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

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ABSTRACT

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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.

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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

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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.