CAPACITATION OF NGUNI SEMEN FOR *IN VITRO* FERTILIZATION OF BOVINE OOCYTES

by

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Submitted in partial fulfilment of the requirements
for the degree of
Master of Science in Agriculture

in the
Department of Animal Science and Poultry Science
Faculty of Agriculture
University of Natal
Pietermaritzburg
1995

PREFACE

The experimental work described in this dissertation was carried out in the Department of Animal Science and Poultry Science, Faculty of Agriculture, University of Natal, Pietermaritzburg, from March 1992 to December 1994, under the supervision of Professor Arthur W. Lishman.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it has been duly acknowledged in the text.

L. J. Durrans.

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ACKNOWLEDGEMENTS

I would like to express my sincere thanks and appreciation to the following institutions and persons for their invaluable contribution to the work presented in this thesis:

Prof. A.W. Lishman for tremendous patience during the course of experiments and supervision of this project;

University of Natal (University Research Fund), Dr. G. Harwin Trust and the Department of National Education for financial assistance without which this project would have been impossible;

Dallas Shaw, Sylvia Goedecke, Annari Faurie and Michelle Cole for invaluable help in the laboratory;

Philip Jack (Taurus), Suzette Foss (Irene), Dr. Steyn (Ramsen) for practical advice and donation of materials;

Dr. Ronchietto for generous donation of Nguni bull semen;

Stockowners for providing heifers for implantation of embryos;

The staff of Cato Ridge abattoir (especially Cliff Walker, Charles Barnes, Gonaseelan Munsamy and John Johnson) for help with ovary collection and without whose help this project would not have been possible;

Jonathan Levin, Margaret Cashman and Eric Grau for help in experimental design and statistical analysis with a tolerant understanding of the practical problems involved;

Prof. R.M. Gous for providing me with the opportunity to study in South Africa;

Hylton Adie, Annari Faurie, Gail Bradford and Vivien Langley for proof-reading;

All of the masters students, technical staff, new friends in South Africa and the friends I have left behind, for tremendous support and helpful advice throughout my time in this country;

My parents for encouraging me in everything that I have wanted to do, especially my dad for giving me the freedom and support to study overseas;

Hylton, for always being there.

ABBREVIATIONS

AI	-	artificial insemination
BOEC	-	bovine oviduct epithelial cell
BSA	-	bovine serum albumin
BSA (FAF)	-	fatty acid free bovine serum albumin
DMSO	-	dimethyl sulfoxide
E_2	-	oestradiol-17ß
ECS	-	oestrous cow serum
FCS	-	foetal calf serum
FSH	-	follicle stimulating hormone
FSH-p	-	porcine follicle stimulating hormone
g	-	gravities
hep	-	heparin
HEPES	-	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IVC	-	*in vitro* culture
IVF	-	*in vitro* fertilization
IVM	-	*in vitro* maturation
LH	-	luteinizing hormone
LH-o	-	ovine luteinizing hormone
PBS	-	phosphate buffered saline
PHE	-	penicillamine, hypotaurine and epinephrine
rpm	-	revolutions per minute
s.e.m.	-	standard error of the mean
TCM 199	-	Tissue Culture Medium 199

CONTENTS

ABSTRACT

In vitro maturation, fertilization and culture is a technique which is increasingly utilised in the animal science industry for embryo production. However, optimal conditions for *in vitro* maturation, fertilization and culture have not been clearly defined and much research is being done to improve this situation. If these techniques are to be valuable in the production of large numbers of animals in short periods, they must be efficient and reliable.

The first part of this investigation aimed to produce comparable rates of fertilization from each of 5 Nguni bulls tested in an *in vitro* fertilization trial. It was hypothesised that each bull would respond favourably to one or more of the treatments tested. A complete randomised block design was set up to account for block and day effects. A total of 2422 cumulus-oocyte complexes were matured and fertilized with swim-up separated frozen-thawed Nguni semen incubated for 1 min in Ca-ionophore A23187 (0.1, 0.2 or 0.4 μM) or for 15 min in heparin (0.05, 10 or 25 μg/ml). Controls for parthenogenesis and capacitation or acrosome reaction in the fertilization medium were included. Presumptive zygotes were transferred to CR1aa medium and supplemented with 10% and 5% FCS on days 4 and 7 post-insemination, respectively. Bull performance was assessed using four criteria: embryo cleavage, a score based on a cleavage index (Score 1; minimum -1, maximum 6) and one which gave greater weight to morulae and blastocysts (Score 2; minimum -1, maximum 10) and blastocyst production. Day effects were highly significant ($P<0.001$). Parthenogenetic activation of 31.6% of oocytes occurred. Depending on the bull used, cleavage rates (%±s.e.m.) varied from 29.5±2.2 to 40.6±2.6, Score 1 from 0.30±0.07 to 0.65±0.07, Score 2 from 0.30±0.07 to 0.72±0.08 and % blastocysts from 0.6±0.6 to 4.8±1.1. Treatment did not significantly affect performance and there were no bull x treatment interactions. However, bull differences in performance were observed. The IVF system employed was not stable and did not produce repeatable results. It was concluded that treatment concentrations tested may have been too low or, alternatively, that treatment effects were

being masked by a factor influencing the IVF system more strongly than the treatments tested.

In the second part of this investigation, laboratory tests to determine semen quality were examined. It was hypothesised that semen quality would correlate with performance determined during the *in vitro* trial and, thus, provide predictors for bull fertility *in vitro*. A dual staining procedure which detects live/dead or acrosome reacted/not acrosome reacted sperm was also utilised to determine whether bull x treatment interactions may have existed in the *in vitro* trial but were obscured by other factors influencing the IVF system.

Sperm motility and abnormal morphology were assessed using light microscopy on whole frozen-thawed semen samples. Statistical analysis could not be done because of lack of replication. Percentages of immotile sperm were high (60.16% to 78.51%), with considerable variation in progressive motility (13.90% to 34.35%) between bulls. A large variation in numbers of morphologically normal sperm was observed between bulls (66% to 90%), with major deformities (9% to 19%) accounting for most of the abnormalities. High negative correlations with performance (as defined in the *in vitro* trial) were found between % normal sperm and % proximal droplets ($r=-0.66$ to -0.88). Percentage of minor abnormalities, distal droplets and coiled tails correlated positively with fertility ($r=0.67$ to 0.91). Motility did not correlate highly with any of the criteria used to assess bull performance. Correlations of semen quality to performance were contradictory to expected results and this may have been due to swim-up separation of sperm for the *in vitro* trial which was not carried out for semen quality assessment. At present, these semen quality tests do not allow prediction of bull fertility *in vitro*.

Assessment of sperm stained for evaluation of live/dead and acrosome reacted/not acrosome reacted was a lengthy procedure. Again, statistical analysis was not possible due to the lack of replication. High percentages of sperm were characterised as dead (52.4% to 100%). Bulls did respond differently to the various treatments, as determined by the proportions of acrosome reacted sperm and live acrosome reacted sperm. Thus,

bull x treatment interactions were apparent, suggesting that the IVF system was more strongly influenced by other factor(s) which reduced sensitivity to the treatments tested.

In summary, more research is needed to stabilise the IVF system if production of large numbers of embryos is to become economically viable.

GENERAL INTRODUCTION

Small scale farmers (subsistence farming) will become increasingly common in South Africa as land redistribution occurs. There are potentially thousands of prospective farmers, with each farmer attempting to feed his/her family from the land. In this context, the importance of fresh milk has been well emphasised. In view of the difficulties of transporting fresh milk to remote areas, and the need for cold storage of such a product, requirements for fresh milk could be provided for by, say, two milking cows.

A need has, thus, been defined for a breed of dairy cow which is adapted for production in the developing areas of southern Africa (Ramsay and Lubout, 1994). These animals should display disease resistance coupled with milk production and a short calving interval. Of the European dairy breeds, the Jersey has been shown to be better adapted to the harsh African climate than the Friesian and, furthermore, has superior reproductive performance (Tibbo *et al.*, 1994). Improved performance (milk yield, lactation length, age at first calving and calving interval) has been observed from F_1 crosses of Jerseys with local breeds when compared to F_2 crosses. Thus, to obtain optimal performance, crossing of pure-bred stock is necessary.

The Nguni is a breed indigenous to South Africa and it has been suggested that crossing this breed with the Jersey will provide animals which can tolerate environmental conditions in sub-tropical regions and, at the same time, have a desirable milk yield (Ramsay and Lubout, 1994). However, establishing a new cross is a lengthy process, as natural methods of reproduction do not allow for rapid population growth within a short time. One way to solve this problem would be to employ *in vitro* fertilization techniques which provide a means whereby considerable numbers of embryos can be produced for implantation into cows of immaterial breed. Herds could, therefore, be built up more rapidly than would normally be the case, with sustained production of the F_1 cross.

With an initial herd of 120 Jersey cows (Ramsay and Durham, 1994), it would take 19 years with natural mating to establish a herd of 1000 F_1 cows (assuming that the cows were already at calving age and a calving interval of 416 d; Tibbo *et al.*, 1994). This time could effectively be reduced to the gestation length of the recipient cows (approximately 282 d; Hunter, 1982) if *in vitro* fertilization techniques were employed, depending on the availability of oocytes from Jersey females (abattoir material or obtained via ultrasonic aspiration from live donors).

Approximately 75 000 oocytes exist in the heifer ovary at birth (Craplet, 1963) and are potentially available for maturation and eventual ovulation. However, only a small proportion of these oocytes are actually ovulated and are available for fertilization. What happens to those oocytes which are not ovulated?

Many oocytes are destined to follow the line of atresia for reasons that have not been identified. In a study of ovarian follicular dynamics, Sirois and Fortune (1988) determined that follicular growth may occur in two or three waves, each using up many more follicles than necessary for ovulation. On average, a two wave pattern of follicular growth involved recruitment of 9 follicles per ovulation. Thus, at best, a cow would be able to produce 8 333 ovulatory oocytes from all potential oocytes. Regardless of this fact, there are insufficient oestrous cycles in a cow's lifetime to allow all of these oocytes to be ovulated. Even if a cow ovulates every 21 days from the age of 6 months until she is 10 years old, she will only have ovulated 165 times. The shortfall is 8 168 oocytes which are lost through atresia and non-recruitment.

If *in vitro* fertilization techniques could utilise the oocytes which would otherwise be lost, efficiency of animal production would be immensely enhanced. With this in mind, the aim of the experimental work contained in this thesis was to assess the *in vitro* performance of individual Nguni bulls for subsequent use in *in vitro* production of embryos. This was achieved by carrying out *in vitro* fertilization tests alongside laboratory tests which attempted to characterise bulls without the need for lengthy and expensive *in vitro* fertility tests.

CHAPTER 1

REVIEW OF LITERATURE

1.1 INTRODUCTION

Innovations in reproductive technology will have a tremendous impact on possible future developments in animal science. The importance of these techniques to the animal scientist cannot be over-emphasised and include:

a) animals, by utilisation of their gametes, can effectively be transported world-wide, allowing international access to superior genetic stock,
b) gametes and embryos may be transported with simpler needs for disease control,
c) multiple ovulation and embryo transfer (MOET) techniques allow genetically superior female animals to produce large numbers of offspring without having to carry the young themselves,
d) cryopreservation permits storage of embryos for long periods of time, allowing reintroduction of genetic material to the gene pool at a much later date,
e) *in vitro* maturation, fertilization and culture of follicular oocytes allows the use of material from live and slaughtered animals.

The technology of *in vitro* maturation, fertilization and culture of follicular oocytes has become a reality for use in animal production. However, these techniques must be reliable, efficient and economical if animal science is to benefit in the long term. An understanding of the basic principles of *in vivo* reproduction is necessary if manipulation of *in vitro* reproduction systems is to be carried out to full advantage.

1.2 *IN VIVO* REPRODUCTION

1.2.1 The female

1.2.1.1 Ovary and oocyte development

The details of oogenesis have been elucidated by Johnson and Everitt (1980) and Baker (1982) and will not be discussed here. However, a brief overview is necessary to gain an understanding of the events that the use of *in vitro* techniques aims to replicate.

Early in embryonic and foetal development, the number of germ cells (oogonia) within the ovaries increases rapidly (Hunter, 1982). These oogonia give rise to primary oocytes within the ovary by a meiotic division and extrusion of a polar body. The development of these oocytes is arrested at the diplotene stage of the first meiotic prophase (Byskov, 1982). In this way, the young heifer foetus produces her full quota of oocytes before she is born. However, many of the follicles containing these oocytes will become atretic and a large proportion of the oocytes will never be ovulated (Baker, 1982).

1.2.1.2 Follicular growth and resumption of meiosis

Follicular growth begins before birth, but only after puberty does this follicular growth lead to ovulation of oocytes. The final growth phase of a follicle is under hormonal control which is initiated at puberty. A fundamental role in oocyte maturation is played by the follicle (Masui and Clarke, 1979). Schultz (1986) stated that an inhibitory effect is exerted on the oocyte by the follicle through granulosa-cumulus cell contact, which is only overcome under the influence of gonadotropins. A surge of luteinizing hormone (LH) occurs, approximately 30 to 36 h before ovulation (Hunter, 1982). This surge induces the granulosa cells within the dominant follicle to produce progesterone and terminates the follicle's inhibitory effect on the oocyte. The LH surge also acts on the oocyte, causing disintegration of the nuclear membrane surrounding the chromosomes arrested at the first meiotic prophase. Both cytoplasmic and chromosomal maturation now take place. During this time, the Golgi apparatus synthesises lysosomal-like granules, called cortical granules, which migrate to the surface of the oocyte (Johnson and Everitt, 1980). Chromosomal maturation continues through the stages of meiosis

until metaphase II is reached, at which time the second polar body is extruded. Once again, meiotic development is arrested.

1.2.1.3 Ovulation

Once the oocyte has reached the second meiotic stage of development, ovulation is induced by a precise ratio of follicle stimulating hormone (FSH) and LH (Baker, 1982). The oocyte is gently expelled from the follicle, caught by the fimbria of the oviduct and continues through the oviduct until it is reached by the spermatozoa. The ruptured follicle develops into a corpus luteum, producing progesterone for maintenance of pregnancy.

1.2.1.4 Oocyte ageing

Austin (1982) reports that the fertile life of the bovine oocyte terminates 22 to 24 h after ovulation. After this time, the ability of the ovulated oocyte to be successfully fertilized by a spermatozoon is lost, although sperm-egg fusion may still take place. As the oocyte ages, chromosomes, which have aligned along the equator of the second meiotic spindle, become disordered. These chromosomes are lost from the metaphase plate due to the escape of microtubules, laterally from the spindle (Hunter, 1985). A further event involved in oocyte ageing is the migration of the cortical granules from the oolemma surface deeper into the oocyte (Gulyas, 1980). The position of the cortical granules just under the oolemma surface is part of the mechanism which is designed to prevent polyspermy (the penetration of an ovum by more than one spermatozoon). This mechanism fails once the cortical granules become disorganised and leaves the ageing oocyte susceptible to multiple penetration by spermatozoa. This polyspermic condition is lethal and the resulting embryo fails to develop (Johnson and Everitt, 1980).

1.2.2 The male

1.2.2.1 Spermatogenesis

Spermatogenesis, formation of mature sperm within the male reproductive tract, has been described in detail by Johnson and Everitt (1980) and Setchell (1982) and will only be briefly described here.

The male primordial germ cells are known as gonocytes and are surrounded by Sertoli cells. They remain inactive until puberty, from which time they undergo an indefinite period of multiplication (Setchell, 1982). These dividing gonocytes are called spermatogonia. They are diploid cells, identified as three different types: A, intermediate or B. Spermatogonia in group A give rise to type B spermatogonia through the intermediate stage (Johnson and Everitt, 1980). Each type B spermatogonium then undergoes DNA replication to produce short-lived primary spermatocytes, which divide to form four haploid spermatids. It is at this stage that the spermatozoon is almost ready to be released from the Sertoli cell (Setchell, 1982). Condensation of the nuclear material is followed by formation of the acrosome and, lastly, the tail. The bonds between the spermatozoon and the Sertoli cell are broken and the immotile spermatozoon travels along the rete testis via the efferent ducts to the epididymis (Harper, 1982).

1.2.2.2 Sperm transport

During passage through the epididymis, the spermatozoa gain motility. This motility appears to be a function of the ageing of the sperm and is accompanied by a gain in fertilizing ability (Harper, 1982). Seminal fluid is added to the spermatozoa as they pass the seminal vesicle. This fluid aids in the transport of sperm into the female reproductive tract, as well as providing nutrients for the sperm (Johnson and Everitt, 1980). Seminal osmolarity may be maintained by inositol in the seminal fluid and, amongst other constituents, citric acid chelates Ca^{2+} ions to prevent coagulation and fructose is provided for energy metabolism (Johnson and Everitt, 1980). The average pH of bull semen is 7.2 to 7.8 and the fertile life of bovine sperm varies from 28 to 50 h (Harper, 1982) in the female reproductive tract.

1.2.3 Fertilization

1.2.3.1 Deposition of sperm in the female tract

Sperm is deposited in the vagina of the cow (Hunter, 1982). From here, the sperm has to negotiate the reproductive tract to reach the oocyte at the site of fertilization, the oviduct. Under the influence of oestrogen, conditions in the cervix are favourable for

the sperm motility, as the cervical mucus becomes more dilute (Harper, 1982). Muscular activity in the vagina and cervical walls aid sperm transport to the oviduct. However, not all sperm are transported at once. Many sperm get trapped in the walls of the crypt of the cervix, thus allowing transport of sperm in waves. This ensures that mature sperm will reach the matured oocyte and ideally leads to successful fertilization.

1.2.3.2 Capacitation and the acrosome reaction

These events will be discussed in more detail later. Briefly, capacitation has been defined as maturation of the sperm once in the female reproductive tract (Yanagimachi, 1981) and before the acrosome reaction takes place (Bavister, 1973). Capacitation seems to involve removal of some or all of the molecules attached to the sperm plasma membrane and dilution out of the seminal plasma which inhibit the acrosome reaction (Green, 1978; Yanagimachi, 1981). Once sperm have completed capacitation, their fertile life is short (36 h in bovine: Dukelow and Riegle, 1974). Capacitation enables the acrosome reaction to take place. This reaction entails fusion of the outer acrosomal membrane of the sperm head with the inner plasma membrane (Yanagimachi, 1981), which causes release of the acrosomal enzymes necessary for breakdown of the outer oocyte investments. Sperm acrosomes contain abundant amounts of hyaluronidase which has been implicated in cumulus penetration (Yanagimachi, 1981). Corona radiata penetrating enzyme aids movement through to the zona pellucida (Hamner and McLaughlin, 1974) and penetration of the zona pellucida is achieved by acrosin (Hunter, 1982).

Capacitation of sperm *in vivo* takes place in the oviducal fluid (Gould, 1974). A heparin-like glycosaminoglycan present in bovine oviduct fluid has been described as a possible *in vivo* capacitator of spermatozoa (Parrish *et al.*, 1989b).

1.2.3.3 Oocyte penetration

Once the sperm has successfully penetrated the cumulus cell investment and entered the zona pellucida, fusion with the vitelline membrane of the egg occurs (Johnson and Everitt, 1980). The sperm head decondenses and is incorporated into the egg cytoplasm.

Extrusion of the second polar body takes place and the male pronucleus is formed. Fusion of the male and female pronuclei complete the fertilization process.

1.2.3.4 Block to polyspermy

In unpenetrated oocytes, the cortical granules form a monolayer just under the plasma membrane (Gulyas, 1980). On sperm penetration of the oocyte, the cortical granules release their contents into the perivitelline space. This enzyme release from the cortical granules initiates the zona reaction (Johnson and Everitt, 1980). The zona reaction comprises two events: a reduction in sperm-binding capacity to the zona and zona hardening (Wolf, 1981). Both of these events reduce the likelihood of further sperm penetration of the oocyte.

1.2.4 Embryonic development

Embryonic development within the female occurs in the oviduct and the uterus. By 24 h post-insemination, the bovine embryo will be at the 2-cell stage (Hunter, 1982). Bovine embryos enter the uterus at the 8- to 16-cell stage, after 3 to 4 days in the oviduct (Hunter, 1982; McLaren, 1982). An embryo of 16 cells or more is termed a morula. Compaction of the cells of the morula lead to formation of a blastocoele cavity and the blastocyst at 7 to 8 days post-insemination (McLaren, 1982). The blastocyst hatches from the zona pellucida at 9 to 11 days post-insemination and forms placental attachment after 22 days (Hunter, 1982). The nutrient requirements of the developing embryo have to be met by the fluids of the oviduct and uterus, until placenta formation allows nutrients to pass to the foetus via the maternal blood supply.

1.3 *IN VITRO* REPRODUCTION

Reproduction *in vitro* aims to replicate the events which occur *in vivo*. The exact requirements for *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) have not yet been determined and most culture systems rely on nutritional supply by animal protein sources which are not precisely defined.

1.3.1 The oocyte

1.3.1.1 Oocyte recovery and selection

Follicular oocytes are collected from follicles less than 6 mm in diameter (Wahid *et al.*, 1992; Hamano and Kuwayama, 1993). Brackett and Zuelke (1993) suggested that follicles with a diameter larger than 7 mm are already committed to atresia, while small follicles may contain under-developed oocytes that are incapable of meiotic division (Schultz and Wasserman, 1977; Tan and Lu, 1990). Strict criteria are obviously necessary to determine which liberated oocytes are likely to complete meiotic maturation *in vitro.*

Leibfried and First (1979) characterised bovine oocytes from differently sized follicles. They obtained superior maturation from oocytes originating from small follicles and which had a complete, multilayered cumulus investment compared to those in which the cumulus investment was absent. Support for this work comes from Yang and Lu (1990), where significantly higher proportions of cleaved embryos and blastocysts were obtained from oocytes with 2 to 3 layers of cumulus cells. A dark rim of corona cells surrounding the oocyte was judged to aid maturation by Laurinčík *et al.* (1992). The condition of the oocyte cytoplasm has also been shown to have an influence on the frequency of maturation, with improved maturation occurring in those oocytes whose cytoplasm was uniform regardless of its granular nature (Hawk and Wall, 1994).

1.3.1.2 Maturation media

Successful *in vitro* maturation of follicular oocytes is a prerequisite for successful *in vitro* fertilization. It is, therefore, essential to identify the important elements of *in vitro* maturation if successful *in vitro* fertilization is to follow.

Culture media employed for *in vitro* maturation of follicular oocytes vary between laboratories. The most commonly used defined media include modified Tyrode's medium (Ball *et al.*, 1983; Lenz *et al.*, 1983a) and the most popular, Tissue Culture Medium 199 (TCM 199) (Parrish *et al.*, 1988; Sirard *et al.*, 1988; Yang *et al.*, 1993). Parrish *et al.* (1986) achieved 90% fertilization after maturation in a modified Tyrode's medium. High maturation rates were achieved using TCM 199 for IVM of bovine

follicular oocytes (88 to 93% by Saeki *et al.*, 1990; 90 to 95% by Yang *et al.*, 1993). All of these investigators added foetal calf serum and hormones to the maturation medium.

The addition of sera to maturation medium has been investigated. Of most importance to bovine IVF systems are oestrus cow serum (ECS) and foetal calf serum (FCS). Many researchers routinely incorporate 10% FCS into the maturation medium (Camous *et al.*, 1984; Aoyagi *et al.*, 1990). Prokofiev *et al.* (1992) obtained 24.8% blastocysts using 10% ECS in the maturation medium. Fukui *et al.* (1989) supplemented maturation medium TCM 199 with 20% ECS to produce 12.2% blastocysts. In further investigations, Fukui and Ono (1989) tested ECS and FCS (20% in TCM 199 maturation medium) and found that maturation rates, cleavage and development to blastocysts were not different for the two sera but that FCS produced significantly higher fertilization rates and polyspermy. Rexroad and Powell (1991) working with ovine embryos found that there was no difference in development when bovine serum albumin was substituted for FCS.

Investigation into hormone supplementation of maturation media has led to conflicting data. Moor and Trounson (1977), while testing the effects of hormones (FSH, LH and oestradiol-17ß (E_2)), found that all three of these hormones used in combination significantly increased the proportions of ovine blastocysts produced. Saeki *et al.* (1990) confirmed this effect with bovine oocytes, showing higher fertilization, cleavage and blastocyst production with hormones than without. However, contradictory to this evidence is that of Fukui and Ono (1989), who found that there was no positive effect of hormones on maturation rates or blastocyst production. Supporting this work is the claim by Trounson *et al.* (1994) that gonadotropins (FSH and LH) were not essential for bovine embryo production. However, these investigators did not supply data and there was no indication whether maturation without gonadotropins was as successful as maturation with these hormones.

Co-culture with granulosa cells during maturation has been shown to confer developmental competence on bovine oocytes (Critser *et al.*, 1986; Li and Foote, 1992). Fukui and Ono (1989) also demonstrated that significantly more blastocysts were produced when bovine oocytes were co-cultured with granulosa cells during maturation.

Granulosa cells have also been found to support successful IVM of ovine oocytes (Szöllözi *et al.*, 1988). However, Leibfried-Rutledge *et al.* (1989) reported that co-culture with granulosa cells may delay meiotic maturation. This statement was, however, qualified by the proposal that delayed meiosis may be due to prolonged processing times and that the observed effect may not have been elicited by the granulosa cells.

1.3.1.3 Incubation conditions

A gas atmosphere of 5% CO_2 in air is most commonly used for *in vitro* culture of bovine and ovine oocytes. It has been suggested that high O_2 concentrations inhibit development of both bovine and ovine ova (Tervit *et al.*, 1972). Carney and Bavister (1987) raised the CO_2 concentration from 5% to 10% and almost doubled the number of blastocysts produced in the hamster. However, Wang *et al.* (1992), having tested the effects on bovine blastocyst yield of gas phases of 5% CO_2 in air, 10% CO_2 in air, mixtures of 5% CO_2, 10% O_2 and 85% N_2 or 5% CO_2, 5% O_2 and 90% N_2 found that none of the gas phases tested was superior to 5% CO_2 in air. Bavister (1988), in a review article, suggested that differences in results from different laboratories may be due to the fact that the rate-limiting factor was not the gas atmosphere. There is a genuine need for investigation into these influences within strictly defined IVF systems.

The temperature dependence of oocyte maturation was investigated by Lenz *et al.* (1983a). Temperatures of 35 to 39 °C did not produce significantly different maturation rates, but maturation at 41 °C was detrimental to the resumption of meiosis. This result is not surprising considering that proteins would be denatured at this temperature. These investigators went on to determine that maturation at 39 °C led to significantly increased fertilization rates at 39 °C. Since the body temperature of the cow is in the region of 38 to 39 °C, this finding is not extraordinary.

Determination of optimum incubation time in maturation medium is important to ensure that the oocytes have reached metaphase II and are capable of fertilization. Culture for 24, 26 or 28 h did not result in significantly different maturation rates for bovine oocytes (Fukuda *et al.*, 1990).

Culture vessels differ between laboratories and the choice seems to be a personal one. Superior development of >16-cell ovine embryos was achieved in multiwell dishes compared to culture in microdrops under oil (Thompson *et al.*, 1989). In further work, Thompson *et al.* (1992) confirmed that a significantly greater number of blastocysts were produced using multiwells compared to either microwells or microdrops under oil. Successful results have been obtained from culture under oil (Sirard *et al.*, 1988). The type of oil and the culture system employed obviously affects the success of these investigations and, thus, each laboratory must implement procedures which work best under the prevalent conditions.

1.3.2 The spermatozoa

Obtaining a fertile sperm sample is of utmost importance for the successful outcome of the *in vitro* fertilization process. The sample must contain sufficient sperm capable of being capacitated and undergoing the acrosome reaction if successful fertilization is to ensue. Sperm samples may be obtained fresh from the epididymis, or from fresh or frozen ejaculated samples. Fresh epididymal sperm are flushed from the epididymis with a flushing medium and washed by means of centrifugation (Ball *et al.*, 1983). While conducting experiments to determine the effect of epididymal or ejaculated sperm on fertilization rate, Pavlok *et al.* (1988) found that epididymal sperm fertilized more oocytes, but with a higher incidence of fertilization anomalies than ejaculated sperm. However, the epididymal sperm were stored at room temperature for 20 h, whereas the ejaculated sperm were collected 1 to 3 h before the start of the experiment. Consequently, the anomalies produced by the epididymal sperm may have been a function of sperm ageing rather than true differences between epididymal and ejaculated sperm samples. A further weakness in this experiment was the fact that the sperm samples tested were not from the same bull, a difficult objective to achieve when testing ejaculated and epididymal sperm samples. It has been proven that bull differences exist in the ability of sperm to fertilize oocytes *in vitro* (Leibfried-Rutledge *et al.*, 1989; Marquant-Le Guienne *et al.*, 1990; Shi *et al.*, 1990). Therefore, differences observed between epididymal and ejaculated semen may be attributed to differences between bulls

and not to the source of sperm. These facts cast serious doubts over the conclusions of Pavlok *et al.* (1988).

A more practical situation is to use frozen semen. In this way, the bull element may be kept constant while varying other factors. Parrish *et al.* (1986) used a method termed "swim-up" which separates motile from immotile sperm. Thawed sperm are layered under a volume of swim-up medium in a test tube and the solution incubated for an hour or more. The immotile sperm are incapable of swimming from the bottom of the test tube. Therefore, any sperm in the top fraction of the medium are assumed to be motile and free from semen extender debris. This swim-up method increased rates of fertilization and has become a commonly employed sperm separation method.

Washing sperm in a solution of 7.5% Ficoll and subsequent centrifugation, resulted in removal of up to 99.6% of the original medium and maximal recovery of 98% of the sperm from a sample of ram semen (Harrison, 1976). However, this method was not as successful with bull semen, as bull sperm became immotile during the washing procedure. This was attributed to the removal of seminal plasma.

The *in vivo* fertility of bull semen as judged by artificial insemination was significantly lowered when sperm samples were centrifuged for 3 min at forces as low as 270 g (Pickett *et al.*, 1975). Hence, swim-up separation of sperm may be preferable to separation by gradient centrifugation to minimise the centrifugal forces applied to sperm. However, swim-up separated sperm still require centrifugation to concentrate the sperm sample if sufficient numbers of sperm are to be obtained for insemination. Niwa and Chang (1974) determined an optimum concentration of 0.5 to 1.5×10^6 sperm/ml for fertilization of rat oocytes. As a result, concentrations of 1×10^6 sperm/ml are routinely used for IVF (Parrish *et al.*, 1986).

1.3.3 Capacitation and the acrosome reaction

Capacitation and the acrosome reaction are separate events (Bedford, 1970, 1983; Yanagimachi and Usui, 1974). Both must be successfully completed if sperm are to potentially fertilize mature oocytes *in vitro*. The literature gives contradictory discussion on whether glycosaminoglycans capacitate or initiate the acrosome reaction. This would seem to be dependent on the exact definition of capacitation and the acrosome reaction. For the purposes of the present study, glycosaminoglycans are accepted to induce capacitation (Parrish *et al.*, 1988), allowing the acrosome reaction to take place in the presence of Ca^{2+}. Glycosaminoglycans *per se* are not taken to induce the acrosome reaction directly.

1.3.3.1 Capacitation

As mentioned previously, capacitation is defined as the sperm maturation events leading to and in preparation for the acrosome reaction (Bavister, 1973; Yanagimachi 1981). Capacitation is, therefore, a prerequisite for the acrosome reaction and, as such, is a significant element in any IVF protocol. Cumulus cells have been shown to effect capacitation in hamster sperm (Gwatkin *et al.*, 1972). The specific components of the cumulus cells which induced this reaction were present in the cumulus matrix. However, Yanagimachi (1981) maintained that this event is unlikely *in vivo* because sperm do not remain within the cumulus cells long enough to initiate and undergo capacitation before penetration of the oocyte. An alternative mechanism is, therefore, required. Glycosaminoglycans (heparin, chondroitin sulphate A and hyaluronic acid) have been implicated as inducers of capacitation (Parrish *et al.*, 1989b) and have been found in the reproductive tract of the cow (Lee and Ax, 1984). Heparin binds specifically to bovine sperm *in vitro* (Handrow *et al.*, 1984) and, therefore, could reasonably be thought to bring about sperm membrane changes leading to the acrosome reaction. Glycosaminoglycans have also been detected in bovine seminal plasma but in concentrations too high to induce capacitation (Lee *et al.*, 1985).

The capability of glycosaminoglycans to induce capacitation appears to be partly due to the degree of sulphation of the compounds (Handrow *et al.*, 1982). Heparin is highly sulphated (containing almost 30% w/w sulphate) and is, as such, one of the most

effective glycosaminoglycans, compared to chondroitin sulphate or hyaluronic acid, for inducing capacitation in bovine sperm (Handrow *et al.*, 1982; Miller and Hunter, 1986). Delgado *et al.* (1987) found that hydrolysis of follicular fluid glycosaminoglycans greatly increased their ability to elicit capacitation reactions of porcine sperm. They suggested that degradation of glycosaminoglycans in the female reproductive tract may be a mechanism ensuring sperm capacitation.

1.3.3.2 The acrosome reaction

Following capacitation, increased sperm membrane permeability to Ca^{2+} appears to be the main signal for initiation of the acrosome reaction (Brackett and Zuelke, 1993). The acrosome reaction requires Ca^{2+} (Yanagimachi and Usui, 1974; Singh *et al.*, 1978) and factors which can induce Ca^{2+} influx into the sperm head should provoke an acrosome reaction. Tarín and Trounson (1994) reviewed some *in vitro* activators of the acrosome reaction in human sperm. Amongst these were follicular fluid, the zona pellucida and Ca-ionophore A23187, which transports Ca^{2+} across biological membranes (Reed and Lardy, 1972). A particular benefit of Ca-ionophore for biological work is that it induces Ca^{2+}-dependent reactions without affecting monovalent ion balances, such as Na^+ and K^+ (Pressman, 1976).

Ca-ionophore A23187 has been shown to induce a rapid acrosome reaction in ram spermatozoa in the presence of Ca^{2+} (Watson and Plummer, 1986) which was not preceded by capacitation. Because the sperm were not capacitated, these acrosome reactions were abnormally fast (Talbot *et al.*, 1976). The speed of the acrosome reaction in Ca-ionophore-treated sperm is detrimental to motility, probably due to exhaustion of ATP sources (Green, 1978). However, this can be rectified by addition of bovine serum albumin (BSA) which chelates the Ca-ionophore and reduces its effectiveness (Suarez *et al.*, 1987). Russell *et al.* (1979) stated that acrosome reactions induced by Ca-ionophore appeared morphologically normal.

1.3.4 *In vitro* fertilization

In vitro fertilization removes the need for sperm to negotiate the reproductive tract. It is then necessary to induce capacitation by artificial means, as passage in the reproductive tract is not an available method of achieving capacitation in sperm samples.

In vitro capacitation of bovine spermatozoa has been successfully carried out by incubation of sperm with a variety of chemicals, the most common being heparin. Heparin causes sperm capacitation but does not induce the acrosome reaction (Parrish *et al.*, 1988). Using dosages of heparin between 0 and 200 μg/ml and incubation times of 0 to 240 min, Fukui *et al.* (1990) determined that heparin concentrations of 25 to 100 μg/ml and incubation times of 5 to 60 min provided optimal rates of fertilization. Incubation with higher concentrations of heparin resulted in increased polyspermy, whereas incubation times longer than 60 min lowered fertilization rates. These investigators achieved the best development (24.2% 8-cell embryos) with sperm incubated in 50 μg/ml heparin for 60 min. Lu and Gordon (1988) found no difference in fertilization rate when sperm were incubated with heparin for either 15 or 30 min. However, as heparin concentration was increased from 0 to 100 μg/ml, the proportion of fertilized oocytes significantly increased, with a maximum of 83.5% at 100 μg/ml heparin.

Glucose has been shown to reduce the effectiveness of heparin as a capacitating agent (Susko-Parrish *et al.*, 1985). In fact, the presence of glucose inhibits sperm capacitation by heparin and reduces the number of sperm undergoing acrosome reactions (Parrish *et al.*, 1989a). Glucose is routinely omitted from the fertilizing media of some workers (Leibfried-Rutledge *et al.*, 1989) due to the inhibition of sperm capacitation by heparin. The presence of glucose in the medium of Fukui *et al.* (1990) may explain the difference in penetration rate compared to that achieved by Lu and Gordon (1988).

Ca-ionophore A23187 induces the acrosome reaction in bovine sperm (Talbot *et al.*, 1976). Fertilization of 92% of matured oocytes was achieved by Fukuda *et al.* (1990) by incubating sperm for 1 min with 0.1 μM Ca-ionophore. High concentrations of Ca-ionophore (>0.2 μM) reduced sperm motility which was restored on addition to medium

containing BSA (Bird *et al.*, 1989). Yang *et al.* (1993) suggested that sperm incubation with Ca-ionophore may have advantages over heparin when producing embryos *in vitro*. They found significantly more acrosome reacted sperm after incubation with 0.1 μM Ca-ionophore, compared to incubation with heparin concentrations of either 10 or 100 μg/ml.

Other chemicals known to activate sperm are platelet activating factor (Parks and Hough, 1990), chondroitin sulphate (Lenz *et al.*, 1983b) and high ionic strength medium (Bondioli and Wright, 1983). However, these chemicals are not widely used, probably because they have no proven advantage over the more commonly used chemicals, heparin and Ca-ionophore.

Certain chemicals are often incorporated into capacitation media to enhance motility of the capacitated sperm. Sperm motility is not essential for sperm-egg fusion in the hamster (Yanagimachi, 1981), but may aid penetration. Dodds and Seidel (1984) suggested that caffeine induced increased sperm motility, leading to higher proportions of fertilized mouse ova. As stated previously, sperm treated with high concentrations of Ca-ionophore have reduced motility until resuspended in medium containing BSA (Bird *et al.*, 1989). Garbers *et al.* (1971) demonstrated that bovine sperm motility was not only heightened by incubation with caffeine, but also that the increased motility was maintained for at least 4 h. Bovine sperm treated with 0.1 μM Ca-ionophore and 2.5 mM caffeine exhibited significantly increased velocity and penetration of zona-free hamster oocytes (Bird *et al.*, 1989). Sperm incubated with caffeine and heparin in combination penetrated significantly more oocytes than incubation with either chemical alone (Niwa and Ohgoda, 1988; Chian *et al.*, 1991). This effect was attributed to the ability of the two chemicals to act synergistically to cause capacitation or the acrosome reaction with a potential elevation in penetration rates.

Sperm incubated with penicillamine, hypotaurine and epinephrine (PHE) and heparin penetrate oocytes more frequently than sperm incubated in heparin alone (Susko-Parrish *et al.*, 1990). Leibfried and Bavister (1982) noted that epinephrine alone reduced hamster sperm motility but if epinephrine was added 2 h after incubation in hypotaurine,

sperm activation and oocyte penetration increased. They suggested that hypotaurine and epinephrine independently affect the sperm and that epinephrine must be administered after hypotaurine to activate hamster sperm. Fertilization of bovine oocytes on a bovine oviduct epithelial cell (BOEC) monolayer, and in the presence of PHE, increased cleavage rates and the proportion of embryos developing to blastocysts (Miller *et al.*, 1994). Whether fertilization was improved by the monolayer, PHE or a synergistic effect of the two is uncertain, since sperm motility has been enhanced by incubation in oviduct conditioned medium (Ijaz *et al.*, 1994).

Co-incubation of male and female gametes for 20 to 28 h was shown to yield increased fertilization rates but not subsequent development compared to co-incubation from 4 to 12 h (Rehman *et al.*, 1994). Testing co-incubation periods of 6, 12, 24 and 48 h, Younis and Brackett (1991) found that fertilization rates were significantly higher and development to the morula/blastocyst stage was significantly enhanced by co-incubation of gametes for 24 h. Most IVF protocols recommend incubation of oocytes and sperm for a period of 20 to 24 h.

1.3.4.1 Bull effects

Bulls have been shown to perform differently under the same IVF conditions (Parrish *et al.*, 1986; Marquant-Le Guienne, 1990). For production of large numbers of embryos by IVF, the system employed must be reliable and consistent. It is, therefore, necessary to determine methods by which all bulls can perform equally well. Comparable fertilization rates were achieved by all bulls whose sperm were incubated with different dosages of heparin (Leibfried-Rutledge *et al.*, 1989) and by adjusting sperm concentrations (Eyestone and First, 1989b). Bull effects have also been noted after stimulation of the acrosome reaction by Ca-ionophore (Aoyagi *et al.*, 1988). It was inferred that these observed differences might be reduced by sperm treatment with a combination of chemicals. No reports could be found describing capacitation by a mixture of glycosaminoglycans, imitating concentrations found in the reproductive tract, or of mixtures of chemicals with different actions. It is possible that sperm which do not react well to heparin (and, therefore, are not capacitated) may react well to chemicals which induce the acrosome reaction directly.

1.3.5 *In vitro* culture

Culture of *in vitro* produced embryos is complex and the exact requirements for embryo development have still not clearly been defined. Culture media for embryo development contain sources of energy, proteins and salts. Additionally, culture methods may involve growth on a cellular layer to enhance development.

Energy sources have been shown to dramatically alter the developmental capacity of mouse embryos (Brinster, 1965). Two-cell mouse embryos can utilise lactate and pyruvate as energy sources but not glucose. Rieger (1992) suggested that the metabolism of glutamine in bovine embryos provides the early embryo with significant amounts of energy. Glutamine metabolism is high in the 2- to 4-cell embryo and is reduced by the time blastulation is reached. Bovine expanded blastocysts metabolise as much as 30 times more glucose than 2-cell embryos (Rieger *et al.*, 1992). It would, therefore, seem that, during development, the embryo switches from metabolism of glutamine to glucose. Consequently, culture media for development must meet these changing nutritional requirements.

Protein supplementation of media involves addition of sera or serum albumin. However, these compounds vary from one batch to the next and it is, therefore, not possible to determine the exact composition of the additive. This may lead to success one month and failure another. There is obviously a need for a serum-free culture medium which can be reliably replicated from one experiment to the next.

Embryonic development, from the one-cell stage to foetuses, in serum-free (BSA-containing) medium has been shown to be as effective as in medium containing FCS (Rexroad and Powell, 1991). However, development involved co-culture on a monolayer of oviduct epithelial cells in TCM 199 which may have masked the effects of the sera/albumin. Rosenkrans and First (1991) have developed a culture medium (CR1 salts) in which embryos can develop to the blastocyst stage without the need for co-culture or serum. Addition of specific amino acids to the CR1 medium improved rates of blastocyst production. Lim *et al.* (1994) found that addition of FCS to embryos 120 h post-insemination improved the number of blastocycts produced.

It has been suggested by Trounson *et al.* (1994) that autocrine factors are involved in the positive effects exhibited when embryos are cultured together in small volumes of medium. This effect may also explain, in part, why improved development occurs when embryos are co-cultured with other types of cells.

Bovine oocytes exhibit an irreversible developmental block from the 8- to 16-cell stage (Eyestone and First, 1986). However, development past this stage can be achieved by embryo co-culture with oviduct epithelial cells (Eyestone *et al.*, 1987; Eyestone and First, 1989a; Fukui *et al.*, 1989; Aoyagi *et al.*, 1990), trophoblastic vesicles (Camous *et al.*, 1984; Heyman *et al.*, 1987; Aoyagi *et al.*, 1990) and amniotic sac cells (Aoyagi *et al.*, 1990).

Kuzan and Wright (1982) postulated that the mechanism by which uterine fibroblasts convey developmental competence to bovine morulae involved cell contact, since conditioned medium had no effect on development. In an experiment designed to determine if successful co-culture with trophoblastic vesicles required cell contact with the embryo, Heyman *et al.* (1987) established that co-culture and conditioned medium were equally effective in imparting developmental competence on 1- and 2-cell bovine embryos to morulae. Camous *et al.* (1984), in preliminary trials, discovered that culture of bovine embryos in medium conditioned by trophoblastic vesicles resulted in development intermediate to that in medium alone and co-culture. These differing conclusions may be due to the conditioning ability of the different cell types employed. Bovine oviduct epithelial cells have been shown to support growth of *in vitro* matured and fertilized oocytes to the blastocyst stage (Fukui *et al.*, 1991) and are commonly used to sustain embryo development.

It is unclear exactly how co-culture systems exert their positive effects on development. Co-culture systems may provide embryotrophic factors, reduce toxic elements in the medium or they may provide the embryo with metabolites necessary for continued development (Bavister, 1988; Rexroad, 1989). Epithelial cells may release growth factors which are not hormonal and promote embryonic development (Kane *et al.*, 1992). It has been suggested (Eyestone *et al.*, 1991) that conditioned media contain secretory factors (possibly proteins) that enhance development. Gandolfi *et al.* (1993) suggested that

oviduct epithelial cell monolayers may remove developmental inhibitory substances from the medium. Gardner *et al.* (in press, cited by Trounson *et al.*, 1994) recommended that the culture medium is replaced every 48 h so that toxic ammonium does not accumulate. Exposure to light of medium containing N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) may generate toxic photoproducts, such as hydrogen peroxide (Zigler *et al.*, 1985). Toxic photoproducts have been shown to negatively affect mammalian cell activity (Wang *et al.*, 1974) and are thought to be involved in abnormal embryo cleavage (Walker *et al.*, 1992). Incorporating oviduct epithelial co-culture and PHE into the fertilization medium increased cleavage and development to the blastocyst stage (Miller *et al.*, 1994) which may have been due to removal of free radicals.

Cellular co-culture and serum supplementation remain the most effective methods for growth and development of *in vitro* produced embryos. However, the drawback in both of these techniques is that consistency from one batch to the next is not guaranteed. If *in vitro* production of bovine embryos is to be practical, elements which affect the success of such systems must be determined, allowing reproducibility. Defined culture conditions would go a long way to providing the necessary information for repeatable *in vitro* culture.

CHAPTER 2

CAPACITATION OF NGUNI SEMEN FOR *IN VITRO* FERTILIZATION

2.1 INTRODUCTION

Sperm capacitation in the bovine is most commonly carried out by incubation with glycosaminoglycans. Marquant-Le Guienne *et al.* (1990) showed that a bull x dose interaction exists in bovine spermatozoa when capacitation is induced by heparin, the most potent of the glycosaminoglycans. Furthermore, it has been shown that glucose inhibits the capacitating action of heparin (Susko-Parrish *et al.*, 1985; Parrish *et al.*, 1989a). Thus, optimal heparin doses will vary according to the *in vitro* system employed and must, therefore, be determined for each bull according to the culture medium used and the components in it, if successful *in vitro* culture is to follow.

Langlais and Roberts (1985) suggested that heparin may act through one or more of the following mechanisms;

a) stimulating Ca^{2+} entry into the sperm,
b) activating sperm phospholipase A_2, or
c) promoting sperm membrane fusion.

Their experiments provided support for all three of these events. Changes in the cholesterol:phospholipid ratio during the course of capacitation render the sperm plasma membrane sensitive to the effects of sperm phospholipase A_2. An influx of Ca^{2+} ions on completion of capacitation activates endogenous sperm phospholipase A_2 leading to membrane fusion and release of the acrosomal enzymes.

The most widely used heparin concentration for sperm capacitation is 10 μg/ml (Parrish *et al.*, 1985; Susko-Parrish *et al.*, 1985; Niwa and Ohgoda, 1988; Park *et al.*, 1989; Chian *et al.*, 1991, 1994). Marquant-Le Guienne *et al.* (1990) found that capacitation with

0.05 μg heparin/ml resulted in an IVF rate which correlated (r=0.83) to *in vivo* fertility as determined by non-return rate. Fukui *et al.* (1990) achieved good IVF results using 25 to 100 μg heparin/ml and incubation times of 5 to 60 minutes.

An alternative approach has been to bypass capacitation and to induce the acrosome reaction in sperm. This has been achieved by exposing sperm to Ca-ionophore A23187 (Shams-Borhan and Harrison, 1981). This compound promotes uptake of Ca^{2+} ions across the sperm plasma membrane and thereby induces the acrosome reaction (Pressman, 1976; Bird *et al.*, 1989). As with heparin, varying the dosage of Ca-ionophore results in variable rates of fertilization (Yang *et al.*, 1993). It is, therefore, important to determine optimal concentrations of Ca-ionophore which will yield the best fertilization rates when using a particular IVF procedure.

The range of Ca-ionophore concentrations tested by other workers in order to cause the acrosome reaction varies from 0.01 μM to 1.8 mM (Shams-Borhan and Harrison, 1981; Dodds and Seidel, 1984; Bird *et al.*, 1989; Didion *et al.*, 1989). However, the most commonly used concentrations are in the range of 0.1 μM to 2 μM. Bird *et al.* (1989) achieved sperm penetration rates of 63% with Ca-ionophore concentrations of up to 0.4 μM when testing concentrations of 0, 0.1, 0.2, 0.4, 1 and 2 μM. Nevertheless, it was noted that sperm motility decreased with increasing ionophore concentration. Fertilization rates of 92% of matured oocytes were obtained by Fukuda *et al.* (1990), using sperm reacted with 0.1 μM Ca-ionophore. Both groups of investigators used caffeine to promote sperm motility.

If differences in bull performance *in vitro* can be attributed to the reaction of the sperm to different capacitation chemicals, it should be possible to find a chemical and concentration which will yield an acceptable response from any bull. In this trial, the chemicals used to elicit a response were heparin and Ca-ionophore A23187. Both chemicals were used at three different concentrations. Thus, it was hypothesised that acceptable IVF rates (performance) would be achieved by every bull in the test from one or more of the treatments tested.

To test this hypothesis, the following questions were asked:

a) Do the bulls perform equally well with respect to the treatments tested?
b) If (a) does not apply, which treatment elicits the best performance from each bull?
c) Does heparin result in better bull performance than Ca-ionophore?

2.2 MATERIALS AND METHODS

2.2.1 Experimental design

Five bulls and a control were to be tested for performance with each of seven treatments by using *in vitro* fertilization techniques on oocytes (collected from the local abattoir) and frozen Nguni semen. Performance was to be determined by fertilization rates (as exhibited by cleavage) and subsequent embryo development. However, it was not possible to obtain sufficient numbers of oocytes on a given day to allow testing of all bulls on the same day. In addition, the maintenance of a viable population of oocytes during the processing time was problematical and this effectively reduced the numbers of oocytes that could be processed on any one day. Consequently, it was possible to test only two bulls on any given day. A complete randomised block with a factorial design was, therefore, used to minimise day effects. The block design was such that two bulls were tested per day and that a block consisted of three days. As far as possible, 10 good quality oocytes were allocated to each treatment. If there were insufficient oocytes of good quality, the numbers of oocytes per treatment were reduced.

Ca-ionophore has been shown to induce parthenogenesis, the activation of an oocyte to undergo cleavage divisions spontaneously and without the introduction of male gametes, (Marcus, 1990). The extent to which parthenogenesis would occur, thus, had to be evaluated. Accordingly, two sets of control treatments were included. The first consisted of the chemical treatments without semen, effectively a no-bull control. In this control group, oocytes underwent the same treatments as those fertilized with semen, in order to show the effect of the chemical treatments on parthenogenetic cleavage. The second

control tested the effect of treatment on the sperm. Semen was, therefore, added to fertilization medium without prior chemical treatment to induce capacitation or the acrosome reaction.

To avoid ambiguity, terminology in this chapter will be adopted as follows:

a) *Trial:* the whole investigation involving all blocks and replications.
b) *Experiment:* any investigation carried out on one day, that is, the testing of two bulls.

2.2.1.1 Random allocation of bulls to labels

Bulls were randomly labelled A to F at the beginning of the trial (Table 2.1).

Table 2.1. Test bulls assigned to labels

Label	Bull
A	PC 8775 XX009 P/17
B	Lusa
C	S15 XX001 L/5
D	N1 XX002 M/17
E	S11 NG001 M/13
F	Control

2.2.1.2 Random allocation of bulls to days

Because it was possible to test only two bulls per day, experiments were designed so that all combinations of bull pairings were tested. Bulls were randomly allocated to days of the block such that each bull was tested once in an experiment with each of the other five bulls and twice with one bull because there were six blocks (Table 2.2).

Table 2.2. Random allocation of bulls to days within blocks

	Day 1	Day 2	Day 3
Block 1	A	B	C
	D	E	F
	Day 4	Day 5	Day 6
Block 2	A	C	E
	B	D	F
	Day 7	Day 8	Day 9
Block 3	A	B	D
	C	F	E
	Day 10	Day 11	Day 12
Block 4	A	B	C
	E	D	F
	Day 13	Day 14	Day 15
Block 5	A	B	D
	F	C	E
	Day 16	Day 17	Day 18
Block 6	A	C	D
	B	E	F

2.2.1.3 Random allocation of treatments

It was not known whether conditions in the outer wells of the 96-well Microwell® cell culture plate would differ to those in the inner wells. Treatments were, therefore, randomly allocated to the innermost wells of the 96-well plate at the beginning of the trial (Appendix 1) and the outermost wells were filled with sterile MilliQ® water to minimise evaporative losses from the inner experimental wells.

2.2.2 Preparation of stock solutions

Stock solutions of heparin and Ca-ionophore A23187 (Appendix 2) were prepared before the trial and stored at -20 °C until thawed on the day of use. This was done to minimise differences between capacitation treatments on different days.

2.2.3 Treatment concentrations tested

2.2.3.1 Heparin

In this experiment, sperm were incubated in 0.05, 10 or 25 μg/ml heparin in fertilization medium (Appendix 3) for 15 min before being added to matured oocytes previously washed in fertilization medium.

2.2.3.2 Ca-ionophore A23187

In the present investigation, Ca-ionophore concentrations of 0.1, 0.2 and 0.4 μM, incubated for 1 min, were tested (Bird *et al.*, 1989; Yang *et al.*, 1993). These concentrations covered the range of ionophore treatments commonly used by other workers. It was not possible to test the effects of sperm motility factors, such as caffeine and hypotaurine, due to a low availability of oocytes.

Because Ca-ionophore reacts with bovine serum albumin in the fertilization medium (Suarez *et al.*, 1987), solutions of Ca-ionophore were made up in BSA-free fertilization medium. The risk of activating the oocytes with the Ca-ionophore on addition of the sperm was reduced by chelating the Ca-ionophore with BSA (fatty acid free) (Suarez *et al.*, 1987). This was achieved once the sperm and Ca-ionophore mixtures were added to the matured oocytes in fertilization medium containing BSA. All procedures were carried out in tubes covered with aluminium foil and with the laboratory lights switched off to minimise the reaction of Ca-ionophore with light.

2.2.4 Oocyte recovery

Ovaries from cows of unknown origin were collected from the local abattoir at Cato Ridge and, as far as possible, transported to the laboratory within 2 h of slaughter. The

ovaries were transported in physiological saline (Appendix 3) at 30 to 35 °C. At the laboratory, ovaries were wiped free of saline and oocytes were liberated by aspirating all follicles approximately 2 to 6 mm in diameter (Wahid *et al.*, 1992) with an 18 gauge needle attached to a 10 ml syringe. Follicular fluid containing the liberated oocytes was stored in a sterile test tube at 37 °C until all of the ovaries had been processed (approximately 1 h). Oocytes are more dense than follicular fluid and thus sink to the bottom of the tube. A 90 mm petri dish was half-filled with M199 HEPES wash medium (Appendix 3) at 38.5 °C and a portion of the follicular fluid (containing the oocytes) removed from the bottom of the test tube and added to the wash medium. Oocytes were located using a stereomicroscope at 15x magnification. Recovered oocytes were placed in a 60 mm petri dish containing M199 HEPES wash medium and underwent two further washes of M199 HEPES (3 ml each) in 35 mm petri dishes at 33 °C on a heated stage before the next batch of oocytes was diluted out of follicular fluid. Once all of the oocytes had been recovered and washed, they were transferred to a maturation medium wash (3 ml)(Appendix 3). These oocytes then underwent a further maturation wash before final selection. From the first dilution of oocytes out of follicular fluid to final selection took approximately one hour.

Sheets of granulosa cells were also recovered for co-culture with the oocytes. Granulosa cells have been shown to confer oocytes with developmental competence (Critser *et al.*, 1986; Li and Foote, 1992) when co-cultured in maturation medium. The granulosa cells underwent the same washing procedure as the oocytes.

During the first block of the trial, it was noted that one oocyte was parthenogenetically activated after 24 h in maturation medium. Fenton *et al.* (1993) found that oocytes exposed to 1 mg/ml heparin during maturation were not capable of being fertilized. It was, therefore, decided to exclude the heparin from the M199 HEPES wash because this may have caused the parthenogenetic activity.

2.2.5 *In vitro* maturation of bovine oocytes

Oocytes having a complete cumulus and a uniformly granular cytoplasm were selected for maturation (Leibfried and First, 1979). Ten oocytes and additional granulosa cells (one sheet) in approximately 5 μl of maturation medium were placed in 95 μl maturation medium in wells of a 96-well Microwell® plate. The plate had previously been conditioned with a bovine oviduct epithelial cell (BOEC) monolayer (Appendix 4). Co-culture with oviduct epithelial cells has been shown to increase development past the 8- to 16-cell block (Aoyagi *et al.*, 1990; Eyestone and First, 1989a; Eyestone *et al.*, 1987; Gandolfi and Moor, 1987). Oocytes were allowed to mature for 24 h at 38.5 °C and 5% CO_2 in air.

After 24 h maturation, oocytes were removed from the maturation medium and washed in two washes of 3 ml each of fertilization medium. Maturation controls were taken at this point. The washed, matured oocytes were removed from the fertilization medium in groups of ten in as small a volume as possible (approximately 10 μl) and placed in 80 μl of fertilization medium in wells of the 96-well Microwell® plate. The plate was then placed in the incubator at 38.5 °C and 5% CO_2 in air until the sperm were added.

2.2.6 Maturation controls

At least ten oocytes were taken as maturation controls. These were placed in an eppendorf tube (1 ml), in approximately 50 μl of medium, and vortexed for 3 min to remove cumulus cells. The oocytes were then resuspended in 1 ml M199 HEPES wash medium and allowed to settle to the bottom of the tube. The oocytes were then removed and taken up in as small a volume of medium as possible (approximately 10 μl). They were placed on a microscope slide and most of the fluid drained off, leaving the oocytes stranded on the slide. A coverslip with mountant (Appendix 5) on the central part of each side was positioned above the oocytes and pressed down to capture the oocytes between the coverslip and the slide. Rubber cement was placed on the corner between the coverslip and the slide and allowed to dry. The slide was then placed in fixative (Appendix 5) for at least 24 h. The oocytes were stained with aceto-orcein (Parrish *et al.*, 1985; Appendix 5) and evaluated for meiotic activity. Oocytes with a

metaphase plate and a membrane-bound polar body were regarded as having successfully matured.

2.2.7 Capacitation of semen and *in vitro* fertilization

Semen straws (one from each bull) were thawed at 35 °C for 1 min (Parrish *et al.*, 1986). The semen (approximately 0.25 ml) was layered under 1 ml (Parrish *et al.*, 1986) of fertilization medium (Appendix 3) and allowed to swim up for one hour at 38.5 °C and 5% CO_2 in air. The top 0.85 ml of the swim-up medium was removed and divided equally between two eppendorf tubes. The sperm was then centrifuged at 700 g for 1 min at 30 to 35 °C. The supernatant was discarded and the pellets resuspended in 50 μl of fertilization medium (minus BSA for Ca-ionophore treatments), to yield a sperm concentration of approximately $2x10^7$ sperm/ml. Counts were determined using a haemacytometer. The sperm from each bull was then exposed to the capacitation treatments (Table 2.3).

Table 2.3. Capacitation treatments and incubation times for semen

Treatment	Concentration (μg/ml)	Incubation time (min)
Ca-ionophore	0.1	1
	0.2	
	0.4	
Fertilization medium	N/A	15
Heparin	0.05	15
	10	
	25	

Equal volumes (10 μl) of sperm suspension and heparin solutions (Table 2.4) were added to incubation tubes. Thus, solutions of 0.1, 20 and 50 μg/ml heparin were required to

give final concentrations of 0.05, 10 and 25 μg/ml heparin in the incubation tubes. The heparin/sperm mixtures were incubated at 38.5 °C, 5% CO_2 in air for 15 min.

Table 2.4. Preparation of heparin solutions

Volume of 50 μg/ml heparin stock solution (μl)	Volume of fertilization medium (μl)	Concentration (μg/ml)
2	998	0.1
20	30	20
10	0	50

Solutions of 0.2, 0.4 and 0.8 μM Ca-ionophore (Table 2.5) were prepared from a stock solution of 20 μM Ca-ionophore. Manipulations of Ca-ionophore were carried out in tubes covered with foil to prevent reaction with light. Equal volumes (10 μl) of sperm suspension and Ca-ionophore solution (0.2, 0.4 and 0.8 μM) were incubated for 1 min at room temperature and the reaction stopped on addition of sperm to the mature oocytes in medium containing 6 mg/ml BSA (fatty acid free) (Ware *et al.*, 1989).

Table 2.5. Preparation of Ca-ionophore solutions

Volume of 20 μM Ca-ionophore A23187 stock solution (μl)	Volume of fertilization medium (μl)[1]	Concentration (μM)
5	495	0.2
10	490	0.4
20	480	0.8

[1] Ca-ionophore solutions were diluted with fertilization medium without BSA. This was to avoid reduction of the activity of the Ca-ionophore.

After addition of sperm, the final volume in each well of the culture plate was 100 μl. Oocytes and sperm were incubated in fertilization medium for 20 h at 38.5 °C and 5% CO_2 in air.

2.2.8 *In vitro* culture and development

Presumptive zygotes were removed from the fertilization medium after 20 h of culture, placed in eppendorf tubes, in approximately 50 μl of fertilization medium, and vortexed for 2 min to remove cumulus cells. One millilitre of CR1aa medium (Appendix 3) at 38.5 °C was then placed in each eppendorf tube to resuspend the oocytes and allow them to fall to the bottom of the tube. The presumptive zygotes were held at 38.5 °C, 5% CO_2 in air until they could be processed. This involved washing the oocytes in three washes (3 ml each) of equilibrated CR1aa and transfer, in 5 μl of CR1aa medium, to new wells containing 95 μl of CR1aa.

On days four and seven post-insemination, 10 μl and 5 μl, respectively, of sterile-filtered foetal calf serum (FCS) was added to the medium containing the presumptive zygotes. Six days were sufficient for Gandolfi and Moor (1987) to produce ovine blastocysts using co-culture with oviduct cells. Therefore, development was assessed over a period of at least eight days following fertilization to ensure that all expected developmental stages were recorded. Presumptive zygotes were classed according to the number of blastomeres within the zona pellucida on days two, three, five, seven and eight post-insemination. Blastocysts forming after ten days post-insemination were regarded as having degenerated and were not included in the analysis.

2.2.9 Embryo scoring

Cleavage indices, where the index is equal to the number of cleavage divisions undergone (Wright *et al.*, 1976), were calculated for all embryos. The cleavage indices were used to calculate a total score for all of the embryos from one bull in a treatment on a particular day. The scoring system of Williams *et al.* (1992) was adopted. This allocates a sequential score to successive embryonic stages of development from 2-cell embryos

to blastocysts. One-cell embryos were given a score of zero and disintegrating embryos were given a negative score. However, as the morula and blastocyst stages are so important for implantation of embryos, it also was decided to employ a second scoring system which would emphasise the achievement of the later embryonic stages. These scores were called Score 1 and Score 2 for Method 1 and Method 2, respectively (Table 2.6).

Table 2.6. Embryo descriptions and respective scores

Description	Score 1[1]	Score 2[2]
Disintegrating oocyte/embryo	-1	-1
1-cell (not fertilized)	0	0
2-cell (one cleavage division)	1	1
3-cell (uneven cleavage)	1.5	1.5
4-cell (two cleavage divisions)	2	2
6-cell (uneven cleavage)	2.5	2.5
8-cell (three cleavage divisions)	3	3
16-cell (four cleavage divisions)	4	4
Morula	5	7
Blastocyst	6	10

[1] after Williams *et al.* (1992)

[2] Method 2, weighting more heavily for morulae and blastocysts

A number of criteria were employed in an effort to successfully assess bull fertility *in vitro:*

a) *Cleavage rate:* numbers of embryos cleaved as a proportion of the total number of oocytes were expressed as a percentage.

b) *Developmental scores:* the maximum score for a bull and treatment on a given day was expressed as a mean for the number of oocytes tested on that day. This mean was then averaged over all replicates for that bull and treatment to give a mean maximal score for an embryo.

c) *Blastocyst production rate:* the number of blastocysts produced as a proportion of the total number of oocytes was expressed as a percentage.

Cleavage rates were used to provide an indication of fertilization rates without allowing the condition of the oocyte to affect the assessment. Developmental scores were used as a means of assessing overall development. Blastocyst production rates were used to indicate which bulls would successfully support development to the implantation stage.

2.2.10 Statistical analyses

Because of the negative weighting for the disintegrating oocytes, maximum scores were used to indicate the best performance over the time period of each experiment. Thus, data for Scores 1 and 2 were totalled to give a maximum score for any one bull and treatment during an experiment. This maximum score was then averaged over the number of oocytes used on that day. A maximal mean score was thus obtained for both Scores 1 and 2 for all bulls and treatments. Maximal mean score data (developmental scores) were analysed by multiple regression. The terms fitted to the regression were blocks, maturation rates, sperm numbers, bulls, treatments and bull x treatment interactions to determine differences between bulls and treatments. Maximum cleavage rates for the post-insemination period (indicating rates of fertilization) and rates of blastocyst production were analysed by logistic regression. The terms fitted to the logistic regression model were the same as those used for the multiple regression. All data were analysed using Genstat (Version 5.2.2, 1990, Lawes Agricultural Trust, Rothamsted Experimental Station, U.K.).

Comparisons between data groups (fitted terms) that were found to be significant were carried out using Student's t-test for continuous data and Normal tests for proportional data. The results of these tests (comparing results between bulls) must be regarded with

caution due to the large errors involved in using such a large number of comparisons. However, it was possible to consider these statistical results as a descriptive tool rather than a definitive explanation of the experimental results.

2.3 RESULTS

2.3.1 Day effects

As expected, block effects were highly significant ($P<0.001$) for all responses tested (maximal mean Scores 1 and 2, cleavage rates and rates of blastocyst production). Block effects accounted for differences within blocks (day effects) as well as between block variation. Day effects accounted for the fact that all bulls could not be tested on one day. Thus, it would appear that day of experiment had a significant ($P<0.001$) influence on the parameters assessed.

It was noted that, on some days, ovaries possessed large follicles (>6 mm in diameter) containing a dark yellow follicular fluid. On other days, ovaries had many smaller follicles (1-2 mm in diameter) which contained an almost clear follicular fluid.

Towards the end of the trial, it was noted that many of the oocytes did not show cleavage divisions, that is, the proportion of one-cell embryos was higher than at the start of the trial.

Fungal and bacterial contamination occurred in some of the co-culture plates and experimental plates. As the detrimental effect of the contamination on the embryos could not be determined, it was necessary to repeat days 6, 11 and 17. Day 6 was repeated immediately and remained in the correct order within its block. However, the contamination in days 11 and 17 was evident only much later in the development of the embryos. It became impossible to visually assess these embryos as the contamination spread, and thus these two experiments had to be repeated at the end of the trial.

2.3.2 Maturation rates

Maturation rate is defined as the proportion of oocytes (maturation controls) which exhibited metaphase II plates and membrane-bound polar bodies, expressed as a percentage. Over the time period of the trial, the minimum maturation rate was 11.1% (day 6) and the maximum was 72.7% (day 14). Despite this large variation in maturation rates, oocyte maturation rates for different days had no significant effect on any of the responses tested.

2.3.3 Sperm numbers

All of the semen from the bulls except for Bull B was extended in egg yolk and glycerol. The semen from Bull B was collected more recently than the others and was extended in Triladyl®.

As the semen was donated, it was not possible to acquire semen samples from the same collection. The samples for Bulls B and D came from one collection, but the samples from Bulls A, C and E came from two separate collections. This variation in the sample collection date could not be taken into account in the statistical analyses, and led to an introduced and unavoidable experimental error of unknown magnitude.

The minimum sperm concentration was zero (Bull F, control). The maximum number of sperm (1 153 000 sperm/ml final concentration) occurred on day 17 for Bull C and the heparin treatments. However, this combination of treatments and sperm numbers did not lead to the development of any blastocysts. Sperm numbers had no significant effect on the responses for maximal mean scores, cleavage rates or rates of blastocyst production.

2.3.4 Cleavage rates

The minimum cleavage rate was zero for a number of treatments and bulls on different days. The maximum cleavage rate was 100% (Bull D on day 1, capacitated with 25 μg heparin/ml). This treatment combination did not result in the production of blastocysts.

Where cleavage rate was used as a means of assessing fertilization rate, there was no significant treatment effect but cleavage rates did differ significantly ($P<0.01$) between bulls. There were also no significant bull x treatment interactions on cleavage rates. Because there were no significant effects of treatment, the results for each bull were pooled across all treatments (Table 2.7).

Table 2.7. Cleavage rates for bulls (values are actual means ± s.e.m.)

Bull	n[1]	Cleavage rate[2]	
A	400	40.8^{a}	± 2.5
B	396	41.2^{a}	± 2.5
C	417	40.8^{a}	± 2.4
D	397	29.8^{b}	± 2.3
E	402	$31.6^{b,c}$	± 2.3
F	405	$36.8^{a,c}$	± 2.4

[1] n=number of oocytes evaluated for each bull

[2] values with different superscripts differ significantly ($P<0.05$) (Normal test)

No bulls gave significantly higher cleavage rates than the control (Bull F) and no bulls showed a cleavage rate of above 50% (Table 2.7).

Predicted cleavage rates (Table 2.8), obtained by logistic analysis, incorporated block and day effects, maturation rates and sperm numbers. Although treatment was not significant, it was included in the model because it was a variable tested.

Bull C performed better than the other bulls, providing the highest predicted cleavage rate of 40.6%. Bulls D and E were predicted to have cleavage rates falling below that of the control. Predicted cleavage rates for the control indicated a high rate of parthenogenesis, with 31.6% of oocytes expected to show cleavage division.

Table 2.8. Predicted cleavage rates for bulls (values are predicted means ± s.e.m.)

Bull	Cleavage rate
A	37.0 ± 2.9
B	31.9 ± 3.0
C	40.6 ± 2.6
D	29.5 ± 2.2
E	29.8 ± 2.2
F	31.6 ± 3.2

2.3.5 Developmental scores

Block effects significantly ($P<0.001$) affected the scores that were obtained. Maturation rates, sperm numbers and treatment had no significant effect on maximal mean Scores 1 and 2. There were no significant bull x treatment interactions influencing maximal mean Scores 1 and 2. Thus, it was possible to pool the results for the bulls and treat them as a complete bull effect.

There were significant ($P<0.01$) bull effects on maximal mean Scores 1 and 2. This suggests that some bulls yielded better overall development than others. Scoring using the second method, where morulae and blastocysts were weighted more heavily, did not alter the significance of the bull effect.

Actual means ± s.e.m. for maximal mean Scores 1 and 2 are shown in Table 2.9. Values for n here are lower than those for cleavage and blastocyst production because some oocytes had already been taken into account in the evaluation of the mean. Thus, n is the number of observations of the mean and not the total number of oocytes that were used to calculate that mean.

The differences in means between the two methods of scoring were not pronounced, therefore, results are discussed for both sets of means simultaneously. Bull C was the only bull to respond significantly ($P<0.05$) higher than the control (Bull F) for both

methods of scoring (Table 2.9). Bulls D and E did not perform well under either scoring method. The scores for Bulls B and E did not differ between the two scoring methods.

Table 2.9. Average score per embryo (for maximal mean scores 1 and 2) for bulls over all treatments (values are actual means ± s.e.m.)

Bull	n[1]	Mean Score 1[2]		Mean Score 2	
A	41	$0.58^{a,c}$	± 0.06	$0.61^{a,d}$	± 0.07
B	41	$0.55^{a,c}$	± 0.06	$0.55^{a,b,d}$	± 0.07
C	42	0.61^{c}	± 0.06	0.69^{a}	± 0.07
D	41	0.40^{b}	± 0.06	$0.43^{b,e}$	± 0.07
E	41	0.36^{b}	± 0.06	$0.36^{c,e}$	± 0.07
F	41	$0.43^{a,b}$	± 0.06	$0.48^{b,d,e}$	± 0.07

[1] n=number of observations of mean score

[2] values with different superscripts differ significantly ($P<0.05$) (Normal test)

A multiple regression analysis of the data gave predicted values for the bulls accounting for differences in days, blocks, maturation rates and sperm numbers (Table 2.10). The predicted equation of the line of best fit accounted for 63% of the variation in the data for maximal mean Score 1 and 61.2% for maximal mean Score 2.

Table 2.10. Predicted average score per embryo (for maximal mean Scores 1 and 2) for bulls over all treatments (values are predicted means ± s.e.m.)

Bull	Mean Score 1	Mean Score 2
A	0.48 ± 0.08	0.53 ± 0.09
B	0.30 ± 0.08	0.30 ± 0.09
C	0.65 ± 0.07	0.72 ± 0.08
D	0.40 ± 0.07	0.43 ± 0.07
E	0.30 ± 0.07	0.30 ± 0.07
F	0.27 ± 0.10	0.33 ± 0.10

Predicted scores for Bulls B and E varied little between Methods 1 and 2, whereas all other bulls (A, C, D and F) had increased scores with Method 2.

2.3.6 Blastocyst formation

The maximum number of blastocysts produced was four, from 10 oocytes (40%), (Bull C capacitated with 0.1 μM Ca-ionophore on day 5). Many bull and treatment combinations did not yield any blastocysts.

Blocks had a highly significant ($P<0.001$) effect on the rate of blastocyst production. Maturation rates, sperm numbers and treatments were not significant and there was no bull x treatment interaction. Therefore, blastocyst production rates were combined across all treatments to provide an average for each bull.

Only Bulls C and D yielded blastocyst production rates which were significantly ($P<0.05$) higher than the control (Bull F) (Table 2.11). The control (Bull F) also produced blastocysts (treated with 0.1 μM Ca-ionophore, 10 and 25 μg heparin/ml and control treatment with fertilization medium). Bull B was the only bull which had a lower blastocyst production rate than the control, although this was not significant.

Table 2.11. Rates of blastocyst formation (values are actual means ± s.e.m.)

Bull	n[1]	Blastocyst production rate[2]	
A	401	$2.0^{a,c}$	± 0.7
B	396	0.5^{a}	± 0.4
C	417	5.3^{b}	± 1.1
D	398	$3.3^{b,c}$	± 0.9
E	404	$1.2^{a,c}$	± 0.6
F	406	1.0^{a}	± 0.5

[1] n=number of oocytes evaluated for each bull

[2] values with different superscripts differ significantly ($P<0.05$) (Normal test)

Logistic regression of the data produced predicted values ± s.e.m. (Table 2.12) for rates of blastocyst production for all bulls. The only bull predicted to produce lower numbers of blastocysts than Bull F (control) was Bull B, although Bull E barely rated higher than the control. The highest predicted blastocyst production rate was allocated to Bull C with Bulls D and B following in that order.

Table 2.12. Predicted mean blastocyst production rates (values are predicted means ± s.e.m.)

Bull	Blastocyst production rate
A	2.3 ± 1.0
B	0.6 ± 0.6
C	4.8 ± 1.1
D	3.1 ± 0.9
E	1.0 ± 0.5
F	0.9 ± 0.6

2.3.7 Level of performance

To summarise the information for the responses tested, the bulls were given ranks, such that the highest was designated 1 and the lowest 6 (Table 2.13).

Table 2.13. Order of performance for developmental scores, cleavage rates and blastocyst production rates (ranking for predicted values is given in parentheses)

Bull	msc1[1]	msc2	Cleavage rate	Blastocyst production rate
A	2 (2)	2 (2)	3 (2)	3 (3)
B	3 (5)	3 (6)	1 (3)	6 (6)
C	1 (1)	1 (1)	2 (1)	1 (1)
D	5 (3)	5 (3)	6 (6)	2 (2)
E	6 (4)	6 (5)	5 (5)	4 (4)
F	4 (6)	4 (4)	4 (4)	5 (5)

[1] msc1 and msc2 represent maximal mean Scores 1 and 2, respectively

Rankings for predicted scores did not closely follow the actual rankings for developmental scores. Rankings for actual cleavage rates were more accurately estimated by those for predicted values. The order of performance for blastocyst production was identical for both actual and predicted values.

The bull predicted to perform best for all responses was Bull C. Bull D, predicted to produce the next highest number of blastocysts to Bull C, was not expected to show high cleavage rates. Bull A was expected to perform well after Bull C. This bull ranked highly for expected developmental scores, cleavage rates and rates of blastocyst production. Bull E was predicted to do badly in all responses evaluated. The control (Bull F) came last for developmental Score 1, fourth for developmental Score 2 and cleavage rate and fifth for rate of blastocyst production.

Neither of the developmental scores, nor the rates of cleavage were suitable predictors of the order that bulls would perform for blastocyst production.

2.4 DISCUSSION

2.4.1 Block variation

Despite the attempt to reduce the effect of days and blocks, these had a significant effect on the outcome of the results, regardless of the criteria used for assessing success. Within block variation (day effect) was also considerable, as all bulls could not be tested on the same day. Within block variation included factors such as variations in ovary quantity and quality, time from slaughter to aspiration, saline temperature, laboratory temperature, processing duration (searching, washing and selection), maturation rates and variation in sperm numbers.

Variation in the number of ovaries collected from the abattoir may have affected the success of the *in vitro* system in a number of ways. Low numbers of ovaries meant that fewer oocytes were obtained, resulting in faster processing times, but a lower number of good quality oocytes from which to select. Conversely, if large numbers of ovaries were

collected, the time period between time of slaughter and placing oocytes into maturation medium was lengthened. The advantage of increased ovary collection was that there were more oocytes from which to select those of a high quality.

It was impossible to determine which of these two situations was more advantageous to the successful culture of the embryos. Optimal conditions would minimise processing times and maximise the numbers of oocytes obtained to allow a wide choice of oocytes from which to select those suitable for *in vitro* maturation, fertilization and culture.

Ovary quality also played an important role in the oocyte yield. Ovaries collected on some days had many small follicles. With each follicle potentially yielding one oocyte, the total yield from ovaries of this type is expected to be much higher than that from ovaries with fewer, large follicles. It has been shown that differences in cleavage rates and numbers of blastocysts produced can be attributed to the cow from which the ovaries were obtained (Prokofiev *et al.*, 1992). Whether the dark yellow follicular fluid had a positive or negative effect on oocyte development could not be determined. It may be of no significance at all, and may be simply due to a dietary factor. It could be hypothesised that the ovaries of feedlot heifers contain clear follicular fluid whereas those of pasture cows contain yellow follicular fluid and that the different rations produce this effect.

During the aspiration of ovaries, the temperature of the saline in which ovaries were stored dropped dramatically as it equilibrated with the ambient temperature (approximately 25 °C). Temperature fluctuations in the laboratory varied from 20 to 27 °C during the course of the trial. Cell proliferation in rabbit embryos was significantly impaired when exposed to room temperature (23 °C) for one hour (Fischer *et al.*, 1988) and may be due to disruption of the meiotic spindle (Moor and Crosby, 1985). During the processing period, the temperature may not have been sufficiently high to maintain developmental competence in the oocytes. The heated stage was maintained at a maximum of 33 °C so that the oocytes would not become too hot, but perhaps this temperature was too low. Lenz *et al.* (1983a) showed that maturation rates were not significantly different when oocytes were incubated at 35 to 39 °C. If decreased

temperature was important, the length of processing time may also have played a part in the decrease in functional maturation. When cooled below 29 °C for 3 h, oocytes showed disorganisation of chromosomal material and only 6 to 11% of these oocytes developed into blastocysts compared to 44% for controls (Moor and Crosby, 1985).

Schumacher and Fischer (1988) showed that the later stages of embryo development are more susceptible to decreasing temperature than early cleavage stages. In the present trial, it was necessary to examine the presumptive zygotes on a regular (almost daily) basis to collect the results. This involved approximately 20 min microscopic examination per culture plate. Thus, developmental competence may have been impaired due to a difficulty in providing a stable environment in which to work. It is, therefore, possible that decreased temperature could, to some extent, explain the variation in maturation rates and lack of developmental competence in the present trial.

Maturation rate was part of the day effect and its non-significance may have been because it had already been accounted for in the block variation. It is difficult to believe that oocytes showing poor maturation rates could be fertilized and undergo further development equally as well as those with high maturation rates. The fact that only ten oocytes were taken for maturation controls could lead to significant errors in estimating the rate of maturation. Unfortunately, it was not practical to take more oocytes for maturation controls and thus it was recognised from the outset that this measure of maturation rate would be inaccurate. It was hoped that the *in vitro* system would prove to be more stable. If this were the case, maturation controls would then have confirmed that the trial was being carried out using an *in vitro* maturation system which was reliable and effective.

Because oocytes were collected from follicles of varying size, not all oocytes would have been at the same stage of development. Most oocytes would not have initiated meiosis while others would be almost at metaphase II. Those that were further along the path to metaphase II would perhaps be more susceptible to the effects of ageing. Long processing times would thus leave these oocytes incapable of being successfully fertilized and they would spontaneously break down. Spontaneous activation rates were found to

be higher in aged oocytes (6 to 57%) than in young oocytes (0 to 14%) (Precisse and Yang, 1994). Maximal activation with Ca-ionophore occurred once oocytes had been removed from the follicle for more than 26 h (Ware *et al.*, 1989).

The large fluctuations in the rates of maturation from day to day pose problems for further research into *in vitro* fertilization. It is necessary to ensure that the conditions up to the point under test are as stable as possible and can easily be replicated. This was not the case for the current trial, although the small sample numbers involved did not aid interpretation. Before the start of the trial, it was acknowledged that the quality of oocytes would play a large role in the outcome of the investigation. However, it was hoped that the experimental design would overcome this problem. Despite the use of approximately 400 oocytes for each bull, giving a total of 2 422 oocytes used during the course of the trial, this was insufficient to ensure that oocyte differences did not have an effect on the results.

An unavoidable experimental error was introduced because semen from each bull did not originate from one collection. However, if prediction of a bull's fertility is only possible by using single semen collections, the test results would have no predictive value for any subsequent collections. Amann (1989), when discussing the possibilities of predicting fertility potential, suggested that results should be pooled for several semen samples and that a minimum sample size should be three.

In this trial, sample size for some bulls was one while others had a sample size of two. Although, for statistical reasons, it was preferable to use one collection for the present trial, commercial testing of bulls and attempts to predict fertility should look at testing varied samples from bulls. It would also be reasonable to re-test bulls after a period of time, as changes in environmental conditions (nutrition, disease, temperature, physical injury, etc.) may lead to an altered sperm morphology (Barth and Oko, 1989). Alterations in sperm morphology may indicate changes in potential fertility. Thus, if a bull's fertility is to be accurately predicted, any changes occurring, due to the environment, must be incorporated into the prediction equation.

The experimental design for sperm swim-up and subsequent concentration of the semen was not ideal. Sperm numbers were often low and this may have negatively affected fertilization rates. Niwa and Chang (1974) determined an optimal sperm concentration for use in *in vitro* fertilization in rats of 0.5 to $1.5x10^6$ sperm/ml. Sperm concentrations for the present Nguni trial often fell below this lower limit of $0.5x10^6$ sperm/ml. This may have been an influential factor in the observed differences between bulls despite analysis that showed sperm numbers to be non-significant. It is interesting to note that Niwa and Chang (1974) did not use a swim-up method of sperm separation. Higher numbers of sperm would, therefore, be required for fertilization because a certain proportion would be immotile. The use of the swim-up procedure in the current study enabled concentration of motile sperm and minimised the chance of introducing immotile sperm into the fertilization wells. Thus, the concentrations of live sperm which are required for successful fertilization may be much lower than those determined by Niwa and Chang (1974), if the immotile fraction of the sperm used by these workers was high.

If the sperm numbers used in the present trial were below a minimum required for successful fertilization, the criteria would not have assessed the effects of treatments but instead the effects of sperm concentration on bull performance. Conversely, if the sperm numbers had been much higher than a minimum threshold level, sensitivity to the treatments tested would have been lost. Obviously, there is a need to determine threshold levels of sperm concentrations when testing performance between bulls, and this may not be equivalent to the concentrations routinely used to achieve successful fertilization.

2.4.2 High rate of parthenogenesis

Using the present IVF system, 31.6% (minimum 9.1%, maximum 88.9%) of the oocytes used were predicted to show parthenogenetic cleavage. Fukui *et al.* (1989) obtained parthenogenetic activation of only 3% of oocytes when oocytes were inseminated with killed sperm and left to develop in fertilization medium. Controls for parthenogenesis are seldom carried out, with those investigators running parthenogenesis controls

achieving approximately 3% activation (Leibfried-Rutledge *et al.*, 1986; Fukui *et al.*, 1991). The high rate of parthenogenesis observed in the current study was alarming, as it left only 68.4% of the oocytes to exhibit differences when exposed to different treatments. There were a number of possible sources of parthenogenetic activation of these oocytes. A factor which may have contributed to the high rates of parthenogenesis was the rise in pH of the HEPES wash medium while it was exposed to normal atmospheric conditions during oocyte processing. Whether the requirement for CO_2 or the rise in pH had an effect on developmental competence is unknown.

Chemical factors involved in parthenogenetic activation of the oocytes may have been heparin (in the HEPES wash medium during the first experimental block) or ethanol (introduced with the addition of oestradiol (E_2) to the maturation medium). Ethanol (7% solution) has been used to produce parthenogenetically activated oocytes (Mitani, 1993; Minamihashi *et al.*, 1993; Yang *et al.*, 1994). Although this solution is more concentrated than the ethanol found in the maturation medium used here (0.1%), one cannot exclude the possibility that the small amount of ethanol is causing parthenogenesis in the oocytes while in the maturation medium. Rates of activation comparable to those observed here were obtained in bovine oocytes by Yang *et al.* (1994) (37%) and Nagai (1987) (25 to 38%) using 7% ethanol. The fact that treatment did not significantly affect cleavage rates or blastocyst production rates, suggests that there was no one treatment causing the parthenogenesis. In fact, cleavage rates and blastocyst production rates were not significantly different between treatments and the control in fertilization medium. It then seems quite likely that the factor(s) causing this high rate of parthenogenesis are either contained in the basal media or are environmental.

Blastocysts produced by parthenogenetic activation have been observed previously (Fukui *et al.*, 1992; Minamihashi *et al.*, 1993). This casts serious doubt on any experimental work that involves morphological examination of the oocytes/zygotes. Bavister (1981) states that the use of strict criteria for the assessment of success should be central to any *in vitro* fertilization technique. Often, microscopic evaluation, which entails killing the embryo, closes the path to analysis of further development. Thus, small numbers of

embryos do not facilitate use of criteria which involve both terminal microscopic evaluation and assessment of further development.

2.4.3 Criteria for predicting bull fertility

With the sentiments of Bavister (1981) in mind, a number of criteria were used in an effort to successfully assess the *in vitro* fertility of the bulls under test, namely cleavage rates, assessment of relative development and production of blastocysts.

Cleavage rates, as an indicator of fertilization, were not significantly different to the cleavage rate of the control. Thus, there was no way of ascertaining if the cleavage produced by the bulls was a true indicator of fertilization rates. Microscopic examination of fixed oocytes stained with orcein, in contrast to parthenogenetic activation, would have revealed rates of normal fertilization and polyspermy but no further development would have been assessed. To include terminal microscopic evaluation would have doubled the work, effectively requiring the trial to be run twice; once for microscopic evaluation and once for assessment of embryonic development.

Developmental scores gave an indication of how bulls would perform with respect to potential implantation of embryos. There was little difference between the first method of scoring and the second. This was due to the consistently low numbers of morulae and blastocysts obtained. It was hoped that the second scoring system would provide greater differences between those bulls producing embryos suitable for implantation and those whose embryos did not develop fully, but this was not the case. An *in vitro* system which produces large numbers of blastocysts is required to enable detection of these differences. Again, there were few differences between the bulls and the control (due to the high level of parthenogenetic cleavage). However, bulls could be separated from one another on levels of performance. It would be possible to develop a league table of bulls most likely to perform well in the *in vitro* system used in this laboratory.

Bulls B and E produced the same score for Method 1 and Method 2. This was because the maximum observed score was not on the day of blastocyst production. The negative

effect of disintegrating embryos was introduced to include the parthenogenesis factor. This effect was, perhaps, too strong, as the time when the highest rate of disintegrating embryos is observed is likely to coincide with the production of blastocysts. The scoring system could perhaps have been improved if the disintegrating embryos were given a zero score so that they did not affect the final outcome.

Blastocyst production (maximum 5.3%) during this trial was much lower than that reported by other workers using similar culture media (28.6% by Rosenkrans and First, 1991). Even though the treatments tested were not expected to be optimal for all bulls, the rate of blastocyst production was disappointing. However, Bulls C and D did produce a significantly ($P<0.05$) higher number of blastocysts than the control. Thus, bulls could be separated on rates of blastocyst production for further use in *in vitro* fertilization.

The levels of blastocyst production from bulls was not closely related to cleavage rates or developmental scores. The percentage variance accounted for in the evaluation of developmental scores was in the order of 60%. A large amount of the variance in the data is, therefore, not accounted for by the variables included in the model. Further investigation into these unknown variables is obviously necessary if prediction of bull fertility is to be accurate.

Neither cleavage rates nor developmental scores were suitable indicators of blastocyst production rates to the extent that bull performance *in vitro* could be predicted accurately. However, trends could be determined and these criteria may go some way to aiding prediction of bull fertility.

2.4.4 Bulls performance with respect to treatments

The fact that there were no treatment differences and no treatment x bull interactions was surprising. Treatment effects on fertilization rates have been reported previously for Ca-ionophore (Bird *et al.*, 1989; Yang *et al.*, 1993) and heparin (Lu and Gordon, 1988; Fukui *et al.*, 1990). This suggests that other factors influencing the IVF system had a

greater effect on the development of the embryos than the treatments, thereby masking any treatment effects that may exist. Another alternative is that, for the bulls tested here, the concentrations of heparin and Ca-ionophore were too low. It has been noted previously that concentrations of Ca-ionophore for acrosome reaction tests have been as high as 1.8 mM (Dodds and Seidel, 1984) and concentrations of 200 μg/ml heparin are not unheard of (Fukui *et al.*, 1990). The range of concentrations tested here, therefore, may not have been sufficiently high to elicit a response from the bulls in the test. Other factors may also be of importance, that is, incubation times and motility factors. Further work should perhaps concentrate on some of these influences, once the block variation has been reduced.

Differences in fertilization rates between bulls have been noted by Sirard *et al.* (1984), Parrish *et al.* (1986) and Marquant-Le Guienne *et al.* (1990). However, Sirard *et al.* (1984) found that the differences between bulls could be altered by changing the method of capacitation, whereas Leibfried-Rutledge *et al.* (1989) decreased differences between bulls by altering the sperm concentration and levels of heparin. It was hypothesised by Aoyagi *et al.* (1988) that bulls could achieve comparable rates of fertilization using combinations of sperm capacitation chemicals.

Bull F was not consistently the worst performer. The high rate of parthenogenesis may have provided the control with artificially high scores and cleavage rates, thus effectively increasing its performance. It is also possible that bulls which performed worse than the control were providing another measure of the control, although it is not possible to state this with surety because the control was not exposed to sperm.

From the results presented here, it is apparent that bulls did not perform equally well given the range of treatments tested. Neither did the heparin treatments produce better bull performance than the Ca-ionophore treatments. Thus, the hypothesis that bulls would produce acceptable IVF rates with respect to the treatments tested must be rejected, using the present IVF system.

2.4.5 Future work

The variability in maturation rates has already illustrated that the *in vitro* system employed during this trial is not stable. A new system has recently been employed in our laboratory which involves culture under oil. This may be an alternative to the use of culture plates. It would provide a more stable environment for *in vitro* culture without the need for co-culture (BOEC or granulosa cells). However, it will still be necessary to attempt to reduce the daily variation in oocyte populations. Strict time limits on aspiration of ovaries and oocyte processing may go some way towards this. Brackett and Zuelke (1993) suggest that developmental competence is lost in bovine oocytes which are not placed in maturation medium within 45 min of dilution out of follicular fluid. On a more general level, long term observations on oocyte quality may lead to discoveries as to ovary effects on maturation rates.

Rates of parthenogenesis must be reduced to assure developmental competence in experimental oocyte populations. Perhaps reagents within the various media could be systematically removed or replaced and parthenogenetic activation assessed. Much of the failure of blastocyst implantation to produce high pregnancy rates (37.5% by Sirard *et al.*, 1988; 55% by Eyestone and First, 1989a; 50% by Fukuda *et al.*, 1990; 59% by Hamano and Kuwayama, 1993) may be due to implantation of parthenogenetically activated oocytes. The production of parthenotes during this trial emphasises the need for strict criteria for assessment of success if meaningful results are sought. Perhaps future work could limit the number of treatments tested and include terminal microscopic evaluation for normal fertilization.

A study of a wider range of concentrations of the capacitating chemicals and incubation times is warranted. This would obviously be a lengthy procedure, especially if individual bulls are to be characterised accurately but the success of rapid population growth depends on reliable and efficient IVF procedures. Once treatments have been proven to yield successful fertilization, enhancement of the fertilization procedure may be carried out by addition of sperm motility factors (such as caffeine or PHE) to the incubating medium.

If bull fertility in IVF procedures could be accurately predicted by laboratory methods (such as microscopic semen evaluation), the need for lengthy IVF studies, purely as a means of assessing bull fertility *in vitro,* could be eliminated. Parameters exist which are highly correlated with bull fertility and these will be examined in Chapter 3.

CHAPTER 3

SEMEN QUALITY TESTS AND RELATIONSHIP TO IVF PERFORMANCE

3.1 INTRODUCTION

Experiments described in Chapter 2 investigated the performance of bulls in an existing *in vitro* fertilization system. This was a time-consuming procedure, if used purely to predict potential IVF performance, where results were significantly influenced by day effects. Laboratory tests (such as microscopic evaluations, staining techniques), where experimental conditions can be accurately replicated, have been shown to correlate with fertility *in vivo* (Wood *et al.*, 1986; Blottner *et al.*,1990). Such tests, therefore, have the potential to predict the performance of bulls in *in vitro* fertilization systems.

Semen collected for use in artificial insemination (AI) is routinely assessed for sperm concentration, sperm motility and abnormal morphology. These parameters have been shown to correlate with fertility and have been incorporated into prediction models for *in vivo* fertility (Wood *et al.*, 1986). Furthermore, morphology has been implicated as an indicator of sperm motility and zona binding ability (Kaskar *et al.*, 1994). However, correlations alone between gross sperm characteristics and performance do not predict fertility (Amann, 1989), although a combination of correlated factors could perhaps be used to form prediction equations.

The IVF trial described in Chapter 2 posed the question "Did the treatments successfully capacitate the sperm used in the IVF trial?". This question could be answered by use of a dual staining procedure (Didion *et al.*, 1989) which determines the proportion of dead/live and acrosome reacted/not acrosome reacted sperm. It would, therefore, be possible to determine if treatment effects in the IVF trial were being masked by other factors influencing the culture system.

The aim of this investigation, therefore, was to explore laboratory tests with the potential to successfully predict fertility in IVF trials. The following experiments were conducted:

a) Assessment of sperm motility by microscopic evaluation
b) Abnormal sperm morphology assessment by microscopic evaluation
c) Determination of acrosome-reacted sperm by use of a staining technique

It was hypothesised that the results from experiments a) and b) would correlate with the results from the IVF trial in Chapter 2, and, in this way, provide laboratory tests which could be used to predict a bull's performance in the existing IVF system. Experiment c) was expected to show differences in the ability of the treatments tested in Chapter 2 to effect the acrosome reaction in sperm.

3.2 MATERIALS AND METHODS

3.2.1 Assessment of sperm motility by microscopic evaluation

Semen samples were assessed for motility using a modification of the procedure of Naidu (1989) where the sperm were categorised as follows:

Immotile: any sperm which is not displaying movement.
Motile: any sperm displaying movement of any form.
Progressively motile: any sperm displaying directional movement only.

Semen straws were thawed for 1 min at 35 °C. To give a concentration of approximately 10 sperm per haemacytometer square (0.004 mm^3), 40 μl of semen was added to 2 ml of Dulbecco's phosphate buffered saline (Dulbecco's PBS; Appendix 6), previously incubated at 38.5 °C and 5% CO_2 in air. A haemocytometer slide was placed on the heated stage (35 °C) of a light microscope and sufficient diluted semen solution added to fill the chamber. The diluted semen sample was held at 38.5 °C and 5% CO_2 in air for a replicate count. The sperm were immediately counted at 10x magnification and the

procedure repeated for the second haemocytometer chamber. Semen from all bulls tested, as detailed in Chapter 2, was processed in this manner.

3.2.2 Abnormal sperm morphology assessment by microscopic evaluation

Abnormal sperm morphology assessment followed the procedure routinely carried out at Taurus Artificial Insemination Centre (P. Jack, personal communication). Frozen semen was thawed for 1 min at 35 °C and a small drop (approximately 10 μl) was added to 1 ml of 0.2% gluteraldehyde (Appendix 6). The gluteraldehyde was incubated at 38.5 °C to reduce the risk of cold-shock to the sperm and, thus, reduce the incidence of artificially high numbers of abnormal morphological characteristics. Approximately 10 μl of the fixed semen sample was then placed on a microscope slide, a coverslip carefully placed on top and the semen viewed with oil immersion and 100x magnification. Semen were characterised according to the method described by Barth and Oko (1989) (Appendix 7) until 100 sperm had been counted.

3.2.3 Determination of acrosome-reacted sperm by use of a staining technique

Due to time constraints, it was not possible to conduct the staining experiment for all bulls on one day. To minimise day effects, therefore, sperm from Bulls A and B was stained on the first day and that from Bulls C, D and E was stained the following day.

The procedure for thawing sperm, separation by swim-up and centrifugation was exactly as described for *in vitro* fertilization in Chapter 2 (section 2.2.7). Treatments incorporated into the staining procedure for capacitation were the same as those detailed in Chapter 2 (section 2.2.7). The following staining procedure is a modification of the dual staining technique of Didion *et al.* (1989).

3.2.3.1 Ca-ionophore treatments

Ten microlitres of sperm was added to each of three foil-covered eppendorf tubes (1 ml) containing an equal volume of 0.2% (v/v) Trypan blue (Merck, Germany) in BSA-free fertilization medium. These tubes were then incubated at 38.5 °C and 5% CO_2 in air.

After 9 min, 20 μl of 0.1, 0.2 or 0.4 μM Ca-ionophore was added to the tubes. The sperm and Ca-ionophore mixtures were then incubated for 1 min at room temperature and then 1 ml of BSA-free fertilization medium added to each tube. The eppendorf tubes were then centrifuged at 700 g for 6 min at 30 °C and the resulting supernatant discarded. The samples then underwent one further wash in 1 ml of BSA-free fertilization medium and the sample centrifuged once more. The supernatant was discarded to leave approximately 20 μl of sperm in the eppendorf tube. Ten microlitres of this sample was smeared on a glass slide and dried under a stream of warm air on a heated stage (30 °C). The smears were covered with 10% (v/v) Giemsa (Saarchem, Krugersdorp) in distilled water for 40 min. The Giemsa stain was rinsed off with distilled water and the slides air-dried. Coverslips were mounted on the slides with DePeX® mounting medium (BDH, Poole, England).

3.2.3.2 Heparin treatments

Ten microlitres of sperm was added to each of four eppendorf tubes containing an equal volume of one of the following treatments; fertilization medium, 0.05, 10 or 25 μg/ml heparin. These samples were incubated for 5 min at 38.5 °C and 5% CO_2 in air. After this time, 2.2 μl of 1% (v/v) Trypan blue in BSA-free fertilization medium was added to each tube and the tubes incubated for a further 10 min at 38.5 °C and 5% CO_2 in air. This yielded final heparin concentrations of 0.045, 9.01 and 22.52 μg/ml approximating to the *in vitro* fertilization treatments of 0.05, 10 and 25 μg/ml, respectively. It was not possible to incubate the sperm samples for the full time period at the heparin concentrations used in Chapter 2 because of the dilution effect of the Trypan blue stain. However, concentrations closely corresponded to those used in the *in vitro* trial. After this second incubation period, the samples underwent the same washing and Giemsa staining procedures as those described for the Ca-ionophore treatments.

3.2.3.3 Slide examination

Slides were examined under a light microscope at 40x magnification. Every fourth lane of each slide was examined and all sperm counted and categorised into dead/live and acrosome reacted/not acrosome reacted sperm. Sperm taking up Trypan blue in the lower half of the sperm head are dead and those taking up Giemsa in the upper half of

the head are not acrosome reacted. Completely clear sperm are live and acrosome reacted. Percentages of live sperm, acrosome reacted sperm and live/acrosome reacted sperm compared to the total number of sperm, and the proportion of live sperm which were acrosome reacted were evaluated.

3.2.5 Statistical analysis

The semen quality tests described here could not be replicated because of the shortage of semen straws. Differences in percentages of abnormal and motile sperm between bulls within experiments could not be statistically analysed because there were too few data. It was also not possible to analyse differences between treatments for the dual stain procedure because bull x treatment interactions were assumed, thus, preventing bulls to be regarded as blocks. However, the results from the semen quality tests involving motility and morphological examinations were regressed against the results from the IVF trial using Minitab Release 8.2 to determine whether any of the parameters assessed in the semen quality tests could potentially predict a bull's performance in the IVF procedure used in Chapter 2.

3.3 RESULTS

3.3.1 Assessment of sperm motility by microscopic evaluation

Assessment of sperm motility was a simple technique which produced quick results. However, there was an inherent error involved in the procedure. Progressively motile sperm may be counted more than once or not at all during the examination of a slide. This error may cancel itself out but cannot be ignored. Sperm motility assessments displayed differences between bulls (Table 3.1).

Table 3.1. Percentages of immotile, motile and progressively motile sperm in semen straws of each bull.

Bull	Immotile	Motile	Progressively motile	Concentration (x 10^8/ml)
A	74.63	25.37	13.90	2.091
B	60.16	39.84	34.35	2.509
C	72.88	27.12	22.67	2.407
D	76.13	23.87	19.37	2.264
E	78.51	21.49	15.04	3.086

The number of immotile sperm was high compared to the number of motile sperm, for all bulls. Bull B displayed the highest and Bull E the lowest percentage of motile sperm for all bulls tested. Bull B also had a considerably higher percentage of progressively motile sperm compared to all the other bulls. Bulls A and E had particularly low percentages of progressively motile sperm. Bull E, with the highest percentage of immotile sperm and the second lowest percentage of progressively motile sperm, had the highest concentration of sperm. This concentration was 50% higher again than the concentration in the straw from Bull A.

3.3.2 Abnormal sperm morphology assessment by microscopic evaluation

The investigation of abnormal sperm morphology assessed a number of parameters. However, only the parameters expected to be relevant are detailed here (Table 3.2).

Bull C displayed the lowest proportion of morphologically normal sperm. The number of major abnormalities contributing to this was not different from Bulls A or E, but minor abnormalities were considerably higher than for any of the other bulls. Bulls A, D and E produced similar numbers of normal sperm; most of the abnormalities being explained by major abnormalities.

Table 3.2. Percentages of major and minor abnormalities, coiled tail defects, proximal droplets and normal spermatozoa.

Bull	Major	Minor	Coiled tail	Proximal droplet	Normal[1]
A	18	4	6	1	80
B	9	1	5	2	90
C	19	21	10	0	66
D	15	3	5	1	82
E	18	2	7	5	80

[1] percentages of normal sperm do not necessarily equal 100 minus the sum of the major and minor abnormalities, as some sperm displayed more than one abnormality

[2] sperm defined as having coiled tails comprised midpiece reflex and dag deformities

3.3.3 Determination of acrosome-reacted sperm by use of a staining technique

Examination of the stained slides was a lengthy procedure. Sperm were unevenly distributed over the length of the slide and were often found in clumps of one type. The sampling of random fields was, therefore, impossible, as it would have led to a biased result. This led to examination of the full length of the slide to obtain as random a sample as possible. Thus, the total number of sperm examined for any one bull and treatment was determined by the number of sperm encountered during examination of the slide and was, therefore, highly variable (Table 3.3).

A further problem was that some of the sperm had taken up the Giemsa stain over the whole sperm head. These sperm were later categorised as dead and not acrosome reacted. It was not possible to determine if dead acrosome reacted sperm were acrosome reacted before they died or if the acrosome reaction was false due to sperm death.

Table 3.3 Percentages of sperm proportions of live, acrosome reacted (AR) and live acrosome reacted (LAR) in relation to total sperm counted (n), and LAR compared to numbers of live sperm determined.

Bull[1]	Treatment[2]	n	live/dead	AR/n	LAR/n	LAR/live
	fert	664	0.0	0.0	0.0	0.0
	Ca 0.1	308	40.6	34.1	23.0	79.8
	Ca 0.2	107	9.2	15.9	7.5	88.9
A	Ca 0.4	151	19.8	17.9	11.9	72.0
	hep 0.05	197	10.7	20.8	9.1	94.7
	hep 10	1241	2.3	4.6	2.0	89.3
	hep 25	704	17.5	15.5	14.1	94.3
	fert	430	1.6	0.9	0.9	57.1
	Ca 0.1	248	0.4	0.0	0.0	0.0
	Ca 0.2	287	1.1	2.1	0.4	33.3
B	Ca 0.4	256	11.3	25.8	9.8	96.2
	hep 0.05	602	0.5	0.3	0.2	33.3
	hep 10	834	9.6	8.5	6.8	78.1
	hep 25	561	2.4	1.1	1.1	46.2
	fert	1100	11.3	9.6	9.3	91.1
	Ca 0.1	539	3.1	1.9	1.7	56.2
	Ca 0.2	580	0.2	0.2	0.2	100.0
C	Ca 0.4	292	0.3	0.3	0.3	100.0
	hep 0.05	421	5.8	4.5	3.8	69.6
	hep 10	1030	2.9	2.6	2.0	72.4
	hep 25	840	2.4	2.1	2.0	85.0
	fert	1327	47.6	24.6	24.3	75.5
	Ca 0.1	120	7.1	2.5	2.5	37.5
	Ca 0.2	429	0.9	0.7	0.7	75.0
D	Ca 0.4	470	0.0	0.0	0.0	0.0
	hep 0.05	891	3.1	0.7	0.7	22.2
	hep 10	674	0.0	0.0	0.0	0.0
	hep 25	631	7.7	6.5	6.5	91.1
	fert	821	0.1	0.1	0.1	100.0
	Ca 0.1	523	4.6	4.8	4.4	100.0
	Ca 0.2	777	1.6	1.0	0.9	58.3
E	Ca 0.4	376	0.3	0.0	0.0	0.0
	hep 0.05	661	22.4	18.3	17.2	94.2
	hep 10	1095	1.4	1.3	1.3	93.3
	hep 25	659	8.2	7.4	7.4	98.0

[1] bull allocation is defined in Table 2.1

[2] fert=control;Ca 0.1,0.2,0.4=0.1,0.2,0.4μM Ca-ionophore;hep 0.05,10,25=0.05,10,25μg/ml heparin

The survival rate of sperm during the dual stain procedure was low for all bulls tested (Table 3.3). Maximum survival occurred in Bull D in the fertilization medium control, but even this value did not exceed 50%. This treatment also provided the highest number of live acrosome reacted sperm expressed as a percentage of all sperm incubated. The 0.1 μM Ca-ionophore treatment for Bull A paralleled this observation. The lowest survival rate was zero for Bull D treated with 0.4 μM Ca-ionophore and Bull A in the fertilization medium control. Each bull had poor survival with at least one of the treatments tested, but this treatment was not the same for each bull.

The best treatment for Bull A would appear to be 0.1 μM Ca-ionophore which produced the highest survival rate and percentage of live acrosome reacted sperm for the total sperm population (Table 3.3). The fertilization medium control treatment did not yield any live or acrosome reacted sperm for this bull. Bull B performed best with 0.4 μM Ca-ionophore and worst with 0.1 μM Ca-ionophore. Bull C did not react well to any of the treatments, although the control provided the highest proportions of live and acrosome reacted sperm. Bull D produced high proportions of live and acrosome reacted sperm with the control treatment, but did not respond at all to the 0.4 μM Ca-ionophore or 10 μg/ml heparin treatments. Bull D appeared to react most favourably to 0.05 μg/ml heparin and least favourably to 0.4 μM Ca-ionophore.

Thus, bulls were seen to react favourably to different treatments, although it was not possible to determine if these reactions were significantly different to each other.

3.3.4 Correlation of semen quality parameters with IVF trial

Correlations of sperm characteristics with fertility parameters described in Chapter 2 were conducted (Table 3.4). Morphological and motility parameters were expressed as percentages of total sperm counted, sperm concentration was expressed as number of sperm per ml. However, it was appreciated that the IVF trial did not produce repeatable results and day effects were highly significant, therefore, correlating semen quality parameters with unstable fertility parameters was not an ideal situation. Furthermore,

because there were only five bulls, regression lines with only five points were not expected to produce precise data.

Table 3.4. Regression coefficients (r) and percentage of variance accounted for (% var) for abnormal morphology and motility examinations with bull performance criteria.

Sperm characteristic[1]	% Cleavage		Score 1		Score 2		% Blastocyst	
	r	% var	r	% var	r	% var	r	% var
% major abnorm	0.46	20.9	0.61	37.4	0.61	37.7	0.58	33.7
% minor abnorm	0.82	68.1	0.91	82.4	0.90	80.8	0.86	74.4
% normal	-0.73	53.4	-0.88	77.2	-0.87	76.3	-0.86	73.8
% coiled tail	0.73	53.6	0.76	57.3	0.74	55.5	0.67	45.0
% proximal droplet	-0.66	43.0	-0.76	57.0	-0.76	57.9	-0.74	54.8
% distal droplet	0.86	74.0	0.90	80.6	0.89	78.9	0.81	66.1
% motile	0.05	0.2	-0.23	5.1	-0.23	5.4	-0.35	12.3
% prog motile	-0.04	0.1	-0.20	4.1	-0.22	4.6	-0.22	4.7
concentration	-0.45	20.1	-0.51	26.0	-0.52	27.4	-0.46	21.6

[1] abnorm = abnormalities, prog motile = progressively motile

The percentage of major abnormalities correlated positively with bull performance but did not account for a large percentage of the variation in the data (Table 3.4). High positive correlations with performance were obtained with % minor abnormalities, % coiled tails and % distal droplets. High negative correlations with fertility were found between % normal sperm and % proximal droplets. Motility, expressed either as total motile or progressively motile sperm, did not correlate highly with any of the criteria used to assess bull performance and the percentage variance accounted for by the data was low.

3.4 DISCUSSION

3.4.1 Motility and abnormal morphology examinations

The limitations of the laboratory quality tests were recognised before undertaking any of these assessments. Lack of replication was an obvious drawback, unavoidable because of the low numbers of semen straws available. However, the aim was to try to incorporate existing quality assessment techniques into a prediction of how bulls would perform in *in vitro* fertilization studies. If the only way to do this is to use many straws and time-consuming assessments, then the practical application of these assessments is not feasible.

Even though small numbers of sperm were examined, differences in percentage motility and abnormal morphology were observed between bulls. However, the statistical significance of these differences could not be determined and, thus, the observed differences may have been within reasonable limits of variation occurring naturally in sperm populations. Examination of greater numbers of sperm would confer greater precision to the tests conducted. However, as previously stated, the aim was to replicate tests already conducted within the artificial insemination industry to ascertain if these tests could be applied to predict bull performance in IVF systems.

It must be remembered that all of the bulls used in the current tests had been accepted for use in the artificial insemination industry. Thus, the differences between these bulls may not have been sufficiently pronounced to provide high variation between samples.

A high frequency of morphological defects is coupled with reduced fertility and even sterility (Pace, 1980). The correlations determined for morphological examination contradicted this statement, for example, the proportion of normal sperm in a sample was shown to negatively correlate to bull fertility as determined in Chapter 2. There seems to be no reasonable explanation of these results, although the lack of fit due to few plotted points may have contributed to this anomalous result. Perhaps the motility and morphology determinations should have been conducted on swim-up separated samples to give a better approximation to the sperm samples introduced to the matured

oocytes. This may have produced correlations approximating more closely to those expected. Wood *et al.* (1986) observed negative correlations when comparing coiled tails (r=-0.51), proximal droplets (r=-0.31) and distal droplets (r=-0.50) with non-return rate. However, because semen quality tests were reproduced from those carried out in the AI industry, swim-up separation was not done.

The only sperm type correlating as expected with bull fertility was the proportion of proximal droplets in a semen sample. Wood *et al.* (1986) showed that the percentage of proximal droplets in a semen sample was negatively correlated (r=-0.31) to fertility as determined by non-return rate after artificial insemination. The corresponding correlation co-efficient of -0.66 to -0.76 (depending on fertility parameter used) in this investigation was more highly correlated to fertility than found by Wood *et al.* (1986).

Progressive motility has been shown to correlate positively and significantly with sperm function (Liu *et al.*, 1990, cited by Kaskar *et al.*, 1994). However, Blottner *et al.* (1990) found that total motility and forward motility did not significantly correlate with non-return rates. In this study, neither motility nor progressive motility was positively correlated to bull fertility *in vitro*. However, this result is not too surprising considering that sperm underwent a swim-up separation before fertilization *in vitro* which minimised the number of immotile sperm introduced to the oocytes. Sperm examined for motility were not subjected to a swim-up separation, as it was intended that semen quality tests should be performed on neat semen from the straws provided, so that quality tests would be quick and efficient as well as reproducing tests carried out in industry. Using samples which had been washed but not swim-up separated, Ohgoda *et al.* (1988) found no correlation between *in vitro* penetration of bovine oocytes and conception rates following artificial insemination. However, one bull which was fertile *in vivo* did not fertilize oocytes *in vitro*, suggesting that the IVF technique did not adequately capacitate the sperm.

The argument that contradictory correlations could be due to swim-up separation of sperm holds for sperm concentration, which was negatively correlated to bull

performance. The *in vitro* fertilization procedure corrects for low sperm numbers by centrifugation to allow concentration of sperm samples before addition to oocytes. Again, it must be realised that the bulls in this test were acceptable for use in artificial insemination. Therefore, the motility, abnormal morphology and concentrations observed were within acceptable limits to allow successful fertilization *in vivo* and may not have been sufficiently variable to allow detection of differences in the tests conducted here. It was, thus, necessary to reject the hypothesis that fertility *in vitro* could be predicted by evaluation of semen quality parameters and their correlations with fertility parameters.

It would seem plausible that if sperm are swim-up separated before fertilization *in vitro*, even bulls of poor fertility could be successfully used in IVF systems for embryo production. This would mean that bulls showing high growth rates but poor fertility could still contribute to animal production systems.

3.4.2 Examination of stained sperm

Although it was not possible to determine if bull differences between treatments were significant, the differences observed suggest that bull x treatment interactions do exist. This is not unexpected, as other researchers have determined bull x treatment interactions (Parrish *et al.*, 1986; Marquant-Le Guienne, 1990). It would, therefore, be reasonable to speculate that the lack of observation of these differences in the *in vitro* fertility trial was due to some factor(s) which was influencing the culture system to a greater extent than the fertilization treatments tested. In order to substantiate this theory, it would be necessary to replicate staining experiments so that statistical analysis of the results was possible.

It had been hoped that the staining procedure of Didion *et al.* (1989) would provide a quick means of assessing the acrosome reaction in sperm exposed to different treatments. However, the examination of slides was a very lengthy process and, as conducted in this study, this technique does not yield results quickly. However, if the technique could be improved to be more efficient, it may become a useful tool for prediction of *in vitro*

fertility. High and significant correlations of induced acrosome reaction with non-return rate have been indicated (Lenz *et al.*, 1988; Blottner *et al.*, 1990) and sperm penetration of oocytes *in vitro* has been shown to be related to *in vivo* fertility (Hillery *et al.*, 1990).

3.4.3 Future work

The tests carried out in this investigation require refinement if they are to be used practically. Future examinations of motility and morphology could be conducted on swim-up separated sperm to determine if these evaluations would yield improved correlations with bull fertility *in vitro*. It would also be important to determine the minimum numbers of sperm that should be counted to give a representative sample, as the numbers counted in this investigation (and in the AI industry) were small.

The dual stain procedure, in particular, was extremely time-consuming during the slide examination phase. In future, it would be advisable to place a smaller volume of the sperm sample onto the slide, say 5 μl, and to smear this only as far as the area of one coverslip. Examining the area of three coverslips is too time-consuming for this method to be of practical use at the present time.

If a quick and efficient staining protocol could be designed to yield results which correlated with *in vitro* fertility, the need for lengthy and costly testing of bulls using IVF systems could be eliminated. Marks and Ax (1985) determined a method for separating bulls of high and low fertility which involved a differential binding affinity for ^{3}H-heparin. Correlations of acrosome reactions induced by chondroitin sulphates with non-return rates have also been reported (Lenz *et al.*, 1988). It would, therefore, be possible to design simple laboratory analyses where experimental conditions could be precisely controlled for evaluation of bull fertility in IVF systems.

GENERAL DISCUSSION

There is a defined need for a breed of dairy cow adapted for production in the harsh sub-tropical climate of South Africa: the Nguni x Jersey. Large scale embryo production by *in vitro* fertilization may go some way to meeting the requirement for substantial numbers of this cross. However, the use of Nguni bulls for successful *in vitro* fertilization depends on the ability of the sperm to be capacitated or acrosome reacted by chemicals in the culture system. Optimal treatments for each bull must, thus, be determined by experimentation.

Accordingly, the objective of this investigation was to produce acceptable rates of fertilization from all Nguni bulls tested by using a number of treatments to capacitate or acrosome react the sperm. Bull x treatment interactions have been observed previously and it was hypothesised that the sperm from each bull would react favourably with at least one of the treatments tested. However, the IVF system used proved to be unstable and repeatable results could not be obtained. Day effects were highly significant and would need to be reduced for further work in this area to be of commercial value. Although the results obtained suggested poor embryo development, it was noted that on day 5, Bull C produced 40% blastocysts when sperm were incubated in 0.1 μM Ca-ionophore. This level of blastocyst production is comparable to those achieved in established culture systems (28.6%; Rosenkrans and First, 1991). However, this high incidence of blastocyst production was not reproducible during the present trial. At the outset, it was acknowledged that, if bull x treatment interactions existed, not all of the treatments tested would yield optimal sperm capacitation/acrosome reaction. Consistently high rates of blastocysts production were, therefore, not expected. However, other researchers testing different culture methods have also obtained low yields of blastocysts (minimum 5.1%, maximum 14.6%; Applewhite and Westhusin, 1995). This is not greatly different from the yield across all treatments for Bull C (4.8% $\pm$ 1.1) in the current investigation. Results from the current investigation, therefore, are not inconsistent with work of other researchers, although definite problems have been identified and must be investigated.

It was also speculated that the concentrations of the treatments tested were too low. Differences in sperm binding ability for heparin exist (Marks and Ax, 1985) and, therefore, sperm requirements for induction of capacitation by heparin may vary. If a dose x response curve exists for capacitation by heparin, it is possible that, for the bulls tested here, the heparin concentrations may have corresponded to the straight line part of the response curve. Higher concentrations of heparin may be required to examine areas of the curve which are in a growth phase. Fukui *et al.* (1990) have used concentrations of 200 μg/ml heparin for sperm capacitation which is considerably higher than the concentrations tested in this investigation (0.05, 10 and 25 μg/ml heparin).

The possibilities for testing sperm capacitation procedures are endless. Not only is the potential for testing chemical concentrations infinite, but the combinations and permutations with incubation times and motility factors are far greater than can ever be examined. Areas where other researchers have had success are a good starting point. However, it must be remembered that *in vitro* systems vary from one laboratory to the next; a working system in one laboratory may not work in another. Defining optimal sperm capacitation procedures for individual bulls is, however, time-consuming and results must be accurate if *in vitro* systems are to be reliable and efficient.

Semen quality tests were examined as a means of eliminating the need for lengthy *in vitro* fertilization tests, exclusively for characterising bull responses to capacitation treatments. This involved evaluation of motility and abnormal morphology as carried out in the commercial AI industry. However, these characteristics failed to correlate with performance determined during *in vitro* fertilization. Whether this was due to an inadequacy of the semen quality tests or to poor IVF performance is unclear. Bull performance in the IVF system employed here, therefore, could not be predicted by parameters assessed during this investigation.

An additional experiment investigated the proportions of sperm from each bull which underwent the acrosome reaction when incubated with the treatments tested in the IVF trial. This allowed for exact replication of the experimental conditions without the effect of the oocyte, which influences embryonic development (Prokofiev *et al.*, (1992). The

initial intention was to examine treatment differences by staining and to correlate these with treatment differences in the IVF trial. If this had been successful, the need for time-consuming IVF trials to determine optimum levels of sperm capacitators would be eliminated. It would also provide a relatively quick method of determining the most effective capacitator for use in emergency situations. For example, recovery of oocytes from genetically superior cows where euthanasia has been necessary at short notice and *in vitro* fertilization with a bull which has not previously been tested is required by the owner. However, no treatment differences were observed in the IVF trial and the staining experiment, therefore, tested for treatment differences that may have existed but could not be detected by the IVF trial. Bull x treatment interactions were observed using the dual staining technique, suggesting that bulls did have a varied response to different chemicals. It was concluded that the expected interactions during the IVF trial were masked by other factors limiting embryonic development.

The scope of this investigation provided for the study of Nguni bulls which were acceptable for use in artificial insemination. Of importance to the animal science industry, is the potential to use bulls for *in vitro* fertilization which have been rejected by AI stations. It would be easy to obtain semen from such bulls in quantities sufficiently large to conduct quality tests with replication allowing full statistical analysis of the results. If bulls with vastly differing motility and morphological counts could be examined, the semen quality tests might be able to predict bull performance in *in vitro* fertilization. Once trends had been established, the tests could be refined to predict bull fertility for those bulls which were acceptable by AI stations and did not differ widely. Furthermore, it would be invaluable to the animal science industry if bulls of low fertility *in vivo* could be used *in vitro* with high success. Swim-up separation of sperm and removal of the need to negotiate the reproductive tract may indicate the use of *in vitro* fertilization techniques for embryo production from bulls of low fertility.

There is enormous potential for large scale production of embryos by use of IVF at a rate which would not be possible using natural mating methods. The results of this investigation indicate that this is not a feasible option at present, but that further research will be able to solve many of the problems encountered here. Production of

embryos by IVF is, therefore, a future possibility for rapid population growth of desired breeds.

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APPENDIX 1

Random allocation of treatments to wells of the 96-well Microwell plate.

Example of a 96-well Microwell® plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	*	*	*	*	*	*	*	*	*	*	*	*
B	*	M	M	M	F	F	F	C	C	C	-	*
C	*	M	M	M	F	F	F	C	C	C	-	*
D	*	M	M	M	F	F	-	C	C	-	-	*
E	*	M	M	-	F	F	-	C	C	-	-	*
F	*	M	M	-	F	F	-	C	C	-	-	*
G	*	M	M	-	F	F	-	C	C	-	-	*
H	*	*	*	*	*	*	*	*	*	*	*	*

Key:

* sterile MilliQ® water

M maturation wells

F fertilization wells

C culture wells

\- empty except for BOEC monolayer

The oocytes from the maturation wells were pooled and then allocated to fertilization wells. After fertilization, the oocytes were transferred to corresponding positions in the culture wells (columns 8-10).

Example: Treatment of sperm from Bull A with 25 μg heparin/ml on day 1.
The sperm from this treatment was placed in well B5 (already containing matured oocytes). On the following day, the presumptive zygotes were moved to well B8 for culture in CR1aa.

APPENDIX 1 contd.

Treatment allocation

Day 1			Day 2		
A; hep 25	A; Ca 0.4	*	B; Ca 0.4	B; Ca 0.2	*
D; hep 10	D; hep 25	*	E; Ca 0.1	B; hep 0.05	*
A; Ca 0.1	D; Ca 0.1	*	E; Ca 0.4	E; hep 10	*
D; Ca 0.4	D; hep 0.05	*	E; hep 0.05	B; hep 10	*
A; Ca 0.2	D; Ca 0.2	*	E; Ca 0.2	E; hep 25	*
A; hep 10	A; hep 0.05	*	B; Ca 0.1	B; hep 25	*

Day 3			Day 4		
C; Ca 0.2	F; Ca 0.4	C; fert	A; Ca 0.1	A; hep 10	A; fert
C; hep 25	F; Ca 0.2	F; fert	B; Ca 0.1	A; hep 25	B; fert
F; hep 10	C; Ca 0.1	*	B; Ca 0.2	A; Ca 0.4	*
F; Ca 0.1	F; hep 25	*	B; Ca 0.4	A; hep 0.05	*
C; hep 0.05	C; Ca 0.4	*	B; hep 25	B; hep 0.05	*
C; hep 10	F; hep 0.05	*	B; hep 10	A; Ca 0.2	*

Day 5			Day 6		
C; Ca 0.2	C; hep 0.05	C; fert	F; Ca 0.4	E; Ca 0.1	E; fert
C; Ca 0.1	D; hep 10	D; fert	E; hep 25	F; Ca 0.1	F; fert
D; Ca 0.2	C; hep 10	*	F; hep 10	E; Ca 0.2	*
C; hep 25	D; hep 25	*	E; hep 0.05	E; Ca 0.4	*
D; Ca 0.4	D; hep 0.05	*	F; hep 0.05	E; hep 10	*
D; Ca 0.1	C; Ca 0.4	*	F; Ca 0.2	F; hep 25	*

Day 7			Day 8		
A; Ca 0.2	C; fert	A; hep 25	F; hep 10	B; Ca 0.4	B; hep 0.05
C; hep 10	C; hep 25	C; Ca 0.2	B; hep 10	B; Ca 0.1	F; fert
A; Ca 0.1	C; hep 0.05	*	B; Ca 0.2	F; hep 0.05	*
A; Ca 0.4	C; Ca 0.1	*	F; Ca 0.4	F; ca 0.2	*
A; hep 10	C; Ca 0.4	*	F; hep 25	B; fert	*
A; hep 0.05	A; fert	*	F; Ca 0.1	B; hep 25	*

APPENDIX 1 contd.

Day 9			Day 10		
E; hep 25	D; fert	E; Ca 0.4	A; hep 25	A; Ca 0.4	A; hep 0.05
E; Ca 0.1	E; Ca 0.2	D; Ca 0.2	E; hep 0.05	E; Ca 0.2	E; hep 25
E; hep 0.05	D; hep 10	*	A; fert	A; Ca 0.1	*
D; hep 25	E; hep 10	*	A; hep 10	E; Ca 0.1	*
D; Ca 0.4	E; fert	*	A; Ca 0.2	E; hep 10	*
D; Ca 0.1	D; hep 0.05	*	E; fert	E; Ca 0.4	*

Day 11			Day 12		
B; Ca 0.2	B; Ca 0.1	D; hep 25	C; hep 25	F; Ca 0.2	F; fert
B; Ca 0.4	D; Ca 0.4	B; fert	F; hep 25	C; hep 10	F; Ca 0.1
D; Ca 0.1	B; hep 10	*	C; Ca 0.4	F; hep 10	*
D; fert	D; hep 10	*	C; fert	F; Ca 0.4	*
D; Ca 0.2	D; hep 0.05	*	C; Ca 0.1	F; hep 0.05	*
B; hep 0.05	B; hep 25	*	C; Ca 0.2	C; hep 0.05	*

Day 13			Day 14		
F; Ca 0.4	F; Ca 0.2	A; Ca 0.2	C; hep 0.05	B; hep 0.05	B; Ca 0.4
A; hep 0.05	A; hep 25	A; fert	C; Ca 0.2	B; Ca 0.2	B; hep 10
A; Ca 0.4	F; Ca 0.1	*	C; hep 10	C; Ca 0.4	*
A; hep 10	F; hep 0.05	*	B; fert	C; Ca 0.1	*
F; hep 10	F; fert	*	B; Ca 0.1	B; hep 25	*
A; Ca 0.1	F; hep 25	*	C; fert	C; hep 25	*

Day 15			Day 16		
E; Ca 0.1	E; Ca 0.4	D; hep 0.05	A; hep 10	A; Ca 0.4	A; fert
D; hep 10	D; Ca 0.1	E; fert	B; fert	A; Ca 0.2	A; Ca 0.1*
D; Ca 0.2	D; fert	*	A; hep 0.05	B; Ca 0.4	*
E; Ca 0.2	E; hep 10	*	B; hep 10	B; hep 0.05	*
D; Ca 0.4	D; hep 25	*	A; hep 25	B; Ca 0.2	*
E; hep 0.05	E; hep 25	*	B; Ca 0.1	B; hep 25	*

APPENDIX 1 contd.

Day 17			Day 18		
C; Ca 0.2	E; Ca 0.2	C; fert	F; CA 0.2	D; hep 25	D; Ca 0.1
C; hep 0.05	E; Ca 0.1	E; hep 0.05	F; Ca 0.4	D; Ca 0.4	F; hep 25
E; fert	E; hep 10	*	D; hep 10	D; fert	*
C; Ca 0.4	E; hep 25	*	F; Ca 0.1	F; hep 0.05	*
C; hep 25	C; Ca 0.1	*	F; hep 10	D; hep 0.05	*
E; Ca 0.4	C; hep 10	*	F; fert	D; Ca 0.2	*

Key for treatments:

hep 0.05 - 0.05 μg heparin/ml

hep 10 - 10 μg heparin/ml

hep 25 - 25 μg heparin/ml

Ca 0.1 - 0.1 mM Ca-ionophore A23187

Ca 0.2 - 0.2 mM Ca-ionophore A23187

Ca 0.4 - 0.4 mM Ca-ionophore A23187

APPENDIX 2

Heparin and Ca-ionophore stock solutions.

Heparin

- A stock solution of 10 mg heparin (Sigma Chemical Co., USA; H-3393, 187 USP units/mg)/ml was made up in physiological saline and filtered through a 0.22 μ acetate filter (Micron Separations, Inc.).
- 25 μl of this stock solution was then added to 4.975 ml filter sterilised NaCl (pH 5.35), which yielded a final working stock solution of 50 μg heparin/ml.
- 100 μl aliquots were put into eppendorf tubes, snap frozen in liquid N_2 and stored at -20 °C until required.

Ca-ionophore A23187

- Ca-ionophore reacts to light, therefore, all manipulations were carried out in red light.
- 1 mg Ca-ionophore A23187 (Sigma C-7522) was dissolved in 1.91 ml DMSO (Holpro Analytics, Johannesburg; filter sterilised) to give a 1 mM solution.
- 100 μl of this stock was then added to a further 4.9 ml DMSO to give a final working stock solution of 20 μM.
- 100 μl aliquots were put into eppendorf tubes, snap frozen in liquid N_2 and stored at -20 °C until required.

APPENDIX 3

Stock solutions and culture media for *in vitro* maturation, fertilization and culture.
(Recipes for media are from the Laboratory Manual, Dept. Animal Science, University of Natal, Pietermaritzburg (1994), unless otherwise stated).

All media were filter sterilised through a 0.22 μ acetate filter (Micron Separations, Inc.) and equilibrated for a minimum of 2 h at 38.5 °C and 5% CO_2 before use.

Physiological saline
9.21 g NaCl (Saarchem, Krugersdorp)/litre of distilled water plus 500 μl gentamicin stock (Genta 50, Phenix SA, Randburg).

Pyruvate stock solution
11 mg pyruvate (Sigma P-5280)/5 ml TL stock. This was made up fresh daily.

Gentamicin stock solution
76.3 mg of gentamicin sulfate (Sigma G-3632)/ml of MilliQ® water.

Heparin 10x stock solution
10 mg heparin (Sigma H-3393)/ml physiological saline. Stored at 0-5 °C.

FSH stock solution
4 mg FSH-p (Sigma F-8001)/ml BSA saline (5 mg BSA fraction V/ml physiological saline). This solution was transferred in 50 μl aliquots to eppendorf tubes and snap frozen in liquid N_2. The frozen FSH-p was then stored at -20°C until required.

LH stock solution
1 mg LH-o/ml BSA saline. This solution was transferred in 25 μl aliquots to eppendorf tubes and snap frozen in liquid N_2. The frozen LH-o was then stored at -20 °C until required.

APPENDIX 3 contd.

Oestradiol-17$_\beta$ (E_2) stock solution

1 mg E_2 (Sigma E-8875) was thoroughly mixed with 1 ml 100% ethanol by vortexing. This solution was then stored in a dark bottle at -20 °C before use.

M199 Hepes wash medium

Reagent	Source	Amount	Final concentration
M199 Hepes	Sigma M-2520	99 ml	N/A
BSA fraction V	Sigma A-8022	100 mg	1 mg/ml
Pyruvate stock	Sigma P-5280	1 ml	0.2 mM
Gentamicin stock	Sigma G-3632	50 µl	25 µg/ml
Heparin 10X stock	Sigma H-3393	50 µl	5 µg/ml

After the first block of the trial had been completed, heparin was excluded from the M199 Hepes wash medium because it was thought that it may be causing parthenogenesis in the matured oocytes.

Maturation medium

Reagent	Source	Volume	Final Concentration
TCM 199	Sigma M-5017	9.0 ml	N/A
FCS (heat-inactivated)	Highveld Biological, SA	1.0 ml	N/A
Pyruvate stock	Sigma P-5280	100 µl	0.2 mM
LH stock	Sigma L-9773	25 µl	2.5 µg/ml
FSH-p stock	Sigma F-8001	50 µl	20 µg/ml
E_2 stock (added after filtering)	Sigma E-8875	1 µl	1.0 µg/ml
Gentamicin stock	Sigma G-3632	5 µl	25 µg/ml

APPENDIX 3 contd.

TL-stock

Reagent	Sigma Cat no.	mg/100 ml	Final concentration (mM)
NaCl	S-5886	584	100
KCl	P-5405	23	3.1
$NaHCO_3$	S-5761	210	25
$NaH_2PO_4H_2O$	S-9638	4	0.29
Hepes	H-3375	238	10
Phenol red	P-5530	1	1 mg/100 ml
Na lactate	L-4263	0.368 ml	21.6
$CaCl_22H_2O$	C-7902	31	2.1
$MgCl_26H_2O$	M-2393	8	0.4

(after Parrish, Susko-Parrish and First, 1985)

Fertilization medium

Reagent	Source	Volume	Final Concentration
TL-stock	N/A	10 ml	N/A
BSA (fatty acid free)	Sigma A-6003	60 mg	6 mg/ml
Pyruvate stock	Sigma P-5280	100 µl	0.2 mM
Gentamicin stock	Sigma G-3632	5 µl	25 µg/ml

APPENDIX 3 contd.

CR1aa medium

Reagent	Sigma cat. no.	mg/10 ml	mg/25 ml	Final concentration (mM)
NaCl	S-5886	67	167.5	114.7
KCl	P-5405	2.3	5.8	3.1
$NaHCO_3$	S-5761	22	55	26.2
L-glutamine	G-3126	1.5	3.8	1.0
Pyruvate	P-5280	0.4	1.0	0.4
L(+)-lactate	L-4388	5.5	13.8	5.0
Gentamicin stock	G-3632	5 µl	12.5 µl	25 µg/ml
BSA (FAF)	A-6003	30	75	3 mg/ml
The remaining ingredients were added using sterile technique after filtering the above solution.				
MEM amino acids	M-7145	100 µl	250 µl	
BME amino acids	B-6766	200 µl	500 µl	

(after Rosenkrans and First, 1991)

APPENDIX 4

Bovine oviduct epithelial cell monolayer culture.

Bovine oviducts were collected from the abattoir at Cato Ridge. They were selected for ovaries displaying a dominant follicle, to indicate that the cow was ready to ovulate and that the oviduct cells would be prepared to receive oocytes. Oviducts were transported in physiological saline at 30-35 °C. At the laboratory, oviducts were trimmed free of connective tissue to yield clean, straight oviducts. Epithelial cells were dislodged by gently scraping the outside of the oviduct with the end of a glass slide. A clamp was placed 1-2 cm into the uterine horn from the oviduct end. A syringe was used to flush the dislodged cells, with BOEC flushing medium, through the oviduct into a 10 ml sterile test tube. Cells were allowed to settle at room temperature for 10 min, after which time the supernatant was discarded and the cells washed in two further rinses of flushing medium (10 ml each). The remaining cells were resuspended in approximately 1.5 ml BOEC culture medium. Five microlitres of the cell suspension was added to 95 µl of BOEC culture medium previously placed in the wells of a 96-well Microwell® plate. The cells were cultured at 38.5 °C and 5% CO_2 in air. They began to form monolayers after 2-3 days in culture.

After three days of culture, 50 µl of fresh BOEC culture medium was added to each well. Every three days after this, 50 µl of medium was removed and 50 µl of fresh BOEC culture medium added.

BOEC flushing medium

Reagent	Source	Amount	Final Concentration
TCM 199	Sigma M-5017	48.5 ml	N/A
FCS (heat-inactivated)	Highveld Biological (SA)	1.0 ml	2%
Pyruvate stock	Sigma P-5280	0.5 ml	0.2 mM
Gentamicin stock	Sigma G-3632	25 µl	25 µg/ml

BOEC culture medium is the same as BOEC flushing medium but contains 10% FCS instead of 2% FCS (Laboratory Manual, 1994).

APPENDIX 5

Staining solutions.

Mountant

1 part paraffin wax was mixed with 3 parts vaseline and heated.

Once well mixed, the mountant was loaded into 5 ml syringes for use.

Fixative

Fixative solution was made by adding 1 part glacial acetic acid to 3 parts absolute ethanol.

Aceto-orcein stain

0.5 g orcein (Hopkin and Williams Ltd., London) was added to 30 ml MilliQ® water, heated briefly and stirred overnight. 20 ml glacial acetic acid was added and the solution filtered through a Whatman number 1 (7 cm diameter), qualitative filter.

APPENDIX 6

Solutions used in abnormal sperm morphology assessments.

Dulbecco's phosphate buffered saline

9.85 g Dulbecco's phosphate buffered saline powder (Highveld Biological, SA) was dissolved in one litre of MilliQ® water and 0.5 ml Genta 50 (Phenix SA, Randburg) added.

Phosphate buffered saline (PBS)

Reagent	Source	Amount required
$NaH_2PO_4H_2O$ - 8 g/1000 ml dH_2O	Sigma S-9638	30 ml
Na_2HPO_4 - 9.47 g/1000 ml dH_2O	Sigma S-5136	70 ml
NaCl	Saarchem, Krugersdorp	0.45 g

0.2% gluteraldehyde

0.8 g gluteraldehyde (25% w/w solution) was weighed and added to 100 ml PBS solution (Barth and Oko, 1989).

APPENDIX 7

Abnormal morphology evaluation.

Date:	Bull:

MAJOR DEFECTS	Total
1. Teratoid	
2. Knobbed Acrosomes	
3. Pyriform Heads	
4. Nuclear Vacuoles	
5. Folded Head	
6. Macrocephalic	
7. Microcephalic	
8. Abnormal Loose Head	
9. Double Forms	
10. Degenerative Heads	
11. Corkskrew Head	
12. Stump Tail	
13. Midpiece Reflex	
14. Other Midpiece Defects	
15. Dags	
16. Broken Tails	
17. Proximal Droplet	
18. Pseudo Droplet	
MINOR DEFECTS	
19. Loose Normal Heads	
20. Degenerative/Loose Acrosomes	
21. Abaxial Implantation	
22. Single Absent Mitochondria	
23. Curved Headpiece/Endpiece	
24. Distal Droplet	

	% Cells	Total
Major defects		
Minor defects		
Normal		