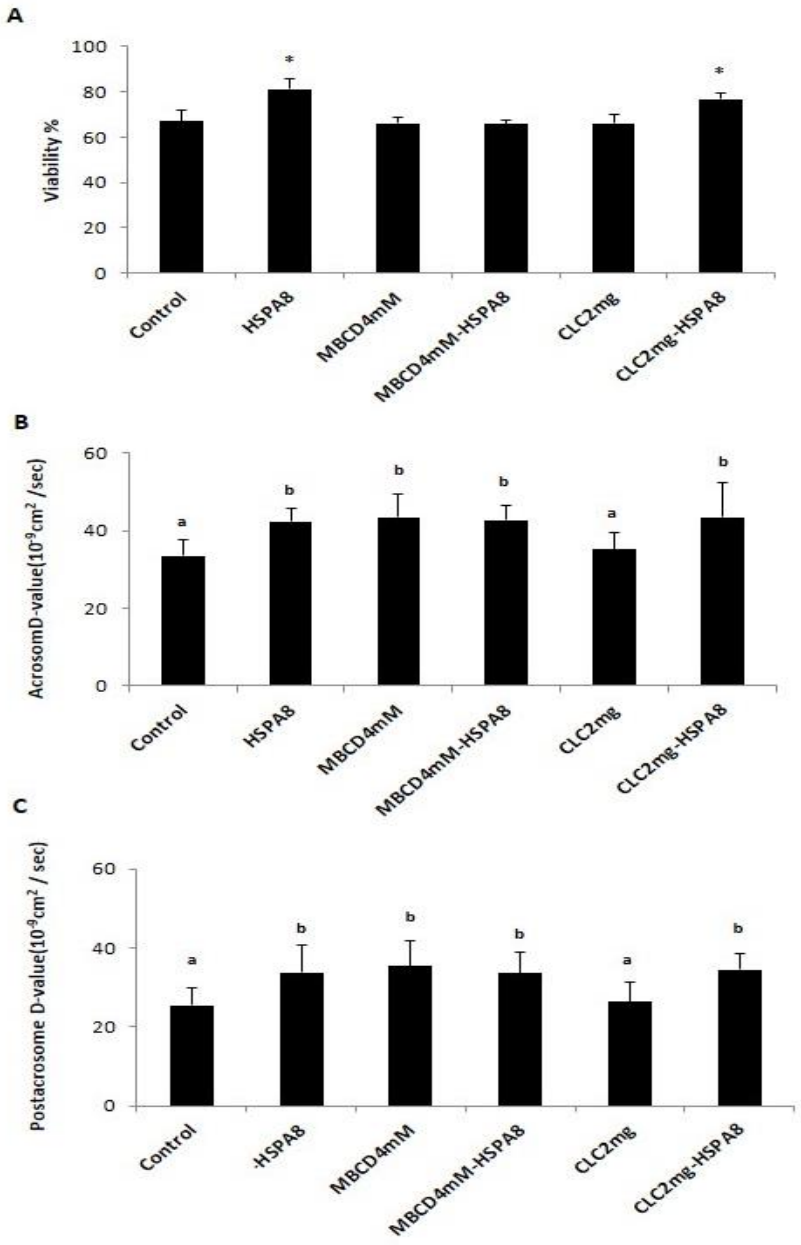

Figure 5.4 Effect of membrane cholesterol repletion on HSPA8-mediated effects on sperm membrane integrity and fluidity. (**A**) Viability (membrane integrity) %, (**B**) acrosome D-values ($10^{-9}cm^2$/sec) and (**C**) postacrosome D-values ($10^{-9}cm^2$/sec) of boar spermatozoa (30 spermatozoa) after addition of CLC (2mg/ 10^8 spermatozoa) to spermatozoa that were already depleted of their membrane cholesterol by MβCD 4 mM / 10^8 spermatozoa and 15 min exposure to 0 and 0.5 µg/ml HSPA8. $P < 0.05$, n = 5, mean ± s.e.m.

5.5 Discussion

The body of research presented in this chapter, aimed to progress from simply understanding that HSPA8 interacts with sperm membrane through to identifying the actual molecular candidate, membrane cholesterol as a key mediator of HSPA8 effect on sperm membrane integrity and fluidity.

The significant enhancement of sperm membrane lipid fluidity after exposure to HSPA8 which we observed in our study and the capacitation-induced rise in sperm membrane fluidity as the consequence of cholesterol loss which has been reported previously (Gadella, Lopes-Cardozo et al. 1995; Kadirvel, Kumar et al. 2009), led to the proposal that HSPA8 might function as a potential cholesterol acceptor.

Capacitation is one of the well-studied causes of sperm membrane fluidity increases. Capacitation-induced membrane hyperfluidity is an early stage in the process and this takes place as a consequence of cholesterol efflux from sperm membrane (Gadella, Lopes-Cardozo et al. 1995; Kadirvel, Kumar et al. 2009). Oviduct and uterine fluids contain a number of proteins known as sterol binding proteins whose role is to facilitate the process of capacitation by removing sperm membrane cholesterol (Shadan, James et al. 2004). Similar effects can be replicated *in vitro* by cholesterol-depleting agents such as methyl beta-cyclodextrine (MβCD) or bovine serum albumin (Kilsdonk, Yancey et al. 1995; Muller, Muller et al. 2008). Hence, it would be plausible to hypothesise that HSPA8 as an oviductal component which has known membrane impacts is able to act as a lipid modifying agent. Nevertheless, this hypothesis was proven wrong, as HSPA8 had no effect on sperm membrane cholesterol content.

The concept that cholesterol might be involved in the observed effects of HSPA8 on the sperm membrane led to the next series of experiments. These used MβCD as an effective cholesterol depleting agent or the combination of MβCD with cholesterol as a potent cholesterol donor to modify sperm membrane cholesterol and investigate the influence which these modifications have on HSPA8-induced membrane alterations.

Measuring the residual cholesterol in MβCD-treated spermatozoa indicated that there was a safe window for MβCD-mediated cholesterol efflux, during which there was no measurable disruption to membrane integrity and significant rise in the incidence of sperm capacitation. MβCD concentrations beyond the safe window caused major cholesterol loss, disturbance in normal membrane status to the extent that allowed Propidium iodide to enter the cells. Most damage probably occurred through uncontrolled leakage of intracellular ions which resulted from loss of membrane integrity, since cholesterol reinforces the permeability barriers by condensation and thickening of membrane lipid bilayer (Stockton and Smith 1976; Ipsen, Mouritsen et al. 1990). Basically, cholesterol integrates in between the phospholipids and glycolipids of membrane rafts in the interior hydrophobic end of the bilayer and act as biological glue in the spaces between the lipid chains. By packing the basic lipid structure tightly against each other, cholesterol molecules maintain the raft assembly in a tight order and reduce membrane permeability to water and small water-soluble molecules (Bloom, Evans et al. 1991; Corvera, Mouritsen et al. 1992; Crockett 1998).

Data obtained from FRAP analysis showed that there was a significant increase in the measured lipid diffusion coefficients of sperm membrane after non-detrimental MβCD-induced cholesterol removal. However, excessive cholesterol efflux induced by 8 mg CLC induced a substantial drop in lipid fluidity parameters and the formation of immobile membrane planes over the sperm head which are consistent with a loss of membrane integrity (Ladha, James et al. 1997; Wolfe, James et al. 1998). This was consistent with previous reports which have shown that decreases in membrane cholesterol enhance lipid fluidity by allowing phospholipids to flow more easily along the two dimensional membrane plane, and that excessive reductions in membrane cholesterol have opposite effects and result in membrane rigidity and disturbance in membrane integrity (Muller, Muller et al. 2008).

The observation that the depletion of membrane cholesterol abolished the effect of HSPA8 on both membrane integrity and fluidity led to the proposition that there was a biologic interaction between cholesterol and HSPA8 molecules, without which the influence of HSPA8 on sperm membrane parameters diminished. Studies presented earlier demonstrate that similar results

were obtained when the effect of sperm capacitation on HSPA8 function on membrane was investigated, i.e. there was gradual loss of HSPA8 enhancing effect on membrane integrity and fluidity as capacitation progresses. Considering that membrane cholesterol efflux is a pivotal phase in sperm capacitation process (Cross 1998; Travis and Kopf 2002), this supported our conclusion that the diminishment of HSPA8 effect in either experiments resulted from significant reduction in sperm membrane cholesterol, either induced by capacitation process or MβCD treatment. Evidence in the literature also shows that a number of membrane-bound HSPs, HSPA8 in particular, associated with membrane lipid rafts in pre- and post-synaptic spaces of neurons (Tytell, Barbe et al. 1994; Chen, Bawa et al. 2005). Treatment with MβCD caused major lipid raft disruption and dissociation of membrane-bound HSPs from brain cells (Chen, Bawa et al. 2005). Cholesterol depletion in tumour cells has also been shown to correlate with a major loss of membrane-associated HSPA (HSP70) isoforms (Gehrmann, Liebisch et al. 2008).

Having provided insight into the mediating role of cholesterol in HSPA8 effects, membrane cholesterol repletion experiments were used to validate the above findings. Although MβCD is a very well-known cholesterol acceptor, any potential unknown secondary effects, caused directly by MβCD *per se* or indirectly by lipid structure rearrangement consequent to MβCD treatment and therefore, interference with final results had to be ruled out. In other words, it was expected that HSPA8-mediated effects on membrane integrity and fluidity would be restored after cholesterol was reloaded to cholesterol-depleted spermatozoa, unless additional molecules or other membrane components were involved.

In the current study, the addition of cholesterol to sperm membrane led to a decrease in lipid diffusion coefficients (D-value $10^{-9}cm^2$ / sec) which was consistent with the notion that by intercalating in between phospholipid molecules and orienting them in a position closer to each other, cholesterol immobilises the lipid plane and reduces its deformability. So cholesterol at high concentrations tends to reduce membrane fluidity and increase its stiffness (Buffone, Verstraeten et al. 2009; Simons and Sampaio 2011). The fact that HSPA8 influence on sperm membrane was restored consequent to repletion of cholesterol to sperm membrane was a reassuring step in concluding that cholesterol definitely mediated the observed HSPA8 effects.

Overall, it is very important to bear in mind that there is no absolute safe range for modification of cholesterol in sperm membranes, as its concentration is variable among different species, within individuals of a species and even in sperm subpopulations produced from a single individual. Bull sperm membrane cholesterol has been enhanced by use of 0.75 mg CLC only (Purdy and Graham 2004), whereas higher CLC concentrations are required for significant membrane cholesterol enhancement in boar sperm. Consequently, interpretation of results should be cautiously done according to experimental conditions, applied methods and the species under study. Cholesterol depletion can create paradoxical effects in lipid diffusion rate (Kabouridis, Janzen et al. 2000; Kwik, Boyle et al. 2003). Shadan *et. al*. has reported a safe window of MβCD-mediated cholesterol efflux which was compatible with boar sperm capacitation and had minimal adverse effects on membrane integrity (Shadan, James et al. 2004), quite similar to our findings. However, they observed very little change in lipid diffusion coefficients after MβCD treatments within non-detrimental MβCD concentrations and detected a significant decrease only after use of higher MβCD concentrations.

Little is known about the association of HSPs with cellular membranes and this is partially because extracellular HSP is a concept at its early stages. Nonetheless, the certain conclusion thus far is that membrane cholesterol mediates the influence of HSPA8 on the integrity and fluidity of sperm membranes. However, it is yet unknown whether cholesterol molecules act as the binding sites for HSPA8, or if cholesterol merely plays a supporting role by maintaining the raft structures so that HSPA8 molecules can attach to their binding sites / receptors on the organised lipid plane.

Investigations into the localisation of stress-induced HSPA1A (HSP70) on tumour cell membranes indicated that HSPAs (HSP70) are abundantly present in membranes of GB3-positive tumour cells (Hightower and Guidon 1989; Broquet, Thomas et al. 2003; Gehrmann, Liebisch et al. 2008). Selective binding of HSPA1A to globoyltriaosylceramide (Gb3), a marker on tumour cell surface, has concluded that Gb3 is an essential interaction partner for HSPAs on tumour cell membranes (Gehrmann, Liebisch et al. 2008).

Although membrane Gb3 is exclusive to tumour cells, it provides an interesting concept on which the determination of the interaction partners for HSPA8 on membranes of normal healthy cells including spermatozoa can be based. Co-staining of HSPA8 and the molecules of suspect on a cell's membrane surface, as well as application of single-tracking particle methods or fluorescent labelled antibodies could be helpful by enabling us to follow single molecules as potential mediators for the membrane association of HSPA8.

This study provided valuable information towards understanding the molecular basis behind the interaction of HSPs with cell membranes. We demonstrated here that HSPA8 does not influence the level of membrane cholesterol, but requires cholesterol to enhance sperm membrane fluidity and integrity. This discovery expands the application of exogenous HSPA8 from preservation of fresh spermatozoa to improving sperm cryosurvival technology. Cholesterol efflux from the sperm membrane is the major cause of premature capacitation and short life span of sperm after cryopreservation. Increases in membrane cholesterol content before freezing decreases post-thaw cryocapacitation and its resulting short sperm longevity (Purdy and Graham 2004; Moore, Squires et al. 2005). Hence, it is highly possible that adding HSPA8 along with cholesterol to spermatozoa prior to freezing could boost the cryosurvival rates, prolong the life span of spermatozoa after cryosurvival procedure and, most importantly, enhance their fertilising outcome. Therefore, it would also be interesting to test the effect of exogenous HSPA8 on *in vivo* and *in vitro* fertilising performance of frozen thawed spermatozoa before and after loading membrane cholesterol.

To the best of our knowledge, this is the first report on extracellular HSP-membrane cholesterol association in the reproductive system, and this study creates the necessary fundamental information for further investigations on the precise binding sites and potential HSP receptors on sperm membranes.

Chapter 6

Extracellular HSPA8 associates with membrane of a minor subpopulation of spermatozoa

6.1 Introduction

Heat shock response was first discovered in 1962 during a study on the effect of temperature on Drosophila embryos and was described as a dramatic change in the puffing pattern of chromosomes in Drosophila salivary glands (Ritossa 1964). Later it was characterised as a series of defensive cellular activities which was initiated by elevated temperature. Heat shock response was aimed to avert stress-induced cell death through expression of a specific set of proteins collectively known as heat shock proteins (HSPs) (Tissieres, Mitchell et al. 1974; Lindquist 1986; Schlesinger 1986). Not long after that discovery, it became clear that the expression of HSPs was neither restricted to the fly nor to temperature rise. Heat shock proteins were present in all living organisms from *E. coli* to human (Kelley and Schlesinger 1978; Lemaux, Herendeen et al. 1978; Schlesinger 1986) and were produced in response to a range of environmental physical and chemical stressors such as amino acid analogues, radiation, cold, heavy metals, oxidation, infection and inflammation (Kelley and Schlesinger 1978; Schlesinger 1990; Gehrmann, Pfister et al. 2002). Stress damages cellular protein structure and causes detrimental accumulation of denatured proteins in the cell. Intracellular HSPs mediate cytoprotection through refolding or scavenging denatured proteins and thus preventing protein aggregation under stressful conditions (Lindquist 1986).

With the growth in the understanding of the roles of major HSPs in cell physiology it became clear that the function of HSPs extended beyond stressful situations. These are multi-faceted proteins which are also involved in maintaining intracellular protein homeostasis in normal physiological conditions (Craig, Gambill et al. 1993; Bukau, Weissman et al. 2006), as well as in the mediation of vital physiological events such as cell cycle regulation (Helmbrecht, Zeise et al. 2000), intracellular signalling, inflammatory and immunologic responses (Malhotra and Wong 2002) and resistance to stress-induced apoptotic or necrotic cell death (Mosser, Caron et al. 1997; Lanneau, Brunet et al. 2008).

Initially, the general belief in the field was that HSPs are intracellular proteins that were only passively released to extracellular space after cell degradation due to infection, inflammation or necrotic cell lysis (De Maio 2011). However, this concept was challenged when researchers demonstrated that endotoxin-free recombinant HSPA1A (HSP70) was able to activate immune

cells (Srivastava 1997; Asea, Kraeft et al. 2000; Vega, Rodriguez-Silva et al. 2008), thereby suggesting a possible role for extracellular HSPs.

Consistent with those observations, other laboratories reported the presence of HSPs in the serum of normal healthy humans (Pockley, Shepherd et al. 1998), the synovial fluid (Luo, Zuo et al. 2008), the culture medium of antigen presenting cells (Schmitt, Gehrmann et al. 2007) or integrated into the outer surface of cell membrane (Multhoff, Botzler et al. 1995; Theriault, Mambula et al. 2005; Schmitt, Gehrmann et al. 2007).

Recently, identification of HSPA1A in the pig oviductal secretions (Georgiou, Sostaric et al. 2005) and HSPA8 on the oviductal apical plasma membrane (Elliott, Lloyd et al. 2009) has prompted scientists to investigate the role of these extracellular HSPs in physiology of reproduction. Exogenous recombinant HSPA8 has proven to have prolonging effects on sperm viability from a number of species (Elliott, Lloyd et al. 2009; Lloyd, Elliott et al. 2009). However, the nature of the interactions of exogenous HSPA8 with sperm and its localisation in sperm remain unclear. It is plausible to speculate that localisation of HSPs in different systems might vary according to the type and role of HSP, type of the cell and the circumstances in which the cell and exogenous HSP are placed. For instance, Johnson and Tytell (1993) reported that exogenous HSPA1A protected the viability of stressed arterial smooth muscle cells through interactions with the cell surface rather than internalisation (Johnson and Tytell 1993). In contrast, Browne and his colleagues showed that exogenous HSPA1A accelerated the cell cycle of fertilised sea urchin eggs by internalisation and then its distribution into the zygotes' cytoplasm, being completely excluded from the cell nucleus (Browne, Swan et al. 2007).

Previously, we provided evidence that brief exposure of an exogenous recombinant HSPA8 to spermatozoa significantly enhanced boar sperm viability (membrane integrity) over a brief exposure time *in vitro* (Chapter 2). Further investigations strongly suggested that the HSPA8 effect on sperm plasma membrane protection was mediated through membrane cholesterol (Chapter 5). Following those findings, we hypothesised that exogenous HSPA8 directly

associated with sperm plasma membrane within a short incubation time. We aimed to prove this hypothesis using confocal imaging techniques. In addition, we investigated the membrane integrity and capacitation status of spermatozoa which bound to HSPA8 using flow cytometry to gain insight into the characteristics of spermatozoa that associated with exogenous HSPA8.

6.2 Materials and Methods:

All chemicals used for preparation of Tyrode's medium were purchased from BDH Laboratory Supplies, Poole, UK. Other chemicals were from Sigma-Aldrich company Ltd, Dorset, UK, unless otherwise stated.

6.2.1 Semen Preparation

Boar semen diluted and stored for 24 hours in Beltsville Thawing Solution (BTS) (Pursel and Johnson 1975) was obtained from a commercial artificial insemination station (JSR Healthbred Limited, Thorpe Whillougby, Yorkshire,UK). On the day of experiment, diluted boar semen was washed using Percoll gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Briefly, aliquots of 2-4 ml of diluted semen were layered over a Percoll density gradient. The iso-metric Percoll gradient consisted of 2 ml 70% (v/v) Percoll overlaid with 2 ml 35% (v/v) Percoll. The gradient was centrifuged at 200 g for 15 min followed by 15 min centrifugation at 900 g (Holt and Harrison 2002). The supernatant was removed and the pellet was resuspended in Tyrode's medium consisting of 3.1 mM KCL, 0.4 mM $MgCl_2.6H_2O$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, 0.3 mM $NaH_2PO_4.2H_2O$ (Elliott, Lloyd et al. 2009). Thereafter, semen samples were centrifuged at 900 g for 15 min and resuspended in Tyrode's medium. Sperm concentration was measured in duplicate using an improved Neubauer chamber (Marienfield, Germany) and adjusted to the required concentration in Tyrode's or TALP (Tyrode's medium supplemented with 12 mg/ml bovine serum albumin, 21.6 mM sodium lactate and 1 mM sodium pyruvate), based on the experimental conditions.

6.2.2 Sperm confocal microscopy

Images were obtained with a confocal laser scanning microscope (CLSM 510, Carl Zeiss, Jena, Germany) based on a Nikon inverted microscope equipped with an argon laser. A 63x / 1.40NA

oil immersion objective coupled with a 130 µm pinhole was used. Images were processed on a steady silicon workstation placed in a flow chamber with stable lateral (x-y-axis and vertical z-axis) medium substitution. The plane Z-axis was set at 0.1 µm. During image acquirement the chamber temperature was maintained at 20-22°C room temperature. Samples were evaluated within 1 hour of preparation.

6.2.3 Protein labelling with Atto^{488} for confocal microscopy

Recombinant HSPA8 or HSPA1A (StressMarq, Victoria, BC, Canada) as control protein was labelled with Atto^{488} fluorescent dye according to the manufacturer's instructions (Lightning-Link^{TM} Atto^{488} conjugation kit, Innova Biosciences, Cambrige, UK). This kit allows fluorescent conjugations of a protein in a short time simply by adding a solution of the desired protein to the lyophilised mixture containing an activated fluorescent ligand. Atto^{488} is a coumarin derivative with Ex / Em of 501 nm / 523 nm and high photostability suitable for confocal scanning.

Initially, before adding HSPA8 or HSPA1A to the designated tube containing the lightning Link Atto^{488} mix, 1 µl of LL-modifier reagent (provided in the kit) was added for each 10 µl of HSPA8 and mixed gently. Then 300 µg of HSPA8 or HSPA1A (400 µg/ml from original stock) was pipetted directly onto the mix. The mix was resuspended gently by withdrawing and re-dispensing the liquid using a pipette. The vial was left in the dark at 20-25°C for 3 hours. Finally, 1 µl of LL-quencher reagent was added for each 10 µl of HSPA8 in the mix. The conjugate was ready to use after 30 min or could be stored at -20°C for future use.

6.2.4 Loading spermatozoa with nuclear fluorescent probe for confocal microscopy

Nuclei of spermatozoa were loaded with DRAQ5 (Biostatus Limited, Leicestershire, UK), a highly cell permeable and photostable DNA-interactive agent with fluorescence emission from 665 nm into the low infrared. For staining the cells, 2.5 µl dye was added to 200 µl (5 x 10^6 / ml) washed sperm aliquots in Tyrode's medium for 30 min at room temperature. Staining with DRAQ5 is usually the final step in sample preparations as there is no need for centrifugations

or any further steps. The DRAQ5 fluorochrome did not generate background fluorescence in the sperm nucleus and enabled clear distinction between cells and artefacts.

6.2.5 Flow cytometric analysis of sperm

Flow cytometric analysis was performed on a Beckman Coulter Epics cytometer (Beckman Coulter Inc, Miami, Florida, USA) equipped with a 20-mW argon laser (488 nm) for fluorochrome excitation. Data were collected from 10,000 events in each sample and analysis was performed by a general purpose flow cytometry data analysis software, WEASEL 3.0.2. (Bio-Soft Net website; http://en.bio.-soft.net). To exclude aggregates and debris in the analysis and restrict data acquisition to a pure sperm population, a live gate in using side and forward light scatter characteristics (SSC and FSC respectively) was used.

6.2.6 Assessment of sperm viability

Sperm viability was evaluated using Propidium iodide / SYBR-14 (Live / Dead Sperm Viability Kit, Molecular Probes, Eugene, Oregon, USA) as described previously.

6.2.7 Assessment of sperm capacitation

capacitation status of boar sperm was assessed using the Chlortetracycline Hydrochloride (CTC) staining method, as described by Fazeli et al. (Fazeli, Duncan et al. 1999).

6.3 Experimental design

6.3.1 Localisation of exogenous HSPA8 in spermatozoa

The aim of these investigations was to determine the localisation of HSPA8 in sperm after a short co-incubation period. On the day of the experiment, Atto^{488}-labelled-HSPA8 or HSPA1A were added at 0.5 µg/ml to 200 µl of DRAQ5-loaded spermatozoa ($5x10^6$ / ml) for 15 min at room temperature. A 50 µl droplet was placed on the smear and evaluated by confocal

microscopy. The 488 nm argon laser was used for excitation and the emitted fluorescence was detected through 500 - 550 and 650 - 710 bandpass filters for Atto^{488} and DRAQ-5, respectively. All cells appeared red due to DRAQ staining and those interacting with HSPA8 showed green fluorescence as well. Ten to fifteen sequential z-sections were performed over the focal plane through the cell depth based on the size of sperm head and position.

6.3.2 Differentiation of HSPA8-bound spermatozoa based on membrane integrity status

This experiment was conducted to identify the membrane integrity status of spermatozoa which were bound to HSPA8. Fresh Percoll-washed spermatozoa from 6 different semen samples were adjusted to 5 x 10^6 / ml in Tyrode's, stained with 12 µM Propidium iodide and then incubated with Atto^{488}-labelled-HSPA8 (0.5 µg/ml) for 15 min at room temperature. The final volume of the samples were adjusted to 500 µl with flow cytometry buffer and analysed by flow cytometry. Concomitantly, viability (membrane integrity) of each sample was assessed microscopically after 15 min treatment with 0 and 0.5 µg/ml HSPA8.

6.3.3 The effect of capacitation on HSPA8-sperm binding pattern

Here, we aimed to study if sperm capacitation had any effect on exogenous HSPA8-sperm binding pattern observed in the previous experiment. A total of 6 boar semen samples (5 x 10^6 / ml) were incubated in TALP at 39ºC, 5% v/v CO_2 (capacitating condition) for 0 and 5 hr. At each time point, samples were split into three aliquots to be assessed for viability (membrane integrity), capacitation status as well as HSPA-sperm binding pattern. Membrane integrity and capacitation were assessed microscopically, as described above. Sperm-HSPA8 binding was evaluated by flow cytometry after spermatozoa were stained with 12 µM Propidium iodide and treated with Atto^{488}-HSPA8 (0, 0.5 µg/ml) for 15 min at room temperature.

6.3.4 Statistical analysis

Data were analysed by analysis of variance (ANOVA) and posthoc Fisher least significant difference (LSD) test was used to examine the effect of treatments within experimental designs

by Statistica software, version 7 (WWW.StatSoft.com, 1984-2004). Data were expressed as mean ± s.e.m. A probability of $P \leq 0.05$ was considered to be statistically significant.

6.4 Results

6.4.1 Exogenous HSPA8 associated with sperm membrane surface

Confocal images exhibited two different fluorescent patterns of spermatozoa; cells which merely showed red fluorescence due to DRAQ5 nucleus stain and those which displayed Atto^{488}-HSPA8 related green fluorescence in conjunction with the red nucleus. In samples treated with HSPA8, images revealed that only a minor subpopulation of spermatozoa were positive for green signal representing exogenous HSPA8. HSPA8 was mainly visible on the sperm head and particularly covered the acrosome region after 15 min incubation at room temperature (Figure 6.1A). In contrast, HSPA1A did not display any specific interaction with spermatozoa after 15 min exposure (6.1B).

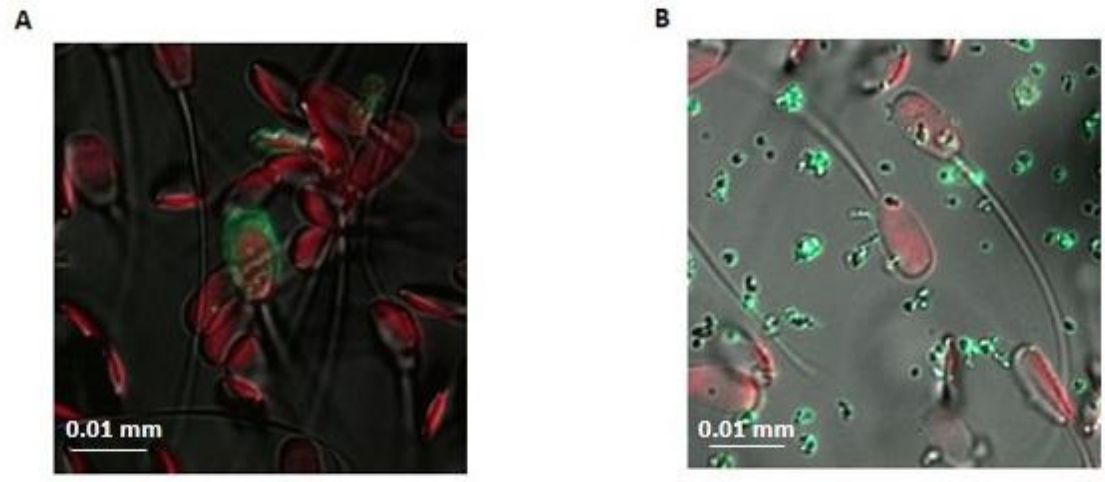

Figure 6.1 Confocal microscopy imaging of the interaction of DRAQ5-stained fresh boar spermatozoa (red fluorescence) with; (**A**) exogenous Atto^{488}-labelled HSPA8 (green fluorescence), (**B**) exogenous Atto^{488}-labelled HSPA1A (green fluorescence). HSPA8 associated with a minor subpopulation of spermatozoa and was distinctively distributed over the outer surface of the sperm acrosome region after 15 min co-incubation at room temperature. On the contrary, HSPA1A did not display any specific interaction with spermatozoa after 15 min exposure.

A series of sequential sectioning over the focal plane through the cell depth and 3D imaging revealed that HSPA8 was located on the sperm outer surface rather than inside the cell (Figure 6.2).

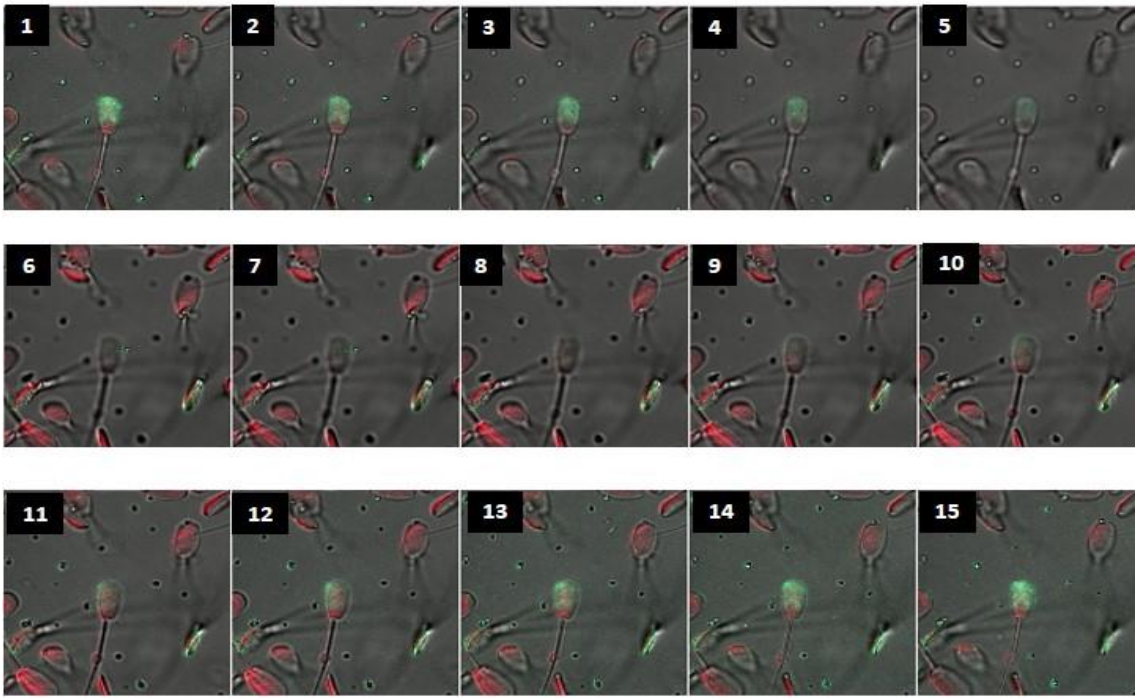

Figure 6.2 Confocal microscopy imaging of interaction between DRAQ5-stained fresh boar spermatozoa (red) and exogenous HSPA8 (green). Confocal sequential z-sectioning over the focal plane through the sperm depth confirmed that exogenous HSPA8 was localised on the outer surface of sperm head and was not internalised after 15 min co-incubation at room temperature. Numbers indicate the order of the images taken from top to bottom.

6.4.2 HSPA8 predominantly interacted with spermatozoa with damaged membranes

The viability of fresh spermatozoa was significantly increased by 12% ($P \leq 0.002$) after 15 min treatment with HSPA8. Flow cytometric analysis (Figure 6.3) showed that an average of 41 ± 5.5% of the total sperm population associated with HSPA8 and among the attached spermatozoa only 13 ± 5.5 % had intact membranes. The remaining majority of bound cells were all membrane disrupted, Propidium iodide positive cells (Figure 6.4). However, the 6 individual samples expressed wide variability for HSPA8-bound spermatozoa and the variability was more noticeable among Propidium iodide positive spermatozoa and ranged from 11% to 42% of the bound population.

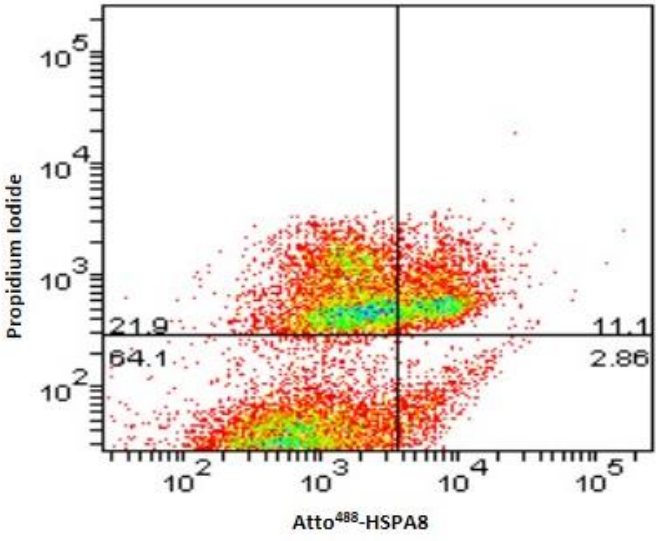

Figure 6.3 Flowcytomtric analysis of sperm-HSPA8 binding pattern with respect to membrane integrity status of the cells. Fresh Percoll-washed spermatozoa (5 x 10^6 / ml) were stained with 12 µM Propidium iodide and then incubated with Atto^{488} labelled-HSPA8 (0.5 µg/ml) for 15 min at room temperature. Dotplot quadrants represent; UL: PI + / Atto488-HSPA8 -, UR: PI + / Atto^{488}-HSPA8 +, LL: PI - / Atto^{488}-HSPA8 – and LR: PI - / Atto^{488}-HSPA8 + spermatozoa.

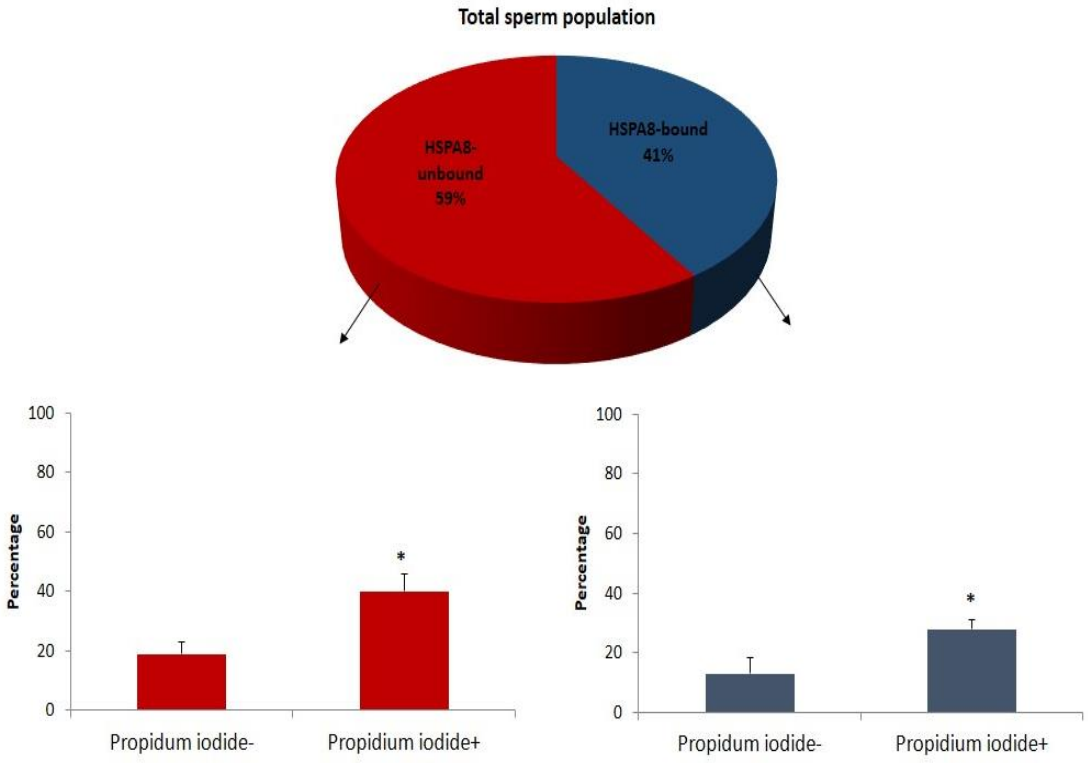

Figure 6.4 Binding pattern of exogenous HSPA8 to fresh boar spermatozoa in respect with the status of sperm membrane integrity. Fresh Percoll-washed boar spermatozoa were stained with Propidium iodide and treated with Atto^{488}-labelled HSPA8 (0.5 µg/ml) for 15 min at room temperature. Mean proportion of spermatozoa which were bound ■ or unbound ■ to HSPA8 with respect to their membrane integrity, n = 6, * < 0.05, mean ± s.e.m.

6.4.3 The pattern of HSPA8-sperm binding alters with sperm capacitation

This experiment was carried out to investigate the effect of capacitation on pattern of sperm-HSPA8 binding. Semen samples were assessed for their viability, capacitation status and HSPA8 binding pattern before and after 5 hr incubation in capacitating conditions. The percentage of viable (membrane intact) spermatozoa significantly dropped after 5 hr incubation (58 ± 7%) as compared with fresh samples (86.5 ± 5.5%) (P = 0.001) (Figure 6.5A). CTC staining of fresh and capacitated samples showed that 5 hr incubation of spermatozoa in TALP medium significantly increased the proportion of sperm capacitation from 4 ± 1.5% in control to 51 ± 5.6% in incubated samples (Figure 6.5B). Meanwhile, despite the fact that there was not a significant difference between the mean proportion of spermatozoa which were bound to HSPA8 in the two groups, HSPA8 binding intensity significantly declined by 40% in capacitated samples (P = 0.005) (Figure 6.5C and D).

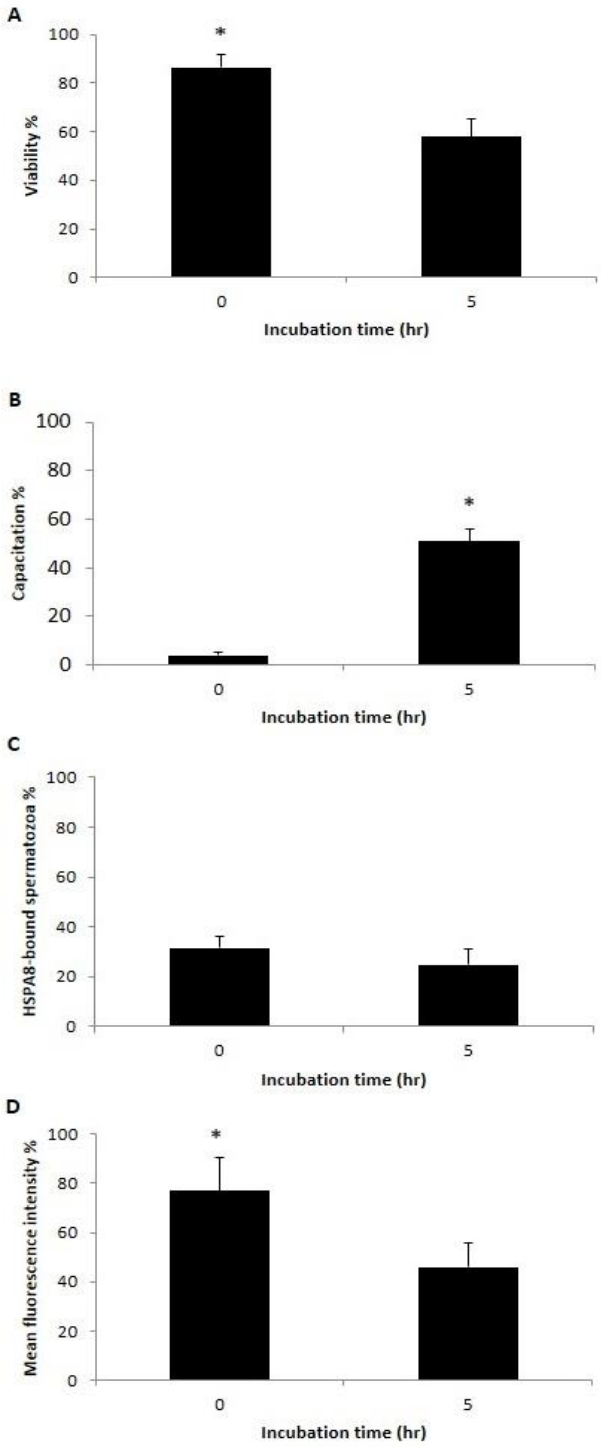

Figure 6.5 Effect of capacitation on the pattern of sperm-HSPA8 binding. (**A**) Mean viability % (membrane intactness) of spermatozoa, (**B**) mean capacitation % of spermatozoa and (**C**) mean proportion of spermatozoa bound to HSPA8, (**D**) mean fluorescent intensity % of Atto^{488}- labelled HSPA8 bound to spermatozoa before and after 5hr incubation in capacitation conditions (TALP medium at 39°C), n = 6, * < 0.05, mean ± s.e.m.

6.5 Discussion

The classic concept of HSPs being exclusively intracellular in their distribution and function is no longer accepted (Johnson and Fleshner 2006). The description of a HSP-like protein as a glia-axon transfer protein in the squid giant axon along with other reports on the roles of extracellular HSPs in different physiologic systems revealed that these proteins remain functional when released in the extracellular space (Tytell, Greenberg et al. 1986; Multhoff, Botzler et al. 1995; Pockley, Shepherd et al. 1998; Georgiou, Sostaric et al. 2005; Luo, Zuo et al. 2008; Schilling, Gehrmann et al. 2009). These findings have led to substantial increase in the application of naturally extracted HSPs from body tissues / secretions or their recombinantly produced forms in *in vitro* investigations (Srivastava 1997; Asea, Kraeft et al. 2000; Boilard, Reyes-Moreno et al. 2004; Browne, Swan et al. 2007; Vega, Rodriguez-Silva et al. 2008; Elliott, Lloyd et al. 2009). Having proven the functionality of a recombinant HSPA8 in porcine sperm and its enhancing effect on sperm membrane integrity and fluidity, we were encouraged to investigate the localisation of HSPA8 in sperm cells which is a poorly studied subject.

The differentiation between intracellular and extracellular location of extracellular HSPA8 and its precise localisation relative to cell membrane was performed by confocal laser scanning microscopy. Confocal laser scanning microscopy is the best method to visualise intracellular or extracellular localisation of proteins in viable cells (Miyashita 2004). Confocal Z sectioning has been proven to be highly efficient in differentiating between intra and extracellular location of *Ureaplasma Urealyticum* bacteria in bovine spermatids (Buzinhani, Yamaguti et al. 2011).

On the other hand, staining the sperm DNA and labelling HSPA8 in distinct colours of red and green respectively, provided a contrast to determine the cell-HSPA8 association when they co-occurred. DRAQ5 is very suitable for chromatin visualisation in cells and can easily be used with other fluorophores with blue to orange emission. In addition, as photo-stable fluorochromes, Atto^{488} and DRAQ5 facilitated examination of relatively higher numbers of microscopic fields compared to other fluorescent probes such as TOPRO-3, TOTO-3 or Propidium iodide which photobleach quickly after light exposure (Smith, Blunt et al. 2000;

Martin, Leonhardt et al. 2005; Yao, Uttamapinant et al. 2012).

Spermatozoa were all positive for red DRAQ nucleus stain, but only a small number of them displayed green fluorescence which represented exogenous HSPA8. The association of a small number of spermatozoa with HSPA8 indicated that some level of sperm selection might be involved. HSPA8 molecules distinctively associated with the sperm head and most predominantly with acrosomal region. Lack of fluorescent signals inside sperm cytoplasm confirmed that HSPA8 was not internalised into the cell. Immunolocalisation studies have detected abundant amounts of HSPA (HSP70) isoforms in sperm membranes over the acrosome from a number of species such as bull and boar (Miller, Brough et al. 1992; Boulanger, Faulds et al. 1995; Kamaruddin, Kroetsch et al. 2004; Spinaci, Volpe et al. 2005; Naaby-Hansen and Herr 2010). This fact conveys the idea that extracellular HSPA8 supplements the action of the existing membranous HSPs. Co-localisation of membranous and exogenous HSPs in sperm by using specific antibodies and immunofluorescence methods would be a useful complement to our observations.

Moreover, the specificity of HSPA8 in sperm membrane binding was confirmed by comparing the obtained result with that of exogenous HSPA1A as the control protein. Inducible isoform HSPA1A exhibits more than 84% sequence homology with its constitutive counterpart, HSPA8 (Daugaard, Rohde et al. 2007; Mouguelar and Coux 2012) and therefore is a suitable factor for proof of HSPA8's functional specificity.

Despite valuable information that confocal imaging studies provided, there was a major point which was missing in our experimental context. DRAQ5 nuclear stain is a permeant dye which enters all the spermatozoa regardless of the status of their membrane integrity and therefore made it impossible to differentiate between membrane intact and damaged cells. Thus, the outstanding question remained as to what cells (in regards with their status of membrane integrity) attracted HSPA8 towards themselves. To answer that question, we applied flow cytometry which easily enabled us to determine the integrity of spermatozoa membranes and quantify the proportion of HSPA8-bound spermatozoa, simultaneously. Flow cytometric analysis identified that a very small percentage of bound spermatozoa had intact membranes

and the remaining were all Propidium iodide positive, membrane disrupted cells. In a parallel experiment, brief exposure of fresh spermatozoa to HSPA8 resulted in an average of 12% increase in sperm viability (membrane integrity), reasonably comparable with the viable HSPA8-bound sperm proportion.

The exponential growth in our understanding of HSPs over the past decade has clarified that extracellular HSPs sense minor membrane lipid defects caused by mild stressors through alteration in membrane lipid structure and stabilise chief membrane lipid attributes such as fluidity and permeability (Vigh, Escriba et al. 2005; Nakamoto and Vigh 2007). Hence, extracellular HSPs play important role in stabilisation of the membranes through direct interaction with membrane lipid structure (Horvath, Glatz et al. 1998; Torok, Goloubinoff et al. 2001; Nitta, Suzuki et al. 2005).

The fact that only a limited number of spermatozoa in any given sample were responsive to the reparative effects of HSPA8, indicated that there was a selective process to HSPA8 restorative function. Correlating the proportion of viable HSPA8-bound spermatozoa to the percentage of increase in sperm viability (membrane integrity) after HSPA8 treatment, implies that the viable bound spermatozoa, represented the target cells for the selective HSPA8-mediated effect. The target cells were spermatozoa with reversible minor membrane disruptions which had been fixed by the time of examination and excluded the impermeant Propidium iodide stain. However, the membrane damage in the remaining cells was too extensive and beyond HSPA8 repairing abilities.

The observed variability in sperm-HSPA8 binding between samples indicated that there was a significant degree of variation among semen. This finding might provide a clue to an efficient method for predicting the fertility outcome of a given sample based on the level of sperm-HSPA8 interaction in combination with the level of sample responsiveness to the reparative functions of HSPA8.

Previously we showed that HSPA8 enhancing effect on sperm membrane integrity and fluidity was abolished by sperm capacitation (Chapter 2). Hypothesising that the diminishment of the

HSPA8 effect could result from capacitation-induced membrane changes and decrease in membrane-HSPA8 attachment, we investigated the influence of capacitation on sperm-HSPA8 binding pattern. Flow cytometric analysis showed that HSPA8 binding intensity significantly declined in capacitated samples compared with fresh ones. Earlier experiments strongly suggested membrane cholesterol as the mediator of HSPA8 function on sperm membrane (Chapter 5). Furthermore, it is known that the depletion of membrane cholesterol is an integral aspect of sperm capacitation process (Harrison, Mairet et al. 1993; Ashworth, Harrison et al. 1995; Harrison, Ashworth et al. 1996; Osheroff, Visconti et al. 1999). Taken together, these findings suggest that capacitation-induced membrane reorganisation and reduction in membrane cholesterol might decrease the potential binding / acting sites for HSPA8 on sperm membrane. In line with those findings, investigating the HSPA8-sperm binding pattern after depletion of membrane cholesterol would provide additional confirmation to the obtained body of knowledge.

The specific binding partner for HSPA8 in the sperm plasma membrane has to be identified. Globoyltriaosylceramide (Gb3) in cholesterol-rich microdomains has been reported as the HSPA1A binding mediator in some tumour cell lines, as membrane HSPA1A is lost with depletion in membrane Gb3 molecules (Gehrmann, Liebisch et al. 2008). On the other hand, Toll-like receptors 2 and 4 (TLR2 and 4) have also been considered as membrane receptors for extracellular HSPAs (former HSP70s) in immune cells (Multhoff 2007). Co-immunolocalisation of exogenous HSPA8 with each of these known molecules using specific antibodies along with a concentration-dependent association of HSPA8 with these molecules would be one method for confirming the potential receptors for extracellular HSPA8 on sperm membranes. However, since spermatozoa are highly differentiated cells with distinctive cellular characteristics from other somatic cells, the presence of the above molecules in sperm membrane has to be first confirmed. Moreover, due to the high diversity in HSP cellular targeting and functionality, the existence of other receptor molecules (lipid, protein or carbohydrate) is highly possible. Membrane-based co-immunoprecipitation assays could be one practical method to identify the partner for HSPA8 in sperm plasma membranes (Horn, Lalowski et al. 2006).

The current findings reveal that exogenous HSPA8 exerts its restorative effects via associations with the sperm membrane over the acrosome domain. Furthermore, the repairing effect of HSPA8 appeared to occur by selective binding to a distinctive subpopulation of spermatozoa with reversibly damaged membranes. However, it is still unclear whether the participation of HSPA8 in membrane repair is enabled via direct physical interactions with sperm membranes or if HSPA8-membrane association is the initial trigger to intracellular restorative pathways. Discovering the answer would have significant implications in application of restorative effects of HSPA8 in different systems. The correlation of other basic sperm characteristics such as mitochondrial activity or motility with sperm-HSPA8 binding pattern remains to be determined. Capacitation-induced decreases in HSPA8 binding intensity implicated cholesterol in the interaction of HSPA8 with the sperm membrane and its physiological consequences. It would be interesting to determine the influence of capacitation on the localisation of exogenous HSPA8 on the sperm outer surface.

Due to high variability in semen samples from different animals, conventional methods for semen assessment such as measurement of concentration, motility and viability are poor predictors of sperm fertilising capacity in artificial insemination or IVF methods for livestock breeding. Thus, the study of relation between sperm characteristics and exogenous HSPA8 may be a good marker to design new evaluation assays with high predictive values for sperm fertilisation outcome.

Chapter 7

The role of cAMP/PKA signalling pathway in HSPA8-mediated enhancement of sperm membrane fluidity

7.1 Introduction

Spermatozoa are exposed to numerous oviductal factors which are potential sources to regulate sperm characteristics immediately on arrival in the oviduct. Despite the fact that considerable attention has been paid to the apparent physiologic effects that oviductal secretions elicit on sperm, fewer studies have examined the regulatory mechanisms behind the induced effects. During the past chapters in this thesis, as well as investigations from other laboratories, it became clear that extracellular HSPA8, a conserved oviductal protein, possessed the ability to elicit physiological and functional responses in boar sperm.

One of the highlights of our study was the novel discovery of the ability of extracellular HSPA8 to repair sperm membrane integrity. Following that, our major focus has been dedicated to mechanistic approaches towards deciphering the molecular details of the observed effect. In Chapter 4, we showed that HSPA8 immediate effect on sperm membrane integrity was exerted through alterations in lipid fluidity which is a major attribute of cellular plasma membrane. The studies presented in Chapter 5 elaborately described the role that cholesterol played in mediating HSPA8 impact on sperm membrane fluidity. Although both findings together created valuable insight towards molecular basis of sperm-HSPA8 interaction in spermatozoa, none of them provided information on the mechanisms underpinning the immediate HSPA8-induced alterations in lipid fluidity. It is yet not clear how spermatozoa respond to HSPA8: does exogenous HSPA8 directly stimulate enhancement in membrane lipid fluidity through interactions with membrane lipids / cholesterol or are there intracellular signalling events downstream to the membrane which mediate HSPA8 effect on membrane.

In principle, cellular response to external stimuli are controlled by signalling pathways (Harrison and Miller 2000; Baxendale and Fraser 2003). It was therefore hypothesised that alterations in membrane lipid fluidity and improvements in membrane integrity in sperm that were mediated by HSPA8 involved activation of transmembrane signalling events. The apparent difficulty in studying the potential HSPA8-related transduction pathway was the fact that no previous reports on exogenous HSP-induced membranous responses and associated mechanisms in sperm existed in the literature. Therefore, as a preliminary step towards testing

our hypothesis, we resorted to an already well-studied intracellular pathway which was associated with regulation of membrane lipid phase in sperm. A number of studies have reported major alterations in sperm membrane lipid packing induced by bicarbonate (HCO_3^-) in *in vivo* and *in vitro* biologic systems. Bicarbonate-induced effects on membrane is documented to be under the regulation of a adenosine 3',5'-cyclic monophosphate (cAMP)-protein kinase A (PKA)-dependent pathway (Harrison, Ashworth et al. 1996; Harrison and Miller 2000) and is described as membrane hyperfluidity (Figure 7.1). The hyperfluidity state involves a shift from a highly packed to a loosely packed state in membrane lipid structure (Harrison, Ashworth et al. 1996) and can be detected through increased ability in sperm membrane to bind merocyanine 540 (Harrison and Miller 2000).

Merocyanine 540 is a lipophilic fluorescent cyanine dye which is widely used as an optical probe to monitor structural changes in biological lipid membranes (Lelkes, Bach et al. 1980). The lipophilic property of merocyanine enables it to penetrate into the loosely packed lipid membrane. Merocyanine contains a charged sulfonate group which restricts its membrane permeability and allows selective binding to the outer lipid membrane leaflet (McEvoy, Schlegel et al. 1988). Therefore, merocyanine can be employed as a useful means of measuring level of lipid packing in the exoplasmic leaflet of plasma membrane. Ordered tightly packed lipid membrane allows so little amount of merocyanine in the outer leaflet, exhibiting low levels of fluorescence in membrane. In contrast, a disordered, loosely packed state allows higher intercalation of merocyanine into the hydrophobic environment in between the loosely packed phospholipid molecules and increases fluorescence intensity in membrane (Harrison, Ashworth et al. 1996; Harrison and Gadella 2005). Hence, the level of fluorescence intensity detected in cell membrane of the cells stained with merocyanine positively correlates with the level of membrane lipid fluidity.

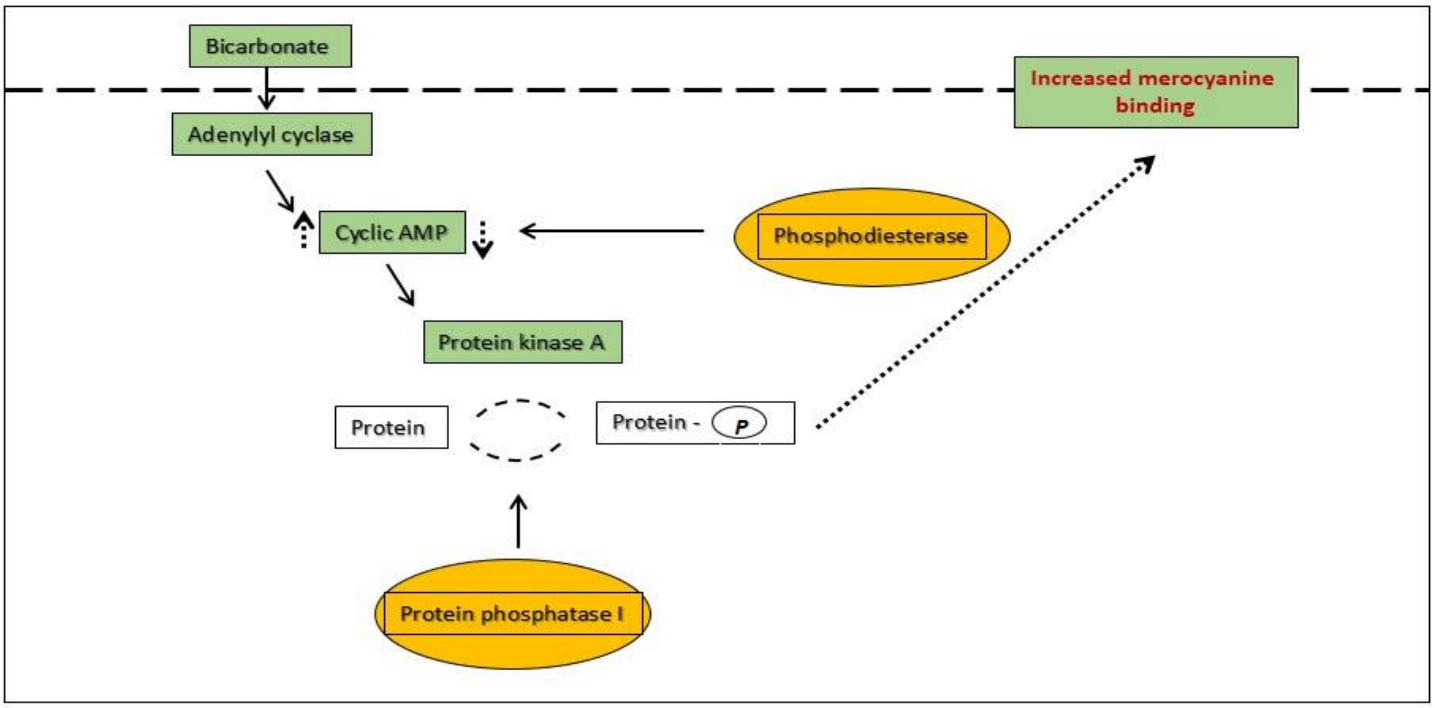

Figure 7.1 Schematic diagram of intracellular pathway regulating merocyanine binding ability in boar sperm membrane.

Previous studies have indicated that bicarbonate-induced shift in membrane lipid organisation contributes to the very early phase of sperm capacitation (Visconti, Galantino-Homer et al. 1998) which forms the basis for regulation of the later phases of capacitation process. The associated pathway appears to be the universal mode of signal transduction for capacitation-mediated lipid reorganisations in membrane of mammalian spermatozoa and occurs very quickly over a short (1 - 10 min) period of time (Harrison and Miller 2000; Fraser 2010).

The major question at this point was whether HSPA8-induced membrane effects observed in our study shared a common pathway with membrane events associated with bicarbonate stimulation. Interestingly, the time scales of development of membrane response to both external stimuli, either HSPA8 in our investigations (Chapter 4) or bicarbonate from other reports (Harrison and Miller 2000; Fraser 2010) are rapid. Moreover, the nature of the cellular response in both systems is a shift in lipid fluidity. The evidence mentioned above was convincing enough to hypothetically assume that the effect of HSPA8 on sperm membrane fluidity was elicited through the same cAMP-dependent pathway as observed with bicarbonate stimulation.

Accordingly, the aim of the present study was to determine the role of the cAMP / PKA-dependent pathway in HSPA8-induced increase in sperm membrane lipid fluidity. The initial rise in cyclic AMP level, 15 min after adding HSPA8 to spermatozoa implied a causal relationship and reflected the efficacy of HSPA8 in igniting this intracellular pathway. This provided the base on which a body of experiments were designed to test the proposed hypothesis.

First we validated the efficacy of cAMP analogue, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole 3',5'-cyclic monophosphothioate (cBIMPS) as the stimulator, 2-1H-benzimidazol-2-ylthio)-2-[(5-bromo-2-hydroxyphenyl) methylene hydrazide, propanoic acid (soluble adenylyl cyclase inhibitor; KH7) and H89 (PKA-blocker) as the inhibitors of the cAMP / PKA-dependent pathway by merocyanine staining and CTC staining of spermatozoa, respectively. Next, by application of the above functional pathway effectors, we examined

whether HSPA8-induced enhancement in membrane fluidity was mediated by cAMP / PKA pathway.

7.2 Materials and methods

All chemicals used for preparation of Tyrode's medium were purchased from BDH Laboratory Supplies, Poole, UK. Other chemicals were from Sigma-Aldrich company Ltd, Dorset, UK, unless otherwise stated.

7.2.1 Semen Preparation

Boar semen diluted and stored for 24 hours in Beltsville Thawing Solution (Pursel and Johnson 1975) was obtained from a commercial artificial insemination station (JSR Healthbred Limited, Thorpe Whillougby, Yorkshire,UK). On the day of experiment, diluted boar semen was washed using Percoll gradient (Holt and Harrison 2002). Briefly, aliquots of 2-4 ml of diluted semen were layered over a Percoll density gradient. The iso-metric Percoll gradient consisted of 2 ml 70% (v/v) Percoll overlaid with 2 ml 35% (v/v) Percoll. The gradient was centrifuged at 200 g for 15 min followed by 15 min centrifugation at 900 g. The supernatant was removed, and the pellet was resuspended in PBS (136.9 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4.12H_2O$ and 1.5 mM KH_2PO_4) (Harayama, Okada et al. 2003). Thereafter, semen samples were once again centrifuged at 900 g for 15 min and resuspended in PBS. Sperm concentration was measured in duplicate using a Neubauer chamber (Marienfield, Germany) and adjusted to desired concentration in either Tyrode's medium consisting of 3.1 mM KCL, 0.4 mM $MgCl_2.6H_2O$, 100 mM NaCl, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, 0.3 mM $NaH_2PO_4.2H_2O$ (Elliott, Lloyd et al. 2009) or non-capacitating medium (NCM) consisting of 2.7mM KCl, 1.5 mM KH_2PO_4, 81 mM Na_2HPO_4, 137 mM NaCl, 5.55 mM glucose and 1 mM sodium pyruvate, pH=7.4. (Kumaresan, Johannisson et al. 2012), depending on the experimental conditions. The tubes containing spermatozoa in NCM were tightly closed during incubation periods to avoid HCO_3 formation.

For inducing capacitation, spermatozoa were incubated in TALP which consisted of Tyrode's medium supplemented with 12 mg/ml bovine serum albumin, 21.6 mM sodium lactate and 1 mM sodium pyrovate (Elliott, Lloyd et al. 2009). Stimulation of merocyanine binding in sperm

membrane was done by adding bicarbonate in the form of 15 mM aqueous solution of $NaHCO_3$ in NCM. The media was maintained at 38°C in equilibrium with 5% (v/v) CO_2 (Harrison, Ashworth et al. 1996). Bicarbonate-containing media has pH of 7.4 when kept at 38°C in equilibrium with 5% v/v CO_2. To prevent CO_2 loss, incubations were done in capped poly-styrene tubes and the experimental steps were performed quickly as far as practically possible. In case tubes were uncapped for removal or addition of agents, the 5% v/v CO_2 gas was replenished before continuing the experiment. It is noteworthy to mention that NCM was specifically used in the experiments where we wanted to avoid the interference of capacitating agents and activation of capacitation-associated intracellular pathways.

7.2.2 Evaluation of Sperm viability

Sperm viability was evaluated by fluorescent viability assay composed of the combination of Propidium iodide & SYBR-14 (Live / Dead Sperm Viability Kit, Molecular Probes, Eugene, Oregon, USA), as described previously.

7.2.3 Evaluation of sperm capacitation

The Chlortetracycline Hydrochloride (CTC) staining method was used to assess the capacitation status of boar sperm as described by Fazeli *et al.* (Fazeli, Duncan et al. 1999).

7.2.4 Flow cytometric analysis of merocyanine binding to sperm using merocyanine540 / YO-PRO staining

The ability of sperm to bind merocyanine was assessed according to the merocyanine staining protocol described by Harrison *et al.* (Harrison, Ashworth et al. 1996). For this, 1 mM Merocyanine (Molecular Probes, Eugene, OR, USA) and 25 µM Yo-Pro (Molecular Probes) working solutions were prepared in dimethyl sulphoxide (DMSO). Sperm samples in NCM were incubated with Yo-Pro (25 nM) at 38°C and 5% v/v CO_2 for 10 min in closed tubes to enable differentiation between live and dead cells. Merocyanine (3 µM) was added just before the analysis to enable detecting membrane lipid instability. Solutions containing merocyanine were maintained at 38°C, protected from light and stirred continuously as far as practically possible.

Following addition of merocyanine to the sperm samples, flowcytometric analysis was performed as quickly as possible on a Beckman Coulter Epics cytometer (Beckman Coulter Inc, Miami, Florida, USA) equipped with a 20-mW argon ion laser for excitation at 488 nm. Fluorescence data were collected in logarithmic mode. By setting high sensitivity forward (FSC) and side (SSC) light scatter obscuration lines, large events (agglutinated sperm aggregates) and non-sperm events (debris) were gated out and analysis was restricted to pure sperm population by obtaining the typical L-shaped forward/side scatter distribution for sperm population. Yo-Pro and merocyanine-540 fluorescence were detected by FL-1, 525 nm band-pass filter and FL-2, 575 nm band-pass filter, respectively. Quadrants were plotted on the flow dotplots to differentiate 3 subpopulations with respective percentages; cells unstained with Yo-Pro with low merocyanine signal delineated as live with stable membrane, cells unstained with Yo-Pro with high merocyanine signal defined as live with unstable membrane and Yo-Pro positive or dead cell regardless of their merocyanine signal. Yo-Pro positive cells were disregarded from analysis when histograms were plotted. Data were collected from 10,000 events in each sample and analysis was performed by general purpose flowcytometry data analysis software, WEASEL 3.0.2. (Bio-Soft Net website : http://en.bio.-soft.net)

7.2.5 Assessment of sperm membrane fluidity by FRAP

Mobility of lipid molecules in sperm plasma membrane was measured by FRAP analysis, as described previously.

7.2.6 Cellular cyclic AMP analysis

All steps including cell lysis and quantitative measurement of cAMP concentration in the cell lysate were performed by Cyclic AMP Parameter Assay kit (R&D Systems, Inc. Abingdon, United Kingdom), according to the supplier's instructions. The kit contained 96 well polystyrene microplate (12 strips of 8 wells) coated with a goat anti-mouse polyclonal antibody, cell lysis buffer, cAMP conjugated to horseradish peroxidase, standard cAMP, primary antibody solution (mouse monoclonal antibody to cAMP), wash buffer, colour diluents and cAMP high, medium and low controls.

- ***Cell lysis protocol***

  The first step for determination of intracellular cAMP concentration was to prepare cell lysates. For this, the spermatozoa were resuspended in 1 ml of the 1x cell lysis buffer provided with the kit. For complete cell lysis, the suspension was frozen and thawed at -20°C and room temperature, respectively in three repeated cycles and then centrifuged at 600 g for 10 min. The supernatant was collected and stored at -20°C for future analysis. In order to ensure complete cell lysis had occurred and the whole intracellular cAMP was collected, after collecting the supernatant the pellet was resuspended in 300 µl of PBS and stained with live and dead staining to be examined for presence of cells under the fluorescent microscope.

- ***Direct enzyme-linked immunosorbent assay (ELISA) for cAMP measurement***

  The principle of this cAMP assay was based on the competitive binding technique. The provided micro-plate was coated with goat anti-mouse antibody. Briefly, in the first step the primary cAMP specific monoclonal antibody bound to the coated anti-body onto the microplate. After a wash to remove excess monoclonal antibody, the samples (cell lysate in this study) and horseradish peroxidase (HRP)-labeled cAMP were added to the plates. Cyclic AMP present in the sample competed with a fixed amount of HRP-conjugated cAMP for binding sites on the monoclonal antibody coated plate. Following another wash to remove excess conjugate and unbound cAMP, a substrate solution was added to the wells to measure the bound horse radish peroxide activity through development of a new colour. The colour development was stopped and the absorbance was measured at 450 nm by a Benchmark 96 well-plate reader (Bio-Rad laboratories, Ltd, Herts, UK). The colour intensity was inversely proportional to the cAMP concentration in the sample. To measure cellular cAMP level, a calibration curve was applied.

On the day of experiment, all the reagents were warmed to room temperature and the dilutions were made according to the manufacturer's instructions. Wash buffer 25x and cell lysis buffer 5x were diluted in deionised water to give final concentrations of 1x. A serial dilution of cAMP standard was made in cell lysis buffer 1x to produce the highest (240 pmol/ml) and lowest

(3.75 pmol/ml) standards. A zero standard (0 pmol /ml) was also included by using cell lysis buffer in equal volumes as other standards. Sperm lysate dilutions of 1x and 10x were prepared in cell lysis buffer 1x. Substrate solution was made by mixing equal volumes of A & B colour reagents within 15 min of use. All samples, controls and standards were assayed in duplicate. The assay procedure was performed as summarised in Figure 7.2.

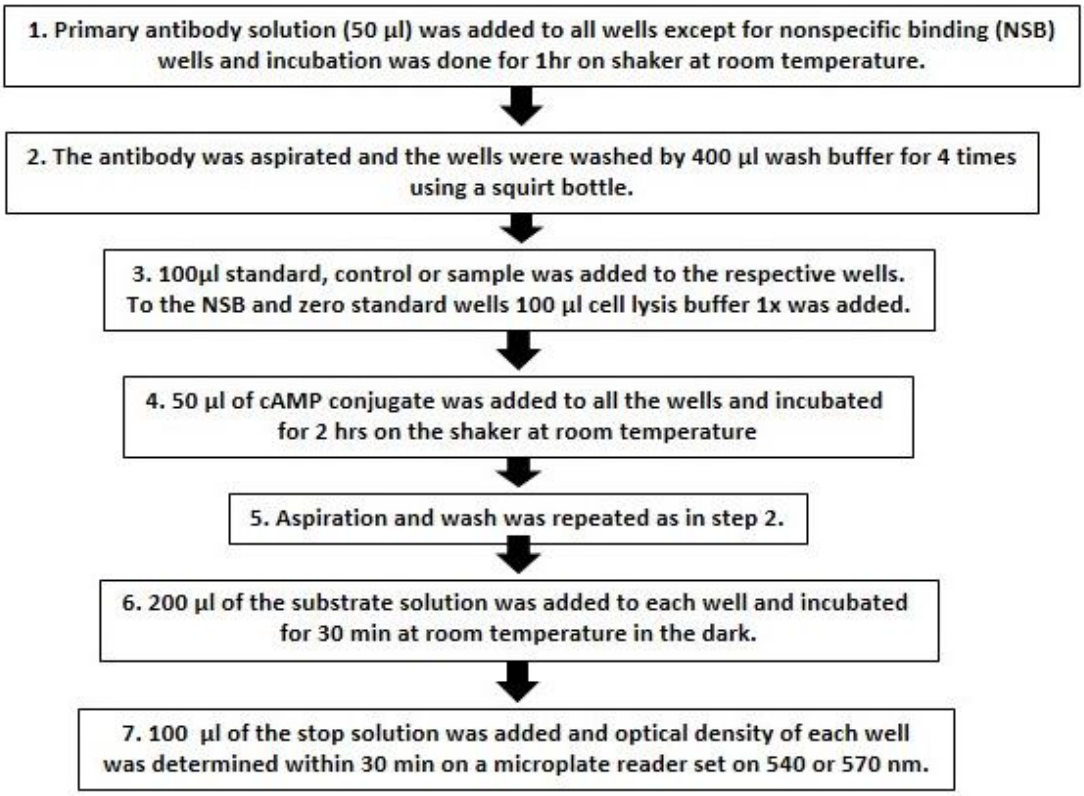

Figure 7.2 Procedure summary of enzyme linked immusorbent assay for quantitative determination of Cyclic AMP in spermatozoa.

7.3 Experimental design

7.3.1 Effect of brief treatment with exogenous HSPA8 on intracellular cAMP level

Fresh spermatozoa from 5 different boars were washed using Percoll gradient, adjusted to 10^7 cell / ml and incubated with HSPA8 (0 or 0.5 µg/ml) in NCM for 15 min at room temperature. The incubation was followed by 3 centrifugation rounds in 1 ml NCM at 600 g for 10 min at room temperature to remove free protein from the medium and avoid protein interference with the assay reagents. After the last wash the supernatant was removed and the pellet was ready for cell lysis and analysis of total cellular cAMP content, as described above. In parallel, a 200 µl aliquot from each treatment was allocated to viability assessment.

7.3.2 Validation of PKA blocker, H89 efficiency in inhibiting the cAMP-dependent capacitation pathway assessed by CTC assay

To validate efficiency of H89 to inhibit the cAMP pathway, fresh Percoll-washed semen samples from 6 different boars were incubated in TALP (5 x 10^6 cells / ml) at 39ºC with 5% v/v CO_2 (capacitating condition) for 0 and 5 hr in the presence of H89 (Enzo Life Sciences, Exeter, UK) at 0, 50 and 100 µM. At each time interval, sperm capacitation status was evaluated using the CTC staining assay. In parallel, sperm viability was assessed by Propidium iodide / SYBR-14 staining after 5 hr incubation with H89 to exclude detrimental effects of H89 on sperm membrane integrity and hence unpredictable interference with the final results.

7.3.3 Effect of PKA blocker, H89 on HSPA8-mediated enhancement in sperm membrane fluidity assessed by FRAP method

To investigate the inhibitory effect of H89 on HSPA8-mediated enhancement in membrane fluidity, 6 different fresh semen samples were washed through Percoll gradient, adjusted to 5 x 10^6 cells / ml in NCM and labelled with ODAF according to the protocol described previously. ODAF-labelled spermatozoa were first incubated with H89 at 0 and 50 µM concentrations for 15 min followed by another 15 min incubation with HSPA8 (0 and 0.5 µg/ml) at room temperature. Consequently, FRAP analysis was performed on acrosome and postacrosome regions of spermatozoa and a minimum number of 30 spermatozoa were

examined in each sample. Control samples of sperm with HSPA8 and sperm with H89 were also prepared for exclusion of potential effects from individual treatments.

7.3.4 Confirmation that the cAMP analogue (cBIMPS) stimulates merocyanine binding in sperm

In order to confirm that the cAMP analogue can stimulate merocyanine binding in sperm, merocyanine and Yo-Pro were added to 500 µl aliquots (n = 3 different boars) of three different experimental media (pre-warmed at 38°C) including NCM, NCM containing bicarbonate and NCM containing 50 µM cAMP analogue (cBIMPS) (Enzo Life Sciences, Exeter, UK) designated as negative control, positive control and test group, respectively. Negative control samples were supplemented with 15 mM NaCl. Experiments began by adding fresh Percoll-washed spermatozoa at 5 x 10^6 / ml concentration to the prepared tubes. Following 5 min incubation, samples were rapidly applied to the flow cytometer for analysis. All suspensions were maintained at 38°C and in 5% v/v CO_2.

7.3.5 Relative effects of HSPA8 and cBIMPS on the ability of spermatozoa to bind merocyanine

To compare the effects of HSPA8 and cBIMPS on the binding of merocyanine by sperm, fresh washed sperm samples (n = 5) at 5 x 10^6 cells / ml were resuspended in equal volumes in tubes containing NCM only, NCM with HSPA8 (0.5 µg / ml) or NCM with cBIMPS (50 µM). At 15 min after incubation at 38°C, samples were stained with merocyanine / YO-PRO for flow cytometric analysis.

7.3.6 Relative effects of HSPA8 and cBIMPS on sperm membrane fluidity

To compare the effects of cBIMPS and HSPA8 on sperm membrane fluidity, fresh washed sperm samples from 5 different boars were resuspended in NCM to give a final concentration of 5 x 10^6 spermatozoa / ml and labelled with ODAF as described before. ODAF-labelled spermatozoa were then split into 4 equal aliquots, one as the control and the rest three to be

incubated with HSPA8 (0.5 µg/ml) or cBIMPS (50 µM) or cBIMPS (100 µM) individually, for 15 min at room temperature. FRAP analysis on acrosome and postacrosome regions of spermatozoa was performed on a minimum number of 30 spermatozoa in each sample.

7.3.7 Confirming the capacity of the adenylyl cyclase inhibitor (KH7) to block cAMP-mediated binding of merocyanine in sperm

In order to validate the blocking effect of KH7 on cAMP-mediated merocyanine binding in sperm, fresh washed sperm samples (n = 3) at 5 x 10^6 cells / ml were resuspended in NCM (pre-warmed at 38°C) with or without 100 µM adenylate cyclase (AC) inhibitor, KH7 (Enzo Life Sciences, Exeter, UK). Following 10 min incubation, resuspended spermatozoa in both samples were activated by adding bicarbonate in the form of 15 mM aqueous solution of $NaHCO_3$ and immediately stained with merocyanine / YO-PRO for flow cytometric analysis. The samples were maintained at 38°C as far as practically possible throughout the experiment.

7.3.8 Effect of adenylate cyclase blocking by KH7 on the enhancement of membrane fluidity by HSPA8

To study the blocking effect of KH7 on HSPA8-mediated enhancement in membrane fluidity, fresh washed sperm samples (n = 5 different boars) in NCM at 5 x 10^6 / ml were initially treated with KH7 (0, 100 µM) for 15 min followed by a 15 min incubation with HSPA8 (0, 0.5 µg/ml) at room temperature. Consequently, FRAP analysis was performed on acrosome and postacrosome regions of spermatozoa and a minimum number of 30 spermatozoa were examined in each sample. Control samples of sperm with HSPA8 and sperm with KH7 were also prepared for exclusion of potential effects from individual treatments.

7.3.9 Statistical analysis

Mean values for cAMP level in two control and treatment groups were analysed by the student t-test. Data obtained from flow cytometry or FRAP analysis were subjected to factorial analysis of variance (ANOVA). When the results were significant in ANOVA, means were further

tested with post-hoc Fisher's least significant difference (LSD) test to examine the effect of treatments within experimental designs by Statistica software, version 7 (WWW.StatSoft.com, 1984-2004). Data were expressed as mean ± s.e.m. A probability of $P \leq 0.05$ was considered to be statistically significant.

7.4 Results

7.4.1 Brief treatment with HSPA8 enhanced intracellular cAMP level in spermatozoa

Inclusion of HSPA8 in the sperm medium for 15 min caused a significant rise in the intracellular cAMP levels in sperm compared to control samples. The number of viable (membrane intact) spermatozoa also increased significantly after 15 min exposure to HSPA8 (Figure 7.3).

7.4.2 H89 efficiently inhibited sperm capacitation

Sperm capacitation assessed by CTC assay was inhibited by H89 in both concentrations (50 and 100 µM) indicating that H89 is an efficient blocker of the cAMP-dependent capacitation pathway in sperm (Figure 7.4). Meanwhile, viability% (membrane intactness) of spermatozoa in samples treated with H89 at both 50 and 100 µM concentrations for 5 hr correlated with control samples (65 ± 5% and 67.5 ± 8% vs 66.5 ± 7.5%, respectively), indicating that H89 did not adversely affect sperm membrane and could be used safely in our studies.

7.4.3 PKA blocking had no inhibitory effect on HSPA8-mediated sperm membrane fluidity

Membrane fluidity was enhanced on both acrosome and postacrosome sperm domains after exposure to HSPA8, even in the samples which were pre-treated with H89. This indicated that specific PKA blocking had no inhibitory effect on HSPA8-mediated enhancement of sperm membrane fluidity (Figure 7.4).

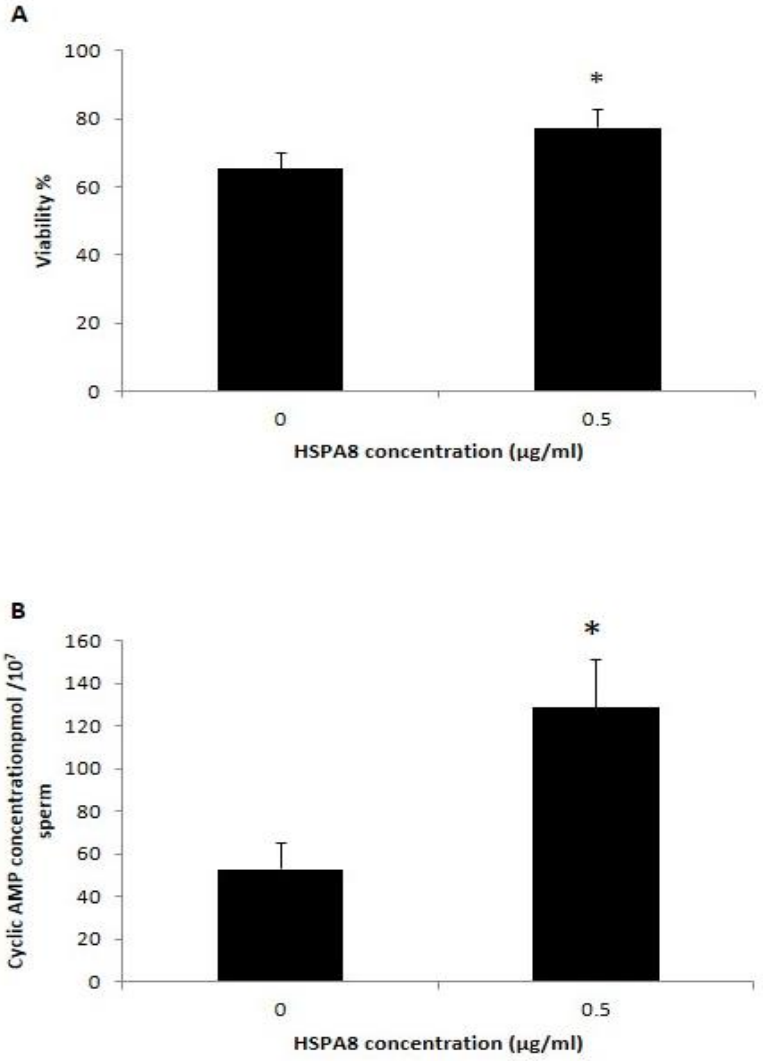

Figure 7.3 Effect of brief treatment (15min) with HSPA8 on viability and intracellular cAMP content of boar spermatozoa. (**A**) Sperm viability% (measured as membrane integrity and (**B**) intracellular cAMP (pmol / 10^7 sperm after 15 min exposure to HSPA8,0 and 0.5µg/ml. n = 5, * $P \leq 0.05$, mean ± s.e.m.

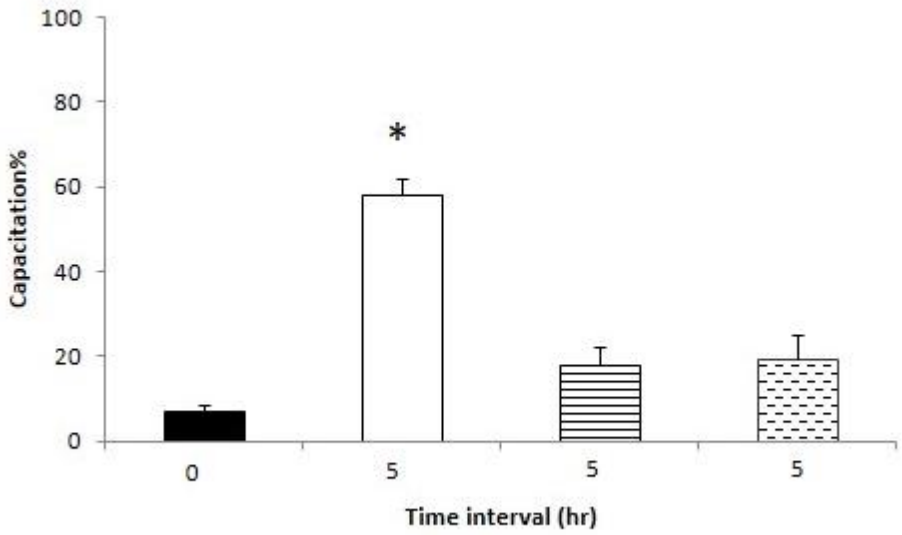

Figure 7.4 Confirmation of the effect of the protein kinase inhibitor, H89 in blocking sperm capacitation. Sperm capacitation % was measured by CTC assay after 5 hrs incubation in capacitation conditions in the presence of H89 at 0 □, 50 ▤ and 100 ▦ µM compared to 0 hr control ■, * $P < 0.005$. n = 6, mean ± s.e.m.

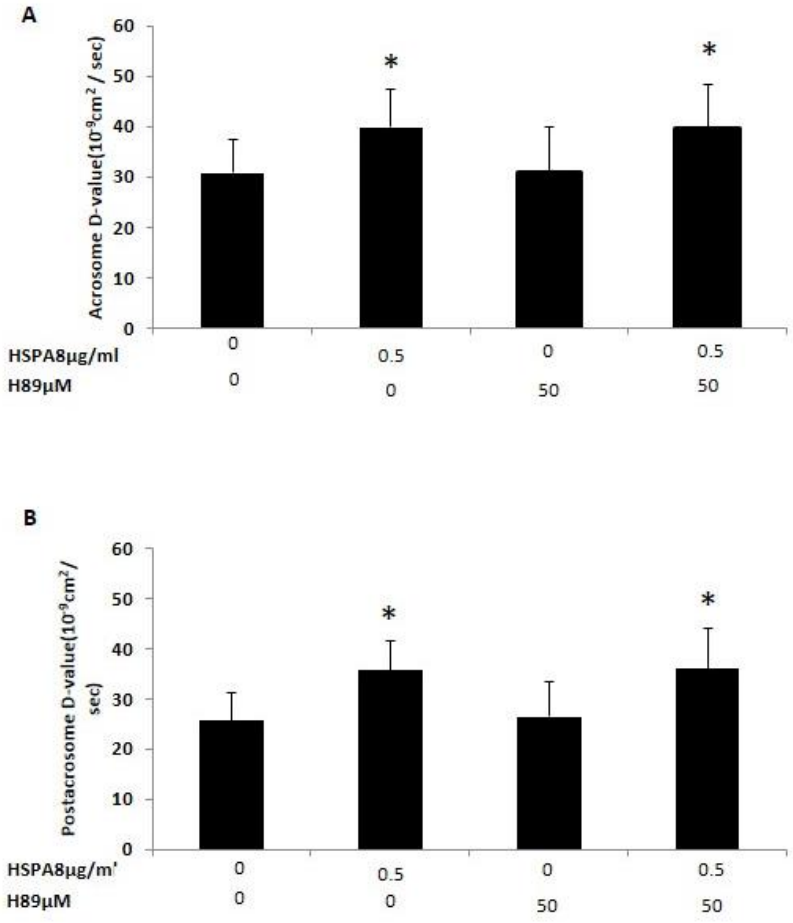

Figure 7.5 Effect of the protein kinase inhibitor, H89 on HSPA8-mediated enhancement in sperm membrane fluidity. (**A**) Acrosome D-values (10^{-9}cm^2 / sec) and (**B**) postacrosome D-value (10^{-9}cm^2 / sec) of boar spermatozoa (30 spermatozoa / sample) in response to HSPA8 (0.5 µg/ml) after pre-treatment with H89 (50 µM). n = 6, * $P < 0.05$, mean ± s.e.m.

7.4.4 cAMP analogue enhanced sperm membrane merocyanine binding

This experiment was carried out to confirm the efficiency of cBIMPS as cAMP analogue, in stimulating merocyanine detectable changes in sperm membrane lipid structure. Similar to HCO_3^-, cBIMPS added to sperm samples immediately stimulated merocyanine incorporation in live sperm subpopulation (15.5 ± 4.5% vs 65 ± 7.25% vs 71 ± 8.5%, control vs cBIMPS-treated vs HCO_3^--treated, n = 3, $P \leq 0.005$, mean ± s.e.m) which was indicative of the cAMP analogue efficiency (Figure 7.6).

7.4.5 HSPA8 had no effect on sperm merocyanine binding ability

Inclusion of HSPA8 in the sperm incubation medium had minimal effect on sperm merocyanine binding ability in contrast to cBIMPD which significantly enhanced the proportion of live spermatozoa with high merocyanine binding (Figure 7.7).

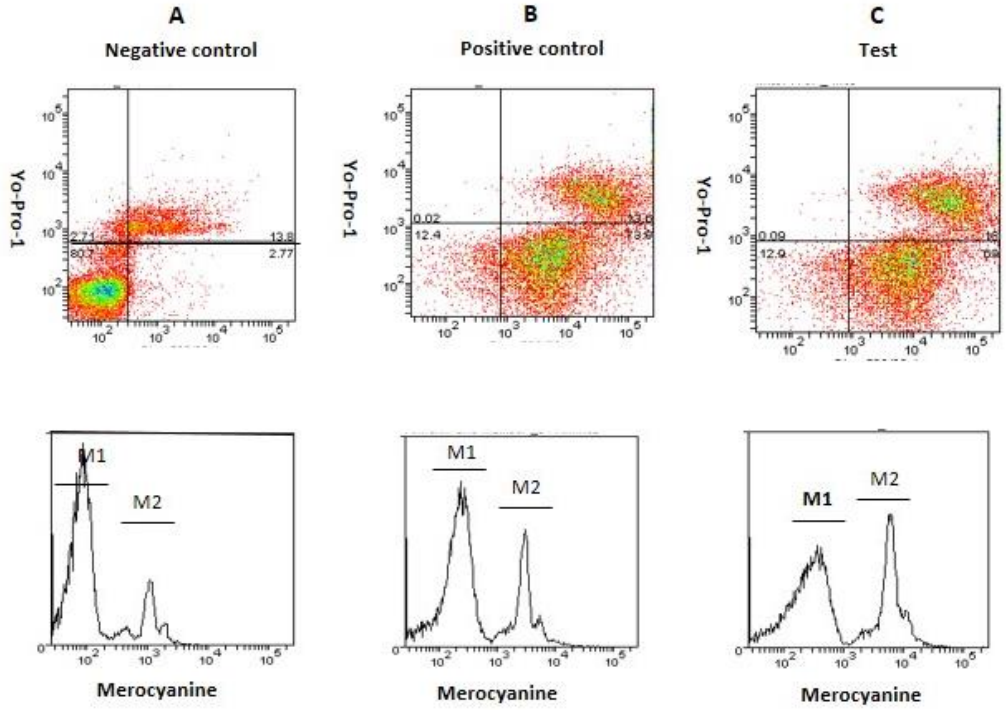

Figure 7.6 Confirmation of the efficacy of cAMP analogue (cBIMPS) with comparison to the stimulatory effect of HCO_3^- in stimulating the merocyanine binding ability in boar sperm membrane by flowcytometry. Dotplots and histograms obtained from a sperm sample after incubation in (**A**) non-capacitating condition (NCM) (negative control), (**B**) NCM with HCO_3 (positive control) and (**C**) NCM plus cBIMPS 50 µM (test), followed by merocyanine / YO-PRO staining. Dotplot quadrants represent; UL: YO-PRO + / merocyanine -, UR: / YO-PRO + merocyanine +, LL: YO-PRO - /merocyanine – and LR: YO-PRO - / merocyanine + spermatozoa. Histograms represent YO-Pro negative spermatozoa with low (M1) and high (M2) membrane-bound merocyanine levels.

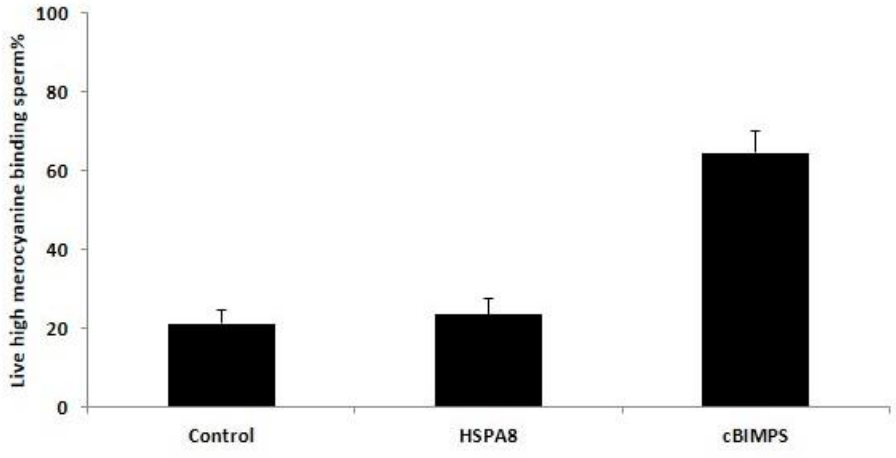

Figure 7.7 Relative effects of HSPA8 and cAMP analogue (cBIMPS) on merocyanine stainability in live boar sperm population. Flowcytometric analysis of fresh washed spermatozoa after incubation in non-capacitating medium (NCM) only (control), NCM containing HSPA8 (0.5 µg/ml) and NCM containing cBIMPS (50 µM) for 15 min at room temperature, followed by merocyanine / YO-PRO staining, n = 5, * $P < 0.05$, mean ± s.e.m..

7.4.6 Cyclic AMP analogue (cBIMPS) had no effect on sperm membrane fluidity assessed by FRAP

This experiment was devised to investigate the effect of cBIMPS on the lipid fluidity of sperm membranes (assessed by FRAP analysis) and compare it with the effect of HSPA8 on sperm membrane. While membrane fluidity significantly increased in response to a brief exposure to HSPA8, cBIMPS failed to stimulate any changes in membrane lipid fluidity (Figure 7.8).

7.4.7 KH7 efficiently reduced sperm membrane merocyanin binding, but had no inhibitory effects on HSPA8-mediated sperm membrane fluidity

As indicated in Figure 7.9, addition of HCO_3^- in the form of $NaHCO_3$ instantly increased merocyanin binding in sperm membrane in live sperm subpopulation. However, this effect was not observed in the samples which included the KH7 (65% ± 7.25 vs 22% ± 5.5, HCO_3^--treated vs KH7-treated, n = 3, $P \leq 0.001$, mean ± s.e.m). This indicated that the AC inhibitor efficiently blocked HCO_3-induced merocyanine-detectable changes in sperm lipid membrane.

On the other hand**,** Figure 7.10 clearly shows that HSPA8 enhanced sperm membrane fluidity in a significant manner even after specific blocking of AC by KH7. This indicated that AC is not involved in mediating HSPA8 effect on sperm membrane.

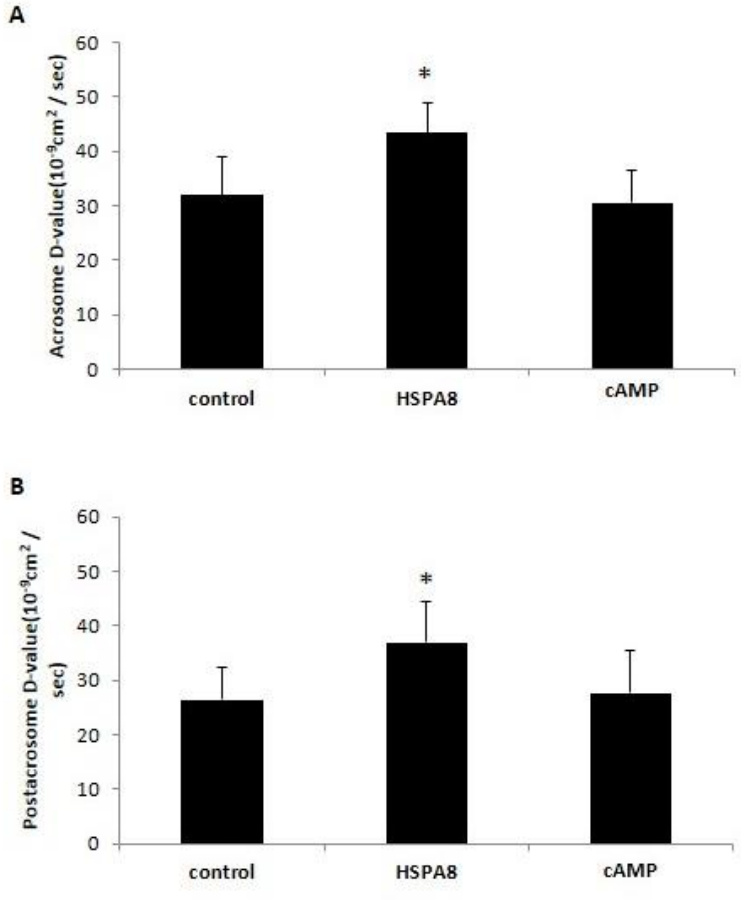

Figure 7.8 Effect of the cAMP analogue, cBIMPS on sperm membrane fluidity evaluated by FRAP analysis. (**A**) Acrosome D-values ($10^{-9}cm^2$ / sec) and (**B**) postacrosome D-value ($10^{-9}cm^2$ /sec) of boar spermatozoa (30 spermatozoa / sample) at 15 min after treatment with cBIMPS (50 µM) or HSPA8 (0 and 0.5 µg/ml), respectively. n = 5, * $P < 0.05$, mean ± s.e.m.

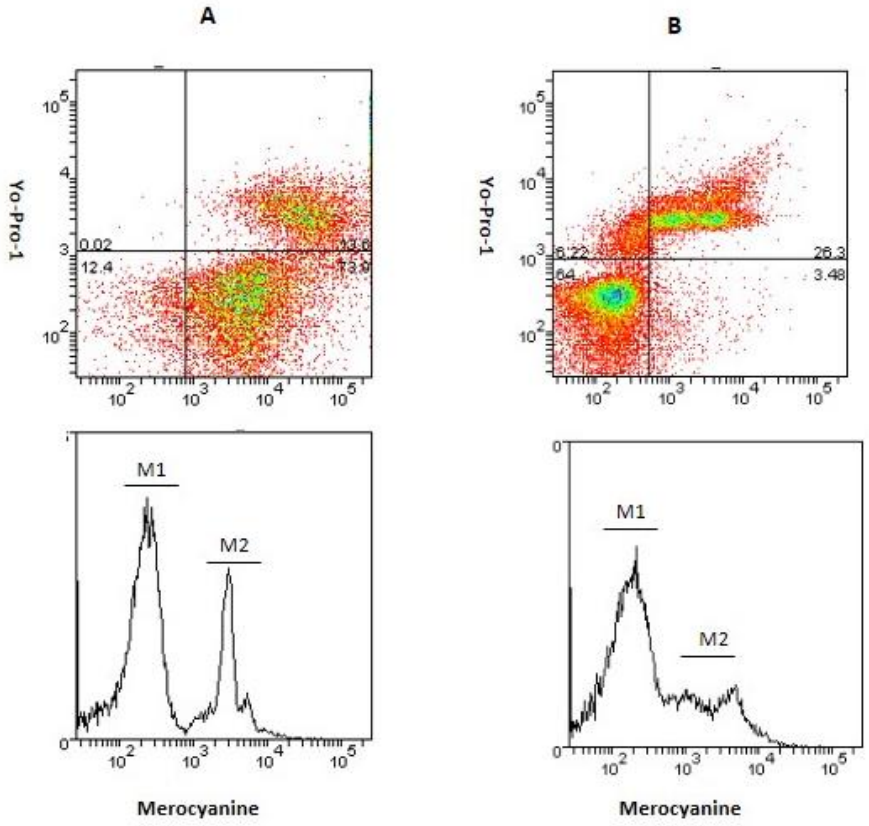

Figure 7.9 Confirmation of the efficacy of soluble adenylyl cyclase inhibitor, KH7 in blocking merocyanine binding ability in boar sperm membrane by flowcytoetry. Dotplots and histograms obtained from a sample sperm sample after incubation in (**A**) non-capacitating medium plus bicarbonate only (control) and (**B**) non-capacitating medium plus bicarbonate containing KH7 100 µM (test), followed by merocyanine / YO-PRO staining. Dotplot quadrants represent; UL: YO-PRO + / merocyanine -, UR: / YO-PRO + / merocyanine+-, LL: YO-PRO - / merocyanine – and LR: YO-PRO - / merocyanine + spermatozoa. Histograms represent YO-Pro negative spermatozoa with low (M1) and high (M2) membrane-bound merocyanine levels.

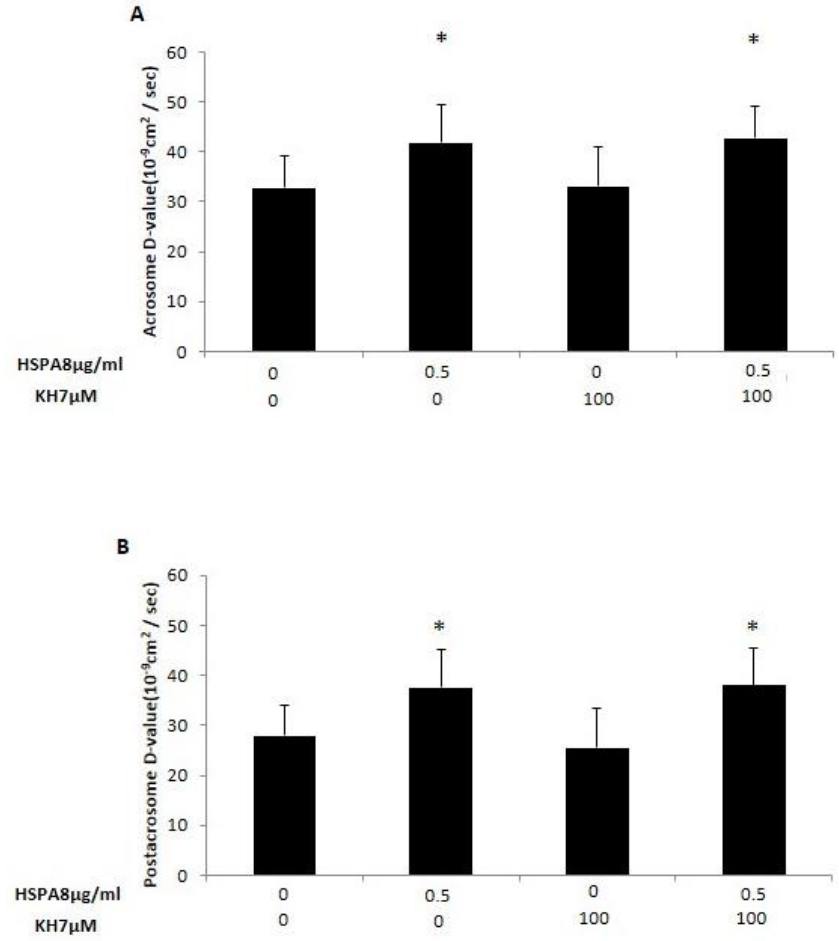

Figure 7.10 Effect of the soluble adenylyl cyclase inhibitor, KH7 on HSPA8-mediated enhancement in sperm membrane fluidity evaluated by FRAP technique. (**A**) Acrosome D-values (10^{-9}cm^2 / sec), and (**B**) postacrosome D-value (10^{-9}cm^2 / sec) of boar spermatozoa (30 spermatozoa / sample) in response to HSPA8 (0.5 µg/ml) after pre-treatment with KH7 (100 µM). n = 5, * $P < 0.05$, mean ± s.e.m.

7.5 Discussion

The cyclic AMP signaling pathway plays a pivotal role in regulating many physiologic characteristics critical for spermatozoa to gain fertilisation potential such as motility, capacitation and gamete fusion (Holt and Harrison 2002; Baxendale and Fraser 2003). Cyclic AMP (cAMP) is synthesised from ATP and its production in the cell is mainly regulated by the enzyme adenylyl cyclase (Visconti, Galantino-Homer et al. 1998). In capacitation-related sperm membrane remodeling, the signalling cascade downstream to cAMP involves the critical step of protein kinase A (PKA) activation. Protein kinase A, a ubiquitous cellular kinase belongs to a superfamily of kinase enzymes which regulates signal transductions through phosphorylation of the serine and threonine residues of the protein substrate (Lochner and Moolman 2006). PKA activity is chiefly dependent on cellular cAMP levels and hence it is also known as cAMP-dependent protein kinase.Two other enzymes named phosphodiesterase and protein phosphatase I negatively influence the pathway as the rate-limiting steps, through the act of dephosphorylation at cAMP and PKA level, respectively (Harrison and Miller 2000) (Figure 7.1).

Following our preliminary findings regarding elevations in sperm intracellular cAMP in response to HSPA8 treatment, we examined the role of PKA in the increases in sperm membrane lipid fluidity that were induced by HSPA8. For this, a selective, high affinity and cell permeable PKA inhibitor, H89 was used. H89 has been extensively used for evaluating the role of PKA in various cell systems including heat muscle cells, osteoblasts, hepatocyte, neural tissue (Lochner and Moolman 2006) as well as spermatozoa (Holt and Harrison 2002; Miro-Moran, Jardin et al. 2012). The inhibitory effects of H89 are due to its competitive binding to the ATP binding site on the catalytic subunit of PKA (Lochner and Moolman 2006).

The potency and functionality of H89 were determined by evaluating its inhibitory effect on the process of sperm capacitation. H89 markedly inhibited the capacitation of boar spermatozoa, but did not inhibit HSPA8-mediated enhancement in sperm membrane fluidity. This was suggestive of the fact that the effect of HSPA8 on membrane fluidity was not PKA-dependent. However, since PKA is not the only cAMP substrate, an involvement of a cAMP-

dependent pathway as the mediator of HSPA8 effect could not be excluded. Recently, it has been reported that cAMP also exerts its effect through a PKA-independent pathway known as Epac proteins (exchange proteins activated directly by cAMP) (Miro-Moran, Jardin et al. 2012) in mammalian spermatozoa. Investigating the presence and role of Epac components including guanine-nucleotice-exchange factors and hyperpolarisation-activated cyclic nucleotide-gated channels (Harayama, Okada et al. 2003) in our system using commercially available specific antibodies could definitely provide useful information regarding HSPA8 pathway effectors.

Moreover, although H89 is recognised as a specific PKA inhibitor, a recent study by Bain et al. (Bain, McLauchlan et al. 2003) showed that even protein kinase inhibitors with the highest affinity frequently share a degree of sequence resemblance. For instance, despite its high specificity, H89 inhibits at least 8 other kinases besides PKA in varying degrees (Davies, Reddy et al. 2000). The overlapping PK inhibitory effects of blocking agents should be considered to avoid misinterpretation of the results.

In the next step, we examined whether a rise in cellular cAMP content could bring about similar changes in membrane fluidity as HSPA8. This was based on the speculation that similar results would be observed with both treatments if cAMP was involved as a mediator of the pathway. Initially, the merocyanine binding assay was applied to determine the potency of a cAMP analogue, in activation of the cAMP / PKA pathway against bicarbonate which is the known activator of the pathway. Similar to bicarbonate, addition of cBIMPS to spermatozoa elevated sperm merocyanine intensity indicating that cBIMPS was highly membrane permeable, metabolically stable to enzymatic degradation and a potent mediator of the cAMP / PKA dependent pathway regulating membrane lipid organisation.

Although direct stimulation of the cAMP pathway by cBIMPS enhanced membrane merocyanine binding ability, it had no effect on lipid fluidity parameters measured by FRAP. On the other hand, HSPA8 was unable to stimulate sperm membrane merocyanine binding ability. Taken together, these two observations provided direct evidence on the independence of HSPA8-mediated effects on cAMP pathway, and were further supported by blocking adenylyl cyclase (AC) as the main effector molecule of cAMP-related cascade.

Among 10 identified AC isoforms in mammalian cells, 9 are membrane-bound and regulated by G-protein. This classical trans membrane AC regulation consists of a G-protein coupled receptor upstream of AC which can be stimulated by a range of hormones, proteins and external signal substances (Tresguerres, Levin et al. 2011). The tenth AC type is a non membrane-bound cytoplasmic soluble isoform which appears to be insensitive to G-protein or other common AC activators and is believed to be stimulated by bicarbonate and calcium ions *in vitro* and *in vivo* (Zippin, Levin et al. 2001; Fraser 2010). In mammalian spermatozoa soluble AC (sAC) is physiologically important and regulates major sperm physiological process of capacitation and acrosome reaction (Fraser 2010; Tresguerres, Levin et al. 2011).

Using a specific sAC blocker (Kumar, Kostin et al. 2009), KH7 whose potency was confirmed by successful suppression of sperm merocyanine binding ability, we demonstrated that blocking sAC did not inhibit the rise of lipid fluidity in response to HSPA8. This last piece of experimental data convincingly ruled out any relation between HSPA8-membrane effects and cAMP-dependent pathway.

During sperm storage in the oviduct prior to ovulation, sperm cAMP-dependent pathways and related sperm physiologic functions (capacitation, motility) remain quiescent due to diminished levels of bicarbonate. That window period provides the opportunity for spermatozoa to establish a close contact with oviductal epithelium and attain physiologic fitness under the influence of oviduct protein factors such as HSPA8. Upon ovulation the surge in oviductal bicarbonate concentrations activates cAMP production by soluble AC which results in sperm hypermotility, detachment from oviduct epithelium and capacitation (Holt and Harrison 2002). The fact that HSPA8-mediated and capacitation-mediated membrane changes did not share a common pathway emphasises that HSPA8-repairing effect on sperm membrane has important role in providing high quality sperm for fertilisation and needs to precede capacitation and fertilisation processes. Therefore, even if the two stimulators, HSPA8 and bicarbonate share cAMP as the effector molecule, they induce two chronologically different entities with independent cAMP-related pathways.

Furthermore, FRAP analysis revealed that neither pathway stimulation by cAMP analogue nor

its inhibition by KH7 or H89 interfered with the effects which HSPA8 elicits on the sperm membrane. Combining this with the earlier observation which displayed the ineffectiveness of HSPA8 in inducing sperm merocyanine binding ability provided complementary data in support of the assumption that two entirely distinct physiologic entities in sperm membrane are being addressed. Basically, membrane merocyanine binding reflects lipid disorder and destabilisation (Aboagla and Terada 2003; Kadirvel, Kumar et al. 2009; Coy, Lloyd et al. 2010) which is mostly considered as an early stage of capacitation (Harrison, Ashworth et al. 1996; Harrison and Gadella 2005). The FRAP technique used in the current study determined two dimensional dynamics of sperm membrane lipids (Ladha, James et al. 1997; Wolfe, James et al. 1998) as a measure of membrane stability under HSPA8 effect.

Our observations could also be an indirect reflection that cAMP production in sperm in response to HSPA8 was the result of activation of the classical G protein-dependent AC isoforms rather than the soluble AC. However, definite conclusions could be drawn by further investigating the effect of specific trans-membrane adenylyl cyclase inhibitors on HSPA8-induced cAMP production in sperm. Furthermore, co-localisation of HSPA8 and G protein-coupled receptors by immunohistochemistry or confocal imaging techniques would yield critical information.

Yet, two major questions arise from our presented results; 1) what are the potential effectors downstream of cAMP produced in sperm response to HSPA8 and 2) What biological role the HSP8-induced cAMP pathway plays in sperm physiology?

Considering the data presented in earlier chapters, we demonstrated that a number of other sperm physiologic characteristics other than membrane fluidity, such as mitochondrial activity, capability to bind epithelial cells and *in vitro* fertilisation performance were immediately influenced by HSPA8 (Chapter 2 and 4). On the other hand, the discovery in Chapter 6 regarding HSPA8 localisation on the outer acrosomal surface, introduced the possibility of the presence of intermediate intracellular pathways for HSPA8 to elicit its intracellular effects from outside the cell. In view of the existing evidence it is reasonable to postulate that HSPA8-induced cAMP rise in spermatozoa could be involved in pathways regulating any of the above

mentioned characteristics. The application of membrane permeant cAMP / PKA pathway stimulators / inhibitors along with proteomic technologies would provide useful tools to understand more about the underlying cellular mechanism of the cAMP-dependent pathway and associated cellular physiologic activities. Alternatively, the possibility that exogenous HSPA8 is involved in cellular anti-apoptotic pathways provides grounds for further research on HSP-mediated signalling pathway (Multhoff 2007; Lanneau, Brunet et al. 2008). Figure 7.11 illustrates a schematic model for the potential HSPA8-mediated sperm signaling pathway and associated cellular activities.

In conclusion, observations in the current study regarding the rapid rise in cAMP level under HSPA8 effect strongly suggested the involvement of cAMP-dependent intracellular signalling cascades in sperm physiologic responses to HSPA8. However, the nature of the response is yet to be elucidated. Moreover, it was clarified that HSPA8-induced increase in sperm membrane lipid fluidity is a distinct entity from the already documented cAMP / PKA pathway involved with membrane remodelling in sperm capacitation. Unlike intracellular HSPs, the published information on the influence of exogenous HSPs on modulation of membrane lipid phase in scarce. This study provides grounds for further conclusive research on the role of exogenously applied HSPs in cell membrane stabilisation and may reveal new therapeutic approaches for treatment of cell membrane-associated pathologies.

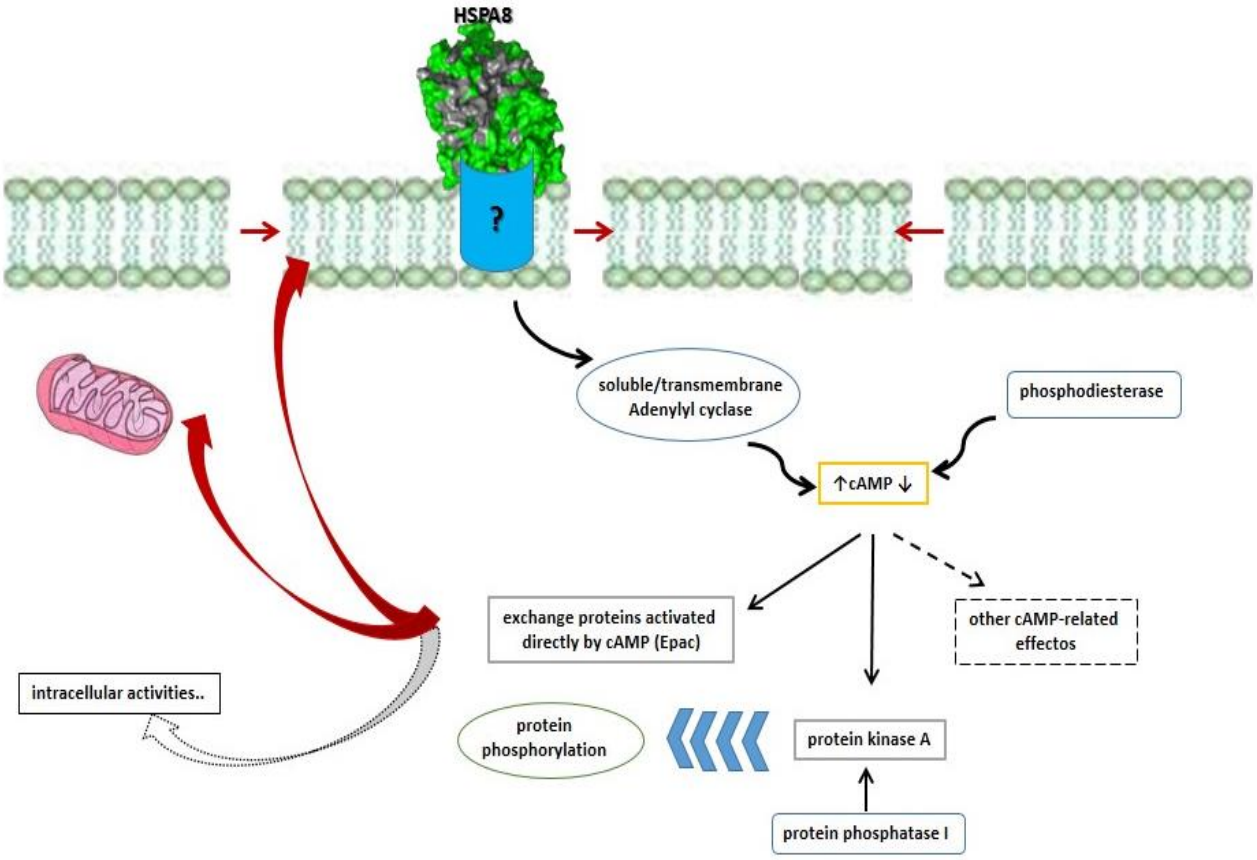

Figure 7.11 Proposed model of cAMP signalling pathway involved in HSPA8-mediated effects on sperm physiology. Direct or receptor-dependent activation of adenylyl cyclase by HSPA8 results in cAMP production. Increase in cAMP leads to either PKA or Epac activation. The net result is regulation of sperm physiologic activities influenced by HSPA8 such as membrane lipid fluidity, mitochondrial activity, mitochondrial apoptosis, etc.

Chapter 8

General discussion

8.1 Summarising discussion

Despite the existence of a wealth of studies focussing on characterising the oviductal sperm reservoir and the individual components that are involved in regulating sperm fertilising capacity within the oviduct, the oviductal sperm reservoir is far from being totally characterised. For instance, there remains minimal knowledge on the particular factors and their related cellular / molecular mechanisms which are responsible for maintaining sperm longevity for long term inside the female oviduct. This thesis aimed to focus on the *in vitro* approaches to elucidate the effect of an oviductal protein, HSPA8 (HSC70) on sperm physiology and conclusively demonstrates the ability of HSPA8 to regulate sperm properties which are crucial in their fertilisation mission. It also describes in detail, the molecular basis and cellular mechanisms behind the remarkable biologic ability of exogenous HSPA8 to immediately enhance sperm viability that has not been reported previously. The aim of this final chapter is to provide an integrated summary of the major findings of this thesis, consider their potential implications and propose directions for future investigations.

8.2 Thesis findings

The early phase of the study described in Chapters 2 and 3, was dedicated to the characterisation of the role of a bovine recombinant HSPA8 in sperm physiology. This included identifying the functionality and optimal working concentrations of HSPA8, as well as descriptive studies to discover impacts of exogenous HSPA8 on some of the most important cellular and functional characteristics of sperm including viability, motility, sperm ability to bind oviductal epithelial cells (OEC) and *in vitro* fertilisation performance. On one hand, our results supported previous reports from other laboratories on long term beneficial effects of HSPA8 on the survival of boar spermatozoa after 24 or 48 hr of treatment (Elliott, Lloyd et al. 2009; Lloyd, Elliott et al. 2009). However, we observed that exogenous HSPA8 had the ability to enhance sperm viability in short exposure periods. In what was a novel finding, this impact was manifested in spermatozoa after only 15 min exposure to HSPA8. When faced with such an interesting observation, explorations on whether this immediate effect of HSPA8 extended to other physiologic properties in sperm revealed that exogenous HSPA8 decreased sperm

mitochondrial activity, increased the ability of spermatozoa to bind to oviductal cells and enhanced the rate of *in vitro* monospermic fertilisation after a brief (15 min) exposure time span.

Reduced mitochondrial activity was detected by JC-1 mitochondrial staining, thereby indicating effects on mitochondrial activity, energy production and motility (Garner, Thomas et al. 1997). Although *in* vitro, the HSPA8-induced reduction in sperm mitochondrial activity was attributed to the transient quiescence that occurs to spermatozoa shortly after exposure to the oviductal environment (Overstreet and Cooper 1978; Overstreet, Katz et al. 1980; Suarez 1987; Grippo, Way et al. 1995; Lefebvre, Chenoweth et al. 1995; Coy, Lloyd et al. 2010) for formation of oviductal sperm reservoir *in vivo*. This observations suggests the possible role of HSPA8 as an important oviductal component in the formation of the sperm reservoir in the oviduct by rapidly reducing mitochondrial activity and motility.

An *in vitro* primary oviductal cell monolayer provided the opportunity to reveal the enhancing effect of HSPA8 on the ability of sperm to bind to oviductal epithelial cells which is an important functional aspect of sperm physiology in respect with fertilisation (Chapter 3). Although the epithelial purity of the culture content were not identified and the presence of non-epithelial cells could not be ruled out, we attempted to use the cells from very early subculture passages in order to avoid cellular de-differentiation and maintain the closest possible model to *in vivo* conditions in terms of cell status. Moreover, this system created the ideal situation for investigating the pure effect of HSPA8 on its own and unaffected by other hormonal factors on sperm-OEC binding ability. Given the lipid binding property of extracellular HSPs and 70kDa HSPs in particular (Chen, Bawa et al. 2005; Gehrmann, Doss et al. 2011), it is highly likely that the increased attachment of spermatozoa to individual OECs was brought to sperm membrane due to the influence of HSPA8 on the membrane lipid moieties. Interpreting the *in vitro* enhanced attachment of HSPA8-treated spermatozoa to OECs, in the *in vivo* context once again supports the modulatory role of HSPA8 in sperm reservoir formation and enhancement of maternal-gamete interaction.

Brief pre-treatment of spermatozoa with HSPA8 significantly enhanced monospermic *in vitro*

fertilisation rate (Chapter 3). Although the exact stage of fertilisation affected by HSPA8 was not pinpointed, this observation strongly supported the influence of HSPA8 as an oviductal protein on multiple stages of the fertilisation process. Furthermore, since sperm membrane plays an integral role through the entire fertilisation process (Foltz, Partin et al. 1993; Coux and Cabada 2006; Wortzman-Show, Kurokawa et al. 2007; Sutovsky 2009), HSPA8-mediated membrane alterations were speculated to be involved.

In our findings, the fact that a brief exposure (15 min) to exogenous HSPA8 significantly enhanced sperm viability was intriguing as it was different from previous reports which demonstrated that prolonging effects of HSPs on the viability of various biologic systems took effect after longer (24 and 48 hr) co-incubation periods (Johnson, Berberian et al. 1990; Houenou, Li et al. 1996; Boilard, Reyes-Moreno et al. 2004; Elliott, Lloyd et al. 2009; Lloyd, Elliott et al. 2009). On the other hand, the short period of action for HSPA8 to enhance sperm viability indicated that the effect was not dependent on gene expression or protein production. Besides, spermatozoa as highly differentiated cell are devoid of transcriptional / translational apparatus (Spinaci, Volpe et al. 2005). These facts prompted us to find out the mechanisms that were mediated by immediate exposure of spermatozoa to HSPA8.

Exclusion dye assays which measure plasma membrane integrity as an attribute to cell viability (Pegg 1989) provided a valuable clue to suggest the sperm membrane as the potential target for HSPA8-mediated effects. This, in conjunction with supporting evidence on the ability of different HSPs to prolong viability via interactions with and stabilisation of cellular membranes in different cell types (Johnson, Berberian et al. 1990; Johnson and Tytell 1993; Torok, Horvath et al. 1997; Horvath, Glatz et al. 1998; Boilard, Reyes-Moreno et al. 2004; Horvath, Multhoff et al. 2008; Elliott, Lloyd et al. 2009) was suggestive of HSPA8 ability to immediately restore membrane integrity via alterations in membrane properties. This speculation directed us to the next level of investigations which established the ground for the forthcoming mechanistic approaches.

Among various cell membrane properties, lipid fluidity became of our particular interest, as according to our findings, there was a rapid mechanism involved in the membrane repair

process mediated by HSPA8. Moreover, it is known that shifts in lipid fluidity happen very quickly over the membrane lipid plane (Ladha, James et al. 1997; Wolfe, James et al. 1998; Balogh, Horvath et al. 2005). Therefore, fast recovery of sperm membrane intactness under HSPA8 effect seemed a highly likely scenario in our investigation. Examination of membrane lipid fluidity using FRAP revealed significant elevations in sperm membrane lipid fluidity after 15 min exposure to HSPA8 (Chapter 4). This presented a mechanism for the immediate repairing effect of HSPA8 on sperm membrane integrity.

During viability studies, only fresh spermatozoa as opposed to capacitated spermatozoa appeared to be responsive to HSPA8-mediated effects on membrane. Further FRAP investigations provided supportive results indicating that capacitation induced changes in sperm suppressed HSPA8 effects on sperm membrane and rendered spermatozoa unresponsive to HSPA8 impacts. Substantial reductions in membrane cholesterol is a hallmark in sperm capacitation process which results in significant remodelling of lipid architecture in sperm plasma membrane (Osheroff, Visconti et al. 1999; Buffone, Verstraeten et al. 2009; Tapia, Macias-Garcia et al. 2012). Considering interactions of HSP isoforms with membrane cholesterol (Mamelak and Lingwood 2001; Mamelak, Mylvaganam et al. 2001; Chen, Bawa et al. 2005) led to a new series of experiments (described in Chapter 5) to explore the role of sperm membrane cholesterol in HSPA8-mediated effects on sperm membrane. Enhancing effects of exogenous HSPA8 on sperm viability (integrity) and membrane fluidity decreased following reduction in sperm membrane cholesterol content by methyl beta-cyclodextrine (MβCD). The role of cholesterol in mediating HSPA8 effects on the sperm membrane was the immediate conclusion drawn from this set of results. However, complementary data were necessary in order o rule out potential unknown secondary effects, caused directly by MβCD *per se* or indirectly by lipid structure rearrangement consequent to MβCD treatment. Cholesterol was conclusively identified as the mediator of HSPA8 effects on sperm membrane after HSPA8 effects on sperm membrane integrity and fluidity were restored by reloading cholesterol to cholesterol-depleted spermatozoa using cholesterol-loaded MβCD,.

For reasons previously discussed, the association of HSPA8 with the sperm membrane during short co-incubation periods seemed a plausible extrapolation from the positive effects of

HSPA8 on the sperm membrane. However, supportive evidence had yet to be obtained. In an attempt to address this, confocal laser scanning microscopy revealed that exogenous HSPA8 molecules distinctively associated with the outer surface of the sperm head and most predominantly with the acrosomal region after 15 min co-incubation (Chapter 6). However, images from different samples consistently showed that this association only occurred in a small number of spermatozoa. Having conclusively established that HSPA8 directly interacted with sperm membrane, our attention was directed to identifying the characteristics of the minor subpopulation of spermatozoa which attached to HSPA8. We chose to use flow cytometry to distinguish the viability (membrane integrity) status of spermatozoa that attached to HSPA8 molecules. Using Propidium iodide as the marker of cell integrity along with fluorescent labelled HSPA8 enabled the concurrent visualisation of sperm membrane integrity status along with their association with HSPA8. It became apparent that among the spermatozoa which associated with HSPA8, a very small percentage had intact membranes and the remaining were all Propidium iodide positive or membrane disrupted cells. This supported the speculation that exogenous HSPA8 selected a subpopulation of spermatozoa with minor reversible membrane injuries and repaired the damage in an immediate process. Moreover, sperm capacitation revealed to attenuate the intensity of HSPA8 molecules attached to sperm cells which affirmed that capacitation-induced membrane reorganisation and namely reduction in membrane cholesterol decreased the potential binding / acting sites for HSPA8 on sperm membrane.

Experimental data presented in Chapter 7 was dedicated to discovering the intracellular mechanism which mediated HSPA8 effects on sperm membrane and examined whether the observed increase in membrane fluidity might be the result of direct HSPA8 stimulation of membrane lipids structure or the activation of an intracellular signalling pathway by HSPA8. A significant rise in sperm intracellular cyclic AMP (cAMP) content after 15 min exposure to HSPA8 indicated the involvement of a cAMP-mediated signalling pathway. Analysis of literature directed us to the cAMP / PKA-dependent pathway which is responsible for capacitation-related hyperfluidity of the sperm lipid membrane (Harrison, Ashworth et al. 1996; Harrison and Miller 2000). After validation and application of a number of functional pathway stimulators and inhibitors including cAMP analogue, KH7 and H89, we determined the role of the above mentioned cAMP / PKA pathway in HSPA8-induced enhancement in

membrane fluidity. The obtained results clarified that the HSPA8-induced increase in sperm membrane lipid fluidity is a distinct entity from the already documented cAMP / PKA pathway involved with membrane remodelling in sperm capacitation. However, in view of the previously discussed evidence regarding ability of HSPA8 to modulate a number of other sperm physiologic properties such as mitochondrial activity, oviductal epithelial cell binding and *in vitro* fertilisation performance (Chapter 2 and 3) in conjunction with its localisation on sperm outer membrane (Chapter 6), it is reasonable to postulate that HSPA8-induced cAMP rise in spermatozoa could be involved in pathways regulating any of the above mentioned characteristics.

8.3 Implications of the major thesis findings

8.3.1 Potential use of exogenous HSPA8 in *in vitro* semen storage, *in vitro* fertilisation and artificial insemination

In practice, semen storage is highly desirable for the purpose of preservation of genetic diversity, maintenance of germplasm of the remarkable genetic traits, providing a germplasm reserve for more efficient breeding and maximising the male reproductive potential by bypassing temporal and spatial constraints (Guthrie and Welch 2005; Alvarez-Rodriguez, Alvarez et al. 2013). Despite great efforts, two main conventional semen preservation procedures including liquid storage (commercial extenders) and cryopreservation have failed to prolong sperm survival without compromising their fertilising ability (Guthrie and Welch 2005; Holt 2011).

Semen extenders are formulated to provide energy sources for sperm metabolism and protect membrane balance over the storage period. However, the most efficient semen extenders have the potential to store sperm for only up to 7 days from the point of collection. This is because co-existence of a large number of spermatozoa in a set volume of extender reduces sperm survival due to increased production of toxic by-products (Robert 2006). Moreover, environmental temperature fluctuations and low ingredient quality severely compromise the efficiency of the extenders in maintaining the sperm survival rate (Robert 2006).

Although cryopreservation has proved the longest successful sperm storage for up to several years (Royere, Barthelemy et al. 1996), mammalian spermatozoa are extremely sensitive to cellular and molecular damages sustained through the process of cryopreservation. Sperm membrane is highly vulnerable to ultrastructural cryoinjury induced as the result of osmotic stress, cold shock and intracellular crystal formation during the cryopreservation procedure (Royere, Barthelemy et al. 1996; Guthrie and Welch 2005; Moore, Squires et al. 2005; Moce, Blanch et al. 2010).

Membrane instability and the resulting decline in sperm viability significantly decrease the success rates of *in vitro* fertilisation and artificial insemination (Guthrie and Welch 2005; Alvarez-Rodriguez, Alvarez et al. 2013). Therefore, improving protocols containing membrane stabilising agents for more efficient and long-term semen conservation appears to be necessary. Recent attention has been drawn to the benefits that semen preservation technology could attain by discovering the physiological capability of female reproductive tract in extending sperm viability *in vivo*. Exogenous HSPA8 whose ability in membrane stabilisation and viability enhancement has been proven in this thesis, may well prove as being a functional and efficient ingredient in the semen preservation biotechnology. Inspiring from *in vivo* biological events, *in vitro* fertilisation and embryonic development significantly benefit from oviductal co-culture with gametes and embryo (Buhi, Alvarez et al. 1997). This indicates that oviductal components such as HSPA8, as protective chaperones, may enhance the efficiency of chemically defined *in vitro* fertilisation and embryo culture media. The viability enhancing effect of HSPA8 is extended to spermatozoa from a number of mammalian species (Elliott, Lloyd et al. 2009; Lloyd, Elliott et al. 2009). This suggests that due to its highly conserved nature and bioactivity across mammalian species (Daugaard, Rohde et al. 2007), application of HSPA8 may as well be useful in human reproduction technologies.

8.3.2 A biological marker for semen selection in assisted reproductive technologies

The success rates of assisted reproductive technologies including *in vitro* fertilisation and artificial insemination significantly rely on the gamete quality. From male gamete point of view, prediction of sperm fertilising capacity and selection of semen samples with optimal

reproductive potential yield great socio-economic benefits in livestock breeding as well as in human reproduction. The ultimate goal in spermatology is development of efficient assays capable of detecting sperm characteristics that are evidence of reduced fertility (Gadea 2005).

The conventional semen analysis is based on a series of descriptive methods which measure the sperm concentration, morphology and the percentage of live and motile cells in a given ejaculate (Dyck, Foxcroft et al. 2011). While useful in detecting overt sperm abnormalities, the existing classical semen evaluation methods are generally inadequate for predicting sperm fertilising ability in semen samples which display normal physical parameters but are defective in subcellular levels (Popwell and Flowers 2004; Collins, Flowers et al. 2008; Dyck, Foxcroft et al. 2011). There is growing evidence that the suboptimal fertility of a given ejaculate despite the acceptable quality determined by conventional methods, is due to presence of incompetent subpopulations of spermatozoa with subcellular aberrations. Highly selective tests with the ability to determine the proportion of abnormal spermatozoa at subcellular levels greatly benefit the reproduction industry (Popwell and Flowers 2004; Waberski, Magnus et al. 2006).

Our observations discussed in Chapter 2 indirectly implied that HSPA8 identified and repaired a minority subpopulation of spermatozoa which appeared to suffer from minor reversible injuries. In Chapter 6, the speculation turned to be reasonable since only a limited number of spermatozoa in any given sample associated with exogenous HSPA8. This pointed to the selective nature of HSPA8-sperm interaction. On the other hand, expression of variability in the proportion of HSPA8-bound spermatozoa between individual semen samples showed that there was different binding tendency for HSPA8 in different semen samples. This finding can be a clue to an efficient method to predict the fertility outcome of a given sample based on the level of sperm-HSPA8 interaction along with the level of sample responsiveness to HSPA8 repairing function.

8.3.3 Potential therapeutic effects of HSPA8 as a membrane stabilising drug

Enclosing cells and the organelles within them, it is not surprising that the disruption of membrane function brings about detrimental pathological consequences. Cell membrane diseases are life-threatening disorders that disrupt the normal functions of the cells by simply

affecting the cell membrane and are caused by a range of different mechanisms (Goldberg and Riordan 1986). A number of fatal diseases have been described to be caused by environmental hazards to the cell membrane. For instance, Alzheimer's disease is the disruption of brain function induced by oxidative stress to the phospholipid structure of brain cells' plasma membrane (Markesbery 1997). Certain chemical neurotoxins or infectious microorganisms contribute to the development of degenerative diseases by causing membrane lipid / protein disruptions (Goldberg and Riordan 1986; Halliwell 1991; Valko, Leibfritz et al. 2007).

This thesis demonstrated that HSPA8 possesses the capacity to improve sperm membrane lipid status and repair damages by direct interactions with sperm plasma membrane. Similar phenomenon has been reported previously with different HSP isoforms in other biologic systems (Carratu, Franceschelli et al. 1996; Vigh, Maresca et al. 1998; Vigh, Horvath et al. 2007). This indicates that the system of HSP-mediated stabilisation in biologic membranes is most probably conserved across the HSP families and between species. If HSPA8 can re-organise membrane lipid structure in spermatozoa in favour of prolonging viability and functionality, one might speculate that as a therapeutic component, it can elicit a similar effect on membranes of other biologic systems and counteract adverse pathogenic processes in plasma membrane bilayer.

8.4 Directions for future study

This research has contributed to a better understanding of the events that are responsible for prolonging sperm longevity inside the female oviduct through uncovering evidence on the function of a particular oviductal component, HSPA8 on boar sperm. However, there remains numerous areas of research where additional experimentation could provide further knowledge in this field.

8.4.1 Sperm cell surface binding partners for exogenous HSPA8

In Chapter 6, we demonstrated for the first time that exogenous HSPA8 established direct attachment with sperm plasma membrane. This in conjunction with the phenomenal short-term effect of HSPA8 on sperm membrane integrity and fluidity shown in the early chapters

(Chapters 2 and 4), reinforced the stipulations on the presence of HSP binding partners / receptor molecules on sperm membrane. Despite having discovered the important role of membrane cholesterol in mediating HSPA8 effect on sperm membrane, the question still remains as to whether membrane cholesterol serves as a direct binding site for HSPA8 or it holds a supporting role by maintaining the membrane lipid raft structures so that HSPA8 molecules can attach to their yet unknown binding sites / receptors on the organised lipid plane. The concept of specific HSPA8 binding sites on sperm is further supported by the the evidence obtained in Chapter 2 which revealed the regulating effect of exogenous HSPA8 on a number of sperm characteristics apart from membrane including mitochondrial activity, OEC-binding ability and *in vitro* fertilisation performance.

Extracellular HSPs and their biologic function is an emerging field and hence there is scarce knowledge on HSP cell-surface receptors. Therefore, the best approach towards identification of sperm membrane HSP receptors is to resort to already studied surface molecules associated with heat shock proteins in other biologic systems. Selective binding of exogenous HSPA1A (HSP70) to globoyltriaosylceramide (GB3), a marker on tumour cell surface concluded that GB3 is an essential interaction partner for HSPAs on tumour cell membranes (Gehrmann, Liebisch et al. 2008). Activation of Toll like receptor 4 (TLR4) on culture human macrophages has been achieved by extracellular HSPA1A (Luong, Zhang et al. 2012). Recently, several groups have reported exhibition of TLR members on surface of rat as well as human sperm (Palladino, Savarese et al. 2008; Fujita, Mihara et al. 2011). In practice, the application of immunolocalisation techniques by targeting potential candidates enables localisation of HSP binding molecules on sperm. In addition, using target protein-specific antibodies in co-immunoprecipitation technology facilitates identification of HSPA8 binding partners, their binding affinities as well as the kinetics of binding.

8.4.2 HSPA8 exogenous modulator of sperm apoptosis

Apoptosis or the process of programmed cell death is the dominant mechanism which regulates production and function of male germ cells throughout their life from early differentiation to the moment of fertilisation. During embryogenesis, apoptosis removes supernumerary spermatogonia to adjust germ cell : sertoli ratio (Koppers, Mitchell et al. 2011; Aitken and

Baker 2013). In adulthood on the other hand, apoptosis is the predominant mechanism to eliminate damaged and fertilisation incompetent spermatozoa. In mature spermatozoa, apoptosis is a default mechanism which means that the fate of every sperm cell is to undergo apoptotic death except for those which successfully fertilise oocytes (Aitken and Baker 2013).

The default position of apoptotic death in spermatozoa poses the question as to what prevents apoptosis in highly differentiated spermatozoa to achieve prolonged sperm storage inside female reproductive tract. Secretion of anti-apoptotic agents from oviductal cell lining and their association with prolonged sperm survival have been reported in bats (Roy and Krishna 2011). Having discovered the enhancing effect of HSPA8, a conserved oviductal secretory protein, on sperm viability, demands investigating HSPA8 role in regulation of sperm apoptotic pathways. Application of immunohistochemical and proteomic analysis for detection of apoptotic and anti-apoptotic molecules in spermatozoa under the influence of HSPA8 provide deeper insight into the potential anti-apoptotic functions of HSPA8 in sperm and may allow for identification of methods to improve sperm preservation technology.

8.4.3 Exogenous HSPA8 and sperm intracellular signalling pathways

In Chapter 7 of this thesis, the involvement of capacitation-related cAMP pathway in HSPA8-mediaed effects on sperm membrane was conclusively ruled out. However, enhanced levels of sperm cAMP in response to HSPA8 treatment justifies further investigations on intracellular pathways responsible for mediating HSPA8-sperm interactions. Data provided in the current thesis demonstrated changes that occurred rapidly following sperm brief exposure to HSPA8 including enhancement in membrane integrity, fluidity and reduced mitochondrial activity. Hence, significant rise of cAMP in HSPA8-treated spermatozoa can be attributed to any of the mentioned observed effects. With advancements in proteomic and immunolocalisation technologies, several key proteins and their related signalling cascades have been identified to be associated with different sperm characteristics. Phosphoinositide 3-kinase (PI3K), mitogen activated protein kinase (MAP kinase) along with their substrates and activators have been assigned to motility and prolonged sperm survival in sperm of a variety of mammalian species, respectively (Koppers, Mitchell et al. 2011; Roy and Krishna 2011). Studying changes in the levels and phosphorylation of proteins characterised in regulation of sperm properties in

response to HSPA8 treatment may well increase the existing knowledge on sperm-HSP interactions.

8.5 Conclusion

The current thesis has characterised the immediate impact of exogenous HSPA8 on sperm physiology. Our results suggest a novel aspect of exogenous HSPA8 as a 'rapid response' extracellular cytoprotector and modifier of cell function which rapidly restores cell membrane integrity by influencing membrane microviscosity via a cholesterol-dependent mechanism. To the best of our knowledge, this study was the first to reveal a mechanism behind prolonged sperm survival in female tract and will have direct positive impacts on the existing body of knowledge of the sperm interaction with female oviductal sperm reservoir.

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