THE EFFECT OF LEUKAEMIA INHIBITORY FACTOR (LIF) ON BOVINE EMBRYO DEVELOPMENT *IN VITRO*

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

ENDANG TRI MARGAWATI

1995

ABSTRACT

Margawati, E.T. 1995. *The effect of Leukaemia Inhibitory Factor (LIF) on bovine embryo development in vitro.* MAgSc thesis, Massey University, Palmerston North, New Zealand, 94 pp.

The aim of the study was to investigate the effect of Leukaemia Inhibitory Factor (LIF) either during in vitro maturation (IVM) or in vitro culture (IVC) on bovine embryo development. Three main experiments were conducted using oocytes aspirated from 2-8 mm diameter follicles collected from cows slaughtered at local abattoirs, Hamilton. The oocytes were matured in a modified TCM-199 containing 10 μg/ml of FSH and LH, and 1 μg/ml E_2, fertilised in TALP and cultured in SOF/AA/BSA.

Experiment 1 examined the effect of LIF (0, 500, 1000 or 2000 U/ml) and various time periods of IVM (18, 22 or 28 h), in a 4 x 3 factorial design on oocyte maturation. Following maturation, oocytes were stripped out of cumulus cells, then denuded oocytes were stained in 1% lacmoid for determination of maturation stage while the cumulus cells were examined for the incidence of apoptosis by observation of DNA fragmentation using gel electrophoresis procedures.

Experiment 2 comprised two parts, (a) the effect of LIF (0, 500, 1000 or 2000 U/ml) at 24 h IVM in a randomised block design on *in vitro* development of embryos, (b) comparison of 20 vs 24 h IVM in the presence of LIF (0, 500, 1000 or 2000 U/ml) in a 2 x 4 factorial experiment on embryo development. In the two studies, the proportion of bovine oocytes that cleaved and developed to blastocyst stage was recorded. In addition, cell numbers of blastocysts after Giemsa staining were counted.

Experiment 3 examined the effect of LIF during IVM (0 vs 1000 U/ml) or IVC (0, 500, 1000 or 2000 U/ml) in a 2 x 4 factorial design on development of embryos. The incidence of cleavage and blastocyst development and cell numbers of blastocysts were recorded. In addition, blastocysts were further categorised into early, expanded and

hatched blastocyst stages and cell numbers of blastocyst inner cell mass (ICM) and trophectoderm (TE) after differential staining with Hoechst 33342 and propidium iodide were determined.

In Experiment One, an interaction of LIF concentration and duration of IVM was not observed for the proportion of immature oocytes reaching metaphase II ($P>0.05$). The presence of LIF (500, 1000 or 2000 U/ml) increased the proportion of oocytes at metaphase II at 18 h (50%, 52% or 58%, respectively, compared to without LIF= 27%), indication that LIF may accelerate the maturation process *in vitro*. Supplementation of LIF during IVM did not affect the incidence of apoptosis of the cumulus cells.

In Experiment Two, compared to 24 h IVM in the presence of LIF, 20 h IVM significantly increased blastocyst rates (Σ blastocysts : Σ cleaved, $P<0.05$). Cell numbers of blastocysts were not different from oocytes matured for 20 or 24 h in the presence of LIF ($P>0.05$), however the data show that treatment groups of 20 h IVM in LIF resulted in higher cell numbers of blastocysts than achieved by 24 h IVM.

In Experiment Three, there was a correlation between LIF during IVM and LIF during IVC in the proportion of blastocysts ($P<0.05$). This finding shows that the proportion of blastocysts decreased when oocytes were matured in the absence of LIF and cultured in LIF. In contrast, more blastocysts developed when the oocytes were matured and then cultured in media containing LIF. There was no effect of addition of LIF during IVM and IVC for cell numbers of blastocysts ($P>0.05$). However, blastocysts derived from oocytes matured without LIF had significantly increased cell numbers (121 cells) compared to those matured in 1000 U/ml LIF (109 cells, $P<0.05$).

Supplementation of LIF both during IVM and IVC did not affect the proportion of blastocyst stages ($P>0.05$). However, a concentration of 2000 U/ml LIF during IVC accelerated blastocyst development with more blastocysts hatching (60%, $P<0.05$).

Cell numbers of inner cell mass (ICM), trophectoderm (TE), and the proportion of ICM were not affected by supplementation of LIF during IVM or IVC ($P>0.05$). A

concentration of 1000 U/ml LIF during IVC resulted in higher cell numbers of ICM ($P<0.05$).

This study suggests that LIF of 500, 1000 or 2000 U/ml increased the proportion of metaphase II bovine oocytes and even reduced the time course of IVM. Supplementation of LIF during IVM may suppress the incidence of apoptosis of the cumulus cells. IVM for 20 h in the presence of LIF resulted in a higher number of blastocysts and 1000 U/ml LIF during IVM and culture in LIF increased the proportion of blastocysts. A higher concentration of LIF is required for reaching the hatched blastocyst stage. A level of 1000 U/ml LIF during IVC promoted higher cell numbers of ICM.

ACKNOWLEDGEMENTS

I realise that this piece of work could not have been completed without the support, encouragement and enthusiasm of a number of people.

First, I wish to express my sincere gratitude to my main supervisor, Associate Professor M.F. McDonald, who allowed me to pursue my own interests in this study. I also appreciate to his valuable time given in editing this manuscript.

My deepest gratitude to my supervisor, Ms. Anne Pugh (AgResearch Ruakura, Hamilton), who has assisted in developing this work. I appreciate her dedication and enthusiasm for this study and invaluable guidance and advice during the preparation of this manuscript.

I extend my sincere appreciation to Dr. H.R. Tervit and Dr. J.G.E. Thompson (AgResearch Ruakura, Hamilton) for their advisory expertise in planning this study.

A special thank you to a number of people in Animal Physiology Centre, AgResearch Ruakura, Hamilton. They include Dr. Lora Hagemann in helping with DNA analysis, Dr. Allan Nixon in setting-up a fluorescence microscope during crucial time of cell observations, M. Donnison, J. Larsen, B. Shane and several people in the Embryology Laboratory have given technical expertise during my hectic work. Without their assistance, the volume and quality of this work would not have been possible.

I am deeply grateful to Professor D.J. Garrick and Dr. P.C.H. Morel for their patience help and advice on statistical analyses. My thanks to Dr. John Campbell and Dr. Muhammad Anwar who have freely given their comments on drafts of material in this thesis.

I gratefully acknowledge the financial assistance and scholarship received from the Ministry of Foreign Affairs and Trade of New Zealand.

A special note -- I would extend my thanks to my friends: Dan Waggoner MAgSc and Bob Leonard D.V.M who assisted in providing important references related to this study. I am thankful to Dr. Frank and Lydia Weilert MSc for translating a Germany reference.

Thank you to all the rest of staff and post-graduate colleagues and friends of the Animal Science Department who were always friendly and ready to lend a hand and made my stay in New Zealand pleasant and memorable.

My sincerest appreciation to Timothy , Suzanne and Patrick Byrne who in one way or another have lightened the yoke on my shoulders with their concern and understanding.

On a more personal note I would like to dedicate the fruit of these struggles to my parents, brothers and a sister for their considerable patience, encouragement and support during the course of this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

The following abbreviations have been used in this text without prior definition:

Units:

°C	degree Celsius
gr	gram (s)
h	hour (s)
mg	milligram (s)
ml	millilitre (s)
mm	millimetre (s)
ng	nanogram (s)
g	microgram (s)
l	microlitre (s)
w/v	weight/volume

Hormones:

E_2	oestradiol
FSH	follicle stimulating hormone
LH	luteinizing hormone

Growth Factors:

EGF	epidermal growth factor
FGF	fibroblast growth factor
IGF	insulin-like growth factor type I and II
LIF	leukaemia inhibitory factor
PDGF	platelet-derived growth factor
TGF	transforming growth factor type α and ß

Solutions or Media:

BSA	bovine serum albumin
B 199	bicarbonate buffered 199
eaas	essential amino acids
FCS	foetal calf serum
H 199	hepes buffered 199
neaas	non essential amino acids
PBS	phosphate-buffered saline
PB1	enriched phosphate-buffered saline
PVA	polyvinyl alcohol
SOF	synthetic oviduct fluid
TALP	Tyrode's medium with albumin, lactate and pyruvate
TCM-199	tissue culture medium-199

Others:

Ana	anaphase
Blst Rate	blastocyst rate
cAMP	cyclic adenosine mono phosphate
conct	concentration
DNA	deoxyribonucleic acid
FAF	fatty acid free
ICM	inner cell mass
IVC	in vitro culture
IVF	in vitro fertilisation
IVM	in vitro maturation
Met I	metaphase I
Met II	metaphase II
MOET	multiple ovulation and embryo transfer
MQH_2O	milli Q water
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
SAS	statistical analysis systems

SEM	standard error of the mean
TE	trophectoderm
Tel I	telophase I
vs	versus

CHAPTER 1. INTRODUCTION

CHAPTER 1.

INTRODUCTION

In recent years, offspring of cattle, sheep, goats and pigs have been produced by techniques involving the in vitro maturation (IVM), in vitro fertilisation (IVF) and in vitro culture (IVC) of ovarian oocytes that have been recovered from animals slaughtered at abattoirs. These techniques are termed as in vitro production. In cattle, this technique was first introduced over 15 years ago and the first IVF calf was born in 1981 (Brackett *et al.*, 1982).

In vitro production technology offers the potential for inexpensive mass production of oocytes and embryos for both research and commercial purposes. Several commercial firms are already marketing in vitro produced embryos. In addition to the recovery of oocytes from abattoir-derived ovaries, oocytes can be recovered from living animals using ultrasound guided transvaginal oocyte recovery techniques. A similar procedure has been described for the recovery of oocytes from prepubertal calves. Using these techniques, oocytes of living animals can be retrieved several times from the same animal and in vitro embryo production from calves can result in increased genetic gain. Oocytes can also be recovered from cows that have been culled due to ill health, or misadventure. For techniques such as pronuclear injection for transgenetics or for cloning purposes, large numbers of precisely staged zygotes or matured oocytes are required. The technique of IVM and IVF enables a large number of embryos to be produced more efficiently than traditional superovulation and surgical oocyte recovery.

Sperm from a number of species can be successfully sorted into X- and Y- bearing population using a fluorescence activated cell sorter. However, current techniques do not produce sufficient numbers of sorted sperm for use by conventional A.I. procedures. This can be overcome by using in vitro fertilisation where as few as 2000 sperm per matured oocyte is required (Wei *et al.*, 1994). In vitro fertilisation techniques can also be successfully used for epididymal sperm, for the hybridisation of related species (e.g.,

certain deer species) and, further, by using techniques such as intra cytoplasmic sperm injection non-motile sperm can be used for fertilisation.

Other possible commercial application for *in vitro* produced embryo include the generation of Bos Taurus and Bos Indicus embryos for export, the production of beef embryos for transfer into dairy herds and the use of *in vitro* derived embryos to produce twins in beef cattle. These applications would result in increased efficiency in the production of desirable quality meat animals.

The development of in vitro production technology has considerably improved in many laboratories. Further improvement in the technique is still required since only 25% to 30% of all oocytes develop into blastocysts. Numerous factors have been identified that affect embryo development *in vitro*, such as inadequate maturation causing a lower percentage of fertilised eggs and subsequently a lower number of producing transferable embryos. A developmental block was also found in bovine embryos cultured *in vitro*. Using co-culture systems with somatic cells or conditioned medium have enhanced the development of IVM/IVF of embryos to the blastocyst stage. Similarly, several growth factors have been added to culture media to overcome the developmental block in early bovine embryos (reviewed by Heyner *et al.*, 1993). Leukaemia Inhibitory Factor (LIF) is one of several growth factors that may overcome the problems in embryo development *in vitro*.

There are many interesting functions of LIF. One function of particular interest to embryologists, is the prevention of differentiation of embryonic stem (ES) cells *in vitro*. In the *in vitro* systems, the source of LIF (also known as Differentiation Inhibitory Factors - DIA) is generally provided by the 'feeder' cell or Buffalo Rat Liver cells (BRL cells). Differentiation of cells can be triggered by many factors and involves the mechanisms of apoptosis (Programmed cell death). Programmed cell death is a normal phenomenon in embryo development - particularly at the blastocyst stage and involves a complex series of biochemical triggers such as the generation of hydrogen peroxide. LIF may act by suppressing apoptosis of ES cells *in vitro*. Recently, apoptosis has been correlated to follicle atresia events in ovine and bovine ovaries. Granulosa cells of atretic

follicles have DNA fragments while healthy cells do not. Apoptosis is evidenced by the presence of 'a DNA fragment' ladder on an agarose gel. This fragmentation of DNA is caused by DNA breakage due possibly to free radical generation.

While many growth factors have been examined for their effects on maturation and/ or development of bovine oocytes and embryos, there are few reports on the effects of and possible actions of LIF. This present study was therefore conducted to examine whether LIF in IVM medium alone or in IVC medium would enhance bovine embryo development *in vitro*. The duration of IVM was also assessed in this study since the presence of LIF in IVM medium may affect the time course of IVM.

CHAPTER 2. REVIEW

CHAPTER 2.

REVIEW OF LITERATURE PERTAINING TO THE RESEARCH TOPIC

2.1 MATURATION OF BOVINE OOCYTES

2.1.1 Meiosis

In mammals, meiosis consists of two cell divisions, each including four stages: Prophase, Metaphase, Anaphase and Telophase (see Figure 1, Tsafriri, 1978). In most mammals, the first meiotic division occurs in foetal life while the second meiotic division is completed in the oviduct only following sperm penetration. First meiotic division (or meiotic prophase I) commences early in development either before or shortly after birth (Baker, 1979; Gondos, 1978). This division is subdivided into 5 subphases: Leptotene, Zygotene, Pachytene, Diplotene and Diakinesis. In rats and mice, an additional Dyctiate (or Dyctiotene) stage follows immediately after Diplotene (Figure 1). The oocytes in meiotic prophase I are found in the prefollicular ovary. The first meiotic division is then arrested at a diffuse diplotene or dyctiate stage (Baker, 1979) and will be completed in the adult at a few hours before ovulation (Gondos, 1978; Tsafriri, 1978). Thus, the duration of the first meiotic arrest of the oocyte persists until a time prior to ovulation when germinal vesicle breakdown occurs.

Maturation is completed when meiosis is resumed (from no. 7 to 11, Figure 1) and ovulation takes place usually at the metaphase II stage (No. 11, Figure 1). The resumption of meiosis *in vivo*, which occurs after dictyate stage, takes place only in oocytes that are in maturing antral follicles, and is dependent on the high levels of gonadotrophic hormones in follicular fluid during proestrus (so-called "LH-surge"). Oocytes removed from antral follicles will progress *in vitro* from the diplotene (germinal vesicle) stage to Metaphase II while those from preantral follicles remain at the diplotene stage. In addition, oocytes from small antral follicles are seemingly "blocked" without further development at Metaphase I (Erickson and Sorensen, 1974). Thus, only those oocytes within normal antral follicles will undergo maturation to Metaphase II of meiosis.

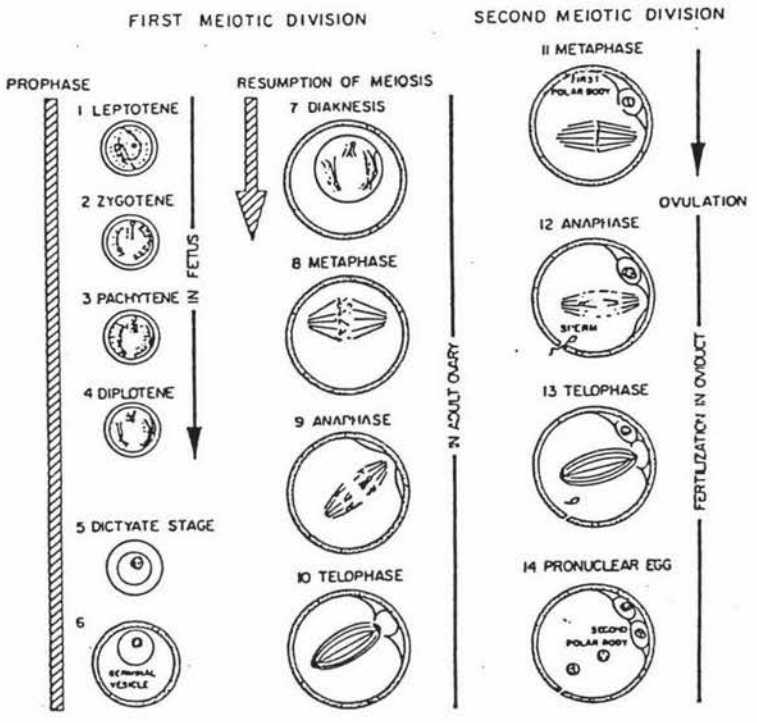

Figure 1. Diagram illustrating oocyte meiosis in foetal and adult ovaries, and in the oviduct following ovulation and fertilisation. For simplicity, only three pairs of chromosomes are described (Tsafriri, 1978)

Oocyte maturation includes both morphological changes, as evidenced by nuclear maturation and expulsion of the first polar body and physiological maturation which is referred to as "cytoplasmic maturation". In cattle, extrusion of the first polar body occurs in 80% of oocytes 12 to 18 hours after onset of maturation (Van der Westerlaken *et al.*, 1994).

2.1.2 Control of Oocyte Maturation *In Vitro*

The transformation of oogonia to oocytes has been investigated for a variety of species, but the factors which initiate meiosis remain obscure. Studies on IVM of mammalian eggs were initiated by Pincus and Enzmann (1935). Later work by Edwards (1965) and Moor and Trounson (1977) has shown that mammalian oocyte meiosis resumes spontaneously in serum-containing culture media without supplementation of hormones, but many of these spontaneously matured eggs failed to be fertilised or to produce viable

embryos (Thibault, 1977; Leibfried and Bavister, 1983; Shalgi, 1984; Fleming *et al.*, 1985). However, recent reports have demonstrated that bovine oocytes can be matured and fertilised and will develop to blastocysts in serum-free medium (Rose and Bavister, 1992).

Nuclear maturation, cytoplasmic maturation and membrane maturation (or membrane competence) are critical components of the maturation process for continued viability of oocytes following fertilisation (Thibault *et al.*, 1987; Younis *et al.*, 1989). In addition, Rose and Bavister (1992) suggested that cytoplasmic maturation is necessary for assessing oocyte maturation since nuclear maturation can occur in oocytes that may not have undergone cytoplasmic maturation.

Many studies have dealt with the role of follicular constituents in the control of oocyte maturation. Leibfried and First (1979), Fukui and Sukuma (1980) and Dahlhausen *et al.* (1981) demonstrated that there was no maturation, or a low maturation rate of bovine oocytes when the cumulus cells were removed prior to oocyte culture *in vitro*. Supplementation of the culture medium with cumulus cells during IVM did not alter the frequency of completing nuclear maturation (Critser *et al.*, 1986). However, compared to nude or corona-enclosed oocytes, IVM of cumulus-enclosed bovine oocytes yielded a significantly higher proportion of embryos following IVF. The presence of intact cumulus cells for at least 12 hours is necessary for normal cytoplasmic maturation of bovine oocytes *in vitro*. Chian and Niwa (1994) demonstrated that the direct communication of cumulus cells within the oocyte may be important for cytoplasmic maturation. This direct communication is not necessary in the later stages of IVM since there was no difference between oocytes that were stripped free of cumulus and intact cumulus at either 16 or 20 hours after onset of maturation in the fertilisation rate and subsequent cleavage and development to morulae and blastocysts (Van der Westerlaken *et al.*, 1994).

Contact between the oocyte and granulosa cells prevents oocyte maturation (reviewed by Tsafriri, 1978). This contact through gap junctions may play a part in the inhibition of meiosis resumption. A reduction in the number of gap junctions may increase the

frequency of oocyte maturation (Larsen *et al.*, 1986). The inhibitory effect of granulosa cells seems to be similar to the effect of follicular fluid on oocyte maturation. It was suggested by Tsafriri (1978), that resumption of meiosis is triggered by the preovulatory gonadotrophin surge and Baker *et al.* (1977) have reported that LH, FSH and oestradiol are all effective in inducing meiotic division and progression to metaphase II. It may be that LH acts by reducing the number of gap junctions.

Growth factors have been proposed to promote oocyte maturation *in vitro* in the presence of steroids and gonadotrophins (Dekel and Sherizly, 1985; Feng *et al.*, 1988; Downs *et al.*, 1988). Several studies have reported that growth factors, their receptors and localized production of growth factors occur within the ovary and granulosa cells (Hammond *et al.*, 1985; Feng *et al.*, 1987; Skimmer *et al.*, 1987; Roy and Greenwald, 1990). Growth factors have also been found in the follicular fluid (Hofman *et al.*, 1990) and they may act as both *autocrine* and *paracrine* regulators of ovarian function (Hammond *et al.*, 1988; Carson *et al.*, 1989), causing granulosa and theca cell proliferation and differentiation (Harper and Brackett, 1993b).

Several studies of particular growth factors on maturation of oocytes have been made. Epidermal growth factor (EGF) has been reported to induce maturation in follicle-enclosed rat oocytes (Dekel and Sherizly, 1985). EGF has also shortened the time required for germinal vesicle breakdown and increased the proportion of oocytes undergoing germinal vesicle breakdown in mice (Downs, 1989; Das *et al.*, 1991). Harper and Brackett (1993a) suggest that there is a physiological role for EGF in regulating bovine oocyte maturation. This positive influence of EGF during IVM of bovine oocytes resulted in an increase in the proportion of oocytes that were able to undergo cleavage and development to the blastocyst stage. Harper and Brackett (1993b) suggested the possibility of collaborative action of gonadotrophins with growth factors occurring in oocyte maturation. They found that a combination of platelet-derived growth factor (PDGF) with FSH increased the proportions of matured and fertilised oocytes developing to blastocysts which contrast with the results obtained with a combination of PDGF and LH. In addition, Insulin-like Growth Factor-I (IGF-I) both in IVM and IVC media can increase the number of morulae and blastocysts (Herrler *et al.*, 1992).

2.2 APOPTOSIS

2.2.1 What is Apoptosis ?

In biological investigations, the effect of some treatments on development has been measured by the incidence of apoptosis. *Apoptosis* is a type of cell death which is distinguished from *necrosis*. Apoptosis is a term to describe the type of cell death frequently observed when cells are deleted from living tissues (Wyllie, 1988). Necrosis is characterised by cellular oedema and terminates in rupture of plasma and internal membrane and leakage of cellular contents into extracellular space, while apoptosis involves progressive contraction of cellular volume, widespread chromatin condensation and preservation of the integrity of cytoplasmic organelles (Wyllie, 1981). The affected cells separate into membrane-bounded fragments which are rapidly *phagocytosed* by adjacent cells. Lately, apoptosis has been referred to as programmed cell death (Arends and Wyllie, 1991).

2.2.2 Incidence of Apoptosis

Programmed cell death occurs in certain circumstances which include normal cell turnover, embryonic development, metamorphosis, hormone-induced atrophy and regression of endocrine-sensitive tumours and is initiated by physiological stimuli (Wyllie and Morris, 1982). Cell death of these circumstances is morphologically distinct from that evoked by nonphysiologic stimuli of the environment which is termed as 'coagulative' necrosis.

Apoptosis is the major mode of death observed from the modelling of tissue from the early blastocyst (Handyside and Hunter, 1986). In mammals, apoptosis occurs in endometrial (Rotello *et al.*, 1989) and ovarian tissue (O'Shea *et al.*, 1978; Zeleznik *et al.*, 1989). Apoptosis occurred in bovine granulosa cells and was most evident in cells from atretic follicles but can also occur in healthy follicles during the luteal phase of the oestrous cycle (Jolly *et el.*, 1994).

The effect of growth factors on the incidence of apoptosis has been reviewed by Arends and Wyllie (1991). Apoptosis can also be caused by physiologic regulatory hormones, such as glucocorticoids acting on lymphocytes (Wyllie and Morris, 1982). As observed under the light microscope, apoptotic cells in tissues are inconspicuous, appearing singly or in small groups and consisting of portions of dark staining cytoplasm, usually with smooth contour (Wyllie, 1981). These apoptotic cells are frequently digested by *macrophages*.

2.2.3 Mechanisms

There have been few studies to elucidate the mechanisms of apoptosis. However, as reviewed by Arends and Wyllie (1991) at least six major events are known. These events are: 1) cell density rises sharply, 2) intracellular calcium concentration undergoes a moderate but sustained rise, 3) total protein and RNA synthesis are discontinued, 4) chromatin is cleaved at *internucleosomal* sites, apparently by an endogenous endonuclease, 5) previously cryptic glycan groups become exposed on the cell membrane and act as recognition signals, permitting binding and absorbtion by phagocytes, 6) cytoskeletal elements become less readily deformable, perhaps as a result of transglutaminase.

Internucleosomal chromatin cleavage is mostly associated with the morphology of apoptosis (Arends and Wyllie, 1991). Evidence from glucocorticoid-treated thymocytes and lymphoid cell lines of rat indicates that the chromatin condensation of apoptosis is associated with endogenous endonuclease activity which destroys nucleosome chains from nuclear chromatin (Wyllie, 1980). Cleavage of internucleosomal linker DNA generates well-organised chains of oligonucleosomes with lengths that are integer multiples of 180-200 base pairs (Wyllie, 1981; Arends and Wyllie, 1991; Jolly *et al.*, 1993) and appear as a ladder after gel electrophoresis (Arends and Wyllie, 1991). This ladder has now been reported along with morphologic chromatin condensation of apoptosis in many cell systems (Rotello *et al.*, 1989; Zeleznik *et al.*, 1989). Evidence has been demonstrated that the characteristic morphologic condensation of chromatin in apoptosis is due to DNA cleavage (Arends and Wyllie, 1991). Recent evidence has

shown that DNA cleavage in apoptosis occurs selectively without associated chromatin proteolysis (Arends *et al.*, 1990).

2.2.4 Agonists and Antagonists of Apoptosis

Cyclic adenosine mono phosphate (cAMP) has a role in cell death (Wyllie, 1981). Dibutyryl cAMP also causes premature deletion of embryonic palatal shelf tissue with the morphology of apoptosis (Pratt and Martin, 1975). Epidermal growth factor (EGF) blocks this deletion which is thought to exert its effect through depletion of endogenous cAMP (Hassell and Pratt, 1977). In some species a decrease in cAMP content of oocytes triggers resumption of meiosis (Thibault *et al.*, 1987)

There have been few studies on the effect of hormones or growth factors on the incidence of apoptosis. Withdrawal of specific growth factors from lymphoid cell lines in culture, or serum withdrawal from fibroblasts initiates apoptosis even though the factors do not necessarily stimulate proliferation (Vaux *et al.*, 1988). It appears that growth factors have important roles in the regulation of cell survival and death. The addition of stem cell factor and Leukaemia Inhibitory Factor (LIF) promoted primordial germ cell survival by suppressing programmed cell death or apoptosis (Pesce *et al.*, 1993). This study suggested that the two cytokines (stem cell factor and LIF) may affect the *in vitro* and possibly *in vivo* development of mammalian primordial germ cells.

2.3 IN VITRO FERTILISATION

2.3.1 Capacitation of Spermatozoa

Mammalian spermatozoa must undergo capacitation and the acrosome reaction before they can penetrate the oocyte. Capacitation is a process involving the spermatozoa in a series of biochemical and physiological reactions (Gordon, 1990). In mammalian spermatozoa, the acrosome reaction is characterised by the dispersion of the acrosomal contents which is preceded by the fusion between the plasma membrane and the outer

acrosomal membrane at multiple sites (Yanagimachi, 1988). This reaction promotes movement of the spermatozoon through the zona pellucida and fusion with the egg plasma membrane. The process of acrosome reaction is illustrated from a study of guinea pig sperm (Figure 2).

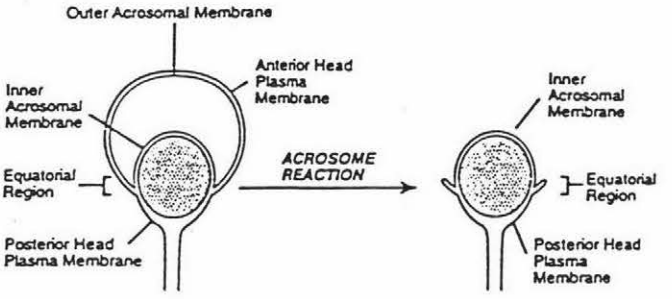

Figure 2. Before and after the acrosome reaction in guinea pig spermatozoa head. During the acrosome reaction, the anterior head plasma membrane (except for the posterior-most equatorial region) is lost after fusion with the outer acrosomal membrane. The equatorial and/or posterior head region initiates fusion with the egg plasma membrane (Myles and Primakoff, 1984).

Numerous capacitation systems have been developed for inducing capacitation of bovine spermatozoa. Several methods of bull sperm capacitation are presented in Table 1. Based on the observations of First and Parrish (1988), sperm capacitation can be achieved by agents which cause Ca^{2+} entry into the sperm acrosome and a pH increase within the spermatozoon. Heparin has been proven as a potent glycosaminoglycan in its ability to induce the acrosome reaction in bovine epididymal spermatozoa (Handrow *et al.*, 1982) and to capacitate ejaculated bovine spermatozoa (Parrish *et al.*, 1985b).

Table 1. Methods of artificial capacitation of bull sperm

Method	Investigator (s)
High Ionic Strength Medium	Brackett *et al.*, 1982
Bovine Follicular Fluid	Fukui *et al.*, 1983
Standard Ionic Strength Medium	Iritani *et al.*, 1984
Ionophore A23187	Hanada, 1985
Heparin	Parrish *et al.*, 1985a
Liposomes	Graham *et al.*, 1986
Percoll Gradients/Hypotaurine	Utsumi *et al.*, 1988
Caffeine	Niwa *et al.*, 1988
TEST-Yolk	Ijaz and Hunter, 1989

2.3.2 Fertilisation Events

Union of the sperm and oocyte normally occurs when spermatozoa have undergone capacitation and the acrosome reaction and the oocyte has matured to metaphase II. The fertilisation process involves a series of sequential events. As described by Parrish and First (1993), the events involve sperm attachment to the zona pellucida of the egg, binding to the zona pellucida, the acrosome reaction, penetration of the zona pellucida, fusion of sperm and egg plasma membranes, activation of the egg to complete meiosis II, the cortical granule reaction to block polyspermy, the zona reaction of hardening the zona in response to cortical granule exudate, sperm head swelling, sperm chromatin decondensation in synchrony with oocyte chromatin decondensation, deposition of a pronuclear envelope around the sperm chromatin and finally syngamy of the two pronuclei and entry into first mitotic cell cycle.

2.3.2.1 Attachment and Binding

Initial sperm attachment to the zona pellucida is loose and sperm may easily be removed by pipetting. After a period of interaction with the zona, sperm form a firmer attachment, called binding (Parrish and First, 1993). Binding occurs when sperm interact

with a receptor protein of the zona. In the mouse, the receptor protein is called ZP_3 (Bleil and Wassarman, 1983) and is one of three glycoproteins found in the zona pellucida. Similar zona proteins also exist for cattle (Florman and First, 1988). Binding of spermatozoa to the oocyte is mediated by the presence of a binding substance on the surface of the spermatozoa (Parrish and First, 1993). As a result of the interaction of the binding substance on spermatozoa, a specific zona protein causes the acrosome reaction to be induced (Bleil and Wassarman, 1983).

2.3.2.2 Penetration of The Zona Pellucida

Based on the review of penetration of mouse oocytes, Parrish and First (1993) described a possible model for mammalian sperm penetration. This includes binding of a capacitated sperm to the zona, induction of the acrosome reaction, rebinding of sperm to a different zona protein (i.e., ZP_2 in mouse) by a sperm protease (perhaps acrosin), cleavage of that zona protein, sperm advancement due to its motility, rebinding of sperm protease to another molecule of the secondary binding site, and the cycle repeated until the sperm reaches the perivitelline space. Only acrosome reacted sperm that are motile cross the zona pellucida.

2.3.2.3 Fusion of Sperm and Oocyte

After a spermatozoon penetrates to the zona and crosses the perivitelline space, it becomes attached to the vitellus and can then fuse with the oocyte. This occurs at the plasma membrane over the equatorial region of the sperm head after it contacts the correct region on the vitellus (Parrish and First, 1993; see Figure 3). The contact place usually contains microvilli and the contact rarely occurs over the meiotic spindle (i.e., a region devoid of microvilli). There are two distinct sites of the mature oocyte plasma membrane: 1) the cortical granule-free domain that lies over the metaphase chromosomes and where the second polar body formation occurs, 2) the rest of the oocyte. A complete fusion of the sperm and oocyte plasma membranes occurs when the entire sperm is incorporated within the oocyte (Parrish and First, 1993).

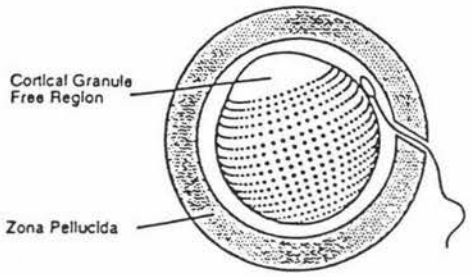

Figure 3. Fusion of mammalian sperm-oocyte. The cortical granule free region lies over the metaphase-arrested chromosomes. After sperm and oocyte fusion this region will produce a second polar body. The first polar body is not shown (Myles, 1993).

2.3.2.4 Oocyte Activation and Formation of Pronuclei

Immediately after contact of the sperm with the oocyte plasma membrane (vitelline membrane), a series of changes in the egg is initiated. Contact of the sperm and the oocyte causes an increase of cytosolic Ca^{2+} (reviewed by Parrish and First, 1993). The elevation of Ca^{2+} is sufficient to trigger a block to polyspermy, to increase cellular metabolism and to initiate the resumption of meiosis. In mammalian oocytes, the elevation of Ca^{2+} can be stimulated by Ca^{2+} ionophore, ethanol and electric voltage (Parrish and First, 1993).

Fusion of cortical granules in the oocyte with the oocyte plasma membrane and deposition of the cortical granule contents in the perivitelline space causes the zona block to polyspermy (Parrish and First, 1993). A block to polyspermy is a crucial early event during oocyte activation. Fertilisation of an immature oocyte (metaphase I) or an ageing oocyte results in an incomplete cortical granule reaction resulting in polyspermy (Soon-Chye *et al.*, 1990). This may be because cortical granules of the oocytes are not correctly distributed around the oocyte periphery .

Following activation, meiosis resumes in the oocyte. Female chromosomes are released from metaphase II and proceed to anaphase II and telophase II which is followed by an

uneven cytoplasmic division and results in extrusion of the second polar body. Chromosomes decondense during prophase II and formation of the female pronucleus then occurs at the same time as the formation of the female pronucleus, the sperm nucleus undergoes a second expansion then condenses again into a male pronucleus. This pronuclear development is completed when the membrane forms around each pronucleus (Parrish and First, 1993).

2.3.2.5 Syngamy

Syngamy is referred as 'union of genetic material from the male and female' and represents the final event in the fertilisation process. After formation of male and female pronuclei, they migrate to the centre of the oocyte and remain for the next 12-18 hours in some mammals (Parrish and First, 1993). The membranes of the pronuclei disintegrate, chromatin recondenses and intermixes. The chromosomes then replicate to restore the diploid state of the zygote in prophase of the first cleavage division.

2.4 EARLY EMBRYO DEVELOPMENT

2.4.1 *In Vivo*

Early embryo development begins soon after fertilisation. In cattle, fertilisation occurs in the upper oviduct within a few hours after ovulation (Salisbury and VanDemark, 1961) or almost immediately after ovulation (Barnes and Eyestone, 1990). The time of ovulation and fertilisation is believed to be nearly the same (Hyttel *et al.*, 1988). After fertilisation, the oocyte undergoes a series of cell divisions, beginning with nuclear division and progressing to cytoplasmic division. At first, a 1-cell zygote divides into two cells of approximately the same size, then, another synchronous division, resulting in four cells. Cellular divisions continue, however they become less synchronous (Salisbury and VanDemark, 1961; Barnes and Eyestone, 1990). The timing of these divisions in the cow and in some other animals are presented in Table 2.

Table 2. Early divisions of fertilised oocytes in mammalian species*

Species	Hours of age (after oestrus) when recovered						
	1-cell	2-cell	3-4 cell	5-8 cell	9-16 cell	Morula	Blast.
Cattle	23-52	40-56	44-66	46-96	71-141	144	190
Goat	30	30-48	60	85	98	120-140	150
Sheep	0-38	38-39	42	44	65-77	96	114-139
Swine	0-51	51-66	66-72	90-110	-	110-114	114

* Salisbury and VanDemark (1961)

Barnes and Eyestone (1990) have characterised development of early bovine embryos through four cell-cycles (Table 3). Each cell cycle consists of G1, S, G2 and M phases and the length of each phase is different. Extrusion of second polar body and subsequent division into 2-cell stage takes place during first cell cycle. The first cell-cycle takes the longest period (28-32 hours) with extrusion of second polar body taking place at 20-24 hours of first cell-cycle. The zygote continues to cleave into 4- and 8-cell stages that occur at approximately 36-50 and 56-64 hours post fertilisation, respectively. These cleavages occur during the second and the third cell-cycles which last 13 and 14 hours, respectively. Asynchronous cleavage of the embryo may be due to a longer G2 phase (4-6 hours in bovine embryos) at the third cell cycle. Subsequently, cleavage into 16-cell stage (fourth cell-cycle) occurs at approximately 80-86 hours after fertilisation and is 21-30 hours long.

Table 3. Duration of the first four cell-cycles in bovine embryos (h)

Cell Cycle	G1	S	G2	M	Total Duration
First	6	8	4	2	28-32
Second	-	8	0-2	2	12-13
Third[a]	-	8	4-6	2	13-14
Fourth[b]	?	?	?	?	21-30

[a] First observed zygotic transcription
[b] First observed rRNA synthesis and genomic activation (Barnes and Eyestone, 1990)

Early development in bovine embryos is governed by mRNA's and proteins that are produced in the oocyte during oocyte growth phase and maturation. This is a period of maternal control of development (Barnes and Eyestone, 1990). Furthermore, changes in protein synthetic patterns begin to appear during the 4-cell stage. A major change in protein pattern occurs in the fourth cell-cycle that is presumed to reflect maternal/zygotic transition for the control of development. During development from the 1-cell to the 8-cell stages, the synthesis of proteins declines while ribosomal RNA synthesis is active from the 8-cell stage. The protein production is believed to correspond to an early developmental block in bovine embryos cultured *in vitro*. Activation of the embryonic genome fully occurs during this transition of the fourth cell-cycle.

At approximately 7 days after fertilisation in the cow, the embryo begins to form a hollow sphere (Salisbury and VanDemark, 1961) resulting in formation of the blastula or blastocyst. During this stage, an inner mass of cells (ICM) accumulates at one side of the hollow sphere. This ICM is destined to become the body of the embryo. The thin layer of trophectoderm cells around the outside of the blastocyst functions primarily to nourish the embryo.

2.4.2 *In Vitro*

2.4.2.1 Developmental Block in Early Bovine Embryos

The birth of calves derived from IVF of oocytes matured both *in vivo* (Brackett *et al.*, 1982, 1984; Sirard and Lambert, 1985) and *in vitro* (Critser *et al.*, 1986) has demonstrated that IVF could be applied in the breeding of domestic animals. However, the efficiency of producing offspring was still less than achieved using Multiple Ovulation and Embryo Transfer (MOET) procedures. One of many problems encountered is the existence of a developmental block following cleavage *in vitro*. For example, bovine and ovine embryos cultured *in vitro* from 1- to 4- cell stages rarely cleaved further than 8- to 16-cells while embryos cultured from the 8- to 16-cell stages frequently develop into morulae and blastocysts (Thibault, 1966; Eyestone and First, 1986).

The causes of the early developmental block in bovine embryos include improper oocyte maturation (Van de Sandt *et al.*, 1990) or an inadequate IVC medium for early embryonic development *in vitro* (First and Parrish, 1987, Flood *et al.*, 1993).
Farell and Bavister (1984) have observed that the block occurred within a few minutes of exposure of 2-cell hamster embryos to artificial conditions, as evidenced by loss of viability. Deficiencies in the conventional culture system compared with the in vivo environment were the cause of early developmental block in most species of animals since neither growth retardation nor developmental blockage were observed in vivo (Natsuyama *et al.*, 1992). Barnes and Eyestone (1990) have stressed that in bovine embryos cleavage past the 8- to 16-cell stage is very sensitive to environmental conditions.

First and Parrish (1987) in a review have suggested that there are two causes of the block in development in early cleavage of mammalian embryos *in vitro*: IVC media are lacking in factors that are conducive to early embryo development and that the oviduct contains specific factors that are required for developmental events that occur during early cleavage (between 1- and 16- cell stage). The time of blocked development is characterised by a prolonged cell-cycle, increased rates of DNA, RNA and protein syntheses, concomitant with a transition from maternal to embryonic control of development for the cow (King *et al.*, 1985) and the sheep (Calarco and McLaren, 1976).

2.4.2.2 Media and Culture Systems

In the past, the embryonic developmental block *in vitro* has been a serious problem encountered for IVF experiments. Recent advances in embryo culture systems using somatic cell co-culture have allowed the continuous development of cow zygotes fertilised either *in vitro* or *in vivo* past the problematic 8- to 16- cell stage to morulae and blastocysts, and has resulted in the birth of calves following non-surgical transfer to recipients (Eyestone and First, 1989; Ellington *et al.*, 1990). Oviduct epithelial cells are generally used for co-culture. Some studies have reported that these epithelial cells supported a better development of sheep embryos than other non-reproductive tract cells

(Gandolfi and Moor, 1987; Rexroad and Powel, 1988).

Culture medium conditioned with oviduct cells also promoted development of bovine embryos. This conditioned medium resulted in an approximately normal pregnancy rate (50%) after transfer into recipient cows (Eyestone and First, 1989). Gandolfi *et al.* (1990) have suggested that the oviduct epithelium of all examined species secretes specific proteins during the passage of the embryo along its lumen and these proteins bind to the embryos exactly as *in vivo* actions. As previously mentioned a variety of cell types and media will enable embryo development to continue. An unidentified bovine serum albumin contaminant and co-culturing embryos with trophoblastic vesicles or with granulosa cells have shown some positive results (Gandolfi *et al.*, 1990).

Besides successful co-culture systems, non co-culture systems have been developed that will overcome the problem of a developmental block in early sheep and cow embryos. Under low oxygen (5%) concentration, Tervit *et al.* (1972) demonstrated that synthetic oviduct fluid medium (SOF) without somatic cell support enabled early cleavage stage (1- to 8- cell) embryos derived *in vivo*, to develop to morulae with the birth of calves after transfer. Further, Thompson *et al.* (1990) demonstrated that under reduced oxygen (4-8%), 2-cell sheep and 2- to 4-cell and 8-cell cow embryos reached morulae stages after culture in a simple medium of SOF containing Bovine Serum Albumin (BSA) without somatic cell support. In recent years, a number of non co-culture media for bovine embryo development has been developed, such as CR1 salts medium containing sodium chloride, potassium chloride, sodium bicarbonate, hemicalcium lactate and sodium pyruvate (Rosenkrans and First, 1991). From various reports of using co-culture and non co-culture systems in embryo development, Bavister (1992) has suggested that the presence of somatic cells in culture media may not be necessary and that since embryos produce their own growth factors (Paria and Dey, 1990), these growth factors may partly replace the function of somatic cells needed for co-culture.

2.5 ROLE OF GROWTH FACTORS IN EMBRYO DEVELOPMENT

2.5.1 *In Vivo*

Studies of preimplantation embryos and the oviduct indicates that there are several factors involved in the cooperative interaction between the embryos and the female genital tract. Gandolfi (1994) has divided these factors into three groups: autocrine, paracrine and environmental factors. Growth factors derived from the preimplantation embryos participate in an *autocrine* pathway while growth factors of the oviduct and uterus act in a *paracrine* manner. Complex interactions may occur, and as reviewed by Simmen *et al.* (1993), the autocrine and paracrine modes of action of insulin-like growth factors (IGFs) are regulated within the uterine microenvironment by IGF-I receptors and IGF binding proteins which are themselves exposed to local control within the uterus and conceptus.

Growth factors are also located within the ovary (Hammond *et al.*, 1985; Skimmer *et al.*, 1987). These growth factors may act as both autocrine and paracrine regulators of ovarian function (Hammond *et al.*, 1988; Carson *et al.*, 1989). For example, epidermal growth factor (EGF) has been found in small and medium preantral follicles (Harper and Brackett, 1993a) and has a function in bovine oocyte maturation.

Growth factor is present in the early developing embryo as it passes through the reproductive tract. It was speculated that uterine secretions (histotrophe) are involved in regulating the attachment, implantation, nutrition and growth of the conceptus (Biggers, 1988; Roberts and Bazer, 1988). The uterine environment with its capacity as a source of nutrition, can provide growth factor and other regulatory proteins critical to growth and survival of preimplantation conceptuses (Robert and Bazer, 1988). As reviewed by Brigstock *et al.* (1989) the involvement of peptide and polypeptide growth factors have been established in the control of cell proliferation. However, recent work has shown that a range of growth factors influence not only proliferation but in some cases also functional differentiation of ovarian follicle cells (Carson *et al.*, 1989) and preimplantation embryos (Heyner, *et al*, 1993). Paria and Dey (1990) stated that there

is cooperative interaction among preimplantation embryos and growth factors in embryo development and growth.

The physiological role of growth factors in mammals has been considered by Gospodarowicz and Moran (1976) and involves controlling normal development, maintaining homeostasis and wound healing and regeneration.

2.5.2 *In Vitro*

2.5.2.1 Action of Growth Factors

Some growth factors have been shown to influence embryo development both *in vivo* and *in vitro*. As reviewed by Brigstock *et al.* (1989), peptide and polypeptide growth factors occur in uterine tissues and fluids. In addition, steroid hormones (oestrogen and progesterone) have a role in the control of growth factor synthesis. These growth factors include Insulin-like growth factors/ somatomedins (IGF), Epidermal growth factor (EGF), Transforming growth factors (TGF-α, -ß), Fibroblast growth factors (Acidic, Basic FGF) and Platelet-derived growth factor (PDGF).

Serum, notably foetal calf serum, is thought to contain a variety of mitogens and growth factors (Carson *et al.*, 1989). Mitogens can make the cells responsive to growth factors. Growth factors in the bloodstream can be extracted from plasma, platelets and serum (Gospodarowicz and Moran, 1976).

Many investigators have reported that growth factors regulate differentiation and morphogenesis. In a review, Mercola and Stiles (1988) concluded that growth factors can function as a mitogen in one context and promote differentiation in another. Furthermore, they suggested that growth factors, like other hormones, regulate gene expression within their target cells. The initial event in growth factor action is binding to specific receptors on embryonic cells (Mercola and Stiles, 1988; Heyner *et al.*, 1993). This is usually a high-affinity receptor which spans the outer cell membrane. Generally, the receptor is a tyrosine-specific protein kinase (Mercola and Stiles, 1988). In addition,

the binding of a growth factor by its receptor induces soluble, intra cellular second messengers which transmit a signal to the nucleus. These second messengers may be phosphoproteins, inositol phosphates, diacylglycerol, cyclic nucleotides, monovalent or divalent ions (Mercola and Stiles, 1988). Within minutes, after formation of the growth factor-receptor complex, changes in gene expression can be detected. There appears to be an association of genes which are expressed across tissue boundaries in response to numerous growth factors.

2.5.2.2 Growth Factors Involved in Embryo Development

Epidermal Growth Factor. There have been a number of studies investigating the role of EGF either in maturation of oocytes or early embryonic development *in vitro*. The EGF was usually provided either alone in a defined medium or in combination with gonadotrophins. The addition of EGF in a defined medium with combination of low concentrations of gonadotrophins during IVM enabled subsequent blastocyst development at a rate comparable to that produced by media with high concentrations of FSH and LH (Harper and Brackett, 1993a). Their findings suggested a possible physiological role of EGF in regulating bovine oocyte maturation. Flood *et al.* (1993) found that compared to chemically-defined medium alone, supplementation with EGF tended to increase the percentage of blastocysts. They concluded that certain growth factors such as EGF, TGF-α,-ß1, IGF-I,-II, PDGF, bFGF, did not improve the development of *in vitro* matured and *in vitro* fertilised bovine embryos.

EGF and other growth factors (TGF-α, PDGF, CSF-I = Colony stimulating factor-I) significantly enhanced trophoblast outgrowth (Haimovici and Anderson, 1993). They suggested, these growth factors may play an important role in the preimplantation process.

Insulin-like Growth Factor. The insulin-like growth factors (i.e., IGF-I and IGF-II) possess a high degree of amino acid sequence homology with insulin (Heyner *et al.*, 1993). Like other growth factors, insulin and IGFs bind to cell-receptors and their actions are receptor-mediated. Receptor-specific binding of IGF-1 and IGF-II was

observed on the cells of inner cell mass and trophectoderm of mouse blastocysts (Mattson *et al.*, 1988). An effect of IGF-I was observed for bovine embryo development following IVM or culture in the presence of IGF-I, however this IGF-I could not replace granulosa cell co-culture (Herrler *et al.*, 1992).

The potential applications of peptide growth factors in particular the IGF family of growth factors have been studied for their biological actions in the maternal uterus and the developing conceptus. These include improved embryo viability, "rescue" of poor embryos, synchronisation of the maternal uterine environment for receptivity, increase embryo survival, and increase pregnancy rates and reproductive efficiency (Pope *et al.*, 1990).

There is little information of IGF-II in domestic animal species. However, IGF-II is an imprinted gene in mice and is involved in the regulation of post implantation growth of mouse embryos (Rappolee *et al.*, 1992).

Transforming Growth Factor. There are two types of Transforming Growth Factors, TGF-α and TGF-ß. TGF-α is structurally analogous to Epidermal Growth Factor (EGF) and others of the TGF-ß family. TGF-α is expressed in bovine thecal cells and has been localised in the thecal cell layer during follicular growth (Lobb and Dorrington, 1992). In addition, a decrease in immunoreactive TGF-α has been found in preovulatory follicles which corresponded to a decrease in mitotic activity of granulosa cells. TGF-ß is also produced by bovine thecal cell culture, however, TGF-ß has different actions to those of TGF-α on bovine granulosa cell proliferation.

TGF-α has the ability to stimulate the rate of blastocoel expansion and it was observed in 70% of mouse embryos (Dardik and Schultz, 1991). It seems that TGF-α could support embryo implantation. Flood *et al.* (1993) have investigated nine growth factors, and demonstrated that TGF-α did not improve the development of bovine IVM/IVF embryos under chemically-defined conditions.

In mouse, addition of TGF-ß1 increased the proportion of 2-cell mouse embryos developing to blastocysts when cultured singly (Paria and Dey, 1990), but in groups, TGF-ß1 did not affect blastocyst development, hatching or the cell number of bovine embryos (Keefer *et al.*, 1994). However, Marquant-LeGuinne *et al.* (1989) reported that supplementation of TGF-ß to a co-culture system stimulated the growth of inner cell mass cells of bovine embryos. These studies show that TGF-ß1 may be necessary for embryo development *in vitro*.

Platelet-derived Growth Factor. Stimulatory effects of endometrial platelet-activating factor (PAF) or embryo-derived PAF (EDPAF) activate platelets resulting in a release of Platelet-Derived Growth Factor (PDGF). Platelets are isolated from within oviduct and uterus (Thibodeaux *et al.*, 1993).

PDGF, added to serum-free medium, promoted development of 2-cell bovine embryos beyond the 16-cell stage (Larson *et al.*, 1992b). However, when added to media for 16-cell embryos, PDGF reduced the proportion of embryos blastulating from 12.7% to 5.8%. In contrast, at the same time, TGF-α increased the proportion of embryos blastulating from 8.6 to 40.6%. Larson *et al.* (1992a) concluded that TGF-α and perhaps basic fibroblast growth factor (FGF) promoted blastulation of the 16-cell embryos during subsequent culture.

Certain growth factors and gonadotrophins may act synergistically on bovine embryo development. Harper and Brackett (1993b) found that addition of the combined PDGF and FSH in IVM medium enhanced bovine oocyte maturation with more matured and fertilised oocytes developing to blastocysts *in vitro* than PDGF or FSH alone. Furthermore, combinations of growth factors such as EGF, IGF-I and PDGF or EGF alone enhanced oocyte maturation.

2.6 LEUKAEMIA INHIBITORY FACTOR

2.6.1 Biological Functions

The biological properties of LIF have been defined in terms of its actions *in vitro*. LIF has a diverse range of actions depending on the target cell (Fry, 1992). For example, observations in mice demonstrated that LIF inhibits embryonic stem cell differentiation *in vitro* (Bhatt *et al.*, 1991), but induces differentiation and inhibits the proliferation of myeloid leukaemia cell line (Gough *et al.*, 1988).

It has been shown that LIF is able to replace feeder layers to maintain embryonic stem cells in their undifferentiated state. LIF also influences the functions of a variety of terminally differentiated cell types such as neurons, hepatocytes and adipocytes (reviewed by Hilton, 1992).

2.6.2 Physiological Roles on Embryo Development

Little is known about the *in vivo* physiological functions of LIF. The analysis of the temporal and spatial expression of LIF during embryogenesis in neonatal and adult mice may help to elucidate understanding of the physiological roles (Bhatt *et al.*, 1991; Hilton, 1992). In mice, LIF is expressed at low levels in many different tissues and the highest levels of LIF mRNA expression occurs in the endometrial glands of the uterine endometrium (Bhatt *et al.*, 1991). This expression appears transiently on day 4 of pregnancy or prior to implantation. It is suggested that a principal function of LIF *in vivo* may be to regulate the growth and to initiate implantation of blastocysts. The expression of LIF by endometrial glands of the uterus is coincident with the time of blastocyst implantation and receptors have been found on the preimplantation blastocyst (Fry, 1992). Conquet and Brulet (1990) demonstrated that LIF transcripts were detected at the preimplantation blastocyst stage and no transcripts were detectable in embryonic stem cells. They suggested that LIF is synthesized in the extraembryonic portion of the embryo and acts on the embryonic tissues during early mouse development. Since LIF

is secreted both by the reproductive tract and uterus, and also by the preimplantation embryo, LIF acts by *paracrine* and *autocrine* pathways in embryonic development. Shen and Leder (1992) reported that LIF transcripts appear only in the uterus of adult tissues where the level fluctuates in the oestrous cycle, with a peak after ovulation.

In a review, Heyner *et al.* (1993) noted that LIF may act to inhibit the differentiation of embryonic stem cells in the mouse by suppressing apoptosis of those cells *in vitro*. Stewart *et al.* (1992) reported that transient expression of LIF in mice is essential for implantation.

In sheep, LIF was able to increase by four-fold the number of blastocysts that hatched from the zona pellucida after 48 hours culture (Fry *et al*, 1992a). This finding suggested that LIF may have a protective effect on early embryo development *in vitro*.

In bovine embryo culture, myeloid LIF not only acted as a differentiation factor but also appeared to have a growth factor role (Hassan-Hauser *et al.*, 1990). In addition, the growth of the inner cell mass increased in the presence of LIF. Fukui and Matsuyama (1994) found that human LIF improved the development of in vitro matured, fertilised and cultured bovine morulae and early blastocysts when bovine embryos were cultured in SOF containing BSA or PVA. However, the addition of LIF in IVC medium did not improve development to the expanded blastocyst stage from 1-, 4-, 8-cell, morula, or early blastocyst stages. A study of Fukui *et al.* (1994) reported that the addition of 5000 U LIF/ml to in vitro-produced day-5 parthenogenetic bovine morulae significantly improved their subsequent development to the blastocyst stage.

LIF may play a role in ICM differentiation into two of the primary germ layers, ectoderm and endoderm (Hilton, 1992). Recent studies found that LIF may play a significant role in the development of ovine (Fry, 1992; Fry *et al.*, 1992a) and bovine (Fry *et al.*, 1992b) embryos by increasing the number of blastocysts that expand or hatch after 48 h culture in LIF.

2.7 EFFECT OF CULTURE SYSTEMS AND GROWTH FACTORS ON EMBRYO SURVIVAL

The effect of certain experimental treatments on early or advanced embryo development can be measured by counting total cell numbers as well as differential staining of inner cell mass (ICM) and trophectoderm (TE) cells. 'Cell death' for apoptosis occurs later in blastocysts.

2.7.1 Blastocyst Cell Numbers

One possibility for the lower survival of *in vitro* produced embryos is that the cultured embryos contain fewer cells and have a greater incidence of chromosomal anomalies (Iwasaki and Nakahara, 1990). Thus, the assessment of total number of nuclei present at the blastocyst stage is often used to determine the quality of *in vitro* produced blastocysts.

The occurrence of cell death is often more recognised by the reduction in cell number than from visualisation of dying cells (Arends and Wyllie, 1991). As indicated by Papaioannou and Ebert (1986), blastocyst cell numbers may be a valid measure of the viability of preimplantation embryos. Blastocyst cell numbers can be counted after a simple squash technique (Ebert *et al.*, 1985) or after Giemsa staining (Lim *et al.*, 1994).

There have been several studies examining the effect of culture conditions on blastocyst cell number. As an example, the total number of cells in bovine blastocyst (early and expanded) recovered from the rabbit oviduct (*in vivo*) was nearly double than that of embryos cultured *in vitro* with cumulus cells (Iwasaki and Nakahara, 1990). In addition, the occurrence of chromosomal anomalies was higher (37.5%) in embryos cultured *in vitro* than from *in vivo* derived embryos (28.0%).

The effect of some growth factors on bovine embryo development has also been examined with regard to the total cell numbers. EGF and PDGF have been reported to increase the proportion of embryos that develop into morulae and blastocysts without

an increase in cell number (Yang *et al.*, 1993). A recent report, Lim *et al.* (1994) demonstrated that the mean cell numbers of blastocysts developed in a chemically defined bovine embryo culture (BEMC) with heat treated or unheated FCS were significantly increased compared with blastocysts grown in PVA supplemented media. It seems that some components of serum are needed to increase blastocyst cell numbers. Another report, Vansteenbrugge *et al.* (1994), found that blastocysts produced from a buffalo rat liver-conditioned medium contained a greater number of cells than for blastocysts obtained from a bovine oviduct-conditioned medium. Transfer of blastocysts obtained from buffalo rat liver-conditioned medium to recipients led to pregnancies and birth of calves. The buffalo rat liver cells produce some growth factors such as LIF, TGF-ß and multiplication stimulating activity (Vansteenbrugge *et al.*, 1994). In addition, Fry *et al.* (1992) reported that ovine embryos cultured in a medium containing LIF have increased cell numbers and viability.

2.7.2 Inner Cell Mass and Trophectoderm

The mammalian blastocyst consists of two distinct cell types, the outer trophectoderm and the inner cell mass. The trophectoderm cells have functions for transportation of fluid during blastocyst formation and for attachment and invasion of the uterine endometrium during implantation. The inner cell mass gives rise to the embryo-proper, yolk-sac and other extra embryonic tissues (Gardner *et al.*, 1973). It may be important to distinguish which of these two cell types is affected by experimental treatments. Total cell number and mitotic index at the blastocyst stage are frequently examined as a means of comparing cleavage rate in control and treated embryos (Handyside and Hunter, 1984). The inner cell mass is relatively more susceptible for certain inhibitor factors such as antimetabolites, RNA and protein synthesis inhibitors (Sherman, 1979). The determination of cell numbers in each of the ICM and TE would be useful for measuring the effect of experimental treatments such as the addition of growth factors to the culture medium. Thus, addition of IGF-I to the culture medium increased development to the blastocyst stage and cell numbers of the ICM of mouse blastocysts (Harvey and Kaye, 1992).

There have been many studies involving preparation of ICM and TE nuclei samples for counting of the cells in blastocysts. Handyside and Hunter (1984) have demonstrated differential labelling *in situ* as a rapid method for visualisation of ICM and TE nuclei in mouse blastocysts. This method has two important advantages: 1) a count of both cell types (ICM and TE) can be determined at once for each individual blastocyst, 2) the spatial relationships between cells are partially preserved and therefore regional interactions can be studied (Handyside and Hunter, 1984). Papaioannou and Ebert (1988) have also demonstrated a technique of immunosurgery and double dye staining for differential ICM and TE cell counts of preimplantation pig embryos. This technique has been adapted by Iwasaki *et al.* (1990) for determining the morphology and proportion of ICM and TE of bovine embryos fertilised either *in vivo* or *in vitro*.

2.8 OBJECTIVES OF THE STUDY

The objectives of this study were to examine the effects of Leukaemia Inhibitory Factor (LIF) on bovine embryo development *in vitro*. Three experiments were conducted to examine the effect of LIF on oocyte maturation, and conditioned development to the blastocyst. In addition, cell numbers were measured in the blastocyst and their distribution in the inner cell mass and trophectoderm.

In IVC experiments, some growth factors (EGF and EGF in combination with gonadotrophins, IGF-I, TGF-ß, PDGF and PDGF in combination with FSH) have been used to increase the numbers of in vitro produced embryos while the use of LIF has been rarely studied. Several studies of Fry *et al.* (1992a), Fry *et al.* (1992b), Fukui and Matsuyama (1994) and Fukui *et al.* (1994) have only used LIF in IVC medium for development of ovine or bovine embryos, but not in IVM medium. Several growth factors such as EGF (Harper and Brackett, 1993a) in maturation medium and IGF-I either in maturation or culture media (Herrler *et al.*, 1992) were found to increase the number of bovine oocytes maturing and subsequently developing to blastocysts. The presence of LIF in IVM medium may influence subsequent IVF and IVC in terms of the

number of oocytes reaching the blastocyst stage. LIF, like other growth factors, may function in an *autocrine* and a *paracrine* fashion. Thus, its presence during embryo development *in vitro* may be necessary to allow early embryo development to the blastocyst stage.

The use of inadequate maturation periods may result in either a lower percentage of matured oocytes (immature oocytes) or 'ageing' of oocytes. There are a number of consequences of oocyte ageing. 'Aged' oocytes have a greater incidence of polyspermic fertilisation with additional impaired cleavage to the 4-cell to 16-cell stages (Chian *et al.*, 1992). Supplementation of LIF in IVM medium might be beneficial for increasing the percentage of matured oocytes and might reduce the time course of IVM. Therefore, the first experiment attempted to determine an optimal concentration of LIF in combination with an optimal period of maturation in the production of matured oocytes. Progression of oocyte maturation was determined by the examination for stages of meiosis and particularly metaphase II. Occurrence of the degree of apoptosis in the cumulus cells from IVM due to the effect of LIF was also assessed by determination of the DNA ladders of the cumulus cells on agarose gels.

The effect of LIF in maturation medium for different periods of maturation of oocytes on embryo development following in vitro fertilisation and in vitro culture was then examined. The second experiment therefore attempted to ascertain whether maturation of oocytes for 20 hours in the presence of LIF would be more beneficial then 24 hours of maturation in terms of incidence of cleavage and development to the blastocyst. The number of blastocyst cells was also counted after IVM with or without LIF.

LIF is reported to stimulate proliferation of the inner cell mass and trophectoderm cells. The third experiment attempted to determine the effect of LIF on both tissues and embryo development in terms of cleavage and blastocyst development rates, and the number of blastocyst cells. In this experiment the effect of the presence of LIF during the 20 hours maturation period and also the subsequent IVC period was examined. Supplementation of LIF in those media was expected to enhance early embryo development and progression to more advanced embryo stages.

CHAPTER 3. MATERIALS AND METHODS

CHAPTER 3.

MATERIALS AND METHODS

3.1. OOCYTE COLLECTION AND IN VITRO MATURATION

Ovaries were collected at three local abattoirs near Hamilton from unidentified cows immediately after slaughter and were transported in saline held at 39°C in a flask thermos within 2 hours (Plate 1). In the laboratory, the ovaries were washed three times in sterile saline (39°C) and kept in warmed saline during the process of oocyte aspiration.

Only follicles with diameters between 3-8 mm and visible on the surface of the ovaries were aspirated. This was done using an 18 or 20 gauge needle attached to a rubber lid of a 15 ml tube and with another needle attached to a vacuum line for aspiration of air. Each aspiration tube contained 2 ml of aspiration medium containing H199, 0.4% BSA and 50 μg/ml Heparin (Appendix 1). The tubes containing follicular fluid were placed on a warmed (39°C) bench (Plate 2). Cellular contents in the bottom of each tube were collected by aspiration into a sterilised pipette and expelled into a 90 mm petri dish. Oocytes were selected under a dissecting microscope that was equipped with a warmed stage. Only oocytes with homogenous cytoplasm and surrounded by cumulus cells were selected and transferred into a 35 mm petri dish containing 2.5 ml of oocyte handling medium (H199 + 10% FCS, Appendix 1). Before transferring into maturation drops, the selected oocytes were washed in a 35 mm petri dish in oocyte washing medium (B199 + 10% FCS, Appendix 1).

A modified TCM 199 supplemented with 10% FCS, 10 μg/ml FSH, 10 μg/ml LH, 1 μg/ml E_2 was used as maturation medium (Appendix 1). Oocytes were matured in IVM drops. Prior to maturation, ten drops (50 μl per drop) were prepared in a 60 mm petri dish overlaid with mineral oil and were equilibrated in a humidified incubator at 39°C in an atmosphere of 5% CO_2 in air for at least 2 h prior to use. Ten oocytes were placed in each maturation drop and incubated for 20 h or 24 h under the same conditions.

3.2 SPERM PREPARATION AND IN VITRO FERTILISATION

Frozen semen from a single Jersey bull was used in these experiments. Prior to insemination, a highly motile fraction of sperm was collected by percoll gradient separation. Two different percoll solutions (90% and 45%) were prepared fresh each day for use. Gradients were prepared in a conical tube by layering 2 ml of 45% percoll solution on the top of 2 ml of 90% percoll solution.

The 90% percoll solution comprised of 4.5 ml percoll, 0.5 ml 10x SPTL, 0.05 ml 100x SPAD and 0.05 ml stock B. This solution was adjusted for the osmolarity of 280-300 mOsm with pH 7.3. The 45% percoll gradient was made up by diluting 2 ml of 90% percoll solution into 2 ml of 1x SPTL. These components of percoll solutions are given in Appendix 1.

Sperm were separated by thawing one or more straws of frozen semen at 35°C and layering the contents on the percoll gradient (Plate 3) followed by centrifugation at 700 gravitation for 20-30 minutes. The pelleted sperm were then washed in Hepes TALP medium (Appendix 1) by centrifuging at 300 gravitation for 5 minutes. The spermatozoa were then resuspended in fertilisation medium (Appendix 1) and counted using a haemocytometer. The volume was adjusted to give a concentration of 10×10^6 sperm/ml.

Ten fertilisation drops (30 µl per drop) were prepared in a 60 mm petri dish overlaid with mineral oil and equilibrated for at least 2 h prior to insemination. Only oocytes with well-expanded cumulus cells were fertilised in these experiments. After maturation, the oocytes were washed in Hepes TALP and rewashed in fertilisation medium and the oocytes individually separated. Five washed oocytes were aspirated into 10 µl volume of fertilisation medium which was added to a fertilisation drop. Before insemination, the final volume of each fertilisation drop was 40 µl. This fertilisation drop was inseminated with 10 µl sperm solution (= 2×10^6 sperm/ml concentration) and then incubated in a humidified incubator at 39°C in atmosphere of CO_2% in air for 24 h.

3.3 IN VITRO CULTURE

Seven culture drops (30 µl per drop) were made in a 35 mm petri dish overlaid with mineral oil and equilibrated for at least 2 h before use. After sperm exposure, the inseminated oocytes were stripped of cumulus cell using a vortex in 0.1% Hyaluronidase (Appendix 1). Denuded oocytes were then washed in Hepes SOF (Appendix 1) and rewashed in culture medium (SOF/AA/BSA, Appendix 1). Five denuded oocytes were then allocated to each culture drop and cultured in a humidified incubator at 39°C in atmosphere of 5% CO_2, 7%O_2 and 88% N_2. The culture medium was changed every 2 days with fresh culture medium. At the first change over (day 2), the incidence of oocytes cleaved was recorded. Blastocyst stage embryos were collected at 7 days after IVF (day 7).

3.4 FIXATION AND STAINING OF OOCYTES AND EMBRYOS

3.4.1 Lacmoid Staining of Oocytes

Fixing of Oocytes. Adherent cumulus cells were removed by mechanical pipetting, using a flame-drawn pipette tip with an inner diameter slightly larger than the egg diameter, or by vortexing. The cumulus cells were collected for DNA fragmentation studies. Denuded oocytes were washed in Hepes SOF and every 10-oocytes placed in a very small volume on a glass slide and covered by a coverslip. The coverslip was pressed down gently until the oocytes were slightly flattened and held in place. The slide and coverslip were then rimmed with rubber cement at the two opposite edges. A fixative solution (acetic acid : ethanol = 1 : 3) was allowed to flow under the coverslip prior to complete immersion of the slide in fixative solution for at least 2 days.

Staining of Oocytes. Slides were stained with 1% lacmoid solution for about 1 to 2 minutes, then washed with 45% acetic acid. As a final step, the chamber formed between the slide and coverslip was sealed with nail paint to prevent the fixative solution from drying out. The oocytes were then examined for maturation stages.

3.4.2 Giemsa Staining of Blastocysts

Fixing of Embryos. Blastocyst stage embryos were placed into a 4-well chamber containing washing solution (Hepes SOF) and then transferred into 0.9% Sodium Citrate for 15 or 20 minutes. One at a time, each blastocyst was picked up and put into cold fixative solution (ethanol : acetic acid : water = 3 : 2 : 1) and then immediately transferred onto a prepared slide in a very small amount of fixative. The slide was then air dried on a slide warmer.

Staining of Embryos. When the slide was dried, 4% Giemsa solution was dropped on each blastocyst for 15 minutes and then rinsed off with water and dried by air, and blastocyst cell numbers were counted under a microscope at 300 x magnification.

3.4.3 Differential Staining of Blastocyst Inner Cell Mass and Trophectoderm Cells

Preparation of Reagents

1. One vial of Rabbit anti-sheep (cow) whole serum (Sigma) was resuspended in 2 ml water and stored frozen in aliquots.
2. One vial of Guinea pig complement (Gibco) was resuspended in 5 ml water and stored frozen in aliquots.
3. Propidium Iodide - 1 mg/ml in water was stored in a dark bottle in fridge.
4. Hoechst 33342 - 1 mg/ml in water was stored in a dark bottle in fridge.
5. 0.5% Pronase was prepared by dissolving 50 mg protease (Sigma Type XIV) in 10 ml PBS, filtered and stored frozen in aliquots (150 µl).
6. Complement solution (1 : 5 dilution) contents of 50 µl complement + 5 µl propidium iodide + 5 µl H33342 + 190 protein-free medium (PBS + 0.1% PVA).

Differential Staining Procedures

The zona pellucida was removed from the embryo by incubation in 0.5% pronase for a few minutes at room temperature and then washed extensively in protein-free medium (PB1, Appendix 1). The embryo was incubated in rabbit anti-sheep whole serum at 39°C for 1 hour and then washed extensively in protein-free medium. The embryo was incubated in complement solution at 39°C for 60 to 120 minutes and washed briefly in PB1 + 0.4% BSA. One at a time, the embryos were mounted on a slide containing 10 µl drop of 50% glycerol (in PBS) supplemented with 0.02% sodium azide. The embryo was covered with a coverslip and directly examined under an inverted microscope equipped with fluorescence, and a video image was recorded on a video cassette.

3.5 DNA PREPARATION AND GEL ELECTROPHORESIS

3.5.1 Extraction of DNA.

Cumulus cells were placed into an eppendorf and spun at 13,000 rpm for 5 minutes. The supernatant was removed and the pelleted cells were resuspended in 50 µl of lysis (digestion) buffer (Appendix 2) then 1.25 µl 20 mg/ml Pronase K was added. The eppendorf was then incubated at 50°C in a waterbath for 3 h and followed by storage at 4°C until processed.

3.5.2 Gel Electrophoresis.

Into each eppendorf, 55 µl phenol was added, gently shaken for 3 minutes and then spun for 10 minutes at 13,000 rpm. The top layer was collected into a new eppendorf and 55 µl isoamyl alcohol (24:1) added, gently shaken and spun at 13,000 rpm. The top layer was collected into another eppendorf. Into this was added 14 µl sodium acetate (3M, pH 7) and 175 µl ethanol. The eppendorf was stored at -20°C for at least 20 minutes and then spun at 13,000 rpm for 25 minutes. After centrifuging, the ethanol was removed, the pellet washed with 70 µl of 70% ethanol and the eppendorf dried by air for 30 minutes. 100 µl of TE solution (Appendix 2) was then added and the DNA was

solubilised overnight at room temperature.

DNA was run on 0.8% agarose gel in 1 x TAE. Into each 25 µl sample was added 4 µl bromphenol blue solution and then loaded into each well of gel and run with low voltage (50 or 70 volt) for 5 -7 hours. DNA in the gel was stained with ethidium bromide for 10-15 minutes. The gel was then washed in water and the DNA was visualised under UV light.

3.6 ASSESSMENT

3.6.1 Meiosis Division

Determination of maturational stages of oocytes was performed under a compound-microscope at x 300 magnification. Some photomicrographs of various meiotic stages were taken by a NIKON automatic camera attached to an inverted-microscope NIKON Diaphot using Ektachrome colour slide film (daylight, ASA 200).

The denuded oocytes were classified by the stages of meiosis division, and based on the illustrations of Tsafriri (1978). The stages of meiosis division observed were: Metaphase I, Anaphase I, Telophase I, and Metaphase II. The matured oocytes were determined by the presence of the first polar body and chromosomes or termed as Metaphase II. Examples of Metaphase I, Telophase I and Metaphase II are presented in Plates 4, 5 and 6.

The proportion of oocytes at metaphase II from each group was converted into percentages: (Σ Metaphase II : Σ Oocytes) x 100%. Similarly, conversion into percentages was also calculated for the observation of Metaphase I, Anaphase I and Telophase I.

3.6.2 DNA Fragmentation

The incidence of apoptosis was determined by examination of the DNA ladders formed on the agarose gel under ultra violet light. The gel was photographed onto polaroid film (Plate 9).

3.6.3 Embryo Development

The same observations were carried out both in Experiment 2 and Experiment 3. Under a dissecting microscope, cleavage was determined by the morphological appearance of embryos that had cleaved into 2 or 4 cells at day 2 after IVF. The blastocyst stage was determined by the presence of a small or large cavity (=blastocoele) in the embryo at day 7 after IVF.

The number of ova classified as cleaved and blastocysts were converted into percentages and a rate of development calculated.

$$\text{Cleavage (\%)} = \frac{\Sigma \text{ Cleavages}}{\Sigma \text{ Oocytes}} \times 100\%$$

$$\text{Blastocyst (\%)} = \frac{\Sigma \text{ Blastocysts}}{\Sigma \text{ Oocytes}} \times 100\%$$

$$\text{Blastocyst Rate} = \frac{\Sigma \text{ Blastocysts}}{\Sigma \text{ Cleaved}} \times 100\%$$

3.6.4 Blastocyst Cell Numbers

Cell numbers were counted under a compound microscope at 300 x magnification. In some blastocysts, accurate determination of all numbers after Giemsa staining could not be made and these blastocysts were excluded from the analyses. An example of a Giemsa stained blastocyst is presented in Plate 7.

3.6.5 ICM and TE Cell Numbers

Following immunosurgery and differential staining, the ICM and TE cells were determined by counting the blue and pink cells, respectively. An example of a differentially stained blastocyst is shown (Plate 8).

3.7 METHODOLOGY OF INDIVIDUAL EXPERIMENTS

3.7.1 EXPERIMENT 1: Effect of LIF on the time course of IVM of bovine oocytes

Two studies were performed. The first study determined the effect of LIF (0, 500, 1000 or 2000 U/ml) on oocyte maturation after 18, 22 or 28 h of IVM. The second study was designed to determine the degree of apoptosis of cumulus cells after these treatments. This experiment involved 12 groups in a 4x3 factorial in a randomised block design. Each group was replicated for 7 or 8 times and used a total of 867 oocytes.

The LIF used for all experiments was recombinant human LIF and its preparation for use in these experiments is presented in Appendix 3.

LIF at concentrations of 0, 500, 1000 or 2000 U/ml was added to the maturation medium (modified TCM-199). The oocytes were matured in these treatment media for 18, 22 or 28 h.

After the assigned duration of maturation, the oocytes were stripped of cumulus cells and the denuded oocytes were examined for the stages of meiotic division following staining. Oocytes that had reached Metaphase II were termed as 'matured'.

3.7.2 EXPERIMENT 2: Effect of LIF During IVM on bovine embryo development following IVF

Two investigations were performed. The first was designed to study the effect of LIF (0, 500, 1000, 2000 U/ml) during 24 h IVM on embryo development following IVF. This study involved four doses of LIF doses in a randomised block design with 8 replications of each treatment and used a total of 1041 oocytes. The same concentrations of LIF during IVM were also used in the second investigation comparing 20 h versus 24 h IVM on embryo development following IVF. The second study was a 4x 2 factorial experiment in a randomised block design and was replicated 5 times for each group using a total of 870 oocytes.

First Study. The maturation medium was supplemented with 0, 500, 1000 or 2000 U/ml LIF as treatments. Bovine oocytes were matured for 24 h in each of these four media. The matured oocytes were fertilised for 24 h with Percoll separated sperm. After IVF, the oocytes were placed in culture medium (SOF/AA/BSA). Observations for embryo development was made at day 2 (cleaved) and day 7 (blastocyst) after IVF. The collected blastocysts were then prepared for determination of cell numbers by Giemsa staining.

Second Study. The same concentrations of LIF during IVM as above were used, but the oocytes were matured for 20 h versus 24 h in a modified TCM-199. Two groups of in vitro matured oocytes were fertilised and cultured *in vitro* using the same procedures of the first study and similar observations on embryo development and cell numbers of blastocysts were made.

3.7.3 EXPERIMENT 3: Effect of LIF during IVM or IVC on bovine embryo development following IVF

The effect of LIF either in IVM or in IVC medium on embryo development after maturation for 20 h following IVF was examined.

Oocytes were matured in either medium containing 0 or 1000 U/ml LIF for 20 h. The matured oocytes from each group were then randomly cultured in SOF/AA/BSA containing of 0, 500, 1000 and 2000 U/ml LIF following IVF. Therefore, the design was a 2x4 factorial experiment in a randomised block design with 8 replications of each group using a total of 1996 oocytes.

As well as recording embryo development and blastocyst cell numbers, the effect of LIF on the cell numbers of the inner cell mass and trophectoderm was also examined. Blastocysts collected at day 7 after IVF were divided into two groups: for Giemsa staining and for differential staining of ICM and TE cells. The cell numbers of blastocysts and the ICM and TE cell numbers were counted.

3.8 STATISTICAL ANALYSES

All data were analyzed using the General Linear Models (GLM) procedure in the statistical analysis systems (SAS) package to calculate means and standard errors for the means. Some data, in particular, the proportion of meiosis stages (Metaphase I, Anaphase I, Telophase I and Metaphase II), and the proportion of embryos reaching cleavage, blastocyst or blastocyst stages (early, expanded and hatched) were not normally distributed. These data were therefore transformed by arc-sine of the square root $\sin^{-1}\sqrt{\text{proportion}}$ and values of 0 and 1 were counted as 1/(4n) and (n-1/4)/n, respectively, where n= number of observations (Snedecor and Cochran, 1980) before being analyzed by GLM procedure. Cell counts (*i.e.*, blastocyst cell numbers, Inner Cell Mass and trophectoderm cell numbers) were assumed normally distributed. These data were then directly analyzed by GLM procedure. Differences between treatments for the

means were compared by Student's t-tests using significance levels of 5% - 1% ($P<0.05$ - $P<0.01$). Means of the transformed data were retransformed as $(Sin^{-1} x)^2$ for inclusion in the results and also for discussion.

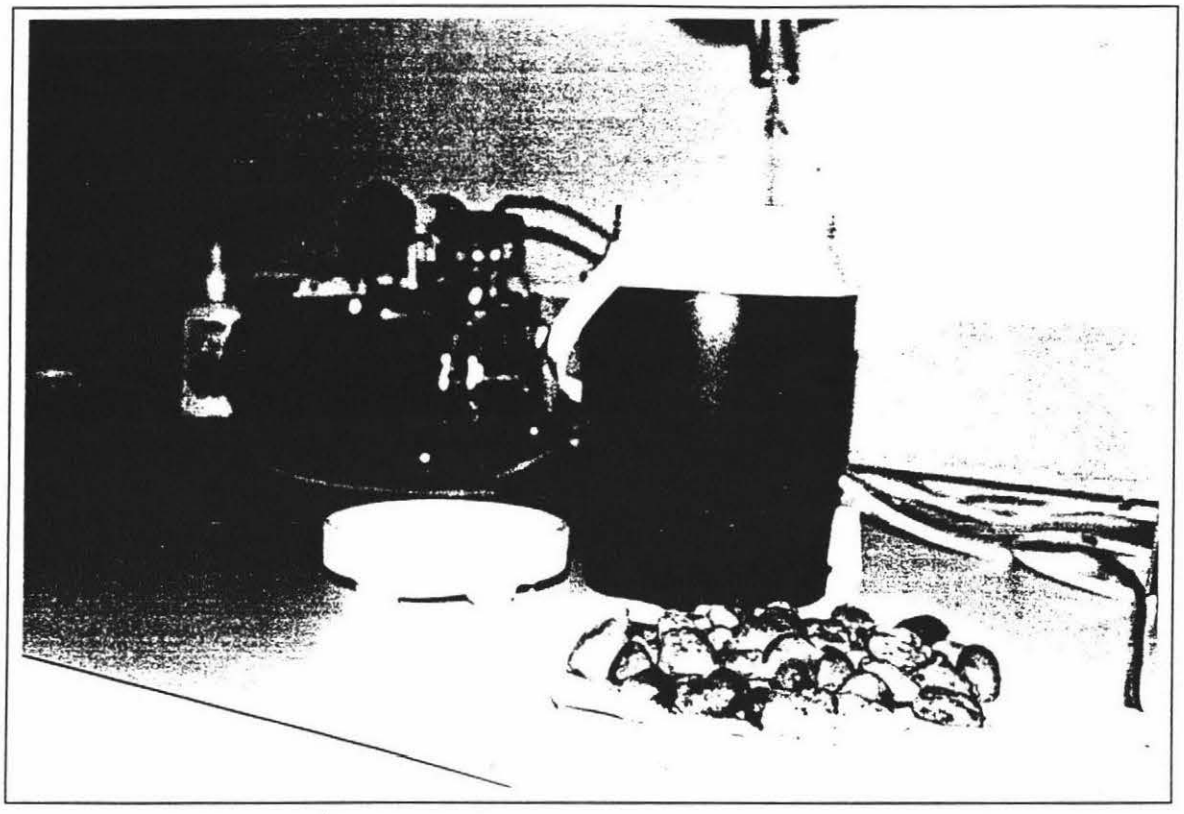

Plate 1. Ovaries, collected from an abattoirs prior to aspiration by vacuum pump (in background)

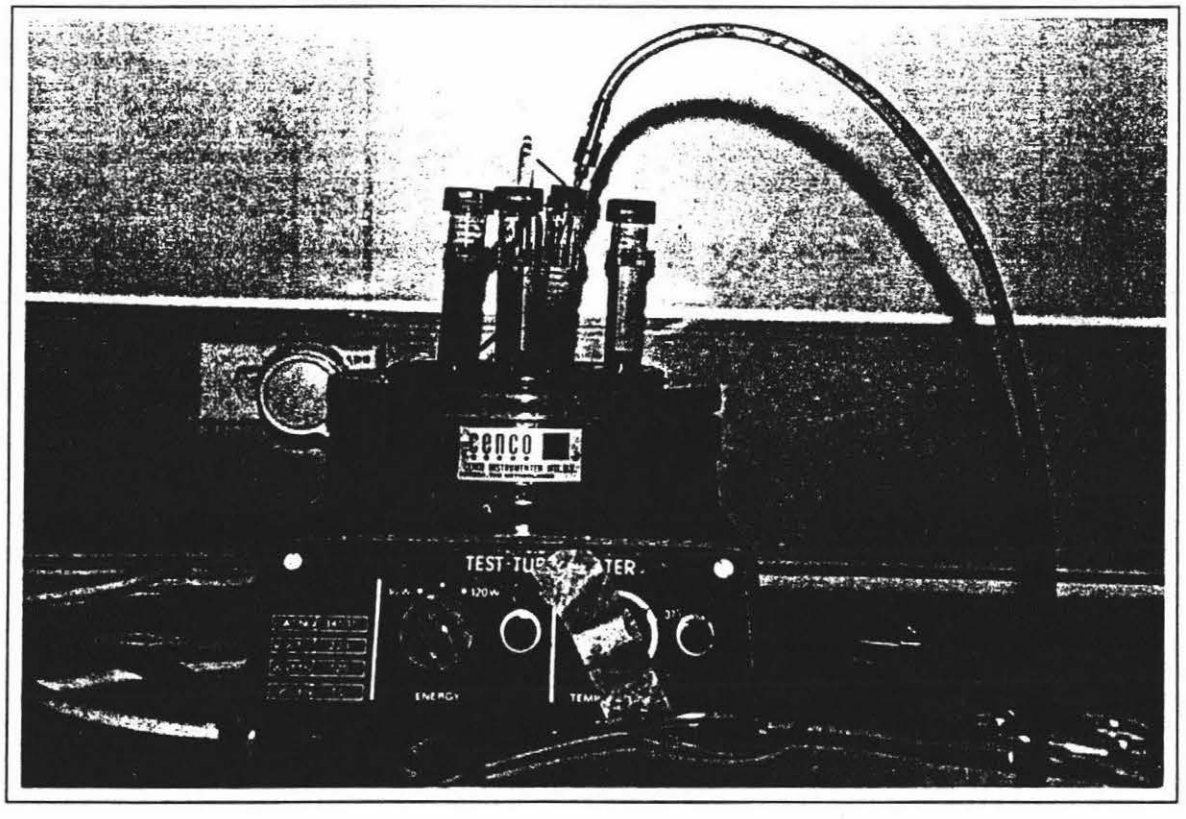

Plate 2. Aspirated follicular contents were kept on a warmed (39°C) bench

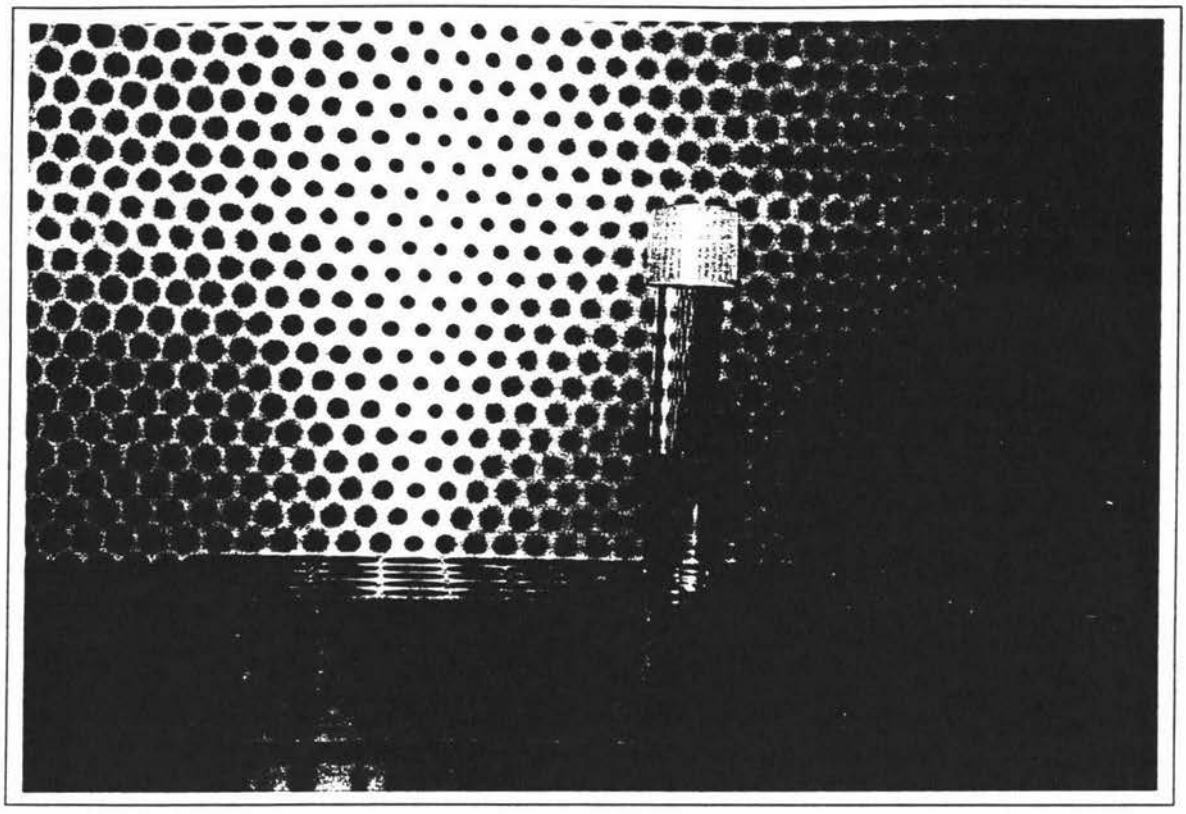

Plate 3. The Frozen-thawed sperm layered on the top of the percoll gradient

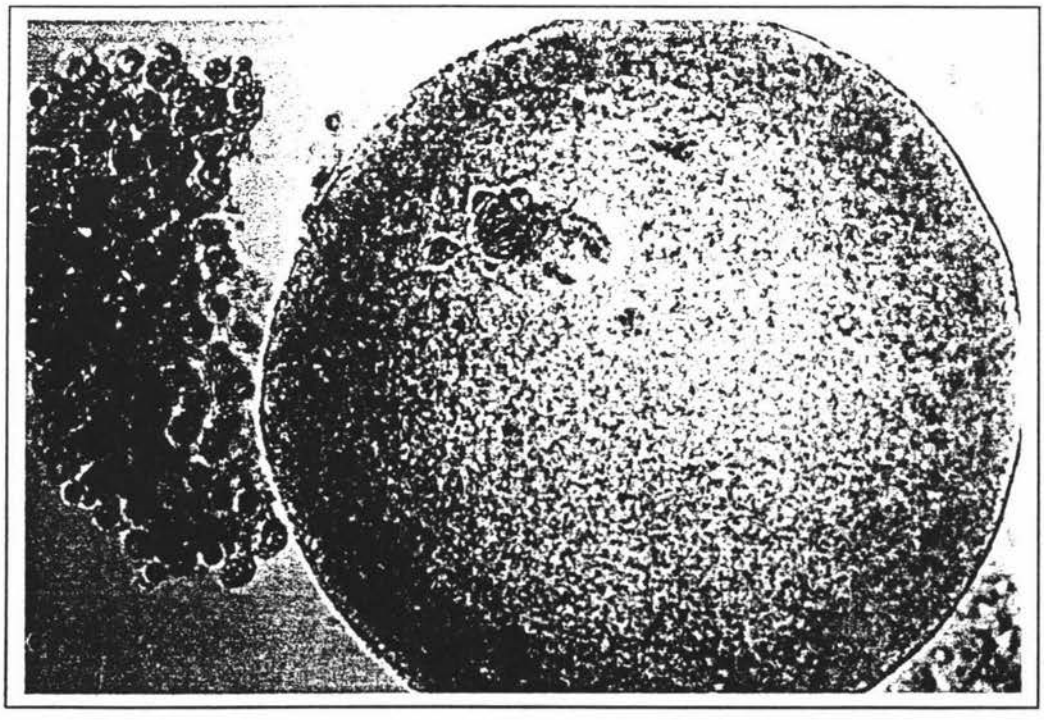

Plate 4. Metaphase I of bovine oocyte maturation (x 300)

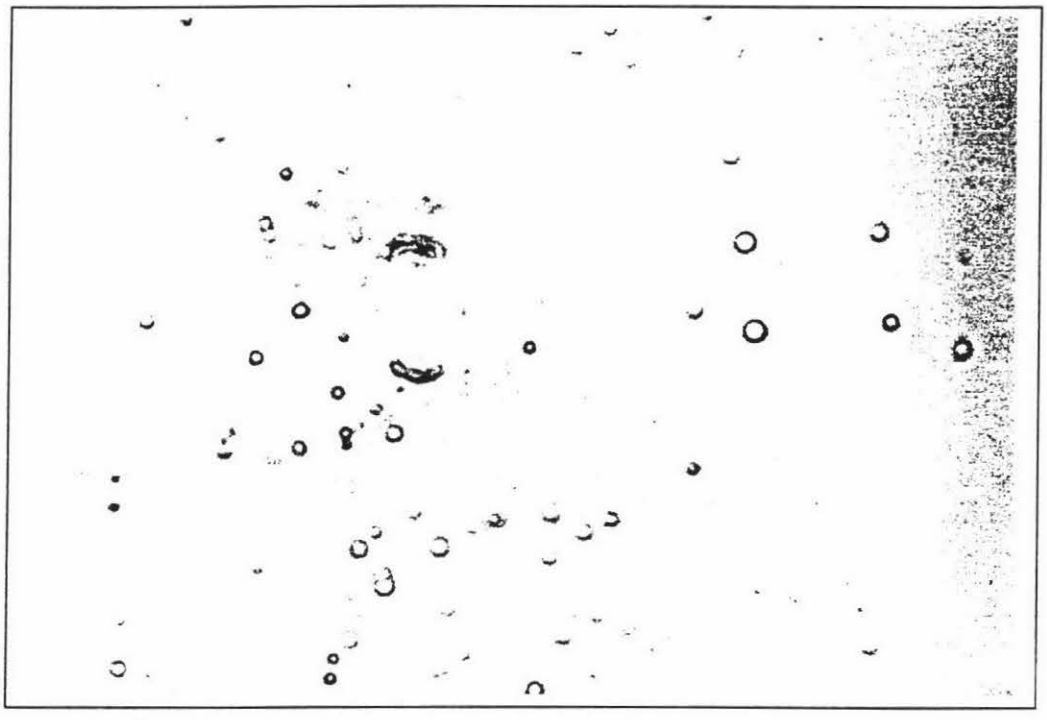

Plate 5. Telophase I of bovine oocyte maturation (x 300)

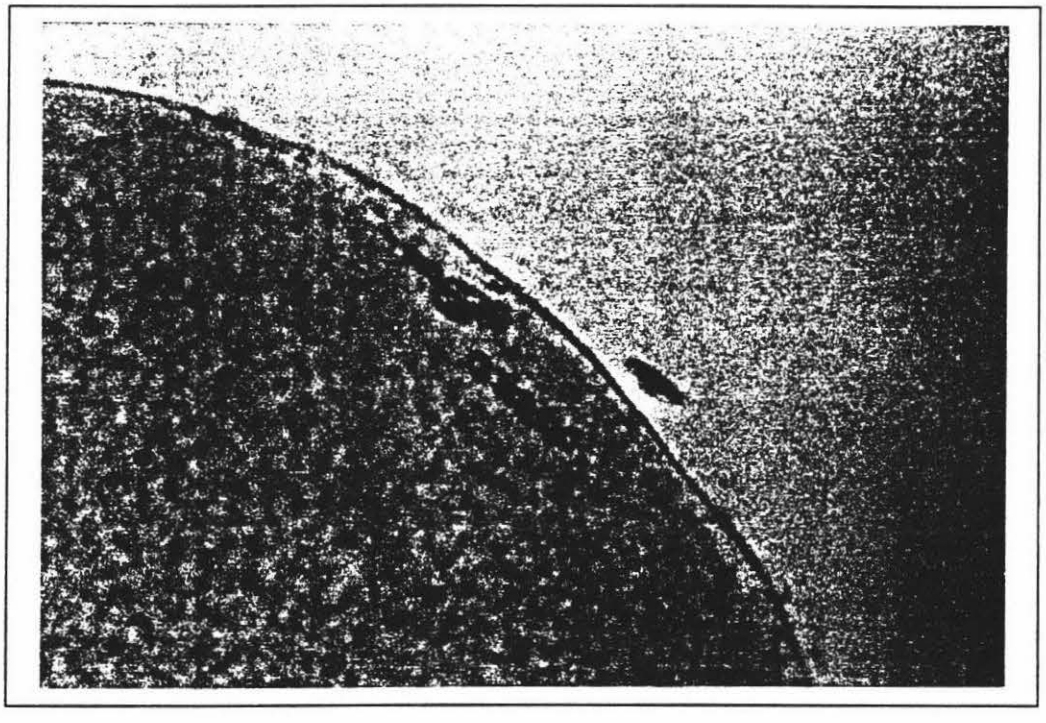

Plate 6. Metaphase II of bovine oocyte maturation (within the cell, top - polar body, bottom - metaphase II plate, x 300)

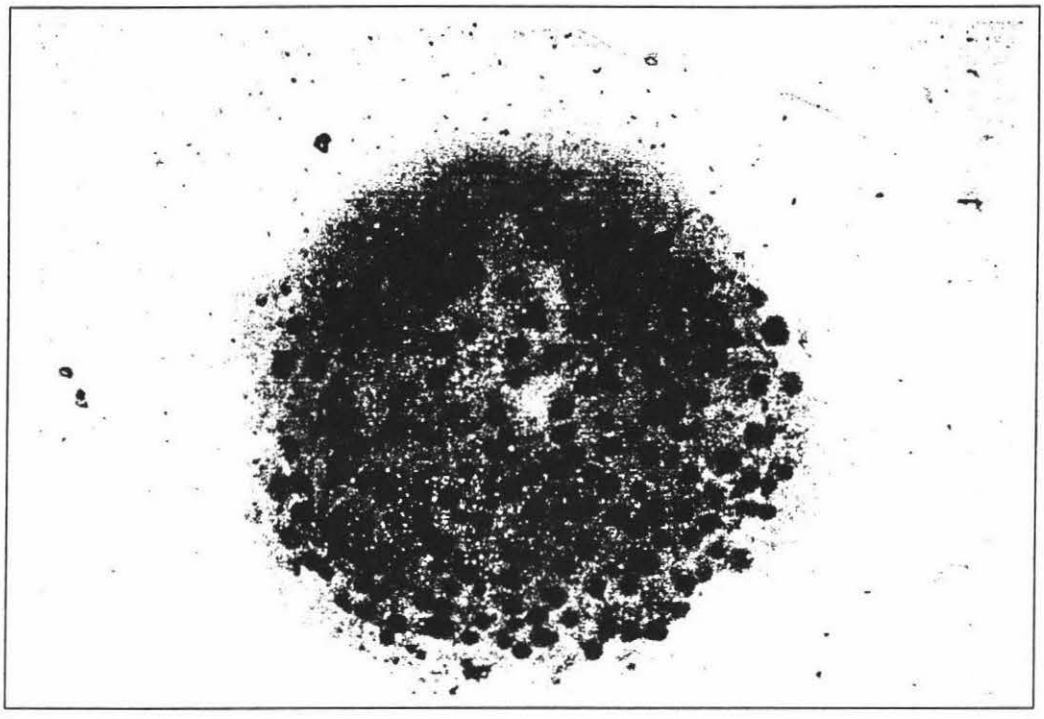

Plate 7. Bovine blastocyst following Giemsa staining (approximately 120 cells, x 300)

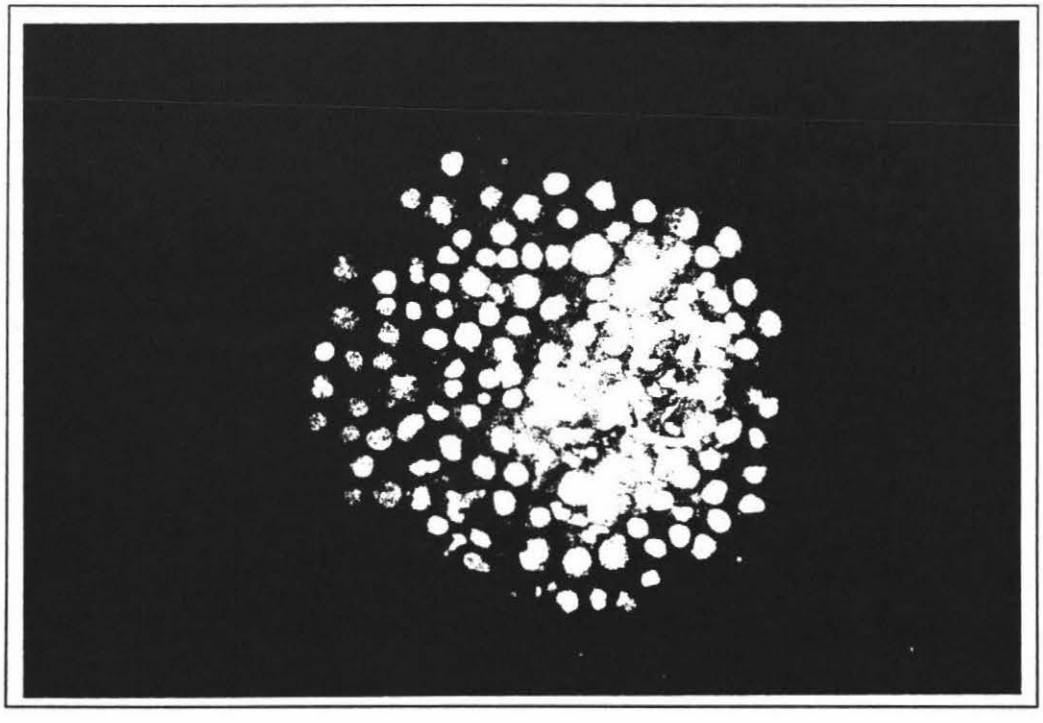

Plate 8. Bovine blastocyst following differential staining with H33342 and propidium iodide: Inner cell mass (ICM, blue) and Trophectoderm (TE, pink), (x 300)

CHAPTER 4. RESULTS

CHAPTER 4.

RESULTS

4.1 MATURATION OF OOCYTES

Experiment 1. Data analyses were not only performed on the percentage of matured (Metaphase II) oocytes, but also on the percentage at other stages of meiosis (i.e., Metaphase I, Anaphase I and Telophase I).

Table 4 shows the percentage of oocytes which had matured to metaphase II. There was no interaction (P>0.05) between the duration of maturation and the dose of LIF for the percentage of oocytes reaching metaphase II. At all periods of maturation, more oocytes had reached metaphase II after IVM in the presence of LIF than in the absence of LIF. However, the beneficial effect of LIF was not observed by 28 h of IVM. By 22 h of IVM, the proportion of oocytes that had reached metaphase II in the presence of LIF was almost similar in its percentages.

Table 4. The effect of LIF during IVM and the duration of IVM on the percentage of oocytes reaching Metaphase II (Mean ± SEM)

Duration of IVM (h)	Dose of LIF (U/ml)			
	0	500	1000	2000
18	26.95±3.52	40.91±3.52	52.00±3.52	58.50±3.52
22	62.24±3.29	79.28±3.52	81.36±3.29	78.77±3.29
28	58.87±3.52	63.96±3.52	73.30±3.52	75.98±3.29

No interaction of LIF dose and the duration of IVM (P>0.05)

Figure 4 shows the distribution of maturation stages at various times of IVM. There were highly significant differences (P<0.001) within the maturation periods of 18, 22 and 28 h for the proportions of metaphase I, anaphase I and metaphase II oocytes but not for the proportion of telophase I oocytes. More oocytes had matured to metaphase II by 22 h and 28 h than at 18 h. In addition, complementary to this observation, fewer oocytes were observed at Anaphase I and Metaphase I after 22 or 28 h of IVM than after 18 h.

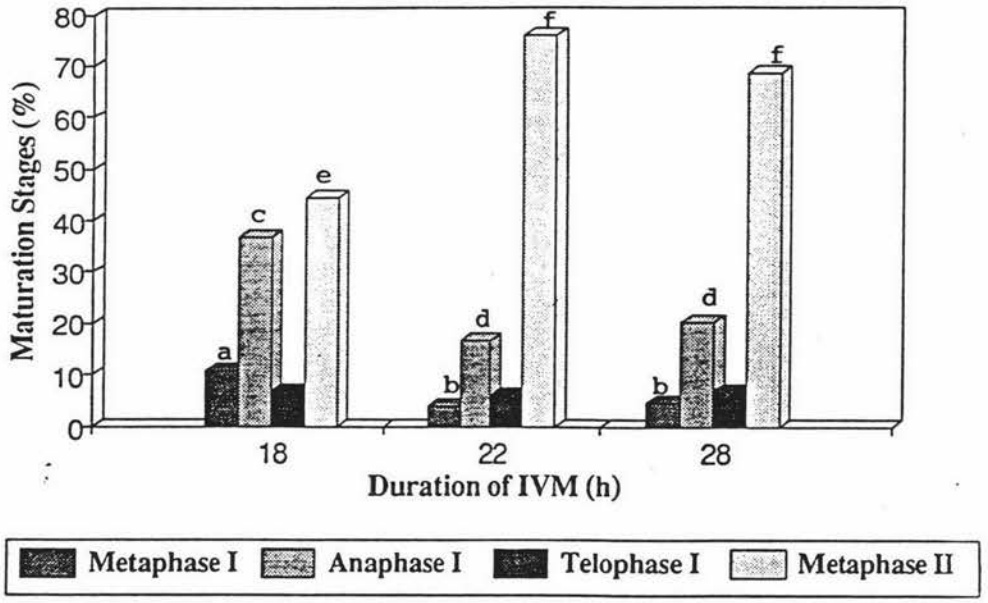

Figure 4. The effect of the duration of IVM on the percentage of Metaphase I, Anaphase I, Telophase I and Metaphase II oocytes (Mean ± SEM). Means with different superscript letters within stage differ significantly (P<0.001)

The percentage of oocytes reaching various maturation stages following IVM with (500, 1000, 2000 U/ml) or without LIF are presented in Figure 5. This table shows that there was a significant effect (P<0.05) within dose of LIF (0, 500, 1000 or 2000 U/ml) for the percentage of oocytes that reached Anaphase I and Metaphase II. Compared to the control (0 LIF), the addition of 1000 or 2000 U/ml LIF to the maturation medium significantly increased the percentage of oocytes that reached metaphase II (70% and 72% vs 50%; P<0.05). However, a LIF dose of 500 U/ml did not significantly increase the percentage of oocytes that reached metaphase II above the control (62% vs 50%;

P>0.05) and was also not significantly different from the responses to 1000 or 2000 U/ml LIF. There was a tendency (P=0.07) for the proportion of oocytes at metaphase I to vary within LIF dose (0, 500, 1000 or 2000 U/ml). The proportion of oocytes at metaphase I for both 0 and 500 U/ml LIF was greater than for 1000 or 2000 U/ml LIF. The percentage of oocytes at telophase I was not influenced by LIF doses. However, more oocytes (P<0.05) were seen at Anaphase I following IVM in the absence of LIF than if IVM included LIF at 500, 1000 or 2000 U/ml.

The results suggest that LIF, in particular at the higher concentrations, increased the percentage of oocytes progressing to metaphase II.

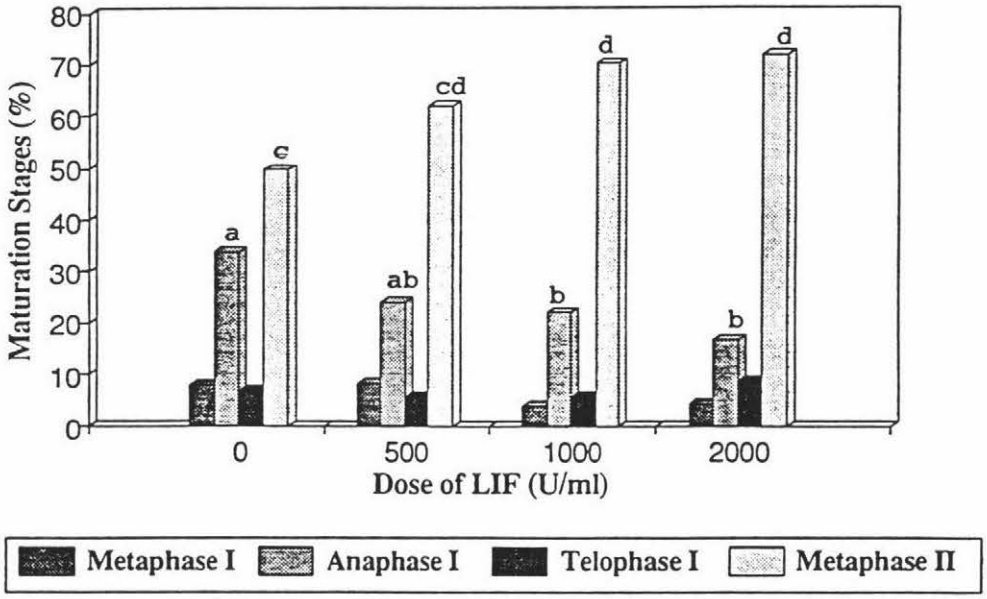

Figure 5. The effect of LIF doses during IVM on the percentage of Metaphase I, Anaphase I, Telophase I and Metaphase II oocytes (Mean ± SEM). Means with different superscript letters within stage differ significantly (P<0.05)

4.2 DNA FRAGMENTATION OF CUMULUS CELLS

Experiment 1. Two methods of DNA preparation for electrophoresis on agarose gel were used to determine DNA fragmentation in cumulus cells. Plate 9 shows that with material prepared by either method there were no distinct DNA ladders observed in sample from any of the treatment groups.

A B

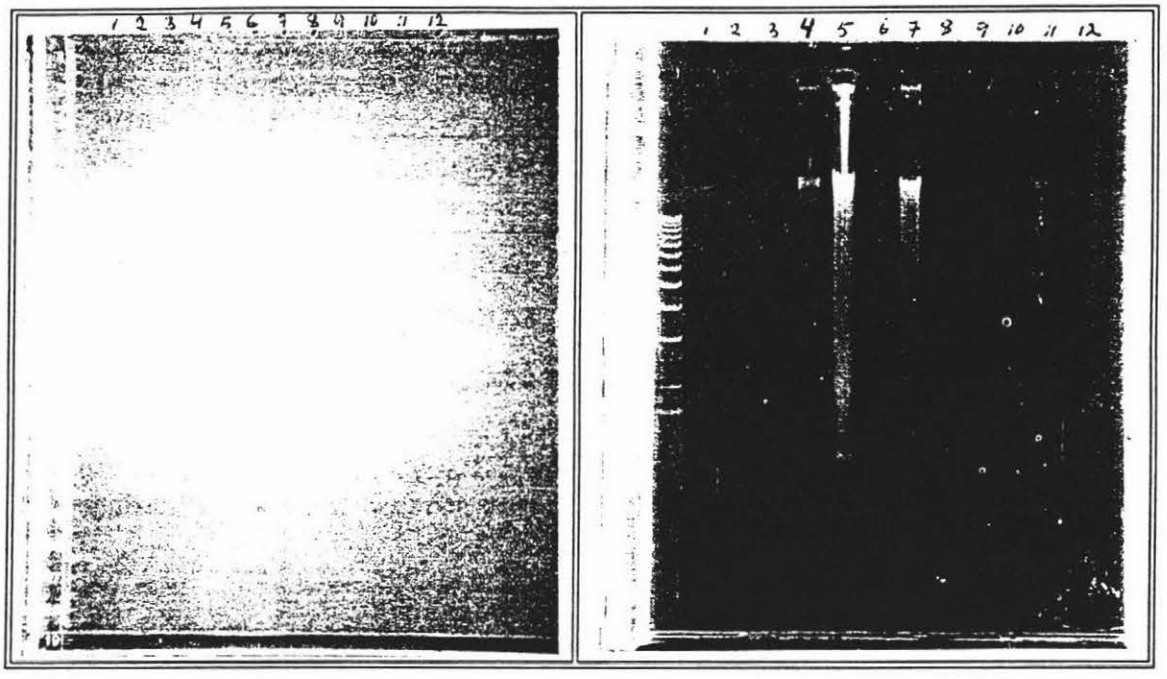

Plate 9. Electrophoretic analysis of DNA fragmentation in cumulus cells during in vitro maturation for different time courses and LIF doses. A, samples were stabilised overnight at room temperature. B, samples were precipitated overnight at -20°C. (0= marker; 1 to 12= treatment groups)

4.3 DEVELOPMENT OF BOVINE EMBRYOS

Experiment 2. The percentages of oocytes which cleaved and those which developed to blastocysts are presented in Table 5. There was no effect ($P>0.05$) of LIF dosage during 24 h of IVM on subsequent cleavage and blastocyst development.

However, when IVM for 20 vs 24 h was compared, the duration of IVM significantly affected blastocyst rate ($P<0.05$), but did not affect ($P>0.05$) the percentages of oocytes that cleaved and developed to blastocysts (Table 6). This Table shows that more oocytes that were matured for 20 h cleaved and continued development to blastocysts than those oocytes matured for 24 h and cleaved (27% vs 20%; $P<0.05$). There was no effect of LIF dosage (0, 500, 1000 or 2000 U/ml) during IVM on the percentage of oocytes that cleaved or developed to blastocysts ($P>0.05$).

Table 5. Development of bovine embryos derived from 24 h IVM in LIF following IVF and IVC (Mean ± SEM)

LIF in IVM (U/ml)	Number of oocytes	Embryo development		
		Cleavage (%) (of oocytes)	Blastocyst (%) (of oocytes)	Blst Rate [1](%) (of cleaved)
0	260	84.05±2.65	15.99±3.15	19.60±3.36
500	259	85.87±2.65	17.44±3.15	20.62±3.36
1000	267	85.46±2.65	18.29±3.15	21.60±3.36
2000	255	85.61±2.65	12.44±3.15	14.70±3.36

[1] Blastocyst rate = (Σ blastocysts/Σ cleaved) x 100%
No significant difference between treatments (P>0.05)

Table 6. Development of bovine embryos derived from 20 or 24 h IVM following IVF and IVC (Mean ± SEM)

Duration of IVM (h)	Number of oocytes	Embryo development		
		Cleavage (%) (of oocytes)	Blastocyst (%) (of oocytes)	Blst Rates (%) (of cleaved)
20	443	78.86±1.97	21.01±1.62	27.22±1.70[a]
24	427	82.63±1.97	16.34±1.62	19.95±1.70[b]

Means with different superscripts differ significantly (P<0.05)

The percentages of oocytes that cleaved and developed to blastocysts following 20 or 24 h IVM at different doses of LIF are presented in Table 7. There was no interaction (P>0.05) between the time course (20 vs 24h) and dose of LIF (0, 500, 1000 or 2000 U/ml) during IVM on the percentage of oocytes which cleaved and developed to blastocysts following IVF and IVC.

Table 7. The effect of LIF during IVM and the duration of IVM on bovine embryo development (Mean ± SEM)

LIF in IVM (U/ml)	Number of oocytes	Embryo development		
		Cleavage (%) (of oocytes)	Blastocyst (%) (of oocytes)	Blst Rate (%) (of cleaved)
20h IVM				
0	112	77.78±4.04	20.84±3.33	27.14±3.53
500	110	80.14±4.04	21.02±3.33	26.79±3.53
1000	111	80.40±4.04	25.34±3.33	32.40±3.53
2000	110	77.04±4.04	17.15±3.33	22.80±3.53
24h IVM				
0	96	77.99±4.04	12.00±3.33	15.20±3.53
500	110	88.70±4.04	20.52±3.33	23.20±3.53
1000	112	86.24±4.04	19.23±3.33	22.47±3.53
2000	109	76.32±4.04	14.24±3.33	19.33±3.53

No significant difference between various treatments ($P>0.05$)

Experiment 3. Table 8 presents data on the development which occurred in embryos following IVM with or without LIF and cultured in LIF. There was an interaction ($P<0.05$) of LIF dose (0 vs 1000 U/ml) during IVM and LIF doses (0, 500, 1000 or 2000 U/ml) during IVC for the percentage of blastocyst and blastocyst rate, but no interaction ($P>0.05$) for the percentage that cleaved. This interaction shows that when LIF was present during IVC only, the number of blastocysts was decreased. In contrast, when LIF was added both during IVM and IVC, the number of blastocysts was increased. Furthermore, a combined treatment of 1000 U/ml LIF during IVM and 1000 U/ml LIF during IVC resulted in the highest percentages of blastocysts (33%) and the blastocyst rate (37%). The interactions of the percentage of blastocysts and the rate of blastocyst are presented in Figure 6 and Figure 7, respectively.

Table 8. The effect of LIF during IVM and IVC on bovine embryo development (Mean ± SEM)

LIF in IVM/IVC (U/ml)	Number of oocytes	Embryo development		
		Cleavage(%) (of oocytes)	Blastocyst(%)[1)] (of oocytes)	Blst Rate(%)[1)] (of cleaved)
0/0	255	86.78±1.79	29.14±2.23	34.46±2.46
0/500	249	85.98±1.79	25.67±2.23	30.90±2.46
0/1000	261	88.32±1.79	22.56±2.23	25.66±2.46
0/2000	259	88.75±1.79	21.89±2.23	24.81±2.46
1000/0	247	87.18±1.85	21.70±2.32	24.60±2.55
1000/500	237	86.76±1.85	20.37±2.32	23.89±2.55
1000/1000	242	90.29±1.85	33.05±2.32	37.05±2.55
1000/2000	246	88.82±1.85	28.50±2.32	32.83±2.55

[1)] Interaction between LIF in IVM and LIF in IVC; P<0.05.

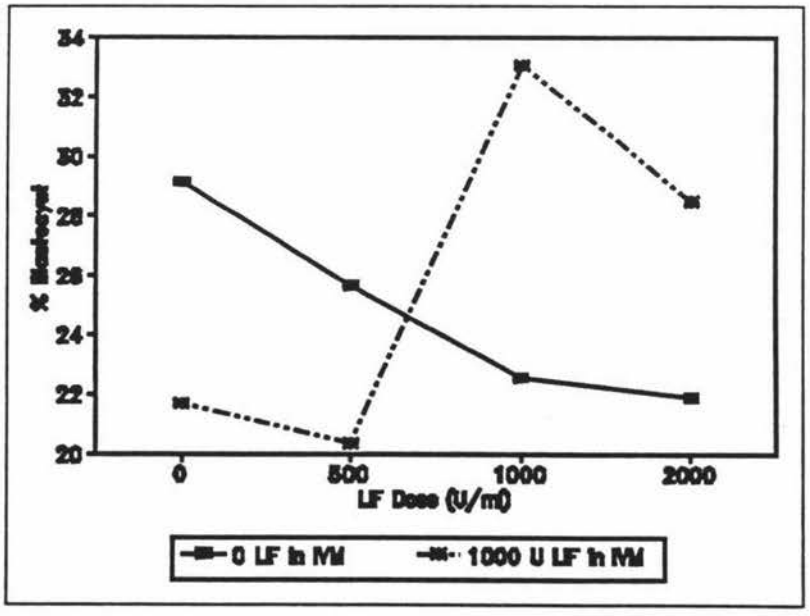

Figure 6. Interaction of LIF in IVM and IVC on the proportion of blastocysts ({Σ blastocyst/Σ oocytes} x 100%), (P<0.05).

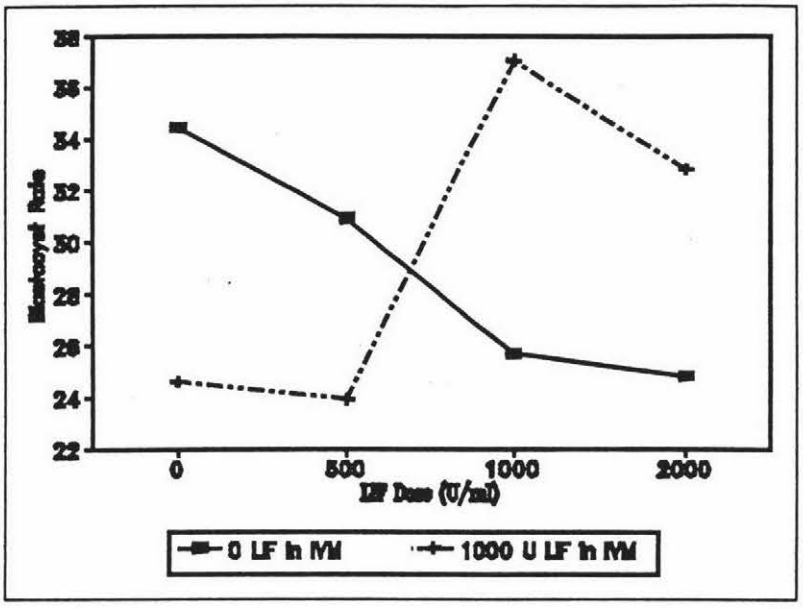

Figure 7. Interaction of LIF in IVM and IVC on the blastocyst rate ({Σ blastocysts/Σ cleaved} x 100%), ($P<0.05$).

4.4 CELL NUMBERS OF BLASTOCYSTS

Experiment 2. Cell numbers of blastocysts derived from 24 h IVM containing LIF dosage are presented in Table 9. There were no differences ($P>0.05$) between LIF doses during IVM in the mean number of blastocyst cells counted.

Table 10 shows data on the number of blastocyst cells after maturation for 20 or 24 h in a variable concentration of LIF. There was no interaction ($P>0.05$) between the duration of IVM (20 vs 24 h) and LIF doses (0, 500, 1000 or 2000 U/ml) during IVM on cell numbers of blastocysts, following IVF and IVC. Similarly, there was no difference ($P>0.05$) either within the IVM treatment time or LIF dosage (0, 500, 1000 or 2000 U/ml) during IVM on the cell numbers of blastocysts.

Table 9. The effect of dose of LIF during IVM on blastocyst cell numbers following IVC (Mean ± SEM)

LIF in IVM (U/ml)	Number of Blastocysts	Blastocyst cell numbers
0	29	114.07±5.89
500	38	117.34±5.15
1000	34	126.06±5.44
2000	21	121.57±6.92

Differences between treatments not significant (P>0.05)

Table 10. The effect of LIF during IVM and the duration of IVM on blastocyst cell numbers following IVC (Mean ± SEM)

Duration of IVM (h)	Dose of LIF in IVM (U/ml)			
	0	500	1000	2000
20	123.20±8.94 (n=24)	131.89±9.42 (n=29)	141.82±9.70 (n=22)	131.59±9.70 (n=26)
24	118.12±14.14 (n=8)	115.80±8.94 (n=20)	128.82±9.70 (n=17)	127.75±11.54 (n=12)

n = Number of blastocysts
No significant difference between treatments (P>0.05)

Experiment 3. Cell numbers of blastocysts derived from IVM and IVC containing LIF following IVF are presented in Table 11. There was no interaction (P>0.05) of LIF (0 vs 1000 U/ml) during IVM and LIF dosage (0, 500, 1000 or 2000 U/ml) during IVC for the mean cell numbers of blastocysts. However, with regard to 0 vs 1000 U/ml LIF during IVM, the cell numbers of blastocysts derived from IVM without LIF were significantly (P<0.05) higher than those derived from 1000 U/ml LIF during IVM, (Table 12). Furthermore, there was no effect (P>0.05) of LIF dose during IVC on the mean cell numbers of blastocysts.

Table 11. The effect of LIF during IVM and IVC on blastocyst cell numbers (Mean ± SEM)

LIF in IVM (U/ml)	Dose of LIF in IVC (U/ml)			
	0	500	1000	2000
0	117.71±6.01 (n=42)	121.67±8.50 (n=21)	133.15±7.64 (n=26)	115.76±8.50 (n=21)
1000	114.32±7.80 (n=25)	106.70±9.45 (n=17)	110.05±6.40 (n=37)	106.88±6.78 (n=33)

n = Number of blastocysts
No interaction of LIF in IVM and LIF in IVC (P>0.05)

Table 12. The effect of LIF during IVM on blastocyst cell numbers following IVC (Mean ± SEM)

Dose of LIF in IVM (U/ml)	
0	1000
121.74±3.69[a] (n=110)	109.56±3.66[b] (n=112)

n = Number of blastocysts
Means with different superscript letters differ significantly (P<0.05)

4.5 PROPORTION OF EARLY, EXPANDED AND HATCHED BLASTOCYST STAGES

Experiment 3. Table 13 shows the percentage of embryos classified as early, expanded or hatched blastocysts. There was no effect (P>0.05) of addition of 1000 U/ml LIF during IVM on the percentage of blastocysts distributed among the stages of development.

Table 13. The effect of LIF during IVM on the percentage of blastocysts that reached the early, expanded or hatched stages following IVC (Mean ± SEM)

LIF in IVM (U/ml)	Number of Blastocysts	Blastocyst stages		
		Early (%)	Expanded (%)	Hatched (%)
0	114	27.54±3.19	39.66±3.08	36.42±3.62
1000	123	40.22±3.49	36.65±3.37	22.75±3.96

No significant difference between treatments (P>0.05)

There was, however, a significant effect of LIF added during IVC on the percentage of blastocysts at the early (P<0.001), expanded (P<0.05) and hatched (P<0.001) stages (Figure 8). In the absence of LIF during IVC, most (56%) blastocysts were classified as expanded while only 12% had hatched. On the other hand, most blastocysts that developed following IVC in 2000 U/ml LIF had hatched (60%). The results show that a higher concentration of LIF during IVC accelerated development through the blastocyst stages.

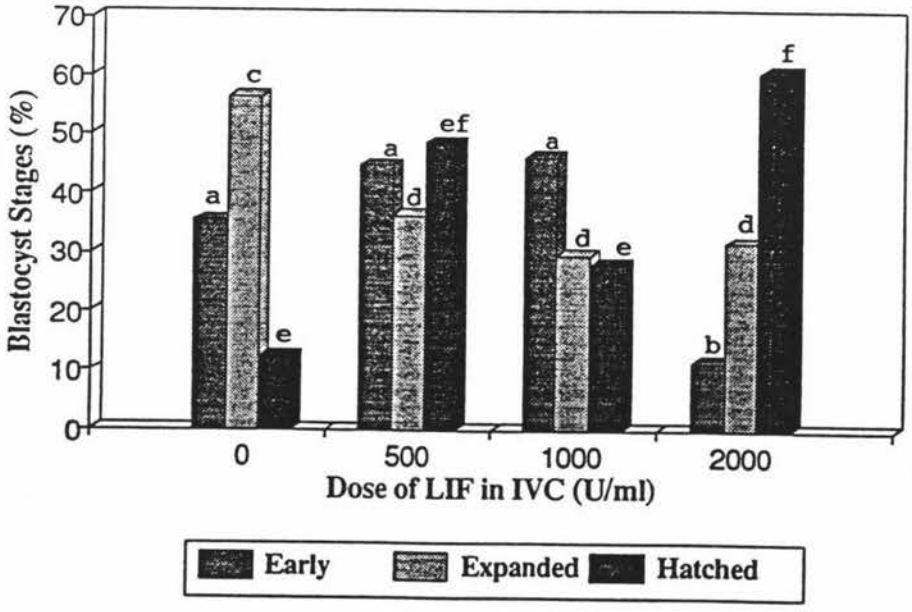

Figure 8. The effect of LIF dose during IVC on the percentage of blastocysts that reached the early, expanded or hatched stages (Mean ± SEM). Means with different superscript letters within stage differ significantly (P<0.05)

There was no interaction (P>0.05) of LIF dose (0 vs 1000 U/ml) during IVM and LIF doses (0, 500, 1000 or 2000 U/ml) during IVC on the percentages of early, expanded and hatched blastocysts (Table 14). When LIF was absent from the IVM media, the addition of LIF during IVC did not affect the proportion of early blastocysts other than at the high LIF (2000 U/ml) dose, however more early blastocysts were observed for low doses of LIF during IVC when LIF was also included in IVM.

Table 14. The effect of LIF during IVM and IVC on the percentage of blastocysts that reached the early, expanded or hatched stage (Mean ± SEM)

LIF in IVM/IVC (U/ml)	Number of Blastocysts	Blastocyst stages		
		Early (%)	Expanded (%)	Hatched (%)
IVM/IVC				
0/0	33	34.56±5.00	52.90±5.82	13.96±5.84
0/500	32	29.95±5.00	44.49±5.82	35.90±5.84
0/1000	26	43.40±5.00	34.79±5.82	29.80±5.84
0/2000	23	10.38±5.00	27.28±5.82	69.78±5.84
1000/0	23	34.53±5.48	60.23±6.38	10.92±6.40
1000/500	25	68.08±5.48	26.72±6.38	11.52±6.40
1000/1000	35	49.63±5.48	24.14±6.38	25.88±6.40
1000/2000	40	12.92±5.48	37.16±6.38	48.68±6.40

No interaction between LIF in IVM and LIF in IVC (P>0.05).

4.6 CELL NUMBERS OF BLASTOCYST INNER CELL MASS AND TROPHECTODERM

Experiment 3. Table 15 shows the number of cells counted in the inner cell mass and trophectoderm. There was no interaction (P>0.05) of LIF dose (0 vs 1000 U/ml) during IVM and LIF doses during IVC (0, 500, 1000 or 2000 U/ml) for the cell numbers of

ICM and TE and the relative proportion of ICM in the blastocysts. However, with regard to the LIF doses during IVC, a dose of 1000 U/ml LIF yielded greater ICM cell numbers (62 cells, $P<0.01$) than did LIF doses of 0, 500 and 2000 U/ml (43, 45, 36 cells, respectively, Figure 9). In addition, there were no differences ($P>0.05$) between LIF doses in IVC either for the TE cell numbers or the proportion of ICM.

Table 15. The effect of LIF during IVM and IVC on the numbers of inner cell mass (ICM) and trophectoderm (TE) cells of bovine blastocysts (Mean ± SEM)

LIF in IVM/IVC (U/ml)	Number of Blastocysts	ICM Cells	TE Cells	ICM (%)
IVM/IVC				
0/0	6	41.50±7.63	69.67±11.08	36.26±5.20
0/500	7	43.14±7.06	68.57±10.26	38.68±4.82
0/1000	6	62.17±7.63	69.50±11.08	46.89±5.20
0/2000	6	30.17±7.63	66.00±11.08	37.19±5.20
1000/0	7	43.57±7.06	81.71±10.26	35.40±4.82
1000/500	6	47.50±7.63	72.33±11.08	40.70±5.20
1000/1000	6	63.00±7.63	85.00±11.08	42.46±5.20
1000/2000	7	40.71±7.06	73.00±10.26	37.13±4.82

ICM (%)= {Σ ICM cell numbers/(Σ ICM + TE cell numbers)} x 100%
Differences between various treatments not significant ($P>0.05$)

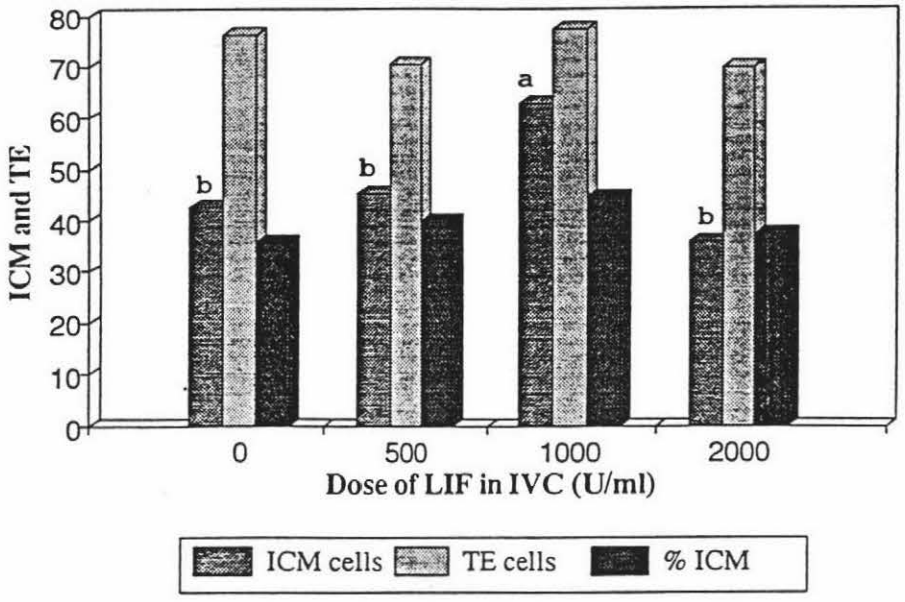

Figure 9. The effect of LIF dose during IVC on the numbers of inner cell mass (ICM) and trophectoderm (TE) cells of bovine blastocysts (Mean ± SEM). Means with different superscript letters differ significantly ($P<0.05$)

CHAPTER 5. DISCUSSION

CHAPTER 5.

DISCUSSION

5.1 MATURATION OF OOCYTES

The results of Experiment 1 indicate that the combined actions of LIF in concentrations of 0, 500, 1000 or 2000 U/ml during IVM and the duration of 18, 22 or 28 h IVM had no beneficial effect on the proportions of oocytes at metaphase II and other meiotic stages (metaphase I, anaphase I, telophase I, Table 4). However, the results show that the presence of LIF (500, 1000 or 2000 U/ml) at 18 h of IVM enhanced the proportion of metaphase II oocytes (41, 52 or 58%, respectively) compared to maturation without LIF (27%). This suggests that the presence of LIF accelerates the process of oocyte maturation *in vitro*. This is a similar finding to that of previous studies where EGF in IVM media shortened the time required for germinal vesicle breakdown (Downs, 1989) to achieve metaphase II stage (Das *et al.*, 1991) in mouse oocytes.

Regardless of LIF in the medium , significantly more oocytes matured for 22 and 28 h reached metaphase II than after 18 h of IVM (Figure 4). Fukui and Ono (1988) reported that after 24 h of IVM in TCM-199 supplemented with granulosa cells, 72% of bovine oocytes had matured and Sirard *et al.* (1988) and Lu *et al.* (1987), found 93% and 92% of bovine oocytes, respectively were matured after 24 h of IVM. Furthermore, immature oocytes that were matured for 24-26 h in groups were capable of yielding more than 90% of bovine oocytes at metaphase II (Lu and Gordon, 1987). Oocytes from immature bovine follicles (1-5 mm) have been matured for 24-27 h *in vitro* to develop from the germinal vesicle stage to metaphase II (Leibfried and First, 1979). The same length of time was also required for the ovine oocytes of 3-6 mm non-atretic follicles, (Moor and Trounson, 1977; Staigmiller and Moor, 1984). Chian *et al.* (1992) reported that a longer period of IVM (28 to 48 h) significantly increased the proportion of polyspermic bovine oocytes (36 to 78%, respectively) compared to those matured for 20 to 24 h (22 to 35%, respectively).

A range of growth factors influences not only proliferation but also functional differentiation of ovarian follicle cells, and perhaps also oocyte maturation (Carson *et al.*, 1989). The results of this study also support the role of growth factors in oocyte maturation since LIF (500, 1000 or 2000 U/ml) during IVM increased the proportion of oocytes reaching metaphase II (62%, 70%, 72%, respectively) compared to the control (0 LIF= 50%, Figure 5). EGF in combination with low concentrations of gonadotrophins (0.5 g/ml of FSH or LH) has also been reported to increase the proportion of matured oocytes *in vitro* (Harper and Brackett, 1993a). They reported that 1, 10 and 100 ng EGF/ml alone yielded 72%, 76%, and 74% matured oocytes, respectively while in combination with 50 g LH/ml, maturation rates were increased to 90%, 93% and 90%, respectively. Similar collaborative actions of gonadotrophins (FSH or LH) with other growth factors (EGF and/or PDGF) in the physiological (functional) maturation of bovine oocyte have been reported (Harper and Brackett, 1993b).

The results of the present study suggest that LIF may be present in the follicular fluid of the ovary because of its effect on oocyte maturation. Several studies have reported that growth factors, their receptors as well as localised production of growth factors occur within the ovary and granulosa cells (Hammond *et al.*, 1985; Feng *et al.*, 1987; Skimmer *et al.*, 1987; Roy and Greenwald, 1990). Growth factors may act as *autocrine* and *paracrine* regulators of ovarian function (Hammond *et al.*, 1988; Carson *et al*,. 1989) causing granulosa and theca cell proliferation and differentiation (Harper and Brackett, 1993b).

Lorenzo *et al.* (1994) reported that gonadotrophins are the primary regulators of nuclear maturation in mammalian oocytes *in vivo*. However, there are other factors that appear to regulate ovarian function. Several growth factors are believed to have roles in ovarian function, such as EGF (Dekel and Scherizly, 1985) and Insulin-like growth factor type I, IGF-I (Hernandez *et al.*, 1988). EGF alone or in combination with IGF-I will stimulate cumulus cell expansion as well as enhance nuclear maturation of bovine oocytes (Lorenzo *et al.*, 1994). Growth factors also may act synergistically with other growth factors or gonadotrophins to influence oocyte maturation. The results of Experiment One add to these observations since LIF used in combination with FSH, LH and E_2,

accelerated the rate of oocyte maturation.

5.2 DNA FRAGMENTATION

Gel electrophoresis of DNA extracted from cumulus cells of oocytes from any one of the twelve treatments in Experiment One did not reveal any DNA ladders characteristic of apoptosis (Plate 9). There are a number of possible explanations for this result. Firstly, it could be that insufficient DNA was loaded to visualise DNA laddering without end labelling with radionucleotides. Alternatively, DNA fragmentation due to apoptosis did not occur during IVM in the conditions used. DNA fragmentation, due to apoptosis in bovine granulosa cells previously has been reported, primarily in cells from atretic follicles (Jolly *et al.*, 1994). There is the possibility that oocytes recovered from atretic follicles were not selected for maturation in the experiments reported here. Alternatively, the gonadotrophins present in the maturation medium either alone or acting synergistically with LIF prevented apoptosis or 'rescued' oocytes from early apoptosis during IVM.

The results of this study are thus inconclusive in determining the presence or effect of apoptosis of cumulus cells during oocyte maturation *in vitro*.

5.3 DEVELOPMENT OF BOVINE EMBRYOS

In Experiment 2, the addition of LIF (500, 1000 or 2000 U/ml) during 24 h IVM did not affect the subsequent development of embryos (the proportion that cleaved or formed blastocysts) after fertilisation *in vitro* (Table 5). This is in contrast to the results of Harper and Brackett (1993a,b) who reported that EGF, and EGF plus PDGF alone, respectively, or in combination with gonadotrophins (LH or FSH) during IVM increased the number of oocytes developing to blastocysts. Reasons for these different results from the findings of the present study may be due to the different growth factors and maturation systems used.

Gonadotrophin hormones are secreted by the ovary during follicular growth and during the period when the oocyte enters metaphase II (maturation). Only matured oocytes will undergo cleavage and subsequent embryo development following fertilisation (Rose and Bavister, 1992). The present study indicates that the amount of LIF added during IVM was probably not enough to enhance the IVM of oocytes and their subsequent ability for further development more than that provided by the gonadotrophins present during IVM.

Since the results of Experiment One indicated that the addition of LIF during IVM could shorten the time course of IVM, the second investigation of Experiment 2 compared 20 h vs 24 h of IVM in the presence of 0, 500, 1000 or 2000 U/ml LIF on the development of embryos. The results showed that there was no interaction effect of these treatments on incidence of cleavage and blastocyst formation (Table 7). However, the duration of IVM significantly affected the rate of blastocyst development (Table 6). A shorter maturation period of 20 h resulted in a higher blastocyst rate (27%) than that of oocytes matured for 24 h *in vitro* (20%). A similar result was reported by Dominko and First (1992) who noted that 24 h of IVM either in the presence of LH (5 g/ml) or FSH (25 g/ml) resulted a lower proportion of blastocyst rates (10% and 12%, respectively) than that achieved by 16 h of IVM (47% and 34%, respectively) or by 20 h IVM (35% and 30%, respectively). In the present study, maturation for 24 h may lead to the oocytes becoming aged or 'over mature'. As reported by Chian *et al.* (1992), aged oocytes showed an increase in the incidence of polyspermic fertilisation and further cleavage to the 4- to 16-cell stages was impaired.

The results of Experiment 3 showed there was interaction between the supplementation of LIF during IVM (0 vs 1000 U/ml) and LIF during IVC (0, 500, 1000 or 2000 U/ml) in the percentage of blastocysts formed, but the proportion of oocytes that cleaved was not affected (Table 8). These data show that oocytes matured and cultured in LIF-free medium had a higher percentage of blastocyst rates (34%) compared to the oocytes matured in 1000 U/ml LIF and cultured in LIF-free medium (25%). The effect of LIF in embryo development may be similar to the ***biphasic effect*** observed for serum. Growth factors are one of the components of the serum (Harper and Brackett, 1993a)

and LIF might be one of those growth factors. Pinyopummintr and Bavister (1991) and Bavister *et al.* (1992) defined a ***biphasic effect*** of serum as one that suppresses first cleavage division and also is needed to increase compaction of the morula and blastocyst formation.

Furthermore, addition of 1000 or 2000 U/ml LIF during IVC of oocytes matured in 1000 U/ml LIF considerably increased the percentage of blastocysts and rate of blastocysts (33% and 37% or 28% and 33%, respectively, Table 10). The highest proportion of blastocysts was reached when LIF of 1000 U/ml was used both during IVM and IVC (33% and 37%, respectively for the percentage and rate of blastocysts). These findings were similar to the study of Herrler *et al.* (1992) who found that supplementation of 50 g/ml IGF-I both in IVM using TCM-199 and IVC on the granulosa cell monolayer increased the number of morulae and blastocysts. In addition, IGF-I has been reported to enhance growth of 2-cell mouse embryos by increasing proliferation of the inner cell mass *in vitro* (Harvey and Kaye, 1992). It is possible that LIF may be acting by two mechanisms: one that enhances the maturation process and the other affecting subsequent development.

The presence of 1000 U/ml LIF during IVM may enhance cumulus cell expansion. The presence of EGF alone or in combination with gonadotrophins (Harper and Brackett, 1993a) or EGF together with IGF-I (Lorenzo *et al.*, 1994) or combination between PDGF and FSH (Harper and Brackett, 1992b) during IVM enhanced cumulus cell expansion. Since no measure of the extent of cumulus cell expansion was made during these studies, the effect of LIF on cumulus expansion remains to be determined.

The present finding is in agreement with the report of Fry *et al.* (1992a) for ovine embryo development. They found that the addition of LIF (1000 U/ml) to the culture medium (SOF) for 48 h significantly improved hatching of ovine blastocysts compared to the control without LIF (64% vs 14%).

Overall, an interaction of LIF during IVM and IVC showed that when LIF was used only during IVC, the proportion of blastocysts decreased as LIF concentrations

increased. On the other hand, when LIF was used both during IVM (1000 U/ml) and IVC, the proportion of blastocysts was enhanced concomitant with the increase in LIF concentrations during IVC. The present study, therefore suggests that the presence of LIF both during IVM and IVC is required to improve the proportion of oocytes developing to blastocysts.

In this study, the presence of LIF during IVM may improve the 'cytoplasmic maturation' of the oocyte allowing it to undergo further development. LIF may act on the oocyte directly or on the cumulus cells, while the presence of LIF during IVC may promote embryo development. As reviewed by Heyner *et al.* (1993), growth factors of the oviduct and uterus are implicated in stimulating cellular proliferation and in some cases, differentiation of preimplantation embryos. Thus, LIF may act along a paracrine pathway by binding to specific receptors on embryonic cells.

5.4 CELL NUMBERS OF BLASTOCYSTS

Experiment 2 provide data that 24 hours of IVM in the presence of LIF (0, 500, 1000 or 2000 U/ml) did not affect the cell numbers of blastocysts (Table 9). However, Table 9 shows that LIF at 1000 U/ml during IVM resulted in the highest number of blastocyst cells (126 cells) compared to the other treatments. Iwasaki and Nakahara (1990) reported that the cell numbers of early and expanded blastocyst stages cultured *in vitro* without supplementation of growth factors were 43 and 80 cells, respectively. Another report has demonstrated that the mean cell numbers in blastocysts cultured *in vitro* with a chemically defined bovine embryo culture medium in PVA-free and without FCS was 119 cells, but addition of heat-treated or unheated FCS yielded 150 and 152 blastocyst cells, respectively (Lim *et al.*, 1994). Different results found in this present study compared with other reports may be due to different culture systems used. In this study, bovine embryos were cultured in SOF containing BSA and under low oxygen.

A similar result was observed in the second study of the Experiment 2. There was no interaction of the duration of IVM (20 vs 24 h) and LIF concentrations (0, 500, 1000 or 2000 U/ml) during IVM on the cell numbers of blastocysts (Table 10). The data of Table 9, however, show that the cell numbers of blastocysts derived from oocytes matured for 20 h in LIF-supplemented medium were higher than those of oocytes matured for 24 h in similar medium, and 20 h of IVM in 1000 U/ml LIF resulted in the highest numbers of blastocyst cells (142 cells). Likewise, there were no differences within the duration of IVM (20 vs 24 h) or within LIF concentrations (0, 500, 1000 or 2000 U/ml) during IVM.

The results of Experiment 3 indicate that the combined application of LIF during IVM and IVC had no beneficial effect on the cell numbers of blastocysts (Table 13). LIF during IVM was the only factor that significantly affected the cell numbers of blastocysts (Table 12), the cell numbers of blastocysts matured without LIF were higher (122 cells) than those matured with 1000 U/ml LIF (109 cells).

While LIF in IVM and IVC appeared to increase the proportion of oocytes developing to blastocysts (Table 8), there was no increase in blastocyst cell numbers (Table 11). These results are in agreement with the findings of Yang *et al.* (1993) who reported that growth factors increased the percentage of embryos that develop into morulae and blastocysts without an increase in the cell numbers. In addition, Fukui and Matsuyama (1994) demonstrated that there was no effect of LIF (500 to 6000 U/ml) during IVC on the cell numbers of hatched blastocysts. However, these results contrast with those of Harvey and Kaye (1992) who found that IGF-I increased the cell numbers of mouse blastocysts.

5.5 STAGES OF BLASTOCYST DEVELOPMENT

There was no interaction between LIF (0 vs 1000 U/ml) during IVM and LIF during IVC (0, 500, 1000 or 2000 U/ml) observed in Experiment 3 on the proportion of early, expanded and hatched blastocyst stages (Table 14). The data of Table 14, however, show

the highest proportion of embryos at early blastocyst stage occurred when they were matured in 1000 U/ml LIF and cultured in 500 U/ml LIF (68%). Higher proportions of expanded blastocysts occurred when the oocytes were matured and cultured in the absence of LIF (53%) or matured in 1000 U/ml LIF and cultured without LIF (60%). A level of 2000 U/ml LIF during IVC accelerated development of embryos into advanced blastocysts (hatched blastocyst stage) after maturation either without LIF (70%) or with 1000 U/ml LIF (48%).

The addition of LIF during IVC was the only factor affecting the proportion of early, expanded and hatched blastocyst stages (Figure 8). A concentration of 2000 U/ml LIF during IVC accelerated blastocyst development stages, fewer were at the early blastocyst stage (11%) while more progressed through to hatching (60%). This finding supports the result of Fukui and Matsuyama (1994) that LIF at 2000 to 6000 U/ml and 1000 U/ml LIF during IVC improved the development of 8-cell and early blastocyst stages, respectively to the hatched blastocyst stage. LIF may be acting by improving blastocyst health *in vitro* allowing them to continue through to hatching (Fry *et al.*, 1994).

However, the addition of LIF during IVM did not affect the proportion of blastocyst stages (Table 13), suggesting that the presence of LIF during IVC is required for enhancement of blastocyst development. Alternatively, LIF at 1000 U/ml during IVM was insufficient to affect subsequent blastocyst formation and progression.

5.6 BLASTOCYST INNER CELL MASS AND TROPHECTODERM

The results of these studies suggest that, overall, LIF during IVM and LIF during IVC did not affect the numbers of cells in the blastocyst inner cell mass (ICM) and trophectoderm (TE) as well as the proportion of the blastocyst comprising ICM (Table 15). LIF concentrations during IVC were the only factor significantly affecting the cell numbers of blastocyst ICM (Figure 9). A dose 1000 U/ml LIF during IVC had a greater effect on the cell numbers of blastocyst ICM (62 cells) while a higher level of 2000

U/ml LIF during IVC caused lower cell numbers in the ICM (36 cells) and as consequence, the proportion of blastocyst ICM was also decreased. It may be explained by referring to the previous statement that the highest level of LIF in this study improved development of advanced blastocysts (hatched blastocysts). Papaioannou and Ebert (1988) found that for pig embryos, the proportion of ICM increased at the early blastocyst stage and then decreased as the blastocyst expanded and hatched. Therefore, the present study suggests that a lower proportion of ICM derived from 2000 U/ml LIF in IVC was probably due to the greater proportion of hatched blastocysts examined compared to a greater proportion of early blastocysts for the 1000 U/ml LIF group. Iwasaki *et al.* (1990) also demonstrated that the proportion of ICM cells increased to the level of embryos fertilised *in vivo* when bovine embryos derived from in vitro fertilisation were transferred to the rabbit oviduct, even though the cell-cell contacts of ICM derived *in vitro* did not improve. Compared to the finding of Iwasaki *et al.* (1990), the proportion of ICM of the embryos in this study was higher at all of the blastocyst stages (early, expanded and hatched) and may be due to different culture systems used. There was no effect of LIF during IVM on the cell numbers in the blastocyst ICM and TE as well as the proportion of ICM relative to the total cells in the blastocysts.

CHAPTER 6. CONCLUSIONS

CHAPTER 6.

CONCLUSIONS

The effect of Leukaemia Inhibitory Factor (LIF) on bovine embryo development *in vitro* was examined and several conclusions can be drawn;

1. There was no interaction of supplementation of LIF during IVM and the duration of IVM in the proportion of oocytes which achieved metaphase II. The presence of LIF can apparently shorten the time required for oocytes to reach metaphase II. A high proportion of oocytes at metaphase II was achieved when bovine oocytes were matured *in vitro* at all periods of maturation and a similar level of metaphase II was observed after 22 h IVM with LIF added. Concentrations of 1000 or 2000 U/ml LIF in a modified TCM-199 containing gonadotrophins improved the proportion of immature oocytes that reached metaphase II.

2. No evidence of apoptosis or rescue from apoptosis by LIF during IVM was found. This may have been owing to an inadequate loading of DNA onto the gel electrophoresis for visualisation of DNA ladders or alternatively, there is no apoptosis occurring during IVM in the presence of gonadotrophins alone or with LIF.

3. Supplementation of LIF during 24 h IVM resulted in a lower proportion of bovine oocytes that cleaved and developed to the blastocyst stage. Regardless of the presence of LIF during IVM, 20 h IVM was better in achieving a number of blastocysts compared to 24 h IVM and supports the results of Experiment One that LIF affects the time course of in vitro maturation.

4. Statistically, there was no effect of LIF during IVM on the cell numbers of blastocysts when oocytes were matured for 20 h or 24 h. However, the data show that 20 h IVM in the presence or absence of LIF resulted in higher blastocyst cell numbers.

5. The proportion of blastocysts was decreased when oocytes were matured in the absence of LIF and then cultured in LIF. On the other hand, more blastocysts developed when bovine oocytes were matured and then cultured in the presence of LIF after IVF. These results indicate that LIF may operate in a 'biphasic' manner.

6. There was no effect on blastocyst cell numbers of LIF, whether it was added to the IVM medium or the IVC medium.

7. The presence of LIF during IVM or IVC did not affect the formation of early, expanded and hatched blastocyst stages. However, supplementation of LIF only during IVC affected the proportion of blastocyst stages and a concentration of 2000 U/ml LIF accelerated the formation of hatched blastocysts.

8. The numbers of cells in the ICM and TE and the proportion of the ICM of the total ICM and TE cells were not affected by the presence of LIF during IVM or IVC. However, LIF during IVC affected the cell number of blastocysts and a concentration of 1000 U/ml LIF resulted a higher cell number of ICM.

The observation made during this study have shown that LIF can influence the maturation of bovine oocytes and the dose-level will have an affect on blastocyst formation and cell numbers. Further research is needed on the effect of LIF on viability of embryos following freezing and/or transfer. This study provided evidence that developing embryos are influenced by the presence of LIF. Further studies using antibodies to LIF or molecular probes for LIF receptor would establish whether bovine embryos synthesise LIF during the course of their preimplantation development.

APPENDICES

Appendix 1. MEDIA RECIPES

1. Laboratory Stock Solutions for IVM, IVF and IVC of Sheep and Cattle Oocytes

Most chemical used for stock preparation were AnalaR grade BDH chemicals

1.1 Stock B (250 mM $NaHCO_3$)

Compound	
$NaHCO_3$	2.101 gr
Phenol red	10.000 mg

Dissolve $NaHCO_3$ in 100 ml MQH_2O and add phenol red. Filter and store in refrigerator (4°C) for up to 2 weeks.

1.2 Stock C (33 mM Pyruvate)

Compound	
Pyruvate	36 mg

Dissolve pyruvate to 10 ml MQH_2O. Filter and store in refrigerator (4°C) for up to 1 week.

1.3 Stock D

Compound	
$CaCl_2.2H_2O$	252 mg

Dissolve $CaCl_2.2H_2O$ in 10 ml MQH_2O. Filter and store in refrigerator (4°C) for up 3 months.

1.4 Stock G (60 mM Glucose)

Compound	
Glucose	108 mg

Dissolve glucose in 10 ml MQH_2O. Filter and store in refrigerator (4°C) for up to 3 months.

1.5 Stock GLN (10 mM Glutamine)

Compound	
Glutamine	14.6 mg

Dissolve glutamine in 10 ml MQH_2O. Filter and store in refrigerator (4°C) for up to 1 week.

1.6 Stock K

Compound	
Kanamycin sulphate	500 mg

Dissolve kanamycin sulphate in 10 ml MQH_2O. Filter and store in refrigerator (4°C) for up to 3 months. Use at 1:1000 dilution to get a final concentration of 50 µg/ml.

1.7 Stock L (330 mM Na Lactate)

Compound	
Na Lactate (60% syrup)	0.47 ml

Dissolve Na Lactate in 10 ml MQH_2O. Filter and store in refrigerator (4°C) for up to 1 month.

1.8 Stock M

Compound	
$MgCl_2\ 6H_2O$	100.0 mg

Dissolve $MgCl_26H_2O$ in 10 ml MQH_2O. Filter and store in refrigerator (4°C) for up to 3 months.

1.9 Stock S_3

Compound	gr/100 ml
NaCl	5.865
KCl	0.534
KH_2PO_4	0.162
Penicillin	0.060
Streptomycin	0.050

Dissolve compounds in NaCl, filter and store in refrigerator (4°C) for up to 3 months.

1.10 TL (10x) stock

Compound	gr/100 ml
NaCl	6.662
KCl	0.238
$NaH_2PO_4H_2O$	0.062
Penicillin	0.060

Dissolve all compounds in 100 ml MQH_2O, filter and store in refrigerator (4°C) for up to 3 months.

1.11 SPTL (10x)

Compound	gr/100 ml
NaCl	4.675
KCl	0.23
$NaH_2PO_42H_2O$	0.045
Penicillin	0.060
Hepes (acid)	1.192
Hepes (salt)	1.302
Na Lactate syrup	3.68 ml

Dissolve all compounds in 100 ml MQH_2O, filter and store in refrigerator (4°C) for up to 3 months.

1.12 Stock Heparin (10 mg/ml)

Compound	
0.9% sterile saline	2 ml
Heparin	20 mg

Dissolve Heparin in 0.9% sterile saline, DO NOT FILTER. Store in refrigerator (4°C) for up to 1 week.

1.13 Stock Hypotaurine (1 mM Hypotaurine)

Compound	
Hypotaurine	1.09 mg

Dissolve Hypotaurine in 10 ml MQH_2O or 0.9% NaCl, filter and store in refrigerator (4°C) for up to 1 week.

1.14 Stock Penicillamine (2 mM Penicillamine)

Compound	
Penicillamine	2.984 mg

Dissolve penicillamine in 10 ml MQH_2O or 0.9% NaCl. Filter and store in refrigerator (4°C) for up to 1 week.

1.15 100x SPAD

Compound	gr/100 ml
$CaCl_2(2H_2O)$	0.308
$MgCl_2(7H_2O)$	0.081

Dissolve all compounds in 100 ml MQH_2O. Filter and store in refrigerator (4°C) for up to 2 months.

1.16 TCM199 stock (2x)

Compound	/500 ml
TCM 199 (Gibco)	1 sachet
Kanamycin sulphate	50 mg

Dissolve TCM199 (Gibco) in 500 ml MQH_2O and add Kanamycin sulphate. Filter and store in refrigerator (4°C) for up to 1 week.

1.17 Bicarbonate buffered 199 or B199 (0.2 mM pyruvate)

Compound	
TCM (2x)	50.0 ml
Stock B	10.0 ml
Stock C	0.6 ml

Add compounds to MQH_2O and bring to 100 ml in a volumetric flask. Adjust osmolarity to 280. Store in refrigerator (4°C) for up to 1 week.

1.18 neaas (Sigma Cat. No. M 7145)

Compound	gr/l
L-Arginine HCL	1.05
L-Cystine	0.60
L-Hystidine (free base)	0.40
L-Isoleucine	1.30
L-Leucine	1.30
L-Lysine HCL	1.849
L-Methionine	0.375
L-Phenylalanine	0.825
L-Threonine	1.20
L-Tryptophan	0.20
L-Tyrosine	0.90
L-Valine	1.175

1.19 eaas (Sigma Cat. No. B 6766)

Compound	gr/l
L-Alanine HCL	0.89
L-Asparagine H_2O	1.50
L-Aspartic Acid	1.33
L-Glutamic Acid	1.47
Glycine	0.75
L-Proline	1.15
L-Threonine	1.05

2. Enriched Phosphate Buffered Saline (PB1)

	Compound	
A.	Oxoid salts	1 tablet
	Phenol red	1.0 mg
	Glucose	0.1 gr
	Stock C, or	1.0 ml
	Na Pyruvate	3.6 mg
	Stock K	0.1 ml
B.	Stock PSA	1.0 ml

A. Dissolve oxoid in 60 ml MQH_2O, add the rest of compounds.
B. Dissolve stock PSA to 35 ml MQH_2O.
Add A solution slowly to B solution, stir vigorously.

PB1 + 0.4% BSA

Compound	
ICP BSA	2 ml

Remove 2 ml of PB1 and replace with 2 ml ICP BSA.

2.1 Stock PSA

Compound	
$CaCl_2.2H_2O$	0.132 gr
$MgCl_2.6H_2O$	0.100 gr

Dissolve $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ in a final volume of 10 ml H_2O. Filter and store in refrigerator (4°C) for up to 3 months.

3. 0.1 % Hyaluronidase in Hepes SOF

Compound	
Hyaluronidase	5 mg
Hepes SOF	5 ml

Dilute Hyaluronidase in Hepes SOF and warm in Incubator before use.

4. Oocyte Holding Media (made up the day use)

4.1 Aspiration Medium (H199 + 0.4% BSA + 50µg/ml heparin)

Compound	
Heparin stock (10 mg/ml)	0.5 ml
ICP BSA (20% w/v)	2.0 ml
H199	97.5 ml

Mix Heparin stock and BSA in H199 and bring to 100 ml in a volumetric flask. Warm in Incubator before use.

4.2 Oocyte Handling Medium (H199 + 10% FCS)

Compound	
FCS	5.0 ml
H199	45.0 ml

Mix FCS in H199 in a volumetric flask. Warm in Incubator before use

4.3 Oocyte Washing Medium (B199 + 10% FCS)

Compound	
FCS	5.0 ml
B199	45.0 ml

Mix FCS in a conical tube with a loose lid and warm in CO_2 Incubator for at least 2 hours before use.

5. Maturation Medium (made up the day use)
(B199 + 10µg/ml FSH, 10µg/ml LH, 1µg/ml E_2 + 10% FCS)

Compound	
FSH (a vial of Ovagen)	0.10 units
B199	8.89 ml
FCS	1.00 ml
LH stock (1 mg/ml)	100.00 µl (a final conct= 10 µg/ml)
E_2 stock (Oestrogen)	10.00 µl (a final conct = 1 µg/ml)

To a vial of ovagen, add all compounds. Equilibrate this medium in drops of 50 µl in a petri dish, overlaid with warmed mineral oil in 5% CO_2 in air for at least 2 hours before use. Ovagen and LH supplied by Immuno Chemical Products, Auckland.

6. Sperm Wash (HEPES TALP)

Compound	
TL (10x)	10.0 ml
Stock B	2.0 ml
Stock H	8.0 ml
Stock D	1.2 ml
Stock M	1.0 ml
Stock L	3.0 ml
Stock C	0.8 ml

Mix compounds in MQH_2O bring to 100 ml, add in stirring 300 mg BSA- FAF. Adjust osmolarity (270 - 290), adjust pH to 7.4. Filter and store in 20 ml aliquots in refrigerator (4°C) for up to 1-2 weeks. This medium contains 5 mM B (Bicarbonate) and 20 mM H (Hepes). BSA-FAF Sigma Cat. No. A 7030

7. Fertilisation Medium, (made up the day use)

Compound	
Fertilisation TALP	10.0 ml
Penicillamine stock	100.0 µl
Hypotaurine stock	100.0 µl
Heparin stock	10.0 µl

Mix compounds in 10 ml Fertilisation TALP, keep with a loose lid and equilibrate in CO_2 Incubator for at least 2 hours before use.

8. Fertilisation TALP

Compound	
10x TL	5.0 ml
Stock B	5.0 ml
Stock D	0.6 ml
Stock M	0.5 ml
Stock L	1.5 ml
Stock C	0.4 ml

Mix compounds to MQH_2O and bring to 50 ml in a volumetric flask. Measure osmolarity (should be 280-300). Add in stirring 300 mg BSA-FAF, filter and store in refrigerator (4°C) for up to 1 week.

9. Fertilised Ova Wash (HEPES SOF)

Compound	
Stock S_2	10.0 ml
Stock B	2.0 ml
Stock H	8.0 ml
Stock C	1.0 ml
Stock D	1.0 ml
Stock M	1.0 ml
Stock L	1.0 ml
Stock G	2.5 ml
Stock GLN	10.0 ml

Mix compounds in MQH_2O and bring to 100 ml in a volumetric flask. Measure osmolarity (should be 265-275), adjust pH to 7.4. Add in stirring 300 mg BSA-FAF. Filter and store in 20 ml aliquots in refrigerator (4°C) up to 1 - 2 weeks. This medium contains 5 mM B (Bicarbonate) and 20 mM H (Hepes).

10. Culture Medium (SOF/AA/BSA)

Compound	
Stock S_3	10.0 ml
Stock B	10.0 ml
Stock C	3.0 ml
Stock D	1.0 ml
Stock L	3.0 ml
Stock M	1.0 ml
Stock G	2.5 ml
Stock GLN	10.0 ml
neaas	1.0 ml
eaas	2.0 ml

Dissolve compounds in MQH_2O and bring to 100 ml, stir the compounds slowly. Measure osmolarity (should be 270 - 280), adjust pH to 7.4. Add in slow stirring 800 mg BSA-FAF. Filter and store in refrigerator (4°C) for up to 1 week. This medium contains 1mM Pyruvate, 10 mM lactate.

Appendix 2. Reagents for DNA Preparation

1. Lysis Buffer (per 10 ml)

Compound	
SDS (10%)	500 µl
1 M TRIS (pH 8.0)	500 µl
0.5 M EDTA	2 ml
H_2O	7 ml

2. TE solution

Compound	
1 M TRIS (pH 8.0)	10 ml
100 mM EDTA	1 ml

Appendix 3. LIF (Leukaemia Inhibitory Factor) Preparation

The LIF used throughout these experiment was recombinant Human LIF (rh-LIF) purchased from the AMRAD Corporation Ltd, Australia. The LIF was resuspended in 500 µl PBS to a concentration of 5 x 10^5 units. The final solution was equivalent to 1 x 10^6 units/ml and were used as a source of LIF levels in medium used (Maturation or Culture medium, MM or CM), i.e., 2000, 1000, 500 units/ml.

To make up 2000 units/ml:

3 ml of MM or CM add 6 µl LIF solution

To make up 1000 units/ml:

1 ml of 2000 units/ml add 1 ml of MM or CM

To make up 500 units/ml:

0.5 ml of 2000 units/ml add 1.5 ml of MM or CM

REFERENCES

REFERENCES

Arends, M.J. & Wyllie, A.H., 1991. Apoptosis: Mechanisms and Roles in Pathology. In: G.W. Richter and K. Solez (eds.), *International Review of Experimental Pathology*. Academic Press, Inc., San Diego. **32**: pp. 223-254.

Arends, M.J., Morris, R.G. & Wyllie, A.H., 1990. Apoptosis: The role of the endonuclease. *American Journal of Pathology* **136**: 593-608.

Baker, T.G., 1979. The control of oogenesis in mammal. In: A.R. Midgley and W.A. Sadler (ed.), *Ovarian Follicular Development and Function*. Raven Press, New York. pp. 353-364.

Baker, T.G., Biggs, J.S.G. & Hunter, R.H.F., 1977. Control of oocyte maturation in mammals. In: V.H.T. James (ed.), *Endocrinology*. Excerpta Medica, Amsterdam. **Vol. 1**: p. 351-361.

Barnes, F.L. & Eyestone, W.H., 1990. Early cleavage and the maternal zygotic transition in bovine embryos. *Theriogenology* **33**: 141-152.

Bavister, B.D., 1992. Co-culture for embryo development: is it really necessary? *Human Reproduction* **7**: 1339-1341.

Bleil, J.D. & Wassarman, P.M., 1983. Sperm-egg interaction in the mouse: Sequences of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Developmental Biology* **95**: 317-324.

Bhatt, H., Brunet, L.J. & Stewart, L., 1991. Uterine expression of leukaemia inhibitory factor coincides with the onset of blastocyst implantation. *Proceedings National Academy of Sciences, U.S.A.* **88**: 11408-11412.

Biggers, J., 1988. Introductory remarks on the milieux of the egg and the early embryo. *Journal of Reproduction and Fertility* **82**: 809-811.

Brackett, B.G., Keefer, C.L., Troop, C.G., Donawick, W.J. & Bennett, K.A., 1984. Bovine twins resulting from in vitro fertilization. *Theriogenology* **21**: 224 (Abstract).

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.

Brigstock, D.R., Heap, R.B. & Brown, K.D., 1989. Polypeptide growth factors in uterine tissues and secretions. *Journal of Reproduction and Fertility* **85**: 747-758.

Calarco, P.G. & McLaren, A., 1976. Ultrastructural observations of preimplantation stages of the sheep. *Journal of Embryology and Experimental Morphology* **36**: 609-622.

Carson, R.S., Zhang, Z., Hutchinson, L.A., Herington, A.C. & Findlay J.K., 1989. Growth factors in ovarian function. *Journal of Reproduction and Fertility* **85**: 735-746.

Chian, R.C., Nakahara, H., Niwa, K. & Funahashi, H., 1992. Fertilization and early cleavage in vitro of ageing bovine oocytes after maturation in culture. *Theriogenology* **37**: 665-672.

Chian, R.C. & Niwa, K., 1994. Effect of cumulus cells present during different periods of culture on maturation *in vitro* of bovine oocytes. *Theriogenology* **41**: 176 (Abstract).

Conquet, F. & Brulet, P., 1990. Developmental expression of myeloid leukaemia inhibitory factor gen in preimplantation blastocysts and in extraembryonic tissue of mouse embryos. *Molecular and Cellular Biology* **10**: 3801-3805.

Critser, E.S., Leibfried-Rutledge, M.L., Eyestone, W.H., Northley, D.L. & First, N.L., 1986. Acquisition of developmental competence during maturation in vitro. *Theriogenology* **25**: 150 (Abstract).

Dahlhausen, R.E, Bonham, J.B. & Ludwick, M.G., 1981. Characterization and maturation of prepuberal calf follicular oocytes in vitro. *Theriogenology* **15**: 111 (Abstract).

Dardik, A. & Schultz, R.M., 1991. Blastocoel expansion in the preimplantation mouse embryo: stimulatory effect of TGF-α and EGF. *Development* **113**: 919-930.

Das, K., Tagatz, G.E., Stout, L.E., Phipps, W.R., Hensleigh, H.C. & Leung, B.S., 1991. Direct positive effect of epidermal growth factor on the cytoplasmic maturation of mouse and human oocytes. *Fertility and Sterility* **55**: 1000-1004.

Dekel, N. & Sherizly, I., 1985. Epidermal growth factor induces maturation of rat follicle-enclosed oocyte. *Endocrinology* **116**: 406-409.

Dominko, T. & First, N.L., 1992. Kinetics of bovine oocyte maturation allows selection for developmental competence and is affected by gonadotropins. *Theriogenology* **37**: 203 (Abstract).

Downs, S.M., 1989. Specificity of epidermal growth factor action on maturation of the murine oocyte and cumulus oophorus *in vitro*. *Biology of Reproduction* **41**: 371-379.

Downs, S.M., Daniel, S.A.J. & Eppig, J.J., 1988. Induction of maturation in cumulus cell-enclosed mouse oocytes by follicle-stimulating hormone and epidermal growth factor: evidence for a positive stimulus of somatic cell origin. *Journal of Experimental Zoology* **245**: 86-96.

Ebert, K.M., Hammer, R.E. & Papaioannou, V.E., 1985. A simple method for counting nuclei in the preimplantation mouse embryo. *Experientia* **41**: 1207-1209.

Edwards, R.G., 1965. Maturation *in vitro* of human ovarian oocytes. *Lancet* **2**: 926-929.

Ellington, J.E., Farrell, P.B. & Foote, R.H., 1990. Comparison of six-day bovine embryo development in uterine tube (oviduct) epithelial cell co-culture versus in vivo development in the cow. *Theriogenology* **34**: 837-844.

Erickson, G.F. & Sorensen, R.A., 1974. *In vitro* maturation of mouse oocytes isolated from late, middle and pre-antral graafian follicles. *Journal of Experimental Zoology* **190**: 123-127.

Eyestone, W.H. & First, N.L., 1986. A study of the 8- to 16-cell developmental block in bovine embryos cultured in vitro. *Theriogenology* **25**: 152 (Abstract).

Eyestone, W.H. & First, N.L., 1989. Co-culture of early bovine embryos to the blastocyst stage with oviductal tissue or in conditioned medium. *Journal of Reproduction and Fertility* **85**: 715-720.

Farell, D.S. & Bavister, B.D., 1984. Short-term exposure of two-cell hamster embryos to collection media is detrimental to viability. *Biology of Reproduction* 31: 104-109.

Feng, P., Catt, K.J. & Knecht, M., 1988. Transforming growth factor-ß stimulates meiotic maturation of the rat oocyte. *Endocrinology* **122**: 181-186.

Feng, P., Knecht, M. & Catt, K.J., 1987. Hormonal control of epidermal growth factor receptor by gonadotrophins during granulosa cell differentiation. *Endocrinology* **120**: 1121-1126.

First, N.L. & Parrish, J.J., 1987. In-vitro fertilization of ruminants. *Journal of Reproduction and Fertility, Supplement* **34**: 151-165.

First, N.L. & Parrish, J.J., 1988. Sperm maturation and in vitro fertilization. *Proceedings of 11th International Congress on Animal Reproduction and Artificial Insemination, Dublin* **Vol. V**: 160-168.

Fleming, A.D., Evans, G., Walton, E.A. & Armstrong, D.T., 1985. Developmental capacity of rat oocytes mature *in vitro* in defined medium. *Gamete Research* **12**: 255-263.

Flood, M.R., Gage, T.L. & Bunch, T.D., 1993. Effect of various growth-promoting factors on preimplantation bovine embryo development in vitro. *Theriogenology* **39**: 823- 833.

Florman, H.M. & First, N.L., 1988. The regulation of acrosomal exocytosis. I. Sperm capacitation is required for induction of acrosome reactions by the bovine zona pellucidae *in vitro*. *Developmental Biology* **128**: 453-463.

Fry, R.C., 1992. The effect of leukaemia inhibitory factor (LIF) on embryogenesis. *Reproduction Fertility and Development* **4**: 449-458.

Fry, R.C., Batt, P.A., Fairclough, R.J. & Parr, R.A., 1992a. Human leukaemia inhibitory factor improves the viability of cultured ovine embryos. *Biology of Reproduction* **46**: 470-474.

Fry, R.C., Purdon, T.L., Squires, T.J. & Parr, R.A., 1992b. The development of bovine embryos cultured in media containing hLIF. *Proceedings Australian Society for Reproduction Biology* **24**: 92 (Abstract).

Fukui, Y., Fukushima, M. & Ono, H., 1983. Fertilization in vitro of bovine oocytes after various sperm procedures. *Theriogenology* **20**: 651-660.

Fukui, Y. & Matsuyama, K., 1994. Development of in vitro matured and fertilized bovine embryos cultured in media containing human leukaemia inhibitory factor. *Theriogenology* **42**: 663-673.

Fukui, Y., Saito, T., Miyamoto, A., Yamashima, H. & Okamoto, Y., 1994. Effect of human leukaemia inhibitory factor on in vitro development of parthenogenetic bovine morulae. *Theriogenology* **42**: 1133-1139.

Fukui, Y. & Sukuma, 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.

Gandolfi, F., 1994. Autocrine, paracrine and environmental factors influencing embryonic development from zygote to blastocyst. *Theriogenology* **41**: 95-100.

Gandolfi, F., Brevini, T.A.L., Modini, S. & Laurina, A., 1990. Embryogenesis in vitro: Role of culture media and co-culture. *Proceedings 3rd Symposium on Advance Topics in Animal Reproduction.* pp. 13-25.

Gandolfi, F. & Moor, M., 1987. Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. *Journal of Reproduction and Fertility* **81**: 23-28.

Gardner, R.L., Papaioannou, V.E. & Barton, S.C., 1973. Origin of the ectoplacental cone and secondary giant cells in mouse blastocysts reconstituted from isolated trophoblast and inner cell mass. *Journal of Embryology and Experimental Morphology* **30**: 561-572.

Gondos, B., 1978. Oogonia and oocytes in mammals. In: R.E. Jones (ed.) *The Vertebrate Ovary*. Plenum Press. New York. pp. 83-120.

Gordon, I., 1990. Laboratory production of cattle embryos. *Proceedings of 3rd Symposium on Advance Topics in Animal Reproduction*. pp. 63-87.

Gospodarowicz, D. & Moran, J.S., 1976. Growth factors in mammalian cell culture. *Annual Review of Biochemistry* **45**: 531-558.

Gough, N.M., Gearing, D.P., King, J.A., Wilson, T.A., Hilton, D.J., Nicola, N.A. & Metcalf, D., 1988. Molecular cloning and expression of the human homologue of the murine gene encoding for myeloid leukaemia inhibitory factor. *Proceedings National Academy of Sciences, U.S.A.* **85**: 2623-2627.

Graber, R. & Losa, G.A., 1994. Early changes of membrane signalling enzymes in glucocorticoid-induced apoptosis of human T-cells. *Experientia* **50**: A1.S01-02 (Abstract).

Graham, J.K., Foote, R.H. & Parrish, J.J., 1986. Effect of dilauroylphosphatidylcholine on the acrosome reaction and subsequent penetration of bull spermatozoa into zona-free hamster eggs. *Biology of Reproduction* **35**: 413-424.

Haimovici, F. & Anderson, D.J., 1993. Effects of growth factors and growth factor-extracellular matrix interactions on mouse trophoblast outgrowth in vitro. *Biology of Reproduction* **49**: 124-130.

Hammond, J.M., Hsu, C.J., Mondschein, J.S. & Canning, S.F., 1988. Paracrine and autocrine functions of growth factors in the ovarian follicle. *Journal of Animal Science, Supplement 2*. **66**: 21-31.

Hammond, J.M., Baranao, J.L.S., Skaleris, D., Knight, A.B., Romanus, J.A. & Rechler, M.M., 1985. Production of insulin-like growth factor by ovarian granulosa cells. *Endocrinology* **117**: 2553-2555.

Hanada, A., 1985. In vitro fertilization in cattle with particular reference to sperm capacitation by Ionophore A23187. *Japanese Journal of Animal Reproduction* **31**: 56-61.

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.

Handyside, A.H. & Hunter, S., 1984. A rapid procedure for visualising the Inner Cell Mass and Trophectoderm nuclei mouse blastocysts in situ using polynucleotide-specific fluorochromes. *Journal of Experimental Zoology* **231**: 429-434.

Handyside, A.H. & Hunter, S., 1986. Cell division and death in the mouse blastocyst before implantation. *Roux's Archives of Developmental Biology* **195**: 519-526.

Harper, K.M. & Brackett, B.G., 1993a. Bovine blastocyst development after in vitro maturation in a defined medium with epidermal growth factor and low concentrations of gonadotropins. *Biology of Reproduction* **48**: 409-416.

Harper, K.M. & Brackett, B.G., 1993b. Bovine blastocyst development after follicle-stimulating hormone and platelet-derived growth factor treatment for oocyte maturation *in vitro*. *Zygote* **1**: 27-34.

Harvey, M.B. & Kaye, P.L., 1992. Insulin-like growth factor-1 stimulates growth of mouse preimplantation embryos *in vitro*. *Molecular Reproduction and Development* **31**: 195-199.

Hassan-Hauser, C., Schellander, K., Korb, H., Kaus, E., Schleger, W. & Mayr, B., 1990. Langzeitkultivierung von rinderembryonen im hinblick auf ie erstellung totipotenter embryonaler stammzellen. *Reproduction in Domestic Animals* **25**: 22-32.

Hassell, J.R. & Pratt, R.M., 1977. Elevated levels of cAMP alters the effect of epidermal growth factor *in vitro* on programmed cell death in the secondary palate epithelium. *Experimental Cell Research* **106**: 55-62.

Hernandez, E.R., Resnick, C.E., Svoboda, M.E., Van Wyk, J.J., Payne, D.W. & Adashi, E.Y., 1988. Somatomedin-C/insulin-like growth factor I as an enhancer of androgen biosynthesis by cultured rat ovarian cells. *Endocrinology* **122**: 1603-1612.

Herrler, A., Lucas-Hahn, A. & Niemann, H., 1992. Effects of insulin-like growth factor-I on in-vitro production of bovine embryos. *Theriogenology* **37**: 1213-1224.

Heyner, S., Shah, N., Smith, R.M., Watson, A.J. & Schultz, G.A., 1993. The role of growth factors in embryo production. *Theriogenology* **39**: 151-161.

Hilton, D.J., 1992. LIF: lots of interesting functions. *TIBS* **17**: 72-76.

Hofman, G.E., Scott, R.T., Brzyski, R.G. & H.W. Jones., 1990. Immunoreactive epidermal growth factor concentrations in follicular fluid obtained from *in vitro* fertilisation. *Fertility and Sterility* **54**: 303-307.

Hyttel, P., Greve, T. & Callesen, H., 1988. Ultrastructure of in-vivo fertilization in superovulated cattle. *Journal of Reproduction and Fertility*. 82: 1-13.

Ijaz, A. & Hunter, A.G., 1989. Evaluation of TEST-yolk buffer capacitated sperm for use in bovine in vitro fertilization. *Biology of Reproduction, Supplement 1*. **40**: 147 (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.

Iwasaki, S., Yoshiba, N., Ushijima, H., Watanabe, S. & Nakahara, T., 1990. Morphology and proportion of inner cell mass of bovine blastocysts fertilized *in vitro* and *in vivo*. *Journal of Reproduction and Fertility* **90**: 279-284.

Iwasaki, S. & Nakahara, T., 1990. Cell number and incidence of chromosomal anomalies in bovine blastocysts fertilized in vitro followed by culture in vitro or in vivo in rabbit oviducts. *Theriogenology* **33**: 669-675.

Jolly, P.D., Tisdall, D.J., Heath, D.A., Lun, S. & McNatty, K.P., 1993. Evidence of apoptosis in bovine granulosa cells. *Proceedings of Australian Society for Reproduction Biology*, University of Otago, Dunedin, New Zealand. p. 5 (Abstract).

Jolly, P.D., Tisdall, D.J., Heath, D.A., Lun, S. & McNatty, K.P., 1994. Apoptosis in bovine granulosa cells in relation to steroid synthesis, cyclic adenosine 3',5'-monophosphate response to follicle-stimulating hormone and luteinizing hormone and follicular atresia. *Biology of Reproduction* **51**: 934-944.

Keefer, C.L., Stice, S.L., Paprocki, A.M. & Golueke, P., 1994. In vitro culture of bovine IVM-IVF embryos: cooperative interaction among embryos and the role of growth factors. *Theriogenology* **41**: 1323-1331.

King, W.A., Niar, A. & Betteridge, K.J., 1985. The nucleolus organizer regions of early bovine embryos. *Journal of Dairy Science, Supplement 2*. **68**: 249 (Abstract).

Larson, R.C., Ignotz, G.G. & Currie, W.B., 1992a. Transforming growth factor ß and basic fibroblast growth factor synergistically promote early bovine embryo development during fourth cell cycle. *Molecular Reproduction and Development* **33**: 432-435.

Larson, R.C., Ignotz, G.G. & Currie, W.B., 1992b. Platelet-derived growth factor (PDGF) stimulates development of bovine embryos during the fourth cell cycle. *Development* **115**: 821-826.

Leibfried, M.L. & Bavister, B.D., 1983. Fertilizability of in vitro matured oocytes from golden hamsters. *Journal of Experimental Zoology* **226**: 481-485.

Leibfried, M.L. & First, N.L., 1979. Characterization of bovine follicular oocytes and their ability to mature in vitro. *Journal of Animal Science* **48**: 76-86.

Lim, J.M., Okitsu, O., Okuda, K. & Niwa, K., 1994. Effects of fetal calf serum in culture medium on development of bovine oocytes matured and fertilized in vitro. *Theriogenology* **41**: 1091-1098.

Lobb, D.K. & Dorrington, J., 1992. Intraovarian regulation of follicle development. *Animal Reproduction Science* **28**: 343-354.

Lorenzo, P.L., Illera, M.J., Illera, J.C. & Illera, M., 1994. Enhancement of 2 cumulus expansion and nuclear maturation during bovine oocyte maturation *in vitro* by the addition of epidermal growth factor and insulin-like growth factor I. *Journal of Reproduction and Fertility* **101**: 697-701.

Lu, K.H. & Gordon, I., 1987. Effect of serum, hormones and cumulus cells on the in vitro maturation of bovine oocytes. *Proceedings Society for the Study on Fertility* 81.

Lu, K.H., Gordon, I., Gallagher, M. & McGovern, H., 1987. Pregnancy established by transfer of embryos derived from in vitro fertilisation of oocytes matured in vitro. *Veterinary Record.* **121**: 259-260.

Marquant-LeGuienne, B., Gerard, M., Solari, A. & Thibault, C., 1989. In vitro culture of bovine egg fertilised either in vivo on in vitro. *Reproduction Nutrition Développement* **29**: 559-568.

Mattson, B.A., Rosenblum, I.Y., Smith, R.M. & Heyner, S., 1988. Autoradiographic evidence for insulin and insulin-like growth factor binding to early mouse embryos. *Diabetes* **37**: 585-589.

Mercola, M. & Stiles, C.D., 1988. Growth factor superfamilies and mammalian embryogenesis. *Development* **102**: 451-46.

Moor, R.M. & Trounson, A.O., 1977. Hormonal and follicular factors affecting maturation of sheep oocyte *in vitro* and their subsequent developmental capacity. *Journal of Reproduction and Fertility* **49**: 101-109.

Myles, D.G., 1993. Molecular mechanisms of sperm-egg membrane binding and fusion in mammals. *Developmental Biology* **158**: 35-45.

Myles, D.G. & Primakoff, P., 1984. Localized surface antigens of guinea pig sperm migrate to new regions prior to fertilization. *Journal of Cell Biology* **99**: 1634-1641.

Natsuyama, S., Noda, Y., Narimoto, K., Umaoko, Y. & Mori, T., 1992. Release of two-cell block by reduction of protein disulfide with thioredoxin from *Escherichia coli* in mice. *Journal of Reproduction and Fertility* 95: 649-656.

Niwa, K., Ohgoda, O. & Yuhara, M., 1988. Effects of caffeine in media for pretreatment of frozen-thawed sperm on in vitro penetration of cattle oocytes. *Proceedings of 11th International Congress on Animal Reproduction and Artificial Insemination, Dublin* **Vol. V**: 346 (Abstract)

O'Shea, J.D., Hay, M.F. & Cran, D.G., 1978. Ultrastructural changes in the theca interna during follicular atresia in sheep. *Journal of Reproduction and Fertility* **54**: 183-187.

Papaioannou, V.E. & Ebert, K.M., 1986. Development of fertilized embryos transferred to oviducts of immature mice. *Journal of Reproduction and Fertility* **76**: 603-608.

Papaioannou, V.E. & Ebert, K.M., 1988. The preimplantation pig embryo: cell number and allocation to trophectoderm and inner cell mass cells of the blastocyst *in vivo* and *in vitro*. *Development* **102**: 793-803.

Paria, B.C. & Dey, S.K., 1990. Preimplantation embryo development *in vitro*: Cooperative interactions among embryos and role of growth factors. *Proceedings National Academy of Sciences, U.S.A.* **87**: 4756-4760.

Parrish, J.J., Susko-Parrish, J.L. & First, N.L., 1985a. In vitro fertilization of bovine oocytes using heparin treated and swim-up separated frozen-thawed bovine semen is repeatable and results in high frequencies of fertilization. *Theriogenology* **23**: 216 (Abstract).

Parrish, J.J., Susko-Parrish, J.L. & First, N.L., 1985b. Effect of heparin and chondroitin sulphate on the acrosome reaction and fertility of bovine sperm in vitro. *Theriogenology* **24**: 537-549.

Parrish, J.J. & First, N.L., 1993. Fertilization. In: G.J. King (Ed.) *Reproduction in Domesticated Animals*. Elsevier Science Publisher B.V., Amsterdam. pp. 195-227.

Pesce, M., Farrace, M.G., Piacentini, M., Dolci, S. & De Felici, M., 1993. Stem cell factor and leukaemia inhibitory factor promote primordial germ cell survival by suppressing programmed cell death (apoptosis). *Development* **118**: 1089-1094.

Pincus, G. & Enzmann, E.V., 1935. The comparative behaviour of mammalian eggs *in vivo* and *in vitro*. *Journal of Experimental Medicine* **62**: 665-675.

Pinyopummintr, T. & Bavister, B.D., 1991. In vitro-matured/ in vitro-fertilized bovine oocytes can develop into morulae/blastocysts in chemically defined, protein-free culture media. *Biology of Reproduction* **45**: 736-742.

Pope, W.F., Xie, S., Broermann, D.M. & Nephew, K.P., 1990. Causes and consequences of early embryonic diversity in pigs. *Journal of Reproduction and Fertility, Supplement* **40**: 251-260.

Pratt, R.M. & Martin, G.R., 1975. Epithelial cell death and cyclic AMP increase during palatal development. *Proceedings National Academy of Sciences, U.S.A.* **72**: 874-877.

Rappolee, D.A., Sturm, K.S., Behrendtsen, O., Schultz, G.A., Pederson, R.A. & Werb, Z., 1992. Insulin-like growth factor II acts through an endogenous growth pathway regulated by imprinting in early mouse embryos. *Genes and Development* **6**: 939-952.

Reichenbach, H.D., Wiebke, N.H., Besenfelder, U.H., Mödl, J. & Brem, G., 1993. Transvaginal laparoscopic guided aspiration of bovine follicular oocytes: preliminary results. *Theriogenology* 39: 295 (Abstract).

Rexroad, C.E. & Powel, A.M., 1988. Co-culture of ovine eggs with oviductal cells and trophoblastic vesicles. *Theriogenology* **29**: 387-397.

Roberts, R.M. & Bazer, F.W., 1988. The functions of uterine secretions. *Journal of Reproduction and Fertility* **82**: 875-892.

Rose, T.A. & Bavister, B.D., 1992. Effect of oocyte maturation medium on in vitro development of in vitro fertilized bovine embryos. *Molecular Reproduction and Development* **31**: 72-77.

Rosenkrans, C.F. & First, N.L., 1991. Culture of bovine zygotes to the blastocyst stage: effects of amino acids and vitamins. *Theriogenology* **35**: 266 (Abstract).

Rotello, R.J., Hocker, M.B. & Gerschenson, L.E., 1989. Biochemical evidence for programmed cell death in rabbit uterine epithelium *American Journal of Pathology* **134**: 491-495.

Roy, S.K. & Greenwald, G.S., 1990. Immunohistochemical localisation of epidermal growth factor-like activity in the hamster ovary with a polyclonal antibody. *Endocrinology* **126**: 1309-1317.

Salisbury, G.W. & VanDemark, N.L., 1961. Gestation. In: *Physiology of Reproduction and Artificial Insemination in Cattle*. W.H. Freeman & Company, San Francisco. pp. 102-154.

Shalgi, R., 1984. Developmental capacity of rat embryos produced by *in vivo* and *in vitro* fertilization. *Gamete Research* **10**: 77-82.

Shen. M.M. & Leder, P., 1992. Leukaemia inhibitory factor is expressed by the preimplantation uterus and selectively blocks primitive ectoderm formation *in vitro*. *Proceedings National Academy of Sciences, U.S.A.* **89**: 8240-8244.

Sherman, M.I., 1979. Developmental biochemistry of preimplantation mammalian embryos. *Annual Review of Biochemistry* **48**: 443-470.

Simmen, R.C.M., Ko, Y. & Simmen, F.A., 1993. Insulin-like growth factors and blastocyst development. *Theriogenology* **39**: 163-175.

Sirard, M.A., Parrish, J.J., Ware, G.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.

Sirard, M.A. & Lambert, R.D., 1985. In vitro fertilization of bovine follicular oocytes obtained by laparoscopy. *Biology of Reproduction* **33**: 487-494.

Skimmer, M.K., Lobb, D. & Dorrington, J.H., 1987. Ovarian theca/interstitial cells produce an epidermal growth factor-like substance. *Endocrinology* **121**: 1892-1899.

Soon-Chey, Ng., Bongso, A., Sathananthan, H. & Ratnam, S.S., 1990. Micromanipulation: Its relevance to human in vitro fertilisation. *Fertility and Sterility* **53**: 203-219.

Snedecor, G.W. & Cochran, W.G., 1980. *Statistical Methods*. Seventh edition. The Iowa State University Press, Awes, Iowa, U.S.A. p. 290.

Staigmiller, R.A. & 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.

Stewart, C.L., Kaspar, P., Brunet, L.J., Batt, H., Gadi, I., Kontgen, F. & Abbondanzo, S.J., 1992. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359**: 76-79.

Tervit, H.R., Whittingham, D.G. & Rowson, E.A., 1972. Successful culture *in vitro* of sheep and cattle ova. *Journal of Reproduction and Fertility* **30**: 493-497.

Thibault, C., 1966. In vitro culture of cow eggs. *Annales de Biologie Animale, Biochemie, et Biophysique* **6**: 159-164.

Thibault, C., 1977. Are follicular maturation and oocyte maturation independent processes? *Journal of Reproduction and Fertility* **51**: 1-15.

Thibault, C., Szollosi, D. & Gerard, M., 1987. Mammalian oocyte maturation. *Reproduction, Nutrition, Développement* **27**: 865-896.

Thibodeaux, J.K., Del Vecchio, R.P., Broussard, J.R., Dickey, J.F. & Hansel, W., 1993. Stimulation of development of in vitro-matured and in vitro-fertilized bovine embryos by platelets. *Journal of Animal Science* **71**: 1910-1916.

Thompson, J.G.E., Simpson, A.C., Pugh, P.A., Donnelly, P.E. & Tervit, H.R., 1990. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. *Journal of Reproduction and Fertility* **89**: 573-578.

Tsafriri, A., 1978. Oocyte maturation in mammals. In: R.E. Jones (ed.) *The Vertebrate Ovary*. Plenum Press, New York and London. pp. 409-442.

Utsumi, K., Katoh, H. & Iritani, A., 1988. Developmental ability of bovine follicular oocytes matured in culture and fertilized in vitro. *Theriogenology* **29**: 320 (Abstract).

Van der Westerlaken, L.A.J., Van der Schans, A., Eyestone, W.H. & de Boer, H.A., 1994. Kinetics of first polar body extrusion and the effect of time of stripping of the cumulus and time of insemination on developmental competence of bovine oocytes. *Theriogenology* **42**: 361-370.

Van de Sandt, J.J.M., Schroeder, A.C. & Eppig, J.J., 1990. Culture media for mouse oocyte maturation affect subsequent embryonic development. *Molecular Reproduction and Development* **25**: 164-171.

Vansteenbrugge, A., Van Langendonckt, A., Scutenaire, C., Maship, A. & Dessy, F., 1994. In vitro development of bovine embryo s in buffalo rat liver- or bovine oviduct-conditioned medium. *Theriogenology* **42**: 931-940.

Vaux, D.L., Cory, S. & Adams, J.M., 1988. Bcl-2 gene promotes haemopoietic cell survival and cooperates with *c-myc* to immortalize pre-B cells. *Nature (London)* **335**: 440-442.

Wei, H., Lu, K.H. & Polge, C., 1994. Separation of X- and Y-chromosome bearing bovine sperm by flow cytometry for use in IVF. *Theriogenology* 41: 183 (Abstract).

Wyllie, A.H., 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature, London* **284**: 555-556.

Wyllie, A.H., 1981. Cell death: A new classification separating apoptosis from necrosis. In: I.D. Bowen and R.A. Lockshin (Eds.), *Cell death in biology and pathology*. Chapman and Hall, London. pp. 9-34.

Wyllie, A.H., 1988. Apoptosis. *ISI Atlas of Science: Immunology* **10**: 192-196.

Wyllie, A.H. & Morris, R.G., 1982. Hormone-induced cell death: Purification and properties of thymocytes undergoing apoptosis after glucocorticoid treatment. *American Journal of Pathology* **109**: 78-87.

Yanagimachi, R., 1988. Mammalian fertilization. In: E. Knobil and J. Neil (Eds.), *The Physiology of Reproduction*. Raven Press, Ltd., New York. pp. 135-185.

Yang, B.K., Yang, X. & Foote, R.H., 1993. Effect of growth factors on morula and blastocyst development of in vitro matured and in vitro fertilized bovine oocytes. *Theriogenology* **40**: 521-530.

Younis, A.I., Brackett, B.G. & Fayrer-Hosken, R.A., 1989. Influence of serum and hormones on bovine oocyte maturation and fertilization in vitro. *Gamete Research* **23**: 189-201.

Zeleznik, A.J., Ihrig, L.L. & Bassett, S.G., 1989. Developmental expression of Ca^{++}/Mg^{++} dependent endonuclease activity in rat granulosa and luteal cells. *Endocrinology* **125**: 2218-2220.