RESEARCH REPORT:
Genetic Predisposition to DTT-induced DNA Decondensation

Anna Aletta Fouché

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INDEX

ABSTRACT

Male infertility may be due to oligozoospermia, asthenozoospermia and teratozoospermia. Intracytoplasmic sperm injection is used to address male infertility. However, the percentage of viable embryos obtained by this technique is very low.

Pronucleus formation has been identified as one of the key events in fertilisation and gamete decondensation is vital for this process to take place. Decondensation can be initiated by chemicals such as DTT that reduce the disulphide groups between the protamine proteins that keep the DNA of the gamete condensed. An increase in decondensation should translate into a higher fertilization rate and a higher yield of embryos. The research from this thesis has compared the decondensation ability via DTT in human spermatozoa and bovine spermatozoa, to study pronucleus formation in bovine zygotes and bovine embryo formation in the presence of DTT; and lastly the cytotoxic effect of DTT using somatic cells in culture has been investigated.

In this study 12 semen samples for either fertile or subfertile subjects were collected, isolated and exposed to 25 mM DTT for 0, 5, 7, and 10 minutes, washed and the morphological changes associated with decondensation was evaluated by phase contrast microscopy. After 5 and 7 minutes 11 of the 12 samples underwent decondensation while after 10 minutes several samples showed a lower rate of decondensation and this was associated with and unusual hypercondensed state, CMA_3 staining revealed all spermatozoa samples evaluated were mature. However, after treatment with DTT for 5, 7 and 10 minutes an increase in fluorescence was observed indicating increased protamine thiol group reduction and subsequently increased CMA_3 accessibility. For some samples reduced fluorescence was observed possible due to the supercoiling of the DNA.

DTT successfully induces decondensation of human spermatozoa, however does this lead to the formation of viable embryos? Due to ethical issues associated with working with human embryos all further studies were done using bovine embryos. Spermatozoa used were derived from Friesian bulls and the samples were pooled to prevent sample bias and interindividual variation. Spermatozoa were exposed to 25 mM of DTT at 5, 7, and 10 minutes as used for human spermatozoa. No decondensation was observed using the same conditions as for human spermatozoa, therefore the ‘swim up’ medium containing heparin and regularly used

in IVF procedures for bovines was used, and this resulted in successful decondensation of bovine spermatozoa after 30 minutes.

The effects of DTT on pronucleus formation and embryo development were evaluated in three bovine specimens. In the first group, DTT had no significant effect on the parameters measured, namely the number of oocytes that were in metaphase II, with one pronucleus, with two pronuclei, with degeneration of the nucleus and polyspermia. In the second group the percentage cleavage and embryo formation was determined on Day 1 (group 2) and 7 (group 3) respectively and statistical differences were obtained between the control and the DTT group. DTT had no significant effect on all the early parameters measured however later in development DTT had a significant adverse effect on cleavage and eventual embryo development.

Cleavage and embryo formation is a process of multiple mitotic divisions resulting in an increase in the number of cells that become smaller with each cell division, while somatic cells also undergo mitotic division although the cell size remains constant. Therefore the L929 cell line, a standardized system used to test toxicity, can be used to investigate the toxic effects of DTT on a dividing cell population. In this study L929 cells were expose to 25mM DTT for 30 minutes, and lysosomal membrane integrity, cell viability and number was determined immediately following exposure and after 48 hours growth. In another experiment the L929 cell line was exposed to all concentrations used in this and other studies for 5, 10 and 20 minutes. At all concentrations and exposure times DTT was found to be cytotoxic to the L929 cell line. How exactly DTT mediates this toxic effect is unknown, however due to its high solubility DTT can cross the cell membranes. The tertiary structure of proteins, enzymes and DNA is vulnerable to the reducing effects of DTT. In conclusion, although DTT induces decondensation in human and bovine spermatozoa, in the bovine model it does not lead to viable embryo formation and this has been confirmed in cell culture where DTT at all concentrations used was found to be cytotoxic.

CHAPTER 1: INTRODUCTION

Successful reproduction is essential for population survival. To understand the processes that determine population increase or decrease, it is vital to understand the external and internal factors which affect reproductive function. In humans infertility is a condition that affects males as well as females. The occurrence of subfertility in couples is approximately 10% to 16% and they subsequently seek medical help. In about 20% to 25% of these cases, the male partner is the cause of the infertility (De Kretser *et al* 1999). Male infertility may be related to oligozoospermia. This is the occurrence of more than 20 million spermatozoa with poor motility (asthenozoospermia) together with a great number of malformed (teratozoospermia) spermatozoa per ml semen. ICSI (intracytoplasmic sperm injection) is one of the techniques used in IVF (*in vitro* fertilization) and it is also one of the methods used to address infertility. ICSI performed with spermatozoa considered morphologically normal spermatozoa, however, does not always result in fertilization.

Although IVF is the answer to many of these problems the percentage of viable embryos obtained by the technique is very low. Gamete decondensation of DNA is an early important event in the process of fertilisation and this process leads to the pronuclear formation just before fusion of the male and female genetic material (Esterhuizen, *et al*. 2002). The causes of defective or incomplete decondensation are either environmental or genetic. Decondensation can be initiated artificially by several different chemicals such as dithiothreitol (DTT) (Chevret, *et al*. 1994)(Martini, *et al*. 1995)(Rousseaux, *et al*. 1995). DTT is a reducing agent of thiol groups and therefore cause double stranded DNA to decondense resulting in pronucleus formation (Perreault, *et al*. 1987) (Rho, *et al*. 1998) (Suttner *et al.* 2000). If DTT is able to induce decondensation then the fertilization success percentage should increase and a higher rate of embryo formation would be obtained. Pronucleur formation in the presence of DTT is an indication of successful fertilization but not necessarily an indication of embryo formation. To study this process and the effect of a decondensing agent such as DTT the use of human tissue is limited due to ethical issues. Animal models will allow the evaluation of decondensing agents on gametes and the process through to embryo formation can be evaluated.

Animal models are therefore the next choice and due to the need to replenish and improve bloodlines in populations of cattle and other farm animals, fertilisation techniques are well established. These animals are genetically similar and gametes are derived from healthy, fertile animals. Furthermore, researchers wishing to develop techniques for human IVF procedures have found that this is a rich source of material to develop methods and procedures as has been seen in the field of animal cloning.

The research from this thesis will therefore attempt to compare the decondensation ability via DTT in human spermatozoa and bovine spermatozoa; study pronucleus formation in bovine zygotes and bovine embryo formation in the presence of DTT and study the cytotoxic effect of DTT using somatic cells in culture.

CHAPTER 2: LITERATURE REVIEW

Background:

Fertilization is a complex and tightly regulated process whereby two haploid gametes eventually form a diploid organism. Artificial insemination has its origins in Russia in 1899 and the first scientific papers on artificial insemination in horses were published in 1922. From 1940-1950 artificial insemination had become an established industry due to improved methods for the freezing and thawing of sperm. Human, *In vitro* fertilization (IVF) was developed in the United Kingdom by Doctors Patrick Steptoe and Robert Edwards. The first so-called "test-tube baby", Louise Brown, was born in Oldham, England, on July 25, 1978 amid intense controversy over the safety and morality of the procedure.

IVF, artificial insemmination and other related processes have found a wide range of applications in agriculture to increase the yield of domestic animals, improve the genetic composition of herds, while in environmental sciences this technique is used to save rare species and to develop disease free animal strains. The application of these techniques in human biology allows individuals with reproductive problems to have healthy offspring. The development of better and new techniques to increase the success rate of in vitro fertilisation is continuously being investigated. The purpose of this study is to determine the effectiveness of DTT as a decondensing agent to promote pronucleas formation and the eventual formation of a diploid organism in order to increase the rate of fertility. Therefore gamete development and fertilisation in several species will be discussed and several animal models used in to study and develop better artificial fertilization techniques will also be discussed.

Development and morphology of spermatozoa:

Development of spermatozoa

Spermatogenesis is the process of spermatozoa formation from spermatogonia and is the process during which the basal cells of the tubuli seminiferi divide and differentiate into spermatozoa cells found in the lumen of this structure. Spermatogonia are the stem cells from which spermatozoa develop and are found near the basement membrane and mitotically divide into spermatogonia type A and type B (Kelly *et al*. 1984)(Sadler 1995)(Fawcett 1986). Type A spermatogonia undergo mitotic divisions and are a source for the provision of constant stem cells. Type B spermatogonia give rise to primary spermatocytes through mitotic cell

division (Sadler 1995) (Kelly *et al*. 1984) (Fawcett 1986). These primary spermatocytes then undergo first meiotic division, to become diploid and are then known as secondary spermatocytes (possessing a haploid number of chromosomes) (Kelly *et al*. 1984) (Sadler 1995) (Fawcett 1986). A second meiotic division then occurs to form spermatids. At this stage cytokinesis is incomplete and cytoplasmic bridges join the successive cell generations. The spermatids remain embedded in deep recesses of Sertoli cells, where these cells (Kelly *et al*. 1984) (Sadler 1995) (Fawcett 1986) provide support, protection and nutrition of the germ cells and eventually assist in the release of mature spermatozoa (Sadler 1995).

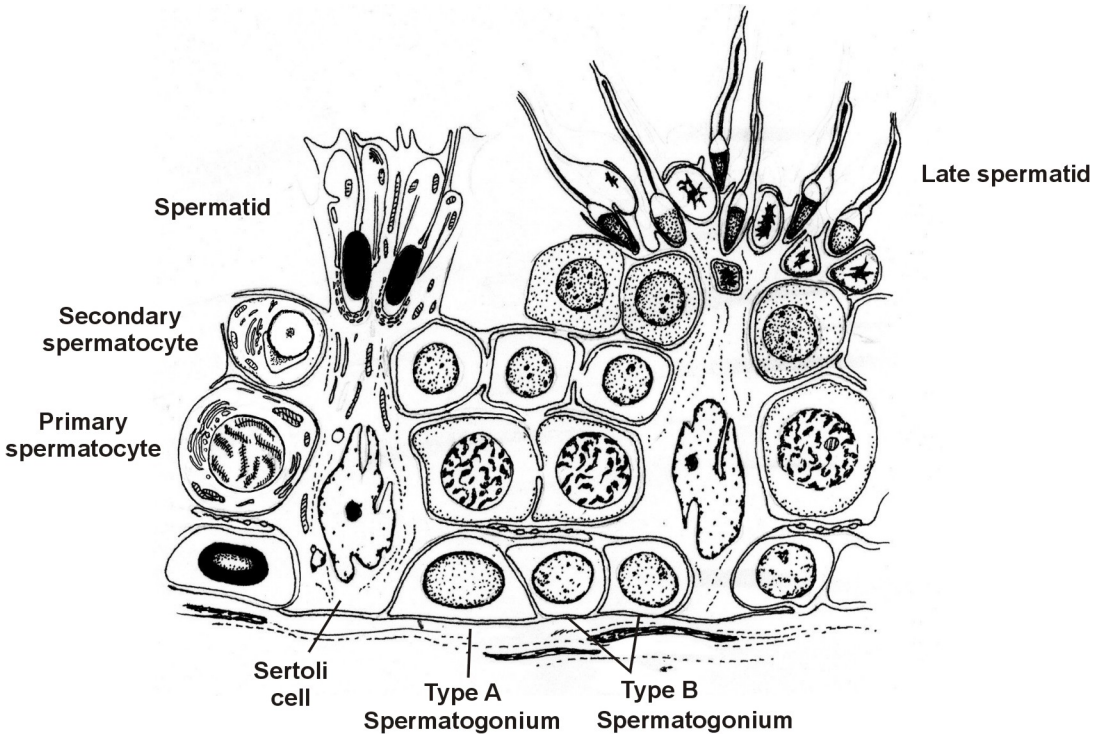

Fig. 1 The process of spermatogenesis modified from Sadler (1995)

The process of spermiogenesis follows spermatogenesis, which is the differentiation of spermatozoa from spermatids (Kelly *et al*. 1984) (Sadler 1995) (Fawcett 1986). To the one side of the spermatid is a well-developed Golgi apparatus, from the Golgi apparatus a number of secretory vesicles form that fuse to form the acrosome vesicle (Kelly *et al*. 1984) (Fawcett 1986) at the anterior pole of the nucleus. The acrosome membrane of the acrosome vesicle begins to grow along with the nuclear membrane to form a cap that encloses two thirds of the nucleus (Nikolettos *et.al.* 1999) (Kelly *et al*. 1984) (Sadler 1995) (Fawcett 1986). The acrosome consists of two parts, the small hemisphere at the anterior pole of the nucleus and the cap that was described

above. Enzymes present in the acrosome enable the spermatozoa to move between the corona radiata cells, which surrounds the ovum. With these enzymes it is possible for the spermatozoa to penetrate the zona pellucida of the ovum (Sadler 1995)(Fawcett 1986).

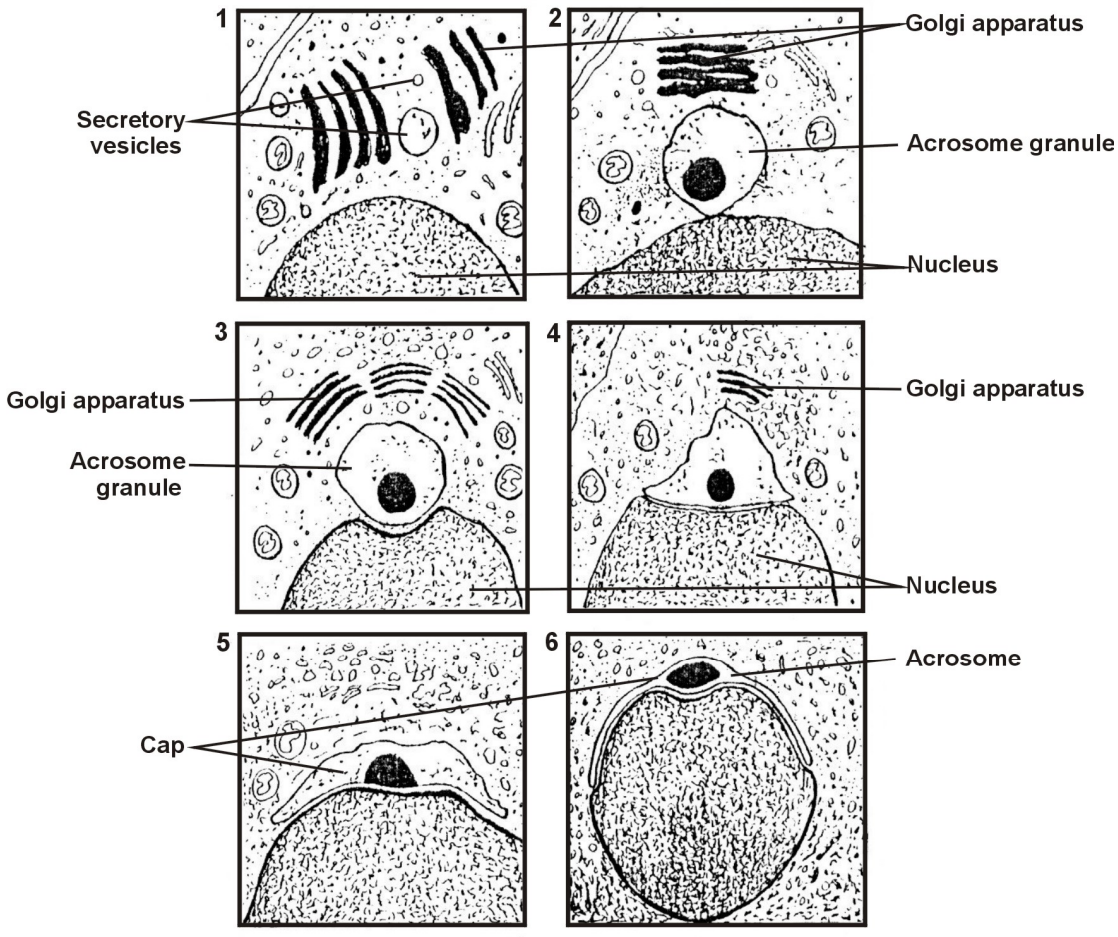

Fig. 2 The acrosome formation process taken from Coetzee, *et al*. 1993

The centrioli of the cell moves from the inner cell surface, to the posterior pole of the nucleus. The microtubules of the spermatozoon's flagellum grow out of the centriole that is situated at right angles to the nuclear membrane as well as the cell membrane (Gilbert 1994), (Fawcett 1986). The microtubules, which project caudally is called the manchette (Kelly *et al*. 1984) and these microtubules project from a ring like structure that surrounds the nucleus. During the formation of the flagellum, a ring-shaped structure called the annulus develops around the centioli and migrates for a short

distance along the flagellum. As the annulus migrates, it is followed by the cell's mitochondria, which arrange themselves in a spiral manner around the microtubules of the flagellum (Gilbert 1994), (Fawcett 1986). Other structures such as dense fibers of the spermatozoa also develop during the formation of the flagellum.

Most of the cytoplasm is now required and accumulates near the proximal part of the flagellum where it is jettisoned (Gilbert 1994). Sertoli cells phagocytize these residual bodies. During spermiogenesis the cytoplasmic bridges between the spermatozoa cells disappear and the spermatozoa are released by the Sertoli cells and are now found in the lumen of the tubuli seminiferi (Gilbert 1994).

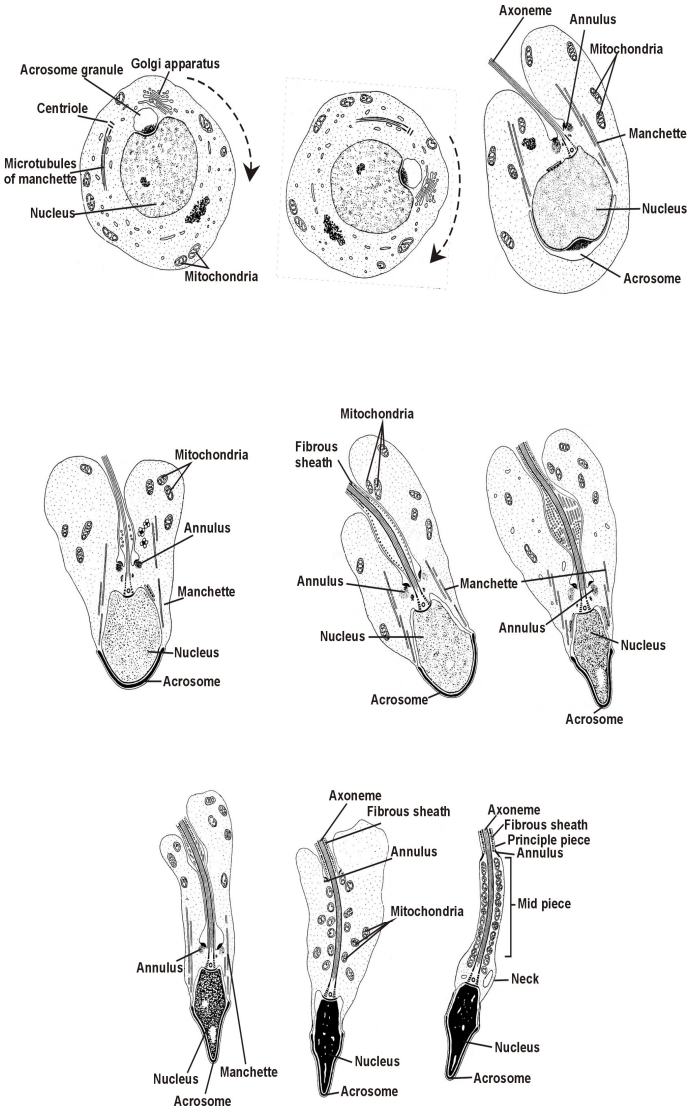

Fig. 3 The process of spermiogenesis modified from Kelly *et al*. (1984)

After leaving the male reproductive tract, the spermatozoa undergoes a final maturation phase called capasitation, before it can fertilize the ovum. This process may occur either in the female reproductive tract or in a defined medium *in vitro* and thereby acquiring the ability to fertilize homologous ova (Gwatkin 1977) (Burks *et.al.* 1992)

Morphology of spermatozoa

Following capasitation each spermatozoon consists of a haploid condensed nucleus, a perinucleur theca, an acrosome and a propulsion system to move the head and nucleus (Gilbert 1994). During the maturation process of the spermatozoon, the haploid nucleus has become streamlined, the DNA becomes tightly compressed and the flattened nucleus takes on a dark, oval shape. The acrosome is found anteriorly. The outer acrosomal membrane occurs immediately beneath the cell membrane and is continuous at the posterior margin of the cap with the inner acrosomal membrane that in turn is close to the nuclear envelope. The two acrosomal membranes enclose a narrow cavity containing enzymes and complex carbohydrates (Fawcett 1975). Between the nucleus and the acrosome, a distinct extraction-resistant cytoplasmic matrix, the perinuclear theca is found (Fawcett 1975) (Courtens, *et al*. 1976)(Longo *et.al.* 1987) (Oko *et.al.* 1988) (Paranko *et.al.* 1995).

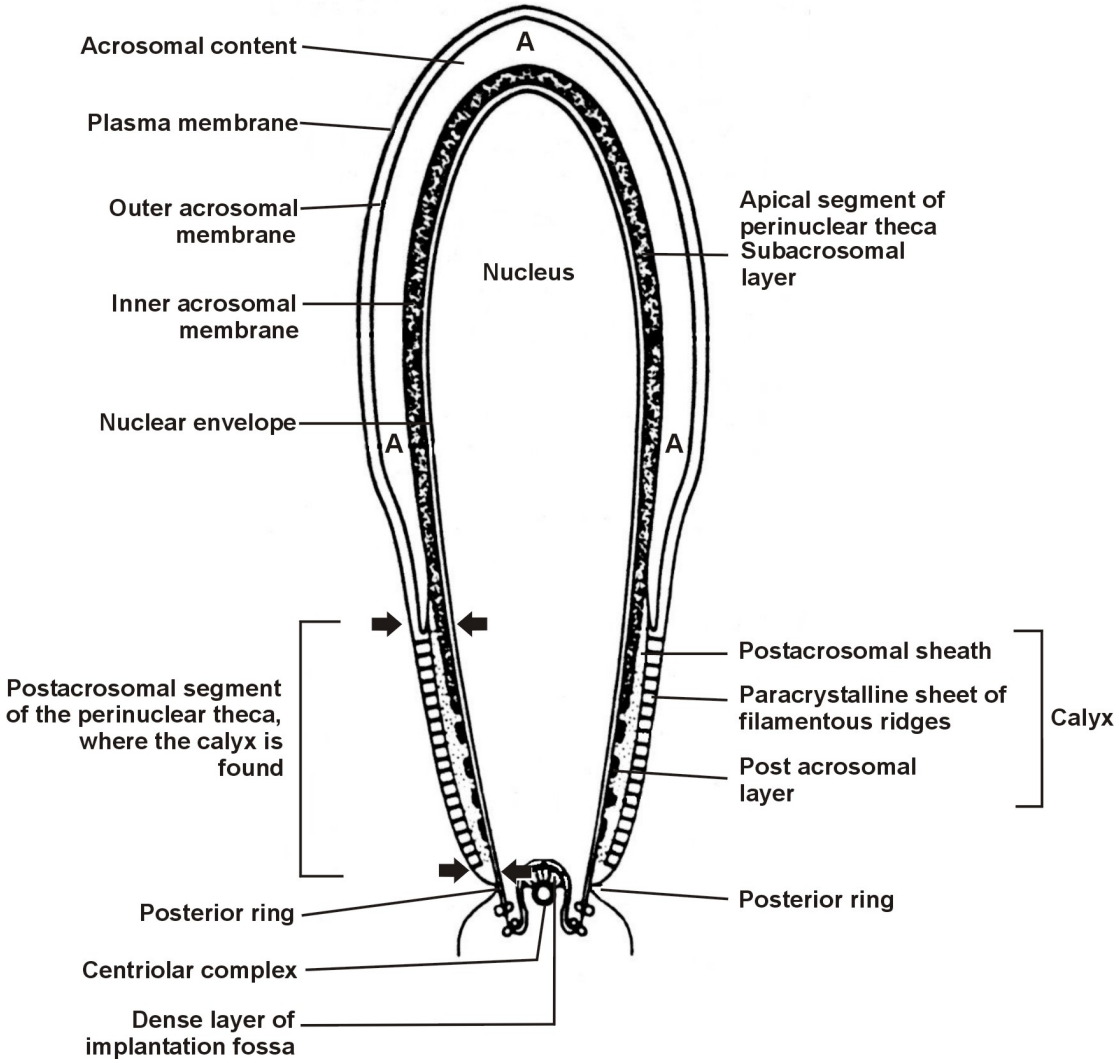

Fig. 4 Composition of the spermatozoon head taken from Longo *et.al.* (1987).

The perinuclear theca can be divided into an amorphous subacrosomal layer sandwiched between the nucleus and the acrosome, and a rigid postacrosomal segment, the calyx. (Longo *et al*. 1987)(Paranko *et al.* 1995) (Longo *et al.* 1991). The calyx is subdivided into three layers. The first is close to the nuclear envelope and continuous with the subacrosomal layer, the second is a lamina, the postacrosomal sheath, which is connected to the plasma membrane by the third layer, the paracrystalline sheet of filamentous ridges. The two compartments, namely the subacrosomal layer and the calyx together form a continuous covering over the nucleus and share a common group of extraction-resistant basic cytoskeletal polypeptides, called multiple-band proteins (Longo *et.al.* 1987)(Longo *et.al.* 1991) (Paranko *et.al.* 1995). Due to the specific biochemical properties and the close association of perinuclear theca proteins with the nuclear envelope and overlying

membranes, there has been interest in their role in the structural modelling of the spermatozoon head and in fertilisation. The plasma membrane covers the whole spermatozoon from head to tail (Nikolettos *et.al.* 1999), The acrosome together with the perinuclear theca, nucleus and plasma membrane constitutes the head of the spermatozoon.

Immediately behind the spermatozoon head is the connecting piece. This complex structure has a dense, convex articular region called the capitulum, which conforms to the concavity of the basal plate lining the implantation fossa of the nucleus (Fawcett 1975).

The spermatozoon is able to move by beating its flagellum that is powered by the axoneme, which is formed by microtubules emanating from the centriole. The protein tubulin forms the basis structure of the flagellum but the protein dynein that is bound to the microtubules in the flagellum provide the force for spermatozoal propulsion (Gilbert 1994).

The flagellum can be divided into 3 different regions, the mid piece or the axoneme, the main/principle piece and the tip.

The core of the mid piece consists of two central microtubules surrounded by a row of nine doublet microtubules (Nikolettos *et.al.* 1999). Mitochondria in the mid piece are arranged tip to tip in a spiral around the microtubules and provide energy for the movement of the flagellum. A layer of dense fibers occurs between the microtubules and the mitochondria. This layer of dense fibers decreases in thickness towards the end of the flagellum and probably stabilises the spermatozoa flagellum preventing the spermatozoon head from detaching from the flagellum. At the annulus, the flagellum narrows to form the longest part of the tail, called the main piece. The main piece may be up to 45µm long and the dark fibers are surrounded by a large number of c-shaped fibers. The tip of the flagellum is about 5µm long and is thinner than the main part since it is not surrounded by any additional structures.

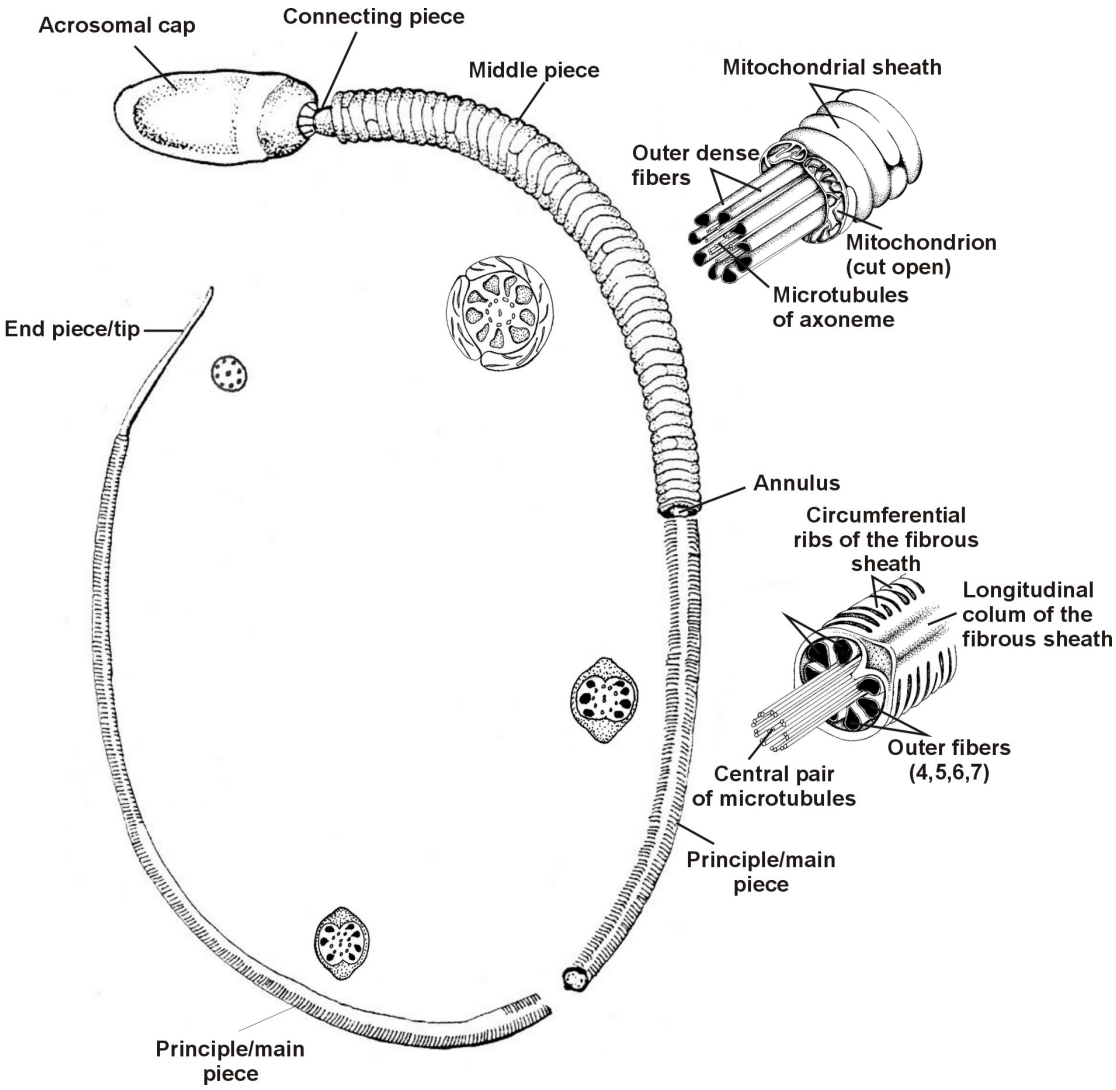

Fig. 5 Spermatozoon morphology modified from Fawcett (1975)

Development and morphology of oocytes

Development of oocytes

Oocytes develop by a process of oogenesis that begins relatively early in fetal development (Wasserman *et al.* 1994) and ends months to years later in the sexually mature adult (Wasserman *et al.* 1994). Oogenesis begins with primordial germ cell formation and encompasses a series of cellular transformations. The development of primordial germ cells into oogonia and from oogonia to oocytes takes place in the fetus. The final stage of the development of oocytes into ova takes place in the adult.

Prenatal maturation

Primordial germ cells reach the surface epithelium of the gonad of a genetic female and move into the cortex. The primordial germ cells, together with some supporting epithelial cells, give rise to the cortical sex cords (Wasserman *et al.* 1994). On completion of migration of primordial germ cells, virtually all the cells differentiate into oogonia in the sex cords (Sadler 1995)(Wasserman *et al.* 1994), undergo a number of mitotic divisions and at the end of the 3rd month of development, become arranged in clusters. A layer of flat/squamous epithelial cells surrounds these clusters (Sadler 1995). All the oogonia in one cluster are probably derived from a single primordial germ cell, but the squamous epithelial cells, known as follicular cells, originate from surface epithelium that covers the ovary (Sadler 1995).

The majority of oogonia continue to divide by mitosis and during the next few months the oogonia increase rapidly in number, and by the 5th month of development, the total number of germ cells in the ovary reaches its maximum, estimated at 7 million (Sadler 1995)(Gilbert 1994). Some of the oogonia differentiate into much larger cells called primary oocytes. Immediately after the formation of the primary oocytes, they replicate their DNA and enter prophase of the 1st meiotic division (Sadler 1995) (Gilbert 1994). Shortly after this process, cell death begins, and many oogonia as well as primary oocytes become atretic and die. The majority of oogonia have degenerated by the 7th month, except for a few oogonia near the surface. All the surviving primary oocytes have entered the 1st meiotic division, and a layer of flat/squamous epithelial cells surrounds most of them individually. A primary oocyte, together with its surrounding flat/squamous epithelial cells, also known as granulosa cells (Fawcett 1986), is known as a primordial follicle (Fawcett 1986)**,** (Gilbert 1994) (Sadler 1995).

Postnatal maturation

Just before birth, all primary oocytes have started prophase of the 1st meiotic division, but instead of proceeding into metaphase they enter the diplotene stage (Sadler 1995) (Gilbert 1994)(Gwatkin 1977). Diplotene is a resting stage during prophase that is characterized by a lacy network of chromatin. The primary oocytes remain in this stage without completing the 1st meiotic division until puberty is reached (Sadler 1995). According to Sadler, a substance secreted by follicular cells, oocyte maturation inhibitor (OMI), are responsible for the remaining of oocytes in the diplotene stage. The total number of primary oocytes at birth is estimated to vary

from 700 000 to 2 million and only about 400 000 of these will still be present at the onset of puberty while fewer than 500 of these will be ovulated in the reproductive lifetime of the individual.

Five to fifteen primordial follicles begin to mature with each ovarian cycle with the onset of puberty. The primary oocyte (still in the diplotene stage) begins to increase in size, while the surrounding flat/squamous follicular cells change to cuboidal shaped cells and proliferate to produce a stratified epithelium of granulosa cells. The follicle is now known as a primary follicle. The granulosa cells rest on a basement membrane, which separate them from the surrounding stromal cells, which form the theca folliculi. The granulosa cells together with the oocyte secrete a layer of glycoproteins on the surface of the oocyte, thus forming the zona pellucida. As the follicles continue to grow, the theca folliculi become organized into an inner layer of secretory cells, the theca interna, and an outer layer of connective tissue containing fibroblast-like cells, the theca externa. Small, finger-like processes of the follicular cells extend across the zona pellucida and interdigitate with microvilli of the plasma membrane of the oocyte. These processes are thought to be important for transport of materials from follicular cells to the oocyte.

Fluid-filled spaces appear between granulosa cells as development continues, and when these spaces coalesce, the antrum is formed. The follicle is now termed a secondary follicle. The antrum is initially crescent shaped, but with time, it greatly enlarges. Granulosa cells that surround the oocyte remain intact and give rise to the cumulus oophorus. The mature follicle, 10 mm or more in diameter, is known as the tertiary, vesicular, or Graaffian follicle. The Graaffian follicle is surrounded by the theca interna, which is composed of cells having characteristics of steroid secretion, rich in blood vessels, and the theca externa, which gradually merges with the ovarian stroma.

A number of follicles begin to develop with each ovarian cycle, but usually only one reaches full maturity. The others degenerate and become atretic. The primary oocyte resumes its 1st meiotic division, as soon as the follicle is mature. This leads to the formation of two daughter cells of unequal size, but each with 23 chromosomes. The secondary oocyte receives most of the cytoplasm while the other oocyte, called the 1st polar body, receives practically none. The latter is located between the zona

pellucida and the cell membrane of the secondary oocyte in the perivitelline space. The 1st meiotic division resumes shortly before ovulation.

At completion of the 1st maturation division, but before the nucleus of the secondary oocyte has returned to its resting stage, the cell enters the 2nd maturation division without DNA replication. The moment the secondary oocyte shows spindle formation with chromosomes aligned on the metaphase plate, ovulation occurs, and the oocyte is shed from the ovary. The 2nd maturation division is completed only if the oocyte is fertilized; otherwise, the cell degenerates approximately 24 hours after ovulation. Whether or not the 1st polar body undergoes a 2nd division is uncertain, but fertilized ova accompanied by three polar bodies have been observed.

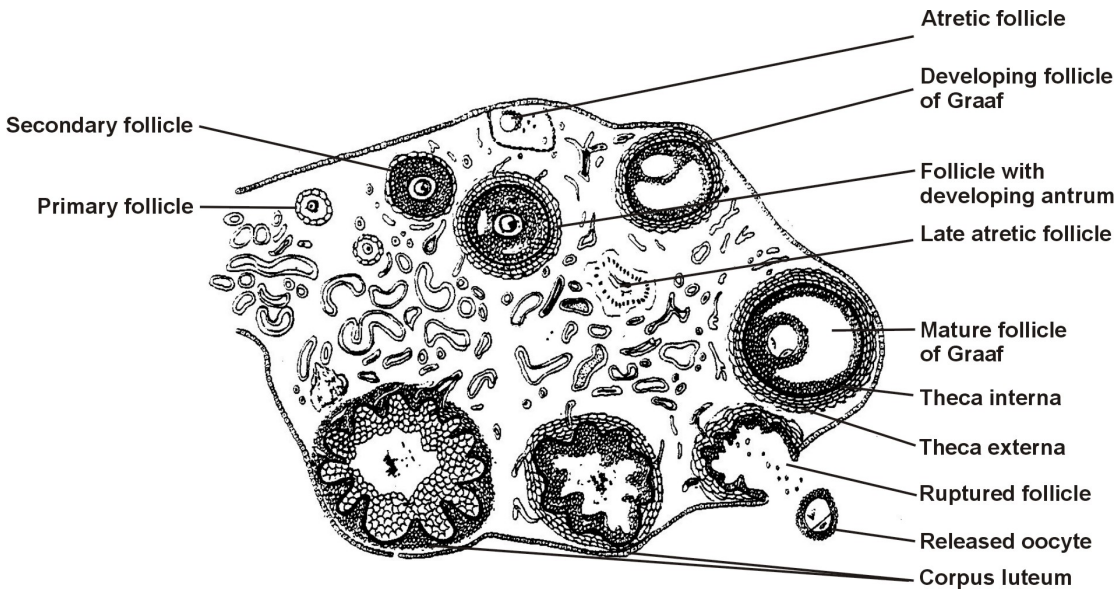

Fig. 6 Ovulation taken from Coetzee, *et al*. 1993

Morphology of oocytes

The primary oocyte is found in the Graaffian follicle and is a round cell of about 120-180µm in diameter. After meiosis II is completed and just before ovulation, this primary oocyte changes into a secondary oocyte, also known as the ovum (cords (Assey *et.al.* 1994)(De Loos *et al* 1991). In contrast to the spermatozoa that has become streamlined during development, all the material required for the beginning

of growth and development after ovulation, is stored in the mature ovum. Besides a haploid nucleus, the ovum cytoplasm includes the following: proteins, ribosomes and tRNA, messenger RNA, morphogenic factors and protective chemicals (Gilbert 1994).

Two protective membranes, the plasma membrane and the zona pellucida, enclose the cytoplasm. The inner plasma membrane regulates the flow of certain ions during fertilization and aids fusing with the spermatozoon plasma membrane (Gilbert 1994). The plasma membrane also has microvilli that protrude through the zona pellucida between the microvilli of the surrounding granulosa cells (Gordon 1994) (Assey *et.al.* 1994) (De Loos *et al.* 1991)**.**

The zona pellucida (ZP) is found outside the plasma membrane where it forms a fibrous weaved area around the ovum. This envelope contains three glycoproteins termed ZP1, ZP2 and ZP3. ZP3 appears to play a key role in the binding of spermatozoa to the surface of the zona pellucida (Gordon 1994) (Burks *et.al.* 1992). The ZP is an open network of fibrils that permits free passage of large molecules. It is supplemented by extensions of membrane glycoproteins from the plasma membrane and by proteinasious vitelline posts that joins the zona pellucida to the membrane. The ovum is further surrounded by a cumulus consisting of granulosa cells, the innermost layer of cumulus cells, immediately adjacent to the zona pellucida, is called the corona radiata that consists of columnar shaped cells (Wasserman *et al.* 1994). In later stages the cytoplasm condenses and then the plasma membrane is a thicker and smoother membrane, known as the vitteline membrane (Wasserman *et al.* 1994)**.**

The ZP is a relatively thick, transparent, perforated, glycoproteinacoius shell with multiple functions (Singh, *et al*. 1986) and contains proteins that allows binding of species specific sperm, blocks and protects the conceptus during early stage of development.

The glycoproteins that comprise the ZP, seems to be secreted exclusively by the oocyte prior to ovulation, with some components from oviductal epithelial cells being added after ovulation (Burks *et.al.* 1992) (Barros *et.al.* 1996)(Riddell, *et al*. 1993)(Stringfellow *et.al.* 1995).
Follicular fluid is a transudate, across the follicle basement membrane. It accumulates in the antrum by coalescing of smaller fluid pockets. The transudate is

modified by follicular metabolic activities and contains specific constituents such as steroids and glycoproteins synthesised by the follicular wall (Betteridge *et.al.* 1988) (Shisong, *et al*. 1989).

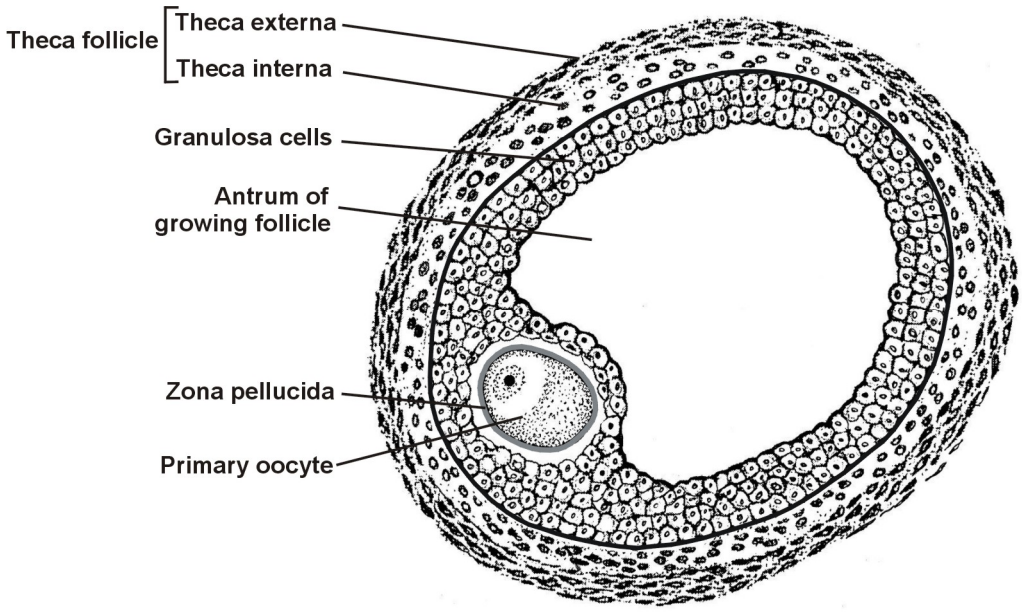

Fig. 7 Diagrammatic representation of an oocyte taken from Coetzee, *et al*. 1993

Fertilization of an oocyte with a spermatozoon

Fertilisation can be divided into six specific processes namely:

1. Spermatozoa capacitation
2. Gamete binding
3. Induction of the acrosomal reaction
4. Secondary binding of spermatozoa to the zona pellucida
5. Fusion of the ovum and spermatozoa plasma membranes
6. DNA decondensation-recondensation processes during fertilization

1. Spermatozoa capacitation

Capasitation is a process that takes place in the female reproductive tract. For *in vitro* fertilization purposes one must be able to perform *in vitro* capasitation. In the next paragraphs *in vivo* capasitation will be discussed. The research done by Austin 1951 will be discussed. They were the first researchers to discuss techniques pertaining to decapacitated spermatozoa and its uses in *in vitro* fertilization. In their technique,

spermatozoa were capacitated in the female repoductive tract (therefore *in vivo*) and were then removed followed by *in vitro* fertilization.

In vivo Capasitation

Capasitation is a sequence of biochemical and molecular changes leading to the destabilization of the spermatozoa membranes, which enables spermatozoa to undergo the acrosome reaction in response to binding to the ZP. The ejaculated spermatozoa are not able to fertilize oocytes without capasitation. The functional changes include the removal of seminal plasma proteins adsorbed to the spermatozoa plasma membrane (Gwatkin 1977) (Johnson *et al*. 1972), and the modification and/or reorganization of spermatozoa surface molecules (Tulsiani *et.al.* 1997).

The exact changes on biochemical and molecular level are still unknown (Saling 1989) (Gilbert 1994). Some of the biochemical changes include: increased adenylate cyclase activity and cAMP levels, an increase in intracellular pH, calcium influx (Tulsiani *et.al.* 1997), the loss of surface components such as proteins and carbohydrates (Gilbert 1994) (Tulsiani *et.al.* 1997), modification/alteration of the spermatozoa plasma membrane and changes in the lectin-binding pattern of spermatozoa. (Tulsiani *et.al.*1997). Other changes include changes in the lipid composition of the cell membrane. The fluidity of the cell membrane of spermatozoa may be altered through this process. According to Gilbert (1994) the concentration of cholesterol in the spermatozoon plasma membrane in several species, is lowered during spermatozoal capacitation (Gilbert 1994). Two proteins found both in serum and in the female reproductive tract (albumin and lipid transport protein 1) have recently been found to remove cholesterol from the human spermatozoal plasma membrane (Gilbert 1994). Phosphorylation of certain proteins involved in binding the spermatozoa to the ZP and mediating the exocytosis of the acrosomal vesicle has been observed concomitant with capacitation (Gilbert 1994). This phosphrylation may convert the inactive forms of these molecules into functional proteins.

Most researchers agree that capacitation results from complex, multiple molecular changes in the proteins/glycoproteins and lipid components of the spermatozoa plasma membrane (Tulsiani *et.al.* 1997). These changes are likely to modify ion channels present in the plasmalemma of spermatozoa and therefore cause a transmembrane flux of ions that are believed to be important in initiating the events of

capasitation, hyperactivation (vigorous movement of the neck and tail) and acrosome reaction. According to Wasserman (Tulsiani *et.al.* 1997), capacitation and hyperactivation in the mouse take place before sperm-zona interaction, and the acrosome reaction is initiated after sperm-ovum interaction. Indications are that these processes occur independently and are likely to involve region-specific changes in spermatozoa plasma membrane. (Tulsiani *et.al.* 1997)

The precise site of capacitation may be different in different species. Most spermatozoa of mammalian species become hyperactive in the isthmus region of the oviduct (Tulsiani *et.al.* 1997). This hyperactive movement of the spermatozoa enables the spermatozoa to break free from the oviductal wall (Van Soom *et.al.* 1996) and migrate from the isthmus to the ampulla and be retained at the site of fertilization (Van Soom *et.al.* 1996). Hyperactivation is seen in rodents; for cattle however, no hyperactivation has been described for spermatozoa, although accelerated progressive spermatozoa movement has been detected after the incubation of spermatozoa with ampullary oviductal fluid (Van Soom *et.al.* 1996). The possible function of hyperactivation could be that the hyperactivated beating pattern of a spermatozoon will enhance its thrust at the site of its binding to ZP. According to Tulsiani *et.al.* (1997) the enhanced thrust is likely to be partially important in order for the spermatozoon to penetrate ZP and fuse with the ovum plasma membrane.

In cattle the spermatozoa form a type of reservoir in the caudal isthmus of the oviduct, while migrating through the female genital tract. Capacitated spermatozoa are released from the reservoir into the ampulla in numbers seemingly adjusted to the number of oocytes ovulated. This implies that the capacitation of spermatozoa is modulated by the events of ovulation (Van Soom *et.al.* 1996). The ampulla is furthermore a destabilizing environment for the spermatozoa and therefore promotes the acrosome reaction and hence the spermatozoa's fertilization ability (Van Soom *et.al.* 1996).

When ovulation takes place, two different matrices envelope the oocyte. The fertilizing spermatozoa must both conquer the cumulus cells and the ZP in order to fuse with the oocyte's membrane. Cattle oocytes, however, recovered from the oviducts within a few hours of ovulation are usually completely devoid of cumulus cells (Van Soom *et.al.* 1996), so the only barrier to be taken by the spermatozoa is

the ZP. The fertilizing spermatozoa bind to the ZP and exocytosis of the acrosome is triggered. According to van Soom, a change in spermatozoa membrane permeability to calcium appears to be the primary signal for the start of the acrosome reaction after capacitation. A glycopeptide component of the zona pellucida is probably the physiological stimulus for this change (Van Soom *et.al.* 1996). The acrosomal enzymes are released and a narrow cleft is produced in the matrix of the zona. This cleft is sufficient for the hyperactivated spermatozoa to gain access to the oocyte plasma membrane, to which it fuses and thereby initiates fertilization (Van Soom *et.al.* 1996)

In vivo Capasitation but in vitro Fertilization

Austin 1951 provided much of the current knowledge on, amongst others, *in vitro* capacitation. They were the first to report successful fertilization of hamster ova *in vitro* by the use of spermatozoa recovered from the female reproductive tract after mating and the use of epididymal spermatozoa preincubated with oviductal secretions. Their studies suggested that there must be one or more factors present in the female genital tract, responsible for the induction of capasitation. Thibault and associates collected uterine spermatozoa and fertilize rabbit oocytes successful *in vitro*. Some authors collected the secretions from the oviduct of oestrous females. This demonstrated to be most efficient in rendering the functional changes in spermatozoa (Gordon 1994). After the initial research by Austin 1951 many authors followed to conduct research on this topic (Tulsiani *et.al.* 1997)(Van Soom *et.al.* 1996).

In vitro Capasitation

Early investigators used biological fluids, for example ocviduct fluid, follicular fluid and blood serum, to capasitate spermatozoa *in vitro* (Gwatkin 1977).

Spermatozoa can also be capacitated *in vitro* in chemically defined medium (Tulsiani *et.al.* 1997). This medium should contain BSA and energy substrates, such as glucose and pyruvate, as well as components used in Krebs-Ringer bicarbonate medium (Tulsiani *et.al.* 1997). Albumin is the major protein in female genital tract secretions and is an important component during *in vitro* and *in vivo* capacitation (Tulsiani *et.al.* 1997). This protein is believed to facilitate capacitation by altering fatty acids and/or cholesterol from the spermatozoa plasma membrane.

As previously mentioned, changes that occur during *in vivo* spermatozoa capacitation takes place in the female genital tract. A possible explanation for these changes could be the association of the ovarian-specific glycoprotein (OGB) secreted from epithelial cells of the oviduct in several species (Tulsiani *et.al.* 1997). Abe *et al.* 1995 (Tulsiani *et.al.* 1997) reported the binding of the glycoprotein to the posterial region of the spermatozoa head, midpiece, and flagellum. This suggests that OGB could have a direct role in spermatozoa capacitation. Recent observations suggested that twice as many bovine spermatozoa were capacitated in the presence of bovine OGB than in its absence (Tulsiani *et.al.* 1997). Another observation was that, spermatozoa capacitated in the presence of OGB showed an increased ability to fertilize homologous oocytes. This result is consistent with the suggestion that the oviduct and its secretions play an important role in spermatozoal function.

The role of proteoglycans and glycosaminoglycans in the induction of capasitation

Substances located in female reproductive tract fluid may be responsible for spermatozoa capacitation. The glycosaminoglycans (GAGs) chondroitin sulfates, heparin-like material, hyaluronic acid and an unidentified GAG (presumably keratin sulphate) have been identified in flushings from female bovine reproductive tracts. GAGs are linear repeating disaccharide polymers, which exist primarily attached to a protein-core by a covalent bond (proteoglycans). Several studies in the last decade suggested that proteoglycans and glycosaminoglycans play a role in the induction of capasitation (Tulsiani *et.al.* 1997) (Parrish *et.al*.1985).

Heparin is the most effective glycosaminoglycan and binds to spermatozoa from several species including human and bovine (Tulsiani *et.al.* 1997) (Delgado *et al* 1982) (Gordon 1994). The binding of heparin to spermatozoa is dependent on pH, ionic strength and temperature (Tulsiani *et.al.* 1997). This binding reaction has characteristics resembling those of a typical receptor-ligand interaction. The molecular mechanisms responsible for these interactions, however, are not known. From the preceding discussion, it is apparent that capasitation reflects multiple changes in the plasma membrane of mature spermatozoa. Although molecular details of these changes vary among species, the end result is the development of hyperactivated motility, or in the bovine, accelerated progressive spermatozoa movement, and responsiveness of spermatozoa to undergo acrosome reaction (Tulsiani *et.al.* 1997).

During *in vitro* incubations, GAGs have been known to induce the acrosome reaction in bovine (Parrish *et.al*.1985) and rabbit spermatozoa (Parrish *et.al*.1985). Heparin, the most highly sulphated GAG, was the most potent inducer of acrosome reacrion (AR) (Parrish *et.al*.1985) (Tulsiani *et.al.* 1997) (Delgado *et al.* 1982)(Gordon 1994). Bovine epididymal spermatozoa treated with chondroitin sulphate A (CS-A) fertilized more ova than spermatozoa incubated only in a control medium (Parrish *et.al*.1985). The GAGs that are sulphated to varying degrees are structurally similar to the fucose polymer of the sea urchin jelly coat, which is known to induce the AR of sea urchin spermatozoa (Parrish *et.al*.1985).

2. Gamete binding

The ZP is the unique extracellular matrix surrounding the mammalian ovum. It is composed of sulphated glycoproteins formed during oogenesis (Barros *et.al.* 1996). During early follicular development, these glycoproteins are deposited between the oocyte and the granulosa cells. The ZP plays two very important roles during fertilization. Firstly, it acts as a binding site for spermatozoa and secondly it initiates the acrosome reaction after the spermatozoa is bound.

As previously mentioned, the zona pellucida is made up of ZP1, ZP2 and ZP3 glycoproteins (Gordon 1994)(Gilbert 1994)(Barros *et.al.* 1996). In the mouse at least it was shown that the carbohydrate moiety of ZP3 is the ligand for receptors located on the spermatozoa plasma membrane. The ZP3 binds to at least three adhesive proteins on the spermatozoal cell membrane (Gilbert 1994). Several proteins have been identified on the spermatozoa cell surface that specifically binds to the ZP3 carbohydrates.

3. Induction of the acrosomal reaction

The acrosomal reaction occurs after the spermatozoon has bound to the zona pellucida. The cross-linking of ZP3 with the receptors on the spermatozoal membrane induces the spermatozoon acrosomal reaction. This cross-linking opens calcium channels to increase the concentration of calcium in the spermatozoon. The mechanism by which ZP3 induces the opening of the calcium channels and the subsequent exocytosis of the acrosome remains controversial, but it may involve the receptor's activation of a cation channel, which would change the resting potential of the spermatozoa plasma membrane. The calcium channels in the membrane would

be sensitive to this change in membrane potential, allowing calcium to enter the spermatozoa. The reason for the acrosomal reaction taking place after binding to the zona pellucida, may be that the zona pellucida is a very thick structure so the spermatozoa is far removed from the ovum, with undergoing the acrosomal reaction directly on the zona, the spermatozoa is able to concentrate its proteolytic enzymes to lyse a hole in this envelope. Therefore, spermatozoa that undergo the acrosomal reaction before they reach the zona pellucida, are unable to penetrate it.

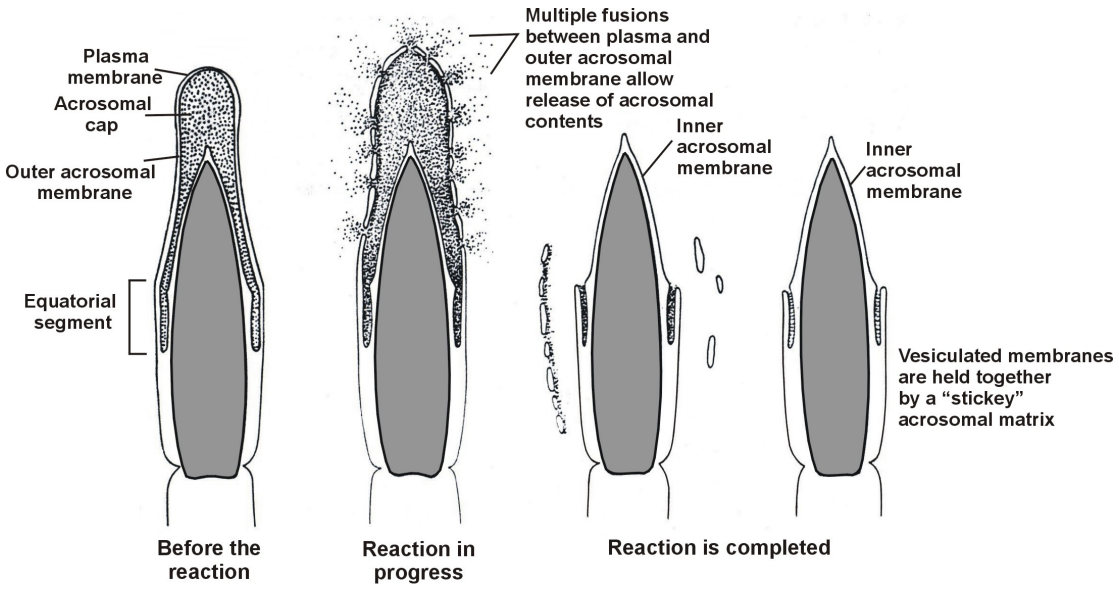

Fig. 8 Acrosome reaction modified from (Burks *et.al.* 1992)

4. Secondary binding of spermatozoa to the zona pellucida

During the acrosomal reaction, the anterior portion of the spermatozoa plasma membrane is shed from the spermatozoa (Gilbert 1994). This region (where the shedding occurred) is where the ZP3-binding proteins are located. The spermatozoa must, however, remain bound to the zona in order to lyse a path through it. It appears that proteins in the inner acrosomal membrane, which bind specifically to ZP2, accomplish the secondary binding to the zona (Bleil *et.al.* 1988). Therefore, acrosome-intact spermatozoa will not bind to ZP2, while acrosome-reacted spermatozoa will, since these proteins are then exposed to the ZP2. Moreover, antibodies against the ZP2 glycoprotein will not prevent the binding of acrosome-intact spermatozoa to the zona, but will inhibit the attachment of acrosome-reacted spermatozoa. The structure of the zona consists of repeating units of ZP3 and ZP2,

occasionally cross-linked by ZP1. It appears that the acrosome-reacted spermatozoa transfer their binding from ZP3 to the adjacent ZP2 molecules. After a spermatozoon has entered the ovum, the ovum cortical granules release their contents. One of the proteins released by these granules is a protease that specifically alters ZP2. This inhibits other acrosome-reacted spermatozoa from moving closer toward the ovum (Gilbert 1994).

De Ioannes (Barros *et.al.* 1996) suggested in 1990 that the spermatozoal mammalian ligand to ZP2 is the proacrosin/acrosin system due to serine-protease inhibitors preventing sperm-zona binding and due to acrosin having a high affinity for zona glycoproteins (Barros *et.al.* 1996)

Jones 1991 (Barros *et.al.* 1996) also report that proacrosin has properties analogous to those described for bindin, the sperm-ovum adhesion protein found within the acrosomal vesicle of sea urchin spermatozoa.

In porcine spermatozoa, secondary zona binding appears to be mediated by proacrosin. Proacrosin becomes the protease acrosin that has long been known to be involved in digesting the zona pellucida. However, proacrosin is also a fucose-binding protein that maintains the connection between acrosome –reacted spermatozoa and the zona pellucida (Gilbert 1994). It is possible that proacrosin binds to the zona and is then converted into the active enzyme that locally digests the zona pellucida.

5. Fusion of the ovum and spermatozoa plasma membranes

After recognition of the spermatozoa by the zona pellucida, the specific portion of the zona in the region of the spermatozoal head is lysed by the acrosomal enzymes (Gilbert 1994). Following the lysis of the zona pellucida is the fusion of the spermatozoa plasma membrane with the plasma membrane of the ovum (Gilbert 1994).

Yanagimachi 1970 (Gilbert 1994) described a similar fertilizasion process for mammals to that of the sea urchin described by Summers 1975 and Schatten 1980 (Gilbert 1994). Sperm-ovum fusion initiates the formation of the fertilization cone through the extension of several microvilli (Gilbert 1994). The fertilization cone in the

ovum extends due to polymerisation of actin. Polymerisation of actin also takes place in the acrosomal process of the spermatozoa. These two separate processes demonstrate the homology between the ovum and the spermatozoa.

The spermatozoa and ovum plasma membranes join, and material from the spermatozoa membrane can later be found on the ovum membrane (Gilbert 1994). The spermatozoa nucleus and tail pass through the resulting cytoplasmic bridge, which is widened by the actin polymerisation. In sea urchins all regions of the ovum plasma membrane are capable of fusing with spermatozoa, but in several other mammalian species, only a particular region of the ovum plasma membrane, is specialized for spermatozoa recognition and fusion.

The fusion of the spermatozoa and ovum plasma membranes is an active process. Specific 'fusogenic' proteins often mediate this fusion process. It is believed that fertilin is such a protein in mammals. The fertilin proteins in the spermatozoa plasma membrane are essential for sperm-ovum plasma membrane fusion (Gilbert 1994). Mouse fertilin is localized to the posterior plasma membrane of the spermatozoon head (Gilbert 1994). It adheres the spermatozoa to the ovum by binding to the □6□1 integrin protein on the ovum plasma membrane (Gilbert 1994). Moreover, like sea urchin bindin (bindin is not structurally related to fertilin), fertilin has a hydrophobic section that could potentially mediate the union of the two membranes (Gilbert 1994). Thus, fertilin appears to bind the spermatozoa plasma membrane to the ovum plasma membrane and then fuse the two plasma membranes. Several spermatozoa defects are noted in mice that are homozygous for mutant fertilin, one of them being the inability to fuse with the ovum plasma membrane (Gilbert 1994). When the membranes are fused, the spermatozoa's nucleus, mitochondria, centriole, and flagellum can enter the ovum.

6. DNA decondensation-recondensation processes during fertilization

Spermatozoa motility and morphology including concentration in a given ejaculate determines the fertility of humans. Recently it was discovered that the decondensation ability of the spermatozoon after entering the ovum is also of major importance in fertility (Esterhuizen, *et al*. 2002). On entering the ovum the DNA in the spermatozoon head is still compact (Samocha-Bone, *et al*. 1998) due to its binding to the nuclear protamines (Wyrobek, *et al*. 1990) (Colleu, *et al*. 1997). These protamines are incorporated into the DNA of the spermatozoon during

spermatogenesis to replace the somatic histones (Montag, *et al*. 1992) and so render greater stability to the mature spermatozoa.

Normal somatic cells contain histones that are involved in the packaging of DNA. The phosphate back bone of the DNA interacts with these small basic (+) proteins which consist of a high arginine and lysine content. The five classes of histones are H1, H2A, H2B, H3 and H4. Two molecules of H2A, H2B, H3 and H4 form a core around which 140 base pairs of double stranded DNA is wound to form a nucleosome. The nucleosome is complexed with H1 and further compacted as strings of nucleosomes wound into helical tubular coils namely the solenoid structure. Other proteins associated with DNA are 'non-histone chromosomal proteins', scaffold proteins and regulatory proteins. The degree to which chromatin is condensed is an important factor in determining whether or not structural genes are expressed and regulatory genes can produce their effect.

Protamines are nuclear proteins specific to human spermatozoa consisting of the amino-acid arginine and cysteine residues (Montag, *et al*. 1992)(Rodriguez, *et al*. 1985). One of their functions is to neutralize the DNA phosphate charges (in DNA of spermatozoa) to form a network of molecules in mature spermatozoa interconnected by intra- and interprotamine disulfide bridges. ((Colleu, *et al*. 1997)(Rodriguez, *et al*. 1985) (Molina, *et al*. 1995). The disulfide bridges originate from the cysteine residues in which they initially present as thiols. These thiols are oxidized to disulfides in the mature spermatozoon (Colleu, *et al*. 1997)(Molina, *et al*. 1995)(Pasteur, *et al*. 1991). This results in the highly compact form of DNA known in the mature spermatozoon (Colleu, *et al*. 1997) (Rodriguez, *et al*. 1985) (Pasteur, *et al*. 1991) (Griveau, *et al*. 1992).

On entering the ovum the spermatozoon nucleus decondenses rapidly and swells to become the male pronucleus. As mentioned above, the DNA in the spermatozoon head is still highly compact on entering the ovum. In order to form the male pronucleus, the protamines are removed from the DNA (Samocha-Bone, *et al*. 1998) (Montag, *et al*. 1992) (Pasteur, *et al*. 1991). This process involves the reduction of the disulfide bonds before binding the polycationic protamine to the nucleoplasmin (Samocha-Bone, *et al*. 1998)(Montag, *et al*. 1992) (Pasteur, *et al*. 1991). Nucleoplasmin is a small, negatively charged protein found in the ooplasm (Samocha-Bone, *et al*. 1998)(Colleu, *et al*. 1997). Protamines are rapidly replaced by

histones and this occurs within 5 minutes (Wasserman *et al.* 1994). The replacement is accomplished by intermolecular competitions between DNA and nucleoplasmin towards protamines. Protamines leave DNA because of their stronger affinity to nucleoplasmin, freed DNA then binds to histones, which are abundant in the ooplasm (Wasserman *et al.* 1994).

When the above processes take place, the spermatozoon's nucleus enlarges (decondensate or swells) and the DNA combines with ovum histones forming the pronucleus. As fertilization proceeds, the male and female pronuclei fuse. Abnormalities in the DNA of the spermatozoon chromatin and the enzymes and proteins involved may delay or prevent chromatin decondensation and can result in abnormal development of the male pronucleus.

Syngamy

This is the migration of the spermatozoa and ovum's pronuclei to the center of the fertilized ovum and their union. The cytoskeletal system plays an important role in this process. Actins occur throughout the cortex of the unfertilized ovum (Wasserman *et al.* 1994) but mostly in the region above the meiotic spindle (Wasserman *et al.* 1994). Actins and fodrin (a spectrinlike protein), which act together during fertilization, are not involved in spermatozoa-ovum fusion but are important in (i) anchoring the meiotic spindle to the ovum's cortex, (ii) determining the axis of cell division (extrusion of the polar-body and cleavages) (Wasserman *et al.* 1994) and (iii) drawing the spermatozoa's nucleus deep into the ovum (Wasserman *et al.* 1994). Microtubules in the ovum are essential for cell division (including polar-body extrusion) and the formation and migration of pronuclei (Wasserman *et al.* 1994). Microtubular organizing centers in fertilized mouse ovums are exclusively of maternal origin. In sheep, human and the cow (Wasserman *et al.* 1994) a centriole is carried into the ovum by the fertilizing spermatozoon. The centriole duplicates and migrates to one pole of the mitotic spindle during the first cleavage. When ovum and spermatozoa pronuclei are in close proximity to each other the proximal surfaces of the pronuclei become highly convoluted. In the rabbit convoluted neuclear envelopes (NEs) interlock and fuse at several places before the entire NE breaks down (Wasserman *et al.* 1994). In the human (Wasserman *et al.* 1994), NEs of spermatozoa and ovum's pronuclei breakdown separately without interlocking. A transient Ca^{2+}, increase can be detected sometimes within 5 minutes before NE breakdown (Wasserman *et al.* 1994). In the hamster, extracellular Ca^{2+} is not essential for the pronuclear development, but without it pronuclear ovums perish before reaching the two-cell stage (Wasserman *et al.* 1994).

Table 2.1: Stages of development

Stage of development
2-cell*
4-cell*
8-cell
16-cell
Morula
Early blastocyst
Blastocyst
Expanded blastocyst
Hatched blastocyst

In vitro fertilisation

Many causes of male infertility have been reported and include low spermatozoa count, abnormal spermatozoa morphology and incomplete decondensation of the pronucleus. Factors that to these problems are genetic, environmental and health related factors. Techniques used to promote fertilisation include artificial insemination ICSI and IVF.

Research in the field of infertility has several benefits in the medical and veterinary sciences. In the medical field it provides infertile couples with children, selection of embryos that do not have genetic defects Shenfield *et.al.* (2003). In veterinary sciences IVF improves herd (Lohuis 1998) by improving genetic quality (Kobayashi *et al*. 1999)resulting in healthy embryos and often preventing the extinction of certain species (Holt *et.al.*2004) and lastly to provide healthy animals for agricultural purposes. Researchers use both human material and animal models. Ethical issues limit the use of human material to study several aspects of human fertility and therefore the use of animals models such as sea urchin, chicken, hamster, swine and bovine has provided insights into the process of fertilisation.

The eventual outcome of all IVF procedures are healthy offspring and this has been a major concern of those involved in human ICSI programs. Meschede *et.al.* (2000) have reported no significant increases in genetic abnormalities children born as a result of these techniques. However, in animal models differences have been observed and Stringfellow and Wrathall 1995 have observed difference between *in vitro*- and *in vivo*-derived bovine embryos.

Comparison of in vitro- and in vivo-derived bovine embryos

There are some obvious differences between day-7 old embryos produced *in vivo* and those produced *in vitro*. These include the compacted appearance of *in vitro*- vs. *in vivo*- derived embryos and the fact that *in vitro* produced morulae and blastocysts often have fewer cells than those collected from a bovine's uterus (Stringfellow *et.al.* 1995).

According to Stringfellow and Wrathall these obvious differences are well known, but important subtle differences, relate to the structure of the zona pellucida. Using monoclonal antibodies produced against zona pellucidae from day seven *in vivo*-derived embryos, it was confirmed that there are differences between zona pellucidae of follicular oocytes, such as would be used in the *in vitro* production of embryos, and the zona pellucidae of developed embryos collected from the bovine's uterus (Stringfellow *et.al.* 1995). Using scanning electron microscopy topographical differences were also observed (Riddell, *et al*. 1993) (Stringfellow *et.al.* 1995)(Van Soom *et.al.* 1996).

Whether the differences between zona pellucidae of *in vivo*- and in vitro-derived bovine embryos are accompanied by differences in their capabilities to resist adherence or penetration by infectious agents has not been investigated adequately (Stringfellow *et.al.* 1995) (Van Soom *et.al.* 1996).

***In vitro* initiation of decondensation**

After the acrosome reaction and spermatozoal penetration, the spermatozoa undergo decondensation before fertilization can take place. Needless to say, if this process does not occur, then fertilization cannot take place and the result is infertility.

Chromatin packaging anomalies in human spermatozoa arise because of defects in the spermatozoa nuclei condensation mechanism in certain patients (Sakkas, *et al*. 1996). It has been proposed that reduced glutathione (GSH) or other thiol reagents such as heparin may participate in the basic mechanism by which spermatozoa-decondensing activity is accomplished. Heparin binds specifically to the spermatozoal membrane and provokes the decondensation of human spermatozoa and the activation of DNA transcription and synthesis. *In vitro* these reagents seem to be inactive and require the presence of other chemicals, usually detergents. Two groups of reagents can be used namely physiological agents like heparin and

glutathione or one can use chemical detergents like Dithiothreitol (DTT), Triton X100, Sodium dodicyl sulphate (SDS) and (N-Cetyl-N,N,N-trimethyl ammonium bromide) CTAB. Perreault and coworkers in 1987(Perreault, *et al*. 1987) reported that hamster spermatozoa nuclei treated with (DTT) decondensed more rapidly when microinjected into hamster oocytes.

Griveau and coworkers in 1992 demonstrated that asthenozoospermic spermatozoa's chromatin (patients displaying a high percentage of spermatozoa with nuclear abnormality) decondenses slowly and partially, compared to normozoospermic in humans. However, following the addition of cytoplasmic extracts from unfertilized *Xenopus laevis* (an amphibian) oocytes decondensation of human spermatozoa occurred

Tateno and Kamifuchi in 1999 reported that chromosome abberations decreased with increased concentrations of DTT suggesting that DTT has a protective function over chromosome damage. Reyes, *et al*. (1989) studied heparin and glutathione (GSH) as potential decondensing agents in human spermatozoa. The author reported that with heparin in combination with GSH, up to 90% decondensation may be obtained and that ten times less heparin was required to achieve decondensation. Furthermore sulphide reducing agents such as DTT alone have no effect, however when used in combination with heparin a rapid and highly significant increase in the number of decondensed nuclein was observed.

Delgado *et al* (2001) studied the combination of heparin and glutathione with some chemicals like DTT and CTAB in bovine spermatozoa. These authors reported that DTT combined with GSH did not induce decondensation and that heparin is essential for the decondensation of bovine spermatozoa. According to Delgado *et al* (2001), this suggests that rupture of disulfide bridges is not the main mechanism to induce nuclei decondensation and perhaps the GSH role resides in potentiating the heparin effect by increasing its negative charge.

Human, mouse, chinchilla, and hamster spermatozoal nuclei decondensed within 30-60 minutes after microinjection into hamster oocytes, whereas bovine spermatozoal nuclei decondensed only after a considerable time lag in the presence of high concentrations of DTT and a detergent and seems to be completely resistant to the decondensing activity of GAGs. This strongly indicates species differences in the

time, concentrations and combinations of decondensing agents required for effective pronuclei decondensation.

CHAPTER 3: THE EFFECT OF DTT ON HUMAN AND BOVINE SPERMATOZOA DECONDENSATION

Spermatozoa decondensation plays an important role in fertility (Esterhuizen, *et al*. 2002). On entering the ovum the DNA in the spermatozoon head is compact (Samocha-Bone, *et al*. 1998) due to its binding to the nuclear protamines (Wyrobek, *et al*. 1990) (Colleu, *et al*. 1997). Protamines are nuclear proteins that are incorporated into the DNA of the spermatozoon during spermatogenesis to replace the somatic histones (Montag, *et al*. 1992). Protamines contain arginine residues that neutralize the negative DNA phosphate while cysteine residues (Montag, *et al*. 1992) (Rodriguez, *et al*. 1985) form intra- and inter-disulfide bridges (Colleu, *et al*. 1997) (Rodriguez, *et al*. 1985) (Molina, *et al*. 1995). The disulfide bridges originate from the cysteine residues that are initially present as thiols. These thiols are oxidized to disulfides in the mature spermatozoon (Colleu, *et al*. 1997)(Molina, *et al*. 1995) (Pasteur, *et al*. 1991). This results in the highly compact form of DNA known in the mature spermatozoon (Colleu, *et al*. 1997) (Rodriguez, *et al*. 1985) (Pasteur, *et al*. 1991) (Griveau, *et al*. 1992).

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Therefore the purpose of the first part of this study was to determine the effectiveness of DTT in inducing decondensation of human and bovine spermatozoa. The latter model was used in further studies to determine whether once effective decondensation had been induced by DTT, does normal embryological development occur (Chapter 4).

The following research questions will be answered in this chapter:

1. **Does DTT induce decondensation in human spermatozoa following exposure for different time intervals?**
2. **Does maturity of human spermatozoa have an influence on decondensation following exposure to DTT for different time intervals?**
3. **Does DTT at different exposure times cause decondesation of bovine spermatozoa?**
4. **Does the inclusion of heparin in the Swim-up method improve decondensation induced by DTT in bovine spermatozoa?**

Materials and Methods

MATERIALS

Human spermatozoa

Semen samples (n = 52) were collected from donors, fertile men and subjects with suspected infertility. The samples were received after a liquefaction period of about 30 minutes and a standard semen analysis was done by Du Buisson Bruinette Kramer and Partners Laboratories. No information was made available about any of the subjects. Ethical clearance was obtained for the study from the Student Ethical Committee of the Medical Faculty of the University of Pretoria, and the participants signed an informed consent form (Appendix 1).

Bovine spermatozoa

Bovine spermatozoa from 6 different bulls were obtained from the Faculty of Veterinary Sciences at Onderstepoort, University of Pretoria.

Media, supplements and reagents

HAM's F10 medium was from Highveld Biological Company, Johannesburg, South Africa. Sartorius cellulose acetate membrane filters 0.22□m were from National Separations, Johannesburg, South Africa.

Fixatives, acids and organic solvents, such as ethanol, methanol and acetic acid were of analytical grade and were purchased from Merck, Johannesburg, South Africa

Streptomycin sulphate, penicillin G (sodium salt), Amphotericim B and trypsin were obtained from Life Technologies Laboratory supplied by Gibco BRL Products, Johannesburg, South Africa. Dithiothreitol (DTT), chromomycin A_3 (CMA_3), 1,4-diazabicyclo[2.2.2.]octane (DABCO), Eosin-nigrosin, ethylene diamine tetra acetate (EDTA), dimethyl sulphoxide (DMSO), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), sodium chloride (NaCl) and sodium hydrogen carbonate ($NaHCO_3$) were from Merck, Johannesburg, South Africa.

The 24 well and 96 well plates, 25cm^2 and 75cm^2 cell culture flasks, 10ml and 5ml pipettes, 15ml and 50ml centrifuge tube, micro centrifuge tubes were from NUNC™ supplied by AEC- Amersham, Johannesburg, South Africa.

Water was double distilled and deionized (ddH_2O) with a Continental Water System and was sterilized by filtration through a Millex 0.2□m filter. Glassware was sterilized at –140^{0}C in a Prestige Medical Autoclave (Series 2100).

Facilities:

All cell culture and human spermatozoa research were done in the Cell Biology Laboratory at the Department of Anatomy, Faculty of Health Sciences, University of Pretoria. All experiments concerning bovine spermatozoa were performed in the In Vitro Fertilization (IVF) Laboratory, Section of Reproduction, Faculty of Veterinary Science, University of Pretoria. All microscopy was carried out at the Laboratory for Microscopy and Microanalysis, University of Pretoria.

Detailed recipes for the preparation of media are preented in Appendix 2.

METHODS

Human spermatozoa

Spermatozoa processing

The HAM's F10 swim-up procedure was used when semen volume and spermatozoa content was considered to be sufficient. If the semen volume and spermatozoa was insufficient the PureSperm® procedure was used. All procedures involved were performed as prescribed for Du Buisson, Bruinette Kramer and Partners Laboratories adapted from Mortimer, D. 1994.

One-step swim-up method:

For the swim-up method the same volume Sperm Washing Medium (SWM namely Hams F10 medium prepared by dissolving 9.8g Hams F10 powdered medium and 2.5g $NaHCO_3$ to a final volume of 1000 ml H_2O, pH 7.4) was added to equal volumes of semen and was then gently mixed. The sample was then centrifuged for 10 minutes at 300xg, the supernatant was then removed and discarded. The pellet was resuspended in 10 µl SWM and gently layered with 1,3ml SWM and incubated for 20 minutes in an incubator at 37°C, 5% CO_2. The upper 0,8 ml was aspirated, 0,5 ml DPBS was added and the sample was gently mixed. Again the sample was centrifuged for 10 minutes at 300xg, the supernatant was removed and the resuspended samples were used as described below. If the swim up method was not successful after 10 minutes the incubation times were extended from 10 minutes to 30 minutes.

The PureSperm® procedure

The PureSperm® procedure was followed when semen volume was perceived as low, and spermatozoa content suspected to be low. Two gradients of 90% and 45% PureSperm® was prepared by adding 9ml or 4.5ml PureSperm® to 1ml or 5.5ml Standard Sperm Medium (SSM), 10% Earles buffered Saline Solution (EBSS), prepared by mixing together 8.7g powdered salts and 2.2g Na_2HCO_3 in 1000 ml H_2O, pH 7.6 respectively. Under aseptic techniques either 1 or 2ml of the 90% gradient PureSperm was pipetted onto the bottom of a centrifuge tube and then carefully overlayed with 1 or 2ml (equal volumes) of the 45% gradient PureSperm®. A volume of 1-2ml of semen were then layered onto the top of the 45% PureSperm® gradient.

Care was taken not to disturb either one of the three layers. The sample was centrifuged for 20min at 300×g. The supernatant was removed and the pellet was resuspended in 2-3 ml of SSM, allowed to stand for 3 minutes and then was centrifuged again, for 10min at 200×g. The supernatant was removed and the washing procedure was repeated twice with Phosphate Buffered Saline Solution (PBS). A 10xPBS was prepared by dissolving 80g NaCl, 2g KCl, 26,8g $Na_2HPO_4.7H_2O$ and 2,4g KH_2PO_4 in 1000ml H_2O, pH 7,4, adjusted with HCl. The DPBS was divide into aliquots and sterilize by autoclaving and stored at room temperature.

Decondensation of human spermatozoa with DTT

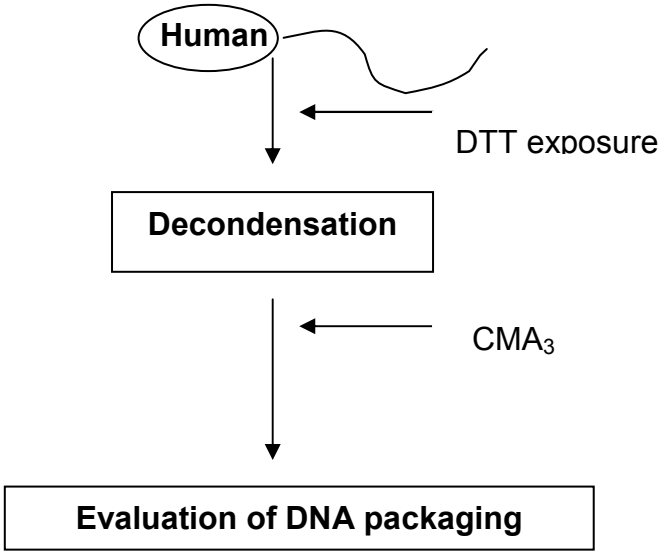


***Figure 3.1**: Schematic representation of the procedures followed to determine the effect of DTT on spermatozoa decondensation*

A fresh 25 mM DTT solution in 1M Tris/HCl, pH 9.5 was prepared fresh for each experiment. Each sample was washed in 2XSSC (Sodium chloride-Sodium Citrate buffer which was prepared dissolving 17.5g NaCl and 8,8g sodium citrate in 1000ml H_2O, pH adjusted to 7.0 with HCl, the buffer was divided into aliquots, sterilize by autoclaving and stored at room temperature) by centrifuging for 10 minutes at 300g and the pellet was resuspended in the 25 mM DTT solution using a volume equal to the initial volume of the semen sample. The contents of the tube were divided into three parts and each was incubated for different time intervals, namely 5, 7 and 10 minutes respectively. After each incubation time interval the spermatozoa were

collected by centrifugation, washed with 2XSSC and twice in a 0,5 ml volume of DPBS at 300xg for 10 minutes.

Preparation of slides

After the isolation of the spermatozoa using either the One-step swim-up or PureSperm® method a control slide was prepared. Following exposure to DTT slides of each incubation period were also prepared.

The slides were dehydrated for one minute in 70%, 90% and 100% ethanol. The slides were then left for 20 minutes to air-dry and then fixed by placing them in a 3:1 methanol: acetic acid solution for 20 minutes and stored.

Phase contrast and fluorescence microscopy

The slides were firstly evaluated using phase contrast microscopy to determine the spermatozoa morphology without or following at different time intervals to DTT. The area evaluated was marked with a diamond point pen, before the spermatozoa in this area was stained with 100µl CMA_3 for ± 20 minutes in the dark. After staining, the slide was washed in Mc Ilvaine's buffer and mounted with DPBS. Slides were mounted with 0.1% DABCO (1,4-diazabicyclo[2.2.2.]octane) a fluorescence, an anti-fade reagent that retards photo-bleaching.

The spermatozoa were evaluated using a fluorescence microscope according to the following 4 classes.

1. No staining – no fluorescence
2. Fluorescent band at the equatorial segment.
3. Fluorescent staining – faintly yellow – of the sperm head.
4. Bright yellow fluorescent staining of the sperm head.

Classes 1 and 2 indicate good quality DNA packaging in the sperm head and indicate mature sperm, while classes 3 and 4 are an indication of poor packaging DNA in the sperm head, thus indicating immature sperm.

Bovine spermatozoa

Spermatozoa processing

Bovine semen (six fresh samples) was treated in exactly the same way as the human samples by using the one-step swim-up method.

Decondensation of bovine spermatozoa with DTT

The source of the human semen was undisclosed and therefore it was necessary to determine the maturity of the spermatozoa with CMA_3 staining. In contrast, the bull semen used in the second part of this study was received from a well controlled and extensively evaluated animal group that have produced healthy offspring and therefore evaluation of sperm maturity was considered unnecessary. Bovine spermatozoa were exposed for time intervals 5, 7 and 10 minutes to 1M Tris/HCl buffer pH 9.5 containing 25 mM DTT.

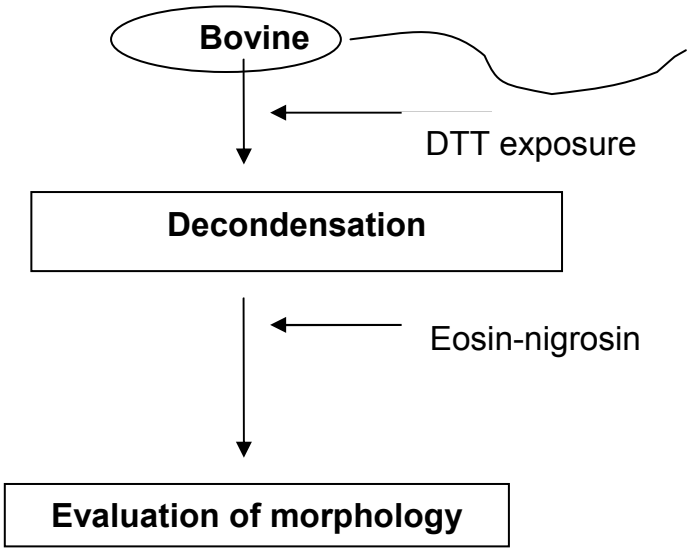


Figure 3.2: *Schematic representation of the procedures followed to determine the effect of DTT on human and bovine spermatozoa decondensation.*

Since no decondensation was observed with 5, 7 and 10-minute exposure using the human procedure for spematozoal preparation the procedure was repeated with a modified method used at the IVF Laboratory, Faculty of Veterinary Science. Frozen samples was thawed by removing frozen semen straws from liquid nitrogen storage, then placed directly into a 30-33°C water bath and swirled gently for 2-3 min. The straws were wiped dry with a paper towel, and then thawed semen was decanted into a separate sterile test tube. The thawed semen was carefully layered on the bottom of the FERT WASH (Appendix 2) filled tube and were placed in the incubator for one hour before the samples were exposed for 5, 7 and 10 minutes to 25mM DTT. The results showed again no decondensation and the exposure times were increased to to 10, 20 and 30 minutes.

Eosin-nigrosin staining

Eosin-nigrosin (E-N) was used to enhance the visibility of the spermatozoa using light microscopy. The E-N solution used was obtained from Onderstepoort, a patented formulation. Equal volumes of liquefied semen and E-N stain were mixed and left for 30 seconds. A 50µl volume of the mixture was transferred onto a clean microscope slide and a smear was prepared. The smear was allowed to air dry and was then mounted with entallen and was stored at room temperature (Mortimer, D. 1994)

RESULTS AND DISCUSSION

Pronucleus formation of the spermatozoa head is essential for fertilization and normal embryonic development. Failure of this process due to no, incomplete or defective decondensation can be found in some cases of infertility (Esterhuizen, *et al*. 2000)(Chung, *et al*. 2000) (Chang 1951). The absence of spermatozoa chromosome decondensation after ICSI with human oocytes results in the failure of fertilization (Rho, *et al*. 1998). Methods and techniques are being sought to improve the male pronucleus formation thereby increasing the success rate of *in vitro* fertilisation. (Hammadeh *et al*. 2001). Chromatin packaging anomalies in human spermatozoa arise because of defects in the spermatozoa nuclei condensation mechanism in certain patients (Sakkas *et al.* 1996). Griveau and coworkers in 1992 reported using cytoplasmic extracts from unfertilized *Xenopus laevis* (an amphibian) oocytes to induce decondensation in human spermatozoa. The authors also demonstrated that asthenozoospermic spermatozoa chromatin (patients displaying a high percentage of spermatozoa with nuclear abnormality) decondenses slowly and partially, compared

to normozoospermic in humans. Two different groups of reagents are used; either physiological agents like heparin and glutathione or chemical agents like DTT and CTAB. Successful decondensation of spermatozoa can be achieved either using a single reagent such as DTT alone or combinations of a chemical and physiological agent such as DTT and heparin (Reyes, *et al*. 1989).

A total of 52 human semen samples were collected but in many the spermatozoa count was very low and only 12 samples could be thoroughly evaluated. These 12 samples were obtained from either fertile or suspected infertile subjects (the fertility status of each subject was not disclosed). The spermatozoa were collected using either the one-step-swim-up or Puresperm methods. The spermatozoa were exposed to 25 mM DTT for 5, 7 and 10 minutes, washed and the morphological changes associated with decondesation compared to a control (not treated) was evaluated by phase contrast microscopy. These results are presented in Figure 3.3a. In different studies different concentrations of DTT together with different time periods were used. In this study a DTT concentration of 25 mM with incubation times of 5, 7 and 10 minutes were used as modified from the protocol Martini et al 1995 used.

Compared to the control, not exposed to DTT, Figure 3.3a (A) different degrees of decondensation was observed following exposure to DTT. This includes cell membranes that appear intact with dark chromatin (Figure 3.3a(B)) as well as a clearly visible acrosome with no decondensation of the acrosome area although towards the basal area of the spermatozoa head, decondensation is clearly visible, Figure 3.3a(C). In Figure 3.3a(D) the acrosome is visible, the membrane is intact and even decondensation has occurred. More extreme effects are that the acrosome is not visible and the spermatozoa head is dispersed (Figure 3.3a(E)) or the spermatozoa head is totally dispersed and only the tail can be distinguished (Figure 3.3a(F)). All morphologies as shown in Figure 3.3aB through to E is considered as successful decondensation. Another pattern of decondensation that is observed is that the spermatozoa appear condensed as similar to the pattern observed for the control however the nuclei and acrosome cannot be observed as distinct entities. Figure 3.3a(G).

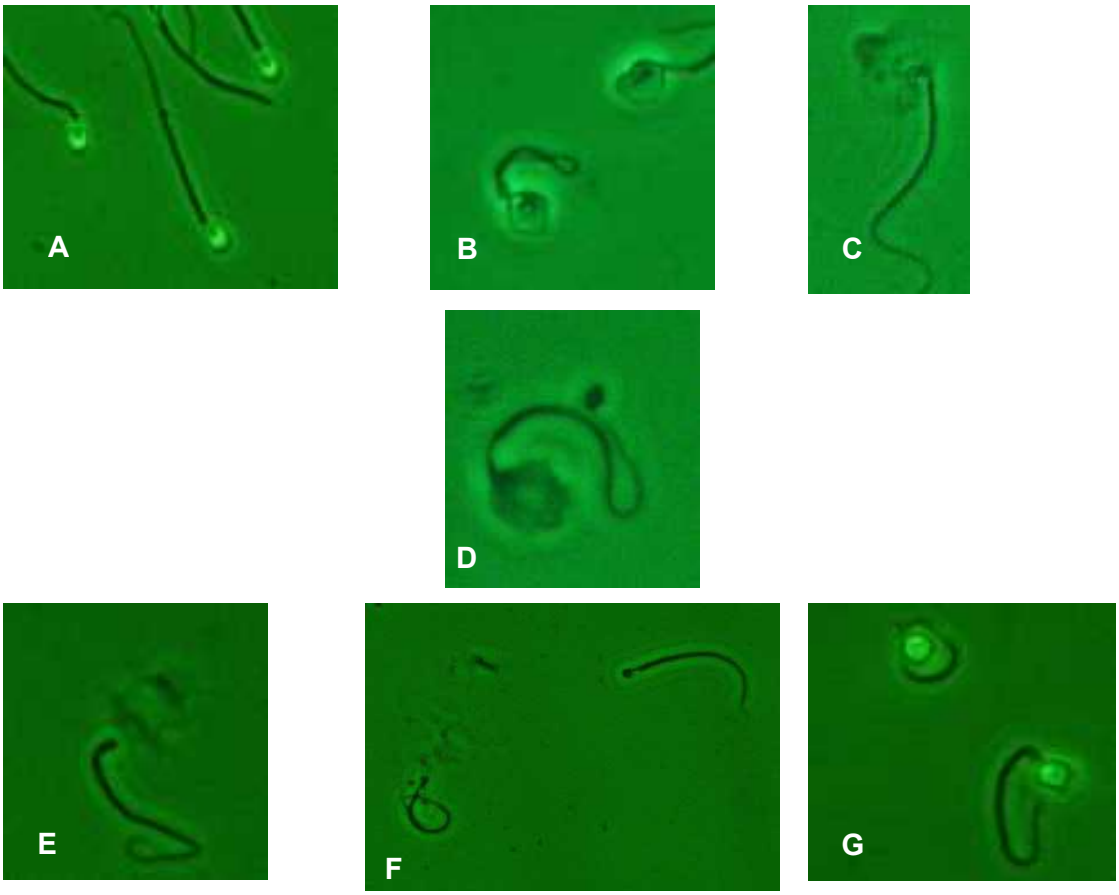


Figure 3.3a: *Different images could indicate decondensed spermatozoa heads (Magnification x 40). (A) Control, (B) spermatozoa with membranes that appear intact with dark chromatin. Visible acrosome in (C) with decondensation only towards the basal area of the spermatozoa head. Membranes are still intact. (D) Acrosome visible, membranes intact, and chromatin evenly decondensed. (E) Membrane not intact, acrosome is not visible and spermatozoa head is dispersed. (F) Spermatozoa head is totally dispersed and only the tail can be distinguished. (G) Similar to control however the nuclei and acrosome cannot be observed as distinct entities.*

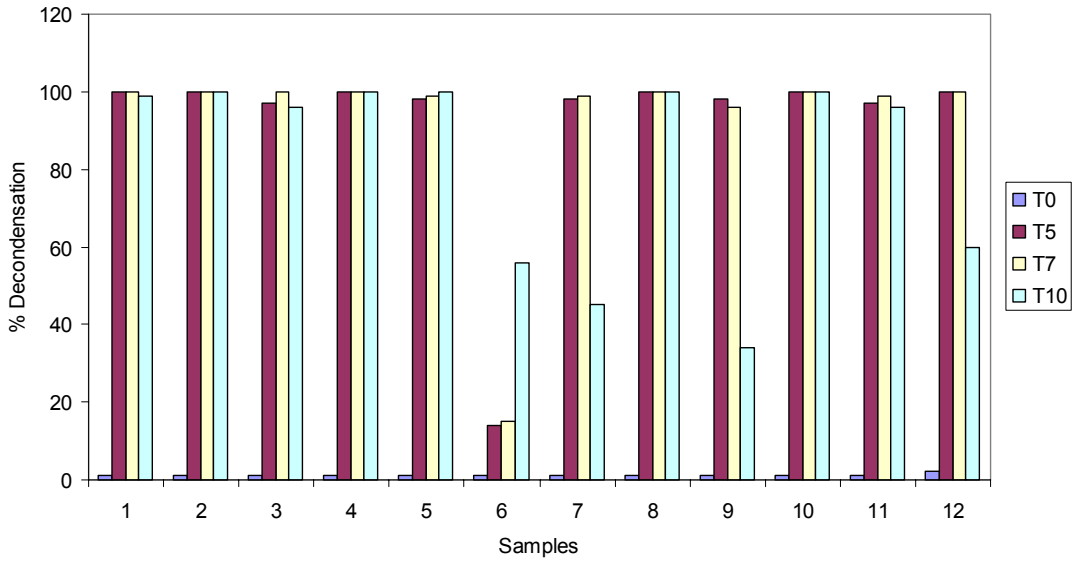
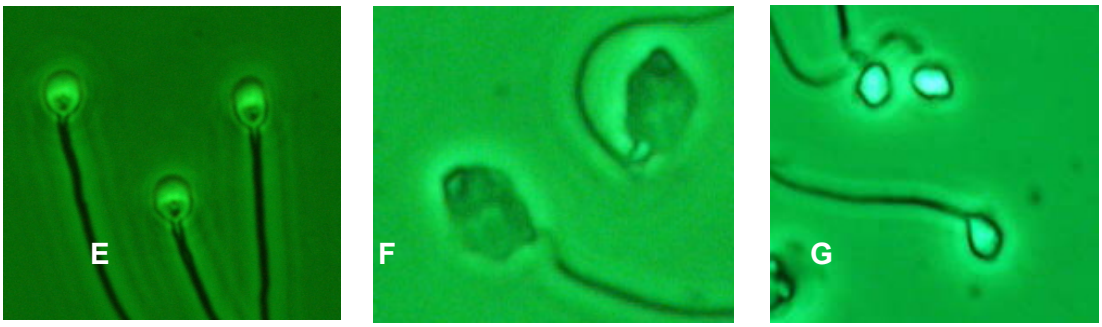


Figure 3.3b. *The percentage of decondensed human spermatozoa determined by phase contrast microscopy after 0, 5, 7 and 10 min exposure to 25mM DTT*

The total number of spermatozoa that underwent decondensation over time was determined and these results are presented in Figure 3.3b. For samples 1-12 (Figure 3.3b) decondensation was observed after 5 and 7 minutes. For all samples expect after 5 and 7 minutes decondensation was 95-100%, while for sample 6, little decondensation was observed with 10% and 12% occurring after 5 and 7 minutes respectively.

Exposure for 10 minutes caused decondensation to increase to 60% in this sample while for samples 7, 9 and 12 a significant decrease in decondensation was observed at 10 minutes and this was associated with a hypercondensation of the nucleus as shown in Figure 3.3c.

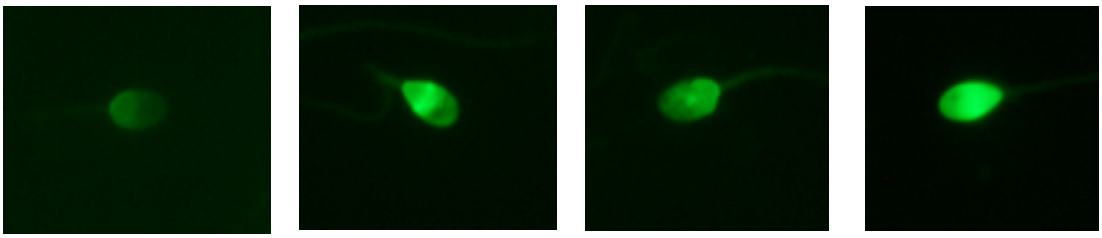


(A) **(B)** **(C)**

Figure 3.3c: *In several subjects, the decondensation process appeared to have had reversed after certain exposure times. This was observed at both the 7 and 10 minute exposure times. (A) shows a normal, condensed spermatozoa, whereas (B) indicates normal decondensation and (C) hypercondensation as is observed in samples 7, 9 and 12.*

Rousseaux, *et al*. (1995) and Chevret, *et al*. (1994) showed that 10 mM DTT induced decondensation of spermatozoa isolated from human donors after 10-50 minutes and the rate of decondensation varied between different samples. Griveau *et al* (1992) induced decondensation of human spermatozoa using 5 mM DTT for 60 minutes. Martini *et al* (1995) induced decondensation in human spermatozoa using 25 mM DTT for 5 minutes. These studies and the results of this study shows that decondensation is a function of DTT concentration, incubation times and the inherent sperm integrity.

During spermatogenesis somatic histone proteins are replaced by protamine proteins to create mature spermatozoa. Maturity of spermatozoa or in other words the stability and packaging of DNA was evaluated with fluorescence microscopy following CMA_3 staining. CMA_3 is a fluorescent dye that binds histones in immature spermatozoa. In addition, CMA_3 accessibility differs during spermatogenesis in the mouse suggesting that it varies according to the level of protamination. In the mouse, testicular spermatids show CMA_3 positive staining, while mature spermatozoa do not stain at all and only following fertilisation where decondensation begins in the oocyte does positive CMA_3 staining occurs. Therefore mature spermatozoa that contain protamines either do not stain (Figure 3.4aA) or only stained at the equator (Figure3.4a B). For immature spermatozoa that have histones, moderately (Figure 3.4a C) or brightly stained nuclei (Figure 3.4a D) are observed.



(A) (B) (C) (D)

Figure 3.4a: *Criteria used to evaluate maturity of spermatozoa. Maturity could be indicated by: (A) unstained; (B) stained on equator. Immaturity is characterized by: (C) moderately stained; (D) bright green stain.*

The maturity of the spermatozoa used in this study was determined. In the absence of DTT no significant staining is observed indicating that in all samples studied the spermatozoa were mature. Therefore the effect of DTT on the decondensation of immature spermatozoa could not be studied.

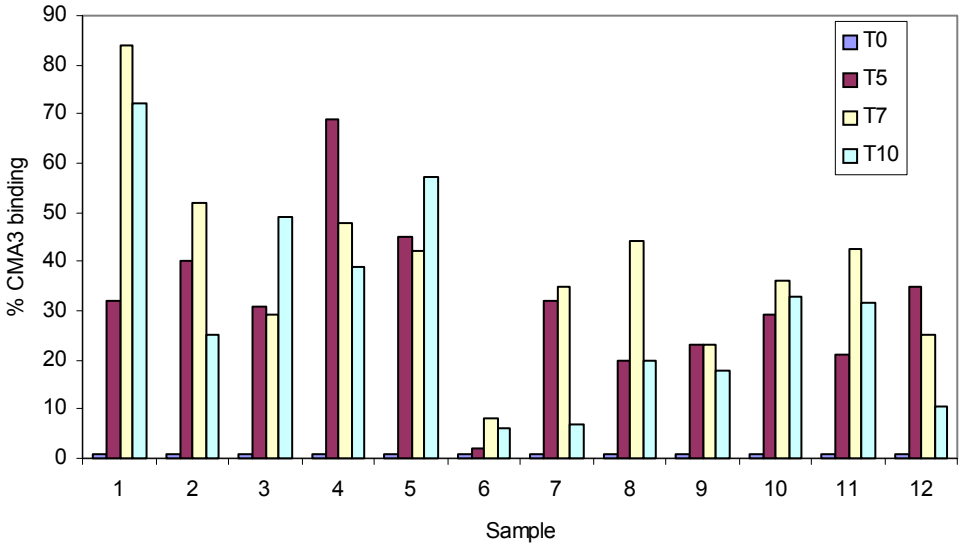
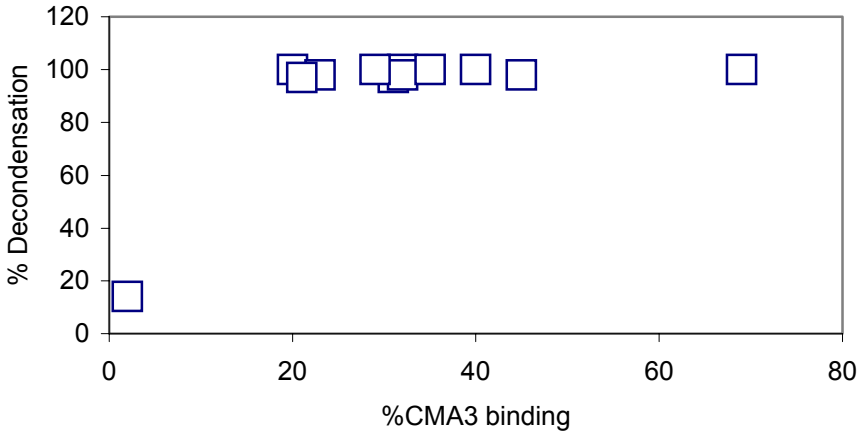


Figure 3.4b *The percentage of CMA_3 positive binding after a) 0, b) 5, c) 7 and d) 10 minutes exposure to 25 mM DTT.*

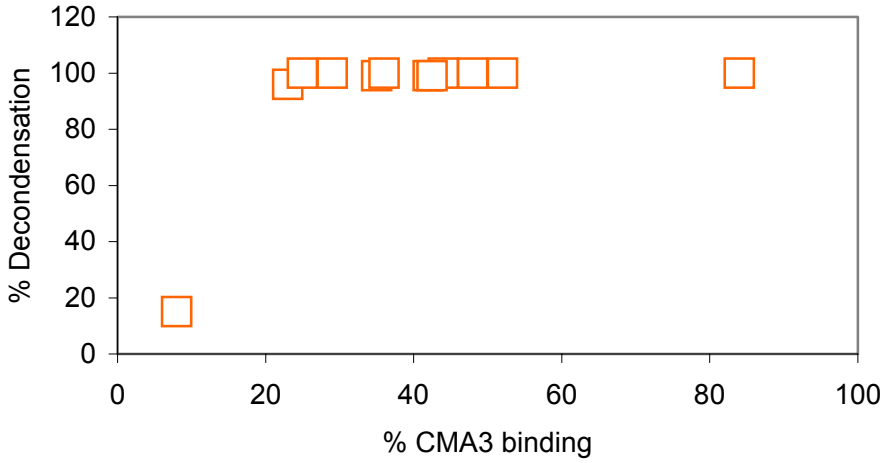
However, following exposure to DTT for 5, 7 and 10 minutes an increase in fluorescence is observed, indicating that the spermatozoa could not possibly have become immature but rather CMA_3 has successfully found new binding sites (Figure 3.4b-d). For all samples, except sample 6 an increase in florescence is observed after 5 minutes exposure to DTT. For samples 1, 2, 6, 8 10 and 11 increased fluorescence is observed after 7 minutes while fluorescence is decreased in samples 3, 5 and 12. For samples 1, 2, 4, 6-12 fluorescence is reduced after 10 minutes of exposure, indicating fewer binding sites for CMA_3 possibly due to DNA supercoiling (Rho, *et al*. 1998).

In Figure 3.4c shows the relationship between the percentage decondensation and the percentage fluorescence due to CMA_3 binding. For all samples except sample 6, there is a direct relationship between the degree of decondensation and CMA_3 staining. At 10 minutes exposure, hypercondensed nucleus (Figure 3.3c) was observed in samples 7, 9 and 12 (Figure 3.3b) which was associated with reduced CMA_3 staining as seen in the lower left hand corner of Figure 3.4c.

a)



b)



c)

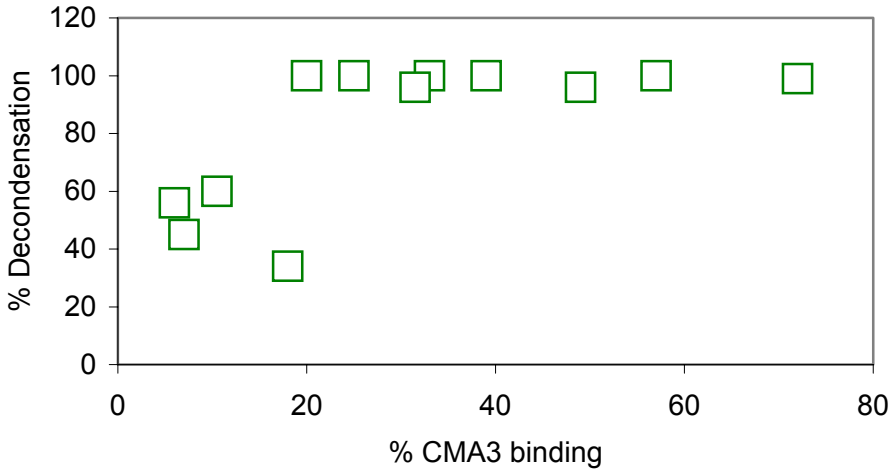


Figure 3.4d: *The relationship between CMA_3 staining and decondensation in samples 1-12 following exposure to DTT for a) 5 (left hand corner, sample 6) b)7 (left hand corner, sample 6) and c) 10 minutes (sample 6 as well as 7,9 and 12 that are hypercondensed)*

DTT is used as a reducing agent for disulphide bonds, dithiotreitol and its isomer, dithioerythritol is capable of maintaining monothiols completely in the reduced state

and of reducing disulphides quantitatively. Protamines and histones contain disulphide bonds that can be reduced by DTT. In these mature spermatozoa, protamines would the predominant protein associated with spermatozoa DNA. According to Bizzaro *et.al.* (1998) CMA_3 has been shown to bind as a Mg^{2+} - coordinated dimer at the minor groove of GC-rich DNA and induces a conformational perturbation in the DNA helix resulting in a wider and shallower minor groove at its binding site. These authors have shown that CMA_3 is a useful tool for the rapid screening of subfertility in man, as it seems to allow an indirect visualization of protamine-deficient, nicked and partially denatured DNA. Therefore, in this study DTT induces decondensation of human spermatozoa pronuclei. However as CMA_3 also indicates nicked and partially denatured DNA the question arises whether decondensation induced by DTT will result in the successful production of embryo's. In contrast, in studies using the Chinese hamster, Tateno and Kamifuchi in 1999 reported that chromosome aberrations decreased with increased concentrations of DTT suggesting that DTT have a protective function over chromosome damage. Due to ethical reasons this question cannot be addressed using human material and therefore the effects of DTT as a decondensing agent will be studied in the bovine model system. Several different models have been used to study IVF and this includes rodent, ovine, porcine and bovine models. The bovine model was chosen as it is a mammalian model and has been extensively researched and every step of the process has been established, standardized and is commonly used in agricultural practices such as Taurus and the IVF program at Onderstepoort In this study spermatozoa were used from fertile bulls that would under normal conditions give rise to viable embryos. In this laboratory the successful production of viable embryos is 60%. In addition to studying the process of decondensation, this study has an added advantage as an increased yield of viable embryos. Furthermore this model gives researchers an opportunity to investigate the possible long-term toxic effects of DTT-induced decondensation, which is not possible using human gametes.

Spermatozoa used were derived from Friesian bulls and the samples were pooled to prevent sample bias and interindividual variation. Spermatozoa were exposed to 25 mM of DTT at 5, 7 and 10 minutes as was used for human spermatozoa in this study. Spermatozoa morphology was studied by light microscopy following N-E staining. Decondensation can be evaluated either by phase contrast microscopy without staining or light microscopy with E-N staining. The latter is an optimised method well established in the laboratory where these studies were undertaken. For this reason this method was used to evaluate the decondensation of bovine sperm.

No decondensation was observed at the same concentration and exposure times as used for human spermatozoa. The use of “swim up” medium containing heparin resulted in successful decondensation of bovine spermatozoa in the presence of 25 mM DTT for 30 minutes with morphological characteristics (Figure 4) similar as was observed for human spermatozoa as shown in Figure 3.3c. Delgado *et al* (2001) studied the combination of heparin and glutathione with some chemicals like dithiothreitol (DTT) and N-Cetyl-N,N,N-trimethyl ammonium bromide (CTAB) in bovine spermatozoa. These authors found that DTT combined with glutathionine (GSH) alone did not induce decondensation. However DTT in combination with heparin induced decondensation indicating that heparin was essential for the induction of decondensation in bovine spermatozoa. Furthermore Delgado *et al* (2001) found that in the presence of heparin lower concentrations of DTT were required to induced successful decondensation.

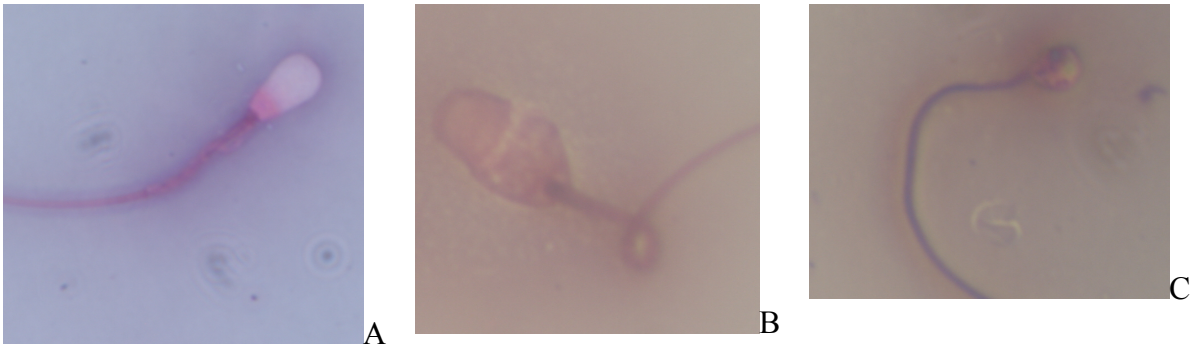


Figure 4: *(A) shows a normal, condensed spermatozoa, whereas (B) indicates normal decondensation and (C) Spermatozoa appear condensed, but no nuclei or acrosome is visible.*

Heparin binds specifically to the spermatozoa membrane via receptorlike functional groups thereby initiating the decondensation of human spermatozoa (Delgado *et al* 1982). Furthermore, mammalian species differ in the type of protamine found bound to chromatin. Mammalian sperm chromatin has been distinguished into two classes according to the types of protamines present in the sperm nucleus (Delgado *et al* 2001). Bull sperm nuclei contain only protamine P1 which is richer in cysteinyl residues and is maximally crosslinked by disulfide bridges, this accounts for its greater stability compared to human spermatozoa and therefore the requirement for both heparin and DTT as well as longer incubation times when compared to human spermatozoa. Perreault and coworkers in 1987(Perreault, *et al*. 1987) reported that

hamster spermatozoa nuclei treated with DTT decondensed more rapidly when microinjected into hamster oocytes. As DTT is a reducing reagent and many important enzymes that participate in the acrosome reaction contain disulphide bonds that are essential for maintaining the activity of catalytical site, DTT may inhibit the acrosome reaction (Tateno *et.al.* 1999). It is therefore important to add the DTT after the acrosome reaction but before the pronucleus formation to insure that the DTT have a chance to influence the pronucleus formation.

In conclusion both human and bovine spermatozoa undergo the same pattern decondesation following exposure to DTT, however the effect on fertilization and the development of viable embryos is unknown and will be investigated further in Chapter 4 using the bovine animal model.

CHAPTER 4: THE EFFECT OF DTT ON BOVINE EMBRYO FORMATION

On entering the ovum the spermatozoon nucleus decondenses rapidly and swells to become the male pronucleus. As mentioned above, the DNA in the spermatozoon head is still highly compact on entering the ovum. In order to form the male pronucleus, the protamines are removed from the DNA (Samocha-Bone, *et al*. 1998) (Montag, *et al*. 1992) (Pasteur, *et al*. 1991). This process involves the reduction of the disulfide bonds before binding the polycationic protamine to the nucleoplasmin (Samocha-Bone, *et al*. 1998)(Montag, *et al*. 1992)(Pasteur, *et al*. 1991). Nucleoplasmin is a small, negatively charged protein found in the ooplasm (Samocha-Bone, *et al*. 1998) (Colleu, *et al*. 1997). When the above processes take place, the spermatozoon nucleus enlarges (decondensate or swells) and the DNA combines with ovum histones forming the pronucleus. As fertilization proceeds, the male and female pronuclei fuse. Abnormalities in the DNA of the spermatozoon chromatin that may delay or prevent chromatin decondensation can result in abnormal development of the male pronucleus.

It has been shown in Chapter 3 that human spermatozoa differ in their ability to undergo decondensation and this is due to the genetic heterogeneity of the population. An advantage to using the bovine model to study the effects of DTT on decondensation is that this is a homogeneous normal population and the presence of genetic variation is absent.

DTT has been shown to cause the decondensation of human and bovine spermatozoahowever little is known whether this will lead to the development of viable embryos.

Therefore the fifth research question is: Does DTT increase the rate of pronucleas formation that results in the formation of viable bovine embryos.

MATERIALS AND METHODS

MATERIALS

Bovine spermatozoa and oocytes

Bovine spermatozoa were obtained from the Faculty of Veterinary Sciences and prepared as described previously in Chapter 3.

Ovaries were obtained from Karan Beef abattoir in Balfour South Africa. All experiments concerning bovine spermatozoa and oocytes were performed in the IVF laboratory of the Section of Reproduction in the Faculty of Veterinary Science (Onderstepoort). All microscopy work was done at the Unit for Electron and Light Microscopy at the main campus of the University of Pretoria.

All media components and culture dishes were purchased from Sigma Aldrich and all the protocols and media formulations are available in Appendix 2.

METHODS

Experimental design

A summary of all procedures is presented in Table 4.1

Table 4.1 Summary of experimental procedures

Isolation of bovine spermatozoa	***Recovery of oocytes***
Insemination of oocytes	
Split into two groups	
Group I: Evaluation of fertilization	***Group 2:*** Evaluation of cleavage and embryo formation
Group Ia: Control, No DTT ***Group Ib***: DTT	***Group 2a:*** Control, No DTT ***Group 2b:*** DTT

Isolation of bovine spermatozoa

The isolation of bovine spermatozoa will be discussed in the paragraph on *in vitro* fertilization after oocyte maturation. This is done to follow the chronological sequence of events.

Recovery of oocytes

Oocyte recovery is the first step in the in vitro *embryo production (IVEP) program. The oocytes are recovered/retrieved from the ovary and not from fallopian tubes. The oocytes are therefore not mature (since no ovulation occurred) and must undergo a maturation process that will be described later.*

Ovaries were cut away from the tip of the uterine horns, as soon as possible after the animal was slaughtered, using aseptic technique. The ovaries were placed directly

into ovary collection/transport medium (pre-warmed to 27-30°C) phosphate buffered saline (PBS), prepared as in Appendix 2.

The ovaries can be kept under these conditions for up to 8-11 hours without reducing the fertilization ability of oocytes and the capacity of early embryos to develop to blastocysts. The ovaries were rinsed with 28-30°C water to remove all excess blood and were kept in H_2O for as short time as possible to reduce the toxic effect of the H_2O.

During ovary processing gloved hands were washed thoroughly with water and/or 70% ethanol to remove all traces of powder, as it is embryocidal. The ovaries were washed 3 times with sterile saline (0.9% NaCl in H_2O) at room temperature. Excess connective tissue was cut away from the ovaries using sterile scissors. The ovaries were placed in a beaker of sterile saline and held at room temperature until aspiration.

Each bovine ovary contains many thousands of oocytes, but with current technology only a relatively small proportion of the total vesicular follicle population is utilized.

Preparation of medium for oocyte aspiration and maturation

At least one-hour prior to use of the different media, MATURATION medium micro-drops and CULTURE medium dishes was prepared and equilibrated at incubator atmosphere and temperature. The 50µl micro-drops were prepared by first placing the droplets on the surface of a small (35 x 10 mm) petri dish. The droplets were well spaced away from each other but not too close to the edge of the dish. The droplets were gently covered with 3-4 ml of washed and serum-equilibrated mineral oil.

One searching dish for every 15 min of searching were prepared and at least 2 final wash dishes for every batch of oocytes both search and wash dishes contained 3-4 ml of MATURATION medium.

Aspiration (syringe and vacuum pump) of oocytes

For the recovery of oocytes from follicles aspiration is used due to the speed of operation. Maintaining an intact cumulus oocytes complex (COC) is important as this will effect the yield of embryos. Aspiration of the ovaries can be done with either 18-

gauge needle syringe coupled either to a syringe or pump. The vacuum pump is preferred as it is faster and the suction level can be controlled and therefore the quality of retrieved oocytes is better.

In syringe aspiration the suction is created via keeping constant pressure on the back of the plunger, through pulling the plunger out of the syringe at a constant pace. To recover follicular oocytes, ovaries were first wiped with sterile gauze to remove any excess saline. The needle was inserted into the ovary penetrating the follicle from the side. (The needle should never be pressed into the follicle itself for the follicle will burst and a loss of follicle fluid will occur resulting in a loss of the oocytes). When inserting the needle into the follicle from the side, the suction created by the constant pressure on the back of the syringe will suck the follicular fluid (containing the oocyte) into the syringe.

After a few follicles were sucked from the ovary, the fluid was flushed into the aspiration tube kept in the water bath (35°C). By piercing the ovary from all different sides and keeping constant suction, the follicles that are on the inside of the ovary and not visible, will also be sucked into the aspiration tube. The aspiration tube was swirled around every now and then to mix and replace the O_2 around the oocytes. The aspiration continued until all of the follicles were aspirated.

The total content in the aspiration tube consists of debris (containing cells and particles collected during the aspiration process) as well as the oocytes. These are arranged in consecutive layers consisting of oocytes/debris. After the aspiration, the debris and oocytes are separated into only 2 layers by turning the tube upside down a few times and then left standing so that the denser oocytes sink to the bottom of the tube with the less dense debris at the top.

The oocytes at the bottom of the tube were collected using a glass pipette attached to a syringe with a rubber tube. The pipetted oocytes were transferred to CULTURE medium in a large petri dish where oocytes were removed individually and taken to the next step, namely maturation. The oocytes are now kept warm at 32-36°C. Therefore, all further procedures were carried out on a warmed surface.

Oocytes were also collected using a vacuum pump is connected to one collection tube via a silicon tube. The same collection tube is then connected to a needle via a

thin silicon line. During the aspiration of oocytes via this method, up to five lines were used, each with a needle this was done by connecting the collection tube to 5 secondary collection tubes by 1 silicon lines and then a silicon line run from each secondary collection tube connected to a 18-gauge needle.

The primary as well as secondary collection tubes were placed in the water bath at 35°C too make sure it stay at a constant temperature. By pressing down on the pedal of the pump it started its suction. This suction formed a vacuum in the primary collection tube which created a vacuum in the secondary collection tube and the fluid was sucked through the silicon line into the secondary collection tube. The rest of the procedure was the same as in aspiration with the syringe.

If there was pressure on the pedal there was suction in the needle. The vacuum pump was used at a suction of 35-40XXX. The tube was also turned up side down a few times and the debris was sucked up and searching the oocytes began.
If the needle is inserted into a follicle, the pump action is activated with a pressure paddle and the suction will create a vacuum in the tube. This will suck the oocyte through the thin line into the collection tube. The pump was first sterilized with 95% ethanol and was then washed by sucking CULTURE medium through the lines of the pump. The cleaning of the pump afterwards follows the same principle. Firstly, medium was sucked through the pipelines to clean the lines from any oocytes that might be trapped in the line; this was followed by sterilization of the pump with 95% ethanol.

When a lot of blood was present in the sample, a filter was used to rid the sample of excess blood.

Oocyte maturation

As mentioned previously, the oocytes were transferred to a heated (39°C) large (20-x 100-mm) petri dish filled with CULTURE medium. The medium was poured in the dish only enough to cover the bottom of the petri dish. Parallel guidelines were drawn on the lid of another dish and placed underneath the searching dish to use as a grid in looking for the oocytes. To search for oocytes, I started at the top of the grid and work from left to right and across and down the plate. This ensured that whole petri dish was searched systematically. A search dish was not kept out of the incubator for

longer than 20 min, since oocytes are temperature sensitive and would dye at too high or too low temperatures.

All the oocytes were transferred to another small petri dish filled with CULTURE medium. The contents of the search dish (containing the debris after oocyte removal) were kept to be cultured for use as monolayers. Good to excellent quality (with at least one complete layer of compact granulosa cells cumulus-oocyte complexes (COC's) were recovered using a stereomicroscope. The COC'si were placed directly into a dish containing 3-4ml CULTURE medium.

The oocytes were then washed twice in CULTURE medium (while still in the dish, the oocytes in the dish were gently swirled). The oocytes were then transferred to a MATURATION dish containing maturation medium for the final wash. The oocytes were now free of any debris and were transferred to a petri dish containing 50 µl droplets MATURATION medium. The oocytes were placed in groups of 10-15 in the 50 µl droplets. The 50 µl droplets were gently covered in 3-4 ml of washed and serum-equilibrated mineral oil. The MATURATION micro-drops were maintained at physiological temperature (38.0-38.5°C) in an incubator for approximately 22 hours. After the incubation period the oocytes were matured.

The oocytes were incubated in 5% CO_2 at a temperature of 39°C for 22 hours, followed by insemination with spermatozoa (for 24 hours); thereby creating presumptive zygotes.

Preparation of the monolayers/feeder layers

Directly after the incubation of the oocytes, monolayers (or feeder layer) were prepared for the presumptive zygotes (fertilized oocytes) to be cultured on. Presumptive zygotes were obtained after 22 hours incubation and 24 hours fertilization.

Before preparation of the monolayers, and at least one hour before use, a CULTURE medium dish and tube of CULTURE medium was placed in the incubator to allow the medium to equilibrate in 5% CO_2. The contents of the search dish were poured into a centrifuge tube and centrifuged for 5 min. The supernatant was poured off and the pellet was re-suspended and filled to 4 ml CULTURE medium. With the addition of the CULTURE medium the re-suspended pellet was sucked up and down with a 500

µl pipette to breakdown the cells. It was centrifuged for 5 min and the process was repeated with 2 ml of CULTURE medium. This re-suspended pellet is left for 5 min to settle. 5µl of the top part of the tube/mixed pellet was added to the CULTURE dish.

The monolayers were made from this dish. 50µl micro-drops were prepared by first placing the droplets on the surface of a small (35 x 10 mm) petri dish. The droplets were well spaced away from each other but not too close to the edge of the dish. 5 µl of the top part of the mixed pellet was added to the 50 µl droplets, which were gently covered in 3-4 ml of washed and serum-equilibrated mineral oil. This grew into a monolayer of cells.

In vitro fertilisation (IVF):

In vitro fertilisation (IVF) is the second step in the *in vitro* embryo production (IVEP) program. As mentioned previously, oocytes were incubated for 22 hours on order to mature. Fertilisation of the oocytes occurs after *in vitro* maturation (IVM). All the media for the fertilisation program were kept in the incubator for at least one hour before use. This ensured that the media were warmed up and equilibrated to 5% CO_2. The incubation of the oocytes takes place in 50 µl FERT medium micro-drops. Each micro-drop contains 30 µl of the FERT medium, 10 µl of medium (transferred with the oocytes) and 10 µl FERT medium containing spermatozoa. 10 oocytes are incubated in each drop. This results in either zygotes or unfertilised oocytes. These zygotes were then be cultured on the monolayers.

In the following paragraphs the *in vitro* fertilization process, in order to obtain presumptive zygotes, will be discussed. One important step during IVF is the swim up procedure that is carried out to select the mature and motile spermatozoa. This procedure involves the layering of semen under medium in a centrifuge tube without mixing the two. The tube containing the semen and the medium is left for 1 hour in an incubator so that the vital spermatozoa can swim to the top of the tube. The supernatant is then taken off and used in IVF.

At least one hour before washing of the oocytes, the IVF droplets were prepared from the FERT medium by placing eight 30 µl droplets of the medium on a small (10x35 mm) petri dish spaced well apart of each other, but not too close to the edge of the

dish. The droplets were gently covered with 3-4 ml of washed and serum-equilibrated mineral oil. The medium droplets and wash dishes were allowed to equilibrate in 5% CO_2 in air for at least one hour before adding the oocytes. The microscope was switched on in advance to warm up the work surface in order to limit heat loss. While working, the oocytes were out of the incubator for as little time as possible, to limit heat and CO_2 loss.

The operating procedures were as sterile and clean as possible. A glass pipette attached to a syringe with a rubber tube was used, keeping aseptic techniques. Aseptic techniques included the rinsing of the glass pipette, syringe and rubber tube in 70% ethanol. The syringe and tip was also rinsed with FERT medium rid them of any ethanol that might be left in the syringe or tip). This equipment were used to aspirate the oocytes from the micro-drops and the oocytes were directly placed into a medium petri-dish (60x15 mm) containing 2 ml of pre-warmed FERT medium (This was done during the swim-up procedures which is carried out to select the mature and motile spermatozoa). The contents of the perti dish was aspirated and dispensed directly into a sterile 15 ml centrifuge tube. The 15 ml centrifuge tube was vortexed for approximately 2 min to remove the excess, expanded cumulus cells, then the contents of the tube was transferred into a second medium (60x15-mm) petri dish. The tube was rinsed thoroughly with the remaining FERT medium and decanted into the medium petri dish. The matured oocytes were searched by using a stereomicroscope and they were placed into the first wash dish containing FERT medium. They were washed once more in a fresh dish of FERT medium, then groups of 10-15 oocytes were transferred into each of the previously prepared FERT medium micro-drops.

At least one hour before beginning the swim-up procedures, the FERT medium were placed in the incubator for it to equilibrate in 5% CO_2 in air. The semen was thawed by removing frozen semen straws from liquid nitrogen storage, then placed directly into a 30-33°C water bath and swirled gently for 2-3 min. The straws were wiped dry with a paper towel, and then thawed semen was decanted into a separate sterile test tube. The thawed semen was carefully layered on the bottom of the FERT medium filled tube. The tubes were put in the incubator for the swim-up that took approximately one hour. The oocytes were washed and transferred to the FERT medium drops and the tubes were taken out of the incubator and the top part of the swim-up was taken off with a Pasteur pipette. The top part was then inserted into a

centrifuge tube and centrifuged for 5min, after which the supernatant was again taken off with a Pasteur pipette. The centrifuge tube containing the pellet was filled with FERT medium until a total volume of 100 µl were obtained.

Separation of motile spermatozoa

At this point, the total number of motile spermatozoa per millilitre was determined by aspirating 5µl of the resuspended spermatozoa pellet (after thorough, but gentle, mixing), and adding this to 95µl of distilled water. The diluted spermatozoa were gently vortexed and then 10µl was placed into each counting chamber and the average was determined. The average was multiplied by 5 million to give the total number of motile spermatozoa per millilitre in the pellet. The volume needed for approximately 1 million motile spermatozoa per millilitre (which will be the volume aspirating from the pellet for inseminating the IVF micro-drops) was determined. The IVF micro-drops were incubated at 38.5°C for 24 hours at 5% CO_2 in air. At the end of this incubation period, 60-805 of the spermatozoa retained some motility; most were, however, bound to the oocytes.

The experimental group of presumptive zygotes were treated with 25mM DTT for 30 minutes after 6 hours of fertilization. After 30 minutes the DTT was washed off with synthetic oviduct fluid (Sof) medium and cultured in the same way as the controls.

In vitro embryo culture (IVC):

In vitro culture is the third step in the *in vitro* embryo production (IVEP) program. Culturing the oocytes occurs 18–20 hours after *in vitro* fertilization. All the media for the fertilisation program was again warmed in the incubator for at least one hour before use, ensuring that the media was warmed up and equilibrated to 5% CO_2. The incubation of the oocytes is in 50 µl drops of synthetic oviduct fluid (Sof) medium on a monolayer of granulosa cells under oil and 30-40 oocytes are incubated per drop.

It is necessary to remove the cumulus and all excess spermatozoa surrounding the now presumptive (fertilized) zygote – this is called stripping. The operating procedures should be as sterile and clean as possible. The syringe used for stripping the zygotes must be rinsed with 70% ethanol and washed through with some medium to limit the contamination. The microscope is once again switched on in advance to warm up the work surface to limit heat loss.

CULTURE medium, is used to wash the presumptive zygotes after IVF. The IVC droplets are the drops with the monolayer of cumulus cells made on day 1.
In the previous paragraphs it was mentioned that the presumptive zygotes must be stripped from the spermatozoa and from the cumulus cells. This is done 24 hours post-insemination (fertilization with spermatozoa), the presumptive zygotes were removed from the IVF droplets, washed once in a wash dish containing CULTURE medium, and placed in groups of 30-40 in the IVC droplets (these droplets were prepared in the same way as the IVF droplets but Sof medium were used in stead of FERT medium, these droplets were prepared on the monolayers made on day 1). A small capillary tube with an inner diameter just larger than the outer diameter of the zygotes (by pulling over a flame), was used to perform these procedures. This effectively removed the majority of spermatozoa and degenerated cumulus cells and debris from the surface of the zygotes. [This was important to allow unimpaired visualization of cleavage development in the early embryo. Any attached clumps of viable cumulus cells often attach to the bottom of the CULTURE dish and may interfere with the normal development of the embryo by pulling it down and flattening it on the surface of the CULTURE dish].

At approximately 72 hours after the embryos were first placed into IVC medium containing cumulus/granulose cells it was necessary to feed the cultures by directly adding 40µl of fresh Sof medium. Again, the one-hour of equilibration of this media in the incubator atmosphere and temperature were allowed before adding it to the cultures. [At this point, the glucose content in the medium were probably entirely depleted by the cumulus/granulose cells; therefore, the adding of fresh medium provides more glucose, and other important factors].

Evaluation methods

"Squashing" of oocytes – Pronuclei count

The squashing (the flattening of oocytes for evaluation under a microscope) of oocytes was performed to evaluate whether fertilization had taken place or not in Group 1a and b. The oocytes were aspirated from slaughterhouse ovaries. The ovaries were transported in PBS at 27-30°C. After aspiration the oocytes were matured in the procedure used at Onderstepoort (discussed previously). They were fertilized after 22 hours of maturation by putting 10µl ($1x10^6$) of spermatozoa to the 30µl FERT micro-drops. The oocytes were fertilized for 6 hours and then exposed to

25mM DTT for 30 minutes. Four hundred and fifty of the presumptive zygotes were cultured to evaluate cleavage and embryo formation. Five hundred and ten presumptive zygotes were studied for pronucleus formation.

The following procedure was performed:
All the spermatozoa and cumulus cells were firstly stripped off from the presumptive zygote and then the medium was changed to make sure the spermatozoa and cumulus cells were washed off. The stripped and washed zygotes were then transferred to a glass microscopy slide, taking care that the minimal amount of medium is transported with the zygote. The glass slide was clearly labelled using a pencil for readability after the staining. Four drops of silicon glue were put around the oocytes and it was then covered by a cover slip. The cover slip was now slowly pressed to compress the oocytes to give them a flat surface in order to study them more clearly under the microscope (they should be pressed very slowly or they might burst). The cover slip was now glued with rubber cement on the sides and the slide left for 24 hours (it might be left for a week) in a Kaplan jar containing 3 X methanol: 1 X acetic acid solution (30ml methanol: 10ml acidic acid) This was followed by staining of the microscopy slide with orcein (the slide should be held on its side to allow the orcein flow through over the oocytes), and care should be taken never to let the slide dry. A microscope with DIC (differential interference contrast) filters was used for the evaluation of the zygotes.

Evaluation of the zygotes:

The following gold standard for evaluation was followed:

Table 4.1: Evaluation of oocytes Group 1a and b

Stage	**Observation**	**Result**
MII	Metaphase plate indicates an unfertilised oocyte	No penetration of the sperm
1PN	Only the female pronucleus has formed	Penetration of the sperm, only one gamete forms a pronucleus
2PN	Fertilisation has taken place and both the male and female pronuclei are visible	Fertilisation
3-9PN	Polyspermia (more than one spermatozoa were able to penetrate the oocyte)	Erroneous fertilisation
Deg	Degeneration	Non viable fertilisation

The formation of two pronuclei (2PN) is an indication that fertilisation had taken place, it does not necessarily means that the zygote will develop into a blastocyst and eventually into an embryo. To see whether the zygote will develop to a full-grown embryo the pre-implantation stages of embryonic development can be viewed under a light microscope. These stages will be discussed in the next paragraph and table 5.

Evaluating pre-implantation stage embryonic development:

Although there will probably be differences in embryonic development between different breeds of domestic cattle, one expects a similar rate of development *in vitro*. The grading of the different cells stages and especially the grading of the unfertilized oocytes and fertilized zygotes are done accordingly to the International Embryo Transfer Society (IETS) standards of grading.(Table 4.3).

Table 4.3: IETS standard grading used for groups 2a and b

Stage of development	Hours post-insemination
2-cell*	28-38
4-cell*	42-48
8-cell	52-70
16-cell	78-100
Morula	120-144
Early blastocyst	144-168
Blastocyst	156-180
Expanded blastocyst	176-192
Hatched blastocyst	192-228
* It is very important to note that the early cleavage stages (i.e. 2-cell and 4-cell) are very sensitive to changes in culture conditions (cooling or pH changes); therefore, the IVC co-cultures should not be removed from the incubator until at least 72 hours post-insemination.	

RESULTS AND DISCUSSION

The effects of DTT on pronucleus formation and embryo development were evaluated in three bovine specimens, numbers 1494, 1260 and 1197. Two groups of were used the first to monitor the pronucleus formation (fixed after 24 hours)(Group 1a and b) and the second embryo formation (fixed after 7 days)(Group 2a and b). In this study the 25 mM DTT was added for 30 minutes after fertilization rather than to the sperm as investigated in Chapter 3. DTT was added after fertilisation as DTT is a powerful reducing agent and therefore may inhibit the acrosomal reaction as reported by Tateno and Kamiguchi (1999) in studies using Chinese hamsters. Intracytoplasmic injection (ICSI) of oocytes with DTT induced decondensed sperm is widely used (Chung, *et al*. 2000) and the oocytes are activated with inomycin and DMAP. Pronucleus formation is the result of two processes the first initiated by gamete binding while the second occurs when the plasma membrane fuses and the

chromatin relaxes. With ICSI both process are eliminated and therefore the addition of chemicals that cause ova activation and sperm decondensation is essential. It was decided not to use microinjection as this technique is not available to all IVF institutions and is time consuming requiring a high level expertise.

In the first group, evaluation was according to Table 4.1, and was as follows MII, 1PN (only one pronuclei), 2PN (2 pronuclei), 3-9PN (3-9 pronuclei), and Deg. The number of oocytes evaluated is shown in Table 4.4 and were statically significant.

In the first group the number of oocytes that were in metaphase II were counted and presented as a percentage of the total number of oocytes evaluated. In the control animals, 1494/C, 1260C and 1197C, 20,83%, 20.41% and 16.36% metaphase plates were identified while in the DTT group 21.37%, 23,85% and 15.75% metaphase plates were identified (Table 4.1 and Figure 4.1). The differences between these two groups were not significant (p=0.7082) (Table 4.2 and Figure 4.2).

Table 4.4 Number of oocytes evaluated

	MII	1PN	2PN	3PN	DEG
1494/C	144	144	144	144	144
1494/DTT	117	117	117	117	117
1260/C	98	98	98	98	98
1260/DTT	109	109	109	109	109
1197/C	110	110	110	110	110
1197/DTT	127	127	127	127	127

In the control animals, 1494/C, 1260C and 1197C, 1.39%, 6.12% and 4.55% presented with one pronucleus (1PN) while in the DTT group 3.42%, 3.67% and 5.51% presented with one pronucleus (Figure 4.2). The differences between these two groups were not significant (p=0.372) Table 4.2.

In the control animals, 1494/C, 1260C and 1197C, 72.22%, 61.22% and 68.18% presented with two pronuclei (2PN) while in the DTT group 68.38%, 64.22% and 66.14% presented with two pronuclei formation (Figure 4.2). The differences between these two groups were not significant (p=0.7926) Table 4.2. This means that both groups, not exposed and exposed to DTT has the potential to undergo cleavage and eventual embryo development.

In the control animals, 1494/C, 1260C and 1197C, 2.78%, 8.16% and 4.55% degeneration of the nucleus were identified while in the DTT group 4.27%, 6.42% and 7.78% degeneration of the nucleus were identified. The differences between these two groups were not significant (p=0.6171) Table 4.2.

In the control animals, 1494/C, 1260C and 1197C, 2.78%, 4.08% and 6.36% poly spermia, (3PN) were identified while in the DTT group 2.56%, 1.83% and 4.72% poly spermia were identified. The differences between these two groups were not significant (p=0.3720) Table 4.2.

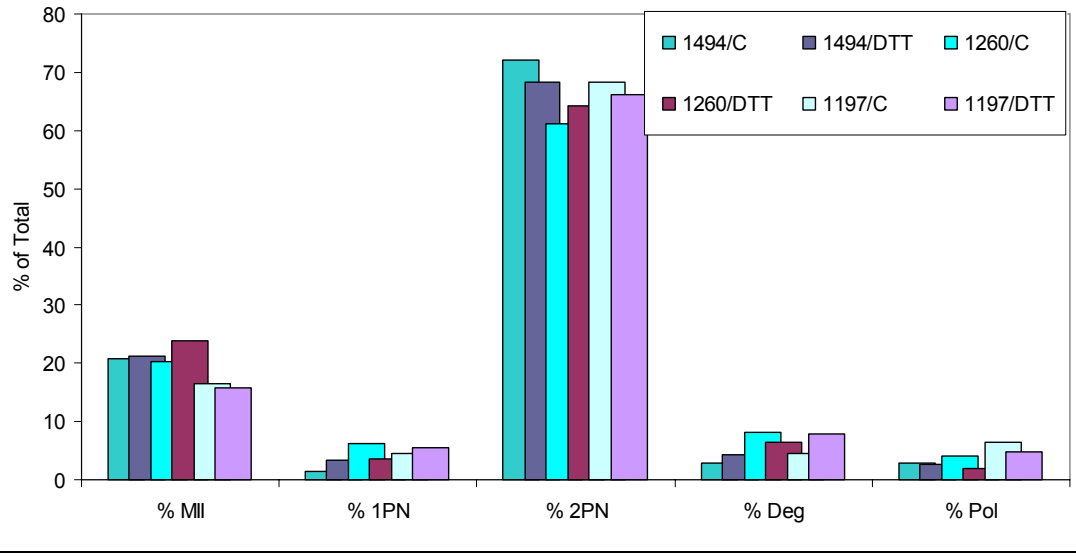


Figure 4.1: *% Pronuclei formation in fertilized oocytes*

In conclusion, a high rate of pronuclei formation (2PN)(Figure 4.1), for control (Group 1a) a mean of 67.20 (SD 4.5) and experimental (Group 1b) a mean of 66.24 (SD 1.7) indicates that zygote formation will occur. Tateno and Kamiguchi (1999) showed that with increasing concentrations of DTT used to induce spermatozoa decondensation successful Chinese hamster pronucleas formation of 72% was obtained following ICSI. The highest degree of pronucleas formation was obtained using 10 mM DTT for 30 minutes. Similarly Rho, *et al*. (1998) using activated bovine ova showed that ICSI with spermatozoa pre-treated with 5 mM DTT for 1 hour and found increased pronucleus formation of 59.6% compared to the control.

In group 2a and b, the percentage cleavage and embryo formation was determined on Day 1 and day 7 respectively. The number of oocytes/zygotes that were evaluated are shown in Table 4.4 and were statistically significant.

Table 4.4: The number of oocytes/zygotes evaluated on day 1 and 7

	Control		DTT	
	1D	**7D**	**1D**	**7D**
Mean	117.33	117.67	156.66	127
SD	23.86	9.02	25.48	9

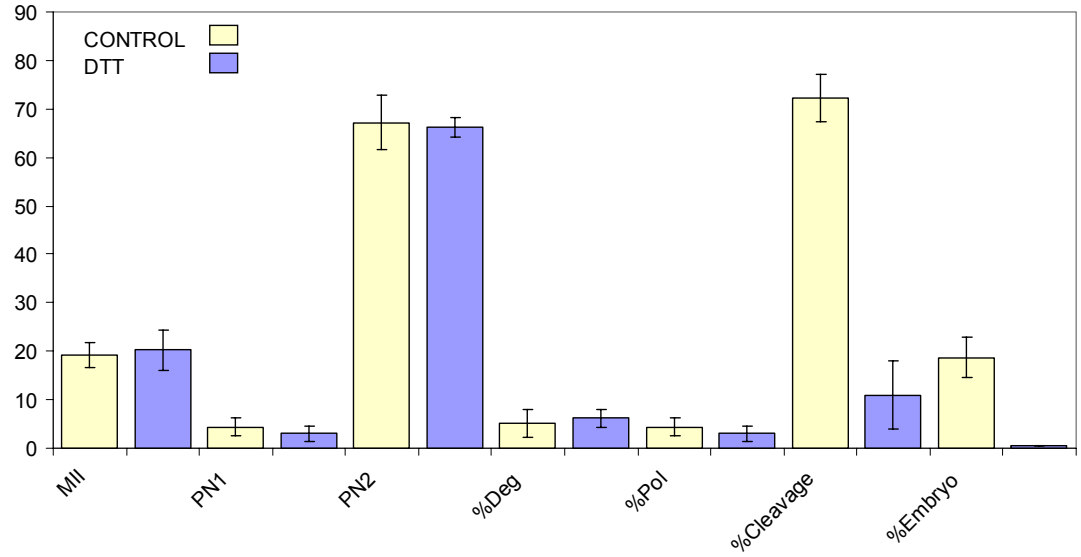


Figure 4.2: *Evaluation of oocytes and zygotes*

Table 4.2 Statistical evaluation of data

Control vs DTT exposed group		
	p-value	**Significance**
Metaphase II (MII)	p=0.7082	ns
1 Pronucleus formed (PN1)	p=0.372	ns
2 Pronuclei formed (PN2)	p=0.7926	ns
Nuclei that undergone degeneration	p=0.6171	ns
% Polyspermia	p=0.3720	ns
% Cleavage	p=0.0003	s
% Embryo	p=0.0016	s
s=significant, ns=not significant		

As shown above DTT had no significant effect on all the early parameters measured (Figure 4.2) however later in development DTT had a significant adverse effect on cleavage and eventual embryo development where for the control animals, 1494/C, 1260C and 1197C, 76%, 67% and 74% cleavage were identified while in the DTT group 10%, 5% and 19% cleavage were identified (Figure 4.3). The differences between these two groups were significant (p=0.0003) Table 4.5. In the control animals, 1494/C, 1260C and 1197C, 23%, 15% and 18% embryo formation occurred while in the DTT group 0%, 0% and 0% embryo formation occurred (Figure 4.3). The differences between these two groups were significant (p=0.0016) Table 4.5. The toxic effects of DTT on cleavage and embryo development will be difficult to investigate due to the time duration of experiments, cost involved and that standard bioassays used to investigate cell toxicity cannot be used. Cleavage and embryo formation is a process of multiple mitotic divisions resulting in an increase in the number of cells that become smaller with each cell division. Somatic cells are also cells that undergo mitotic division although the size of the cells remain constant and therefore this would be an ideal system to investigate the toxic effects of DTT on dividing cell population. The toxicity of DTT will be further investigated in Chapter 5.

CHAPTER 5: THE EFFECT OF DTT ON THE CELLUAR FUNCTION

Cleavage is a process of multiple mitotic divisions resulting in an increase in the number of cells without increasing the volume of the developing embryo. The daughter cells have smaller cytoplasmic mass and the nuclei of the daughter cells are normal in size and contain a full complement of chromosomes. Somatic cells are also cells that undergo mitotic division although the size of the cells remain constant and therefore this would be an ideal system to investigate the toxic effects of DTT on dividing cell population.

DTT is used as a reducing agent of disulphide bonds. It is routinely used in cell biology research to reduce disuphide linkages in proteins prior to electrophoresis as well as in protein structure studies and is known as Clelands reagent. Disuphide linkages occur as intermolecular and intramolecular linkages in many proteins and are essential for maintaining the secondary and tertiary structure of a protein. DTT is highly water-soluble and therefore dissolves easily in medium and taken up into the cell cytoplasm.

DTT is used to reduce the disulphide linkages that occur in histones and or protamines thereby inducing the process of decondensation. In this study DTT was found to be effective in inducing the decondensation of spermatozoa from normal donors and individuals with suspected infertility. Bovine spermatozoa isolated in the presence of heparin and subsequently exposed to DTT undergo decondensation. Subsequent bovine IVF with DTT showed successful pronuclear formation but reduced cleavage and failure of embryo development. The question is then raised whether DTT is having an effect on other cellular proteins and thereby inhibiting cleavage resulting in failure of embryological development. Cleavage is a process of multiple mitotic divisions resulting in an increase in the number of cells that become smaller with each cell division. Somatic cells are also cells that undergo mitotic division although the size of the cells remains constant. The use of a somatic cell such as the L929 cell line would be an ideal system to further investigate the toxic effects of DTT on cell division. Standard bioassays such as the Crystal Violet, MTT and Neutral Red that measure cell number, viability and lysosome membrane function respectively can be used to evaluate the toxic effects of DTT. The aim of this investigation was to determine if DTT at concentrations and exposure times used to

induce spermatozoa decondensation causes a decrease in cell number viability and lysosome membrane integrity in the L929 cell line.

Therefore the sixth research question is to determine whether DTT is cytotoxic to dividing L929 cells *in vitro*.

Material and Methods:

Cultivation, maintenance and preservation of the L-929 fibroblast cell line

The L929 fibroblast cell line was maintained in EMEM supplemented with 5% FCS and 1% antibiotic solution. An antibiotic stock solution was prepared by mixing 10,000U/ml Penicillin G (sodium salt), 10,000□g/ ml Streptomycin sulphate and 25□g/ml Amphotericim B in 0.85% saline. A volume of 10 ml of the working solutions was added to a litre of the prepared medium. The antibiotic solution was kept at -10 ^{o}C and thawed when needed. The media was sterilized by filtration through a 0.22□m membrane filters under aseptic conditions in a laminar flow cabinet. Aliquots of 100 ml were prepared and the medium was stored at 4^{o}C and warmed to 37^{o}C before use.

The cells were plated at a concentration of 5X10^{4} cells per ml in 25cm^{2} and 75cm^{2} cell culture flasks and were maintained at 37°C and 5% CO_2 in a CO_2 -water-jacketed incubator from Forma Scientific. The cell culture medium was changed every three days or when the medium had become acidic. The L929 fibroblasts cells were passaged once confluent with a 0.05% Trypsin solution. A 10X Trypsin/EDTA stock solution of 5g/l Trypsin, 2g/l EDTA.4Na was prepared by mixing 0.25g Trypsin, 0.1g EDTA and 0.425g NaCl in 50ml Hanks buffer. Hanks buffer was prepared by dissolving 9.86g/ l Hanks salt and 0.35g/l $NaHCO_3$ in ddH_2O. A working solution of 1X was prepared by diluting the stock solution with DPBS that was stored at -10 ^{o}C and warmed up at 37^{0}C before use. The solutions were filtered through a 0.22□m membrane filter under aseptic conditions.

The cells were passaged by firstly removing the medium from the confluent monolayer. A volume of 0.08 ml/cm^{2} trypsin working solution was added and the flask was placed at 37^{0}C for 5-10 minutes. The medium containing the detached cells was

added to 10 ml medium. The cells were collected by centrifugation in a BTL Bench centrifuge from Baird and Tatlock at 6000 x g (2000 rpm) for 5 minutes. The medium was removed and the cells were suspended in 10 ml fresh medium and the centrifugation step was repeated. The number of cells was determined by counting a 10 □l aliquot of cells using a haemocytometer from Brand supplied by Merck, Johannesburg, South Africa.

The L929 fibroblasts were either used for experiments described below or stored at –70°C. For storage the cells were suspended in cell culture freezing medium at a concentration of $5x10^6$ cells per ml. The freezing medium was prepared by adding 10% DMSO and 5% FCS to supplemented EMEM. One ml of the cell suspension was transferred to the 1.5ml freezing vials and stored by slow freezing (the vials were wrapped with tissue paper and placed into a large Styrofoam box) in a –70°C freezer. The cells were stored for indefinitely with minimum loss of viability.

The vials containing the L929 fibroblasts were thawed rapidly by stirring the vial in warm water at 37^0C. The cells were suspended in supplemented EMEM to a volume of 15 ml. The cells were collected by centrifugation, the supernatant was removed and the cells were suspended in fresh medium. This step was repeated twice before the cells were suspended in a final volume of 3ml culture medium and plated in $25cm^2$ cell culture flasks.

For each experiment, cells were plated at a cell concentration of $2x10^4$ cells per ml in 24 flat well plates with the culture area of $1.9cm^2$/well and were kept for 24 hours at 37°C and 5% CO_2 before conducting each experiment.

Exposure of L929 cells to DTT

Two different experiments were undertaken the first mimicking the conditions used in Chapter 4 and the second to study the effects of DTT at 5, 10 and 25mM concentrations and time intervals used in this publication and by other authors (Rho, *et al*. 1998)(Reyes, *et al*. 1989).

Two plates of L929 cells plated at a cell concentration of $2x10^4$ cells per ml in 24 flat well plates with the culture area of $1.9cm^2$/well were prepared. After 24 hours at 37°C

and 5% CO_2 a 50ul volume of a 250 mM DTT stock solution was added to 450ul medium to give a final concentration of 25mM. The L929 cells were exposed to the DTT for 30 minutes after which the medium was removed, cells were washed thrice with cell culture medium before lysosomal membrane integrity, cell viability and number was determined immediately or after 48 hours using the Neutral Red, MTT and Crystal Violet assays respectively.

In the second experiment 0, 10, 20 and 50 ul of the 250 mM DTT stock solution was added to 450 ul medium in each well and the final volume was adjusted to 500ul with medium to give a final concentration of 0, 5, 10 and 25 mM. The L929 cell line was exposed to the DTT for 30 minutes. The medium was then removed the cells were washed twice with cell culture medium and were grown for a further 48 hours at 37°C and 5% CO_2 before lysosomal membrane integrity, cell viability and number was determined.

Each concentration point per assay was done in quadruple and all data is an average of three experiments.

The MTT assay

Cell viability was measured using the MTT assay. A 0.1 mg/ml 3-(4,5-Dimethylthiazol-2 -yl) -2,5-diphenyltetrazolium bromide (MTT) solution was prepared in DPBS. A 50µl volume of the MTT solution was added to each well and the cell culture plates were maintained for 20 hours at 37°C and 5% CO_2. The medium was then removed and 200µl of isopropanol: HCl solution (24:1(1M HCl)) was added to each well to dissolve the water insoluble formazan product. After shaking the plates for 20 minutes the solution was transferred into a 96-flat well plate and absorbency at 545nm was measured using an EL900 plate reader.

Combined NR and CV assay

This assay is a combined method for Membrane Integrity Analysis and Cell Number. After exposing the cells to 25mM DTT for a period of 30 minutes, a 100µl of 0,15% (v/v) Neutral Red solution was added into each well. Plates were maintained at 37°C and 5% CO_2 for further 60 minutes for L929 cells. The medium was discarded and the cells were fixed with 200µl of 1% (v/v) acetic acid and 1% (v/v) formalin for 10 minutes. The fixing solution was discarded and the bound dye was dissolved with 200µl of 1% (v/v) acetic acid and 50% (v/v) ethanol solution. The solution was transferred into a 96-welled plate and the absorbency at 570 nm was measured

using a spectrophotometer (EL900) plate reader. The 24 well plates were rinsed with DPBS and then dried overnight at room temperature and then were stained with Crystal violet. A 300µl volume of 0.1% Crystal violet dye solution was prepared in 200mM of formic acid pH 3,5 and was added to the dried plates. The plates were shaken well, washed and dried again. This was followed by dissolving of the bound dye in 300µl of 10% acetic acid by shaking the plates for 10 minutes. Absorbency at 595 nm was determined using a spectrophotometer (EL900) plate reader. The dye solution was transferred to a 96 well plate and the absorbency at 595nm was measured using a spectrophotometer (EL900) plate reader.

Statistical analysis

Differences, single factor either time or concentration was determined using one-way ANOVA analysis while differences between groups, concentration and time was determined using two way ANOVA analysis.

RESULTS AND DISCUSSION

In all species the mechanisms by which cleavage is initiated probably differ depending on the stage of meiosis at which fertilization occurs. The rhythm of cell divisions is regulated by the synthesis and degradation of cyclin, in all species. Cyclin keeps the cells in metaphase, and the breakdown of cyclin enables the cells to return to interphase. Calcium ions appear to initiate the degradation of cyclin and once the cyclin is degraded the cycles of cell division can begin anew (Gilbert *et al.*1994).

Cleavage in the mammalian zygote occurs very slowly. The second characteristic of mammalian cleavage is that unique orientation of mammalian blastomeres with relation to one another. The first cleavage is a normal meridional division, but in the second cleavage one of the two blastomeres divides meridionally and the other divides equatorially. This type of cleavage is called rotational cleavage. A third characteristic is the marked asynchrony of early division which implicates that mammalian embryos do not increase evenly from 2-to 4- to 8-cell stages but frequently contain odd numbers of cells (Gilbert *et al.*1994).

A very distinctive characteristic of mammalian cleavage is compaction. Mammalian blastomeres through the 8-cell stage form a loose arrangement with plenty of space between them. After the third cleavage the blastomeres undergo a change in their behaviour where they suddenly huddle together and by this maximizing their contact with the other blastomeres and forming a compact ball of cells. Tight junctions that

form between the outside cells of the ball stabilize this tightly packed arrangement. The cells within the sphere form gap junctions which enable small molecules and ions to pass between the cells (Gilbert *et al.*1994).

To study the effect of DTT on this process is difficult due to the duration of experiments, ethical issues and cost. Furthermore the standard bioassays used to determine cell viability and number cannot be used. For these reasons the L929 cell line is an accepted cell line system to evaluate the toxicity of a wide range of chemicals was used. The L929 cell line is a dividing cell line and the duration of each experiment was so that the effects of DTT on cell division could also be determined. In this study L929 cells were expose to 25 mM DTT for 30 minutes, washed well to remove all residual DTT and lysosomal membrane integrity, cell viability and number was determined immediately following exposure and after 48 hours growth.

The MTT bioassay measures the ability of mitochondria l succinate reductase to reduce MTT into insoluble formazan crystals. DTT is also a strong reducing agent that readily reduces MTT and although the L929 cells are washed well to remove extracellular MTT any intracellular DTT will reduce the MTT resulting increased absorption that does reflect cell viability. Therefore the effect of DTT was determined using the Neutral Red and Crystal Violet assays.

Differences between controls not exposed and L929 cells exposed to 25 mM differences were significant. For the Neutral Red assay the p-values were 9.48×10^{-5}, and 1.2×10^{-5} at T0 and T48 hrs respectively while for the Crystal Violet assay the p-values were 4.01×10^{-11} and 4.92×10^{-19} at T0 and T48 hrs respectively.

For the Neutral Red Assay L929 cells exposed to 0-25mM DTT for time intervals T5, T10 and T30 differences between each time interval was not significant (p=0.647). In contrast, within each time interval differences between each concentration was highly significant with $p=1.3 \times 10^{-9}$. Difference between 0mM and 5mM, 10mM and 25 mM was highly significant ($p=2.21 \times 10^{-5}$, 2.4×10^{-6}, 8.25×10^{-10}). Differences between 5 mM and 10 mM was not significant (p=0.31), while differences between 5mM and 25mM as well as 10 mM and 25 mM were significant (p=0.0350 and p=0.043).

For the Crystal Violet assay L929 cells exposed to 0-25mM DTT for time intervals T5-T30 differences between each time interval was not significant (p=0.645). In contrast, within each time interval differences between each concentration was highly significant with $p=1.3 \times 10^{-9}$. Difference between 0mM and 5mM, 10mM and 25 mM

was highly significant ($p=9.41x10^{-8}$, $2.54x10^{-8}$, $1.66x10^{-7}$). Differences between 5 mM and 10 mM was not significant (p=0.602), while differences between 5mM and 25mM as well as 10 mM and 25 mM were significant (p=0.00403 and p=0.003697).

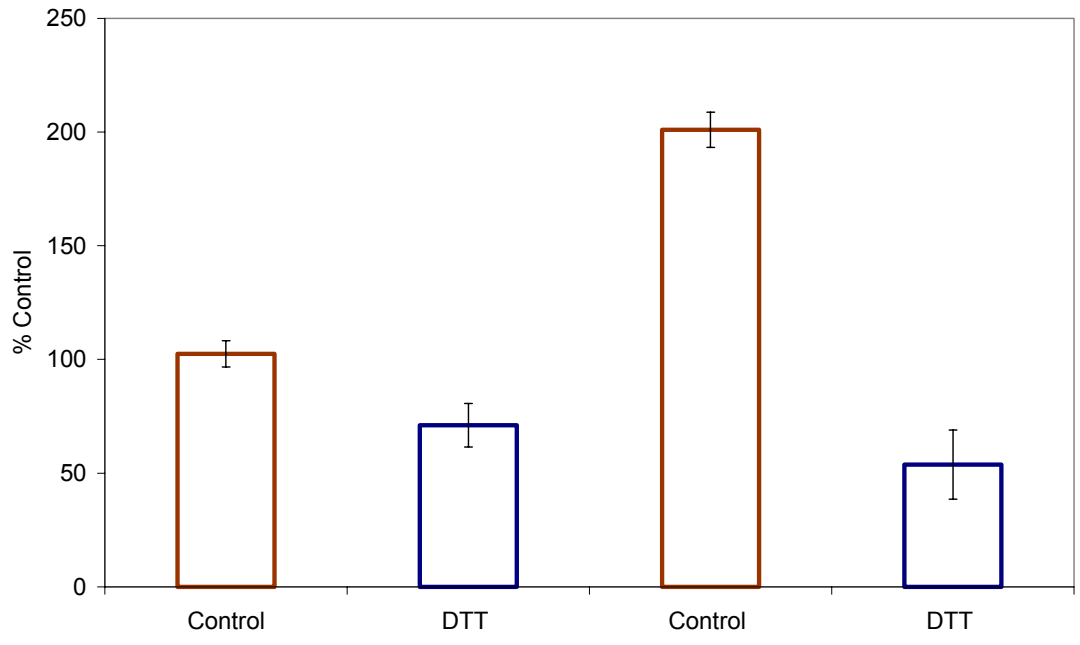


Figure 5.1a: The effect of DTT on lysosomal membrane integrity (a) Control after 30 minutes and 48 hours recovery and (b) exposed to 25mM DTT after 30 minutes and 48 hours recovery

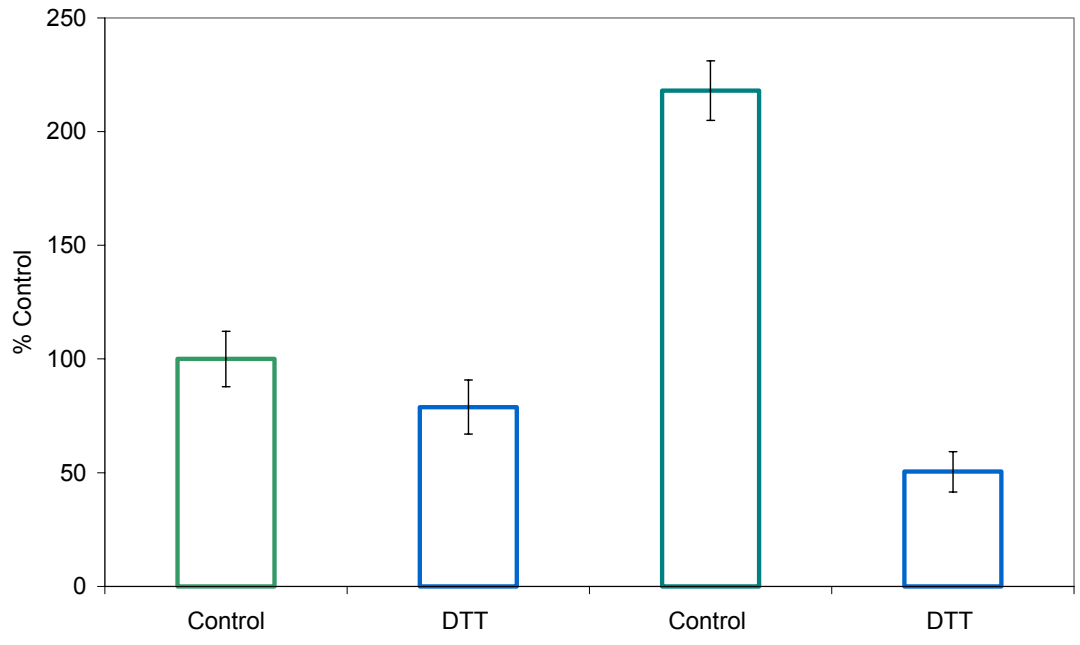


Figure 5.1b The effect of DTT on cell number (a) Control after 30 minutes and 48 hours recovery and (b) exposed to 25mM DTT after 30 minutes and 48 hours recovery

These results show that DTT is cytotoxic to dividing cells at the concentration used in this study after 30 minutes exposure. This effect is irreversible as after 48 hours cell number has decreased further. The question raised is whether lower DTT concentrations and exposure times such as that used to induce decondensation of spermatozoa (Chapter 3) in this study as well that used by Rho, *et al*. (1998) would be as toxic.

The L929 cells were exposed to 0-25mM DTT for 5, 10 and 30 minutes and lysosomal membrane integrity and cell number was determined using the NR and CV assays respectively after a further 48 hours growth in the absence of DTT. Compared to the control not exposed to DTT a significant decrease in lysosomal membrane integrity and cell number was observed at concentrations and exposure times indicating again that DTT is highly toxic to L929 cells (Figure 5.2a and b).

How DTT mediates this toxic effect is unknown, however due to the strong reducing effects of DTT and its ability to cross the cell membranes the tertiary structure of proteins, enzymes and DNA is vulnerable to the effects of DTT.

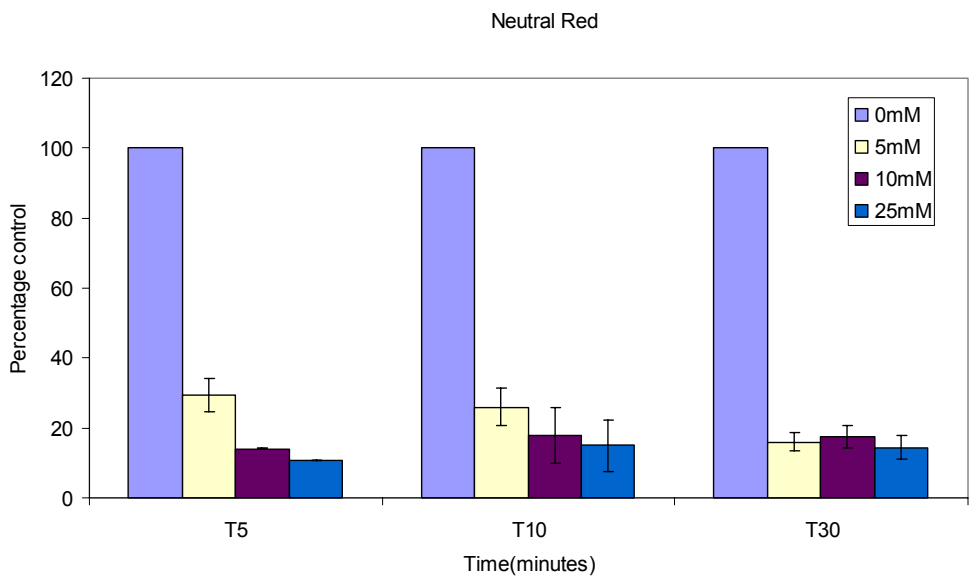


Figure 5.2a: The effects of 0-25mM DTT after 5, 10 and 30 minutes exposure on lysosome membrane integrity measured using the NR assay.

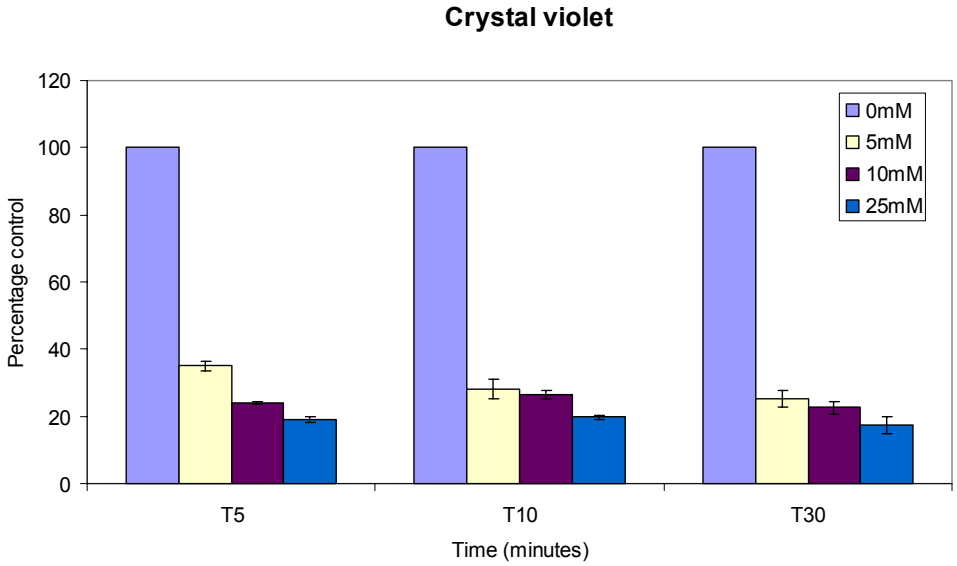


Figure 5.2b: The effects of 0-25mM DTT after 5, 10 and 30 minutes exposure on cell number measured using the CV assay.

Using the MTT CV and NR bioassays Ishiyama *et al.* showed that detergents such as the ionic detergent SDS is cytotoxic at 0.1mg/ml. Chemical compounds that induce decondensation but also have a wide range of other denaturing effects such as SDS, DTT and Triton X100 are not ideal decondensing agents. Therefore newer chemicals that specifically induce decondensation without other effects should be researched and designed.

CHAPTER 6: CONCLUDING DISCUSSION

Infertility in humans affects males as well as females, with an occurrence of approximately 10% to 16% where in about 20% to 25% of these cases, the male partner is the cause of the infertility (De Kretser *et al* 1999). Male infertility may be due to oligozoopermia, asthenozoopermia and teratozoopermia spermatozoa. ICSI (intracytoplasmic sperm injection) is one of the techniques used in IVF to address male infertility however the percentage of viable embryos obtained by the technique is very low.

Pronucleaus formation has been identified as one of the key events in fertilisation and gamete decondensation is vital for this process (Esterhuizen, *et al*. 2002). Defective or incomplete decondensation may be due to environmental or genetic factors. In such cases decondensation can be initiated by chemicals such as DTT that reduce the disulphide groups between the protamines that keep the DNA of the gamete condensed and this initiate pronucleus formation. (Chevret, *et al*. 1994) (Martini, *et al*. 1995) (Rousseaux, *et al*. 1995)(Perreault, *et al*. 1987)(Rho, *et al*. 1998) (Suttner *et al.* 2000). An increase decondensation should translate into a higher fertilization rate and a higher yield of embryos. The research from this thesis has compared the decondensation ability of DTT in human spermatozoa and bovine spermatozoa. Pronucleus formation in bovine zygotes and bovine embryo formation in the presence of DTT was investigated as well as the cytotoxic effect of DTT in somatic cell cultures.

Chromatin packaging anomalies in human spermatozoa arise because of defects in the spermatozoa nuclei condensation mechanism in certain patients (Sakkas et al. 1996). Griveau and coworkers in 1992 reported using cytoplasmic extracts from unfertilized Xenopus laevis (an amphibian) oocytes to induce decondensation in human spermatozoa. The authors also demonstrated that asthenozoospermic spermatozoa chromatin decondenses slowly and partially, compared to normozoospermic spermatozoa in humans. Decondensing agents can be divided into two groups: physiological agents like heparin and glutathione or chemical agents like DTT and CTAB used either alone or in combination (such as heparin in combination with DTT)(Reyes, *et al*. 1989).

In this study 12 of the semen samples for either fertile or subfertile subjects were collected and evaluated. The spermatozoa were collected using either the one-step-

swim-up or Puresperm methods. The spermatozoa were exposed to 25 mM DTT for 5, 7 and 10 minutes, washed and the morphological changes associated with decondesation compared to a control was evaluated by phase contrast microscopy. Compared to the control different degrees/patterns of decondensation was observed following exposure to DTT. These degrees/patterns were as follows; (i) cell membranes that appear intact with dark chromatin, with a clearly visible acrosome and with no decondensation of the acrosome area although towards the basal area of the spermatozoa head, decondensation is visible, (ii) the acrosome is visible, the membrane is intact and even decondensation has occurred, (iii) the acrosome is not visible and the spermatozoa head is dispersed (iv) the spermatozoa head is totally dispersed and only the tail can be identified. Another but unusual pattern of decondensation that is observed is that the spermatozoa appear condensed as in the control, but no nuclei or acrosome is visible in the spermatozoa head. The total number of spermatozoa that underwent decondensation over time was determined, 11 of the 12 samples underwent decondensation after 5 and 7 minutes. After 10 minutes several samples showed a lower rate of decondensation and this was associated with the unusual hypercondensed state described above.

Decondensation is a function of DTT concentration, incubation times and the inherent sperm integrity. Rousseaux, *et al*. (1995) and Chevret, *et al*. (1994) showed that 10 mM DTT induced decondensation of spermatozoa isolated from human donors after 10-50 minutes and the rate of decondesation varied between different samples. Griveau et al (1992) induced decondensation of human spermatozoa using 5 mM DTT for 60 minutes. Martini et al (1995) induced decondensation in human spermatozoa using 25 mM DTT for 5 minutes. In this study a DTT concentration of 25 mM with incubation times of 5, 7 and 10 minutes were used and decondensation was induced in 11 of 12 samples.

During spermatogenesis somatic histone proteins are replaced by protamine proteins to create mature spermatozoa and this aspect was investigated with fluorescence microscopy following CMA_3 staining. CMA_3 accessibility to DNA differs during spermatogenesis in the mouse suggesting that it varies according to the level of protamination. In the mouse, testicular spermatids show CMA_3 positive staining, while mature spermatozoa do not stain at all and only following fertilisation where decondensation begins in the oocyte does positive CMA_3 staining occurs. In this study, human spermatozoa not exposed to DTT did not stain indicating the mature (histone proteins replaced with protamines) status of all samples. Following exposure to DTT for 5, 7 and 10 minutes an increase in fluorescence is observed indicating

that, the spermatozoa could not possibly have become immature but rather CMA_3 has successfully found new binding sites ie the thiol groups of the protamines are reduced and CMA_3 has access to new binding sites. At longer exposure times of 7 and 10 minutes reduced fluorescence is observed for most of the samples possibly due to the supercoiling of the DNA due to the absence of proteins that create a stable three dimensional structure for DNA. DTT is used as a reducing agent for disulphide bonds, dithiotreitol and its isomer, dithioerythritol is capable of maintaining monothiols completely in the reduced state and of reducing disulphides quantitatively. According to Bizzaro *et.al.* (1998) CMA_3 has been shown to bind as a Mg^{2+} -coordinated dimer at the minor groove of GC-rich DNA and induces a conformational perturbation in the DNA helix resulting in a wider and shallower minor groove at its binding site. These authors have shown that CMA_3 is a useful tool for the rapid screening of subfertility in man, as it seems to allow an indirect visualization of protamine- deficient, nicked and partially denatured DNA. Furthermore in this study it has been found that CMA_3 staining can also be used to determine the degree of DNA decondensation.

In conclusion, in this study it was found that DTT induces decondensation of human spermatozoa pronuclei however as CMA_3 also indicates nicked and partially denatured DNA the question arises whether decondensation induced by DTT will result in the successful production of embryo's. In contrast, in studies using the Chinese hamster, Tateno and Kamifuchi in 1999 reported that chromosome aberrations decreased with increased concentrations of DTT suggesting that DTT have a protective function over chromosome damage. To test this hypothesis that decondensation with DTT will lead to the development of viable embryos, it was necessary to change from using human tissue to bovine tissue due to the ethical issues involved. The bovine model was chosen as it is a mammalian model and has been extensively researched and every step of the process has been established, standardized (eg N-E staining for morphology) and is commonly used in agricultural practices such as Taurus and the IVF program at Onderstepoort. In this study spermatozoa were used from fertile bulls that would under normal conditions give rise to viable embryos. The added advantage was that if this study was successful the percentage of viable bovine embryo's obtained could be increased due to an increase in pronucleus formation that should result in higher embryo yield.

Spermatozoa used were derived from Friesian bulls and the samples were pooled to prevent sample bias and interindividual variation. Spermatozoa were exposed to 25

mM of DTT at 5, 7 and 10 minutes as was used for human spermatozoa in these studies. Spermatozoa morphology was studied by light microscopy following N-E staining. Decondensation can be evaluated either by phase contrast microscopy without staining or light microscopy with N-E staining. The latter is an optimised method well established in the laboratory where these studies were undertaken. For this reason this method was used to evaluate the decondensation of bovine sperm.

No decondensation was observed at the same concentration and exposure times as used for human spermatozoa. The use of “swim up” medium containing heparin resulted in successful decondensation of bovine spermatozoa in the presence of 25 mM DTT for 30 minutes with morphological characteristics (Figure 4) similar as was observed for human spermatozoa as shown in Figure 3.3c. Delgado et al (2001) studied the combination of heparin and glutathione with some chemicals like dithiothreitol (DTT) and N-Cetyl-N,N,N-trimethyl ammonium bromide (CTAB) in bovine spermatozoa. These authors found that DTT combined with GSH alone did not induce decondensation however DTT in combination with heparin induced decondensation indicating that heparin was essential for the induction of decondensation in bovine spermatozoa. Furthermore Delgado et al. (2001) found that in the presence of heparin lower concentrations of DTT were required to induced successful decondensation.

The effects of DTT on pronucleus formation and embryo development were evaluated in three bovine specimens, numbers 1494, 1260 and 1197. Two groups of were used, the first to monitor the pronucleus formation (fixed after 24 hours)(Group 1a and b) and the second embryo formation (fixed after 7 days)(Group 2a and b). In this study the 25 mM DTT was added for 30 minutes after fertilization rather than to the sperm as investigated in Chapter 3. DTT was added after fertilisation as DTT is a powerful reducing agent and therefore may inhibit the acrosomal reaction as reported by Tateno and Kamiguche (1999) in studies using Chinese hamsters. Intracytoplasmic injection (ICSI) of oocytes with DTT induced decondensed sperm is widely used (Chung, *et al*. 2000) and the oocytes are activated with inomycin and DMAP. Pronucleus formation is the result of two processes the first initiated by gamete binding while the second occurs when the plasma membrane fuses and the chromatin relaxes. With ICSI both process are eliminated and therefore the addition of chemicals that cause ova activation and sperm decondensation is essential. It was decided not to use microinjection as this technique is not available to all IVF institutions and is time consuming requiring a high level expertise.

In the first group the number of oocytes that were in metaphase II were counted and presented as a percentage of the total number of oocytes evaluated. In the control animals, 1494/C, 1260C and 1197C, 20,83%, 20.41% and 16.36% metaphase plates were identified while in the DTT group 21.37%, 23,85% and 15.75% metaphase plates were identified (not significant p=0.7082). In the same group of animals 1.39%, 6.12% and 4.55% presented with one pronucleus (1PN) while in the DTT group 3.42%, 3.67% and 5.51% presented with one pronucleus (not significant p=0.372). Likewise in these control animals, 72.22%, 61.22% and 68.18% presented with two pronuclei (2PN) while in the DTT group 68.38%, 64.22% and 66.14% presented with two pronuclei formation (not significant p=0.7926). Degeneration of the nucleus animals was observed in both groups, namely 2.78%, 8.16% and 4.55% in the control and 4.27%, 6.42% and 7.78% in the DTT exposed group (not significant p=0.6171). In the control animals, 2.78%, 4.08% and 6.36% poly spermia, (3PN) was identified while in the DTT group 2.56%, 1.83% and 4.72% poly spermia was identified (not significant p=0.3720). The high percentage of two pronuclei formation indicates that zygotes not exposed and exposed to DTT have the potential to undergo cleavage and eventual embryo development.

Tateno and Kamiguchi (1999) used DTT to induce spermatozoa decondensation following ICSI and obtained a success rate of 72%. Similarly Rho et al (1998) using activated bovine ova showed that ICSI with spermatozoa pre-treated with DTT resulted in an increase in pronucleus formation of 59.6% when compared to the control. Rho and co workers were able to produce blastocysts but when these were transferred to heifers no pregnancies were carried to term .

Therefore this aspect was investigated in this study where in group 2a and b, the percentage cleavage and embryo formation was determined on Day 1 and Day 7 respectively. In this study it was found that DTT had a significant adverse effect on cleavage and eventual embryo development. For the control animals, 1494/C, 1260C and 1197C, 76%, 67% and 74% compared to the DTT group 10%, 5% and 19% cleavage rates were significantly different (p=0.0003). In the control animals, 1494/C, 1260C and 1197C, 23%, 15% and 18% embryo formation occurred while in the DTT group 0%, 0% and 0% embryo formation occurred (significantly different p=0.0016). These results show that DTT has a significant toxic effect on embryo development. To investigate the toxic effects of DTT on cleavage and embryo development will be

difficult due to the time duration of experiments; the cost involved; ethical issues furthermore standard bioassays used to investigate cell toxicity cannot be used.

Cleavage and embryo formation is a process of multiple mitotic divisions resulting in an increase in the number of cells that become smaller with each cell division. Somatic cells also undergo mitotic division although the size of the cells remains constant. For this reason somatic cells in cell culture are ideal to study the toxic effects of DTT and therefore the L929 cell line an accepted cell line system to evaluate the toxicity of a wide range of chemicals was used. L929 cells were expose to 25 mM DTT for 30 minutes, the monlayers were washed well to remove all residual DTT and lysosomal membrane integrity, cell viability and number was determined immediately following exposure and after 48 hours growth (a time period chosen to determine if DTT has an adverse effect on cell division). Cell viability was determined using the MTT bioassay that measures the ability of mitochondria l succinate reductase to reduce MTT into insoluble formazan crystals. DTT is also a strong reducing agent that readily reduces MTT and although the L929 cells are washed well to remove extracellular MTT any intracellular DTT will reduce the MTT resulting increased absorption that does reflect cell viability. The effect on lysosomal membrane integrity and cell number was determined using the Neutral Red and Crystal Violet assays respectively. Following 30 minutes exposure a highly significant decrease in lysosomal membrane integrity and cell number was found indicating that DTT is cytotoxic to L929 cells *in vitro.*

Different authors used different concentrations of DTT for different time intervals to induce deconcdensation. For example, Rousseaux, *et al*. (1995) and Chevret, *et al*. (1994) used 10 mM DTT for 10-50 minutes, Griveau et al (1992) used 5 mM DTT for 60 minutes, Martini et al. (1995) used 25 mM DTT for 5 minutes therefore experiments were designed to determine the toxic effects of DTT at all concentrations previously used by other authors. L929 cells were exposed to 0, 5mM, 10mM and 25 mM DTT for 5, 10 and 30 minutes and lysosomal membrane integrity and cell number was determined following exposure.

For the Crystal Violet assay L929 cells exposed to 0-25mM DTT for time intervals T5, T10 and T30 showed the same pattern of toxicity and differences between each time interval was not significant ($p=0.645$). However compared to control toxicity was significant at 5mM, 10mM and 25mM DTT ($p=1.3 \times 10^{-9}$). Likewise for the Neutral Red assay L929 cells exposed to 0-25mM DTT for time intervals T5, T10 and T30

also showed the same pattern of toxicity and differences between each time interval was not significant (p=0.647). However compared to control toxicity was significant at 5mM, 10mM and 25mM DTT ($p=1.3 \times 10^{-9}$).. These experiments clearly indicates that DTT is cytotoxic at all concentrations and exposure times.

DTT is a strong reducing agent and with its ability to cross the cell membranes the tertiary structure of proteins, enzymes and DNA is vulnerable to the effects of DTT. Therefore other commonly used chemicals such as the ionic detergent SDS, non-ionic detergent, Triton X-100 and chelating agents such as EDTA are not ideal decondensing agents due to their wide range of possible effects on enzyme activity, protein and membrane structure. Therefore a newer generation of chemicals that specifically induce decondensation without other effects should be developed and researched. The use of standard cell culture models for cytotoxicity should be used for the initial evaluation of agents used for decondensation. This would save time, valuable resources and would allow the rapid and cost effective evaluation of different agents and combinations thereof at different concentrations and time intervals. There also exists a wide range of bioassays that specifically be used to determine the effect of these agents on DNA structure (TUNEL assay), membrane integrity (Propidium Iodide assay) and enzyme activity (Fluorescien diacetate bioassay). Other smaller animal models such as the chick embryo model will also allow rapid evaluation of the effects of these agents on cleavage and embryo formation. Although these processes do differ between animal species, these models will give a general indication of their effectiveness.

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APPENDIX 1

Information sheet to sperm donors:
DECONDENSATION OF HUMAN SPERMATOZOA AND MALE INFERTILITY; MORPHOLOGICAL STUDIES AND GENETIC FACTORS.

Dear Participant,

The Cell Biology laboratory of the Department of Anatomy, University of Pretoria strives to provide opportunities for our post graduate students to do relevant research, not only to gain maximum experience but to, through our research gain knowledge to improve the health of the nation and contribute valuable information to the world research fraternity.

One of the major problems of this century is infertility. Although it was previously believed that infertility is associated mostly with men, research now show that 55% to 60% of infertility can be ascribed to men.

The aim of the study:
The research goals are to study the process of DNA decondensation in sperm from normal and infertile donors. The following research question must be addressed.

- What time of DTT exposure will best initiate decondensation of the DNA in infertile and fertile spermatozoa?
- Can morphological abnormalities in spermatozoa from infertile males following DTT induced decondensation be observed using light and transmission electron microscopy?
- Is it possible to develop a technique using scanning high-resolution electron microscopy to study the internal morphology of spermatozoa from infertile males with DTT induced decondensation?
- Although DTT will be used to induce DNA decondensation, little is known regarding the effects of this agent on the chromatin structure. Does DTT induce single or double strand nicks in DNA of spermatozoa from infertile males when compared to spermatozoa from fertile males?
- Does the presence of the Δ F508 (the cystic fibrosis gene mutation) contribute significantly to infertility in the South African population?

Thank you for your participation.

Dr E. Pretorius
Researcher and Senior Lecturer
Department of Anatomy, University of Pretoria

INFORMED CONSENT

Title of study: DECONDENSATION OF HUMAN SPERMATOZOA AND MALE INFERTILITY; MORPHOLOGICAL STUDIES AND GENETIC FACTORS.

Research study:
I……, willingly agree to participate in this study which is briefly described in the cover letter. The Department of Anatomy of the University of Pretoria, together with the pathologists, Du Buisson and partners are conducting this study.

Purpose of this study:
As laid out in the information sheet attached to this form.

Risks and discomforts:
There are no risks to myself involved in this study other than those explained to me by my general practitioner or by Du Buisson and partners.

Contact person:
For more information concerning this research I can contact Dr E. Pretorius at (tel.) 012 319 2533.

Benefits:
In understand that the information obtained from this study may not be of direct benefit to my family members or me. The information obtained from this study may provide generally useful knowledge.

Voluntary participation:
Participation to this study is voluntarily. No compensation for participation will be given. I understand that I am free to withdraw my consent to participate in this study at any time.

Confidentiality:
I understand that no record of my personal details will be held in any form by the researcher and furthermore, no information by which I can be identified will be released or published.

I have read all the above and understand the reason for the research and I am willing to give my consent to participate in this research program.

…….. ……
Signature of participant **Date**

…….. ……
Signature of Witness **Date**

APPENDIX 2

PREPARATION OF SOLUTIONS, MEDIA AND BUFFERS USED FOR IN VITRO FERTILIZATION (CHAPTER 4)

TABLE 1: PREPARATION OF STOCK, WASH, CULTURE AND MATURATION MEDIA	
STOCK MEDIA	
Milli Q H_2O	1000 ml
$NaHCO_3$	2.2 g
TCM 199 powder	15 g
pH	7.4
Osmolarity	290-310
WASH MEDIA	
Stock media (prepared above)	300 ml
BSA	0.9 g
Gentamycin	150 □l
CULTURE MEDIA	
Stock media (prepared above)	80 ml
Steer serum 5%	4 ml
Gentamycin	40 □l
MATURATION MEDIA (MM)	
Wash media	8 ml
Mercaptoethylamine	0.0096 g
To 350 □l of the above add 35 ml culture medium	

TABLE 2: PREPARATION OF SPERM TALP (STOCK), FERT WASH AND FERT MEDIA	
SPERM TALP	
Milli Q H_2O	1000 ml
NaCl	6.66 g
KCl	0.238 g
$NaHCO_3$	2.090 g
$NaHPO_4$ ($1H_2O$)	0.055 g
HEPES	2.4 g
$CaCl_2$	0.294 g
$MgCl_2$	0.1 g
Phenol Red	1 ml
pH	7.4
Osmolarity	275-280
FERT WASH	
Sperm talp stock	125 ml
FAF BSA	0.75 g
Pyruvate	0.0065 g
Lactate syrup	157 □l
Gentamycin	62 □l
pH	7.4
FERT MEDIA	
FERT WASH	50 ml

Heparin	0.0015 g
PHE	2 ml
pH	7.8

TABLE 3: PREPARATION OF PHE MEDIA		
STOCK MEDIA		
A	Milli Q H_2O	33 ml
	NaCl	0.3 g
B	A	20 ml
	Penicillamine	0.003 g
	Hypotaurine	0.00109 g
C	Milli Q H_2O	50 ml
	Metabisulphate	0.05 g
	Lactate syrup	110 □l
	pH	4.0
	Epinephrine	0.00228 g
To prepare PHE media, Combine 6ml A, 10 ml B and 4 ml C		

TABLE 4: PREPARATION OF PBS STOCK SOLUTION AND SOF MEDIA	
PBS STOCK	
Milli Q H_2O	100 ml
NaCl	0.6294 g
KCl	0.05338 g
KH_2PO_4	0.0162 g
$CaCl_2$	0.0251 g
$MgCl_2$	0.00996 g
$NaHCO_3$	0.2106 g
MEDIA	
PBS stock	9.5 ml
Lactate	2.25 □l
Pyruvate	0.00033 g
Glutamine	0.00146 g
Gentamycin	5 □l
Serum	500 □l
Filter	
MEM (amino acids)	100 □l
BME (amino acids)	200 □l

APPENDIX 3
List of abbreviations

1PN – 1 - pronucleus

2PN - two pronuclei

3-9PN – 3 to 9 pronuclei

CMA_3 - chromomycin A_3

COC - cumulus oocytes complex

CTAB - N-Cetyl-N,N,N-trimethyl ammonium bromide

CV – Crystal Violet

DABCO - (1,4-diazabicyclo[2.2.2.]octane

ddH_2O - double distilled and deionised water

Deg – degenerative

DIC - differential interference contrast

DMSO - dimethyl sulphoxide

DTT - Dithiothreitol

EBSS - Earles buffered Saline Solution

EDTA - ethylene diamine tetra acetate

E-N - Eosin-nigrosin

IETS - International Embryo Transfer Society

IVC - In vitro embryo culture

IVEP - in vitro embryo production

IVEP - in vitro embryo production

IVM - in vitro maturation

KCl - potassium chloride

KH_2PO_4 - potassium dihydrogen phosphate

MII – metaphase plate

MTT - 3-(4,5- Dimethylthiazol-2 -yl) -2,5-diphenyltetrazolium bromide

Na_2HPO_4 - disodium hydrogen phosphate

NaCl - sodium chloride

$NaHCO_3$ - sodium hydrogen carbonate

NR – Neutral Red

PBS - Phosphate Buffered Saline Solution

SDS - Sodium dodicyl sulphate

Sof - synthetic oviduct fluid

SSM - Standard Sperm Medium