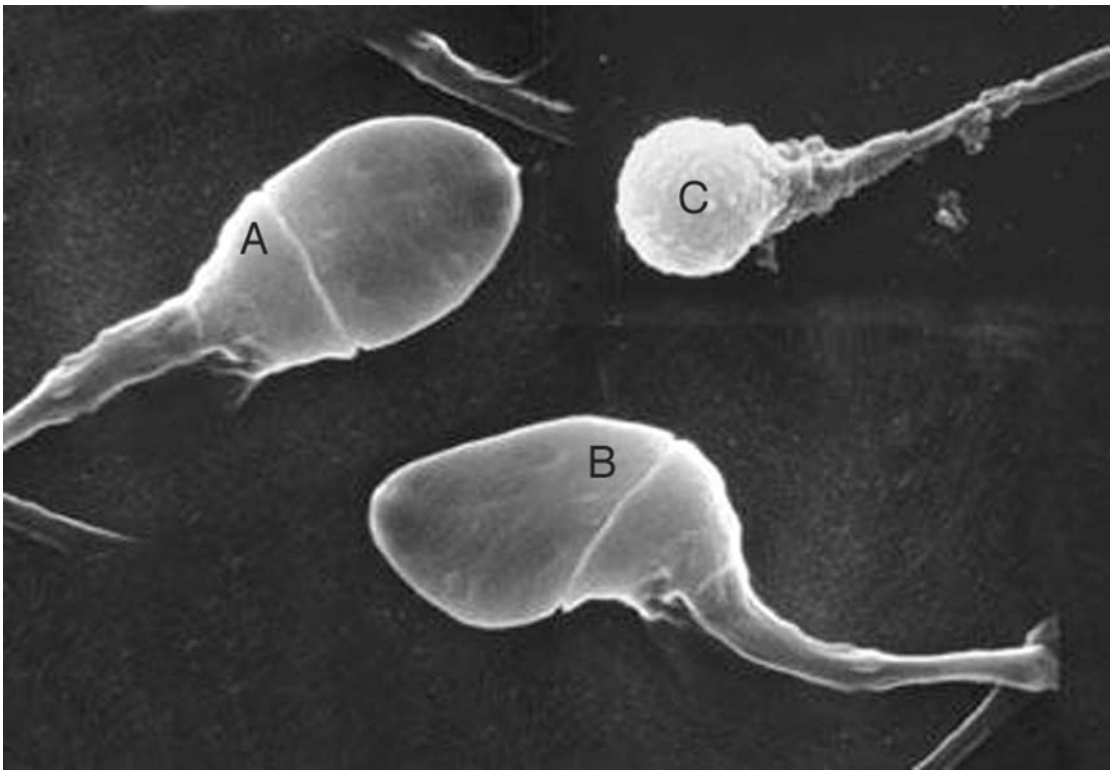
Acrosome deficiency and male infertility; causes and treatment.



The picture on the cover (Hirsh, 2003) shows differences in morphology in spermatozoa. Male infertility can be caused by several quantitative and functional defects. Patients suffering from globozoospermia have a rare form of male infertility based on acrosomeless spermatozoa. Globozoospermia are characterized by round-headed cells, due to their lack of acrosome (c). Additional coiled-coil tails can be observed in these sperm cells. All these morphological changes contribute to their inability to fertilize. Normal sperm cells (a) are elongated and show a cap-structure around the nucleus, the acrosome. Besides globozoospermia other forms of abnormal head development (b) are possible.

Marleen Theunissen
Biology of disease
3075818
Master thesis
Department Cell Biology
Supervisor: Bart Gadella
Daily supervisor: Jason Tsai

List of abbreviation

AOA	=	Assisted Oocyte Activator
ARPR	=	Acrosome Reaction Promoting Region
AR	=	Acrosome Reaction
ART	=	Assisted Reproductive Technology
cAMP	=	cyclic AMP
CK2	=	Casein Kinase 2
DAG	=	Dyacylglycerol
DIgA	=	Drosophila disc large tumor suppressor
DIGE	=	Difference gel electrophoresis
FISH	=	Fluorescence in situ hybridization
GOPC	=	Golgi associated PDZ and coiled coil motif
Hrb	=	HIV-1 Rev-binding/Rev-interacting protein
IAM	=	Inner Acrosomal Membrane
ICSI	=	Intracytoplasmic sperm injection
IMT	=	Intramanchette transport
INM	=	Inner Nuclear Membrane
IP3	=	Inositol triphosphate
IUI	=	Intrauterine injection
IVF	=	In Vitro Fertilization
KASH	=	Klarsicht/Anc-1/syne-1 homology
KO	=	Knock-out
LINC	=	Linkage of the nucleoskeleton and the cytoskeleton
MOAT	=	Mouse oocyte activation test
MyRIP	=	Myosin-Va-Rab Interacting protein
NSF	=	N-ethylmaleimide sensitive factor
OAM	=	Outer Acrosomal Membrane
ODF-2	=	Outer Dense Fiber 2
ONM	=	Outer Nuclear Membrane
PAG	=	Pro-Acrosomal Granules
PAS	=	Post acrosomal sheath
PCC	=	Premature chromosome condensation
PDZ-domain	=	PSD95, DiGA , Zo-1 proteins
PICk1	=	Protein interacting with C kinase 1
PKC	=	Protein Kinase C
PLC	=	Phospholipase C
PM	=	Plasma Membrane
PSD95	=	Post synaptic density protein
PT	=	Perinuclear theca
SAMP-1	=	Spermatozoa acrosome membrane protein 1
SNARE	=	SNAP-receptors
SNAP	=	soluble NSF attachment proteins
SOC	=	Store-operated channels
SPANX	=	Sperm protein associated with the nucleus on the X-chromosome
TEM	=	Transmission Electron Microscopy
TGN	=	*Trans*-Golgi Network
TPR	=	Tetratricopeptide repeat
TUNEL	=	TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end-labeling (TUNEL)
VAMP	=	Vesicle-associated membrane protein
WHO	=	World Health Organization
ZBR	=	Zona Binding Region
Zo-1	=	zonula occludens-1 protein
ZP	=	Zona Pellucida

Introduction

Infertility is a disease affecting women and men, constrain the possibility of conception and pregnancy. 50% of the couples suffering from infertility are caused by male infertility. Several forms of male infertility are analyzed. A rare form (incidence 0.1 %) of male infertility, globozoospermia, will be discussed in this thesis. Globozoospermia, a form of teratozoospermia, is characterized by round-headed cells which show defects in their morphology and function. The aim of this thesis is to reveal the underlying mechanism causing this form of infertility.

First the process of spermatozoa development, spermatogenesis, is discussed. Then the essential component of the spermatozoon, the acrosome is explained. This is important because globozoospermia patients produce spermatozoa without an acrosome. Interesting is how these few patients are diagnosed and distinguished from other infertility forms. Subsequently the pathology of this disease and the possibilities of treatment are investigated by studied literature.

Ch. 1 Spermatogenesis

The process of the formation of spermatogenic stem cells into mature spermatozoa cells is called spermatogenesis. The main event during this process is the reduction of the genome of the spermatogenic stem cell; developing haploid cells from diploid cells. Next to that, other structural changes will take place, forming spermatogonia into mature spermatids which have the right shape and equipments to fertilize an oocyte. See figure 1. Spermatogenesis is a process that takes place in the testis, which can be divided over three phases, proliferation phase, meiosis and differentiation phase (spermiogenesis). In the beginning, germ cells are located in the epithelium which surrounds the seminiferous tubuli in the testis. Different spermatogonia are known (Oehninger and Kruger, 2007;Hermo *et al.*, 2010) and divided in subclasses (spermatogonia $A_{0,\ 1\text{-}4}$ and B). Spermatogonia $A_{(1\text{-}4)}$ are initiated to differentiate into intermediate (In) and B spermatogonia, which divide and additional undergo the second phase, meiosis.

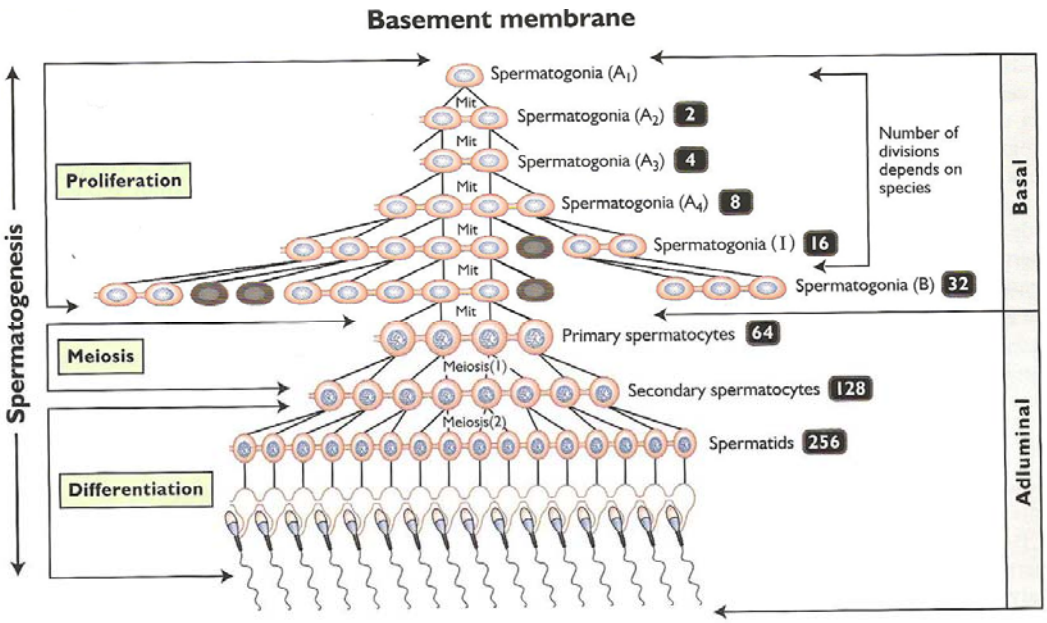


Figure 1. A schematic overview of the process of spermatogenesis. Three phases can be distinguished; the proliferation phase, meiosis (meiosis I and meisosis II) and the differentiation phase, also known as spermiogenesis. During the proliferation phase spermatogonia differentiate from the A subclasse into intermediate and B spermatogonia which are able to undergo meiosis. During meiosis the genetic content of the gametes is reorganized (meiosis I) and divided (meiosis II). During the last phase, spermiogenesis, the spermatocyte undergoes structural changes . (Senger P.L., 2003)

Meiosis organizes cell division and results in the formation of gametes required for sexual reproduction. There are two cell divisions, namely meiosis I and meiosis II. Firstly the prophase of meiosis is entered by preleptotene primary spermatocytes, spermatocytes that just underwent their last DNA replication. These preleptotene spermatocytes move to the adluminal compartment of the seminiferous tubuli, where the following steps of meiosis occur, respectively DNA condensation and alignment of the homolog chromosomes. During this conformation homologous recombination is possible, which results in genetic alterations of the genome. Subsequently segregation of the two sister chromatids arrange and two haploid spermatocytes are formed. During meiosis II division of the two haploid spermatocytes will complete the second phase of spermatogenesis by arranging four haploid cells (Senger P.L., 2003;Oehninger and Kruger, 2007).

During the third phase of spermatogenesis, spermiogenesis, the haploid round cell undergoes radical reorganization. This final process occurs in the passage from the testis via the epididymal duct to the distal end of the epididymis (Yoshinaga and Toshimori, 2003). The structural alterations include formation of the acrosome (an organelle containing enzymes which are required for fertilization), elongation of the nucleus, formation of a long tail including an axoneme (relevant in the motility of the tail) and the removal of majority of the cytoplasm, see figure 2.

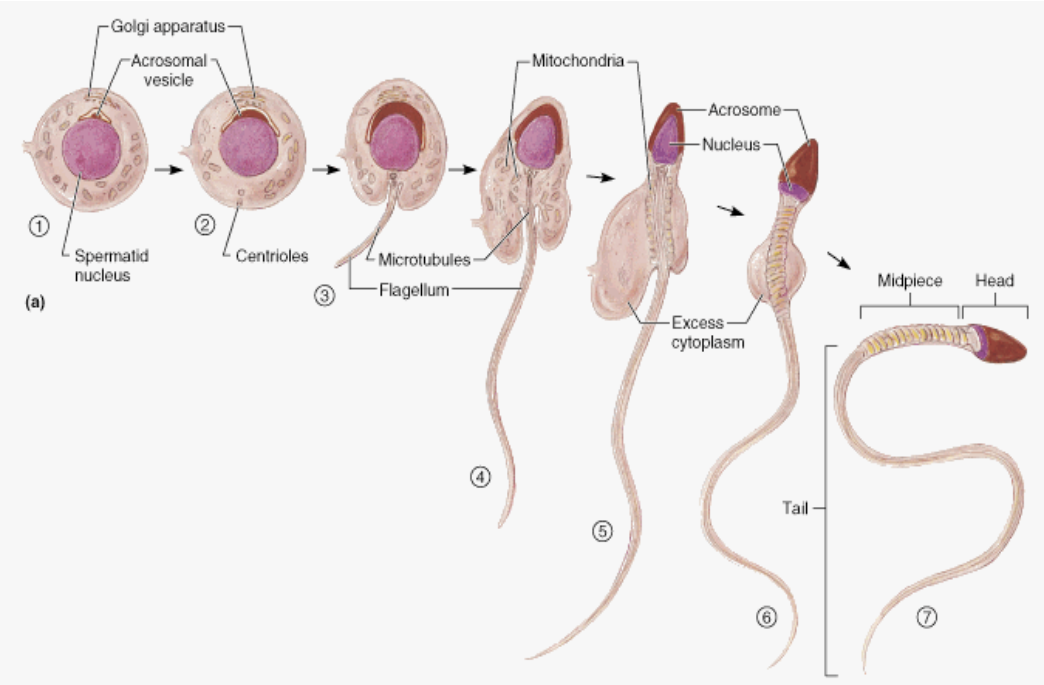


Fig. 2. Spermiogenesis is the third phase of spermatogenesis. In this phase the spermatocyte undergoes structural changes. First the acrosome is formed, then the tail develops and additional a majority of the superfluous cytoplasm is removed and taken up by sertolli cells. Finally a mature sperm cell is developed. (Benjamin Cummings, 2001)

At the end a mature spermatozoon is developed and stored in the epididymis, waiting on the moment it will release the male body to fulfill its reproductive function by fertilizing an oocyte.
Considering this long process of germ cell maturation, the probability of developing any defects during this process is present and can cause infertility. Globozoospermia is a form of infertility caused by a defect in spermatogenesis. This defect is the absence or malfunction of the acrosome, an essential organelle for fertilization, discussed in chapter 2.

Ch. **2 Acrosome**

New life can be obtained by the fusion of a spermatozoon and an oocyte. An important component to make this fusion possible is a structure present in the sperm head, the acrosome. This structure is localized around the nucleus and forms together with the nucleus the long-shaped structure of a spermatozoon. The acrosome is an organelle shaped through fused vesicles, containing hydrolytic enzymes, derived from the Golgi network. The amount of vesicles transported from the Golgi network determines the content and volume of the acrosome, which differs between several species. Although the differences in morphology, the function of the acrosome is thought to be similar between those several species (Yoshinaga and Toshimori, 2003). The description of acrosome biogenesis discussed below is particularly based on information gathered from mice studies.
The acrosome gets functional after ejaculation in the female reproductive tract; there it will release its content via exocytosis during the acrosome reaction (AR). This AR is required for the penetration of the sperm cell through the zona pellucida (ZP), a thick extracellular layer surrounding the oocyte. After successful penetration the sperm cell reaches the perivitellin space where it has the capacity to fertilize the oocyte.
In this chapter first the acrosome biogenesis will be discussed, then the different processes involved in AR.

2.1 Acrosome biogenesis
Much research is done to discover and understand the process of acrosome formation. The most information is obtained by mice and rat studies. Mature spermatids contain an acrosome that covers 40 – 70 % of the nucleus. This organelle can be divided in two domains, the anterior segment and the equatorial (posterior) segment (Toshimori, 1998;Yoshinaga and Toshimori, 2003;Oehninger and Kruger, 2007). The anterior segment contains the content of enzymes that are released during the AR.
Already in 1990 (Anakwe and Gerton, 1990) data showed that biogenesis of the acrosome starts early in meiosis and develops during spermiogenesis. The formation of the acrosome can be divided in different phases: Golgi, cap, acrosome and maturation phase (Toshimori, 1998;Ramalho-Santos *et al.*, 2002a;Moreno and Alvarado, 2006)
Acrosomal biogenesis starts with the Golgi phase, in which the acrosomal vesicle is formed. Firstly, pro-acrosomal granules (PAGs) are formed in the endoplasmic reticulum. These vesicles contain several components, including acrosomal proteins like proacrosin and acrogranin. Secondly, these PAGs are transported to the Golgi network via an exocytic pathway. The transport of the PAGs to the perinuclear organelle will be established from the *trans*-Golgi Network (TGN). Ho et al. showed in 1999 that during spermiogenesis the cisternae of the Golgi network undergo dynamically changes. The *trans*-Golgi network is

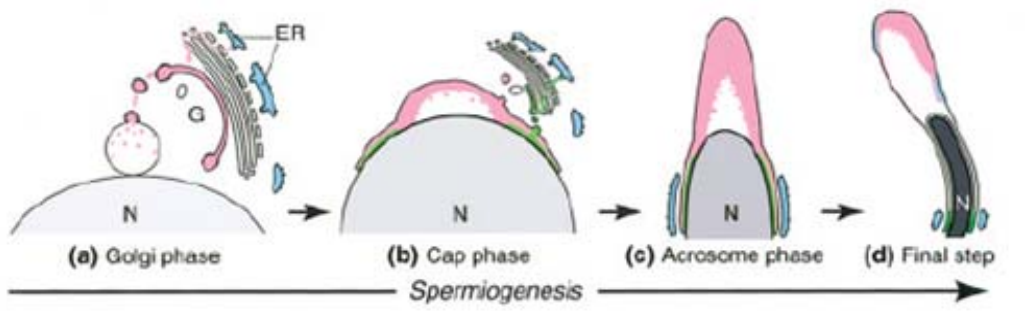


Fig. 3. Acrosomal biogenesis divided in four phases, the Golgi, Cap, Acrosomal and maturation phase. The latter is also called the final step. During the Golgi phase PAGs are formed in the ER and transported via the trans-Golgi-network to the developing organelle, located on the overlying anterior part of the sperm nucleus. During the Cap phase the acrosome increases in size via the delivery of more PAGs from the Golgi network. The acrosome undergoes structural changes during the acrosome phase and gets its final shape in the maturation phase. (Yoshinaga and Toshimori, 2003)

faced to the acrosome and involved in the transport of vesicles (Ho *et al.*, 1999). The transported PAGs are transported and bind an actin-keratin containing cytoskeletal plate, aligned on the acrosome, termed the acroplaxome acroplaxome (Kierszenbaum and Tres, 2004), discussed in chapter 2.2. in detail. The PAGs will fuse with each other and form one acrosomal vesicle.
During the cap-phase, the acrosome increases in size due to the constant delivery and fusion of Golgi-derived vesicles and starts spreading over the anterior nucleus, forming a 'cap'. Subsequently the structure of the spermatozoon rearranges in the third phase, the acrosome phase. The nucleus elongates and the flagellum translocates to the caudal side. At the end of spermiogenesis, in the maturation phase, the equatorial segment of the acrosome is completely formed. The manchette, a microtubule structure is tightly involved in this last step(Peterson *et al.*, 1992;Moreno and Alvarado, 2006), discussed in chapter 2.2
The acrosome is enclosed by a membrane, divided in two parts, the Inner Acrosomal Membrane (IAM), and the Outer Acrosomal Membrane (OAM). The IAM is the membrane located at the nuclear surface, which anchors the acrosome solid to the nuclear envelope via cytoskeletal components, called the perinuclear theca (PT) (Yoshinaga and Toshimori, 2003). This anchorage is necessary in further adaptations of the sperm morphology during spermiogenesis. The OAM includes the part of the membrane that locates to the plasma membrane (PM) of the spermatozoon. During the acrosome reaction, the OAM fuses with the plasma membrane, but the IAM stays intact till the spermatid fuses with the oocyte (Ramalho-Santos *et al.*, 2002a;Yoshinaga and Toshimori, 2003).

2.2 Acrosome–Acroplaxome-manchette complex

The transformation of the sperm head is an important process during spermiogenesis. Successful sperm head shaping is tightly controlled via the Acrosome–Acroplaxome-manchette complex (Yoshinaga and Toshimori, 2003;Kierszenbaum and Tres, 2004). This complex contains two structures, the acroplaxome and the manchette, both connected to the spermatid nucleus via respectively the marginal ring and perinuclear ring. The binding of these structures to the nucleus is also indicated as the endogenous clutch, see figure 4.
The acroplaxome is a cytoskeletal plate, containing actin (F-actin) and keratin (Sak57, a keratin ortholog), positioned as a ring-structure around the developing acrosome. This complex is involved in the development of the acrosomal sac, the anchorage of the acrosome to the nuclear envelop and the nuclear head shaping. PAGs derived from the Golgi network can tether to this cytoskeletal ring network of actin and fuse subsequently with the acrosomal vesicle (Kierszenbaum and Tres, 2004). This membrane trafficking network transport the PAGs via motor proteins like myosin Va, dynein and kinesin. Receptors on the PAGS, Rab27a bind myosin Va and are transported to the acroplaxome via a motor recruiter MyRIP (myosin-Va-Rab interacting protein) (Kierszenbaum *et al.*, 2003b).

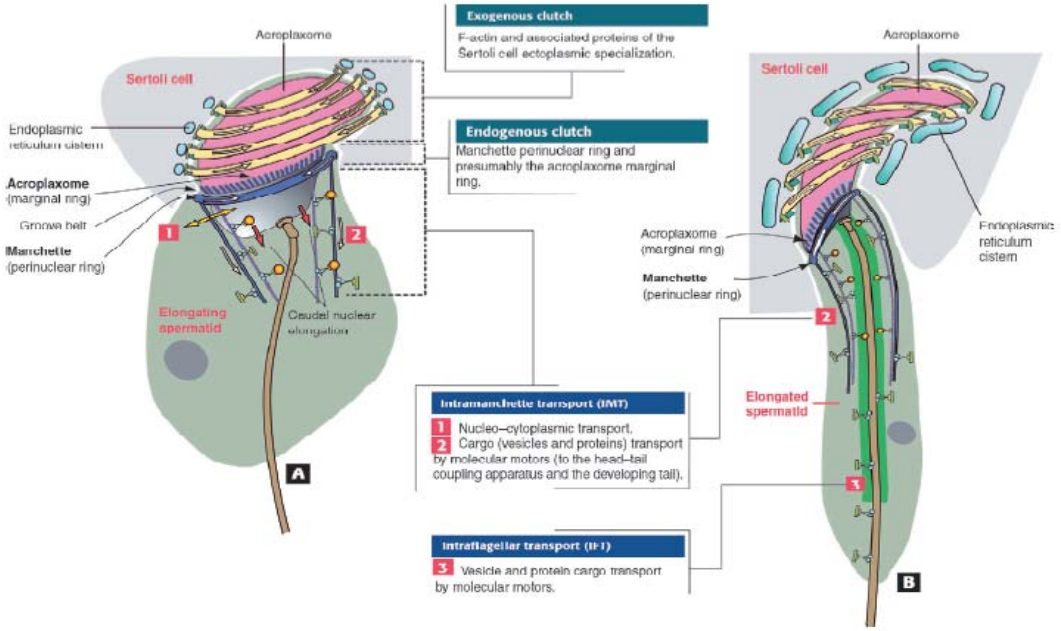


Fig.. 4. The acroplaxome-manchette complex.
The developing spermatid contains two cytoskeletal components, the acroplaxome and the manchette, which are involved in the acrosome formation and the elongation of the spermatid. The acroplaxome is a cytoskeletal plate, containing actin and keratin positioned as a ring-structure around the developing acrosome. The acroplaxome is connected to the nucleus with the marginal ring. This structure is acquired for the formation of the acrosome. PAGs from the golgi-network tether to this structure and fuse with the acrosomal sac. The acroplaxome is also involved in the binding of the acrosome to the nucleus and the nucleus-head shaping. The other cytoskeletal structure is the manchette. This structure starts from the perinuclear ring, the anchorage of the manchette to the nucleus, and runs caudal to the developing spermatid. The manchette regulates the elongation and condensation of the nucleus during spermiogenesis via the intramanchette transport (IMT), which contains nucleo-cytoplasmic transport and cargo transport by molecules. (Kierszenbaum and Tres, 2004)

The second structure, the manchette, is also involved in spermiogenesis. The manchette develops during the acrosomal phase and its function is to regulate the elongation and condensation of the nucleus during spermiogenesis. This is executed via the intramanchette transport (IMT) molecules involved in the elongation process (Kierszenbaum and Tres, 2004). Non-acrosomal vesicles derived from the Golgi-network, proteins developed in the cytoplasm and proteins derived from adjacent Sertolli cells are transported via a network of to the centrosome and tail region. The manchette adheres at the perinuclear ring and moves to the caudal part of the forming spermatozoon, see figure 4. Later will be discussed how this Acrosome-Acroplaxome-manchette complex is involved in the process of sperm head shaping and what the link is between this complex and globozoospermia. See chapter 4.

2.3 Acrosome Reaction

2.3.1 Initiation

After completing spermiogenesis in the epididymis, capacitation is the following action including structural changes in spermatozoa. Capacitation occurs after ejaculation, in the female reproductive tract. This event results in reorganization of membrane lipids and proteins of the spermatozoon causing the ability to bind and penetrate the zona pellucida (ZP), a glycoproteinaceous matrix surrounding the oocyte (Senger P.L., 2003) Later will be discussed that capacitation plays a second role in AR.

The ZP in humans contains 4 types of glycoproteins, ZP1, ZP2, ZP3 and ZP4. All these proteins interact with spermatozoa at different time points. Data (Mayorga *et al.*, 2007) showed that ZP3 is the physiological initiator of AR in mice. Recently is shown, by Gupta *et al.* (Gupta and Bhandari, 2011), that in humans the glycoproteins ZP1 and ZP4 are additional involved in the AR. Two regions of the sperm plasma membranes are concerned in the binding and

penetration of the ZP. First, the primary zona binding region (ZBR) is responsible for the binding of the sperm to the protein ZP3. Subsequently ZP3 interacts with the second region of the sperm plasma membrane, the acrosome reaction promoting region (ARPR) (Senger P.L., 2003).
This binding results in the signal transduction that will lead to the AR. See figure 5.
During the AR, fusion of the OAM with the overlying PM results in the release of acrosomal

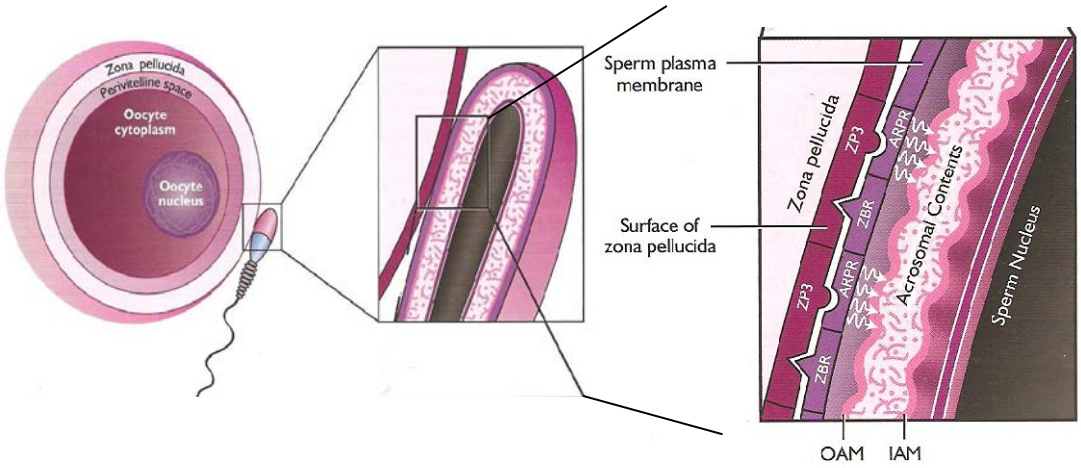


Fig. 5. The interaction of the sperm cell with the zona pellucida (ZP) which initiates the AR. First the Zone Binding Region (ZBR) in the plasma membrane of the sperm cell interact with the ZP3 of the ZP. Then the ZP3, is able to bind another part of the PM of the sperm cell, the acrosome reaction promoting region (ARPR). This binding triggers the signal transduction pathway in the spermatid, resulting in the increase of calcium, inducing the fusions of the PM with the OAM. (Senger P.L., 2003)

content. The released hydrolytic enzymes prepare a way for the spermatozoon through the ZP to position next to the perinuclear envelope of the oocyte. See figure 6.

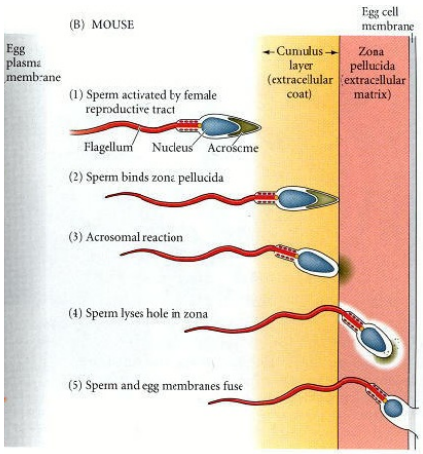


Fig. 6. The binding and penetration process of a mouse spermatid to the ZP, undergoing the acrosome reaction, lysis of hydrolytic enzymes and the penetration of the oocyte membrane. (Gilbert SF, 2000)
Developmental Biology. 6th edition.Gilbert SF. Sunderland (MA) 2000

2.3.2. Signal transduction pathway acrosomal exocytosis

The final fusion of the OAM with the PM is established via an underlying signal transduction pathway initiated by binding of ZP3 to two different receptors (Breitbart, 2002). This induces two pathways which together results in membrane fusion and acrosomal exocytosis via an increased intracellular calcium level. One target for ZP3 is the G_i-coupled receptor. This receptor activates phospholipase C (PLC), increasing the levels of cyclic AMP (cAMP). cAMP, on its turn induces Protein kinase A activity. High levels of protein kinase A opens channels in the OAM, which result in a little increase in cytosolic calcium. Together with Tyrosine kinase activity (also induced via ZP3) the increase in calcium will result in a second calcium influx,

via different steps. Due to the activation of phospholipase C inositol triphosphate (IP3) and diacylglycerol (DAG) are produced, which both open different kind of calcium channels. DAG activates protein kinase C (PKC), opening calcium channels in the PM and IP3 binds to the IP3 receptor, opening store-operated channels (SOC) (Breitbart, 2002). Together opening of SOC and PM channels will finally result in a rapidly high influx of cytosolic calcium, the initiator of the final event before the acrosomal content is released, the multiple fusions of the OAM with the PM.

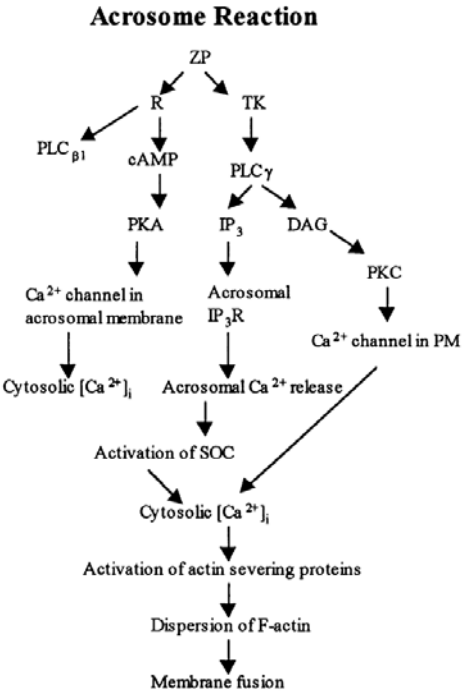


Fig.. 7. The acrosome reaction signal pathway. Binding of the PM of the sperm cell to the ZP initiates the AR. Via two ways (opening SOC-channels and calcium channels in the PM) a rapidly high influx of Ca2+ is established. This increase initiates the fusions of the OAM with the PM. (Breitbart, 2002)

2.3.3. Acrosome membrane fusion

The fusions of the OAM and the PM of the spermatozoon are regulated via different proteins and receptors. Tsai *et al*. demonstrated, in boars, that multiple fusions occur to get mixed vesicles of OAM and PM, causing acrosomal exocytosis. The first fusion already occurs before the AR, during the capacitation of the spermatozoon. Proteins known to be required for the membrane fusions that complete the acrosomal exocytosis are Rab3A, N-ethylmaleimide sensitive factor (NSF) and N-ethylmaleimide sensitive factor attachment proteins (SNAPs) (Michaut *et al.*, 2000;Ramalho-Santos *et al.*, 2002a;Mayorga *et al.*, 2007). Rab3A is a protein from the Rab-family, containing small GTPases. Rab3A is known to activate SNAP-receptors (SNAREs), present on the acrosome vesicle (v-SNARE) and on the PM, the target membrane (t-SNAREs).

Tsai *et al*. (Tsai *et al.*, 2010) demonstrated a model of the multiple fusions leading to acrosomal exocytosis. They demonstrated that during capacitation the proteins syntaxin 1B and VAMP3 on the PM form a complex with the protein SNAP23 on the OAM. Fusion of the two membranes is not established because of the presence of complexin, which stabilizes this complex. During acrosome reaction an influx of calcium, noticed by the calcium receptors synaptotagmin and calmodulin, results in the transformation of the trans-SNARE complex into a cis-SNARE complex, which causes fusion of the PM with the OAM. In figure 8, the model of the multiple point fusion is shown.

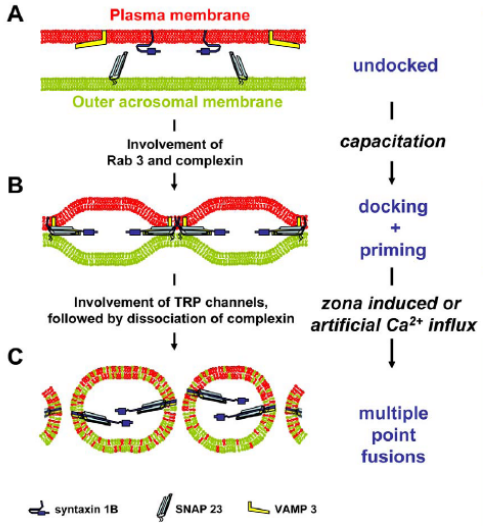


Fig 8. Model for multiple point fusion involved in fusion of the PM and OAM, causing acrosomal exocytosis. The docking of the two membranes occurs already during capacitation. However membrane fusion is then not accomplished by the presence of the stabilizer complexin. After binding of the sperm cell to the ZP proteins in the ZP, the acrosome reaction is initiated and an influx of calcium results in the transformation of the trans-SNARE-Complex into a cis-SNARE-complex. This causes fusion of the membranes. (Tsai *et al.*, 2010)

Ch. 3 Globozoospermia

3.1 Total and partial globozoospermia

As noticed in the previous chapter, the formation of a functional acrosome is essential for oocyte fertilization. Spermatozoa lacking this acrosome are unable to reach the oocyte because they cannot make their way through the ZP. The failure to produce acrosome containing sperm is called globozoospermia and has been described for the first time in 1971 by Schirren *et al.* The lack of an acrosome coincides with abnormal sperm morphology. Most pronounced is the characteristic morphology of round-headed cells, the result of no acrosome. The lack of a postacrosomal sheath (PAS), and a non-elongated, not completely condensed nucleus may attribute to the morphology of the round-headed cells, see figure 9. Beyond this, the presence of a coiled-coil tail is noticed is semen of patients with globozoospermia (Dam *et al.*, 2007a). The amount of globozoospermia in the semen of men is correlated to (sub)-infertility. 100% of globozoospermia will result in complete infertility and is termed total globozoospermia. In addition, also cases are known of semen containing a combination of normal spermatozoa and globozoospermia. When semen consists of more than 50% of round-headed cells they are categorized as partial globozoospermia, causing a strong reduction in fertility. Besides the round-headed cells, patients suffering from partial globozoospermia contain also oval spermatozoa. Although these oval spermatozoa contain an acrosome its functionality is most of the time lost as a secondary result of malformed sperm heads. The oval sperm do not have any fertilizing capacity. Even in healthy, fertile men a little amount (6%) of round-headed cells was recognized. These round-headed cells showed no acrosin, an acrosomal marker, and also no outer acrosomal membrane what indicates the lack of the acrosome. However this amount of acrosomeless spermatozoa in the semen had no negative effect on the fertility of the men (Dam *et al.*, 2007a)

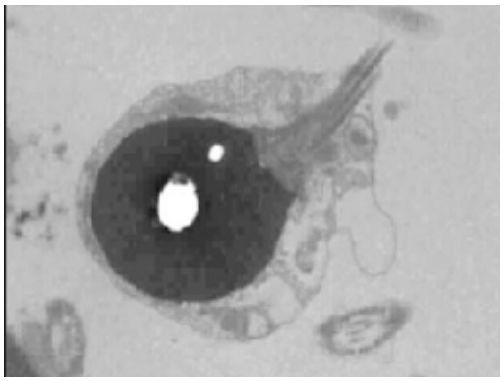


Fig. 9. An example of a round-headed cell, lacking the acrosome and with a round headed nucleus, the main characteristics of globozoospermia. (Dam *et al.*, 2007a)

3.2 Analysis globozoospermia

Failure of pregnancy occurs in couples where one of the partners suffers with any kind of aberration leading to infertility. In case of male infertility several abnormalities in the semen characteristics can result in infertility. In 1999 the World Health Organization (WHO) set up a protocol to provide in vitro fertilization clinics with a diagnostic tool to identify possible aberrations in semen properties that may be linked with male based infertility. (World Health Organization, 1999) The quantity and quality (motility and morphology) of spermatozoa in semen can be analyzed (For all the characteristic values measured in a semen analysis see appendix 1). Because human spermatozoa show heterogeneity two semen samples are needed to get reliable results out of the analysis. The samples are required to be analyzed within an hour after receiving the specimen. The second sample should be collected more than one week after the first and both samples should be acquired after a period of abstinence of at least 48 hours and at last seven days (World Health Organization, 1999;Samplaski *et al.*, 2010). A shorter period of abstinence results in a decreased amount of mature sperm cells in the ejaculate due to the short maturation time. Total abstinence is also discouraged because this will lead to changes in the quantity and quality of the spermatozoa. Spermatozoa cannot be stored for a long period in the cauda

epididymis, otherwise they lose their functionality and go down. These adaptations in the quantity and quality, due to no or too long abstinence, causes subfertility, something you want to avoid in semen analysis, therefore these criteria are appointed (Amann, 2009).
In case of morphology, three parts of a sperm cell are scored, namely; the head, midpiece and tail. All these parts are scored separately. Total and partial globozoospermia have mainly alterations in the head part, showing oval and round-headed cells. Alterations in the tail will be seen in a so called coiled-coil tail (Suzuki-Toyota *et al.*, 2004;Dam *et al.*, 2007a), which is curled during tail development.

3.2.1 *Techniques to analyze acrosome malformation*
During semen analysis the presence of globozoospermia can be distinguished using the Papanicolaou (World Health Organization, 1999), and the diff-quick staining (Dam *et al.*, 2010). These are two techniques to analyze the morphology of spermatozoa. These techniques are standard procedure in a semen analysis. Additional other analysis techniques are done to investigate specific globozoospermia. A large amount of acrosome markers are known which can be used to indicate the lack of acrosome in these spermatozoa. Fluorescent labeled lectins are often used which bound to the OAM or acrosomal contents and indicate presence of an acrosome (Aitken, 2006;Dam *et al.*, 2007a). An investigated acrosome protein which can be used for analysis with immunocytochemistry is VAMP, a vesicle-associated membrane protein (also known as synaptobrevin) (Ramalho-Santos *et al.*, 2002b). Proacrosin and acrosin are components present in the acrosome which can also be visualized via immunostaining (Dam *et al.*, 2007a).

3.2.2 Techniques to analyze other globozoospermia defects
There are also techniques which investigate other alterations in globozoospermia. Transmission electron microscopy (TEM) is used to distinguish abnormal chromatin condensation. Fluorescence in situ hybridization (FISH) is a technique used to analyze aneuploidy in these round-headed cells. And more details about the presence of DNA fragmentation can be obtained by a TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end-labeling (TUNEL) assay (Taylor *et al.*, 2010;Samplaski *et al.*, 2010;Dam *et al.*, 2010).

Additional to all these described techniques, functional tests are executed to distinguish the amount of functional sperm cells present in the semen which have the ability to capacitate, underwent AR and even fuse with an oocyte. The two latter events are interesting to analyze in patients that are supposed to suffer from globozoospermia. Most used viability tests are the hemizona assay and the sperm penetration assay. The hemizona assay analyzes the capacity of the spermatozoa to bind the ZP. This is compared to the binding capacity of semen from healthy people. For this purpose one fresh human oocyte is divided in two, using both halves to compare the binding capacity of the patient's semen to the positive control (healthy) semen. The ability of spermatozoa to fuse with an oocyte is measured with a sperm penetration test, using hamster oocytes without ZP (Aitken, 2006;Samplaski *et al.*, 2010). The percentage of spermatozoa that can fuse with these oocytes indicates the amount of qualitative good sperm cells. These functionality test will especially be used in patients suffering from partial globozoospermia, total globozoospermia are completely infertile and will not show any positive results.

Based on these semen analyses, alterations in quantity, morphology, viability and fertilizing competence can be recognized and thus the potential cause of male infertility. In the case of globozoospermia additional techniques are used to demonstrate the lack of acrosome in

these round-headed cells and their lack of ability to bind the ZP or either fuse with an oocyte.
The progresses in these analysis technologies make it possible to assess the cause of earlier unexplained infertility cases. These earlier diagnosed idiopathic infertility patients can now be analyzed. This is very important to determine which treatment will have the most benefit.

Ch. **4 Pathology globozoospermia**

Several studies have tried to investigate the causes of acrosomal defects in people suffering from globozoospermia. Probably different defects will eventually lead to the development of round-headed cells. This chapter will explain all the defects that are known to be associated with globozoospermia. First, a study will be mentioned in which defects in proteins were reported to deregulate the anchorage of the acrosome to the nucleus, a process regulated in the begin phase of spermiogenesis.
Secondly, up- and down regulated proteins in globozoospermia patients will be mentioned.
Third, genetic defects associated with globozoospermia will be discussed, based on cases of families with a number of infertile men with all round-headed cells (Kilani *et al.*, 2004;Dirican *et al.*, 2008). Several genetic components are believed to play an important role in the development of globozoospermia and are studied extensively using adapted mouse models and gene screening in globozoospermia patients.
Also environmental factors are a factor of debate involving the development of round-headed cells.

4.1 Acrosome anchorage defects

Recently investigators have found an event, very early in the procedure of acrosome formation, which could be deregulated in globozoospermia.

4.1.1 SPAG4L/L-2 defects

Recently a study (Frohnert *et al.*, 2010) is done on transmembrane proteins, located in the inner nuclear membrane in developing spermatids, which could play a role in sperm biogenesis in which they regulate the acrosome anchorage to the nucleus. Deregulation of these proteins is associated with defects in the linkage of the acrosome to the nuclear membrane, resulting in fragmentation of the acrosome, causing globozoospermia. With the discovery of the proteins SPAG4L and its isoform SPAG4L-2, novel testis-specific proteins involved in spermatid biogenesis are identified. SPAG4L/4L-2 belong to the SUN (Sad1p, UNC-84) domain proteins, which are investigated to mediate in different processes like nuclear shaping during neurogenesis in the brain (Koizumi and Gleeson, 2009;Zhang *et al.*, 2009), chromosomal interaction during meiosis and sperm head shaping during late spermiogenesis. Different genes are known to encode for SUN domain proteins in mammals, with the proteins SUN1 and SUN2 studied extensively. SUN1 and SUN2 are transmembrane proteins present in somatic cells, located on the inner nuclear membrane, interacting with their C-terminal to KASH (Klarsicht/Anc-1/Syne-1 homology) domains, located on the Outer Nuclear Membrane (ONM). The SUN domain proteins and KASH domain proteins together form a bridge, between the two membranes of the nuclear envelope, creating linkage of the nucleoskeleton and the cytoskeleton (LINC) complexes (Zhou and Hanna-Rose, 2010). See figure 10.
With the identification of the proteins SPAG4L and SPAG4L-2, two more SUN domain proteins are

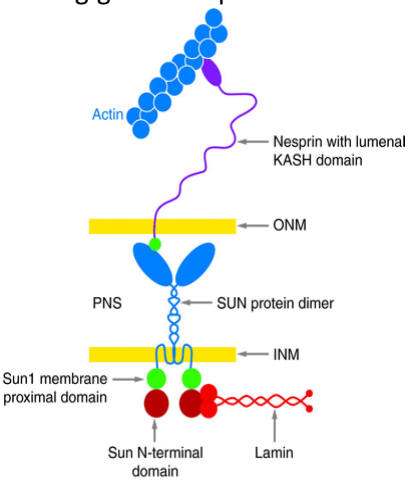


Fig.. 10. The process of LINC-complex formation. SUN domain proteins (including the discovered proteins SPAG4L/SPAG4L-2) in the INM interact with the KASH domain proteins located in the ONM. The KASH domain has the ability to interact with cytoskeletal components. In the case of acrosome formation the LINC-complex play a role in the anchorage of the acrosome to the nucleus via the PT. (Burke and Stewart, 2006)

discovered. The relevance of these proteins in globozoospermia should become apparent when they are lacking or dysfunctional mutated in the testis of such patients. It is established that SPAG4L/L-2 are integral membrane proteins which are localized on the apical nuclear site of developing spermatids, facing the acrosome. These testis-specific proteins show the ability to form LINC complexes with KASH domains. This complex is associated to be involved in the connection of the acrosome to the nucleus via the PT, a cytoskeletal element present in the space between the acrosome and nucleus,
In earlier studies it has been hypothesized that SUN domain proteins SUN1 and SUN2 have such a role (Padmakumar *et al.*, 2005;McGee *et al.*, 2006;Razafsky and Hodzic, 2009). In case of gametogenesis SUN1 seems to be very important. A study with SUN1 deficient mice (Ding *et al.*, 2007) showed viable mice, with impaired fertility. The viability of these mice is assigned to the compensatory role of SUN2 domain protein in somatic cells. Based on the absence of domain protein expression in meiotic cells (Stone *et al.*, 2000;Ding *et al.*, 2007) the infertility of SUN1 deficient mice could be explained by a lack of SUN domain protein redundancy in germ cells (Frohnert *et al.*, 2010). An important finding of the SUN1 and SUN2 function during gametogenesis.
Despite of these findings the lack of SPAG4L/L-2 can cause globozoospermia since this protein is found in the mice testis and shows to be functionally in the acrosome-nucleus connection. Impaired acrosome nucleus connection may result in destruction and removal of the acrosome.
At any rate more research to this protein and its appearance in globozoospermia is needed.

4.2 Deregulation in the production of proteins
Due to the assumption that several proteins are involved in spermiogenesis and especially in the acrosome development, some investigators hypothesized that globozoospermia could be caused by a combination of defects in several proteins. Therefore the expressions of a broad scale of proteins in patients suffering from globozoospermia were compared to the expression pattern of these proteins in normal healthy volunteers (Liao *et al.*, 2009). Liao *et al*. investigated that 61 proteins showed a two times different protein expression both up or down regulated expression, in the patients suffering from globozoospermia compared to normal, healthy volunteers. With the use of two-dimensional fluorescence difference gel electrophoresis (DIGE) coupled with mass spectrometry an analysis of the behavior of these 61 proteins was made (Liao *et al.*, 2009). Because patients suffering from globozoospermia show diverse semen characteristics it is possible that globozoospermia is caused by a combination of defects in several proteins and not caused by one defect. Therefore this protein analysis was done to see more candidates that could involved in globozoospermia.
From the 61 selected proteins which showed a twofold difference in volume ratio, 35 proteins showed still difference when the volume ratio was set on threefold difference. Some of these proteins were up regulated (9 proteins) and other down regulated (24 proteins). Off all these altered produced proteins the most striking alterations were the down regulation of spermatozoa acrosome membrane-associated protein 1 (SAMP1), sperm protein associated with the nucleus on the X chromosome (SPANX) and outer dense fiber protein2 (ODF2). These proteins will be discussed separately.
1. SAMP1 is a glycoprotein receptor, related to the glycosylphosphatidylinositol-anchored Ly-6/uPAR family, located on the membrane of the acrosome. These receptors are known to mediate in multiple physiological processes (Gumley *et al.*, 1995). Based on studies that analyzed the proteins SAMP13 (Shetty *et al.*, 2003) and SAMP32 (Hao *et al.*, 2002), Samp1 may function on the acrosomal membrane in adhesive and proteolytic action (Liao *et al.*, 2009). Liao *et al*. analyzed that the down regulation of the production of SAMP1 could possibly be related to three events, associated with globozoospermia; 1. the absence of an

acrosome, 2. dysfunctional fusion of vesicles to the acrosome, and 3. an impaired skeleton which results in impaired acrosome and nucleus shaping (Liao *et al.*, 2009).
2. SPANX is also down regulated in globozoospermia patients. This protein is found to be expressed in the nuclear envelope, localized to the post acrosomal perinuclear theca, and is functionally in the binding of the acrosome to the nucleus (Liao *et al.*, 2009). Since the function of SPANX is associated with the linkage of the acrosome to the nucleus, the lower production of this protein in globozoospermia patients may be associated as a cause of the lack of an acrosome.
3. The function of ODF2 is studied to be involved in tail-associated strength. This protein can interact with the microtubule network surrounding the sperm tail, and therefore can be established as a coiled-coil-protein (Donkor *et al.*, 2004). Round-headed cells are often scored in semen analysis to have malformed tails and to have a decreased level of motility. The latter could be associated with the down regulated level of the ODF2 proteins in globozoospermia (Liao *et al.*, 2009).
These three proteins that are down regulated in globozoospermia are examples of proteins that each have a function in another part of the spermatogenesis and are altered in globozoospermia. This analysis shows that there are several proteins found to be involved and possibly impaired in globozoospermia.

4.3 Genetic defects in mouse models
According to several cases of globozoospermia known in one family (Carrell *et al.*, 1999;Kilani *et al.*, 2004;Juneja and van Deursen, 2005) a genetic etiology is not excluded. Several genes play an important role in the development of sperm head biogenesis. Deficiency in these genes can result round-headed cells, correlated to an amount of infertility. The previous paragraph already discussed some proteins which are up or down regulated proteins in globozoospermia. Probably these alterations in protein production are due to defects in their underlying genes.
To investigate specific genes which could be impaired in globozoospermia adapted mouse models are used. These mouse models lack a specific gene. The outcome of these modifications mostly resulted in the development of globozoospermia and infertility in male mice.
The paragraph below will discuss the genes that are studied in literature which are likely to be impaired in globozoospermia. The genes *Csnk2a2, GOPC, Hrb* and *Pick1* are demonstrated to produce round headed cells when knocked out in mice. The genes *Pick1* and *SPATA16* are furthermore discovered to be mutated in humans.

4.3.1. *Csnk2a2*
The *Csnk2a2* gene is the first gene studied to be involved in the regulation of sperm head morphogenesis (Xu *et al.*, 1999). *Csnk2a2* is expressed in late spermatogenesis, it encodes for the protein casein kinase II (CK2). This protein contains two catalytic subunits, α and α' and two regulatory β-subunits. This serine-threonine kinase plays an important role in the cell growth of germ cells, primarily in the signal transduction of the nuclear matrix during spermatogenesis.
Mice lacking the *Csnk2a2* gene showed malformations during nuclear and acrosome development. Different forms of malformations in the acrosome are noticed, such as completely acrosomeless spermatozoa, spermatozoa with acrosomal remnants, possibly detached from the nucleus (Xu *et al.*, 1999).
Another characteristic investigated in *Csnk2a2* null mice is a higher amount of apoptotic cells in the testes, indicating that CK2 has anti-apoptotic properties, which is also related to oligozoospermia (Xu *et al.*, 1999). Patients suffering from oligozoospermia have a decreased

(less than 20 x 10^6/ml (World Health Organization, 1999)) concentration of spermatozoa in their semen.

4.3.2. GOPC

Secondly discussed is the *GOPC* gene, expressing a Golgi- associated PDZ (PSD95, DlgA and Zo-1 protein (see abbrev. list)) and coiled-coil motif containing protein. This gene is extensively studied and categorized as a candidate gene for globozoospermia. The GOPC protein contains different domains, a PDZ domain, two coiled-coil domains and two other domains whose functions are unknown. The coiled-coil domains are involved in the acrosome development due to the ability that these domains bind to the Golgi network, analyzed with immune electron microscopy (Yao *et al.*, 2002). Golgi-bound GOPC results in the ability of vesicle transport from the Golgi-network to the acrosome (Yao *et al.*, 2002). The lack of this gene will result in malfunction of PAG transport from the Golgi to the acrosomal vesicle as is demonstrated in generated GOPC-/- mice, where no acrosomal vesicles formation was noticed.

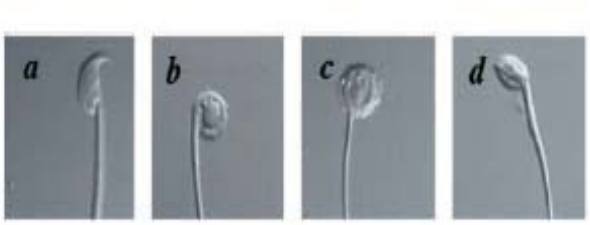


Fig. 11. Morphology of spermatid from WT GOPC mice (a) and GOPC deficient mice (b-d). (Yao et al., 2002)

It is also noticed that when the acroplaxome is not completely formed it causes defects in the anchorage of the acrosome tot the nucleus. Additionally, with the use of antibodies and electron microscopy is demonstrated that the round-headed cells in such mice lack the post acrosomal sheath (PAS) (Ito *et al.*, 2009) a structure which ought to be involved in the spermatid-head shaping and intramanchette transport (Ito *et al.*, 2009;Oko and Sutovsky, 2009). Lacking the PAS causes malformation of the posterior ring. Due to the absence or malformation of the posterior ring, the perinuclear cytoplasm becomes one with the cytoplasm of the cytoplasmic droplet (Suzuki-Toyota *et al.*, 2004). This results in nuclear malformation, deregulated mitochondria arrangement and coiled-coil tails during tail formation (Yao *et al.*, 2002;Suzuki-Toyota *et al.*, 2004). All these factors result in infertility of the mice. Altogether, these defects found in GOPC deficient mice show that this GOPC gene plays an important role in the biogenesis of spermatozoa and that it might be a candidate of globozoospermia related gene.

4.3.3. Hrb

Another gene, which is involved in acrosome formation and therefore essential for fertilization, is coding for the HIV (human immunodeficiency virus)-1 Rev-binding/Rev-interacting protein, *Hrb*. The gene encodes for a cofactor of the HIV-1 Rev protein, which regulates nucleocytoplasmic transport of proteins and RNA (Doria *et al.*, 1999). This Hrb protein, also known as the Rev/Rex activation domain-binding protein, Rab (Bogerd *et al.*, 1995), or human Rev-interacting protein, hRIP, can interact with EPs15 homology (EH) domain-containing proteins. These EH domain-containing proteins are involved in endocytosis and sorting (Doria *et al.*, 1999). Hrb forms a complex with the connections of the EH domain-containing proteins, which result in a role for Hrb in the vesicle to vesicle docking, the fusion of connected pro-acrosomal vesicles to the acrosomal sac, forms of nucleocytoplasmic transport. Events which are essential during acrosome formation (Kang-Decker *et al.*, 2001;Juneja and van Deursen, 2005). Mouse models lacking this gene are used to determine the function of this gene in acrosome biogenesis. With the use of electron microscopy the presence of an acrosome was detected in WT mice, but was absent in *Hrb* deficient mice. More experiments have demonstrated that the Hrb deficient mice cannot

form an acrosome because of a defect in the docking and fusion machinery of pro-acrosomal vesicles.
Additionally, semen analyses of *Hrb* -/- mice showed beyond the presence of acrosomeless spermatozoa also malformation of the acroplaxome plaque, which results in impaired elongation of the sperm nucleus, resulting in round-shaped spermatids. The malformation in the acroplaxome is explained by presence of F-actin, but lack of keratin 5 filament bundles in this component. Kierszenbaum *et al*. (Kierszenbaum *et al.*, 2004) showed no immunoreactivity of keratin 5 in the acroplaxome of *Hrb* deficient mice. Without this component the marginal ring misses filament bundles resulting in a diminished strength of the acroplaxome (Kierszenbaum *et al.*, 2004). In summary, *Hrb* seems to encode for protein which is involved in spermiogenesis, specifically in the acrosome biogenesis and secondary in the sperm head shaping. Therefore mice lacking this protein show an impaired acrosome and altered sperm head shaping due to the missing keratin in the marginal ring of the acroplaxome.

4.3.4 *Pick1*

Recently another protein, protein interacting with C kinase 1 (*Pick1*), is discovered playing a pivotal role in spermatogenesis. The expression of Pick1 is found mainly in the brain, involved in protein trafficking in neurons, in the pancreas and the testis. To distinguish the role of *Pick1*, again a mouse model is used. *Pick1* knock out (KO) mice are all completely infertile and show the same characteristics as found in patients with globozoospermia (Xiao *et al.*, 2009).
The Pick1 protein is established to be functional in the pro-acrosomal vesicular transport in early spermiogenesis. Localization of the protein has resulted in hypothesis that this protein is functional in several events of this transport process. Pick1 is localized at the trans-Golgi-network and is involved in the budding process of pro-acrosomal vesicles from the Golgi-network, see chapter 2.1. It is identified that Pick 1 also has a PDZ domain, which binds traffic related membrane proteins and therefore Pick1 is strongly involved in membrane transport.
Interestingly, recent data (Xiao *et al.*, 2009) showed that both the protein Pick1 and the protein GOPC are located on the same place in the Golgi-complex. Besides the same localization also protein-protein interaction was noticed between these two proteins by using yeast two hybrid analysis (Xiao *et al.*, 2009). However there are differences in characteristics of mice lacking the Pick1 gene compared to the GOPC gene, the function of these co-localized proteins during vesicle transport could differ. In figure 12 the cooperated function of the two proteins is illustrated.
Xiao *et al*. considered that after delivery of the pro-acrosomal vesicles to the mature acrosome the Pick1 protein could be recycled back again to the Golgi network (step 3 in figure 9) to fulfill its function several times. This is not established with data and should be studied more in detail in the future.
Furthermore there is found that Pick1 also interacts with the primary catalytic subunit of CK2. This because both *Pick1* deficient and *CK2* deficient mice show a large amount of apoptotic spermatozoa. How these protein functions together is not established yet (Xiao *et al.*, 2009).

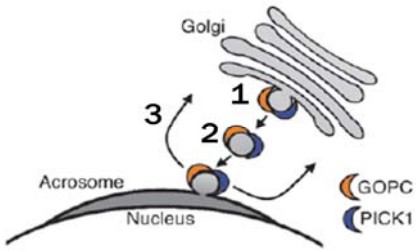


Figure 12. Schematic view of the function of the two proteins, GOPC and PICK1, which are demonstrated to be localized at the same place and showed protein-protein interactions. The numbers in the picture indicate different places were Pick1 , and probably also GOPC, is functional. Number 1 indicates the budding process of PAGs from the golgi-network. Number 2 shows the function of Pick as PAGs transporter in the assumption that the PDZ domain binds trafficking related membrane proteins. Xiao et al. considers that Pick1 could be recycled back to the Golgi to execute recycling of the protein. An adapted picture from (Xiao *et al.*, 2009)

From these data Pick1 is considered to be an essential candidate for causing globozoospermia, as it seems to be involved primarily in the acrosome vesicle transport and secondarily in apoptosis of germ cells.

Together all these genes discussed seem to play a role in fertility. Studies have used KO mice models to reveal their specific role in the development of globozoospermia. Notable was that only the male mice became infertile. All female mice with a mutant allele showed normal fertility (Xu *et al.*, 1999;Kang-Decker *et al.*, 2001;Suzuki-Toyota *et al.*, 2004;Juneja and van Deursen, 2005). The genes discussed above are therefore all involved in male gametogenesis.

The above discussed genes were identified as globozoospermia candidate genes since they showed globozoospermia in mouse KO models. Beyond this method to identify globozoospermia candidate genes, other studies (Dam *et al.*, 2007b;Liu *et al.*, 2010) used gene screenings on globozoospermia patients to identify mutated genes which could be an underlying cause of the round-headed cells. The genes *SPATA16* and *Pick1* are found to be mutated in globozoospermia patients and will be discussed in section 4.3.5.

4.3.5. *Genetic background in human globozoospermia*

The first study that analyzed the gene profile of globozoospermia patients was conducted on a Jewish family containing three fertile brothers and three infertile brothers showing globozoospermia. The underlying defect of globozoospermia was probably related by the homozygous mutation in the spermatogenesis-specific gene *SPATA-16* (Dam *et al.*, 2007b). This gene, also known as *NYD-SP12 (Xu et al., 2003;Zhang et al., 2007)* is primarily expressed in the human testis. The mouse ortholog of this gene is found to be expressed in the mouse spermatocytes and spermatids. The function of the gene *SPATA-16* is associated to be involved in spermatogenesis. The protein SPATA16 produced by *SPATA-16* contains a tetratricopeptide repeat (TPR) domain, which can establish protein-protein interaction with its helix-turn-helix component. By using a GFP-(Green Fluorescent Protein) fusion protein it is established that SPATA16 is localized in the Golgi apparatus and in the proacrosomal vesicles which are transported from the Golgi to the acrosome (Dam *et al.*, 2007b). The mutation in *SPATA-16* has been identified to be that cause of globozoospermia in the studied family. In all cases the TPR domain was disrupted. Due to the localization and the ability of the protein SPATA16 to bind other proteins, a possible interaction and cooperative function with the proteins GOPC and Hrb is not excluded. The GOPC protein contains an α-helix and could interact with helix-turn-helix component of the SPATA16 protein. Studies have to confirm these speculations.

In function this protein is not especially different than the other vesicle transport related proteins; however the *SPATA16* gene is the first gene that is discovered to be mutated in the three brothers of an examined family. This approves a possible heritable genetic defect causing globozoospermia (Dam *et al.*, 2007b).

Recently another study showed a globozoospermia gene which were mutated in the genome of the patient. One globozoospermia patient showed a homozygous missense mutation in the *Pick1* gene. This autosomal recessive genetic mutation resulted in a completely lack of the acrosome seen in humans (Liu *et al.*, 2010). Just as seen in the *Pick1* deficient mice (Xiao *et al.*, 2009).

More genetic screening is needed to identify more globozoospermia related mutations.

4.4 *Environmental defects.*

Beside inheritable effects other factors are associated with the incidence of malformation in spermatozoa causing globozoospermia. One detrimental environmental factor is smoking. Rubes *et al*. (Rubes *et al.*, 1998) analyzed semen of 10 smoking and 15 non-smoking males and investigated for sperm abnormalities in these groups. All participants were 18 years old males and the group 'smokers' consumed 20 cigarettes a day. This study found a statistically significant increase in the amount of 'round headed cells' present in the semen of smokers compared to that of non-smokers (Rubes *et al.*, 1998). Elshal *et al*. in 2009 associates the high amount of round headed cells in the semen of smokers to increased amount of oxidative stress and decreased level of antioxidant enzymes produced by smoking (Elshal *et al.*, 2009). The increased amount of oxidative stress could result in damage in the chromatin and structure breaks in the endogenous DNA in human sperm. A decreased level of antioxidant enzymes diminishes the effect of their own protection mechanism against reactive oxygen species (ROS). This could result in defects in DNA structure and cell damage (Elshal *et al.*, 2009).

CH. **5 Treatment Globozoospermia**

Couples where fertilization is not achieved after a period of two years of having regular intercourse without using contraceptive are classified infertile and can be qualified for therapeutic intervention. With the advent of Assisted Reproductive Technology (ART) the problem of infertility can be bypassed and fertilization can be accomplished. Of all the ARTs, In vitro Fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are the most executed methods for treating infertile couples.
Since in 1978 the first IVF-baby was born, the method of fertilization outside the woman's body was a fact. IVF is commonly used in couples where the woman suffers from a form of infertility. In vitro fertilization is accomplished as it name mentions already to arrange fertilization of an achieved oocyte out of the body by adding sperm of the partner into a tube or dish (Palermo *et al.*, 2009).
Couples that do not benefit by IVF, deal mostly with male infertility. The poor quality or quantity of the semen cannot result in conception. But in 1992 a new method is developed, by Palermo *et al*., which bypasses some steps in the fertilization process and makes conception possible for those infertile couples (Palermo *et al.*, 1992). Fertilization is established by injection of a sperm cell directly into an achieved oocyte with ICSI. For patients suffering from globozoospermia fertilization is not possible via IVF or intrauterine injection (IUI) due to sperm cells lacking the acrosome and having malformed tails, preventing successful zona penetration and thus blocking conception. The successful effect of ICSI in specifically globozoospermia patients was established in 1998 by hamburger *et al*. (Hamberger *et al.*, 1998). The injection of a sperm cell into the oocyte bypasses the zona penetration and thus the acrosome dependent steps onwards to (in vitro) fertilization.

5.1 ICSI method

ICSI is a form of in vitro fertilization with an almost similar protocol, excepted for the process of fertilization. During IVF a sperm cell has to penetrate through the oocyte itself and during ICSI the sperm cell is injected into the oocyte.
First, the woman gets hormones to induce ovulation. Mature oocytes are then collected via transvaginal follicle aspiration by the use of ultrasound application to locate the oocytes. In one ICSI cycle a number of oocytes are collected to increase the pass rate of fertilization. For each cycle the procedure is as follows; the cumulus layer around the collected oocyte is stripped and the oocyte is kept in place via a holding pipette, causing the oocyte to be immobile. The used sperm cells are collected from the ejaculate or directly from the testes. A solution containing polyvinylpyrrolidone is added to the semen to immobilize the sperm, after which one sperm cell can be aspirated into a micro needle, with its tail first. Then the injection of the sperm cell into the oocyte takes place, see figure 13. Finally the injected oocytes were placed back in their normal culture. Here the fertilization procedure continues. After a period of 17 hours the situation of fertilization settings is controlled. The fertilization

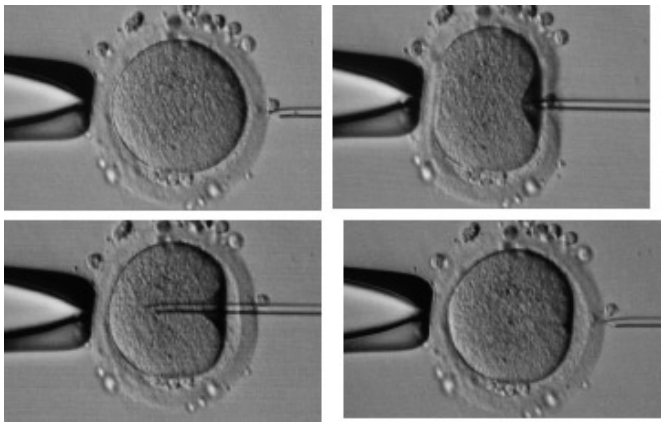


Fig. 13. The injection process of ICSI in four fragments. With a holding pipette the collected oocyte is kept in place. Then a micro needle with an aspirated sperm cell is injected into the oocyte. The sperm cell is pushed out of the micro needle and then the micro needle is removed from the oocyte.(Bringham and Women's Hospital, 2011)

went correct when two pronuclei and two polar bodies are present. Fertilized oocytes that develop into a 4-stage embryo under right conditions can be placed back into the woman. Based on several factors the conception can result in a newborn, but there is also a chance that the conception can be interrupted prematurely.

The next paragraph will discuss studies that show the use of ICSI in patients with globozoospermia.

5.2. *ICSI used for patients with globozoospermia*

Different case reports (Kilani *et al.*, 2004;Dirican *et al.*, 2008;Egashira *et al.*, 2009;Kyono *et al.*, 2009) are published which show successful pregnancies after the use of ICSI in couples suffering from globozoospermia. This demonstrates that fertilization is possible with round-headed acrosome free sperm from globozoospermic patients with the use of ICSI. Although only very low fertilization rates were observed (Battaglia *et al.*, 1997;Nardo *et al.*, 2002;Dirican *et al.*, 2008), which were much lower than the fertilization rate after ICSI treatments using normal shaped sperm from healthy men. The low fertilization rate in globozoospermia is suggested to be caused by additional dysfunctions of the round-headed cells. Because this low fertilization rate multiple cycles of ICSI are often needed, within each cycle a large amount of acquired oocytes.

The underlying aspects of this low fertilization rate is still under debate. Different factors are investigated which could play a role, see section 5.3. According to these investigations, studies suggested that ICSI could be improved by using extra fertilization stimuli like oocyte activators (Kahraman *et al.*, 1999;Dirican *et al.*, 2008;Kyono *et al.*, 2009;Ito *et al.*, 2009;Taylor *et al.*, 2010) or tools to increase the amount of calcium intracellular (Battaglia *et al.*, 1997;Rybouchkin *et al.*, 1997).

5.3 Possible factors that cause a low fertilization rate in ICSI attempts in round-headed cells.

One of the aspects that cause a low fertilization rate is the failure in oocyte activation. Oocyte activation is necessary to complete conception. The mouse oocyte activation test (MOAT) (Heindryckx *et al.*, 2005;Kyono *et al.*, 2009) is a tool to analyze the capacity of human sperm to activate oocytes. The mice oocytes are collected from the oviducts. Sperm of patients is injected and after several hours fertilization is analyzed. When fertilization of these mice oocytes fails with the patient's semen, assisted treatment is given additional during the ICSI procedure to improve fertilization. Studies have investigated different conditions which can explain the underlying cause of the failure of oocyte activation in globozoospermia patients.

5.3.1. Failure in oocyte activation due to chromosomal dysfunction

One of the factors that results in failure of fertilization and therefore a low fertilization rate is the presence of a high rate of premature chromosome condensation (PCC). PCC is induced when the oocyte is not activated after ICSI and remains in arrest at metaphase II. Ooplasmic chromosome condensing factors are then able to damage the DNA in the sperm nucleus (Stone *et al.*, 2000). The phenomenon of high rate of PCC in the sperm cell is several times noticed in patients with globozoospermia. In all cases fertilization did not occur (Stone *et al.*, 2000;Nakamura *et al.*, 2002;Schmiady *et al.*, 2005).

Vicari *et al.* (Vicari *et al.*, 2002) hypothesized that fragmented DNA also could play a role in dysfunctional oocyte activation. They studied the amount of DNA fragmentation with a TUNEL-assay and found a significantly increased percentage of sperm containing DNA fragmentation in globozoospermia patients compared to patients with normal spermatozoa. These globozoospermia patients showed lower fertilization rates after ICSI than the patients with normal spermatozoa.

5.3.2. Failure in oocyte activation due to deregulated calcium influx

Battaglia *et al*. (Battaglia *et al.*, 1997) assumed that failure of the attempts of ICSI treatment could also be associated with a deficiency in oocyte activation due to impaired calcium level during fertilization. In normal healthy cells, an intracellular calcium amount is released in the oocyte after the entrance of the sperm cell in the oocyte. This event regulates the further process of successful fertilization. However in the study of Battaglia *et al*. with globozoospermia, only one fertilization procedure was obtained naturally. Because of the hypothesis of anomaly levels of calcium as the underlying cause of the failure of fertilization, additional a calcium inducing drug was used. The process of the ICSI-cycles was executed as followed. In the first cycle only one oocyte, of the 17 received intact oocytes, were fertilized. After this assumption the unfertilized oocytes were treated with a drug that could induce assisted intracellular calcium levels. This resulted after 38 hours in nine two to four cells embryos. Including with the first normal fertilized oocyte, 5 embryos were implanted in the woman, however no pregnancy was obtained. For the second ICSI cycle eight received oocytes were treated with the calcium drug immediately after sperm injection, besides the 20 oocytes which were treated normally. Six of the calcium drug treated oocytes underwent properly fertilization besides the two of the 20 untreated injected oocytes. However again after implantation of a good developed embryo, only a short term chemical pregnancy was obtained, no delivery happened.

Normal ICSI, without additional drugs, showed a very low fertilization rate with globozoospermia semen. With the addition of a calcium-inducing-drug a higher fertilization was achieved. The calcium release after sperm penetration of the oocyte is demonstrated to be regulated via soluble sperm factors. Globozoospermia could therefore have a deficiency in one of the sperm factors. Battaglia *et al*. considered the failure of calcium release in their fertilized oocyte by the deficiency of the sperm factor oscillin in globozoospermia (Battaglia *et al.*, 1997). This would result in deregulated Ca2+ fluctuations during fertilizations, which contribute to failure in oocyte activation. PLC ζ-1 is another soluble sperm specific factor, a form of phospholipase C which induces levels of the second messenger InsP3, increasing Ca2+ levels in the oocyte during activation of the oocyte (Swann *et al.*, 2006). Mice studies have shown that this protein is located at the sperm nucleus, releasing the amount into the oocyte cytoplasm in a process of 15 minutes. Yoon *et al*. demonstrated (Yoon *et al.*, 2008) that this protein is present in lower levels in patients suffering from globozoospermia. These levels were investigated using antibodies against PLCζ-1. These detected low PLCζ-1 levels were associated with the failure of fertilization in globozoospermia undergoing ICSI.

5.4 Adjustment of ICSI

Now it is established that the low fertilization rate in ICSI partly can be applied to the deficiency of globozoospermia to activate the oocyte, additional treatment could bypass this problem. Several case studies used assisted oocyte activators (AOA) to increase the fertilization rate. Dirican *et al*. (Dirican *et al.*, 2008) performed ICSI in two infertile couples suffering from globozoospermia (familial), one treated with mechanical oocyte activation and one treated without oocyte activation. Mechanical oocyte activation was performed right after the implantation of the sperm into the oocyte by using a microneedle to make a little break in the plasma membrane to induce calcium influx. With this additional event the fertilization rate was three times higher (33.3% -- 9.1% respectively), however in both the treatments, with and without oocyte activation, pregnancy was established with successful gestation.

Another form of oocyte activation during ICSI is acquired by adding a calcium ionophore, a mobile ion-carrier that results in increased intracellular calcium levels in the fertilized oocyte. Rybouchkin *et al*. and Battaglia *et al.* showed already in 1997 improvement in the

fertilization rate among globozoospermia patients compared to treatments of ICSI without oocyte activation. Assisted oocyte activation (AOA) via a calcium ionophore is executed in more ICSI treatments among globozoospermia patients (Kim *et al.*, 2001;Heindryckx *et al.*, 2005;Tejera *et al.*, 2008;Kyono *et al.*, 2009;Taylor *et al.*, 2010) and showed high fertilization rates. However, pregnancies were not established in all cases (Dam *et al.*, 2007b;Taylor *et al.*, 2010).
Finally, another form of AOA used during ICSI to increase the fertilization rate among globozoospermia patients is electrical stimulation (Egashira *et al.*, 2009;Taylor *et al.*, 2010) with successful pregnancy as result.

More research is needed to find additional treatments which optimize oocyte activation during ICSI, to improve this form of ART.

Discussion

Globozoospermia is a rare form of male infertility. Because of the low incidence of globozoospermia patients, the possibilities of doing research are limited. Due to the specific morphologic characteristics of globozoospermia, this form of teratozoospermia can be distinguished from others with extensive semen analysis techniques. Strikingly, the morphology of globozoospermic cells is indicated by the lack of the acrosome. Related to this these globozoospermic cells often posses a non elongated nucleus as well as a coiled-coil tail. These defects result in 'round-headed' cells with decreased motility and a dysfunctional capacity to fertilize the oocyte: These cells lack the property to penetrate through the ZP once they interact with this structure prior to fertilization. Additional globozoospermia show a decreased fertilization rate during the artificial insemination method ICSI because these globozoospermia have a decreased ability to activate an oocyte, possibly because they lack essential proteins to initiate calcium influx, essential for oocyte activation (see chapter 5.3.2)

Based on morphology analysis, male patients can be categorized by having total or partial globozoospermia. Patients suffering from total globozoospermia are completely infertile, while patients suffering from partial globozoospermia, in which at least more than 50% of the spermatozoa are round-headed, have major decreased infertility. It is important to diagnose in semen samples to which of these categories the patient belongs prior to further treatment.

Nowadays, not one specific element deregulated in globozoospermia patients can be assigned as the main cause of this disease. Since a number of different components were found to be deregulated in globozoospermia, it is thought that the impairment of round-headed cells to fertilize could be the result of a combination of defects during the spermatogenesis.

Based on KO mouse models, the defects that were thought to be associated to globozoospermia can be roughly divided in two different groups namely those that cause acrosomal abnormalities and those that organize the shaping of the nucleus and tail.

Related to acrosomal formation, Frohnert *et al*. (Frohnert *et al.*, 2010) demonstrated that the proteins SPAG4L/SPAG4L-2 are essential during the anchorage of the acrosome to the nucleus. SPAG4L and SPAG4L-2 form a bridge with KASH domains present in the outer nuclear membrane, which result in the formation of LINC complexes (see figure 10). This study hypothesizes that lack of SPAG4L and SPAG4L-2 could be the underlying cause of the globozoospermia, because these proteins are functional in the linkage of the acrosome to the nucleus. Absence of SPAG4L and SPAG4L-2 possibly causes an impaired development of the acrosome. Other studies discussed other components involved in acrosome anchorage and therefore a possible target for globozoospermia. Ito *et al.* (Ito *et al.*, 2009) demonstrated that round-headed cells which lack the acrosome also miss the PT. The PT is a cytoskeletal disc, which is located between the IAM and the ONM. The PT is thought to also play a role in the anchorage of the acrosome to the nucleus. A few years earlier Ito and colleagues already demonstrated that the marginal ring, which is a part of the cytoskeletal acroplaxome, is impaired in KO mice lacking the expression of the transport protein GOPC and those mice have globozoospermia as specific genetically altered phenotype (Ito *et al.*, 2004).

Defects in the transport of GAPs are also resulting in impaired acrosome formation. Several proteins are known to play a role in this transport mechanism. The function of these GAP transport proteins (Csnk2a2, GOPC, Hrb, and Pick1) was investigated in KO mice which lack the gene for these specific proteins. All these mice showed impaired acrosome formation and characteristics of globozoospermia (Xu *et al.*, 1999;Kang-Decker *et al.*, 2001;Yao *et al.*, 2002;Xiao *et al.*, 2009).

Secondary to the acrosome formation, malformations in the nucleus and tail are also detected in case of globozoospermia. These deficiencies are caused by defects during spermatozoon shaping, which occurs in late spermiogenesis. Studies (Kierszenbaum *et al.*, 2003a;Kierszenbaum and Tres, 2004;Liao *et al.*, 2009) have demonstrated that these malformations are associated with dysfunction in the manchette. This is a cytoskeletal component that surrounds the caudal part of the developing spermatid. A dysfunctional manchette results in impaired IMT, no elongation of the nucleus and deregulated shaping of the late spermatid. These studies show that many spermatozoal components are involved during spermiogenesis and that defects in these components eventually lead to globozoospermia.

Several cases of human familial globozoospermia are known and have been screened on genetic abnormalities. As discussed above, several genes are classified as globozoospermia candidate genes in mice, since KO models demonstrate globozoospermia features. In contrast only a couple of globozoospermia candidate genes were found to be mutated in humans. Recently, Liu *et al*, showed that one member of a Chinese family showed, after a gene screening of the discussed candidate genes, a homozygous missense mutation in the Pick1 gene. In 2007, Dam *et al*. (Dam *et al.*, 2007b) investigated another genetic cause for globozoospermia in humans. They found a mutation in the SPATA16 gene. In a family of six brothers (three healthy brother and three brothers suffering from globozoospermia), all the three affected brothers showed a mutation in SPATA16 (van dam spata16) gene. Additional, 29 other globozoospermia patients were examined on this mutation but did not show corresponding similarities.

Since several studies showed mutations in the genome, related to proteins involved in spermiogenesis, a genetic defect could be a possible cause of globozoospermia. However the origin of these mutations is still under debate. According to a congenital deviation one would expect the same mutation of that gene in other studies. The only study that found the same mutation in multiple globozoospermia patients was done by Dam *et al*. (Dam *et al.*, 2007b). The patients showing the same mutation were relatives. More research to familial cases of globozoospermia should be done to see if globozoospermia actually could be caused by an inheritable genetic defect. Conceivable is that these defects also could be caused by environmental factors, like smoking, as Elshal *et al*. and Rubes *et al*. (Rubes *et al.*, 1998;Elshal *et al.*, 2009) showed earlier (see chapter 4.4).

Further gene screenings of patients could be useful to detect more globozoospermia candidate genes and also to diagnose and to determine if gene therapy belongs to one of the possibilities to treat this form of infertility.

Since 1992, a new artificial insemination technique was developed, called ICSI. Fertilization is via this method possible for globozoospermia patients. Via ICSI a sperm cell is injected directly into the oocyte. The ZP is via this method penetrated by the micropipette tip rather than the sperm cell which in case of globozoospermia is inapt to do this by the lack of an acrosome and impaired motility. Therefore the acrosome reaction and fusion of the spermatozoon membrane with the oocyte membrane does not take place after natural mating. Thus theoretically fertilization should be possible for these patients via ICSI. However, the sperm cells of patients suffering from globozoospermia are not only dysfunctional in ZP penetration due to their lack of acrosomes, these cells also have a decreased ability of oocyte activation, an essential step during the fertilization, by missing essential proteins. Studies (Battaglia *et al.*, 1997;Nardo *et al.*, 2002;Dirican *et al.*, 2008) noticed a lower fertilization rate in these patients compared to patients which also underwent ICSI. To solve the problem of oocyte activation, additional treatments are given. An important step which is impaired during oocyte activation is the influx of calcium in the oocyte after injection of the sperm cell. Studies used additional a calcium ionophore (Rybouchkin *et al.*, 1996;Kim *et al.*, 2001;Heindryckx *et al.*, 2005;Tejera *et al.*, 2008;Kyono *et*

al., 2009;Taylor *et al.*, 2010) or induce a calcium influx by creating a breakage of the membrane with a microneedle (Dirican *et al.*, 2008). Another method of AOA that is used is electrical stimulation (Egashira *et al.*, 2009). Egashira *et al.* showed an increased fertilization after using electrical stimulation during ICSI. Without AOA the fertilization rate was 6.0% and with AOA this fertilization rate increased into 78.9 %. Of all AOAs, the calcium ionophore is several times discussed in studies (Kim *et al.*, 2001;Heindryckx *et al.*, 2005;Kyono *et al.*, 2009) to have possible toxic effects on oocyte or embryo development. For now limited information of the possible negative effect of this additional treatment is accessible.
ICSI is an extensively operated method of ART and is examined to be one of the best treatments related to male infertility, and the only method, at this moment, for patients suffering from globozoospermia to realize fertilization. This method also has risks and uncertainties with it. Therefore, the use of ICSI in globozoospermia patients brings up some ethical related discussions. The literature indicates that ICSI can lead to a successful conception (Nardo *et al.*, 2002;Dirican *et al.*, 2008;Tejera *et al.*, 2008;Kyono *et al.*, 2009), but multiple attempts are needed in most cases and that these interventions are executed with possible complications for the female. The ethical question also rises, whether it is reliable to induce fertilization with these impaired round-headed spermatozoa? The fertilization rates are very low, which is related with the capacity of the sperm cell to fertilize. It has to be taken into account that the sperm cells in case of globozoospermia are so much impaired that they need assistance for each step in fertilization via drugs (AOA) and an advanced method (ICSI). Because the first ICSI pregnancy was established in 1992, there is too little data from the offspring to determine the risk of developing fertility defects in this following generation. Also no data are known of the risk for the offspring to develop genetic defects. It's very important to determine if male suffering from globozoospermia can be responsible for passing through their defects onto their offspring.
All these arguments and insecurities have to be considered. Couples have to think about whether or not all the effort, costs and possible emotional damage will compensate for the outcome of the treatments which are not always positive. Besides this, it is very important for physicians to notify the couples of all options that are possible next to ICSI-AOA treatments, like donor sperm insemination and adoption (Javed *et al.*, 2010), and the pass rate of such a treatment.

In conclusion, studies have indicated that spermiogenesis is a complex process with several components that show a possible participation in the development of globozoospermia. However, from all these components not one specific element is indicated as the main cause of globozoospermia, but a combination of these impaired components. Further research should focus on the relation of the appearance of several deregulated components. A genetic cause should not be excluded but more evidence of patients having the same mutation in specific globozoospermia candidate genes has to be collected.
Globozoospermia patients are infertile; the only way fertilization can be established is by the use of ICSI. Besides that this technique is not without risks and ethical objections it is also not always effective. Research has tried to find the underlying cause of this incidental low fertilization rate and tried to bypass this with additional methods. Unfortunately this also did not always result in a successful fertilization. For the future, better additional treatments during ICSI therapy or even another method for fertilization could improve the chances of fertilization for globozoospermia patients.

Appendix 1 (World Health Organization, 1999)

Table 1. Normal values of semen variables, WHO standard.

Volume	2.0 ml or more
pH	7.2–7.8
Sperm concentration	20×10^6 spermatozoa/ml or more
Total sperm count	40×10^6 spermatozoa or more
Motility	50% or more with forward progression (i.e. categories (a) and (b); Section 2.4.2) or 25% or more with rapid linear progression (i.e. category (a)) within 60 min after collection
Morphology	50% or more with normal morphology
Viability	50% or more live, i.e. excluding dye
White blood cells	Fewer than 1×10^6/ml
Zinc (total)	2.4 micromol or more per ejaculate
Citric acid (total)	52 μmol (10 mg) or more per ejaculate
Fructose (total)	13 micromol or more per ejaculate
MAR test	Fewer than 10% spermatozoa with adherent particles
Immunobead test	Fewer than 10% spermatozoa with adherent beads

Table 2. Nomenclature for Semen abnormalities

Normozoospermia	Normal ejaculate as defined above
Oligozoospermia	Sperm concentration fewer than 20×10^6/ml
Asthenozoospermia	Fewer than 50% spermatozoa with forward progression (categories (a) and (b)) or fewer than 25% spermatozoa with category (a) movement (see Section 2.4.2)
Teratozoospermia	Fewer than 50% spermatozoa with normal morphology
Oligoasthenoterato-zoospermia	Signifies disturbance of all three variables (combinations of only two prefixes may also be used)
Azoospermia	No spermatozoa in the ejaculate
Aspermia	No ejaculate

The term globozoospermia is not included in this table. The terms 'total globozoospermia' and 'partial globozoospermia' should be placed below the term teratozoospermia. Total globozoospermia is characterized by 100% of round headed spermatozoa, lacking the acrosome. In the case of partial globozoospermia the semen consist at least 50 % of acrosome lacking round headed spermatozoa. Also spermatozoa with an oval shape were recognized.

Reference List

Aitken,R.J. (2006). Sperm function tests and fertility. Int. J. Androl *29*, 69-75.

Amann,R.P. (2009). Considerations in evaluating human spermatogenesis on the basis of total sperm per ejaculate. J. Androl *30*, 626-641.

Anakwe,O.O. and Gerton,G.L. (1990). Acrosome biogenesis begins during meiosis: evidence from the synthesis and distribution of an acrosomal glycoprotein, acrogranin, during guinea pig spermatogenesis. Biol. Reprod. *42*, 317-328.

Battaglia,D.E., Koehler,J.K., Klein,N.A., and Tucker,M.J. (1997). Failure of oocyte activation after intracytoplasmic sperm injection using round-headed sperm. Fertil. Steril. *68*, 118-122.

Benjamin Cummings. Human Anatomy & Physiology . 5th edition. 2001. San Fransisco.
Ref Type: Generic

Bogerd,H.P., Fridell,R.A., Madore,S., and Cullen,B.R. (1995). Identification of a novel cellular cofactor for the Rev/Rex class of retroviral regulatory proteins. Cell *82*, 485-494.

Breitbart,H. (2002). Role and regulation of intracellular calcium in acrosomal exocytosis. J. Reprod. Immunol. *53*, 151-159.

Bringham and Women's Hospital. Infertility Treatment Options: In Vitro Fertilization (IVF). 2011.
Ref Type: Generic

Burke,B. and Stewart,C.L. (2006). The laminopathies: the functional architecture of the nucleus and its contribution to disease. Annu. Rev. Genomics Hum. Genet. *7*, 369-405.

Carrell,D.T., Emery,B.R., and Liu,L. (1999). Characterization of aneuploidy rates, protamine levels, ultrastructure, and functional ability of round-headed sperm from two siblings and implications for intracytoplasmic sperm injection. Fertil. Steril. *71*, 511-516.

Dam,A.H., Feenstra,I., Westphal,J.R., Ramos,L., van Golde,R.J., and Kremer,J.A. (2007a). Globozoospermia revisited. Hum. Reprod. Update. *13*, 63-75.

Dam,A.H. *et al.* (2007b). Homozygous mutation in SPATA16 is associated with male infertility in human globozoospermia. Am. J. Hum. Genet. *81*, 813-820.

Dam,A.H., Ramos,L., Dijkman,H.B., Woestenenk,R., Robben,H., van den,H.L., and Kremer,J.A. (2010). Morphology of Partial Globozoospermia. J. Androl.

Ding,X., Xu,R., Yu,J., Xu,T., Zhuang,Y., and Han,M. (2007). SUN1 is required for telomere attachment to nuclear envelope and gametogenesis in mice. Dev. Cell *12*, 863-872.

Dirican,E.K., Isik,A., Vicdan,K., Sozen,E., and Suludere,Z. (2008). Clinical pregnancies and livebirths achieved by intracytoplasmic injection of round headed acrosomeless spermatozoa with and without oocyte activation in familial globozoospermia: case report. Asian J. Androl *10*, 332-336.

Donkor,F.F., Monnich,M., Czirr,E., Hollemann,T., and Hoyer-Fender,S. (2004). Outer dense fibre protein 2 (ODF2) is a self-interacting centrosomal protein with affinity for microtubules. J. Cell Sci. *117*, 4643-4651.

Doria,M., Salcini,A.E., Colombo,E., Parslow,T.G., Pelicci,P.G., and Di Fiore,P.P. (1999). The eps15 homology (EH) domain-based interaction between eps15 and hrb connects the molecular machinery of endocytosis to that of nucleocytosolic transport. J. Cell Biol. *147*, 1379-1384.

Egashira,A., Murakami,M., Haigo,K., Horiuchi,T., and Kuramoto,T. (2009). A successful pregnancy and live birth after intracytoplasmic sperm injection with globozoospermic sperm and electrical oocyte activation. Fertil. Steril. *92*, 2037-2039.

Elshal,M.F., El-Sayed,I.H., Elsaied,M.A., El-Masry,S.A., and Kumosani,T.A. (2009). Sperm head defects and disturbances in spermatozoal chromatin and DNA integrities in idiopathic infertile subjects: association with cigarette smoking. Clin. Biochem. *42*, 589-594.

Frohnert,C., Schweizer,S., and Hoyer-Fender,S. (2010). SPAG4L/SPAG4L-2 are testis-specific SUN domain proteins restricted to the apical nuclear envelope of round spermatids facing the acrosome. Mol. Hum. Reprod.

Gilbert SF. Developmental biology. 6th edition. 2000. Sunderland (MA).
Ref Type: Generic

Gumley,T.P., McKenzie,I.F., and Sandrin,M.S. (1995). Tissue expression, structure and function of the murine Ly-6 family of molecules. Immunol. Cell Biol. *73*, 277-296.

Gupta,S.K. and Bhandari,B. (2011). Acrosome reaction: relevance of zona pellucida glycoproteins. Asian J. Androl *13*, 97-105.

Hamberger,L., Lundin,K., Sjogren,A., and Soderlund,B. (1998). Indications for intracytoplasmic sperm injection. Hum. Reprod. *13 Suppl 1*, 128-133.

Hao,Z., Wolkowicz,M.J., Shetty,J., Klotz,K., Bolling,L., Sen,B., Westbrook,V.A., Coonrod,S., Flickinger,C.J., and Herr,J.C. (2002). SAMP32, a testis-specific, isoantigenic sperm acrosomal membrane-associated protein. Biol. Reprod. *66*, 735-744.

Heindryckx,B., Van der,E.J., De,S.P., and Dhont,M. (2005). Treatment option for sperm- or oocyte-related fertilization failure: assisted oocyte activation following diagnostic heterologous ICSI. Hum. Reprod. *20*, 2237-2241.

Hermo,L., Pelletier,R.M., Cyr,D.G., and Smith,C.E. (2010). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: background to spermatogenesis, spermatogonia, and spermatocytes. Microsc. Res. Tech. *73*, 241-278.

Hirsh,A. (2003). Male subfertility. BMJ *327*, 669-672.

Ho,H.C., Tang,C.Y., and Suarez,S.S. (1999). Three-dimensional structure of the Golgi apparatus in mouse spermatids: a scanning electron microscopic study. Anat. Rec. *256*, 189-194.

Ito,C., Akutsu,H., Yao,R., Kyono,K., Suzuki-Toyota,F., Toyama,Y., Maekawa,M., Noda,T., and Toshimori,K. (2009). Oocyte activation ability correlates with head flatness and presence of perinuclear theca substance in human and mouse sperm. Hum. Reprod. *24*, 2588-2595.

Ito,C., Suzuki-Toyota,F., Maekawa,M., Toyama,Y., Yao,R., Noda,T., and Toshimori,K. (2004). Failure to assemble the peri-nuclear structures in GOPC deficient spermatids as found in round-headed spermatozoa. Arch. Histol. Cytol. *67*, 349-360.

Javed,M., Esfandiari,N., and Casper,R.F. (2010). Failed fertilization after clinical intracytoplasmic sperm injection. Reprod. Biomed. Online. *20*, 56-67.

Juneja,S.C. and van Deursen,J.M. (2005). A mouse model of familial oligoasthenoteratozoospermia. Hum. Reprod. *20*, 881-893.

Kahraman,S., Akarsu,C., Cengiz,G., Dirican,K., Sozen,E., Can,B., Guven,C., and Vanderzwalmen,P. (1999). Fertility of ejaculated and testicular megalohead spermatozoa with intracytoplasmic sperm injection. Hum. Reprod. *14*, 726-730.

Kang-Decker,N., Mantchev,G.T., Juneja,S.C., McNiven,M.A., and van Deursen,J.M. (2001). Lack of acrosome formation in Hrb-deficient mice. Science *294*, 1531-1533.

Kierszenbaum,A.L., Rivkin,E., and Tres,L.L. (2003b). The actin-based motor myosin Va is a component of the acroplaxome, an acrosome-nuclear envelope junctional plate, and of manchette-associated vesicles. Cytogenet. Genome Res. *103*, 337-344.

Kierszenbaum,A.L., Rivkin,E., and Tres,L.L. (2003a). Acroplaxome, an F-actin-keratin-containing plate, anchors the acrosome to the nucleus during shaping of the spermatid head. Mol. Biol. Cell *14*, 4628-4640.

Kierszenbaum,A.L. and Tres,L.L. (2004). The acrosome-acroplaxome-manchette complex and the shaping of the spermatid head. Arch. Histol. Cytol. *67*, 271-284.

Kierszenbaum,A.L., Tres,L.L., Rivkin,E., Kang-Decker,N., and van Deursen,J.M. (2004). The acroplaxome is the docking site of Golgi-derived myosin Va/Rab27a/b- containing proacrosomal vesicles in wild-type and Hrb mutant mouse spermatids. Biol. Reprod. *70*, 1400-1410.

Kilani,Z., Ismail,R., Ghunaim,S., Mohamed,H., Hughes,D., Brewis,I., and Barratt,C.L. (2004). Evaluation and treatment of familial globozoospermia in five brothers. Fertil. Steril. *82*, 1436-1439.

Kim,S.T., Cha,Y.B., Park,J.M., and Gye,M.C. (2001). Successful pregnancy and delivery from frozen-thawed embryos after intracytoplasmic sperm injection using round-headed spermatozoa and assisted oocyte activation in a globozoospermic patient with mosaic Down syndrome. Fertil. Steril. *75*, 445-447.

Koizumi,H. and Gleeson,J.G. (2009). Sun proteins enlighten nuclear movement in development. Neuron *64*, 147-149.

Kyono,K., Nakajo,Y., Nishinaka,C., Hattori,H., Kyoya,T., Ishikawa,T., Abe,H., and Araki,Y. (2009). A birth from the transfer of a single vitrified-warmed blastocyst using intracytoplasmic sperm injection with calcium ionophore oocyte activation in a globozoospermic patient. Fertil. Steril. *91*, 931-11.

Liao,T.T., Xiang,Z., Zhu,W.B., and Fan,L.Q. (2009). Proteome analysis of round-headed and normal spermatozoa by 2-D fluorescence difference gel electrophoresis and mass spectrometry. Asian J. Androl *11*, 683-693.

Liu,G., Shi,Q.W., and Lu,G.X. (2010). A newly discovered mutation in PICK1 in a human with globozoospermia. Asian J. Androl *12*, 556-560.

Mayorga,L.S., Tomes,C.N., and Belmonte,S.A. (2007). Acrosomal exocytosis, a special type of regulated secretion. IUBMB. Life *59*, 286-292.

McGee,M.D., Rillo,R., Anderson,A.S., and Starr,D.A. (2006). UNC-83 IS a KASH protein required for nuclear migration and is recruited to the outer nuclear membrane by a physical interaction with the SUN protein UNC-84. Mol. Biol. Cell *17*, 1790-1801.

Michaut,M., Tomes,C.N., De,B.G., Yunes,R., and Mayorga,L.S. (2000). Calcium-triggered acrosomal exocytosis in human spermatozoa requires the coordinated activation of Rab3A and N-ethylmaleimide-sensitive factor. Proc. Natl. Acad. Sci. U. S. A *97*, 9996-10001.

Moreno,R.D. and Alvarado,C.P. (2006). The mammalian acrosome as a secretory lysosome: new and old evidence. Mol. Reprod. Dev. *73*, 1430-1434.

Nakamura,S., Terada,Y., Horiuchi,T., Emuta,C., Murakami,T., Yaegashi,N., and Okamura,K. (2002). Analysis of the human sperm centrosomal function and the oocyte activation ability in a case of globozoospermia, by ICSI into bovine oocytes. Hum. Reprod. *17*, 2930-2934.

Nardo,L.G., Sinatra,F., Bartoloni,G., Zafarana,S., and Nardo,F. (2002). Ultrastructural features and ICSI treatment of severe teratozoospermia: report of two human cases of globozoospermia. Eur. J. Obstet. Gynecol. Reprod. Biol. *104*, 40-42.

Oehninger,S. and Kruger,T. Male Infertility; Diagnosis and Treatment. 2007. Informa UK Ltd. Ref Type: Generic

Oko,R. and Sutovsky,P. (2009). Biogenesis of sperm perinuclear theca and its role in sperm functional competence and fertilization. J. Reprod. Immunol. *83*, 2-7.

Padmakumar,V.C., Libotte,T., Lu,W., Zaim,H., Abraham,S., Noegel,A.A., Gotzmann,J., Foisner,R., and Karakesisoglou,I. (2005). The inner nuclear membrane protein Sun1 mediates the anchorage of Nesprin-2 to the nuclear envelope. J. Cell Sci. *118*, 3419-3430.

Palermo,G., Joris,H., Devroey,P., and Van Steirteghem,A.C. (1992). Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet *340*, 17-18.

Palermo,G.D., Neri,Q.V., Takeuchi,T., and Rosenwaks,Z. (2009). ICSI: where we have been and where we are going. Semin. Reprod. Med. *27*, 191-201.

Peterson,R.N., Bozzola,J., and Polakoski,K. (1992). Protein transport and organization of the developing mammalian sperm acrosome. Tissue Cell *24*, 1-15.

Ramalho-Santos,J., Schatten,G., and Moreno,R.D. (2002a). Control of membrane fusion during spermiogenesis and the acrosome reaction. Biol. Reprod. *67*, 1043-1051.

Ramalho-Santos,J., Terada,Y., and Schatten,G. (2002b). VAMP/synaptobrevin as an acrosomal marker for human sperm. Fertil. Steril. *77*, 159-161.

Razafsky,D. and Hodzic,D. (2009). Bringing KASH under the SUN: the many faces of nucleo-cytoskeletal connections. J. Cell Biol. *186*, 461-472.

Rubes,J., Lowe,X., Moore,D., Perreault,S., Slott,V., Evenson,D., Selevan,S.G., and Wyrobek,A.J. (1998). Smoking cigarettes is associated with increased sperm disomy in teenage men. Fertil. Steril. *70*, 715-723.

Rybouchkin,A., Dozortsev,D., Pelinck,M.J., De,S.P., and Dhont,M. (1996). Analysis of the oocyte activating capacity and chromosomal complement of round-headed human spermatozoa by their injection into mouse oocytes. Hum. Reprod. *11*, 2170-2175.

Rybouchkin,A.V., Van der,S.F., Quatacker,J., De,S.P., and Dhont,M. (1997). Fertilization and pregnancy after assisted oocyte activation and intracytoplasmic sperm injection in a case of round-headed sperm associated with deficient oocyte activation capacity. Fertil. Steril. *68*, 1144-1147.

Samplaski,M.K., Agarwal,A., Sharma,R., and Sabanegh,E. (2010). New generation of diagnostic tests for infertility: review of specialized semen tests. Int. J. Urol. *17*, 839-847.

Schmiady,H., Schulze,W., Scheiber,I., and Pfuller,B. (2005). High rate of premature chromosome condensation in human oocytes following microinjection with round-headed sperm: case report. Hum. Reprod. *20*, 1319-1323.

Senger P.L. Pathways to Pregnancy and Parturition. second revised edition, 216-280. 2003. Current Conceptions.
Ref Type: Generic

Shetty,J. *et al.* (2003). SAMP14, a novel, acrosomal membrane-associated, glycosylphosphatidylinositol-anchored member of the Ly-6/urokinase-type plasminogen activator receptor superfamily with a role in sperm-egg interaction. J. Biol. Chem. *278*, 30506-30515.

Stone,S., O'Mahony,F., Khalaf,Y., Taylor,A., and Braude,P. (2000). A normal livebirth after intracytoplasmic sperm injection for globozoospermia without assisted oocyte activation: case report. Hum. Reprod. *15*, 139-141.

Suzuki-Toyota,F., Ito,C., Toyama,Y., Maekawa,M., Yao,R., Noda,T., and Toshimori,K. (2004). The coiled tail of the round-headed spermatozoa appears during epididymal passage in GOPC-deficient mice. Arch. Histol. Cytol. *67*, 361-371.

Swann,K., Saunders,C.M., Rogers,N.T., and Lai,F.A. (2006). PLCzeta(zeta): a sperm protein that triggers Ca2+ oscillations and egg activation in mammals. Semin. Cell Dev. Biol. *17*, 264-273.

Taylor,S.L., Yoon,S.Y., Morshedi,M.S., Lacey,D.R., Jellerette,T., Fissore,R.A., and Oehninger,S. (2010). Complete globozoospermia associated with PLCzeta deficiency treated with calcium ionophore and ICSI results in pregnancy. Reprod. Biomed. Online. *20*, 559-564.

Tejera,A., Molla,M., Muriel,L., Remohi,J., Pellicer,A., and De Pablo,J.L. (2008). Successful pregnancy and childbirth after intracytoplasmic sperm injection with calcium ionophore oocyte activation in a globozoospermic patient. Fertil. Steril. *90*, 1202-1205.

Toshimori,K. (1998). Maturation of mammalian spermatozoa: modifications of the acrosome and plasma membrane leading to fertilization. Cell Tissue Res. *293*, 177-187.

Tsai,P.S., Garcia-Gil,N., van,H.T., and Gadella,B.M. (2010). How pig sperm prepares to fertilize: stable acrosome docking to the plasma membrane. PLoS. One. *5*, e11204.

Vicari,E., Perdichizzi,A., De,P.A., Burrello,N., D'Agata,R., and Calogero,A.E. (2002). Globozoospermia is associated with chromatin structure abnormalities: case report. Hum. Reprod. *17*, 2128-2133.

World Health Organization. WHO laboratory manual for the examination of human semen and semen-cervical mucus interaction. 1999. Cambridge U.P.
Ref Type: Generic

Xiao,N., Kam,C., Shen,C., Jin,W., Wang,J., Lee,K.M., Jiang,L., and Xia,J. (2009). PICK1 deficiency causes male infertility in mice by disrupting acrosome formation. J. Clin. Invest *119*, 802-812.

Xu,M., Xiao,J., Chen,J., Li,J., Yin,L., Zhu,H., Zhou,Z., and Sha,J. (2003). Identification and characterization of a novel human testis-specific Golgi protein, NYD-SP12. Mol. Hum. Reprod. *9*, 9-17.

Xu,X., Toselli,P.A., Russell,L.D., and Seldin,D.C. (1999). Globozoospermia in mice lacking the casein kinase II alpha' catalytic subunit. Nat. Genet. *23*, 118-121.

Yao,R., Ito,C., Natsume,Y., Sugitani,Y., Yamanaka,H., Kuretake,S., Yanagida,K., Sato,A., Toshimori,K., and Noda,T. (2002). Lack of acrosome formation in mice lacking a Golgi protein, GOPC. Proc. Natl. Acad. Sci. U. S. A *99*, 11211-11216.

Yoon,S.Y. *et al.* (2008). Human sperm devoid of PLC, zeta 1 fail to induce Ca(2+) release and are unable to initiate the first step of embryo development. J. Clin. Invest *118*, 3671-3681.

Yoshinaga,K. and Toshimori,K. (2003). Organization and modifications of sperm acrosomal molecules during spermatogenesis and epididymal maturation. Microsc. Res. Tech. *61*, 39-45.

Zhang,Q. *et al.* (2007). Rapid evolution, genetic variations, and functional association of the human spermatogenesis-related gene NYD-SP12. J. Mol. Evol. *65*, 154-161.

Zhang,X., Lei,K., Yuan,X., Wu,X., Zhuang,Y., Xu,T., Xu,R., and Han,M. (2009). SUN1/2 and Syne/Nesprin-1/2 complexes connect centrosome to the nucleus during neurogenesis and neuronal migration in mice. Neuron *64*, 173-187.

Zhou,K. and Hanna-Rose,W. (2010). Movers and shakers or anchored: Caenorhabditis elegans nuclei achieve it with KASH/SUN. Dev. Dyn. *239*, 1352-1364.