A STUDY ON THE USE OF UNFROZEN, DILUTED SEMEN FOR THE IN VITRO FERTILISATION OF BOVINE OOCYTES MATURED *IN VITRO*

ANGELA DAWN SEATON

1991

Massey University Library
Thesis Copyright Form

Title of thesis: A STUDY ON THE USE OF UNFROZEN, DILUTED SEMEN FOR THE IN VITRO FERTILISATION OF BOVINE OOCYTES MATURED IN VITRO

(1) (a) I give permission for my thesis to be made available to readers in Massey University Library under conditions determined by the Librarian.

~~(b) I do not wish my thesis to be made available to readers without my written consent for ... months.~~

(2) (a) I agree that my thesis, or a copy, may be sent to another institution under conditions determined by the Librarian.

~~(b) I do not wish my thesis, or a copy, to be sent to another institution without my written consent for ... months.~~

(3) (a) I agree that my thesis may be copied for Library use.

~~(b) I do not wish my thesis to be copied for Library use for ... months.~~

Signed Angela Dawn Seaton
per M. L. McDonald,
Supervisor,
Massey University

Date 8/6/92.

The copyright of this thesis belongs to the author. Readers must sign their name in the space below to show that they recognise this. They are asked to add their permanent address.

NAME AND ADDRESS DATE

A STUDY ON THE USE OF UNFROZEN, DILUTED SEMEN FOR THE IN VITRO FERTILISATION OF BOVINE OOCYTES MATURED *IN VITRO*

A thesis presented in partial fulfilment
of the requirements for the degree of
Master of Agriculture Science
in Animal Science
at Massey University

ANGELA DAWN SEATON

1991

ACKNOWLEDGEMENTS

I gratefully acknowledge the assistance of the following people and organisations:

- My supervisors, Assistant Professor Maurice McDonald (Department of Animal Science, Massey University) and Dr Bob Welch (Grasslands Division, DSIR), for their time, advice and encouragement.
- Staff at Grasslands DSIR, particularly Adrian King and Drs Michael Gurnsey and Lora Hagemann, for many services rendered.
- Staff of the Animal Science Department and my fellow postgraduate students, particularly Ken Greaney, for their support and advice.
- Dr Dorian Garrick (Department of Animal Science) and Bob Fletcher (Division of Information Science, DSIR) for help with statistical analyses - and their patience!
- Livestock Improvement Corporation for generously donating semen and Caprogen. I would also like to thank Dr Pat Shannon and Earle Newey for their interest and advice.
- Management and staff of Waitaki Freezing Co., Aorangi plant, Feilding.
- Grasslands Division, DSIR, for considerable financial support and for supplying research facilities.
- Massey University for financial support through J. A. Anderson and Helen E. Akers scholarships.
- Very special thanks to co-workers and friends, Sally Rhodes and Dr Jim Catt, without the moral support, advice and encouragement of whom this thesis would not have existed.
- Last but not least, my parents, who gave me my very first experience of embryology...

ABSTRACT

The study investigated the use of unfrozen, diluted semen for in vitro fertilisation of bovine oocytes matured *in vitro*. In experiment 1, semen from each of two bulls was used on two consecutive days ("day-old" and "two-day-old" sperm) to explore the effect of sperm concentration on oocyte penetration rates. The sperm concentrations used were 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0×10^6/ml. Penetration rates were uniformly high when day-old sperm was used, but low penetration rates were obtained below 1×10^6/ml with two-day-old sperm. Unfrozen sperm appeared to give better penetration rates than frozen-thawed sperm at concentrations of 0.5-2.0×10^6/ml. There was no relationship between sperm concentration and incidence of polyspermy. In experiment 2, sperm from the same two bulls were used to investigate the presence of Caprogen extender in the fertilisation medium. Caprogen inhibited penetration when present in concentrations greater than 10ml/litre. Experiments 3 and 4 studied the effect of heparin on penetration rates. In experiment 3, sperm from one bull was used to inseminate oocytes in medium containing 0, 1, 5, 10, 20, 30 or 50µg/ml heparin. There was no relationship between penetration rates and heparin concentrations in the medium, and average penetration rates were high for all concentrations. In experiment 4, five bulls were used to investigate penetration rates at heparin levels of 0µg/ml (frozen-thawed and unfrozen sperm) and 10µg/ml (unfrozen sperm). The results obtained in experiment 3 with sperm from one bull were confirmed; penetration was obtained in the absence of heparin with all five bulls.

Good penetration can be obtained *in vitro* with unfrozen sperm, and its greater longevity and viability make it a useful alternative to frozen semen for both commercial and research in vitro fertilisation programmes.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

* Figures on pages following those indicated.

LIST OF ABBREVIATIONS.

AR	acrosome reaction
BO	Brackett's-Oliphant's medium
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
COC	cumulus-oocyte complex
DNA	deoxyribonucleic acid
FCS	foetal calf serum
FSH	follicle stimulating hormone
GAG	glycosaminoglycan
hCG	human chorionic gonadotrophin
HIS	high ionic strength (medium)
IVC	in vitro culture
IVF	in vitro fertilisation
IVM	in vitro maturation
LIC	Livestock Improvement Corporation
LH	luteinising hormone
m199	medium 199
OCS	oestrous cow serum
PBS	phosphate-buffered saline solution
PHE	penicillamine, hypotaurine, epinephrine
SAS	Statistical Analysis Systems
TALP	Tyrode's medium with acetate, lactate, pyruvate

CHAPTER 1. INTRODUCTION.

The in vitro maturation (IVM) and in vitro fertilisation (IVF) of mammalian oocytes and the in vitro culture (IVC) of mammalian embryos have been studied for several decades. However, development of IVM/IVF/IVC techniques in cattle has been slow: it wasn't until 1965 that IVM of bovine oocytes was achieved, and the birth of calves derived from embryos produced wholly *in vitro* was not reported until 1988. This delay in progress relative to other (laboratory) species is due in part to the large size of cattle, which makes them difficult and expensive subjects for research, and in part to the inherent difficulty involved in inducing bovine oocytes to successfully mature, fertilise, and develop *in vitro*.

Bovine oocytes and embryos produced *in vitro* have several advantages over their in vivo-produced counterparts. In vitro oocytes are relatively inexpensive and easily obtainable in large quantities from waste slaughterhouse ovaries. They also represent a source of very early embryos which cannot be harvested or studied *in vivo* without surgery. The developmental stages of in vitro-produced oocytes/embryos are known with more precision since time of insemination is more easily determined *in vitro* than *in vivo*, and subsequent cleavage stages can be ascertained rapidly using a dissecting microscope.

The practical applications of the in vitro technology are now being realised. It can be used in conjunction with embryo transfer to produce twin pregnancies in dairy or beef cattle to increase production. Other possibilities include the production of large numbers of half- or full-sib offspring simultaneously (for sire-testing purposes) or the production of high quality single calves in average cows. Rapid breed conversions (eg from dairy to beef) could also be effected by producing 1/2- or 3/4-bred embryos. Other practical uses include the treatment of certain types of infertility in females, and reproductive salvage - the removal of oocytes from a valuable animal that may be dying or unable to carry young. Another possibility is the use of the in vitro system to test the spermatozoa of prospective sires for in vivo fertility, although some reports suggest that the correlation between in vivo and in vitro fertility may be poor.

In research, in vitro-produced oocytes and embryos are being used for detailed studies on oocytes and gamete interactions. Early embryonic development can be undertaken and explored in the controlled environment of the laboratory, and variables that may influence fertilisation can be investigated without interference from maternal factors. Other uses for in vitro-produced embryos concern the following emerging technologies:

1. Cloning (the production of many individuals that possess identical genetic material) involves the fusion of blastomeres from 8-16 cell embryos with mature, unfertilised, enucleated oocytes. Both of these can be provided by IVM/IVF/IVC.

2. Procedures for the sexing of sperm can have their efficacy determined quickly with oocytes that have been produced *in vitro*. Sperm of a specific sex are used to inseminate oocytes which are then cultured to a stage when cells can be removed and tested for the presence of DNA associated with the Y chromosome.

3. One form of genetic engineering is the production of transgenic animals that possess some new and valuable trait. Transgenesis (the insertion of foreign DNA into an animal's genome) in the bovine is currently restricted to pronuclear injection. This technique requires 24-27 hour-old zygotes. Since survival after gene injection is low and the frequency of successful gene integration is even lower, the technique also requires large numbers of zygotes. The IVM/IVF system is capable of providing embryos at the desired stage of development and in the desired quantity.

While IVM/IVF/IVC techniques offer great potential, further refinements to every stage of the procedure are still required. Oocyte maturation is possibly the least understood part of the procedure, and the most important since its success is vital to the success of the subsequent steps. While many of the factors that affect maturation have been determined, there is still a lack of information concerning the mechanisms that control various aspects of the process, particularly cytoplasmic maturation. The development of improved methods of fertilisation and culture and the extension of knowledge in these areas are ongoing tasks in several countries. Sperm capacitation, oocyte fertilisation and embryo culture can now be successfully performed in the laboratory. However, the procedures used are constantly under review and, like maturation, the underlying mechanisms that effect these events are not known.

CHAPTER 2. FACTORS AFFECTING THE SUCCESS OF IVM/IVF/IVC PROCEDURES.

This section reviews some of the work that has been carried out in the areas of in vitro maturation, fertilisation and culture of ruminant oocytes. The review is concerned mainly with cattle, although some references pertain to other species.

2.1. COLLECTION OF GAMETES.

2.1.1.Oocytes.

Oocytes may be collected from either selected hormonally treated females or from unidentified slaughterhouse animals. The first method involves the superovulation of donor animals which are then slaughtered to obtain the reproductive tract (Bondioli and Wright, 1983; Crosby *et al.*, 1981). If it is preferable to keep the animal alive, surgery can be performed to either aspirate oocytes from follicles or to remove ovulated oocytes from the oviducts (Bondioli and Wright, 1980; Brackett *et al.*, 1982; Lambert *et al.*, 1986). Oocytes collected from the oviducts are mature enough to undergo fertilisation and further development, and therefore do not have to undergo a maturation procedure *in vitro*. In vivo matured oocytes tend to have a greater developmental capacity than those oocytes matured *in vitro* (Crosby *et al.*, 1981; Leibfried-Rutledge *et al.*, 1987). However, oocytes obtained after the hormonal treatment of animals are expensive and the number available per donor is relatively low (Gordon and Lu, 1990).

The second method is the aspiration or rupturing of follicles on ovaries collected at the slaughterhouse, generally from animals of unknown origin and reproductive history (Bondioli and Wright, 1980; Braun, 1988; Fukuda *et al.*, 1990; Fukui, 1989; Leibfried-Rutledge *et al.*, 1987). Large quantities of embryos can be produced very cheaply from slaughterhouse material (Gordon and Lu, 1990). Aspiration of follicles is achieved by puncturing follicles on and under the ovary surface using an hypodermic needle and collecting follicular fluid and oocytes in the attached syringe. Rupturing follicles involves the dissection of follicles out of the ovary, the tearing of the follicular walls with forceps and the subsequent liberation of follicular fluid and oocytes into a sterile dish.

Using aspiration, as many as 10 acceptable oocytes can be removed from an ovary relatively quickly, and for this reason it is now the most common method of oocyte collection (Gordon and Lu, 1990). Rupturing individual follicles can yield a higher number of morphologically normal oocytes, and a higher number of oocytes overall (Katska, 1984; Lonergan *et al.*, 1991). However, this method is time-consuming and therefore not as practical as aspiration (Gordon, 1990; Katska, 1984). The method of oocyte removal used does not appear to affect later cleavage rate (Gordon and Lu, 1990).

Some variables involved in oocyte collection may have an effect on oocyte quality and subsequent embryo production. Gordon and Lu (1990) reported that cow ovaries yielded smaller numbers of acceptable oocytes than ovaries removed from 2- or 3-year-old beef heifers. Katska and Smorag (1984) ascertained that older cows (9-17 years) had lower numbers of 2-6mm follicles than younger cows and heifers; however, they also found that there was no relationship between the number of ovarian follicles and the quality of the recovered oocytes. Greve *et al.* (1984) found that considerable between and within cow variation existed in oocyte morphology and subsequent maturation.

Follicle size (greater than 1mm) and stage of oestrous cycle do not affect the ability of bovine oocytes to undergo nuclear maturation *in vitro* (Fukui and Sakuma, 1980; Leibfried and First, 1979). Leibfried-Rutledge *et al.* (1985) suggested that neither follicle size (from 1-12mm) nor the stage of oestrous cycle at which the oocytes were collected affected the frequency of fertilisation or the ability to form male pronuclei. However, oocytes removed from very small follicles (less than 1mm in diameter) do not appear to be able to undergo spontaneous nuclear maturation (Leibfried-Rutledge *et al.*, 1989), and more recent work indicates that oocytes recovered from follicles below 2mm in diameter yield fewer embryos after in vitro maturation than those oocytes from larger follicles (Tan and Lu, 1990).

Sheep oocyte maturation and fertilisation rates are greatly influenced by the temperature at which ovaries are held and the interval between animal death and removal of oocytes from the ovaries. Moodie and Graham (1989) held sheep ovaries at 5, 22 and 37°C for 4, 8 or 24 hours: they found that 22°C gave the highest maturation rates, and that recovery should occur immediately after animal slaughter. In contrast, Yang *et al.* (1990) stored cattle ovaries at 24-25°C for at least 11 hours without a reduction in either the fertilisation rate of oocytes or the capacity of early embryos to develop into blastocysts. Shea (1976) flushed follicles from cattle ovaries 1 or 2 hours after animal death and found no difference in maturation rates. However, Shioya *et al.* (1988b) obtained higher rates of cleavage and development to blastocyst when oocytes were aspirated within 43 minutes of death (average time 17 minutes) than when they were aspirated within 309 minutes (average time 162 minutes).

2.1.2.Spermatozoa.

Little is known of the possible effects of sperm collection variables on in vitro fertilisation rates. Critser *et al.* (1984) found that the frequency of fertilisation was higher when ejaculated bull semen was extended 1:1 prior to transportation rather than transported as undiluted semen, although the proportions of normally fertilised oocytes did not differ between treatment groups.

Sperm of epididymal and ejaculated origin appear to have differences in fertilising ability: Pavlok *et al.* (1988) obtained a higher fertilisation frequency (with a corresponding higher rate of fertilisation abnormalities) with the former. Whilst Goto *et al.* (1989) reported that no significant variation in fertilisation frequencies was apparent among sires when epididymal sperm were used, many researchers have noted extensive variation with ejaculated sperm (Leibfried-Rutledge *et al.*, 1989).

Several workers have tested frozen-thawed or unfrozen semen from bulls and found that penetration rates and subsequent embryo development differed among individuals (Hillery *et al.*, 1990; Ohgoda *et al.*, 1988; Parrish *et al.*, 1986; Shi *et al.*, 1990). Fukui *et al.* (1988) found similar trends existed with rams: they suggested that the observed differences may be attributable to variations in either composition of the seminal plasma or in the ratio between seminal plasma volume and sperm number. Conversely, Miller and Hunter (1987) showed that variation among bulls was not significant because variation among replicates was high. Interestingly, very few of the above workers mentioned the significance or otherwise of variation among replicates involved in their experiments: only Parrish *et al.* (1986) reported it as being negligible.

2.2. OOCYTE MATURATION.

Oocytes in secondary follicles are arrested at the dictyate (germinal vesicle) stage of nuclear development. In vivo, advancement through the prophase, anaphase and telophase stages to metaphase I and metaphase II is stimulated by the preovulatory surge of luteinising hormone. The oocyte must proceed to the metaphase II stage in order to develop properly after being fertilised by sperm. Oocytes removed from preovulatory follicles must therefore undergo a period of maturation *in vitro*.

Maturation has been defined as those events that render the oocyte capable of fertilisation and able to initiate the programme that directs preimplantation embryonic development (Leibfried-Rutledge *et al.*, 1987). Nuclear maturation is often used as a gauge of oocyte maturity. However, there is much more to maturation than nuclear development. For example, the development of regulatory signals for meiotic events, sperm decondensation, initiation of cleavage, completion of cleavage stages and embryo development probably occurs during oocyte maturation (First and Parrish, 1987). Many researchers speculate that some or all of these signals are dependent on appropriate cytoplasmic maturation, a phenomenon which is at present impossible to quantify because of our lack of knowledge concerning the events involved. Inadequate cytoplasmic maturation is considered to be the most likely cause of developmental problems and later failure of the embryo (Leibfried-Rutledge *et al.*, 1987).

Oocyte quality and maturation culture conditions have been identified as important factors which govern the success of IVM. Some of the research pertaining to these areas is summarised below.

2.2.1.Oocyte Quality.

Oocyte quality can be determined visually by noting the presence/absence of surrounding cellular investments, the relative size of the oocytes, and the appearance of the cytoplasm. Oocytes lacking good cumulus and corona cell layers have a reduced incidence of maturation, fertilisation, and development to blastocyst (Shioya *et al.*, 1988a; Sirard *et al.*, 1988, Yang and Lu, 1990).

Association with intact cumulus-oocyte complexes (COCs) can help corona-enclosed (but not completely nude) oocytes to complete maturation, indicating that corona cells may be necessary to mediate the influence of the cumulus cells (Critser *et al.*, 1986b). Shioya *et al.* (1988a) suggested that nude oocytes probably fail to mature, not because they do not possess cumulus cells, but because they are often already degenerate before beginning maturation culture. In one of their experiments, 62% of nude oocytes had germinal vesicles with indistinct borders, a sign of degeneracy. While degeneracy is a likely contributing factor, Crosby *et al.* (1981) presented strong evidence for the necessity of intact cumulus cells during maturation - deliberately denuded sheep oocytes did not develop into blastocysts, compared to a 30% success rate with intact COCs. Their work showed that cumulus cells must be present continuously during maturation for later developmental competence, an idea supported by Gandolfi *et al.* (1990). The degree of cumulus expansion is often used as a gauge of maturation, since progression in nuclear development is paralleled by progression of cumulus expansion (Leibfried and First, 1979).

Oocytes that are noticeably smaller than others are likely to have come from very small follicles, and therefore are usually too immature to undergo spontaneous maturation *in vitro* (Leibfried-Rutledge *et al.*, 1989). Oocytes with a dark or granulated cytoplasm (often accompanied by dark or clumped cumulus cells) are usually degenerate (Leibfried and First, 1979; Marion *et al.*, 1968).

2.2.2.Maturation Conditions.

A)*Basic Media.* A wide variety of media has been used for in vitro oocyte maturation. Simple balanced saline solutions (Fukui *et al.*, 1981; Leibfried-Rutledge *et al.*, 1987) and follicular fluid obtained from sheep, cattle or pigs (Leibfried and First, 1979; Quirke and Gordon, 1971; Sreenan, 1970) have been used. These simple media allowed meiosis to resume, but further development (such as male pronuclear formation) was not facilitated (Leibfried-Rutledge *et al.*, 1989). The utilisation of more complex, defined media can overcome this problem to some extent (Leibfried-Rutledge *et al.*, 1989). There are now several complex media in use by various laboratories, but as yet no one satisfactory, completely defined medium has been found for ruminant oocyte culture (First and Parrish, 1987). The most popular choices for medium bases are m199, TALP, and Ham's F-10 or F-12.

Several researchers have carried out media comparisons and found no differences in maturation rates (Fukui *et al.*, 1982; Leibfried and First, 1980; Onuma and Foote, 1969). However, Fukui *et al.* (1982) reported that media may behave differently according to supplementation. When oestradiol and progesterone were added to four different media, Ham's F-12 and Kreb's gave better maturation and fewer degenerate oocytes than did m199 and Brinster's. The addition of luteinising hormone (LH) and human chorionic gonadotropin (hCG) had a significant effect in Ham's or Brinster's, but not in m199 or Kreb's.

B)*Protein Supplements.* First and Parrish (1987) claim that blood serum or serum extract must be present in the maturation medium for full maturational development and normal embryonic growth to occur. These protein supplements may be a source of fixed nitrogen for oocytes (Leibfried-Rutledge *et al.*, 1986), but they more likely act as carriers for growth factors and hormones (J. Catt, pers. comm.). Foetal calf serum (FCS), oestrous cow serum (OCS), and bovine serum albumin (BSA) are the most commonly used protein sources in bovine IVM. FCS appears to give better maturation than BSA in sheep (Braun, 1988) and in cattle (Leibfried-Rutledge *et al.*, 1986), with BSA failing to support cumulus expansion or to facilitate completion of nuclear maturation.

Sanbuissho and Threlfall (1990) obtained a significant increase in the percentage of oocytes matured when they added either FCS or OCS to Ham's F-10 medium. However, they reported earlier that protein supplements do not affect maturation rates, although their presence in the maturation media is essential for good post-maturational development (Sanbuissho and Threlfall, 1985). Another suggestion is that protein supplements in the maturation media are not necessary for normal blastocyst development and hatching if there are supplements in the culture medium (Chen-Lu and Lu, 1990). There is conflicting information as to which of the two sera is better; Fukui and Ono (1989) found that 20% FCS gave higher rates of penetration (and polyspermy) than did 20% OCS, whilst Schellander *et al.* (1990), using the same concentrations of sera, found that OCS gave better fertilisation and cleavage.

The concentration of sera in the maturation medium also tends to vary between laboratories, ranging in general between 5% and 20%. Kim *et al.* (1989) tested OCS at 10, 15 and 20% levels and concluded that the best fertilisation rate was achieved with 10%. FCS was tested by Leibfried-Rutledge *et al.* (1986) at levels ranging from 0.1% to 20%. They obtained good results at concentrations of 5% and 10%.

C)*Hormones.* Presumably hormones are added in an attempt to mimic the follicular environment. The most common hormones added to maturation media are LH, follicle stimulating hormone (FSH), and oestradiol. Others that have been utilised include progesterone (Fukui *et al.*, 1982) and hCG (Fukui *et al.*, 1982; Hensleigh and Hunter, 1985; Sanbuissho and Threlfall, 1990).

Research on the requirement for hormones in the maturation media has yielded conflicting results. A few laboratories have reported hormones to have a beneficial effect on maturation: Stubbings *et al.* (1988) found that oestradiol (but not FSH or LH) favoured oocyte maturation, and Fukui *et al.* (1982) noted that oestradiol (but not progesterone) enhanced maturation in their experiment. Fukushima and Fukui (1985) obtained enhanced in vitro maturation with the addition of LH and a combination of LH, oestradiol and progesterone. Hensleigh and Hunter (1985) reported that FSH gave better cumulus expansion than hCG, which in turn was superior to the control treatment of no hormones. Sirard *et al.* (1988) found that maturation was improved in the presence of hormones (FSH + LH + oestradiol), but fertilisation and later developmental competence (measured *in vivo*) was not affected by the presence or absence of hormones in the maturation media.

In contrast, Saeki *et al.* (1990) showed that the inclusion of oestradiol, FSH and LH in the maturation media improved fertilisation and cleavage without providing any increase in the proportion of oocytes matured. Fukui and Ono (1989) reported that FSH and LH had no positive effect on the frequencies of maturation to blastocyst formation, and a negative effect on cleavage rates. All of the studies utilised similar levels of hormones. It is possible that the results of Fukui and Ono (1989) and Sirard *et al.* (1988) may have been confounded by the inclusion of FCS in their maturation media, since this serum contains a certain amount of gonadotropins and steroids. Levels of oestradiol and progesterone vary widely between batches of serum but are generally lower than those normally incorporated in media (Stubbings *et al.*, 1989). However, the possible role of these "hidden" steroids in the maturation of oocytes *in vitro* should not be discounted (Stubbings *et al.*, 1989).

D)*Helper Cells*. The addition of "helper cells", normally granulosa cells, favours the acquisition of developmental competence in cattle (Critser *et al.*, 1986a) and sheep (Crosby *et al.*, 1984; Staigmiller and Moor, 1984). Critser *et al.* (1986a), Fukui and Ono (1989) and McGowan *et al.* (1990) all report enhanced cleavage and blastocyst development (but not maturation rates) when granulosa cells were added to the maturation medium. Fukui and Ono (1989) noted that granulosa cells improved fertilisation when added to the maturation media containing FCS compared to FCS alone, OCS alone, and FCS or OCS with added hormones. Granulosa cells are usually added to the medium at a concentration of $1x10^6$/ml, although concentrations of $3x10^6$/ml or greater have been used (Fukui and Ono, 1989; Lu *et al.*, 1987; Xu *et al.*, 1987).

E)Incubation Conditions. Factors that have been studied include the gas atmosphere in the incubator (Trounson *et al.*, 1977), the use of static versus agitator systems (Braun, 1988; Staigmiller and Moor, 1984), the use of paraffin oil to cover media and the numbers of oocytes per maturation dish (Sirard *et al.*, 1988). These have been found to have little or no effect on the success or otherwise of in vitro maturation.

Lenz *et al.* (1983a) matured bovine oocytes at temperatures ranging from 35 to 41°C. They found that temperatures of 35-39°C had no deleterious effects on resumption and completion of meiosis, but at 41°C the frequency of oocytes progressing to metaphase II significantly decreased. Oocytes matured at 39°C had significantly higher rates of fertilisation than those matured at 35, 37, or 41°C. Cooling sheep oocytes to 20°C at various stages of meiosis induced chromosomal abnormalities, with the most sensitive time being at germinal vesicle breakdown (Moor and Crosby, 1985).

Shea *et al.* (1976) investigated maturation of bovine oocytes in media at pHs ranging from 6.7 to 7.6. The highest maturation rate was achieved between 7.0 and 7.3.

2.3. CAPACITATION AND SPERM PRETREATMENT.

Spermatozoa, like follicular oocytes, are not immediately capable of fertilisation. In either in vivo or in vitro conditions they must first undergo capacitation, a process that provides sperm with the ability to undergo appropriate modification such that sperm-egg fusion can occur (Xu and King, 1990). This modification has come to be known as the acrosome reaction (AR). Capacitation and the AR are so intimately linked that they are generally considered as one process.

The concept of capacitation was first described independently by Austin (1951) and Chang (1951). Since then it has been the subject of many reviews, one of the most recent and comprehensive being that written by Sidhu and Guraya (1989). A review dealing specifically with ruminant capacitation and fertilisation was produced by First and Parrish (1987).

Capacitation apparently involves changes at a molecular level rather than at a morphological level, and it can be brought about by a variety of exogenous and endogenous stimuli (Sidhu and Guraya, 1989). The findings of numerous in vitro studies indicate that two important molecular alterations occur in sperm undergoing capacitation: sperm surface alteration and/or intramembranal molecular mobility to facilitate calcium ion influx, and changes in sperm energetics (via oxygen uptake and glucose utilisation) which are manifested in the form of changes in flagellar beat. These two alterations are not as yet well defined, and they may not be directly correlated with the actual acquisition of fertilising ability by sperm (Sidhu and Guraya, 1989).

However, fertility is correlated with the occurrence of the AR (Sidhu and Guraya, 1989). The AR involves point fusions between the outer acrosomal membrane and the overlying plasmalemma. These fusions allow the release of acrosomal contents (hydrolytic enzymes) that are probably required for penetration of sperm through egg investments (Bedford, 1983). As well as membrane fusions, the AR appears to involve modification of the equatorial or postnuclear cap for attachment to the egg membrane (Sidhu and Guraya, 1989).

Although considerable information has been accumulated concerning the physical and biochemical changes associated with capacitation and the AR, very little is known about the molecular mechanisms that drive these events (Xu and King, 1990). Thus several methods have been developed to capacitate spermatozoa *in vitro* without a full understanding of how these methods achieve their purpose. In vivo, capacitation appears to be attributable to a sequential exposure of spermatozoa to fluids found in the female reproductive tract, with capacitation being completed in the oviduct and the AR occurring when the spermatozoa are in the immediate vicinity of the oocyte (Sidhu and Guraya, 1989).

In vitro, a number of substances and methods of capacitation have been utilised with varying degrees of success. These include pretreatment of sperm with a calcium ionophore (Ball *et al.*, 1983), lysophospholipids or trypsin (Wheeler and Seidel, 1989), heparin or other glycosaminoglycans (Handrow *et al.*, 1982) or high ionic strength medium (Brackett *et al.* 1980). Other workers have experimented with bovine follicular fluid (Fukui *et al.*, 1983), oviduct fluid (Parrish *et al.*, 1989b), and the oviducts or uteri of cows, gilts and rabbits (Iritani and Niwa, 1977; Iritani *et al.*, 1984). More recently some workers have been investigating the use of caffeine in conjunction with BSA (Goto *et al.*, 1988) or heparin (Niwa and Ohgoda, 1988; Park *et al.*, 1989). The rationale behind the utilisation of these various substances will be discussed in sections 2.3.2-2.3.7.

2.3.1.Measurement of capacitation/AR.

Since capacitation of sperm does not involve any discernable morphological changes, it is generally measured as a percentage of oocytes penetrated. There is an inherent difficulty associated with using oocyte fertilisation to evaluate the efficiency of capacitation, since capacitation is only one of several variables involved in fertilisation. A second method involves the exposure of sperm for a short period to substances that will induce the AR only in capacitated sperm (First and Parrish, 1987). The substances could be fusogenic lipids (such as lysophosphatidylcholine) or components of the cumulus-oocyte complex (such as the zona pellucida) (Parrish *et al.*, 1988). Sperm are then fixed with formaldehyde or glutaraldehyde, stained with Naphthol-Yellow S and Erythrosin B, and evaluated by interference optics (Ax and Lenz, 1987). Light microscopy can also be used to assess occurrence of the acrosome reaction (Lenz *et al.*, 1983b). The vast majority of research on bovine *in vitro* capacitation has utilised the first method.

2.3.2.Follicular Fluid, Oviduct Fluid, Female Reproductive Tracts.

In order to obtain conditions *in vitro* as similar as possible to an in vivo environment, early workers attempted capacitation in "natural" surroundings - using fluids from the reproductive organs, or the organs themselves. Iritani and Niwa (1977) capacitated bovine spermatozoa in a reproductive tract isolated from an oestrous cow, in the uteri of live oestrous rabbits, and in modified Kreb's medium in an incubator; rabbit uteri gave the best results, with the bovine tract proving to be superior to the medium. Iritani *et al.* (1984) used reproductive tracts isolated from cows and gilts as capacitatory environments for bovine spermatozoa. While gilt uteri gave better fertilisation rates, they also gave a higher incidence of polyspermy. Both reproductive tracts offered a more favourable capacitating environment than isotonic Kreb's medium.

Fukui *et al.* (1983) examined the possibilities of bovine follicular fluid as a capacitation medium, both alone and in conjunction with other media. Higher rates of fertilisation resulted with procedures involving follicular fluid than with other treatments tested (apart from high ionic strength medium), and the best result was achieved when bovine follicular fluid was used by itself. Parrish *et al.* (1989b) investigated the use of bovine oviduct fluid in TALP medium for capacitation, and they showed that oviduct fluid exhibited capacitating activity in a dose-dependent manner. Further studies designed to isolate the capacitating factor in oviduct fluid indicated that it is a heparin-like glycosaminoglycan (Parrish *et al.*, 1989b).

2.3.3.High Ionic Strength Medium.

Spermatozoa are coated with epididymal and accessory gland secretions during maturation in the epididymus, and also during ejaculation (Sidhu and Guraya, 1989). These coating secretions act as decapacitating factors, inhibiting or totally preventing the spermatozoa from fertilising the oocyte (Sidhu and Guraya, 1989). Evidence from other species suggests that part of capacitation is the removal of coating proteins contained in the seminal and epididymal secretions (Ahuja, 1984; Oliphant *et al.*, 1985). The use of high ionic strength (HIS) medium for capacitation of bovine spermatozoa became relatively common after work with mice (Oliphant and Brackett, 1973a) and rabbits (Brackett and Oliphant, 1975; Oliphant and Brackett, 1973b) indicated that treatment with HIS medium facilitated the removal of inhibitory factors from the sperm surface. Brackett *et al.* (1982) achieved positive results with HIS medium in cattle, although they and others (Sirard and Lambert, 1985) discovered a variability in response to the treatment among bulls. However, Bondioli and Wright (1983) found that bovine spermatozoa pretreated with standard or HIS media were equally capacitated, indicating that the changes occurring during capacitation in their system were not related to any change induced by the HIS medium. Iritani *et al.* (1984) also found that a HIS medium was unnecessary under their conditions; they achieved up to 58% fertilisation with ejaculated bovine spermatozoa using an isotonic medium (290 mosmol/kg).

2.3.4.Calcium.

The primary role of capacitation may be to schedule the influx of calcium ions into the acrosome of the spermatozoa (Sidhu and Guraya, 1989). The AR in mammalian spermatozoa does not require any specific stimulus, but it does require an adequate level of calcium once capacitation has been achieved (Sidhu and Guraya, 1989).

The pretreatment of spermatozoa with calcium-containing media (Didion and Graves, 1989) or with calcium ionophore (Ball *et al.*, 1983; Fukuda *et al.*, 1990) has been used to bypass capacitation and successfully induce the AR. Using ionomycin, a potent calcium-specific ionophore, Ball *et al.* (1983) managed to induce the AR in 72% of bovine spermatozoa within 4 hours. However, the pretreatment proved not to be superior to the control treatment in terms of subsequent penetration rates. Fukuda *et al.* (1990) on the other hand have shown that sperm pretreatment with the ionophore A23187 gave much better penetration rates than their control treatment.

2.3.5.Lysophospholipids.

The role of membrane lipids in capacitation and the AR is not understood completely (Wheeler and Seidel, 1989). Langlais and Roberts (1985) suggested the following model: an unknown modification of the sperm surface causes an efflux of membrane cholesterol. This alters the membrane sterol/cholesterol ratio, resulting in an influx of calcium ions. The calcium activates phospholipase-A2 which catalyses the synthesis and accumulation of fusogenic lysophospholipids such as lysophosphatidylcholine. These fusogenic compounds could induce the AR in the presence of calcium ions and reduced levels of cholesterol by enhancing membrane fluidity.

Graham *et al.* (1987) demonstrated that zona-free hamster oocytes were penetrated more frequently when spermatozoa from the bull, ram, or stallion were treated with phosphatidyl-containing liposomes. Wheeler and Seidel (1989) tested four lysophospholipids and concluded that these substances can rapidly induce capacitation and the AR in bovine spermatozoa. The type of lysophospholipid tested, the dose rate, and the method of administration (long-term, short-term, or interrupted exposure) affected the penetration rates of dead oocytes in their experiment.

2.3.6.Heparin.

The capacitating substance in oviduct fluid is believed to be a heparin-like glycosaminoglycan (GAG) (First and Parrish, 1987). Glycosaminoglycans are unbranched disaccharide polymers which are covalently bonded to a protein core (Ax and Lenz, 1985). They are found on cell surfaces and play roles in cellular adhesion, proliferation, migration and differentiation. Glycosaminoglycans can also assist in cell to cell communication and topographically obstruct receptors to regulate availability of substrates and growth factors (Ax and Lenz, 1987). Handrow *et al.* (1982) have indicated that the active component of GAGs to promote an AR resides in the amino sugar of the sulphate residue, and that the potency of any particular GAG is partially due to its degree of sulphation. Heparin is one of the more highly sulphated of the GAGs that has been investigated, and it is the best stimulator of capacitation or AR in bovine sperm *in vitro* (Handrow *et al.*, 1982).

Capacitation (or the induction of the AR) with heparin or other GAGs has been experimented with and achieved *in vitro* by many workers, particularly Parrish *et al.* (1985, 1986, 1988, 1989a, 1989b). So far, the mechanism by which heparin and other GAGs effect capacitation or the AR is unknown. In humans (Delgado *et al.*, 1976) and cattle (Handrow *et al.*, 1984) heparin has been shown to bind to sperm in a pH, calcium and temperature dependent fashion, a situation suggestive of a receptor-ligand interaction (Miller *et al.*, 1990). In the act of binding, heparin and other GAGs may displace decapacitating proteins from the sperm surface (First and Parrish, 1987). Alternatively they could have a more direct effect on membrane domain formation - a network of GAG molecules and membrane components could induce reorganisation of the membrane by restricting or causing movement of membrane components (First and Parrish, 1987). Meizel (1985) hypothesised that GAGs could stimulate sperm surface enzymes required for the AR.

A fourth possibility involves calcium uptake, which is enhanced in the presence of heparin and heparin-like GAGs. The increased uptake could be related to the membrane changes outlined above, or heparin may act more directly as an ionophore, opening up a calcium channel in the sperm membrane (First and Parrish, 1987; Ax and Lenz, 1987). GAGs could also work by activating phospholipase-A2 (First and Parrish, 1987), the possible importance of which has already been discussed. Activation of a cAMP-dependent protein kinase is also a possibility (Susko-Parrish *et al.*, 1985). It is unlikely that GAGs bring about capacitation or the AR by means of a single, simple effect.

Dose levels and time of exposure of sperm to heparin vary between laboratories. Parrish *et al.* (1986) and Fukui and Ono (1989) incubated frozen-thawed sperm for 15 minutes before insemination, in media containing 20µg/ml and 100µg/ml of heparin respectively. Ejaculated sperm were incubated for four hours in media containing 10µg/ml heparin by Florman and First (1988). This combination also gave the best penetration rate in an experiment carried out by Parrish *et al.* (1988). Parrish *et al.* (1986) found that ejaculated sperm incubated for 6 hours in media containing 10µg/ml heparin gave better penetration rates than those incubated for less time. Miller *et al.* reported that ejaculated and epididymal sperm required 9 and 22 hours of incubation respectively with GAGs to undergo acrosomal exocytosis in the absence of zona pellucida extracts. Handrow *et al.* (1982) cultured epididymal sperm for 22 hours with various GAGs at concentrations of up to 100µg/ml. Heparin at a concentration of 10µg/ml gave the highest number of acrosome-reacted sperm. Fukui et al. (1990) incubated frozen-thawed sperm in media containing heparin at concentrations of 0 to 200µg/ml for periods of 0 to 240 minutes. Heparin was carried over into the insemination droplets at concentrations ranging from 0.3 to 12µg/ml, depending on the heparin levels in the original incubation medium. They found that heparin levels of 25, 50 and 100μg/ml and incubation times of 5, 15, 30 and 45 minutes gave the best penetration.

Some workers omit a preincubation procedure by mixing sperm and oocytes in an heparinised fertilisation medium (Hillery *et al.*, 1990; Leibfried-Rutledge *et al.*, 1986; Sirard *et al.*, 1988). Niwa and Ohgoda (1988) coincubated sperm and oocytes for 20-24 hours in media containing 10μg/ml heparin to obtain a penetration rate of 35%. The addition of caffeine to their media increased penetration to 68%.

2.3.7.Caffeine.

Caffeine increases and maintains respiration and motility of bovine ejaculated (Garbers *et al.*, 1971a) and epididymal (Garbers *et al.*, 1971b) spermatozoa. Treatment of sperm with caffeine has also resulted in accelerated oocyte penetration *in vitro* in mice (Fraser, 1979). While caffeine has been conclusively linked to increased sperm motility, and it appears that nonmotility may be correlated with nonfertility (Nelson, 1967), it is yet to be proven that caffeine increases fertilisation (through induction of capacitation) *per se.*

Caffeine acts as a cyclic nucleotide phosphodiesterase inhibitor and affects the intracellular level of cyclic adenosine monophosphate (cAMP) by inhibiting its enzymatic breakdown (Niwa and Ohgoda, 1988). There is abundant evidence from several species that shows there is a definite relationship between capacitation and endogenous levels of cAMP (Sidhu and Guraya, 1989). High concentrations of intracellular cAMP are thought to act by controlling calcium ion exchange across the plasma membrane via a cAMP plasma membrane pump (Bird *et al.*, 1989). Administration of exogenous cAMP has improved capacitation in rat and rabbit spermatozoa (Sidhu and Guraya, 1989). However, intact sperm cells have limited permeability to cAMP (Sidhu and Guraya, 1989), so other methods of elevating intracellular cAMP levels are required - hence the use of phosphodiesterase inhibitors like caffeine.

Results in the bovine have been variable. Critser *et al.* (1984) treated sperm with cAMP, caffeine or both for 1.5-2 hours prior to insemination of oocytes with no observed advantages (in terms of frequency of ARs or fertilisation) over the control treatment. Both caffeine and cAMP were used at a concentration of 1mM, which may have been too low - other workers have achieved good results with 2mM (Catt *et al.*, 1990) and 5mM caffeine (Catt *et al.*, 1990; Goto *et al.*, 1988; Niwa and Ohgoda, 1988; Ohgoda *et al.*, 1988). Caffeine may act synergistically with heparin to produce higher penetration rates than either of the two alone (Catt *et al.*, 1990; Niwa and Ohgoda, 1988; Park *et al.*, 1989).

Response to caffeine varies among bulls. Garbers *et al.* (1971a) observed an immediate stimulation of sperm motility by caffeine in low motility sperm samples but not in those of higher motility. Ohgoda *et al.* (1988) and Niwa and Ohgoda (1988) both reported great variations in penetration rates for different bulls, but it is not clear how much of this was attributable to a caffeine effect as opposed to individual variations in fertilising ability.

2.4. FERTILISATION.

Sperm capacitation and oocyte maturation are possibly the two most important factors involved in IVF. If either of these processes fails, fertilisation will not occur. In the late 1970's and early 1980's, very little was known about maturation and capacitation. Consequently, rates of penetration and successful development to blastocyst of ruminant oocytes *in vitro* were relatively low. Wright and Bondioli (1981) present a good review of these earlier experiments. As maturation and capacitation systems have become more efficient, however, the success rate of IVF has improved to the point where penetration rates of 80% or better are routine for in vitro matured oocytes (First and Parrish, 1987).

Fertilisation *in vitro* is affected by many variables. For example, penetration rates can be affected by media constituents and incubator conditions. In addition, variables such as the individual sire, sperm treatment, and oocyte quality are important. Factors such as the inclusion of "helper" cells in culture and oocyte quality have been discussed in sections 2.2.1 and 2.2.2.

2.4.1.Media.

As with maturation, each IVF laboratory has its own successful "recipe" for fertilisation media. Fertilisation media generally consist of a modified medium base (for example, Tyrode's or BO) and various additives such as serum albumin and salts. Depending on the system, sperm stimulating or capacitating factors such as PHE (penicillamine, hypotaurine, epinephrine - Gordon and Lu, 1990; Parrish *et al.*, 1988), caffeine (Critser *et al.*, 1984; Fukuda *et al.*, 1990; Goto *et al.*, 1989) or heparin (Hillery *et al.*, 1990; Kim *et al.*, 1989; Sirard *et al.*, 1988) may also be included.

2.4.2.Incubator Conditions.

Generally bovine and ovine IVF is carried out at 39°C in a gas atmosphere of 5% CO_2 in moist air, the same conditions used in oocyte maturation. The temperature of 39°C is a logical choice since it is the deep body temperature of the cow and ewe. Lenz *et al.* (1983a) demonstrated that sperm penetration of cattle oocytes *in vitro* was highest at this temperature. Spermatozoa and oocytes are usually placed together in 50-100ul microdroplets of medium under paraffin oil.

Duration of incubation of oocytes with sperm has varied between 4 hours (Florman and First, 1988; Parrish *et al.*, 1988) and 48 hours (Ball *et al.*, 1983), with a period of 18-24 hours being commonly used. The duration of coincubation is determined by personal laboratory preference and whether or not sperm are pre-capacitated.

2.4.3.Sperm Concentration.

Sperm handling, sperm capacitation, and sperm concentration in the fertilisation environment can all affect the success of in vitro fertilisation.

The number of sperm present in the fertilisation environment has little effect on penetration rates unless the number is very low or capacitation is very poor. However, it can greatly affect further development through the incidence of polyspermy. Assuming an efficient capacitating system is utilised, a high concentration of sperm will lead to the oocyte being surrounded by large numbers of sperm that are capable of fertilisation, thus increasing the probability of several spermatozoa penetrating the zona pellucida and the perivitelline membrane (First and Parrish, 1987). The incidence of polyspermy is greater *in vitro* than *in vivo*; in vitro matured oocytes appear to have an impaired polyspermic blocking mechanism. Polyspermic oocytes are incapable of normal development to blastocyst (First and Parrish, 1987). However, most fertilisation systems generate a low incidence of polyspermy - less than 15% of penetrated oocytes (First and Parrish, 1987).

Ling and Lu (1990) investigated the effect of various sperm numbers on penetration and blastocyst development in cattle. They found that sperm concentrations between 0.64 x 10^6/ml and 4.8 x 10^6/ml gave similar penetration rates (82-85%), but that development to blastocyst tended to decrease at concentrations higher than 1.6 x 10^6/ml. Frozen-thawed sperm is generally used at a concentration of 1-1.5 x 10^6/ml (Ling and Lu, 1990).

2.4.4.Sire Effects.

Sire effects were briefly discussed in section 2.1.2., where it was mentioned that many workers had noted that penetration rates appeared to vary among individual sires. It is known that ejaculated semen from different bulls possesses different sperm characteristics. At the artificial breeding centres of the Livestock Improvement Corporation (LIC), sires are routinely tested for sperm motility, longevity, sperm concentration and percentage live cells. These characteristics differ among and within bulls. These differences may or may not be reflected in subsequent penetration rates. In order to reduce as far as possible the anticipated effects of variation attributable to individual sires and ejaculates, a small sample of each ejaculate can be tested for penetration and further development (Leibfried-Rutledge *et al.*, 1989). In this way the optimum treatment for the remaining sperm in that ejaculate can be determined. Optimisation may involve the addition of PHE or caffeine to stimulate sperm motility, or the reduction of sperm numbers involved in fertilisation if a high level of polyspermy is encountered in the test sample.

2.5. POST-FERTILISATION CULTURE.

IVF can be utilised to test sperm for likely in vivo fertility, to test the viability of in vitro-matured oocytes, or to examine more closely in vitro fertilisation or capacitation. However, the prime commercial reason for performing in vitro fertilisation is to produce embryos.

Post-fertilisation embryo culture can be either *in vivo* or *in vitro*. The in vivo method uses the reproductive tracts of live recipient females as temporary incubators, and until recently this was the only successful procedure. Rabbits, cattle and sheep have been used as culture environments for bovine oocytes. Workers who have utilised this method of culture include Lu *et al.* (1987), Sirard *et al.* (1985) and Sreenan *et al.* (1968).

The second method is the in vitro co-culture of embryos on monolayers of oviduct epithelial cells, trophoblastic vesicles, fibroblasts, granulosa cells or cumulus cells. Medium conditioned with oviduct epithelial cells has also been used (Eyestone and First, 1989; Hillery *et al.*, 1990). In vitro co-culture has gained popularity as results have improved, especially since the use of intermediate hosts such as sheep or rabbits is undesirable and costly if embryos are to be produced on a commercial scale (Gordon and Lu, 1990).

Lambert *et al.* (1986) showed that in vivo incubation of bovine embryos (matured *in vivo* and fertilised *in vitro*) in rabbit oviducts was superior to in vitro culture in a defined medium. However, they did not use co-culture with "helper" cells. In contrast, Ellington *et al.* (1990) used 1- or 2-cell embryos obtained from superovulated cows to compare development in rabbit oviducts with that in an oviduct epithelial cell co-culture system. The in vitro system proved equivalent to the rabbit oviducts in promoting embryo development for all characteristics measured, including pregnancy rates. Using bovine embryos produced *in vitro*, Fukui *et al.* (1989) made a comprehensive comparison of in vivo systems (rabbit or sheep oviducts) with in vitro systems that used either co-culture (granulosa cells or oviduct epithelial cells) or media alone. Results from their work are shown in Table 1. Selected results from other trials involving post-fertilisation culture are shown in Table 2.

TABLE 1. Developmental capacity of bovine oocytes matured and fertilised in vitro. (From Fukui *et al.*, 1989).

culture conditions	oocytes transferred or cultured	oocytes recovered or examined (%)*	development to morulae/ blastocysts (%)†
sheep oviduct	139	49 (35)	8 (16)
rabbit oviduct	379	258 (68)	58 (23)
IVF media	319	319 (100)	5 (2)
BPM/ granulosa cells	253	253 (100)	1 (0.4)
m199/oviduct cells	361	361 (100)	44 (12.2)

* oocytes recovered or examined/oocytes transferred or cultured

† oocytes developed to morulae or blastocysts/oocytes recovered or examined

TABLE 2. Conditions and success of various bovine embryo culture systems.

References	Maturation & Fertilisation		Embryo Culture			Selected Results devt to blastocyst*(%)	Culture Conditions
	in vivo	in vitro	in vivo	media	coculture		
Berg & Brem 1990		x			x	51/60 (32)	granulosa cells
Boccart *et al.* 1990		x		x		2/50 (4)	m199
		x		x		18/99 (18)	oviduct epithelial cells
Catt *et al.* 1991		x		x		29/173 (17)	m199
		x			x	41/200 (20)	granulosa cells
		x			x	34/180 (19)	oviduct epithelial cells
Critser *et al.* 1986a		x	x			8/22 (36)	sheep oviducts
Ellington *et al.* 1990	x		x			4/29 (14)	ligated rabbit oviduct
	x				x	5/37 (14)	granulosa cells
Eyestone & First 1989	x			x		1/27 (4)	Ham's F-10
	x				x	38/82 (46)	oviduct epithelial cells
Fontes *et al.* 1991		x	x			33/190 (17)	rabbit oviduct
		x			x	38/219 (17)	oviduct epithelial cells
Goto *et al.* 1988		x			x	103/684 (15)	cumulus cells
Kim *et al.* 1990		x		x		0/122 (0)	m199
		x			x	14/138 (10)	oviduct epithelial cells
McLaughlin *et al.* 1990	x			x		31/41 (76)	synthetic oviduct fluid
Nakao & Nakatsuji 1990	x				x	23/224 (10)	trophoblastic vesicles
	x				x	2/149 (1)	fibroblast cells
Parrish *et al.* 1986		x	x			7/31 (23)	sheep oviducts
Shi *et al.* 1990	x				x	102/334 (31)	granulosa cells

* number of blastocysts/number of oocytes fertilised

2.6. PURPOSE AND SCOPE OF THE STUDY.

The purpose of this study was to explore the use of unfrozen, diluted semen in bovine IVF experiments. Unfrozen semen was focused on for two main reasons. Firstly, little is known of its role in IVF. When used *in vitro*, unfrozen semen is treated in a similar manner to frozen-thawed semen, implying that the two are assumed to have identical requirements and behaviour. However, evidence supporting this idea is scant. Only a few workers have made unfrozen semen the subject of their research (although many have employed it while investigating other aspects of IVM/IVF/IVC), and even fewer have made direct comparisons of unfrozen and frozen-thawed semen. Secondly, unfrozen semen is used for the majority of artificial insemination of dairy cattle in New Zealand, and therefore information concerning its behaviour *in vitro* could be valuable.

The effects of three fertilisation parameters on the penetration rate of bovine oocytes were investigated in three separate experiments. The factors studied were sperm concentration for fertilisation *in vitro*, and the presence of Caprogen extender and the concentration of heparin in the fertilisation medium.

Sperm concentrations are known to influence fertilisation rates and subsequent embryo development in IVF trials and in artificial insemination of dairy cattle. Use of inadequate sperm numbers results in poor penetration rates *in vitro* and *in vivo*, while excessive sperm numbers can give rise to high rates of polyspermy (and subsequently poor embryo development) *in vitro*. The first experiment in this study attempted to determine an optimal sperm concentration that would give high penetration rates with a low level of polyspermy. The effect of semen age on penetration rates was also investigated. Differences between penetration rates achieved with frozen-thawed and unfrozen sperm were compared.

Caprogen is the egg-citrate extender used with unfrozen semen by New Zealand's LIC. Since one of the main functions of semen extender is to prolong the life of sperm, it is generally accepted that extender is unlikely to be a suitable fertilisation medium. However, there have been few in vitro investigations of this concept, and it could prove advantageous if sperm did not have to be washed out of the extender prior to IVF. Possible benefits include time savings for laboratory staff and better penetration rates through increased sperm viability. The second experiment in this study attempted to ascertain whether penetration of oocytes could be obtained in the presence of Caprogen extender.

Heparin is a glycosaminoglycan that is known to facilitate the capacitation of frozen-thawed spermatozoa. It has been used at various concentrations ranging from 5 or 10μg/ml up to over 100μg/ml. The purpose of the third experiment was to determine whether unfrozen sperm were also capacitated by heparin, and if any particular concentration of heparin in the fertilisation media gave superior rates of oocyte penetration. A fourth experiment which used sperm from five bulls was later carried out to verify results obtained in the third experiment.

CHAPTER 3. MATERIALS AND METHODS.

Composition of media used in these experiments is shown in Appendix 1. The timing of operations involved in the experiments can be found in Appendix 2.

3.1. COLLECTION OF OVARIES.

Ovaries were collected from unidentified cattle at an abattoir approximately 20 minutes after slaughter. For transportation, the ovaries were placed in a dry thermos flask that had been preheated to 39°C with hot water. In the laboratory the ovaries were trimmed of excess fat and tissue and washed three times in 39°C phosphate-buffered saline solution (PBS) containing antibiotics. They were kept in the warmed PBS until processed. Trimmed ovaries are shown in Figure 1.

3.2. COLLECTION OF OOCYTES.

Follicles on and under the surface of the ovaries were aspirated using an 18-gauge needle attached to a 10ml syringe. At this stage no selection criteria regarding follicle size or appearance were employed, except for the exclusion of blood-filled follicles. The aspirated follicular fluid from 2 or 3 ovaries was expelled into a 6cm plastic petri dish and searched for oocytes using a dissecting microscope that was equipped with a warm stage. Oocytes were transferred into a 3cm plastic petri dish containing 2mls modified Hepes 199 medium (H199) supplemented with 10% foetal calf serum (FCS). Heparin (10µg) was added to prevent adhesion of the cumulus oocyte complexes (COCs) to each other and the dish. The dish was kept in a bench incubator at 39°C until all ovaries had been aspirated.

3.3. COLLECTION OF GRANULOSA CELLS.

Granulosa cells were used as "helper cells" in the oocyte maturation culture. A prominent vascular preovulatory follicle of 5-8mm in diameter was selected and dissected out of the ovary. Excess tissue was carefully removed from the follicular surface using watchmaker's forceps. The follicle was then placed in a 3cm petri dish and examined for clarity and good blood supply (see Figure 2). If these were satisfactory the follicle was punctured to release the follicular fluid. If the fluid contained a poor-looking oocyte (black, clumped supporting cells or no supporting cells, grainy cytoplasm) or dark (brown to black) granulosa cells, the follicle was discarded and a new one selected.

The acceptable follicle was then everted and washed twice in H199 supplemented with FCS. Granulosa cells were scraped from the inside surface into a 3cm petri dish containing 1ml of maturation medium (B199+). This medium was modified bicarbonate medium 199 supplemented with 10% 2FCS and 10% oestrous cow serum (OCS). The medium previously had been equilibrated in 5% CO2 in air at 39°C. Healthy granulosa cells are clear to light brown when viewed through a dissecting microscope. Aliquots of these cells were added to 3cm petri dishes containing 2mls of B199+ so that the cell concentration was approximately $1x10^5$/ml. The dishes were kept in the CO_2 incubator until required.

supernatant (approximately 4.5ml) was discarded and replaced with fresh sperm wash medium. The centrifugation was repeated and all but 100µl of the supernatant removed. The sperm pellet was gently resuspended by tapping the side of the tube.

Sperm were counted using a haemocytometer. 5µl of sperm suspension was added to 45µl of hydrochloric acid to kill the sperm. Approximately 5µl of this mixture was placed in one of the counting chambers of the haemocytometer slide and sperm inside 5 squares were counted. The procedure was repeated in the second counting chamber. The two counts were then averaged, and the concentration of sperm/ml in the original sample was calculated.

3.5.2.Unfrozen sperm.

A similar procedure to that employed with frozen-thawed sperm was used with unfrozen sperm, with some modifications. 1ml of Caprogen was diluted up to 6ml with sperm wash medium. The suspension was centrifuged at 185g for 5 minutes, after which the supernatant was reduced to 0.25ml. The sperm was resuspended in 6ml of fresh wash medium. Centrifugation was repeated and the supernatant was aspirated, leaving the sperm in 100µl of medium. The sperm were resuspended in 3ml of medium and recentrifuged (50g for 7 minutes) before the supernatant was again removed to leave only 100µl.

3.6. FERTILISATION.

Enough washed sperm were added to 1ml of warm, equilibrated fertilisation medium to give the desired sperm concentration. The medium was modified TALP supplemented with caffeine (1.39mM), BSA (3mg/ml) and heparin (10µg/ml). The medium was aliquoted into

45µl drops (8 per 6cm petri dish) and covered with 10ml of warm equilibrated paraffin oil. The drops were then examined under high power on a dissecting microscope to check sperm motility. The fertilisation dishes were placed in the CO_2 incubator while the matured oocytes were prepared.

Using a micropipette, the oocytes were removed from their maturation dishes and placed into a 3cm petri dish containing 2ml of H199 (without serum). Cumulus-expanded oocytes (Figure 4) were dissociated from each other by gentle pipetting. Up to 15 oocytes were transferred to each fertilisation drop in 5µl of H199.

The fertilisation drops were incubated for 18 hours at 39°C in a gas atmosphere of 5% CO_2 in air and 100% humidity.

3.7. FIXING AND STAINING OF OOCYTES.

After incubation with sperm for 18 hours, the oocytes were removed from the fertilisation droplets and placed in warm H199. Adhering corona or cumulus cells were removed by vigorous pipetting or by teasing with a pair of 18-gauge needles.

Oocytes (no more than 15) were then placed on a labelled microscope slide in 2-3µl of medium and the oocytes were aligned (for easy location during assessment) using an 18-gauge needle. A coverslip was gently pressed on to vaseline strips placed on two sides of the slide until it contacted the medium. While watching through the microscope, the coverslip was pressed down further until the oocytes bulged slightly and remained distended. The top and bottom of the coverslip were then sealed to the slide using a small amount of rubber cement, or vaseline and paraffin wax (1:1) melted together. The slide was fixed in acid-alcohol (25% glacial acetic acid:75% ethanol) for at least 24 hours. The slide was then cleared in pure ethanol for 30 minutes prior to staining with orcein (1% weight/volume in 60:40 water:acetic acid). The stain was cleared by running ethanol through the slide before the addition of more stain. Oocytes were assessed with phase contrast microscopy.

3.8. ASSESSMENT.

The oocytes were classified by stage of development and evidence of sperm penetration. Oocytes were assumed to be penetrated if they possessed one or more pronuclei with either a detached sperm tail or evidence of decondensing sperm in the cytoplasm. Penetrated oocytes were further classified as "normal", "late", "polyspermic" or "other", depending on their stage of development relative to the expected stage and the number of penetrated sperm visible. "Other" oocytes were those that were plainly penetrated, but for some reason could not be conclusively placed in any of the categories. Unpenetrated oocytes were classified according to whether or not they had reached the metaphase II stage of nuclear maturation. Immature oocytes were excluded from the results. Figures 5-13 depict some of the developmental stages seen during assessment. The timing of oocyte and embryo development can be found in Appendix 3.

3.9. METHODOLOGY OF INDIVIDUAL EXPERIMENTS.

3.9.1.Experiment 1: Effect of Sperm Concentration and Semen Age on Oocyte Penetration and Polyspermy.

The concentration of the washed sperm was determined using a haemocytometer and then the sperm were diluted to the desired concentrations. Initially these were 0.5, 1.0, 2.0, and 4.0 x 10^6/ml. However, after the first week it became apparent that penetration rates from the four treatments were uniformly high. It was therefore decided to omit the two higher concentrations and introduce the lower concentrations of 0.125 and 0.25 x 10^6/ml - in the second week of the experiment. Thus the concentrations of 0.5 and 1.0x10^6/ml were tested eight times each and the concentrations of 0.125, 0.25, 2.0 and 4.0x10^6/ml were tested four times.

Frozen-thawed sperm from either bull 1 or bull 2 was used to inseminate 214 oocytes at sperm concentrations of 0.5, 1.0, 2.0, and 4.0x10^6/ml. The penetration rates obtained were compared with those achieved with the day-old unfrozen sperm (190 oocytes). 60 oocytes were incubated without sperm as a control for parthenogenesis.

3.9.2.Experiment 2: Effect of Caprogen Presence on Oocyte Penetration.

Washed sperm were added to tubes containing Caprogen diluted with modified TALP medium. The initial concentrations used were (ml/litre) 1000 (i.e. neat Caprogen), 500, 100 and 10. However, the concentrations of 1000 and 500 ml/litre consistently gave no penetration so it was decided to replace these with the higher dilutions of 3 ml/litre and 1ml/litre (essentially washed sperm). Thus the concentrations of 10 and 100 ml/litre were tested 6 times and the concentrations of 1, 3, 500 and 1000ml/litre were tested 3 times. Oocytes were inseminated at a concentration of $1x10^6$/ml, a concentration found in experiment 1 to give good penetration rates with an acceptable level of polyspermy. This concentration was also the lowest at which differences between the two semen ages became negligible.

3.9.3.Experiment 3: Effect of Heparin Concentration on Oocyte Penetration (I).

The fertilisation medium was prepared without heparin and then heparin was added from a stock solution just prior to use. Five concentrations were tested initially: 0, 1, 5, 10, and 50μg/ml. Later it was deemed desirable to explore further the trend in penetration rates at the higher heparin concentrations, so 20 and 30μg/ml treatments were introduced. Thus the concentrations of 20 and 30μg heparin/ml had less observations than the other concentrations. Sperm from one bull only were used in this experiment, the second bull having been withdrawn from service by the LIC.

3.9.4.Experiment 4: Effect of Heparin Concentration on Oocyte Penetration (II).

This experiment was a further exploration of the result generated without heparin in the third experiment. Frozen-thawed and unfrozen sperm from 5 bulls (including the bull used in the third experiment) were used to inseminate oocytes in medium without heparin. Unfrozen sperm was also used with media containing 10μg/ml heparin. The 10μg/ml level was selected because it had given good penetration rates in the third experiment.

3.10. STATISTICAL ANALYSIS.

Two methods of data analysis were employed. The first used the statistical programme Vital (licensed to Onstream Systems Ltd, Palmerston North) to perform analyses of variance to evaluate differences in penetration rates obtained with the various treatments. Raw data were untransformed and unweighted. The second method used the Generalised Linear Models procedure of the Statistical Analysis Systems (SAS) package to fit regression equations to the data. Significant effects were determined with F-values, with the level of significance being $p<0.05$. The data were weighted according to the number of oocytes in each cell. Percentage data were transformed using an arcsine transformation. Predicted equations and values were then retransformed for inclusion in the results.

Results obtained using Vital and SAS were similar, with Vital tending to attach greater significance to effects. Only the results of SAS analyses are reported here.

The analyses of the factors affecting penetration rates and rates of polyspermy for experiments 1 and 3 were conducted using the model overleaf:

$y_{ij} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + e_{ij}$, where

y_{ij} = the penetration rate or incidence of polyspermy in the ith concentration at the jth semen age or semen type

μ = the population mean

α_i = the effect of sperm concentration (i = 1-6)

or heparin concentration (i= 1-7)

β_j = the effect of semen age (j=1,2)

or semen type (frozen-thawed or unfrozen) (j=1,2)

$\alpha\beta_{ij}$= the interaction effect

e_{ij} = the sampling error unique to y_{ij}, assumed to be independently and normally distributed and from a population of sampling errors with mean equal to zero.

Bull and week (ejaculate) effects were not included in the analysis because differences between the two bulls and between ejaculates were not the object of this study. Sperm from only two bulls (and two ejaculates) were utilised so no meaningful conclusions concerning the bull population as a whole could be realistically drawn. However, considerable variation between bulls and between ejaculates exists for in vitro and in vivo fertility. In order to minimise these differences, the two bulls that were used in this study were specifically selected for their similar semen characteristics and in vivo fertility; both traits were of high quality.

The analysis of the effect of Caprogen on penetration rates in experiment 2 was conducted using the model overleaf:

$y_i = \mu + \alpha_i + e_i$, where

y_i=the observation in the ith treatment

μ=the general mean

α=the effect of Caprogen dilution (i=1-6)

e_i=the random error peculiar to yi, assumed to be independently and normally distributed and from a population of sampling errors with mean equal to zero.

The variable of semen age was not included in this model because practical difficulties meant that only one set of observations was able to be collected for some treatments with two-day-old semen. However, the trend in penetration rates over Caprogen dilutions appeared to be similar for the two semen ages.

For all experiments, the experimental unit used was a group of oocytes.

FIGURE 1:

FIGURE 2:

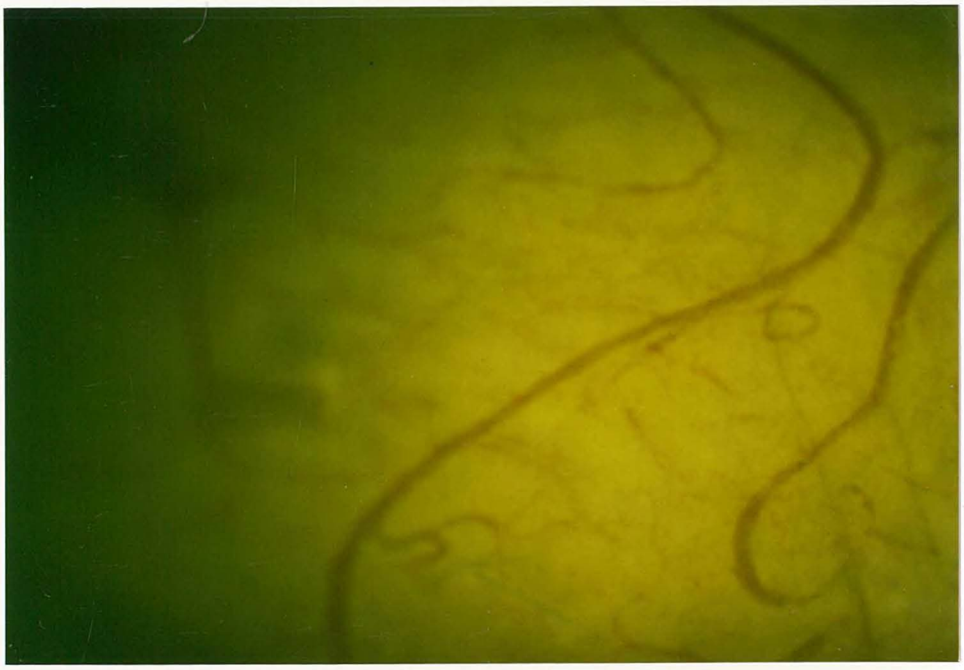


Figure 1: Ovaries collected from the slaughterhouse that have been washed and trimmed of excess tissue.
Figure 2: A healthy follicle with good vascularisation, suitable for providing granulosa cells for maturation culture (x100).

FIGURE 3:

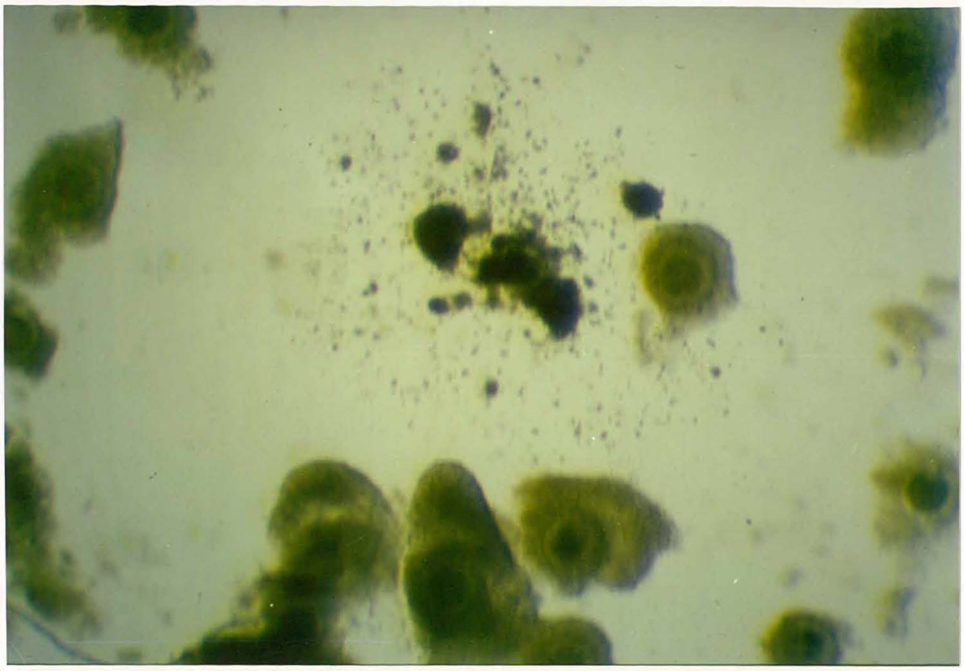


FIGURE 4:

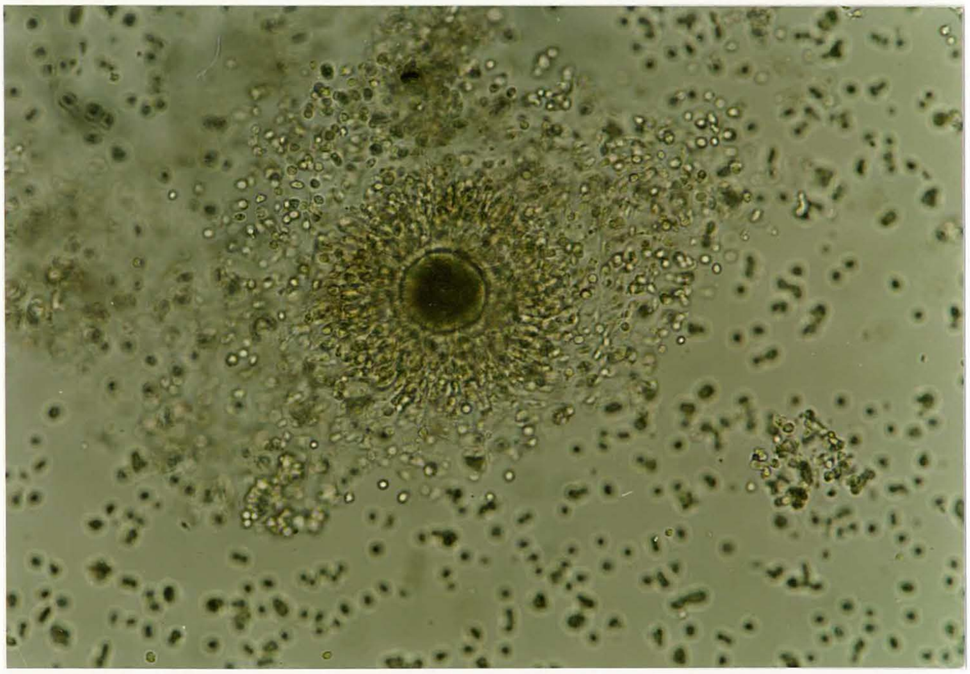


Figure 3: Degenerate oocyte (centre) and healthy immature oocytes with compact unexpanded cumulus layers (x200).
Figure 4: An oocyte that has undergone cumulus layer expansion after maturation culture (x200).

FIGURE 5:

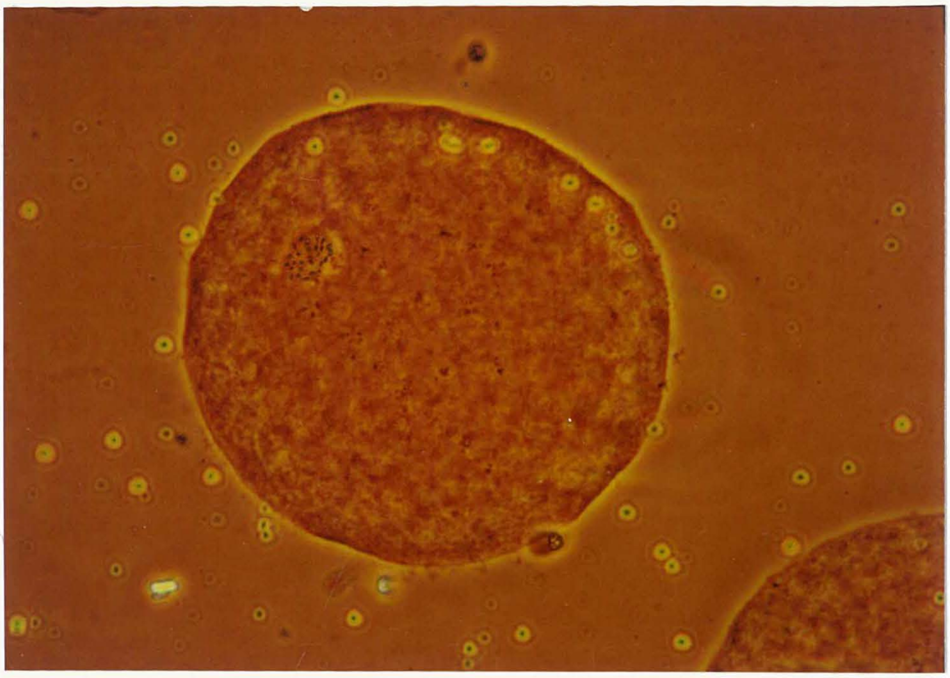
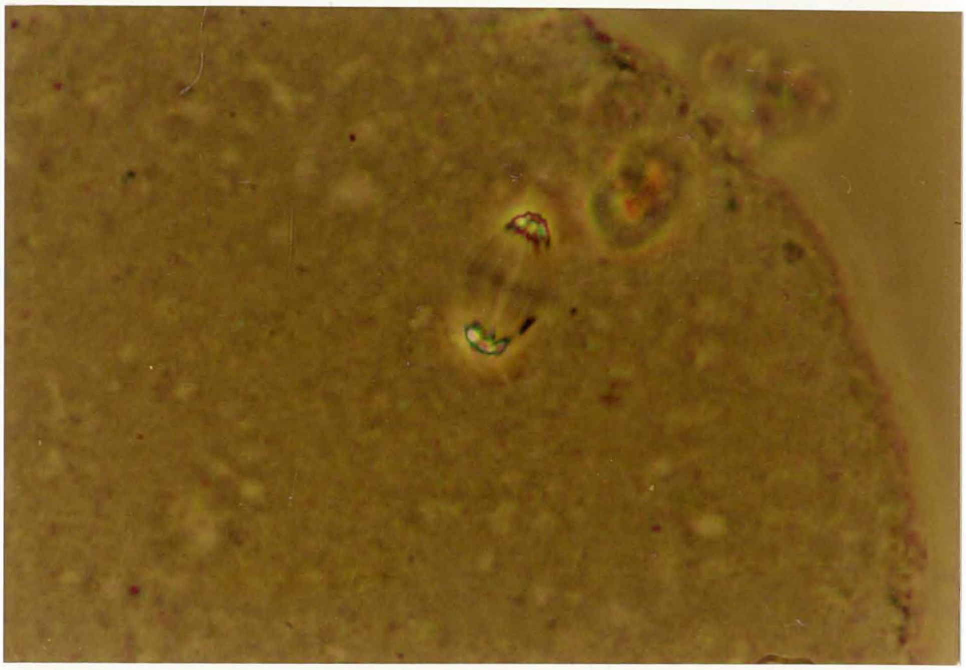


FIGURE 6:



Oocytes at early stages of maturation.
Figure 5: Immature oocyte at nuclear stage metaphase I (x400).
Figure 6: Oocyte in first phase of secondary maturation - nuclear stage anaphase II (x1000).

FIGURE 7:

Figure 7: Oocyte in second phase of secondary maturation - nuclear stage telophase II and the production of the polar body (x1000).

FIGURE 8:

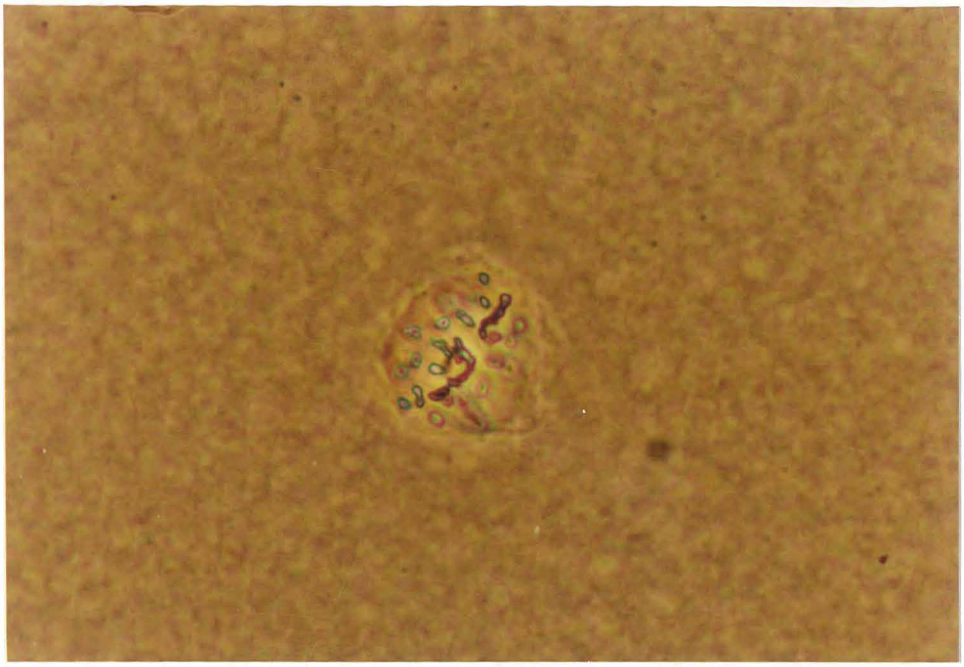
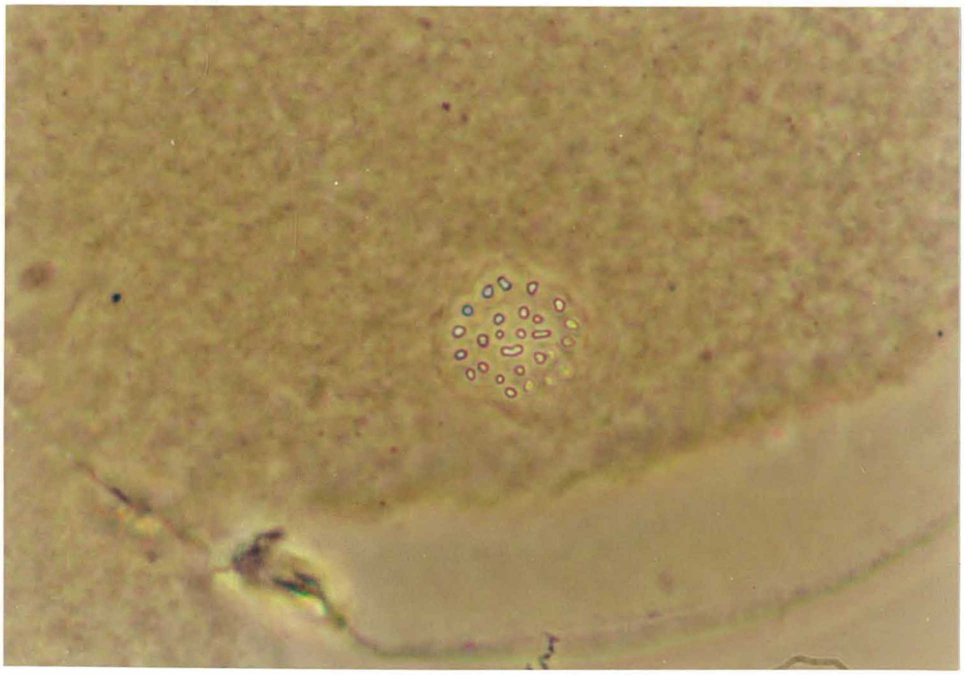


FIGURE 9:



Oocytes in third phase of secondary maturation - metaphase II.
Figure 8: lateral view of spindle (x1000).
Figure 9: cross-sectional view of spindle (x1000).

FIGURE 10:

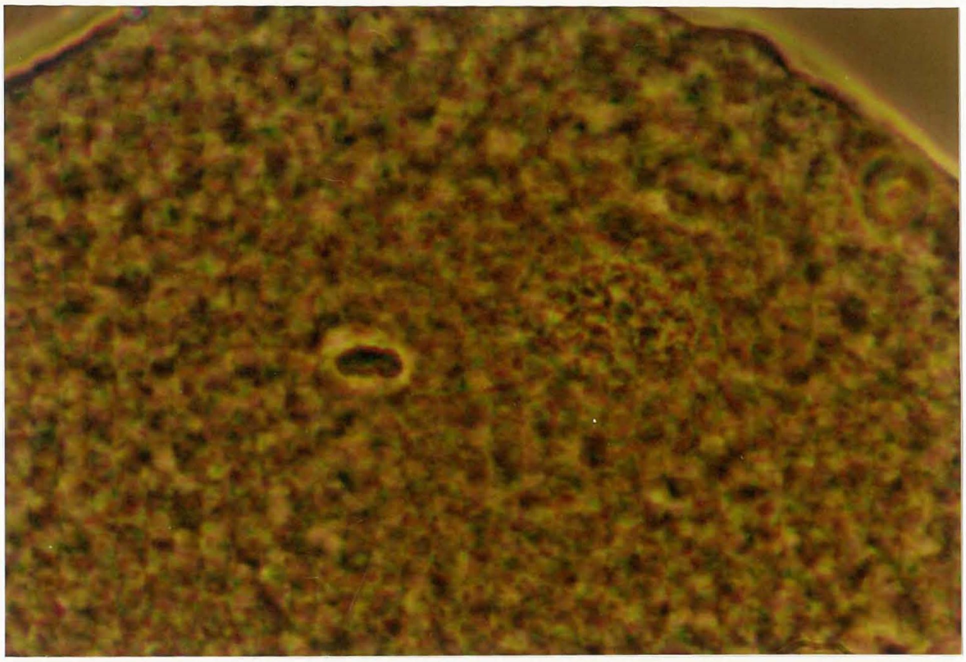


FIGURE 11:

Figure 10: Early penetration with female pronucleus (centre right), decondensing sperm head (centre left) and detached sperm tail (centre) (x1000).
Figure 11: Penetration of two oocytes, both possessing male and female pronuclei. A sperm tail can be seen below the bottom pronucleus in the oocyte on the right (x400).

FIGURE 12:

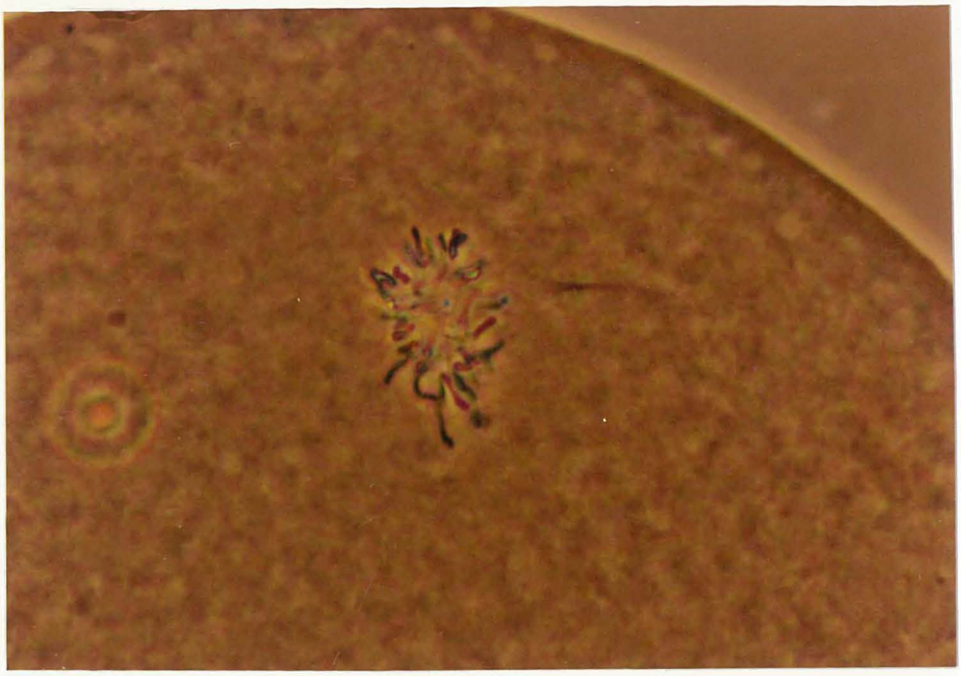


FIGURE 13:

Figure 12: Syngamy - the amalgamation of the male and female pronuclei and the merging of genetic material (x1000).
Figure 13: A polyspermic oocyte with three pronuclei. One of the sperm tails can be seen on the top pronucleus (x400).

CHAPTER 4. RESULTS.

4.1. EXPERIMENT 1: SPERM CONCENTRATION, SEMEN AGE, OOCYTE PENETRATION AND POLYSPERMY.

A total of 470 mature oocytes were incubated with unfrozen, diluted sperm. A further 214 oocytes were incubated with frozen-thawed sperm and 60 oocytes were incubated without sperm as a control for parthenogenesis. None of the 60 oocytes displayed parthenogenetic activation. Equations associated with Figures 14 and 15 and tables containing the statistical analyses for experiment 1 are shown in appendix 4.

4.1.1. Effect of sperm concentration and semen age on oocyte penetration.

There was a significant ($p=0.017$) effect of semen age on the proportion of oocytes penetrated. Consequently, the effects of sperm concentration were analysed within semen age. There was no significant relationship between sperm concentration and penetration rate when day-old sperm were used, with penetration rates ranging from 79-100%. When two-day-old sperm were used, the best-fitting relationship between penetration rate and sperm concentration had significant linear ($p=0.004$) and quadratic ($p=0.026$) components. Penetration rates ranged from 21-94%, with the two lowest sperm concentrations (0.125 and 0.25×10^6/ml) appearing to give lower penetration. At concentrations of 1×10^6/ml or greater there appeared to be little difference in penetration rates obtained with the two semen ages, with rates being uniformly high. Tables 3 and 4 show the penetration rates (untransformed) obtained with day-old and two-day-old sperm respectively. Predicted and observed penetration rates for the two semen ages are depicted in Figure 14.

Table 3. Proportions of oocytes penetrated at different sperm concentrations (day-old sperm).

Concentration ($x10^6$/ml)	Observations - proportions of polyspermic oocytes (%)*	Total(%)	± S.E.†
0.125	17/18 (94), 13/20 (65)	30/38 (79)	7.2
0.25	8/8 (100), 10/11 (91)	18/19 (95)	7.2
0.5	12/17 (71), 10/12 (83), 18/18 (100), 19/22 (86)	59/69 (86)	5.1
1.0	7/8 (88), 15/16 (94), 25/25 (100), 21/21 (100)	68/70 (97)	5.1
2.0	12/14 (86), 10/13 (77)	22/27 (81)	7.2
4.0	15/15 (100), 9/9(100)	24/24 (100)	7.2

* Number of oocytes penetrated/Number of oocytes inseminated. Observations = the number of oocytes assessed on different days. † Standard error of total %.

Table 4. Proportions of oocytes penetrated at different sperm concentrations (2-day-old sperm).

Concentration ($x10^6$/ml)	Observations - proportions of polyspermic oocytes (%)*	Total(%)	± S.E.†
0.125	6/16 (38), 0/12 (0)	6/28 (21)	16.0
0.25	11/13 (85), 2/14 (14)	13/27 (48)	16.0
0.5	13/16 (81), 9/13 (69), 16/17 (94), 6/14 (43)	44/60 (73)	11.3
1.0	14/15 (93), 10/11 (91), 12/13 (92), 8/11 (73)	44/50 (88)	11.3
2.0	15/17 (88), 23/23 (100)	38/40 (95)	16.0
4.0	6/7 (86), 11/11 (100)	17/18 (94)	16.0

* Number of oocytes penetrated/Number of oocytes inseminated. Observations = the number of oocytes assessed on different days. † Standard error of total %.

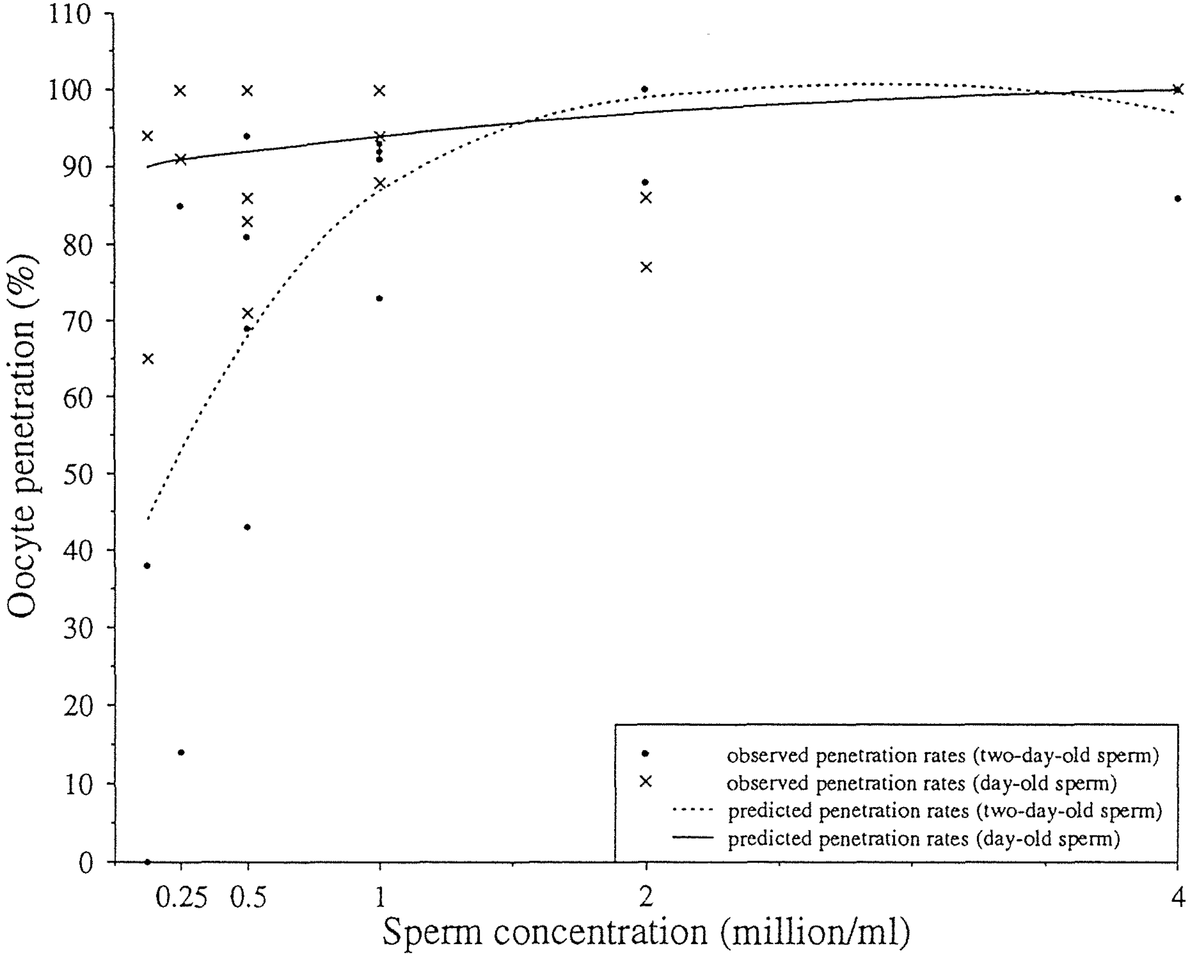


Figure 14. Observed and predicted penetration rates obtained with day-old and two-day-old unfrozen sperm.

4.1.2.Effect of sperm concentration on the proportion of polyspermic oocytes.

Polyspermy ranged from 19-42% for the 6 concentrations. No relationship appeared to exist between sperm concentration and the proportion of polyspermic oocytes. Table 5 shows the proportions of polyspermic oocytes generated by each sperm concentration.

Table 5. Proportion of polyspermic oocytes generated by each sperm concentration.

Concentration (x10^6/ml)	Observations - proportions of polyspermic oocytes (%)[*]	Total(%)	± S.E.[†]
0.125	6/17 (35), 1/6 (17), 0/13 (0)	7/36 (19)	9.8
0.25	4/8 (50), 2/11 (18), 1/10 (10), 1/2 (50)	8/31 (26)	11.7
0.5	1/12 (8), 3/13 (23), 3/10 (30), 1/9 (11), 11/18 (61), 6/16 (38), 1/19 (5), 1/6 (17)	27/103 (26)	7.0
1.0	1/7 (14), 7/14 (50), 2/15 (13), 3/10 (30), 14/25 (56), 7/12 (58), 2/21 (10), 1/8 (13)	37/122 (30)	7.2
2.0	3/12 (25), 10/15 (67), 4/10 (40), 8/23 (35)	25/60 (42)	9.7
4.0	4/15 (27), 2/6 (33), 2/9 (22), 2/11 (18)	10/41 (24)	12.2

[*] Number of oocytes penetrated/Number of oocytes inseminated. Observations = the number of oocytes assessed on different days. [†] Standard error of total %.

4.1.3.Rates of penetration achieved with frozen-thawed versus unfrozen sperm.

There was a significant (p=0.003) difference in the penetration rates achieved with unfrozen and frozen-thawed sperm, so sperm concentration effects were analysed within the two sperm types. There was no significant relationship between sperm concentration and penetration rate when day-old unfrozen semen was used, as was reported in 4.1.1. A significant (p=0.002) linear relationship was apparent when frozen-thawed sperm was used. Penetration rates for 0.5, 1.0, 2.0, and 4.0×10^6/ml concentrations were 30%, 45%, 59% and 84% respectively. Observed and predicted penetration rates for frozen-thawed and unfrozen sperm are shown in Figure 15. Summaries of the penetration rates obtained with frozen-thawed and unfrozen sperm are shown in Tables 6 and 7.

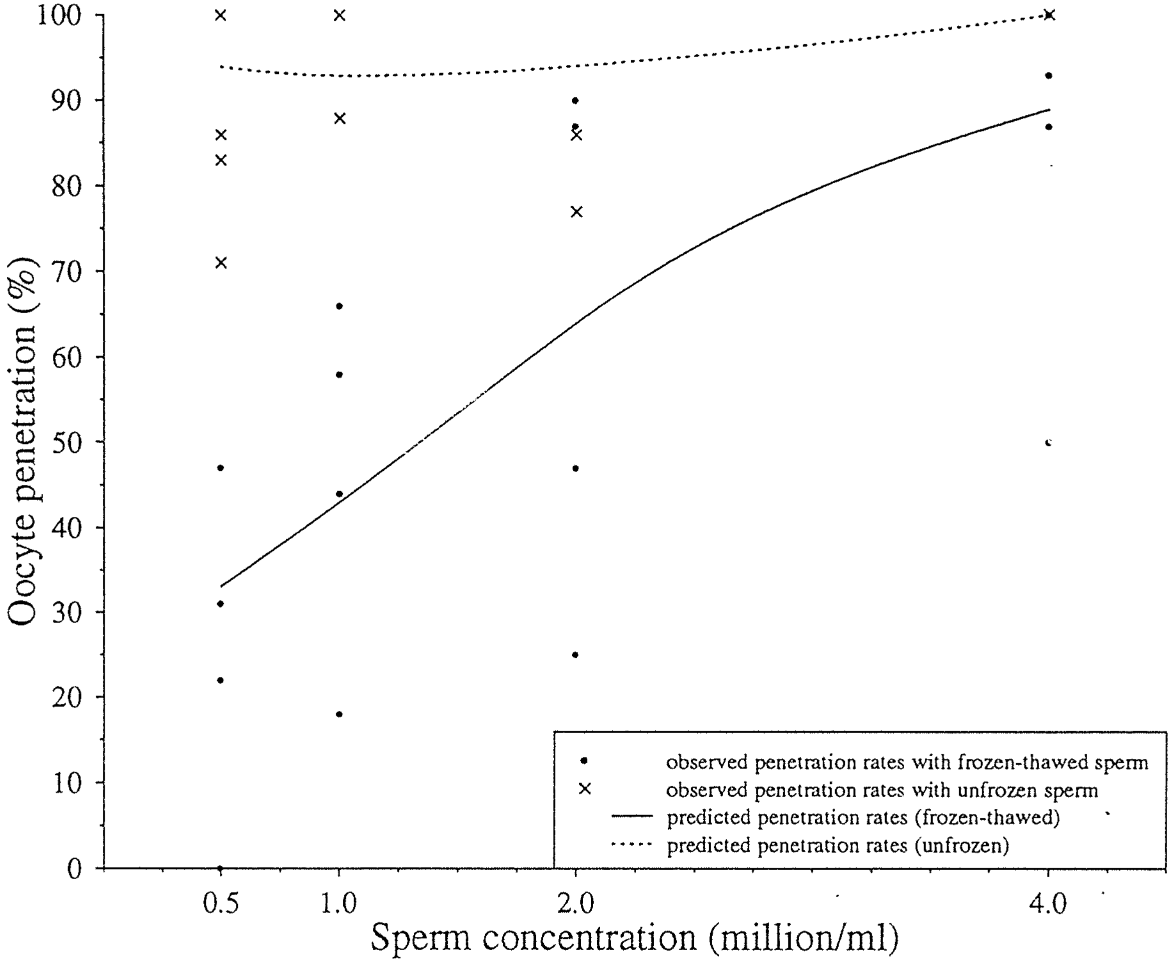


Figure 15. Observed and predicted penetration rates obtained with frozen-thawed and unfrozen sperm.

Table 6. Penetration rates obtained at different sperm concentrations (frozen-thawed sperm).

Concentration ($x10^6$/ml)	Observations - proportions of oocytes penetrated (%)*	Total (%)	± S.E.[†]
0.5	9/19 (47), 5/16 (31), 4/18 (22), 0/7 (0)	18/60 (30)	15.1
1.0	7/12 (58), 4/6 (66), 8/18 (44), 2/11 (18)	21/47 (45)	17.1
2.0	13/15 (87), 9/10 (90), 7/15 (47), 4/16 (25)	33/56 (59)	15.6
4.0	11/11 (100), 14/15 (93), 13/15 (87), 5/10 (50)	43/51 (84)	16.4

* Number of oocytes penetrated/Number of oocytes inseminated. Observations = the number of oocytes assessed on different days. [†] Standard error of total %.

Table 7. Penetration rates obtained at different sperm concentrations (unfrozen sperm).

Concentration ($x10^6$/ml)	Observations - proportions of oocytes penetrated (%)*	Total (%)	± S.E.[†]
0.5	12/17 (71), 18/18 (100), 10/12 (83), 19/22 (86)	59/69 (86)	13.4
1.0	7/8 (88), 25/25 (100), 14/16 (88), 21/21 (100)	67/70 (96)	13.3
2.0	12/14 (86), 10/13 (77)	22/27 (81)	21.4
4.0	15/15 (100), 9/9 (100)	24/24 (100)	22.7

* Number of oocytes penetrated/Number of oocytes inseminated. Observations = the number of oocytes assessed on different days. [†] Standard error of total %.

4.2. EXPERIMENT 2: EFFECT OF CAPROGEN PRESENCE ON OOCYTE PENETRATION RATE.

A total of 447 mature oocytes were incubated with unfrozen sperm ($1x10^6$/ml) in medium containing various dilutions of Caprogen extender.

The model that best fits the spread of data encountered in this experiment had significant linear (p=0.0002) and quadratic (p=0.003) elements. There was also a significant (p=0.01) departure from quadratic fit attributable to the sharp drop in penetration rate observed at the highest dilution of Caprogen. Penetration rates for the Caprogen dilutions (ml/litre) 1000 (i.e. neat Caprogen), 500, 100, 10, 3 and 1 were 0%, 2%, 9%, 46%, 61% and 46% respectively. Table 8 shows the penetration rates obtained with the different concentrations of Caprogen in the fertilisation medium. Observed and predicted penetration rates for this experiment are shown in Figure 16. The statistical analysis of the data and the equation associated with Figure 16 can be found in Appendix 5.

Table 8. Data summary of oocyte penetration rates with different concentrations of Caprogen in the fertilisation medium*.

Concentration of Caprogen (mls/litre)	Observations - proportions of oocytes penetrated (%)*	Total (%)	± S.E.†
1000	0/15 (0), 0/11 (0), 0/20 (0)	0/46 (0)	0
500	0/7 (0), 0/18 (0), 1/17 (6)	1/42 (2)	8.5
100	6/25 (24), 3/21 (14), 0/19 (0), 1/38 (3), 0/6 (0), 0/3 (0)	10/112 (9)	6.0
10	8/12 (67), 10/24 (42), 3/23 (13), 21/35 (60), 6/12 (50), 6/12 (50)	54/118 (46)	6.0
3	27/39 (69), 8/19 (42), 3/4 (75)	38/62 (61)	8.5
1	26/44 (59), 2/13 (15), 3/10 (30)	31/67 (46)	8.5

* Number of oocytes penetrated/Number of oocytes inseminated. Observations = the number of oocytes assessed on different days. † Standard error of total %.

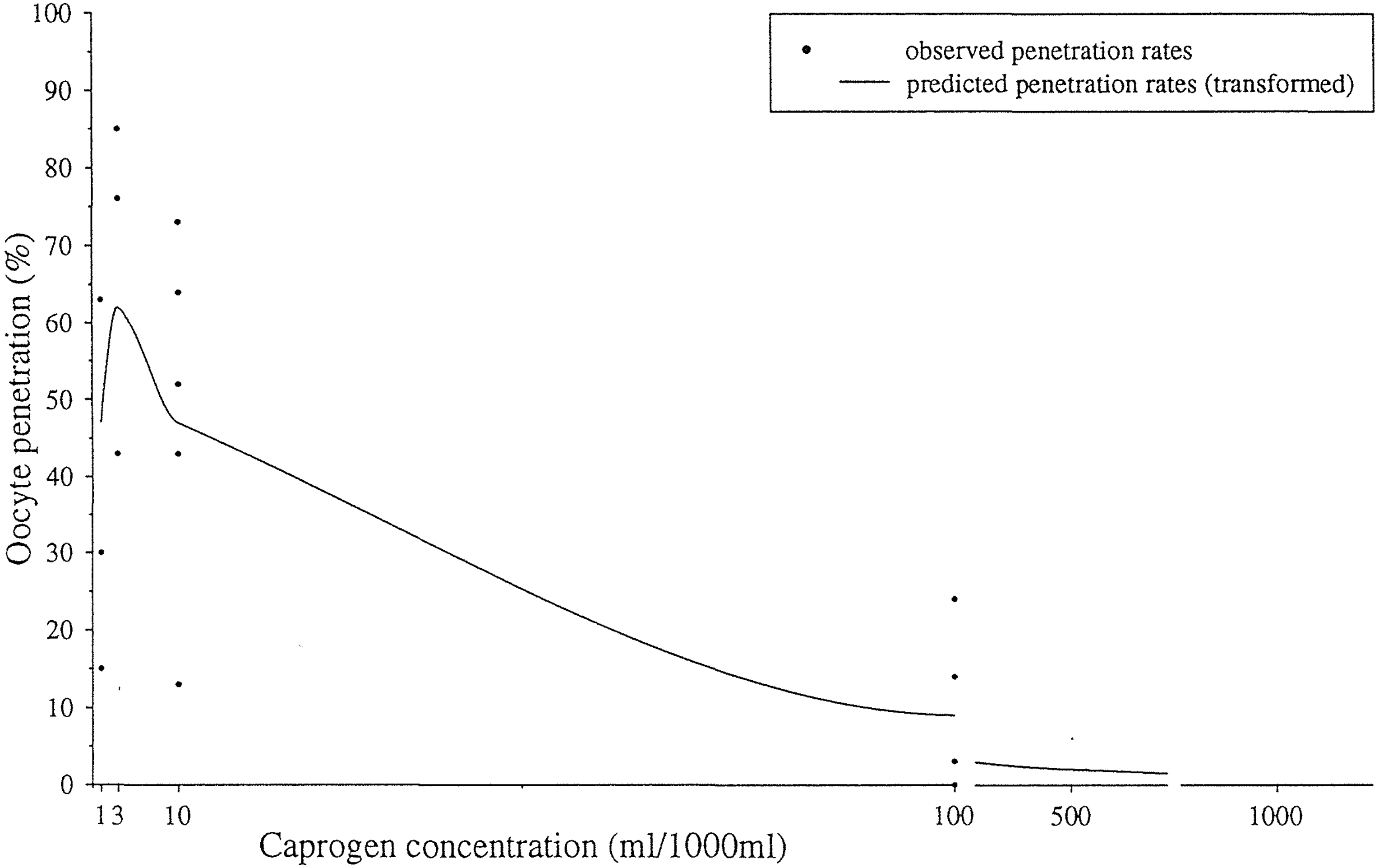


Figure 16. Observed and predicted penetration rates obtained with various concentrations of Caprogen.

4.3. EXPERIMENT 3: EFFECT OF HEPARIN CONCENTRATION ON OOCYTE PENETRATION.

A total of 671 mature oocytes were incubated with unfrozen sperm ($1x10^6$/ml) in medium containing various dilutions of heparin.

In this experiment there was no relationship exhibited between the concentration of heparin in the fertilisation medium and subsequent penetration rates or rates of polyspermy. Penetration rates varied from 3-100%. Average penetration rates for the 7 concentrations examined (0, 1, 5, 10, 20, 30 and 50µg/ml) were 69%, 57%, 82%, 87%, 73%, 75%, and 50% respectively. Tables 9 and 10 present a summary of the penetration rates and the incidence of polyspermy obtained in this experiment. Statistical analyses for the experiment can be found in Appendix 6.

Table 9. Oocyte penetration rates with different concentrations of heparin in the fertilisation medium.

Concentration (mg/ml)	Observations - proportions of oocytes penetrated (%)*	Total (%)	± S.E.†
0	13/42 (31), 51/53 (96), 12/15 (80)	76/110 (69)	16.9
1	19/49 (39), 26/28 (93), 13/25 (52)	58/102 (57)	16.9
5	26/33 (79), 26/26 (100), 36/48 (75)	88/107 (82)	16.9
10	29/38 (76), 37/37 (100), 24/28 (86)	90/103 (87)	16.9
20	16/28 (57), 40/49 (82)	56/77 (73)	20.7
30	7/8 (88), 44/60 (73)	51/68 (75)	20.7
50	1/29 (3), 24/26 (92), 24/29 (83), 3/20 (15)	52/104 (50)	14.7

[*] Number of oocytes penetrated/Number of oocytes inseminated. Observations = the number of oocytes assessed on different days. [†] Standard error of total %.

Table 10. Incidence of polyspermic oocytes for different concentrations of heparin in the fertilisation medium.

Concentration (mg/ml)	Observations - proportions of polyspermic oocytes(%)*	Total (%)	± S.E.†
0	4/13 (31), 8/51 (16), 2/12 (17)	14/76 (18)	10.4
1	5/19 (26), 4/26 (15), 0/13 (0)	9/58 (16)	10.4
5	3/26 (11), 8/26 (31), 5/36 (14)	16/88 (18)	10.4
10	2/29 (7), 15/37 (41), 8/24 (33)	25/90 (28)	10.4
20	3/16 (19), 5/40 (13)	8/56 (14)	12.9
30	2/7 (29), 14/44 (32)	16/51 (31)	12.9
50	0/1 (0), 15/24 (63), 10/24 (42), 0/3 (0)	25/52 (48)	9.1

* Number of oocytes penetrated/Number of oocytes inseminated. Observations = the number of oocytes assessed on different days. † Standard error of total %.

4.4. EXPERIMENT 4: EFFECT OF HEPARIN CONCENTRATION ON OOCYTE PENETRATION.

Data collected in this experiment are summarised in Tables 11, 12 and 13. Penetration rates achieved in the absence of heparin ranged from 47% (37/78) to 86% (59/69) for unfrozen sperm, and from 23% (7/31) to 73% (27/37) for frozen-thawed sperm. Rates of penetration obtained with unfrozen sperm in medium containing 10µg/ml heparin ranged from 53% (10/19) to 100% (20/20). There appeared to be considerable variation among the bulls for all three treatments.

Table 11. Penetration rates obtained with unfrozen sperm from five bulls in medium without heparin.

Bull	Observations - number of oocytes penetrated (%)*	Total (%)
1	5/31 (16), 32/47 (68)	37/78 (47)
2	15/18 (83), 31/38 (82)	46/56 (82)
3	26/51 (51), 33/45 (73)	59/69 (61)
4	29/50 (58), 19/32 (59)	48/82 (59)
5	38/42 (90), 32/78 (41)	70/120 (58)

* Number of oocytes penetrated/Number of oocytes inseminated. Observations = the number of oocytes assessed on different days.

Table 12. Penetration rates obtained with unfrozen sperm from five bulls in medium containing 10µg/ml heparin.

Bull	Observations - number of oocytes penetrated (%)*	Total (%)
1	10/19(53), 6/11 (55)	16/30 (53)
2	11/11 (100), 7/7 (100)	18/18 (100)
3	10/10 (100), 10/10 (100)	20/20 (100)
4	15/15 (100), 11/15 (73)	26/30 (87)
5	12/12 (100), 10/16 (63)	22/28 (79)

* Number of oocytes penetrated/Number of oocytes inseminated. Observations = the number of oocytes assessed on different days.

Table 13. Penetration rates obtained with frozen-thawed sperm from five bulls in medium without heparin.

Bull	Observations - number of oocytes penetrated (%)	Total (%)
1	6/14 (43), 12/35 (34)	18/49 (37)
2	13/28 (46), 4/11 (36)	17/39 (44)
3	2/6 (33), 25/31 (81)	27/37 (73)
4	1/22 (5), 6/9 (67)	7/31 (23)
5	8/10 (80), 37/41 (90)	45/51 (88)

* Number of oocytes penetrated/Number of oocytes inseminated. Observations = the number of oocytes assessed on different days.

CHAPTER 5: DISCUSSION

The effects on in vitro oocyte penetration of three factors associated with IVF were investigated. The three factors were the concentrations of sperm, Caprogen extender and heparin present in the fertilisation medium. Two experimental designs were contemplated; a complex factorial design involving all three variables, and a sequential design with the factors being studied individually in three experiments. The latter was selected for four main reasons:

1. The study was to be conducted concurrently with the development of a reliable IVM/IVF/IVC system in the new DSIR laboratory. Simple experiments only were being carried out at this time so that emphasis could be placed on the development of repeatable procedures.

2. A small and inconsistent supply of oocytes (due to low cow kills at the slaughterhouse) made a large factorial design impractical.

3. Three separate experiments allowed the factors to be studied in more depth than was possible with a factorial design. A factorial design studying penetration rates at four sperm concentrations, four dilutions of Caprogen extender and five heparin concentrations across two semen ages would have required 160 cells. Restrictions on labour and oocyte availability rendered such a large scale experiment difficult to accomplish.

4. A study involving separate experiments appeared to afford greater flexibility than did a factorial design. This was important because it was likely that treatment levels would have to be adjusted, since little was known about the requirements and behaviour of unfrozen sperm in IVF.

5.1. SEMEN AGE AND SPERM CONCENTRATION.

5.1.1.Effects of semen age on penetration.

A.I. trials conducted by the LIC have indicated that unfrozen semen stored in Caprogen can be used up to 96 hours after processing with little decline in fertility *in vivo* (G. Morris, *pers. comm.*). Therefore differences between the two semen ages were not expected. However, when day-old semen was utilised, penetration was uniformly high across all sperm concentrations. In contrast, penetration rates exhibited a curvilinear response to increasing sperm concentration when two-day-old semen was used, with depressed penetration rates occurring at concentrations below $1x10^6$/ml. These results suggest that the older semen samples had a reduced capacity to penetrate oocytes, a phenomenon that was masked at higher concentrations by the higher numbers of fertile sperm.

The depressed fertility exhibited at low sperm concentrations by two-day-old sperm could be attributed to ageing effects. As sperm age they may lose their acrosomal caps, and thus be prevented from undergoing the acrosome reaction necessary for penetration. Ageing also results in reduced motility and increased mortality of sperm, both of which decrease the fertility of a semen sample.

Alternatively the sperm may have utilised all of the Caprogen's available nutrients, leading to nutrient starvation and reduced viability. Neither of these theories seems very likely given the LIC's assertion that sperm stored in Caprogen retains its original fertility for up to 96 hours (G. Morris, pers. comm.).

Another possibility concerns the nitrogen saturation of the Caprogen extender. Flasks containing sperm in Caprogen are saturated with nitrogen prior to being sealed and delivered. The nitrogen excludes oxygen from the flask, thereby reducing the activity and metabolic output of spermatozoa. In this experiment one flask of sperm was used over two days. The seal was broken on day 1, and it is possible that the flask was not sufficiently resaturated with nitrogen. This may have lead to a build-up of toxic substances such as hydrogen peroxide, causing reduced motility and increased rates of mortality in the sperm to be used on day 2.

Further experimentation should be carried out to explore the cause of the low penetration rates achieved with two-day-old semen at low sperm concentrations. The questions of nutrient starvation and sperm ageing could be addressed with a series of tests. The series could involve a) an analysis of nutrient levels and toxic by-products of sperm metabolism in Caprogen that has contained sperm for various periods of time, b) a comparison of motility of one-day-old versus two-day-old sperm, and c) histological examinations of one- and two-day-old sperm. The third test would ascertain whether there is greater acrosomal loss in the older sperm, and whether the losses in motility and fertility could be considered large enough to significantly affect penetration rates. The splitting of the ejaculate into two separate flasks (to be used on day 1 or day 2), or the use of straws of unfrozen sperm would negate the need to resaturate a flask.

If the problem proved to be the methodology (ie, flask resaturation) rather than the Caprogen itself, a subsequent experiment could examine penetration rates obtained using sperm from the same ejaculate over several consecutive days. One of the main inconveniences associated with the use of unfrozen semen for in vitro work is that it is not as readily available as frozen semen, thousands of straws of which can be stored in the laboratory under liquid nitrogen. The time and expense involved in daily or twice weekly collections from animals could be reduced if unfrozen semen from the same ejaculate could be used for four or five consecutive days. Day-old and two-day-old sperm only were used in the current study, because although in vivo fertility is good for at least 96 hours, the in vitro fertility of older sperm is unknown. Since oocyte availability was low, it was decided not to risk reducing the numbers available for studying sperm concentrations by using older sperm with possible poor fertility.

5.1.2. Effects of sperm concentration on penetration.

It was noted above that penetration rates did not differ between sperm concentrations when day-old sperm was utilised. Just as many oocytes were penetrated at relatively low sperm concentrations ($0.125\text{x}10^6$/ml) as there were penetrated at the more usual concentrations of $1\text{-}4\text{x}10^6$/ml. Penetration rates may exhibit a curvilinear response to sperm numbers, with rates increasing with increasing sperm concentration until a plateau of maximum penetration is reached between 80% and 100%. If this is correct it is possible that all the concentrations tested lie in the plateau region of the curve for the day-old sperm used. Further experimentation should be carried out to verify the existence of a relationship between sperm concentration and penetration rates, and to determine the minimum concentration that can be used without reducing penetration below a satisfactory level.

It must be stressed that the minimal sperm concentration for IVF undoubtedly varies widely with individual bulls. Parrish *et al.* (1986) reported reducing sperm:egg ratios to 2000:1 for frozen-thawed sperm in some cases, and as low as 500:1 for one bull. In this study sperm:egg ratios ranged from 470:1 ($0.125\text{x}10^6$/ml) to 15000:1 ($4.0\text{x}10^6$/ml). Penetration *in vivo* is likely to occur when sperm:egg ratios are close to unity, based on numbers found in the reproductive tract (First and Parrish, 1987). Ratios must be much higher for IVF (at least with frozen-thawed sperm) because at present the media used for IVF are unable to maintain the viability of sperm at very high dilutions (First and Parrish, 1987). Literature on the effect of sperm concentration on oocyte penetration by unfrozen sperm is scant.

5.1.3. Effects of sperm concentration on polyspermy.

No significant relationship was apparent between the incidence of polyspermy and the concentrations of unfrozen sperm. This was unexpected, since polyspermy tends to increase with increasing sperm numbers (First and Parrish, 1987). The absence of a relationship may be due to the variability that was exhibited between observations; individual recordings of polyspermy ranged from 0-67%. Alternatively, polyspermy may exhibit a curvilinear response to sperm concentration similar to that suggested for penetration rates: levels of polyspermy generated at the various concentrations used here may have been at a maximum and in the plateau region of the response curve. In support of this, levels of polyspermy obtained here (24-42%) for concentrations above 0.25×10^6/ml were considerably higher than is normally reported (<15%, First and Parrish, 1987). To the author's knowledge there has been no investigation of the maximum level of polyspermy achievable with bovine sperm *in vitro*. Extremely high levels (for example, 75-100%) would seem to be unlikely in systems where predominantly mature oocytes with adequate polyspermic blocks are being utilised.

The fact that both penetration rates and levels of polyspermy were high in this experiment might suggest that the counting procedures used were underestimating the sperm numbers present. However, regular assessments by other laboratory members yielded similar counts, and the counting procedures were as specified by the manufacturers of the haemocytometer. In addition, counts for frozen-thawed sperm resulted in expected levels (based on laboratory experience) of penetration and polyspermy. Given this, the high levels of polyspermy are probably due to the longevity and motility of unfrozen sperm; high numbers of live, motile sperm increase the chance of more than one sperm entering an oocyte before the polyspermic block can be completed.

To further explore the incidence of polyspermy in IVF, the experiment should be repeated with polyspermy as the main factor of interest. Larger numbers of oocytes in the experimental units could be used (to increase the reliability of the results), and sperm concentrations above and below those reported here should be incorporated to explore the possibility of a curvilinear or other relationship.

5.1.4. Penetration achieved with frozen-thawed and unfrozen sperm.

The relationships between penetration rate and sperm concentration were significantly different for frozen-thawed and unfrozen (day-old) sperm. In section 5.1.2. it was suggested that the sperm concentrations used in the first part of this study were in the plateau region of a response curve for day-old sperm, and thus penetration rates were uniformly high for the sperm concentrations. In contrast, a strong linear relationship was exhibited between penetration rate and sperm concentration when frozen-thawed sperm was utilised. An average penetration rate of 84% was obtained at the concentration of $4x10^6$/ml, suggesting that the plateau of a curve for the frozen-thawed sperm used may begin around this level.

Penetration rates obtained with frozen-thawed sperm at concentrations below 4×10^6/ml appeared to be lower than those obtained with unfrozen sperm. This was expected since the viability and longevity of unfrozen samples of sperm is greater than that of sperm that have been frozen and thawed. The percentage of live cells in unfrozen semen after dilution is generally between 80-90% for sires used by the LIC. Sperm motility on average rates 7 out of 10 on a subjective scale. Motility and "percentage live" can remain at these levels for 3-5 days, depending on the packaging of the semen. In contrast, the percentage of live cells and motility of frozen sperm immediately after thawing are 55% and 8 respectively. Both of the characteristics degenerate with time: typical figures are 35% and 4 (4 hours after thawing) and 25% and 2 (after 24 hours) (E. Newey, pers. comm.).

Penetration rates obtained with frozen-thawed sperm in this experiment were slightly lower than the majority of results gained previously in the laboratory, an effect which is probably attributable to the fact that a reliable and repeatable IVM/IVF/IVC system was still being developed.

5.2. PRESENCE OF CAPROGEN EXTENDER IN THE FERTILISATION MEDIUM.

Little or no penetration occurred in media containing more than 10% Caprogen. The inability of sperm to penetrate oocytes in the presence of Caprogen could be attributable to one or more of the factors outlined below.

a) Sperm-directed inhibition: some constituent of the extender could act as a decapacitating agent or as a membrane stabiliser, thus inhibiting capacitation. Egg yolk lipoproteins are a possibility as they are known to adhere tenaciously to sperm, even after several washings (Cookson *et al.* 1984, Foulkes 1977). A coating of lipoprotein could effectively prevent capacitating agents such as heparin from binding to sperm membranes.

Another sperm inhibitor could be catalase, an enzyme that is added to semen extender to reduce levels of hydrogen peroxide. Hydrogen peroxide is a by-product of sperm metabolism which can be toxic in high concentrations. However, Bize *et al.* (1991) reported that hydrogen peroxide appeared to play a significant role during the capacitation of hamster sperm. Catalase, by inhibiting hydrogen peroxide, effectively prevented capacitation and the AR. In their experiment, catalase was used at a concentration of 10-20µg/ml, which is comparable to the 9µg/ml in the neat Caprogen extender used for this experiment.

b) Oocyte-directed inhibition: i. Some constituent of Caprogen may prevent capacitated sperm from binding to the zona pellucida, thus blocking penetration.

ii. High concentrations of Caprogen may actually be toxic to oocytes, causing their death and consequently preventing penetration.

c) High concentrations of Caprogen may create suboptimal conditions in the insemination environment by inducing shifts in pH or salt balances.

The third possibility could be eliminated by making the appropriate dilutions of fertilisation medium and Caprogen; salt concentrations and pHs of the various mixtures could then be identified at regular intervals.

Differentiating between possible sperm- and oocyte-directed effects is more difficult. To investigate possible toxic effects on oocytes, the oocytes could be inseminated and then transferred into a Caprogen-containing medium. Subsequent viability and cleavage rates could later be assessed. To study the effects of Caprogen on capacitation a series of tests could be conducted, based on the ability of lysophosphatidylcholine to induce the AR in capacitated sperm. Samples of sperm could a)be stored in Caprogen extender, b)undergo a wash procedure to remove the extender, c)be washed and then incubated in fertilisation medium, and d)be washed and then incubated in a fertilisation medium containing various amounts of Caprogen. The samples could then be exposed to lysophosphatidylcholine. Results would indicate whether Caprogen was decapacitating sperm in the fertilisation environment, and whether the action was direct or indirect.

It is unclear why penetration was low at the highest dilution tested (1ml/litre). This result was unexpected, since the dilution was essentially washed sperm and as so was part of the routine laboratory procedure. The depressed rate of penetration is attributable to two very low observations (see Table 8), but these could not be related to any particular bull, ejaculate or semen age.

Overall, penetration rates were much lower in this experiment than in the first. This could be a function of several factors, such as different bull ejaculates, poor batches of ovaries, or the fertilisation medium. Batches of media can differ slightly regardless of the care taken in their production.

5.3. PRESENCE OF HEPARIN IN THE FERTILISATION MEDIUM.

In this study no relationship existed between penetration rates and concentrations of heparin in the medium. Penetration rates were good (with the exception of a few rather low observations) and 69% penetration was obtained in the absence of heparin. These results imply that heparin was not a necessary part of the medium in this experiment. However, because only one bull was involved in this study, only limited conclusions can be drawn since the result may have been specific to that individual. A fourth experiment (using sperm from five bulls, including the bull used in the third experiment) was therefore conducted to further investigate oocyte penetration in the absence of heparin. Unfrozen sperm was also used at 10µg/ml heparin to ensure that any depression of penetration encountered in the absence of heparin was attributable to this variable rather than to a fault in the sperm. Frozen-thawed sperm from the same ejaculates were also tested in heparin-free medium to ascertain whether penetration in the absence of heparin was limited to unfrozen sperm.

Penetration rates with unfrozen sperm in the absence of heparin ranged between 47% and 86%, confirming that high penetration can occur without heparin under the conditions of the study. Rates of penetration obtained with frozen-thawed sperm without heparin ranged between 23% and 88% for the five bulls, implying that the effect is not confined to unfrozen sperm. Rates achieved in the presence of heparin appeared to be slightly higher than those without heparin (although a smaller number of oocytes was assessed for the heparin treatment), which may suggest that heparin, although not essential, may improve penetration rates for some bulls.

First and Parrish (1987) suggested that the investigation of capacitation techniques (such as the use of HIS medium) may often be confounded by a carryover of heparin from the oocyte collection medium. This is unlikely to have been the cause of the good results obtained here in the absence of heparin. Although heparin was used in the collection medium (to prevent agglutination of oocytes with themselves and the dish), carryover of heparin into the fertilisation environment probably minuscule - somewhere in the region of $5x10^{-5}$µg/ml - and much lower than levels normally incorporated into capacitation systems.

The lack of a relationship between heparin concentration and oocyte penetration in the third experiment was unexpected. Work in this laboratory has shown that rates with frozen-thawed sperm increase with heparin concentration before declining around 50µg/ml (J. Catt, pers. comm.). Furthermore, Lu and Gordon (1988) preincubated frozen-thawed sperm in media containing 0, 10, 50 and 100µg/ml heparin and found that oocyte penetration rates increased with increasing heparin concentration. The lack of a relationship seen here may be attributable to a large variability between observations. Alternatively, it may indicate that heparin is not a necessary component of fertilisation media for unfrozen sperm.

Sperm stimulating factors (caffeine, epinephrine and hypotaurine) were present in the medium. These may have created an environment conducive to capacitation and subsequent penetration. Combined with large numbers of motile, unfrozen sperm, they may have removed any requirement for heparin. Heparin may have an effect when conditions are suboptimal: this could be tested by inseminating oocytes at very low concentrations of unfrozen sperm, or by removing other sperm stimulating factors from the medium.

There is clearly a need to further investigate the interactions of heparin, caffeine and other sperm stimulants. Niwa and Ohgoda (1988) and Ohgoda *et al.* (1988) have carried out some studies on heparin and caffeine. However, a single, comprehensive experiment has not yet been performed. To the author's knowledge, no work has been reported that involves the combined effects of heparin, caffeine and PHE on frozen-thawed or unfrozen sperm. As a suggestion, a large-scale factorial experiment involving all combinations of caffeine, heparin and PHE might be carried out using frozen-thawed and unfrozen sperm from five bulls. Approximately 4500 oocytes would be required if 50 oocytes were to be assessed for each of the 80 (8x2x5) factorial cells - 4000 oocytes plus approximately 10% for losses throughout the IVM/IVF procedure. This would be achievable over two or three weeks in a large laboratory with access to a consistent supply of ovaries.

To summarise the findings from this study, good penetration rates can be obtained with unfrozen sperm. In vitro fertility may be depressed if older sperm are used at concentrations below $1x10^6$/ml. For best results, sperm must be washed out of the Caprogen extender. Unfrozen sperm do not appear to require the presence of heparin in the fertilisation medium. However, further investigation of the interactions between heparin and sperm stimulants need to be carried out. Unfrozen sperm may differ from frozen-thawed sperm in certain respects, notably their requirements for medium constituents and the concentration of sperm that may be successfully used.

The greater longevity and viability of unfrozen sperm makes it a useful alternative to frozen semen in both research and commercial IVF situations. The use of unfrozen bovine sperm is of particular relevance in New Zealand, since unfrozen semen is used for the majority of artificial inseminations of dairy cattle in this country.

REFERENCES

AHUJA, K.K.; (1984). Lectin-coated agarose beads in the investigation of sperm capacitation in the hamster. Developmental Biology 104:131-142.

AUSTIN, C.R.; (1951). Observations on the penetration of sperm into the mammalian egg. Australian Journal of Scientific Research 34:581-589.

AX, R.L.; LENZ, R.W.; (1987). Glycosaminoglycans as probes to monitor differences in fertility of bulls. Journal of Dairy Science 70:1477-1486.

BALL, G.D.; LEIBFRIED, M.L.; LENZ, R.W.; AX, R.L.; BAVISTER, B.D.; FIRST, N.L.; (1983). Factors affecting successful in vitro fertilization of bovine follicular oocytes. Biology of Reproduction 28:717-725.

BEDFORD, J.M.; (1983). Significance of the need for sperm capacitation before fertilization in eutherian mammals. Biology of Reproduction 28:108-120.

BERG, U.; BREM, G.; (1990). Developmental rates of in vitro produced IVM-IVF bovine oocytes in different cell co-culture systems. Theriogenology 33:195 (abstract).

BIRD, J.M.; CAREY, S.; HOUGHTON, J.A.; (1989). Motility and acrosomal changes in ionophore-treated bovine spermatozoa and their relationship with in vitro penetration of zona-free hamster oocytes. Theriogenology 32:227-241.

BIZE, I.; SANTANDER, G.; CABELLO, P.; DRISCOLL, D.; SHARPE, C.; (1991). Hydrogen peroxide is involved in hamster sperm capacitation *in vitro*. Biology of Reproduction 44:398-403.

BOCCART, C.; MERMILLOD, P.; DELECOEUILLERIE, C.; DESSY, F.; (1991). Bovine oviduct cell monolayers for supporting the blastocyst formation of bovine embryos. Theriogenology 35:187 (abstract).

BONDIOLI, K.R.; WRIGHT, R.W.; (1980). Influence of culture media on in vitro fertilization of ovine tubal oocytes. Journal of Animal Science 51:660-667.

BONDIOLI, K.R.; WRIGHT, R.W.; (1983). In vitro fertilization of bovine oocytes by spermatozoa capacitated *in vitro*. Journal of Animal Science 57:1001-1005.

BRACKETT. B.G.; BOUSQUET, D.; BOICE, M.L.; DONAWICK, W.J.; EVANS, J.F.; DRESSEL, M.A.; (1982). Normal development following in vitro fertilization in the cow. Biology of Reproduction 27:147-158.

BRACKETT, B.G.; OH, Y.K.; EVANS, J.F.; DONAWICK, W.J.; (1980). Fertilization and Early Development of Cow Ova. Biology of Reproduction 23:189-205.

BRACKETT, B.G.; OLIPHANT, G.; (1975). Capacitation of rabbit spermatozoa *in vitro*. Biology of Reproduction 12:260-274.

BRAUN, J.; (1988). Influence of protein supplement and culture conditions on cumulus cell expansion and nuclear maturation of sheep follicular oocytes. Theriogenology 29:228 (abstract).

CATT, J.W.; RHODES, S.L.; GURNSEY, M.P.; WELCH, R.A.S.; SEATON, A.D.; (1990). Optimal treatment of sperm for in vitro production of embryos. Proceedings of the 8th conference of the Australian Association of Animal Breeding and Genetics. p549 (abstract).

CATT, J.W.; RHODES, S.L.; WELCH, R.A.S.; (1991). Viable embryos produced by in vitro maturation, fertilisation and culture of bovine oocytes. Journal of Reproduction (submitted).

CHANG, M.C.; (1951). Fertilizing capacity of spermatozoa deposited in the fallopian tubes. Nature 168:697.

CHEN-LU, H.B.; LU, K.H.; (1990). Effect of protein supplements on in vitro maturation, fertilization and culture to blastocyst and hatching stage of bovine oocytes. Theriogenology 33:205 (abstract).

COOKSON, A.D.; THOMAS, A.N.; FOULKES, J.A.; (1984). Immunochemical investigation of the interaction of egg-yolk lipoproteins with bovine spermatozoa. Journal of Reproduction and Fertility 70: 599-604.

CRITSER, E.S.; LEIBFRIED, M.L.; FIRST, N.L.; (1984). The effect of semen extension, cAMP and caffeine on in vitro fertilization of bovine oocytes. Theriogenology 21:625-631.

CRITSER, E.S.; LEIBFRIED-RUTLEDGE, M.L.; EYESTONE, W.H.; NORTHEY, D.L.; FIRST, N.L.; (1986a). Acquisition of developmental competence during maturation *in vitro*. Theriogenology 25:150 (abstract).

CRITSER, E.S.; LEIBFRIED-RUTLEDGE, M.L.; FIRST, N.L.; (1986b). Influence of cumulus cell association during in vitro maturation of bovine oocytes on embryonic development. Biology of Reproduction 34:192 (abstract).

CROSBY, I.M.; OSBORN, J.C.; MOOR, R.M.; (1981). Follicle cell regulation of protein synthesis and developmental competence in sheep oocytes. Journal of Reproduction and Fertility 62:575-582.

CROSBY, I.M.; OSBORN, J.C.; MOOR, R.M.; (1984). Changes in protein phosphorylation during the maturation of mammalian oocytes *in vitro*. Journal of Experimental Zoology 221:459-466.

DELGADO, N.M.; HUACUJA, L.; PANCARDO, R.M.; MERCHANT, H.; ROSADO, A.; (1976). Changes in the protein conformation of human spermatozoal membranes after treatment with cyclic adenosine 3':5'-monophosphate and human follicular fluid. Fertility and Sterility 27:413-420.

DIDION, B.A.; GRAVES, C.N.; (1989). Influence of calcium on the true acrosome reaction and capacity of bull spermatozoa to penetrate oocytes *in vitro*. Journal of Dairy Science 72:1540-1546.

ELLINGTON, J.E.; FARRELL, P.B.; SIMKIN, M.E.; FOOTE, R.H.; GOLDMAN, E.E.; McGRATH, A.B.; (1990). Development and survival after transfer of cow embryos cultured from 1-2 cells to morulae or blastocysts in rabbit oviducts or in a simple medium with bovine oviduct epithelial cells. Journal of Reproduction and Fertility 89:293-299.

EYESTONE, W.H.; FIRST, N.L.; (1989). Co-culture of early cattle embryos to the blastocyst stage with oviducal tissue or in conditioned medium. Journal of Reproduction and Fertility 85:715-720.

FIRST, N.L.; PARRISH,J.J.; (1987). In vitro fertilization of ruminants. Journal of Reproduction and Fertility, Supplement 34:151-165.

FLORMAN, H.M.; FIRST, N.L.; (1988). The regulation of acrosomal exocytosis. Developmental Biology 128:453-463.

FONTES, R.S.; ECTORS F.J.; DELVAL, A.; TOUATI, K.; BECKERS, J.F.; ECTORS,F.; (1991). Rabbit and bovine oviductal epithelial cell culture media are able to support early development from zygote to the blastocyst stage. Theriogenology 35:201 (abstract).

FOULKES, J.A.; (1977). The separation of lipoproteins from egg-yolk and their effect on freezing of bovine spermatozoa. Journal of Reproduction and Fertility 49:277-284.

FRASER, L.R.; (1979). Accelerated mouse sperm penetration *in vitro* in the presence of caffeine. Journal of Reproduction and Fertility 57:377-384.

FUKUDA, Y.; ICHIKAWA, M.; NAITO, K.; TOYODA, Y.; (1990). Birth of normal calves resulting from bovine oocytes matured, fertilized and cultured with cumulus cells *in vitro* up to the blastocyst stage. Biology of Reproduction 42:114-119.

FUKUI, Y.; (1989). Effects of sera and steroid hormones on development of bovine oocytes matured and fertilized *in vitro* and co-cultured with bovine oviduct epithelial cells. Journal of Animal Science 67:1318-1323.

FUKUI, Y.; FUKUSHIMA, M.; ONO, H.; (1983). Fertilization *in vitro* of bovine oocytes after various sperm procedures. Theriogenology 20:651-660.

FUKUI, Y.; FUKUSHIMA, M.; TERAWAKI, Y.; ONO, H.; (1982). Effect of gonadotropins, steroids and culture media on bovine oocyte maturation *in vitro*. Theriogenology 18:161-175.

FUKUI, Y.; GLEW, A.M.; GANDOLFI, F.; MOOR, R.M.; (1988). Ram-specific effects on in vitro fertilization and cleavage of sheep oocytes matured *in vitro*. Journal of Reproduction and Fertility 82:337-340.

FUKUI, Y.; ISHIDA, S. TERWAKI, Y.; (1981). Effects of synthetic media and nutritional sources on in vitro maturation of bovine follicular oocytes. Research Bulletin of Obihiro University, series I12:185-193.

FUKUI, Y.; ONO, H.; (1989). Effects of sera, hormones and granulosa cells added to culture medium for in vitro maturation, fertilization, cleavage and development of bovine oocytes. Journal of Reproduction and Fertility 86:501-506.

FUKUI, Y.; SAKUMA, Y.; (1980). Maturation of bovine oocytes cultured *in vitro*: relation to ovarian activity, follicular size and the presence or absence of cumulus cells. Biology of Reproduction 22:669-673.

FUKUI, Y.; SONOYAMA, T.; MOCHIZUKI, H.; ONO, H.; (1990). Effects of heparin dosage and sperm capacitation time on in vitro fertilization and cleavage of bovine oocytes matured *in vitro*. Theriogenology 34:579-591.

FUKUI, Y.; URAKAWA, M.; SASAKI, C.; CHIKAMATSU, N.; ONO, H.; (1989). Development to the late morula or blastocyst stage following in vitro maturation and fertilization of bovine oocytes. Animal Reproduction Science 18:139-148.

FUKUSHIMA, M.; FUKUI, Y.; (1985). Effects of gonadotropins and steroids on the subsequent fertilizability of extrafollicular bovine oocytes cultured *in vitro*. Animal Reproduction Science 9:323-332.

GANDOLFI, F.; BREVINI, T.A.L.; MODINA, S.; LAURIA, A.; (1990). Embryogenesis *in vitro*: role of culture media and co-culture. Proceedings of the 3rd Symposium on Advanced Topics in Animal Reproduction. pp13-25.

GARBERS, D.L.; FIRST, N.L.; SULLIVAN, J.J; LARDY, H.A.; (1971a). Stimulation and maintenance of ejaculated bovine spermatozoan respiration and motility by caffeine. Biology of Reproduction 5:336-339.

GARBERS, D.L.; LUST, W.D.; FIRST, N.L.; LARDY, H.A.; (1971b). Effects of phosphodiesterase inhibitors and cyclic nucleotides on sperm respiration and motility. Biochemistry N.Y. 10:1825-1831.

GORDON, I.; (1990). Laboratory production of cattle embryos. Proceedings of the 3rd Symposium on Advanced Topics in Animal Reproduction. pp63-87.

GORDON, I.; LU, K.H.; (1990). Production of embryos *in vitro* and its impact on livestock production. Theriogenology 33:77-87.

GOTO, K.; KAJIHARA, Y.; KOBA, M.; KOSAKA, S.; NAKANISHI, Y.; OGAWA, K.; (1989). In vitro fertilization and development of in vitro matured bovine follicular oocytes. Journal of Animal Science 67:2181-2185.

GOTO, K.; KAJIHARA, Y.; KOSAKA, S.; KOBA, M.; NAKANISHI, Y.; OGAWA, K.; (1988). Pregnancies after co-culture of cumulus cells with bovine embryos derived from in vitro fertilization of in vitro matured follicular oocytes. Journal of Reproduction and Fertility 83:753-758.

GRAHAM, J.K.; FOOTE, R.H.; HOUGH, S.R.; (1987). Penetration of zona-free hamster eggs by liposome-treated sperm from the bull, ram, stallion and boar. Biology of Reproduction 37:181-188.

GREVE, T.; BOUSQUET, D.; KING, W.A.; BETTERIDGE, K.J.; (1984). In vitro fertilization and cleavage of in vitro matured oocytes. Theriogenology 22:151-165.

HANDROW, R.R.; LENZ, R.W.; AX, R.L.; (1982). Structural comparisons among glycosaminoglycans to promote an acrosome reaction in bovine spermatozoa. Biochemical and Biophysical Research Communications 107:1326-1332.

HANDROW, R.R.; BOEHM, S.K.; LENZ, R.W.; ROBINSON, J.A.; AX, R.L.; (1984). Specific binding of the glycosaminoglycan ^{3}H-heparin to bull, monkey and rabbit spermatozoa *in vitro*. Journal of Andrology 5:51-63.

HENSLEIGH, H.C.; HUNTER, A.G.; (1985). In vitro maturation of bovine cumulus-enclosed primary oocytes and their subsequent in vitro fertilization and cleavage. Journal of Dairy Science 68:1456-1462.

HILLERY, F.L.; PARRISH, J.J.; FIRST, N.L.; (1990). Bull specific effect on fertilization and embryo development *in vitro*. Theriogenology 33:249 (abstract).

IRITANI, A.; KASAI, M.; NIWA, K.; SONG, H.B.; (1984). Fertilization *in vitro* of cattle follicular oocytes with ejaculated spermatozoa capacitated in a chemically defined medium. Journal of Reproduction and Fertility 70:487-492.

IRITANI, A.; NIWA, K.; (1977). Capacitation of bull spermatozoa and fertilization *in vitro* of cattle follicular oocytes matured in culture. Journal of Reproduction and Fertility 50:119-121.

KATSKA, L.; (1984). Comparison of two methods for recovery of ovarian oocytes from slaughter cattle. Animal Reproduction Science 7:461-463.

KATSKA, L.; SMORAG, Z.; (1984). Number and quality of oocytes in relation to age of cattle. Animal Reproduction Science 7:451-460.

KIM, C.I.; ELLINGTON, J.; FOOTE, R.H.; (1989). In vitro maturation, fertilization and development of bovine oocytes. Theriogenology 31:210 (abstract).

KIM, C.I.; ELLINGTON, J.E.; FOOTE, R.H.; (1990). Maturation, fertilization and development of bovine oocytes *in vitro* using TCM199 and a simple defined medium with co-culture. Theriogenology 33:433-440.

LAMBERT, R.D.; SIRARD, M.A.; BERNARD, C.; BELAND, R.; RIOUX, J.E.; LECLERC, P.; MENARD, D.P.; BEDOYA, M.; (1986). In vitro fertilization of bovine oocytes matured *in vivo* and collected at laparoscopy. Theriogenology 25:117-133.

LANGLAIS, J.; ROBERTS, K.D.; (1985). A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. Gamete Research 12:183-224.

LEIBFRIED, L.; FIRST, N.L.; (1979). Characterization of bovine follicular oocytes and their ability to mature *in vitro*. Journal of Animal Science 48:76-86.

LEIBFRIED, L.; FIRST, N.L.; (1980). Effect of bovine and porcine follicular fluid and granulosa cells on maturation of oocytes *in vitro*. Biology of Reproduction 23:699-704.

LEIBFRIED-RUTLEDGE, M.L.; CRITSER, E.S.; EYESTONE, W.H.; NORTHEY, D.L.; FIRST, N.L.; (1987). Development potential of bovine oocytes matured *in vitro* or *in vivo*. Biology of Reproduction 36:376-383.

LEIBFRIED-RUTLEDGE, M.L.; CRITSER, E.S.; FIRST, N.L.; (1985). Fertilization potential of follicular oocytes classified by stage of cycle and size of follicle. Theriogenology 23:753-759.

LEIBFRIED-RUTLEDGE, M.L.; CRITSER, E.S.; FIRST, N.L.; (1986). Effects of fetal calf serum and bovine serum albumin on in vitro maturation and fertilization of bovine and hamster cumulus-oocyte complexes. Biology of Reproduction 35:850-857.

LEIBFRIED-RUTLEDGE, M.L.; CRITSER, E.S.; PARRISH, J.J.; FIRST, N.L.; (1989). In vitro maturation and fertilization of bovine oocytes. Theriogenology 31:61-74.

LENZ, R.W.; BALL, G.D.; LEIBFRIED, M.L.; AX, R.L.; FIRST, N.L.; (1983a). In vitro maturation and fertilization of bovine oocytes are temperature-dependent processes. Biology of Reproduction 29:173-179.

LENZ, R.W.; BALL, G.D.; LOHSE, J.K.; FIRST, N.L.; AX, R.L.; (1983b). Chondroitin sulfate facilitates an acrosome reaction in bovine spermatozoa as evidenced by light microscopy, electron microscopy and in vitro fertilization. Biology of Reproduction 28:683-690.

LING, Z.J.; LU, K.H.; (1990). Frequency of cleavage and development *in vitro* of bovine oocytes fertilized in different numbers in drops with different sperm concentrations. Theriogenology 33:275 (abstract).

LONERGAN, P.; VERGOS, E.; KINIS, A.; SHARIF, H.; GALLAGHER, M.; GORDON, I.; (1991). The effect of recovery method on the type of bovine oocyte obtained for in vitro maturation. Theriogenology 35:187 (abstract).

LU, K.H.; GORDON, I.; (1988). Effect of heparin on the capacitation of frozen-thawed bovine spermatozoa used in the in vitro fertilization (IVF) of oocytes matured in vitro. Proceedings of the 11th International Congress on Animal Reproduction and Artificial Insemination (Volume 3):339 (short communication).

McGOWAN, L.T.; JAMES, R.W.; FUKUI, Y.; PUGH, P.A.; TERVIT, H.R.; (1990). Factors affecting the in vitro maturation, fertilisation and culture of bovine oocytes. Proceedings of the 22nd Annual Meeting of the Australian Society of Reproductive Biology. p87 (abstract).

McLAUGHLIN, K.J.; McLEAN, D.M.; STEVENS, G.; ASHMAN, R.A.; LEWIS, P.A.; BARTSCH, B.D.; SEAMARK, R.F.; (1990). Viability of one-cell bovine embryos cultured in synthetic oviduct fluid. Theriogenology 33:1191-1199.

MARION, G.B.; GIER, H.T.; CHOUDARY, J.B.; (1968). Micromorphology of the bovine follicular system. Journal of Reproduction and Fertility 27:451-465.

MEIZEL, S.; (1985). Molecules that initiate or help stimulate the acrosome reaction by their interaction with the mammalian sperm surface. American Journal of Anatomy 174:285-302.

MILLER, D.J.; HUNTER, A.G.; (1987). Individual variation for in vitro fertilization success in dairy bulls. Journal of Dairy Science 70:2150-2153.

MILLER, D.J.; WINER, M.A.; AX, R.L.; (1990). Heparin-binding proteins from seminal plasma bind to bovine spermatozoa and modulate capacitation by heparin. Biology of Reproduction 42:899-915.

MOODIE, G.R.; GRAHAM, E.F.; (1989). The effect of incubating sheep ovaries for various times and temperatures on oocyte maturation rates *in vitro*. Biology of Reproduction 40:53 (abstract).

MOOR, R.M.; CROSBY, I.M.; (1985). Temperature-induced abnormalities in sheep oocytes during maturation. Journal of Reproduction and Fertility 75:467-473.

NAKAO, H.; NAKATSUJI, N.; (1990). Effects of co-culture, medium components and gas phase on in vitro culture of in vitro matured and in vitro fertilised bovine embryos. Theriogenology 33:591-600.

NELSON, L.; (1967). Sperm Motility. In: Fertilisation (edited by Metz, C.B. and Monroy, A., published by Academic Press, N.Y.). pp27-97.

NIWA, K.; OHGODA, O.; (1988). Synergistic effect of caffeine and heparin on in vitro fertilization of cattle oocytes matured in culture. Theriogenology 30:733-741.

OHGODA, O.; NIWA, K.; YUHARA, M.; TAKAHASHI, S.; KANOYA, K.; (1988). Variations in penetration rates *in vitro* of bovine follicular oocytes do not reflect conception rates after artificial insemination using frozen semen from different bulls. Theriogenology 29:1375-1381.

OLIPHANT, G.; BRACKETT, B.G.; (1973a). Capacitation of mouse spermatozoa in media with elevated ionic strength and reversible decapacitation with epididymal extracts. Fertility and Sterility 24:948-955.

OLIPHANT, G.; BRACKETT, B.G.; (1973b). Immunological assessment of surface changes of rabbit sperm undergoing capacitation. Biology of Reproduction 9:404-414.

OLIPHANT, G.; REYNOLDS, A.B.; THOMAS, T.S.; (1985). Sperm surface components involved in the control of the acrosome reaction. American Journal of Anatomy 174:269-283.

ONUMA, H.; FOOTE, R.H.; (1969). In vitro development of ova from prepuberal cattle. Journal of Dairy Science 52:1085-1087.

PARK, C-K.; OHGODA, O.; NIWA, K.; (1989). Penetration of bovine follicular oocytes by frozen-thawed spermatozoa in the presence of caffeine and heparin. Journal of Reproduction and Fertility 86:577-582.

PARRISH, J.J.; SUSKO-PARRISH, J.L.; FIRST, N.L.; (1985). Effect of heparin and chondroitin sulfate on the acrosome reaction and fertility of bovine sperm *in vitro*. Theriogenology 24:537-549.

PARRISH, J.J; SUSKO-PARRISH, J.L.; FIRST, N.L.; (1989a). Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. Biology of Reproduction 41:683-699.

PARRISH, J.J.; SUSKO-PARRISH, J.L.; HANDROW, R.R.; SIMS, M.M.; FIRST, N.L.; (1989b). Capacitation of bovine spermatozoa by oviduct fluid. Biology of Reproduction 40:1020-1025.

PARRISH, J.J.; SUSKO-PARRISH, J.L.; LEIBFRIED-RUTLEDGE, M.L.; CRITSER, E.S.; EYESTONE, W.H.; FIRST, N.L.; (1986). Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 25:591-600.

PARRISH, J.J.; SUSKO-PARRISH, J.L.; WINER, M.A.; FIRST, N.L.; (1988). Capacitation of bovine sperm by heparin. Biology of Reproduction 38:1171-1180.

PAVLOK, A.; TORNER, H.; MOTLIK, J.; FULKA, J.; KAUFFOLD, P.; DUSCHINSKI, U.; (1988). Fertilization of bovine oocytes *in vitro*: effect of different sources of gametes on fertilization rate and frequency of fertilization anomalies. Animal Reproduction Science 16:207-213.

QUIRKE, J.F.; GORDON, I.; (1971). Culture and fertilization of sheep ovarian oocytes - I. Effect of culture medium on resumption of meiosis. Journal of Agricultural Science, Cambridge 76:369-372.

SAEKI, K.; LEIBFRIED-RUTLEDGE, M.L.; FIRST, N.L.; (1990). Are fetal calf serum and hormones necessary during in vitro maturation of cattle oocytes for subsequent development? Theriogenology 33:316 (abstract).

SANBUISSHO, A.; THRELFALL, W.R.; (1985). The effects of estrous cow serum on the maturation and fertilization of the bovine follicular oocyte *in vitro*. Theriogenology 23:226 (abstract).

SANBUISSHO, A.; THRELFALL, W.R.; (1990). The influence of serum and gonadotropins on in vitro maturation and fertilization of bovine oocytes. Theriogenology 34:341-348.

SCHELLANDER, K.; FUHRER, F.; BRACKETT, B.G.; KORB, H.; SCHLEGER, W.; (1990). In vitro fertilization and cleavage of bovine oocytes matured in medium supplemented with estrous cow serum. Theriogenology 33:477-485.

SHEA, B.F.; LATOUR, J.P.A.; BEDIRIAN, K.N.; BAKER, R.D.; (1976). Maturation *in vitro* and subsequent penetrability of bovine follicular oocytes. Journal of Animal Science 43:809-815.

SHI, D.S.; LU, K.H.; GORDON, I.; (1990). Effects of bulls on fertilization of bovine oocytes and their subsequent development *in vitro*. Theriogenology 33:324 (abstract).

SHIOYA, Y.; KUWAYAMA, M.; FUKUSHIMA, M.; IWASAKI, S.; HANADA, A. (1988a). In vitro fertilization and cleavage capability of bovine follicular oocytes classified by cumulus cells and matured *in vitro*. Theriogenology 30:489-496.

SHIOYA, Y.; KUWAYAMA, M.; UEDA, S.; SAITOU, S.; OOTA, H.; HANADA. A.; (1988b). Effect of the time between slaughter and aspiration of follicles on the developmental capability of bovine oocytes matured and fertilised in vitro. Japanese Journal of Animal Reproduction 34:39-44.

SIDHU, K.S.; GURAYA, S.S.; (1989). Cellular and molecular biology of capacitation and acrosome reaction in mammalian spermatozoa. International Review of Cytology 118:231-280.

SIRARD, M.A.; LAMBERT, R.D. (1985). In vitro fertilisation of bovine follicular oocytes obtained by laparoscopy. Biology of Reproduction 33:487-494.

SIRARD, M.A.; LAMBERT, R.D.; MENARD, D.P.; BEDOYA, M.; (1985). Pregnancies after in vitro fertilization of cow follicular oocytes, their incubation in rabbit oviduct and their transfer to the cow uterus. Journal of Reproduction and Fertility 75:551-556.

SIRARD, M.A.; PARRISH, J.J.; WARE, C.B.; LEIBFRIED-RUTLEDGE, M.L.; FIRST, N.L.; (1988). The culture of bovine oocytes to obtain developmentally competent embryos. Biology of Reproduction 39:546-552.

SREENAN, J.; (1970). In vitro maturation and attempted fertilization of cattle follicular oocytes. Journal of Agricultural Science, Cambridge 75:393-396.

SREENAN, J.; SCANLON, P.; GORDON, I.; (1968). Culture of fertilized cattle eggs. Journal of Agricultural Science, Cambridge 70:183-185.

STAIGMILLER, R.B.; MOOR, R.M.; (1984). Effect of follicle cells on the maturation and developmental competence of ovine oocytes matured outside the follicle. Gamete Research 9:221-229.

STUBBINGS, R.B.; BETTERIDGE, K.J.; BASRUR, P.K.; (1988). Investigations of culture requirements for bovine oocyte maturation *in vitro*. Theriogenology 29:313 (abstract).

STUBBINGS, R.B.; LIPTRAP, R.M.; BASRUR, P.K.; (1989). Estradiol and progesterone concentrations in fetal bovine serum and their implication in maturation and fertilization *in vitro*. Theriogenology 31:260 (abstract).

SUSKO-PARRISH, J.L.; PARRISH, J.J.; HANDROW, R.R.; (1985). Glucose inhibits the action of heparin by an indirect mechanism. Biology of Reproduction 32 (supplement 1):80 (abstract).

TAN, S.J.; LU, K.H.; (1990). Effects of different oestrous stages of ovaries and sizes of follicles on generation of bovine embryos *in vitro*. Theriogenology 33:335.

TROUNSON, A.O.; WILLADSEN, S.M.; ROWSON, L.E.A.; (1977). Fertilization and development capability of bovine follicular oocytes matured *in vitro* and *in vivo* and transferred to the oviducts of rabbits and cows. Journal of Reproduction and Fertility 51:321-327.

WHEELER, M.B.; SEIDEL, G.E.; (1989). Capacitation of bovine spermatozoa by lysophospholipids and trypsin. Gamete Research 22:193-204.

WRIGHT, R.W.; BONDIOLI, K.R.; (1981). Aspects of in vitro fertilization and embryo culture in domestic animals. Journal of Animal Science 53:702-729.

XU, K.P.; GREVE, T.; CALLESEN, H.; HYTTEL, P.; (1987). Pregnancy resulting from cattle oocytes matured and fertilised *in vitro*. Journal of Reproduction and Fertility 81:501-504.

XU, K.P.; KING, W.A.; (1990). The biology of mammalian fertilization and embryo development. AgBiotech News and Information 2:25-28.

YANG, Y.B.; LU, K.H.; (1990). The influence of bovine oocyte type on in vitro fertilization and subsequent development *in vitro*. Theriogenology 33:355 (abstract).

YANG, N.S.; LU, K.H.; GORDON, I.; (1990). In vitro fertilization (IVF) and culture (IVC) of bovine oocytes from stored ovaries. Theriogenology 33:352 (abstract).

APPENDICES

APPENDIX 1 - MEDIA RECIPES.

1.1.PBS

	g/5L
NaCl	40
KCl	1
KH_2PO_4	1
Na_2HPO_4	10.8

Dissolve compounds in 5L RO (reverse osmosis) water. Adjust pH to 7.2-7.4. Adjust osmolarity to 275-285. Aliquot in to glass Schott bottles (loose tops) and autoclave on wet cycle for 20min. When cool after autoclaving, add 1ml antibiotics (see below) per 100ml PBS and store for up to 3 months at 4°C.

1.2. Antibiotics

	mg/100ml
Penicillin	3
Streptomycin	5
Neomycin	10

Dissolve in stirring ddH_2O and make up to 100ml in a volumetric flask. Filter for use immediately or store frozen (-20°C).

2. Maturation Media

2.1. B199 Stock (10X)

ddH_2O	100ml
M199 (Gibco, Earles Salts)	1L packet
Kanamycin	0.075g
Hepes	4.0g

Filter and store in 10ml aliquots in refrigerator (4°C) for up to 2 months.

2.2. Additives (20X)

	mg/100ml
Vitamin B_{12}	2
Insulin	20
Polyvinyl Alcohol	100
Ascorbic acid	150
Inositol	10
Na Acetate	200
Glucosamine	400

Dissolve compounds in stirring ddH_20 and bring to 100ml in a volumetric flask. Filter and store in 5ml aliquots in freezer (-20°C) for up to 3 months.

2.3. B199 (1X) (made up weekly)

	/100ml
B199 stock (10X)	10ml
Additives (20X)	5ml
Pyruvate	30mg
$NaHCO_3$	240mg
Glutamine	10mg

Add compounds to stirring ddH_2O and bring to 100ml in a volumetric flask. Adjust pH to 7.9 with 1M NaOH. Measure osmolarity (should be 275-285). Filter and store in plastic universal containers in 18ml aliquots at 4°C for up to 1 week.

2.4. B199+ (made up daily)

B199 (1X)	18ml
Oestrous cow serum	1ml
Foetal calf serum	1ml

Equilibrate universal container overnight in CO_2 incubator. Or equilibrate 2 ml aliquots in 3cm dishes for 15 min in CO_2 incubator.

3. Bench Top Media (H199)

		g/1000ml
	M199 (Gibco, Earles Salts)	1L packet
or	M199 (10X stock)	100ml
	Kanamycin	0.075g
	Hepes	7.08g

Dissolve compounds in stirring ddH_2O making up to 1000ml. Adjust pH to 7.2-7.4. Adjust osmolarity (275-285). Filter and store at 4°C for up to 3 months.

3.1. Heparin Stock (100X)

	mg/10ml
Heparin	5

Dissolve in 10ml ddH_2O. Filter and store in 0.5ml aliquots at -20°C.

3.2. H199+ (made up daily)

H199	18ml
Foetal calf serum	2ml

Warm for 1hr in bench top incubator before use.

NOTE:For oocyte collection - add 20µl Heparin Stock to 2ml H199+.

4.Equilibration of paraffin oil

Place 25ml of non-equilibrated sperm wash media in 250ml plastic flask and add 200ml of oil. Shake slowly for 15 seconds and equilibrate with loose top for at least 2 days in CO_2 incubator.

4.1. Sperm Wash (TALP)

	g/1000ml
NaCl	5.3
KCl	0.23
Na_2HPO_4	0.04
$MgCl_2.6H_2O$	0.31
$NaHCO_3$	2.1
Na lactate (60% syrup)	370µl
Phenol red	0.005
Kanamycin	0.075
BSA (fraction V)	6.0
Na pyruvate	1.0
Caffeine	0.1

Dissolve compounds in 1000ml stirring ddH_2O. Adjust pH to 7.8-7.9. Adjust osmolarity (275-285). Filter and store at 4°C for up to 3 months.

Equilibrate 10ml in a centrifuge tube with a loose top overnight in incubator prior to sperm washing.

5. IVF Media

5.1. IVF Stock (1X) (made up monthly)

	mg/50ml
KCl	12
NaCl	272
$CaCl_2.2H_2O$	39
$MgCl_2.6H_2O$	5
$NaHCO_3$	110
Na_2HPO_4	2.3
Kanamycin	3.8
Na lactate (70% syrup)	93µl
Glucose	50
BSA (fatty acid free)	300
Hypotaurine	25µl (from fresh stock 1mg/ml)
Epinephrine	25µl (" " ")
Heparin	50µl (from fresh stock 10mg/ml)
Caffeine	13.5
Phenol red	0.5

Dissolve in stirring ddH_2O and make up to 50ml in a volumetric flask. Adjust pH to 7.8-7.9. Adjust osmolarity to 275-285. Filter and store in 10ml aliquots at 4°C for up to 5 weeks.

5.2. IVF Medium (made up weekly)

Dissolve 3mg pyruvate in 10ml IVF Stock (1X). Store in 2ml aliquots at 4°C. Equilibrate 2ml overnight in CO_2 incubator.

6. Orcein Stain

1% weight/volume of orcein stain in a solvent of 60/40 water/glacial acetic acid. Filter before using, keep tightly capped.

7. Caprogen Diluent

	ml/litre
buffer solution	900
1.4% citric acid	10
2.5% caproic acid	10
0.05% chloromycetin	10
catalase solution	20
fresh egg yolk	50

Following addition of the egg yolk mix the Caprogen thoroughly to ensure that the egg yolk is evenly suspended throughout the Caprogen. Chill Caprogen to 2-5°C and saturate with nitrogen by bubbling dry nitrogen through the solution for 30 minutes. Seal to exclude air. Allow caprogen to come to room temperature.

7.1. Buffer Solution

	g/litre
tri-sodium citrate	20
glycine	10
D-glucose	3

Add 250ml boiling glass-distilled water and shake to dissolve chemicals. Immediately add cold distilled water to ensure rapid cooling and bring volume to 960ml. Add 12.5ml glycerol, 0.025g sodium sulphacetamide, 1.25×10^6 units penicillin and 1.25×10^6 units streptomycin in 25ml of distilled water. Bring the final volume to 1 litre with the addition of more distilled water. The pH should be 7.35-7.50. Store in refrigerator for up to 7 days.

7.2. Catalase Solution

Dissolve 4.5mg of beef liver catalase in 100ml of buffer solution.

APPENDIX 2 - TIMING OF OPERATIONS

Day	Time		
	8-10am	10-12	1-5pm
Monday	media preparation	media preparation	collect and process ovaries (d1)
Tuesday	collect semen from Awahuri	fertilise oocytes (d1)	collect and process ovaries (d2)
Wednesday	fix oocytes	fertilise oocytes (d2)	collect and process ovaries (d3)
Thursday	collect semen from Awahuri assess oocytes (d1) fix oocytes (d2)	fertilise oocytes (d3)	collect and process ovaries (d4)
Friday	assess oocytes (d2) fix oocytes (d3)	fertilise oocytes (d4)	laboratory duties
Saturday	assess oocytes (d3) fix oocytes (d4)		
Sunday	assess oocytes (d4)		

APPENDIX 3 - TIMING OF OOCYTE AND EMBRYO DEVELOPMENT

3.1.In Vitro Maturation.

STAGE	TIME AFTER MATURATION INITIATION (HOURS)
germinal vesicle breakdown	0-6
metaphase I	6-13
anaphase-telophase I	13-16
metaphase II	16-24

3.2.In Vitro Fertilisation.

STAGE	TIME AFTER INSEMINATION (HOURS)
metaphase II/sperm capacitation	0-6
penetration	6-8
telophase II/head decondensation	8-11
pronuclear formation	11-16
pronuclear development	16-22
syngamy	22-26
mitosis	26-28

APPENDIX 4. STATISTICAL ANALYSES FOR EXPERIMENT 1.

Table 1. Regression analysis of the proportion of oocytes penetrated on semen age and sperm concentration (transformed data). Full model.

source	df	ss	ms	F	Pr>F
model	12	51.905	4.325	2.95	0.0171
error	19	27.826	1.465		
corrected total	31	79.730			
partitioned:					
semen age	1	10.023	10.023	6.84	0.0170
treatment	1	17.058	17.058	11.65	0.0029
treatment x treatment	1	5.366	5.366	3.66	0.0708
semen age x treatment	1	3.913	3.913	2.67	0.1186
semen age x treatment x treatment	1	6.251	6.251	4.27	0.0527
dose	4	5.959	1.490	1.02	0.4235
semen age x dose	3	3.334	1.112	0.76	0.5309

Tables 2.1. and 2.2. Regression analyses within semen age of the proportion of oocytes penetrated on sperm concentration after removing non-significant effects.

Table 2.1. Day-old Semen.

source	df	ss	ms	F	Pr>F
model	2	2.945	1.472	0.91	0.4252
error	13	20.940	1.611		
corrected total	15	23.885			
Partitioned:					
treatment	1	2.942	2.942	1.83	0.1996
treatment x treatment	1	0.002	0.002	0.00	0.9695

Predicted equation for penetration rates obtained with day-old sperm:

$$y = 0.896 + 0.109x - 0.003x^2$$

TABLE 2.2. 2-day-old Semen.

source	df	ss	ms	F	Pr>F
model	2	27.119	13.560	9.42	0.003
error	13	18.704	1.439		
corrected total	15	45.822			
Partitioned:					
treatment	1	17.964	17.964	12.49	0.0037
treatment x treatment	1	9.155	9.155	6.36	0.0255

Predicted equation for penetration rates obtained with 2-day-old sperm:

$$y = 0.339 + 0.764x - 0.156x^2$$

Table 3. Regression of proportion of polyspermic oocytes generated on sperm concentration and semen age.

source	df	ss	ms	F	Pr>F
model	12	4.908	0.409	0.51	0.8808
error	19	15.144	0.797		
corrected total	31	20.051			
Partitioned:					
semen age	1	0.449	0.449	0.56	0.4622
treatment	1	0.403	0.403	0.51	0.4852
treatment x treatment	1	2.434	2.434	3.05	0.0967
dose	4	0.682	0.170	0.21	0.9275
semen age x treatment	1	0.065	0.065	0.08	0.7781
semen age x treatment x treatment	1	0.656	0.656	0.82	0.3758
semen age x dose	3	0.218	0.073	0.09	0.9640

Table 4. Regression of the proportion of oocytes penetrated on sperm concentration and type (frozen-thawed or unfrozen) of semen. Full model, transformed data.

source	df	ss	ms	F	Pr>F
model	5	22.682	4.536	9.94	0.0001
error	22	10.038	0.456		
corrected total	27	32.719			
Partitioned:					
treatment	1	3.124	3.124	6.85	0.0158
treatment x type	1	0.023	0.023	0.05	0.8241
treatment x treatment	1	5.195	5.195	11.39	0.0027
treatment x treatment x type	1	9.079	9.079	19.90	0.0002
type	1	5.260	5.260	11.53	0.0026

Tables 5.1. and 5.2. Regression analyses within semen type of the proportion of oocytes penetrated on sperm concentration (transformed data).

Table 5.1. Frozen-thawed sperm.

source	df	ss	ms	F	Pr>F
model	2	19.133	9.567	7.56	0.0066
error	13	16.448	1.265		
corrected total	15	35.581			
Partitioned:					
treatment	1	19.066	19.066	15.07	0.0019
treatment x treatment	1	0.067	0.067	0.05	0.8210

Predicted equation for penetration rates obtained with frozen-thawed sperm:

$$y = 0.173 + 0.284x - 0.014x^2$$

Table 5.2. Unfrozen sperm.

source	df	ss	ms	F	Pr>F
model	2	2.121	1.061	0.61	0.5634
error	9	15.598	1.733		
corrected total	11	17.719			
Partitioned:					
treatment	1	1.702	1.702	0.98	0.3476
treatment x treatment	1	0.420	0.420	0.24	0.6345

Predicted equation for penetration rates obtained with unfrozen sperm:

$$y = 0.954 - 0.110x + 0.044x^2$$

APPENDIX 5. STATISTICAL ANALYSIS FOR EXPERIMENT 2.

Table 1. Regression analysis of oocyte penetration on caprogen concentration in the fertilisation medium. Full model, transformed data.

source	df	ss	ms	F	Pr>F
model	5	27.013	5.403	9.85	0.0001
error	18	9.875	0.549		
corrected total	23	36.888			
Partitioned:					
treatment	1	12.350	12.350	22.51	0.0002
treatment x treatment	1	6.465	6.465	11.78	0.0030
dose	3	8.197	2.732	4.98	0.0109

Predicted equation for penetration rates obtained in the presence of Caprogen extender.

$$y= 0.568 - 0.0006x + 0.0000001x^2$$

APPENDIX 6. STATISTICAL ANALYSES FOR EXPERIMENT 3.

Table 1. Regression of oocyte penetration rates on heparin concentration. Full model, transformed data.

source	df	ss	ms	F	Pr>F
model	12	70.033	5.836	1.11	0.4619
error	7	36.671	5.239		
corrected total	19	106.704			
Partitioned:					
semen age	1	25.340	25.340	4.84	0.0638
treatment	1	8.836	8.836	1.69	0.2352
treatment x treatment	1	1.067	1.067	0.20	0.6655
semen age x treatment x treatment	1	16.999	16.999	3.24	0.1147
semen age x treatment	1	5.038	5.038	0.96	0.3594
dose	4	11.879	2.970	0.57	0.6952
semen age x dose	3	0.873	0.291	0.06	0.9814

Table 2. Regression of proportion of polyspermic oocytes generated on heparin concentration. Full model, transformed data.

source	df	ss	ms	F	Pr>F
model	12	4.330	0.361	0.22	0.9892
error	7	11.491	1.642		
corrected total	19	15.821			
Partitioned:					
semen age	1	0.004	0.004	0.00	0.9600
treatment	1	0.874	0.874	0.53	0.4894
treatment x treatment	1	0.002	0.002	0.00	0.9749
dose	4	1.166	0.292	0.18	0.9429
semen age x treatment	1	0.434	0.434	0.26	0.6230
semen age x treatment x treatment	1	0.642	0.642	0.39	0.5514
semen age x dose	3	1.207	0.402	0.25	0.8623