FACTORS IN THE PRODUCTION OF IDENTICAL ANIMALS BY NUCLEAR TRANSFER

by

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A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

January 1991

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ABSTRACT

The following thesis addresses the practical aspects of using nuclear transfer for the production of identical animals.

A procedure was established for the production of ovine and bovine nuclear transfer embryos incorporating the use of in vitro culture in synthetic oviduct fluid medium. The culture system was initially validated from the development of one-cell bovine and split eight-cell caprine embryos and subsequently applied to nuclear transfer experiments using ovine embryos. The viability of these embryos was demonstrated with the production of identical lambs and second generation ovine embryos cultured in vitro to the sixteen cell stage. Live offspring were obtained from bovine nuclear transfer embryos cultured in vitro for five to six days. It was found that the supply of oocytes from in vivo sources could be replaced with oocytes matured in vitro.

From these studies several factors were identified as responsible for the reduced development of ovine nuclear transfer embryos. These were (1) failure to enucleate one fifth of the oocytes (2) losses due to the nuclear transfer manipulations (3) transfer of the eight to sixteen cell stage embryos to uterus (4) the effect of an increased nucleocytoplasmic ratio. Solutions to at least two of these problems were provided with the visualisation of oocyte chromosomes for enucleation and transfer of embryos to the oviduct.

Results provided from these experiments improved understanding of the technical constraints of nuclear transfer for the production of identical animals. Also the flexibility of the methodology was increased with the use in vitro culture and/or in vitro matured oocytes.

DECLARATION

I hereby declare that this thesis contains no material which has been submitted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, no material described herein has been previously published or written by another person, except where due reference is made in the text.

If accepted for the award of a Ph.D. degree I consent to this thesis being available for loan and photocopying.

K.J. McLaughlin

ACKNOWLEDGEMENTS

I wish to thank my supervisor R.F. Seamark for advice and support throughout the course of this work. I am grateful to the department of Obstetrics and Gynaecology for their assistance, cooperation and provision of facilities for the work contained in this thesis.

The provision of goats, sheep and surgery facilities was supported by the staff and facilities of Australian Sheep Artifical Breeders/EMTECH. Particular thanks go to Lynn Davies, Alan Cotton and Bronte Mawson. Sheep embryos, oocytes and recipients were also provided by the Reproduction section, Ministry of Agriculture and Fisheries, Ruakura, New Zealand and particular thanks go to Robin Tervit and Anne Pugh.

Cattle and surgical procedures were supported by the South Australian Department of Agriculture. Completion of the studies pertaining to bovine embryos were made possible by the assistance of Doug McLean, Phil Lewis, and Greg Stevens.

Department of Obstetrics and Gynaecology staff contributing major assistance in surgery, embryo collection and laboratory preparation include Leonie Hicks and Rod Ashman, to whom I am grateful.

Financial support during this thesis was provided by a University of Adelaide Postgraduate Research Scholarship.

PUBLICATIONS

Publications derived from this thesis and accepted for publication before November 1990.

McLaughlin, K.J., L. Davies and R.F. Seamark (1990) *In vitro* embryo culture in the production of identical merino lambs by nuclear transplantation. *Reprod Fertil. Dev.* **2**:619-22.

McLaughlin, K.J., D.M. McLean, G. Stevens, R.A. Ashman, P.A. Lewis, B.D. Bartsch, and R.F. Seamark (1990). Viability of one-cell bovine embryos cultured in synthetic oviduct fluid medium· *Theriogenology* **33**:1191-1199.

McLaughlin, K.J., S.K. Walker, L. Davies, B. Mawson and R.F. Seamark (1989). Culture and manipulation of early goat embryos. *Theriogenology* **31**:227.

McLaughlin, K.J., A.P. Pugh, K. Logan and H.R. Tervit (1991). Assessment of oocyte source for ovine nuclear transfer. *Theriogenology* **35**:

McLaughlin, K.J., R.A. Ashman, D.M. McLean, G. Stevens, B.D. Bartsch, R.F. Seamark (1989). Viability of one-cell bovine embryos cultured in synthetic oviduct fluid medium. *Aust. Soc. Reprod. Biol. Proc.* **21**:36.

McLaughlin, K.J., L. Davies and R.F. Seamark (1990). Sequential cloning of ovine embryos in vitro. *Aust. Soc. Reprod. Biol. Proc.* **22**:53

ABBREVIATIONS

Chemicals and Hormones

BSA	bovine serum albumin
CB	cytochalasin B
FCS	fetal calf serum
FSH	follicle stimulating hormone
G	glucose
GnRH	gonadotropin releasing hormone
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
LH	luteinising hormone
PHA	phytohaemagglutinin
PMS	pregnant mares serum

Symbols and Units

AC	alternating current
cm	centimetre (s)
DC	direct current
g	gram (s)
h	hour (s)
HAU	haemagglutination unit (s)
i.u.	international unit (s)
kHz	kilohertz
kV	kilovolt (s)
l	litre (s)
mg	microgram (s)
mg	milligram (s)
ml	microlitre(s)
ml	millilitre (s)
mm	micrometre (s)
mm	millimetre (s)
mOsm	milliosmolar
ms	microseconds (s)
nMol	nanomole (s)
V	volt (s)
$^{\circ}$C	degrees centigrade

Media

BM	Brinster's medium
FM	fusion medium
HBSS	Hanks balanced salt solution
HTFM	human tubal fluid medium
PBS	phosphate buffered saline
SOF	synthetic oviduct fluid

SOFM-C	bicarbonate buffered SOFM
SOFM-CB	SOFM with cytochalasin B
SOFM-H	HEPES buffered SOFM
SOFM-T	HEPES/bicarbonate buffered SOFM
T6	T6 medium
TCM-199	tissue culture medium 199

Others

B	blastocyst
CIDR	controlled internal drug release device
DIC	differential interference contrast
DNA	deoxyribonucleic acid
e.g.	for example
EB	early blastocyst
et al.	et alia (and others)
GS	goat serum
HB	hatched blastocyst
HIGS	heat inactivated goat serum
HIHS	heat inactivated human serum
HISS	heat inactivated sheep serum
HS	human serum
I.D.	inside diameter
i.m.	intramuscular
i.v.	intra-venous
IVF	in vitro fertilisation
IVM	in vitro maturation
LWD	long working distance
MII	metaphase II
mRNA	messenger RNA
O.D.	outside diameter
RNA	ribonucleic acid

SECTION I

INTRODUCTION

1. INTRODUCTION

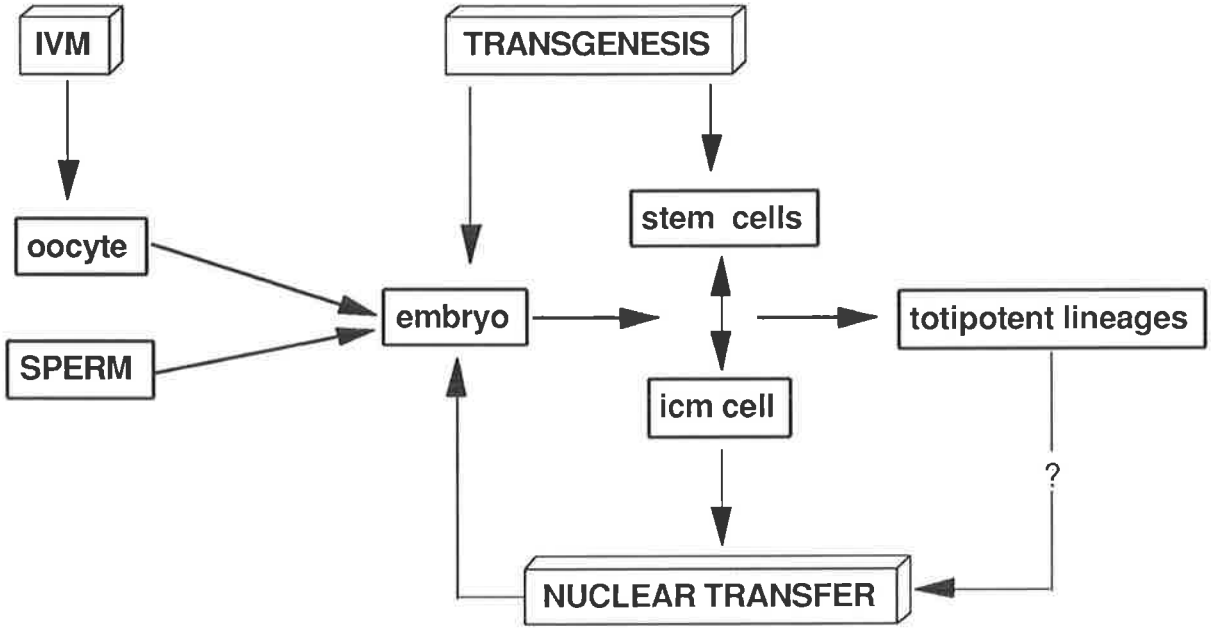
1.1 RELEVANCE OF NUCLEAR TRANSFER TO ANIMAL REPRODUCTION

Nuclear transplantation has major application to the fields of developmental biology and animal production. Initially developed as a means of examining the nucleocytoplasmic interactions of mouse embryos, it has only recently been applied to the production of identical animals. Already, the possibility of repeated cloning from a single embryo has been realised in domestic species. Future potential exists in the use of nuclei sources from the more numerous cell populations such as embryonic stem cells and in later stage embryos.

Nuclear transfer applied to mammalian embryos could potentially be the most rapid means for spreading a chosen genotype within a population (Wooliams 1989). When nuclear transfer is combined with existing advanced breeding technology, it will not only increase breeding flexibility but also produce novel approaches previously unattainable (Figure 1.1). A notable example of this is the combination of nuclear transplantation and transgenesis. Used together it will be possible to improve the accessibility to unique transgenic genotypes (Seamark 1989) by manipulating either transgenic embryos or using genetically modified embryonic stem cells.

This thesis concerns factors which determine the success of creating identical animals through nuclear transplantation in mammalian species, particularly domestic farm animals. This section reviews experiments carried out primarily as a means of exploring the totipotency of embryonic nuclei which also describe interactions between different stage nuclei and cytoplasm. Particular consideration is given to those factors which may limit the success of nuclear transfer in domestic farm species.

Figure 1.1 Existing and potential pathways of embryo manipulation utilising nuclear transfer



2. NUCLEAR TRANSFER IN MOUSE EMBRYOS AND OOCYTES

Nuclear transfer embryos are constructed from two components, the nucleus, and the cytoplasm. Whilst many different sources of nuclei have been tested, only two cytoplasm types have been used, namely the fertilised or unfertilised oocyte. In non-mammalian species the capacity of these two cytoplasms to support nuclei have been shown to differ (DiBerardino 1980, Hoffner and DiBerardino 1980). Therefore the following discussion of nuclear transfer experiments in mouse and non-murine embryos is separated according to the source of cytoplasm.

2.1. MURINE OOCYTES

2.1.1 **Nuclear remodelling**

The unique properties of the oocyte cytoplasm are generated as a consequence of a series of interactions which occur between the meiotic oocyte and the sperm nuclei. Progression of the meiotic oocytes from its arrested state is initiated with sperm penetration resulting in the sperm chromatin and metaphase nuclei simultaneously decondensing and swelling with subsequent pronuclear formation. In the amphibian oocyte, nuclear swelling is considered a prerequisite for nuclear reprogramming and is associated with pluripotent and totipotent development of transplanted nuclei (DiBerardino 1980). In the mouse, thymocyte nuclei introduced into oocyte cytoplasm undergo decondensation and swell to sizes in excess of the female pronucleus (Czolowska *et al* 1984). However, if the nuclei are introduced less than one hour post activation, they fail to decondense as extensively as those introduced prior to activation and exhibit subsequent retarded development (Czolowska *et al* 1984, Szöllözi *et al* 1988). Factors within the germinal vesicle which are released upon germinal vesicle breakdown (GVBD) are thought to contribute to this nuclear remodelling (Czolowska *et al* 1984), as ultrastructural studies have shown that nuclear envelope breakdown and reformation can only be fully effective between metaphase II and telophase of meiosis (Szöllözi *et al* 1986).

2.1.2 **Oocyte somatic cell fusion**

Information on the interaction of foreign nuclei in the oocyte cytoplasm was initially obtained from experiments in which oocytes were fused to nucleated somatic cells. Baranska and Koprowski (1970) fused zona-free meiotic oocytes with several somatic cell types including immortal cell lines and primary embryonic fibroblast cells. The hybrid cells created were all capable of undergoing cleavage with varying success up to early morula stage.

When enucleated oocytes were first used as a recipient cytoplasm for transferred nuclei, the resultant development was very limited. McGrath and Solter (1986) transferred 16-cell stage mouse nuclei into oocytes less than three hours after they had been activated by exposure to ethanol but only 0.5% (1/198) of the resulting embryos developed into blastocysts. However it should be noted that nuclei transferred later than one hour after activation may not have been representative of metaphase oocytoplasm. Poor development (2/62) was also observed with the transfer of embryo carcinoma cell (EC) nuclei to enucleated oocytes and was associated with an abnormal distribution of nuclei (Modlinski *et al* 1990).

2.1.3. **Transfer of embryonic nuclei into metaphase oocytes**

The development of nuclei in the cytoplasm of the enucleated metaphase oocyte has had limited examination, largely due to the difficulties in visualising metaphase chromosomes. However following the development of DNA specific fluorescent dyes it was found that the requisite manipulations could be performed without loss in viability (Tsunoda *et al* 1988). Donor nuclei could then be introduced into oocytes which are subsequently activated by ethanol exposure. As these procedures introduced the nucleus prior to activation, it may be expected to undergo some degree of nuclear condensation in the period prior to activation (Balakier and Czolowska 1977, Czolowska *et al* 1986), but also result in subsequent exposure to the critical remodelling period which would occur subsequent to activation (Czolowska *et al* 1984).

2.1.4 **Development of embryonic nuclei**

Embryonic nuclei obtained from the two-cell through to primordial germ cells, transferred to enucleated metaphase oocytes undergo pronuclear formation (>90%; Tsunoda *et al* 1989). Early two cell stage nuclei transferred to oocytes result in 80% undergoing the first cleavage and 36% reaching the blastocyst stage. By contrast, nuclei derived from the late two-cell and

eight-cell stage nuclei showed lower cleavage rates (50-60%) with no blastocysts forming (Ushijima and Tsunoda, 1989). However this reduction in developmental capacity is not characteristic of all stages as when cells obtained from the inner cell mass are fused to enucleated oocytes, some blastocyst formation results (3%). Fused primordial germ cells also show similar developmental capacity with 50-100% cleaving to the two-cell stage and 6-20% resulting in blastocysts (Tsunoda *et al* 1989). Furthermore the rate of development has been found to increase by transferring nuclei at the two cell stage into the cytoplasm of fertilised two-cell stage blastomeres.

2.1.4 **Conclusion**

Apart from the latter study by Tsunoda *et al* (1989), no other evidence has been presented to show that murine oocytoplasm can support transplanted nuclei. There still remains a large scope for research to determine the importance of the mouse oocytoplasm in reprogramming nuclei. Currently it can be concluded that certain embryonic cell lineages may retain totipotency upon transfer to the oocyte cytoplasm, however term offspring from such nuclei has yet to be observed.

2.2 NUCLEAR TRANSFER TO FERTILISED EGGS.

2.2.1. **Hybrid fusions**

The first experiments relevant to nuclear transfer in fertilised mouse embryos were the production of heterokaryons between cleavage stage embryonic cells and somatic cells (Graham 1969). Somatic cells were fused with one and two cell embryos using inactivated sendai virus, however the failure of these heterokaryons to develop *in vitro* was at least in part due to the culture medium used (Graham 1971). Similar experiments attempted fusion of somatic cells with zona-intact four and eight-cell mouse embryos, but fusion events predominated between the blastomeres rather than with the somatic cells (Lin *et al* 1973).

Heterokaryon fusion was also used to determine the effect of interaction between the cytoplasm and the foreign nucleus by measuring RNA synthesis. (Bernstein and Mukherjee 1972). RNA transcription with somatic cell nuclei decreased when fused with two-cell cytoplasm but remained high when fused to that of the four-cell. The amount of RNA synthesized depended on the number of cells fused, and/or the total volume of cytoplasm contributing to the heterokaryon. Conversely the rate of RNA synthesis by embryonic nuclei increased with the number of somatic cell nuclei fused. Significantly, it was found that the embryo was capable of initiating RNA transcription when fused with transcriptionally dormant erythrocyte cells (Bernstein and Mukherjee 1972,1973), clearly indicating that the two-cell embryo can control the RNA synthesis of nuclei in somatic cells. However as this appeared to be a volume titrated effect, it may be the larger size of the embryonic cells that determines the overall control of transcription rates.

The capacity of transplanted nuclei to contribute genetically to the preimplantation development of fertilised eggs was first observed by fusing morula stage cells with the fertilised egg. These embryos developed into tetraploid blastocysts with evidence of gene expression from the morula cell nuclei (Modlinski 1978). This ability was also dependent on origin of the cell, as nuclei derived from the inner cell mass were capable of supporting development whilst those of the trophectoderm failed (Modlinski 1981). Thus, exogenous nuclei can contribute to subsequent gene expression in the host embryo despite the presence of the endogenous nucleus.

2.2.2 **Nuclear transfers into enucleated cytoplasm**

To study the interaction between a transplanted nucleus and the cytoplasm requires enucleation of the recipient embryo. This was first implemented by Illmensee and Hoppe (1981) who reported term development of enucleated embryos with transplanted nuclei from the inner cell mass but not trophectoderm cells. Later stage nuclei isolated from the embryonic

ectoderm and proximal endoderm were also shown to be capable of supporting similar development (Illmensee *et al* 1981). This result presented the first evidence that some mammalian cell nuclei remain totipotent upon transfer to fertilised egg cytoplasm.

The low proportion of embryos surviving (38%) in the studies of Illmensee and Hoppe (1981) should be considered in relation to the methodology used. Their technique involved puncture during enucleation and injection of nuclei and subsequent attempts to repeat these results have resulted in similarly low success with no embryo development to term (Modlinski 1981, Markert 1982).

Further progress in the nuclear transfer technique has been required to allow further examination of nuclear cytoplasmic interactions. In a procedure described by McGrath and Solter (1983a) cytochalasin B was used to increase the flexibility of the plasma membrane thus enabling removal of the nucleus in a small amount of membrane bound cytoplasm. This fragment could then be fused to the host cell with either inactivated virus or electrofusion (Kubiak and Tarkowski 1986, Tsunoda *et al* 1987b, Kono and Tsunoda 1988). Survival rates after the micromanipulation were 91%, with 96% developing to the blastocyst stage *in vitro* and with no observed difference in the term survival of embryos compared with controls (McGrath and Solter 1983b).

2.2.3. **Cytoplasmic contamination**

The nuclear transfer method described by McGrath and Solter (1983a) differed from that used Illmensee and Hoppe (1981) in that there was a simultaneous transfer of cytoplasm. Therefore the former technique may not have been able to reproduce similar results if contributing factors are present in the transferred cytoplasm.

Cytoplasmic factors have been shown to dominate over nuclear function in nuclear transfer studies. For example the paternally inherited hairpin tail (*Thp*) mutation (Johnson 1975) can be reversed when *Thp* nuclei

are transferred to normal cytoplasm (McGrath and Solter 1984). Similarly the preimplantation lethal DDK mutation is reversed by transfer of DDK pronuclei to a non-DDK cytoplasm along with a non-DDK pronuclei (Mann 1986). Conversely, the transfer of the DDK cytoplasm to non DDK embryos reduces development (Renard *et al* 1988).

2.3.4. **Development of preimplantation nuclei**

2.3.4.1. *Pronuclear egg cytoplasm*

Following the introduction of the nuclear transfer technique described by McGrath and Solter (1983a), it has been demonstrated that embryonic karyoplasts or blastomeres from later than the two-cell are unable to support more than limited preimplantation development in the zygote cytoplasm (McGrath and Solter 1983b, Surani *et al* 1986; Howlett at al 1987; Smith *et al* 1988). This stage corresponds closely to the time of activation of the mouse embryonic genome (Epstein, 1975, Bolton *et al* 1984; Sawicki *et al* 1981) and the time beyond which blastomeres are no longer capable of supporting independent development (Tsunoda and McLaren, 1983; Rossant 1976; Tarkowski and Wrobleska 1967). However the totipotency of nuclei from preimplantation mouse embryos is not lost until at least the eight-cell stage (Kelly 1975,1979). Therefore the inability of the fertilised cell cytoplasm to support development is not limited by nuclei totipotency but from the interaction between the donor nuclei and the one-cell cytoplasm.

2.3.4.2. *2-cell cytoplasm*

Unlike the one cell, the two cell cytoplasm can support development of transplanted nuclei obtained from up to the eight cell stage (Robl *et al* 1986) and term (Tsunoda *et al* 1987). This may be a consequence of genomic activation at the mid to late two cell stage (Epstein 1975, Bolton *et al* 1984) resulting in a cytoplasmic environment more similar than found at the eight-cell stage. This is supported by the observation that eight cell stage nuclei do not undergo swelling in the 2-cell cytoplasm to the degree observed upon

transfer to the one-cell cytoplasm (Barnes *et al* 1987). Furthermore nuclear transfer embryos with late two-cell cytoplasm do not undergo compaction and blastocyst formation earlier than control 2-cell stage embryos (Howlett *et al* 1987; Robl *et al* 1986). Also, if the nuclei were being reprogrammed, the cell numbers in the subsequently forming blastocysts would be expected to be the same as control two-cell embryos and not lower as observed (Kono and Tsunoda 1989).

2.3.5. **Cell cycle synchrony**

The disruption of the synchrony between the nucleus and the cytoplasm, subsequent to nuclear transplantation has been suggested as a limiting factor in developmental capacity (Smith *et al* 1988). Cytoplasmic control over the cell cycle in early embryo development has been established for Xenopus embryos (Newport and Kirschner 1984,1982a,1982b, Dabauville *et al* 1988). and evidence that a similar autonomous cytoplasmic control may exist in mammalian embryos has been indicated from observation of cyclic cortical granule activity in mouse eggs (Waksmundska *et al* 1984). Cell cycle synchrony between nucleus and cytoplasm is of particular relevance in experiments where exchanges of nuclei between the two cell and one cell stages show compelling evidence that extreme asynchrony of cell cycle inhibits preimplantation development (Smith *et al* 1988). However the current evidence for mouse nuclear transfer is inconclusive as other observations of inter-stage nuclear transfer between the late two cell and late one cell cytoplasm have not as yet resulted in development despite the stage of cell cycle being synchronised (Howlett *et al* 1987).

Whilst cell cycle synchronization may be important to transplanted nuclei, the dominating factor for determining subsequent development still appears to be the overall age difference between the nuclei. This is indicated in experiments where late one-cell stage cytoplasm is used as a recipient for the early and late two-cell stage nuclei, and it is found that both nuclei

result in equivalent early cleavage patterns despite a larger asynchrony in the cell cycle (Smith *et al* 1990).

2.3.6 **Nucleocytoplasmic ratio**

Another factor inhibiting the development of transferred nuclei may relate to change experienced in cytoplasmic volume which occurs along with the change in cytoplasmic content. This has been observed when eight-cell nuclei were transferred to 2-cell blastomeres and inadvertent fusions occurred between the two cell blastomeres. Fewer of the resulting embryos with the greater cytoplasm develop to blastocysts than nuclei in single blastomere cytoplasm (Howlett *et al* 1987). Two-cell stage nuclei with a double nucleocytoplasmic ratio in two cell cytoplasm can develop to form blastocysts and support term development but at a lower frequency than unmanipulated two cell embryos (Barra and Renard, 1988). Conversely, the reduction of the cytoplasmic volume of a haploid one cell embryo, increased the initial preimplantation development (McGrath and Solter 1986). Whilst the developmental events of compaction and blastocyst formation in some of these reconstructed eggs can vary from unmanipulated embryos, there is no evidence from expression of stage specific proteins that any reprogramming occurs (Petzoldt and Muggleton-Harris 1987).

3. NUCLEAR TRANSPLANTATION IN NON-MURINE EMBRYOS.

3.1. Pronuclear embryos as nuclei recipients.

3.1.1. Pronuclear exchange

Nuclear transplantation in species other than mice has mainly involved meiotic oocytes rather than fertilised eggs as a nuclei recipient. The few exceptions to this have been when pronuclear cytoplasm has been used as a recipient cytoplasm in studies with the rat (Kono *et al* 1988), porcine (Prather *et al* 1989) and bovine embryos (Robl *et al* 1987), principally because of the ability to visualise nuclei for enucleation. Few of these

embryos underwent development to term upon transfer to recipients (bovine;2/29, Robl *et al* 1987, porcine 7/56, Prather *et al* 1989, rat;6/28, Kono *et al* 1988) and that compares poorly with the nuclear transfer of mouse embryos (66%; Mann and Lovell-Badge 1984). However, this large difference between species may be partly due to the lesser developed culture systems for non-murine embryos.

3.1.2. **Later nuclei stages into pronuclear eggs**

Nuclei derived from the two-, four- and eight-cell stage fused to the zygote cytoplasm exhibit poor development with no greater than two cleavages observed in bovine embryos (Robl *et al* 1987) and no term development from two-cell stage rat embryos transferred to oviducts (Kono *et al* 1988). This failure of early preimplantation stage nuclei to support development can not be attributed to the genomic activation of the nucleus as bovine embryos do not undergo activation of the genome until around the eight-cell stage (King *et al* 1988). Furthermore, single blastomeres from early cleavage stage bovine embryos and other species can produce blastocysts and support term development (reviewed by Papaioannou and Ebert 1986). More likely the loss of viability is due to either the manipulation procedure or incompatibility between nuclei and the zygote cytoplasm.

3.2. OOCYTES AS NUCLEI RECIPIENTS

3.2.1. **Totipotency and reprogramming**

Transfer of nuclei into oocytes of non-murine species was first attempted using intact rabbit oocytes with morula stage cells, however only a few of the resulting hybrid embryos developed to the morula stage (Bromhall 1975). A similar stage of development also resulted from the use of enucleated human oocytes (n=3) for spermatogonia cells (Shettles, 1979).

Nuclear transfer experiments for domestic farm animals were first reported in manipulation studies on identical pig embryos (Robl and First, 1985) however the first live offspring resulted from experiments by Willadsen (1986) who transferred ovine eight and 16-cell stage nuclei into enucleated oocyte cytoplasm. Subsequent success in producing live offspring has been achieved from eight to 16-cell stage nuclei in bovine embryos (Prather *et al* 1987), 8-cell rabbit embryos (Stice and Robl 1988) and 4-cell pig embryos (Prather *et al* 1989). These results provided the first evidence of enucleated oocytes supporting term development of cleavage stage nuclei in mammals.

The finding that sheep 16-cell stage nuclei supported term development upon nuclear transfer (Willadsen 1986) provided clear evidence that nuclei could retain totipotency post activation (Crosby *et al* 1988; Calarco and McLaren, 1976). Term development provided unequivocal evidence of nuclear reprogramming, verified by the synchrony of blastocyst formation and similar cell numbers in nuclear transfer and one-cell stage embryos (Willadsen 1986). The fact that synchrony in development was observed this early in development indicated that the reprogramming events must be occurring immediately after exposure of the nuclei to the oocyte cytoplasm (Prather *et al* 1987; Prather *et al* 1989; Stice and Robl 1988; Willadsen 1986). Further evidence for nuclei reprogramming has been demonstrated by the ability of nuclei derived from cloned embryos to be reused as a nuclei source for second generation nuclear transfer (Willadsen 1986; Bondioli *et al*, 1990) to produce blastocysts for up to eight repeated cycles of nuclear transfer (Willadsen pers. comm.)

The transfer of later stage preimplantation nuclei has been as successful as earlier stages in the production of live offspring (Bondioli *et al* 1990). The limit of this potential is unknown for the domestic species for which nuclei from at least the inner cell mass in sheep (Smith and Wilmut 1989) and blastocysts in cattle (Bondioli *et al* 1990) have produced live offspring. Evidence of the totipotency of later embryonic and fetal stage nuclei in non mammalian species (reviewed by Gurdon 1986 and

DiBerardino 1987) supports the probability that later stage mammalian cells will also be totipotent.

3.3 FACTORS INFLUENCING THE SUCCESS OF NUCLEAR TRANSFER.

The efficiency of nuclear transfer is largely dependent on the success of the manipulation procedures which result in the loss of embryos at each of the manipulation stages.

3.3.1. **Nuclear isolation and enucleation**

Initial embryo losses with the nuclear transfer technique are associated with the isolation and removal of nuclei from both the donor embryo and recipient oocyte. The first occurs with the rupture of cells in isolating nuclei from the embryo (Smith and Wilmut 1990). Secondly enucleation often fails because the chromosomes cannot be easily visualised, making it difficult to assess the success of the enucleation with normal light microscopy. Failure to enucleate the oocyte may result in the remaining endogenous nuclei causing postimplantation mortality as a result of a haploid or triploid genotype.

As practised to date enucleation of an oocyte involves locating the metaphase plate, either by removing the area adjacent to the polar body, or removing the clear patch of cytoplasm in which the metaphase chromosomes can sometimes be visualised. As the success of latter approach is dependent on the density of the cytoplasm the enucleation rate is relatively high for species with clearer cytoplasms such as the rabbit (92%; Stice and Robl, 1988) and decreases for the ovine (67 and 75%; Smith, 1989, Willadsen, 1986), bovine (60%; Prather *et al* 1987) and porcine embryos (74% Prather *et al* 1989).

3.3.2 **Fusion**

Fusion of the karyoplast or blastomere to the oocyte has been attempted using both viral mediated fusion or electrofusion. Viral fusion has been used with rabbit (Bromhall 1975), ovine (Willadsen 1986), bovine (Robl *et al* 1987) and rat embryos (Kono *et al* 1988) but has been less effective when compared with electrofusion for bovine and ovine embryos (Ovine: 50% vs 90%, bovine: 8% vs 75%). Another factor that has been reported to decrease fusion efficiency is the use of smaller cells (Prather 1987), which results in a decrease in efficiency for more advanced stage nuclei, however this appears to be limited to particular conditions and has been overcome using alternative electrofusion systems (Smith *et al* 1988; Bondioli *et al* 1990).

3.3.3 **Activation, age and maturation**

When cells or karyoplasts are fused using electrofusion, the electrical stimulation involves a combination of an AC pulse to align and polarise the egg and a DC pulse to initiate membrane fusion (Zimmerman and Vienkel 1982) and cytoplasmic activation. Independently the DC pulses can activate the intact oocyte and enable parthenogenetic development (Ware *et al* 1989). The efficiency of the DC pulse on activating oocytes has been examined for rabbit (Stice and Robl 1989, Onodera and Tsunoda 1989) and bovine oocytes (Ware *et al* 1989) and tested in relation to age and source of the oocyte. Extending the period of in vitro maturation of bovine oocytes from 26 to 30 hours, increases the proportion of oocytes activating (Ware *et al* 1989) and similarly with *in vivo* matured rabbit oocytes between 16-20 and 20-24 hours post HCG (Stice and Robl, 1988).

In vivo and *in vitro* sources of enucleated oocytes have been compared to determine their ability to support the development of donor nuclei. When bovine oocytes are matured *in vitro* for 22 hours there is a lower rate of blastocyst development (5.6%) than oocytes matured *in vivo* for 36 hours (20%; Prather *et al* 1987). The potential success of using *in vitro* matured

material in nuclear transfer has been shown by demonstrating the use of both nuclei and oocytes derived from *in vitro* matured material to produce blastocysts (Kinis *et al* 1989).

In vivo matured bovine oocytes have been reported to loose their developmental capacity to form blastocysts from transplanted nuclei as age increases from 36 to 48 hours post HCG (20% and 6.7%; Prather *et al* 1987). However other observations of bovine oocytes collected from 0 to 48 hours post HCG show no overall difference in development with transplanted nuclei (16% morula and blastocyst; Marek *et al* 1990) but this study differs in that both nucleated and enucleated halves of the oocyte were utilised for nuclei recipients. The age of oocytes used for nuclear transfer in sheep embryos have been between 30 to 36 hours post HCG and has resulted in a similar rate of development for all studies (Willadsen 1986, Smith *et al* 1988). It is therefore inconclusive as to whether specific age of the oocyte effects development with a transplanted nucleus, this is particularly evident when considering observations for bovine embryos.

3.4 DEVELOPMENT OF NUCLEAR TRANSFER EMBRYOS.

3.4.1 **Preimplantation development**

Assessment of the preimplantation development of nuclear transfer embryos in domestic animals has largely been made from embryos successfully recovered after temporary *in vivo* culture in recipient oviducts. Typically a large proportion of embryos recovered have failed to develop with a significant proportion not cleaving (23% in sheep; Willadsen 1986, 45% in pigs ; Prather *et al* 1989) and many only undergoing a few cleavages (Prather *et al* 1987, Willadsen 1986, Smith and Wilmut 1989). Surviving embryos that result in morphological morulae and blastocysts do so at a variable rates for different nuclei sources. In the bovine this occurs in 20% of eight to sixteen-cell stage nuclei (Prather *et al* 1987) and 35% of nuclei derived from 32 to 64 cell stage embryos (Bondioli *et al* 1990) For sheep embryo nuclei 38% derived from the inner cell mass form morulae or blastocyst (Smith and

Wilmut 1989) compared with 40% from the eight-cell, and 48% from the sixteen-cell (Willadsen 1986). These totals represent approximately 60 % of the embryos undergoing initial cleavage after fusion.

There has been one study that has utilised *in vitro* oviductal cell culture for nuclear transfer embryos, resulting in 13% of attempted fusions forming morulae or blastocysts after seven days culture. However this was lower than the overall rate of development reported for *in vivo* culture in a similar study (16 to 35%; Bondioli *et al* 1990).

3.5.2. ***In vivo* development**

The development of nuclear transfer embryos subsequent to being returned to the recipient has been low. Rabbit nuclear transfer embryos returned to the oviduct after 20 hours culture resulted in only 6/164 developing to term (Stice and Robl 1988). Rat embryos transferred at the two cell stage resulted in no term development compared to 9/41 (22%) for control manipulated embryos (Kono *et al* 1988). Transfer of morula or blastocyst stage clones after *in vivo* culture was first reported with bovine embryos but only 2/29, transferred, survived to term. Transfer of ovine embryos has also been low with 3/22 surviving to term (Smith and Wilmut 1989), however in this study, lower survival was attributed partly to embryo recipient asynchrony and seasonality. Larger scale nuclear transfer trials with bovine embryos have recorded an overall pregnancy rate of 22.5%, which includes frozen embryos and serial nuclear transfers (Bondioli *et al* 1990). This represents about half that observed with commercial embryo transfer where pregnancy rates of 55% are achieved (Massey and Oden 1984).

The overall survival rate from fusion to term can best be determined from the extensive study with bovine nuclear transfer reported by Bondioli *et al* (1990), in which successful preimplantation development of 13 to 35% produces a term survival of 8% of the total manipulated embryos.

The production of identical offspring as a result of nuclear transfer embryos was established with the birth of two identical lambs from nuclei derived from the eight cell-stage embryo (Willadsen 1986), and up to seven bull calves have resulted from a single 32-cell stage embryo (Bondioli *et al* 1990). However many studies have failed to produce identical animals as a result of low embryo survival and small experimental numbers (Robl *et al* 1987, Prather *et al* 1987, Smith and Wilmut 1989, Prather *et al* 1989).

4. IDENTITY OF IDENTICAL ANIMALS PRODUCED BY NUCLEAR TRANSFER

In describing the nuclear transfer methods for production of identical domestic animals, it should be recognized that there are several underlying assumptions. Firstly, the identical animals produced are only similar in the genetic content of the nuclei at the time of isolation ("embryo clones"). Subsequently, the individual is a product of its genotype and its environment, which is unique for each individual. The genotype, although potentially identical at the time of transfer, is also associated with nucleoplasm, nuclear membrane, cytoplasm and plasma membrane (Seidel 1983). Apart from the unknown possible epigenetic factors, cytoplasmic contents have been shown to control the fate of transplanted nuclei. Therefore identical animals produced via nuclear transfer, in a similar environment would be expected to have a greater similarity than siblings but less so than natural twins.

5. SUMMARY

The meiotic oocyte provides the best prospect as a recipient cytoplasm for both supporting development, and the reprogramming mammalian embryonic nuclei. Data primarily from experiments with murine and domestic animals has shown that nuclei from several preimplantation stages

are capable of supporting development to term, with some evidence for later stage nuclei in the mouse.

The methodology for the investigation of nuclear transfer in larger domestic animals has been basically confined to the manipulation, *in vivo* culture and transfer of embryos. The results to date have demonstrated the possibilities of nuclear transfer and the totipotency of nuclei. However limited investigations have been initiated into the interaction between the transplanted nucleus and cytoplasm in relation to subsequent developmental capacity

Significant losses of nuclear transfer embryos occur at three stages of nuclear transfer. These are (1) the manipulation procedures, (2) preimplantation development, and (3) subsequent to transfer and implantation. In studies of larger livestock species, a primary objective is to improve embryo survival by establishing an effective *in vitro* culture system to reduce losses incurred during manipulation procedures and preimplantation development. Effectively this will enable observation of preimplantation development and circumvent the use of intermediate recipients.

The following studies establish the use of *in vitro* culture for manipulated and in particular nuclear transplant embryos. Further investigations evaluate aspects of nuclear transfer such as oocyte source, enucleation rates and preimplantation and post transfer losses, for which embryos from both ovine and bovine species are tested. Also, to determine the effect of increased volume in nuclei transplanted to oocytes, nuclear transfer experiments are conducted with mouse nuclei using artificial control of cytoplasmic volume.

SECTION II

GENERAL METHODS

1. **CULTURE**

1.1 SYNTHETIC OVIDUCT FLUID MEDIUM

1.1.1. **Composition of SOFM in stocks.**

SOFM was prepared according to the formulation of Tervit *et al* (1972) in five stock solutions (Table 2.1).

Table 2.1 Concentration of components and stock solutions of SOFM (Walker *et al* 1988)

Stock	Component	g/100 ml	mM (in mixed medium)
A	NaCl	6.29	107.7
	KCl	0.534	7.16
	KH_2PO_4	0.162	1.19
	$MgSO_4.7H_2O$	0.182	0.74
	Na.lactate	0.6ml	3.3
	glucose	0.27	1.5
	penicillin	0.06	-
B	$NaHCO_3$	0.210	12.5
	phenol red	0.001	-
C	Na.pyruvate	0.051	0.33
D	$CaCl_2.2H_20$	0.262	1.78
E	HEPES	0.651	12.5
F	HEPES	0.596	10.79
	phenol red	0.001	-

1.1.2 **Composition of SOFM-H, SOFM-T and SOFM-C.**

Table 2.2 Composition of SOFM-H, SOFM-T and SOFM-C.

Stock	Holding medium SOFM-H	Transport medium SOFM-T	Culture medium SOFM-C
A	1.0	1.0	1.0
B	0.16	0.5	0.72
C	0.07	0.07	0.07
D	0.1	0.1	0.1
E	-	0.05	-
F	0.84	-	-

(Final volume 10 ml)

1.1.3 **Serum**

1.1.3.1. *Supply*

Human serum was collected from laboratory staff. Ovine and caprine serum was selected from female animals located at Nairne, South Australia. Fetal calf serum was obtained from Commonwealth Serum Laboratories (Australia). Blood was refrigerated overnight and serum separated by centrifugation at 2000 g for 20 minutes followed by heat inactivation at 56°C. Aliquots were stored at -20°C until required.

1.1.3.1 *Addition to media*

Except where stated in the text, all media was supplemented with 20% v/v heat inactivated human serum. Serum was filter sterilised before addition to the media.

1.2 HUMAN TUBAL FLUID MEDIUM

1.2.1. **Composition of Human Tubal fluid medium**

Table 2.3 Composition of HTF medium (Quinn *et al* 1985, modified)

Component	Concentration (mM)
NaCl	101.6
KCl	4.69
$MgSO_4.7H_2O$	0.20
KH_2PO_4	0.37
$NaHCO_3$	25.00
$CaCl_2.2H_20$	2.04
Na.lactate	21.40
Na.pyruvate	0.33
glucose	2.78
penicillin	100 U/ml
phenol red	0.001% (w/v)

1.2.2 **HEPES-HTF medium**

This medium was identical to HTF medium except that sodium bicarbonate was reduced to 4 mM and 21 mM HEPES was added (Quinn *et al* 1984).

1.3 CULTURE SYSTEM

All embryos were cultured in microdrops of media (10µl) overlaid with approximately 2.5 ml of light paraffin oil (BDH, Australia) in 35 mm petri dishes. These dishes were placed inside a humidified chamber (95%) with a constant flow of prewarmed, humidified special gas mixture (5% O_2, 5% CO_2, 90% N_2). This chamber was placed inside an incubator at a temperature of 37°C (mouse and goats) or 39°C (sheep and cattle) with 95% humidity and a gas atmosphere of 5% CO_2 in air.

2. PREPARATION OF INACTIVATED SENDAI VIRUS

2.1 SUPPLY

An initial batch of sendai virus was obtained from Flow Laboratories (Sydney), however this was not effective for fusion even at high concentrations. Subsequently live viral seed stocks and inactivated sendai virus was supplied by Dr. Jeff Mann, Roche Institute of Molecular Biology, New Jersey.

2.2 PRODUCTION

Viral solutions were prepared as follows based on the procedure described by Giles and Ruddle (1973).

Seed virus was inoculated with a dose ranging from 200 µl of undiluted seed stock (12 eggs) to 10 and 100x dilutions (6 eggs each). Infected allantois fluid was harvested with a pasteur pipette. A solution of 0.1% beta-propiolactone was used for inactivation.

Harvested infected fluid was concentrated to one tenth volume by centrifugation and resuspended in Hanks buffered salt solution minus glucose (HBSS-G) containing 3 mg/ml Bovine Serum Albumin. BSA was necessary to prevent loss of fusion efficiency upon storage of the inactivated virus at -70°C (Neff & Enders, 1968).

Before inactivation the virus preparation possessed approximately 30,000 HAU/ml. After inactivation, it possessed about 15,000 HAU/ml. Assays were conducted using 1% (v/v) guinea pig cells in PBS. Aliquots of 300 µl of the inactivated virus were stored in liquid nitrogen. For use, one aliquot at a time was thawed and 20 µl aliquots of this stored at -70°C in sterilised tubes.

3. **NUCLEI STAINING.**

Nuclei identification and counting was determined using the DNA specific dye Hoechst 33342 (Sigma, USA). The procedure for use on embryos was basically as described by Pursel *et al* (1985).

Dye solution was prepared to a concentration of 1 mg/ml in distilled water and stored away from direct light at room temperature. Dye maintained activity for up to six months as such. Embryos or material to be stained were placed a solution of the same media or PBS with dye stock added to a final concentration of 5 μg/ml. Material was incubated in this solution for 5 minutes. Material was then placed through one 30 second wash of media and one 5 minute wash of media. Material was then mounted under a coverslip with or without paraffin waxed edges depending on the degree of cell flattening desired. Fluorescence microscopy was performed with minimum exposure as the dye faded noticeably within several minutes of UV exposure.

4. MICROMANIPULATION

4.1 MICROMANIPULATION SYSTEM

Two three-dimension movement micromanipulators (NT-8, Narishige Scientific Pty. Ltd., Japan) were attached to an inverted microscope (Diaphot, Nikon, Japan) fitted with DIC optics. The objective turret was fitted with a LWD 20x, LWD 40x, and 10x DIC objectives and a 4x bright field objective. To control meniscus movement in pipettes, two micrometer syringes (Gilmont Instruments Inc. U.S.A.), 2.0 ml and 0.2 ml were attached to the enucleation and holding pipette instrument holders respectively via thick walled plastic tubing (Masterflex Tubing 6409-16, Norton Performance Plastics, U.S.A.). The entire system was filled with silicon oil (200 fluid/20 centistokes, Dow Corning, U.S.A.). Microphotography was taken with a 35 mm automatic camera (Nikon) attached directly to a 35 mm camera bayonet

fitting on the microscope, using 50% of the total transmitted light. Photographs taken with 100 and 400 asa colour slide film.

4.2 MANIPULATION PIPETTES

Pipettes were manufactured with glass capillary tubing directly from packaging (Clark Electromedical, U.K.). Capillary tubing was 150 mm long and was pulled on a pipette puller (P-77B, Brown-Flaming, U.S.A.) to produce 2 pipettes with gradually tapered tips.

Holding pipettes were pulled from thick walled tubing (GC100-15) cut on a microforge (Defonbrune, France) to an O.D. of 150 to 180 μm. It was then polished with the microforge filament to an I.D. of 80 to 100 μm. Enucleation/blastomere manipulation pipettes were manufactured from thin walled tubing (GC100T-15) This was cut to an O.D. of 25 to 45 μm depending on the size of the cell being manipulated or enucleated. Cut pipettes were ground on a capillary grinder (Bachofer Laboratoriumsgerate, West Germany) to produce a 45° bevel. Pipettes were then washed in 25% hydrofluoric acid to sharpen the bevelled edge followed by spiking of the distal tip of the bevel on the microforge filament.

All pipettes were then bent twice 80° at 6 mm and 14 mm from the tip to facilitate positioning into the manipulation chamber. They were then stored in glass jars until required. Prior to use, the enucleation pipettes were washed briefly in a 1.25 % (v/v) of detergent (Tween-80, Sigma, USA).

5. **ELECTROFUSION**

5.1 FUSION METHODS

The theory of electrofusion of mammalian cells is described in detail by Zimmerman and Vienken (1982).

5.1.1 **Non-polarised fusion**

5.1.2.1 *Fusion Medium*

The medium used for non-polarised fusion was either HEPES buffered culture media, or PBS.

5.1.2.2 *Procedure*

Cell fusion for cells with natural cleavage divisions were fused essentially as described by Kubiak and Tarkowski (1985). The fusion pulse was delivered by an electrostimulator (S48, Grass Instruments, U.S.A.) at 1.5 kV/cm for 100 μs, twice with a 100 ms interval. The electrodes were manufactured from glass rods containing insulated wire leads connected to either a 100 μm or 300 μm platinum wire. The electrodes were fitted to the micromanipulation system and the fusion was executed whilst viewed on the microscope at 100x. The cells were manipulated with the electrode tips to orientate the cell cleavage plane to be 90° to the direction of the electrodes and the field line.

5.1.2 **Polarised fusion**

5.1.2.1 *Fusion medium.*

Fusion medium was basically as described by Willadsen (1986). The concentration of constituent salts and preparation are as follows;

0.3 M mannitol,

0.1 mM $MgSO_4$,

0.05 mM $CaCL_2$,

pH adjusted to 7.4,

Osmolarity adjusted to 280 mOsm,

H_20 volume adjusted with Milli-Q water,

Filter sterilised and refrigerated (up to 3 months)

Alternatively fusion medium could be produced using a 0.3 M mannitol solution could be supplemented with a 1/30 part by volume of PBS.

Embryos were equilibrated in fusion medium for 15 minutes prior to electrical treatment.

5.1.2.2 *Procedure*

To fuse discreet cells or cell fragments, it is necessary to polarise the fragments with an AC field to firstly align the mutual plane of the two cells at 90° to the electrodes and secondly to achieve a tight junction between the fragments. For the majority of electrofusions a modified AC function generator (Topward Instruments Pty. Ltd. Taiwan) was used. The modifications to the generator were such that the available DC shift in the AC wave generator was engineered to be triggered independently by the DC stimulator. This enabled a continuous AC field to operate with a net DC shift in the AC wave. The duration and strength of the AC field and the DC pulse were controlled manually. The number and duration of the DC pulses were preset.

The specifications and ranges of the individual parameters use were;

<u>AC stimulus</u>

500-1000 kHz

sine wave

0-1000 V/cm

<u>DC pulse</u>

0-675 V/cm

1-6 x 100 μs duration

50-100 ms interval

A commercial electrofusion device (BTX 200 Electro Cell Manipulator, Biotechnologies and Experimental Research, Inc., U.S.A.) was used for some experiments with ovine embryos. The fusion specifications are defined in the appropriate chapter.

5.2 FUSION CHAMBERS

5.2.1 **Single cell chamber**

The single fusion chamber was used with both fusion devices for fusing single embryos. It was 35 mm glass or plastic petri dish. The glass petri dishes were used for improved microscope observation with DIC optics. The dish was filled with fusion medium once for the duration of the fusion. Electrodes were lowered into the dish with the micromanipulators and the cells were manipulated as per non polarised fusion.

5.2.2 **Multiple cell fusion**

The multiple fusion chamber was used exclusively in conjunction with the commercial device due to the larger output voltage required. The chamber consisted of two parallel platinum electrodes, 1.7 mm apart on a microscope slide. The gap between the wires was filled with a small volume of fusion medium prior to fusion. Up to 20 embryos were placed in the chamber at one time. The fusion sequence was initiated immediately and the cells were allowed to self orientate in the AC field.

SECTION III

CULTURE, MANIPULATION AND NUCLEAR TRANSFER IN SMALL RUMINANTS

1. SPLITTING AND CULTURE OF GOAT EMBRYOS

1.1. INTRODUCTION

Identical goat embryos can be potentially produced by dividing the cell mass of preimplantation embryos. This has already been achieved using a variety of mammalian embryo types at different preimplantation stages, however the capacity of blastomeres recovered from various developmental stages varies between species. Viable offspring in the mouse (Tsunoda & McLaren 1983) and rat (Matsumoto *et al* 1989) can only be produced from blastomeres up to the two cell stage. In the rabbit this extends up to the 4 to 8-cell stage (Moore *et al* 1968) while the blastomeres of larger domestic animals can produce live offspring up to the 4- and 8-cell stage from horse (Allen 1982, Allen & Pashen 1984) and sheep (Trounson & Moore 1974, Willadsen and Polge 1981) embryos.

One determinant of blastomere survival is volume of individual blastomeres, which decreases with the stage of development. The viability of divided embryos should therefore be improved by increasing the cell mass or number of blastomeres in each embryo fraction. This has been demonstrated in the ovine and bovine, using groups of blastomeres, from which up to three identical animals have been obtained from a single eight-cell stage embryo (Willadsen 1982).

Manipulation procedures with early stage embryos causes substantial damage to the zona pellucida which can compromise subsequent development *in vivo* (Trounson & Moore, 1974). One option to overcome this is to encase the embryo in agar. These encapsulated embryos are then placed into interim culture in the oviduct of a temporary host, before being recovered, excised and transferred to recipients (Willadsen 1979). Alternatively, embryos can be cultured *in vitro* for an equivalent period and transferred directly to recipients.

However the successful *in vitro* culture of domestic animal embryos is limited particularly with respect to the caprine. Preliminary attempts at *in vitro* culture of one to eight-cell stage caprine embryos using Hams F-10 or Whittens medium supplemented with either BSA or heat treated fetal calf serum (Wright & Bondioli, 1981) have failed. In the caprine, *in vitro* culture appears to be limited to early cleavage stages due to a stage specific block (reviewed by Wright & Bondioli 1981). Similar problems exist with sheep embryos but have been partly resolved using oviductal cocultures (Gandolfi & Moor, 1987) and synthetic oviduct fluid medium (SOFM; Tervit *et al* 1972; Walker *et al* 1988). The similarity between ovine and caprine embryos encourages potential adaptation of these culture systems to caprine embryos.

The objective of this chapter was to establish an *in vitro* culture system for caprine embryos in order to simplify production of animals from manipulated early cleavage stage embryos. Using SOFM as a basic culture media, several serum supplementations were used to test and determine a culture system suitable for caprine embryos. A proven media was then used to support manipulated embryos and the impact on their viability assessed.

1.2. METHODS

1.2.1 **Embryo collection**

Embryos were obtained from cashmere-bearing feral goats at Nairne, South Australia. Donors were superovulated with FSH (Schering), administered twice daily, a primer dose of 4 mg, followed by six doses each of 2 mg at 12-hour intervals. To synchronize the donors, intravaginal progestagen sponges (Repromap, Upjohn) were placed for 10 days and removed 48 hours after the first injection. Goats were injected with prostaglandin analogue, cloprostenol (Estrumate, ICI; 75 μg, i.m.), 12 hours before sponge removal. Donors were mated at 12 and 24 hours after onset of estrus (estrus=day 0). Embryos were collected between 24 to 48 hours after

estrus by mid ventral laparotomy depending on the embryo stage required. Oviducts were flushed using phosphate buffered saline with 10% heat inactivated goat serum and the embryos isolated were placed in SOFM-H. Embryos were transported to the laboratory at 37°C and placed immediately into culture.

1.2.2 **Micromanipulation**

One to four cell-stage embryos were cultured in medium with heat inactivated human serum to the eight-cell stage and then used for splitting. Only eight cell-stage embryos that had regular blastomeres were used in the manipulation procedures to minimise embryo loss as a result of utilising anucleate cytoplasmic fragments. Empty zonae pellucida were produced from either hatched caprine blastocysts or from evacuated porcine oocytes. Embryos were placed in SOFM-H without calcium or magnesium for 15 minutes prior to manipulation. A 90 to 120° cut was made in the zona pellucida using a glass needle. A pipette of similar diameter to the blastomeres was then used to transfer blastomeres from embryos into evacuated zonae (Plate 1: photographs 1 and 2). Embryos were subsequently washed in SOFM-H before being returned to the culture environment.

1.2.3 **Culture**

All embryos were cultured in SOFM-T supplemented with 20% heat inactivated serum at 37°C in 95% relative humidity. The number of nuclei were determined using Hoechst 33342 (Section 2.3).

1.2.4 **Statistics**

Statistical analysis tests used were the Kruskal Wallis test and Fishers exact t-test.

1.3. EXPERIMENTAL

1.3.1. **FCS vs. HS as a protein supplement**

1.3.1.1. *Experimental outline*

The serum sources chosen were heat inactivated fetal calf and human serum. Embryos were collected and separated into two developmental stages, the first being the one to two cell and the second, three to eight-cell stage. Embryos from each stage were then placed into culture medium supplemented with serum. Development was assessed by the number and the time required for embryos to reach the blastocyst stage.

1.3.1.2 *Results*

From 13 donor animals, 59 embryos were collected at the 1 to 2-cell stage and 82 at the 3 to 8-cell stage. Some cultures were contaminated resulting in the final numbers listed in Table 3.1. The majority of embryos developed to the blastocyst stage indicating no evidence of any stage specific block. The number of embryos reaching the blastocyst stage and the mean time required for various stages and serum types is listed in Table 3.1. The difference in the number of blastocyst forming was not different between the embryos placed in culture at the 1 to 2-cell stage and the 3 to 8-cell stage. However the time required for blastocyst formation was less for embryos cultured from the 3 to 8-cell stage($P<0.001$). Embryos cultured in SOFM supplemented with FCS compared with HS did not result in fewer embryos forming blastocysts, however there was a significant difference in time to form blastocysts, with up to two days difference in the time of blastocoel formation ($P<0.001$).

Table 3.1 Development of early cleavage stage embryos in SOFM supplemented with either 20% FCS or HS

Stage of embryos	Serum source	Number	Blastocysts (%)	Days to blastocyst#
1→2-cell	FCS	28	13 (46)	8.92 ± 0.61[a]
1→2cell	HS	11	8 (73)	6.71 ± 0.36[b]
3→8-cell	FCS	31	20 (66)	8.25 ± 0.43[c]
3→8-cell	HS	41	28 (70)	6.34 ± 0.32[d]

Values in same column with different superscripts differ (P<0.05)
#Mean ± standard deviation.

1.3.2 **In vitro development of manipulated embryos**.

1.3.2.1. *Experimental outline*

To determine the feasibility of manipulating goat embryos at early cleavage stages, embryos were cultured to the eight-cell stage from the two to four cell in SOFM-T with 20% heat inactivated human serum. Embryos were to subject to splitting and cultured for a further period of seven days and observed for blastocyst formation daily. Some of the embryos reaching the blastocyst stage were transferred to synchronised recipients.

1.3.2.2 *Results*

A total of 19 embryos were selected from embryos cultured from the two to four-cell stage *in vitro*. The resulting split embryos cultured in human serum developed to blastocyst at similar rates to embryos in experiment 1 with 27/37 (73%) forming blastocysts or blastocyst like structures. The time required for blastocyst formation was 6.71 days (±0.46). Embryos from manipulated embryos were subject to premature hatching (Plate 1: photograph 3). Twelve embryos were chosen for transfer to 6 synchronised recipients late in the breeding season (July) however none became pregnant.

1.3.3. *In vitro* development of pronuclear embryos in SOFM supplemented with either FCS, HS and GS.

1.3.3.1. *Experimental outline*

To determine the effect of serum source on *in vitro* development of embryos cultured in SOFM one-cell embryos were cultured SOFM-T containing either FCS, HS or homologous GS. Embryos were collected 24 hours after the onset of oestrus and allocated to culture treatments. Morphological development was assessed every 24 hours and all the embryos being ultimately stained for nuclei after 168 hours culture.

1.3.3.2. *Results*

From 10 superovulated donors only 6 responded from which 27 embryos were collected. Blastocysts first developed after 96 hours culture in media supplemented with GS. More blastocysts developed in GS and HS (Table 3.2) than FCS however was not significant for the number of embryos tested. The cell numbers of embryos cultured with FCS were lower than for embryos cultured with other serum types ($P<0.001$). The embryos cultured with GS resulted in the highest cell numbers, with some blastocysts in excess of 200 nuclei (Plate 1: photograph 4).

Table 3.2 *In vitro* development of pronuclear embryos cultured in SOFM supplemented with either HS, FCS or GS.

Serum type	N	Blastocysts (hours cultured) 96	120	144	168	Nuclei#
human	10	0	1	4	6	108 ± 47^{a}
fetal calf	8	0	1	2	2	25 ± 18.3^{b}
goat	9	2	4	6	7	146 ± 69.7^{a}

Values with different superscripts differ (t-test; $P<0.01$).
#Mean ± standard deviation.

Photograph 1.

Injection of blastomeres into an evacuated porcine oocyte zona pellucida

(Optics: DIC 200x)

Photograph 2

Blastomeres in zona pellucidae at various times from 5 minutes to 1 hour after manipulation.

(Optics: DIC 200x)

Photograph 3

Blastocysts derived from unmanipulated (upper row) and manipulated half embryos (lower row) subsequent to *in vitro* culture.

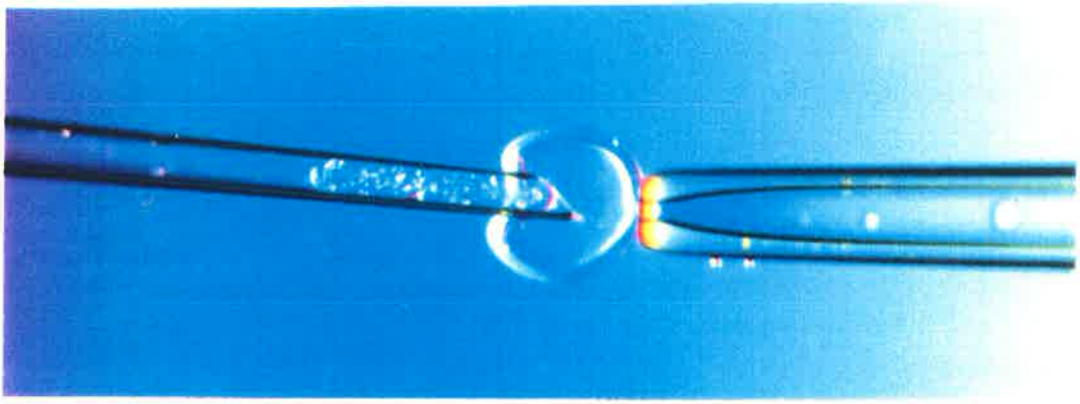
(Optics: DIC 200x)

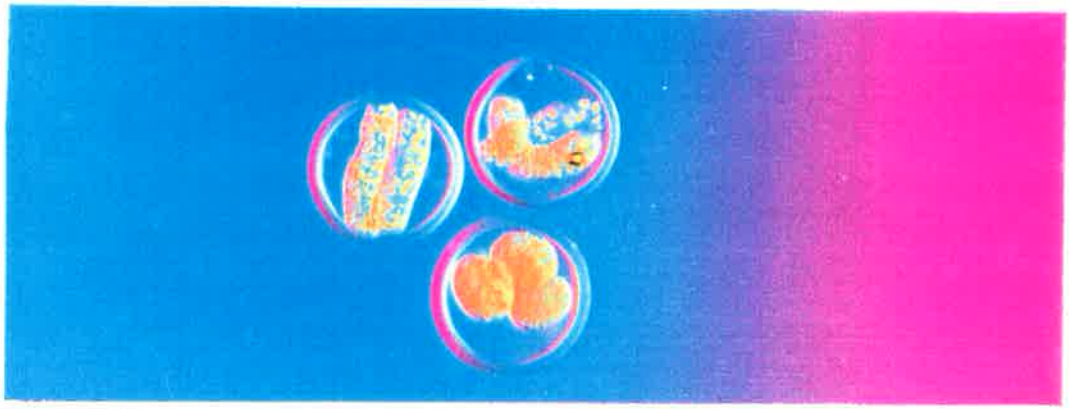
Photograph 4

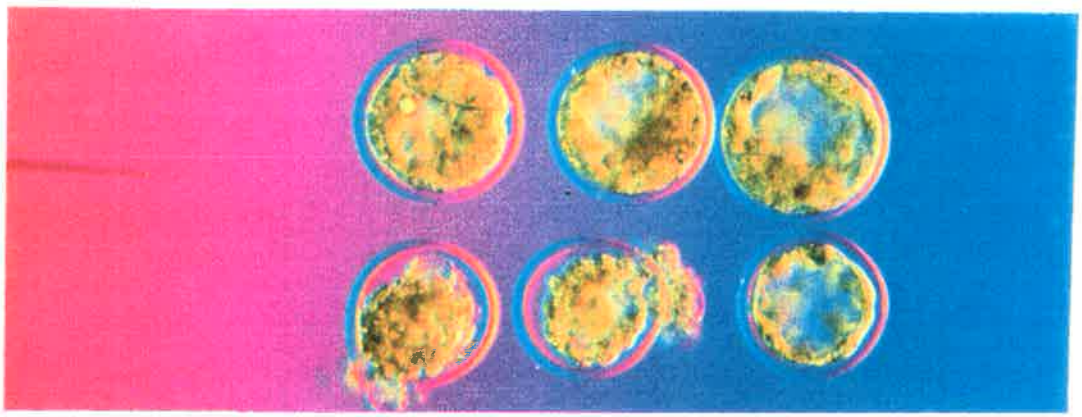
A single blastocyst derived from a pronuclear zygote cultured in SOFM with goat serum for 168 hours. (Stained with Hoechst 33342)

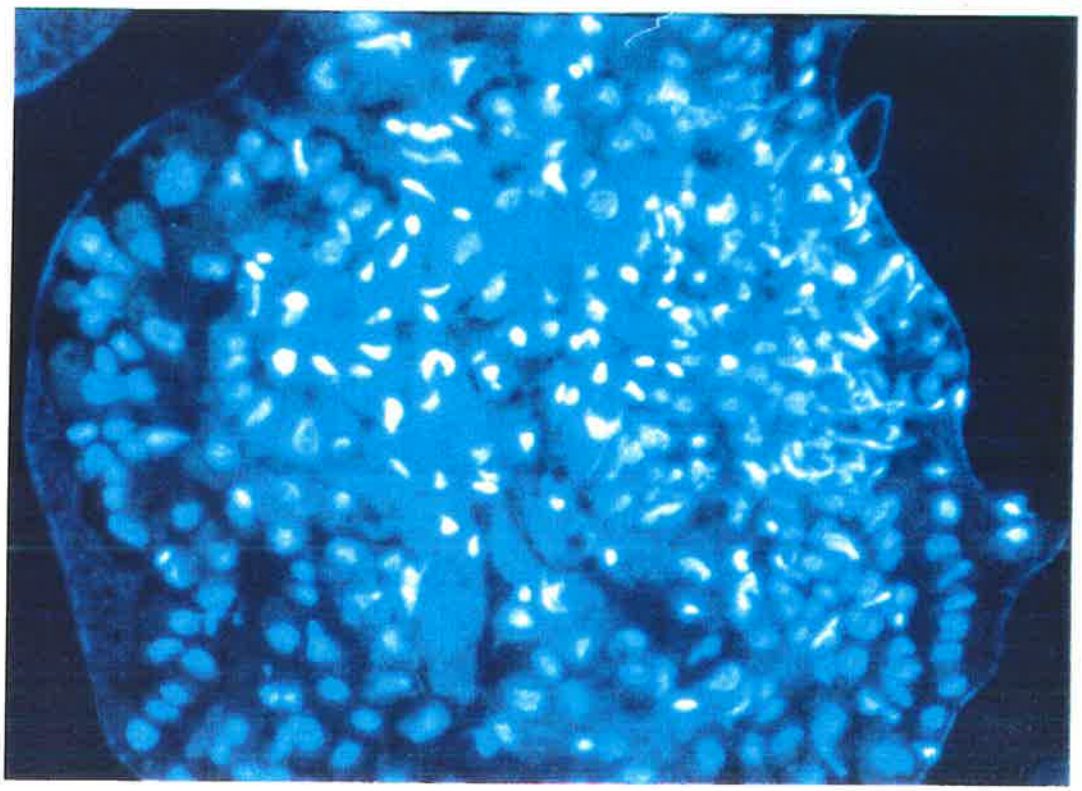
(Optics: UV Flourescent 400x)

PLATE 1









1.4 DISCUSSION

The results represent the first effective culture of early goat zygotes to blastocyst *in vitro* using a simple medium (SOFM) supplemented with serum. However the effectiveness of SOFM was determined by the source of serum. A second observation is that the medium is capable of supporting embryos after micromanipulation procedures involving blastomere disruption.

The initial results of experiment 1.3.1 indicated that FCS was less suitable as a culture supplement than HS. Subsequent testing of one-cell stage embryos in experiment 1.3.3 indicated that FCS resulted in much lower cell numbers after 168 hours culture. Previous studies on the culture of domestic animal embryos with SOFM have utilised BSA as a protein source (Tervit *et al* 1972, Tervit & Rowson 1974). FCS has been used more widely as a supplement for several species and has been used previously for early caprine zygote culture (Wright & Bondioli 1981). Whilst FCS was unsuitable in this trial, batch variation within serum types does not exclude it potential use given the high levels of growth factors in FCS (Möller 1978). If homologous serum is as effective as HS as it was in this trial, it would be preferrable because of cost and availability.

Recent report on culture of early stage caprine embryos has demonstrated successful *in vitro* culture in the presence of oviductal cells. However only 8/20 of one-cell embryos achieved blastocysts after 168 hours culture compared with morula stage embryos in which 13/18 formed blastocysts (Sakkas *et al*, 1989). The cell numbers of the embryos cultured *in vitro* in SOFM supplemented with HS (108±47) or GS (146±69.7) were lower than reported recently for embryos cultured *in vivo* (238±27; Sakkas *et al* 1989). Previous attempts to culture goat zygotes in simple medium (BM, HTF, T6) supplemented with 10% GS (Wright & Bondioli, 1981) resulted in less than 10% of embryos developing past the 8 to 16-cell stage. Therefore the success in culturing early caprine embryos must be explained in terms of either coculture specific growth factors or serum specific factors. Possibly, SOFM is critical for culture but only in combination with particular serum

sources. The use of higher serum levels in this study (20%) may have been advantageous over the levels used in previous studies (10%; Wright and Bondioli 1981, Sakkas *et al* 1989). Factors present in oviductal coculture may supplement the embryo culture environment in combination with the lower (10%) serum levels. Alternatively, physical attributes of the culture system such as humidity, temperature stability and gas mix may be more critical than realised.

The rate of blastocyst formation (22/26) from ovine embryos split at the eight-cell stage and cultured *in vivo* (Willadsen 1980) was higher than this study with caprine embryos cultured *in vitro* (27/37). The number of blastocysts forming from the cultured split embryos was similar to the unmanipulated 3 to 8-cell stage. As a system for the production of identical twins, embryo splitting at the morula and blastocyst stage would be a more efficient and simpler approach than eight-cell stage blastomere splitting (Williams *et al* 1984). However, splitting embryos from zygotes cultured from early cleavage stages demonstrates potential application of the culture system to manipulation procedures such as gene injection and nuclear transfer. However to evaluate such prospects more thoroughly, transfer of cultured zygotes is necessary to determine the effects of culture on production of offspring.

2. NUCLEAR TRANSFER AND IN VITRO CULTURE IN THE PRODUCTION OF MULTIPLE LAMBS FROM A SINGLE EMBRYO.

2.1 INTRODUCTION

Production of genetically identical sheep is possible by the transplantation of cleavage stage nuclei into enucleated oocytes (Smith and Wilmut 1989, Willadsen 1986). During the manipulations required to achieve this, substantial damage is caused to the zona pellucida which necessitates that the zygotes be transferred to ovine oviducts encased in agar and recovered at the morula-blastocyst stage (Willadsen 1986). Recently, several procedures have been developed, allowing *in vitro* culture of ovine zygotes for up to three days (Walker *et al.* 1988). This same system (SOFM-T with 20% HIHS) has been successfully used for the culture of manipulated caprine embryos at the eight-cell stage. More recently it has been shown that SOFM without any HEPES (SOFM-C with 20% HIHS) supplementation is superior for development of zygotes to the blastocyst stage (Walker *et al* 1989).

It is not known if ovine oocytes, subject to the extensive manipulations associated with nuclear transfer, can be cultured *in vitro*, and remain viable. The following experiments examine (1) development of various aspects involved in producing ovine embryos by nuclear transfer, which to date has been limited (Willadsen 1986, Smith *et al* 1989), and (2), the viability of nuclear transfer embryos using an *in vitro* culture system.

2.2 METHODS

2.2.1 **Oocyte and Embryo Collection**

2.2.1.1 *In vivo material*

Superovulation was induced in Peppin strain Merino ewes using a 12-day progestagen pessary (Upjohn Pty Ltd, Australia; 60 mg medroxyprogesterone acetate) and 22 mg per ewe of follicle stimulating hormone (Heriot Agvet Pty Ltd, Australia). Synthetic gonadotrophin releasing hormone (Intervet Australia Pty Ltd; 50 μg per ewe) was administered i.m. 30 hours after pessary removal. Ewes were inseminated laparoscopically with a minimum of 20 x 10^6 motile fresh spermatozoa per uterine horn 48 hours after pessary removal (Walker *et al* 1984).

Oocytes and embryos were collected by midventral laparotomy approximately 12 to 17 hours and 60 hours respectively after the expected median time of ovulation. Oviducts were flushed with 10 ml Dulbecco's phosphate buffered saline (Flow Laboratories, Australia) containing 10% heat inactivated sheep serum. Oocytes and embryos were recovered from the flushings within 5 minutes of collection.

2.2.1.2 *In vitro matured oocytes*

Oocytes were flushed from sliced ovaries of slaughtered ewes. They were matured for 27 hours at 39°C under 5% CO_2 in air in TCM 199 supplemented with 20% FCS, 0.5 μg/ml FSH and LH, and 5 μg/ml estradiol. Oocytes were then denuded of any cells attached to the zona by manual pipetting. Under 100x magnification on a dissecting microscope, oocytes were selected as suitable if the perivitelline space and/or a polar body was present.

2.2.2 **Culture and Transport**

Embryos were cultured in synthetic oviduct fluid medium (SOFM-C) with 20% heat-inactivated human serum (HS). Transportation of embryos was in tubes containing SOFM-T supplemented with 20% HS. The transport media was previously equilibrated in an atmosphere of 5% CO_2 in air at 37°C and transported between the collection site and the laboratory at 37°C. The time between collection and initiation of manipulations was 2 to 4 hours.

2.2.3 **Manipulation procedures.**

Manipulations were carried out at room temperature (25°C). The manipulation medium for donor embryos and oocytes was SOFM supplemented with 25 mM HEPES, 5 mM $NaHCO_3$, 7.5 µg/ml cytochalasin B (Sigma, USA) and 10% heat inactivated sheep serum, (SOFM-CB). Donor nuclei embryos and oocytes were placed in SOFM-CB for 15 minutes prior to manipulation. Isolation of donor nuclei blastomeres, enucleation of oocytes and blastomere transfer was essentially as described by Prather *et al.* (1987). Briefly, donor nuclei blastomeres (Plate 2: photograph 1) and oocyte chromosomes were removed with a 35 µm O.D. enucleation pipette, and the donor blastomeres reinserted beneath the zona of the oocyte to produce pre fusion embryos (Plate 2: photograph 3).

Electrofusion was performed using the single fusion chamber. Embryos were equilibrated in fusion medium for 10 minutes and positioned between platinum electrodes placed 200 µm apart. Manipulated embryos were then aligned in an AC field of 150 V/cm, 500 kHz followed by a 650 V/cm D.C. pulse of 100 µsec. duration followed by a reduction of the A.C. field strength to zero volts over 15 secs. The embryos were cultured in SOFM-CB for 1 hour before fusion rates were recorded after which embryos were transferred to SOFM-HS microdrops.

2.2.4 **Embryo Transfers**.

Manipulated embryos were selected from culture at 66 hours post fusion on the basis of morphological appearance and cleavage regularity during culture (Plate 1: photograph 4). One to five embryos were transferred per ewe according to recipient availability, condition of embryos and number of clones derived from each donor embryo. Recipient animals were tested by ultrasound on day 45 of pregnancy to determine the number of fetuses.

2.2.5 Statistical analysis

Data were analysed using either χ^2 analysis or grouped t-test.

2.3. EXPERIMENTAL

2.3.1. **Enucleation of oocytes**

2.3.1.1 *Experimental outline*

This experiment was designed to determine the efficiency of chromosome removal in oocytes for nuclear transfer. Metaphase oocytes were obtained from both *in vitro* and *in vivo* sources The enucleation method involved removal of cytoplasm adjacent to the polar body (approximately 10% of total oocytoplasm) and/or the area of clear smooth cytoplasm usually associated with the polar body.

For the comparison of oocytes source on enucleation efficiency, only oocytes with relatively normal morphology were used. Particularly in the case of *in vitro* matured oocytes, oocytes were rejected if there was no polar body and/or perivitelline space.

In vitro matured oocytes were firstly stained to determine the proportion of oocytes with the metaphase plates proximal and distal to the polar body. Secondly oocytes from *in vitro* and *in vivo* sources were enucleated and subsequently stained for presence of nuclear material and compared for enucleation frequency.

2.3.1.2 *Results*

A total of 190 *in vitro* matured oocytes were stained. From these, there was approximately 87%(153) with visible metaphase plates of which 74%(138) were associated with the polar body. There were also a small proportion (6.3%;12) of oocytes with non-metaphase plate nuclear material. These included post metaphase nuclei and immature germinal vesicle stages. The remaining oocytes showed no evidence of any nuclear material (7.9%;15).

There was no difference in enucleation rate between oocyte sources This resulted in similar rates of enucleation for both oocyte sources (Table 3.3).

Table 3.3 Enucleation of *in vivo* and *in vitro* matured ovine oocytes

Oocyte source	Number manipulated	Number enucleated(%)
In vitro matured	167	119 (71)
In vivo matured	30	23 (77)

2.3.2. **Production and development *in vitro* of embryos following nuclear transfer.**

2.3.2.1. *Experimental outline*

The production of nuclear transfer embryos was carried out in two trials, one being at the end of the breeding season (June; Trial 1) and early season (December;Trial 2).

Nuclei were collected as blastomeres from 8 to 16-cell stage embryos and transferred to oocytes. The successfully fused embryos were cultured for 66 hours *in vitro* and if they had divided at least twice and had a normal morphological appearance,(see Plate 2:photograph 4) were transferred to recipient ewes. To compare the survival of the nuclear transfer embryos, embryos collected at the 8 to 16-cell stage were transferred the oviducts of synchronised recipients within four hours of collection. To determine the number of viable embryos remaining after selection by morphological criteria, embryos from the last three trials were cultured a further 56 hours and stained to determine the cell number using fluorescence microscopy. A group of cloned embryos were also cultured for 120 hours post fusion to determine the proportion of clones unsuitable for transfer, but capable of developing to the blastocyst stage.

2.3.2.2. *Results*

A total of 44 donor embryos at the 8-16 cell stage used to produce 293 manipulated embryos of which 268 fused (91%). The two trials differed ($P<0.05$) in the proportion of embryos cleaving at 24 hours post fusion (Table 3.4) but not in development to the 8-16 cell stage at 72 hours.

Table 3.4 *In vitro* development of nuclear transfer embryos prior to transfer.

Trial	Embryos	Clones	Fused (%clones)	24hr clv. (% fused)	8/16-cell (% fused)
1(June)	13	84	78 (93)	49 (63)[a]	37 (47)
2(Dec.)	31	209	190 (91)	153 (81)[b]	86 (45)

[a,b]Values differ ($P<0.05$)

Comparison of the embryos that did not reach the 8 to 16 cell stage in Trial 2 (December) with cloned embryos cultured *in vitro* (Table 3.5) indicated that a similar proportion of cloned embryos were arrested at the two to 16 cell stage. A similar number of embryos also developed to greater than the

16-cell stage but did not form blastocysts. The percentage of embryos that formed blastocysts was lower for embryos from Trial 2 than control embryos (14.2 vs. 25%; $P<0.05$). The cell numbers of blastocysts from Trial 2 were less than control nuclear transfer embryos.

Table 3.5 Development in SOFM-C of embryos not suitable for transfer after 72 hours culture (Untrans; Trial 2) and nuclear transplant embryos cultured for 120 hours post fusion

Source	Clones	2-16 cell (%)	>16-cell (%)	B'cysts (%)	Nuclei in b'cysts#
Untrans.	84	33 (39)	7 (8)	12 (14)	14 ± 7^{a}
Control	44	22 (50)	3 (7)	11 (25)	32 ± 8.1^{b}

[a,b] Values differ ($P<0.05$).
#Mean ± standard deviation.

The *in vivo* development of embryos subsequent to transfer is presented in Table 3.6. Heterozygous twin lambs resulted from one of the pregnant recipients in Trial 1. The other recipient died of suspected snakebite during the third trimester. In Trial 2, on day 45, ultrasonography indicated that there were six sets of twins and two singletons from eight pregnancies. Each set of twins was derived from an single embryo with two of the sets of twins, originating from the same embryo. At term there was one surviving set of twins and three of the identical quad embryos. The remaining five lambs were singletons. The pregnancy survival rates for both trials was significantly lower than for the unmanipulated control embryos.

Table 3.6 *In vivo* development of nuclear transfer and unmanipulated uncultured embryos subsequent to transfer at the 8/16 cell stage.

Trial	Embryos	Recips	Pregs. (% recip)	Fetuses (%embryos)	Lambs (%embryos)
1 (June)	37	11	2 (18)	3 (8)	2 (5)
2 (Dec.)	86	36	8 (22)	14 (16)	8 (9)
Control	19	7	7 (100)	15 (79)	14 (74)

2.3.3. **Effect of *in vitro* culture on the early preimplantation development of cloned embryos.**

2.3.3.1 *Experimental outline*

The development of nuclear transplant embryos cultured either *in vitro* or vivo for 72 hours post fusion was tested. Nuclear transplant embryos were produced as in experiment 2.3.2.1. Embryos were either transferred to recipient synchronised ewes 12 hours post fusion or maintained in SOFM-HS. The *in vivo* cultured embryos were transferred without any agar encasing. At 68 hours post fusion embryos were flushed from temporary recipient ewes and their development was compared with embryos cultured *in vitro* for the same period.

2.3.2.2 *Results*

Recovery of embryos from *in vivo* culture found the majority of embryos were totally devoid of any cellular material (Table 3.7). There was no obvious presence of any foreign cells (e.g. lymphocytes) in or attached to the any of the zonae or embryos. The two intact embryos recovered were at the eight cell stage and morphologically normal and absent of foreign cells.

Table 3.7 *In vivo* and *in vitro* development of nuclear transfer embryos for 72 hours post fusion.

Culture group	Recip-ients	Embryos	Recov-. ered	Empty Zonae	8/16-cells
in vivo	6	32	26	24	2
in vitro	-	21	-	-	15

2.3.4. *In vitro* culture and serial nuclear transplantation.

2.3.4.1. *Experimental outline*

The viability of 8 to 16 cell stage nuclear transfer embryos as blastomere/nuclei donors was tested. Donor nuclei were derived from either first generation clones or freshly collected 8/16 cell embryos. Nuclear transfer embryos were cultured a further 66 hours in culture and those reaching the 8 to 16-cell stage were transferred to recipients. The embryos in this experiment were transferred to the tip of the uterine horn rather than the oviduct. The recipients were subsequently monitored for pregnancy.

2.3.4.2. *Results*

A single embryo derived from *in vitro* culture in Experiment 2.3.2 was utilised as the second generation nuclei donor. From this embryo three blastomeres fused to oocytes and the resulting embryos were cultured for 69 hours. Two of these embryos developed to the 16-cell stage and were both utilised to produce a further 30 manipulated embryos, of which 22 were successfully fused and placed in culture. The development of second generation nuclear transplant embryos to the 8 to 16-cell stage and fetuses did not differ from first generation clones cultured simultaneously (Table 3.8). Only one recipient receiving first generation clones was pregnant with identical twin fetuses at day 45.

Table 3.8 Development of nuclear transfer embryos with donor nuclei derived from fresh or nuclear transfer embryos.

Embryo source	Donor embs.	Embs. fused	2nd gen.	8/16 (%)	Recips	Preg.	Fetus
N/T	1	3	31	17 (55)	6	0	-
fresh	2	16	-	10 (63)	5	1	2

PLATE 2

Photograph 1

Isolated blastomeres from a 16-cell stage ovine embryo.

(Optics: DIC 400x)

Photograph 2

A zona pellucida subsequent to blastomere removal, showing the extent of zona damage as a result of manipulations.

(Optics: DIC 400x)

Photograph 3

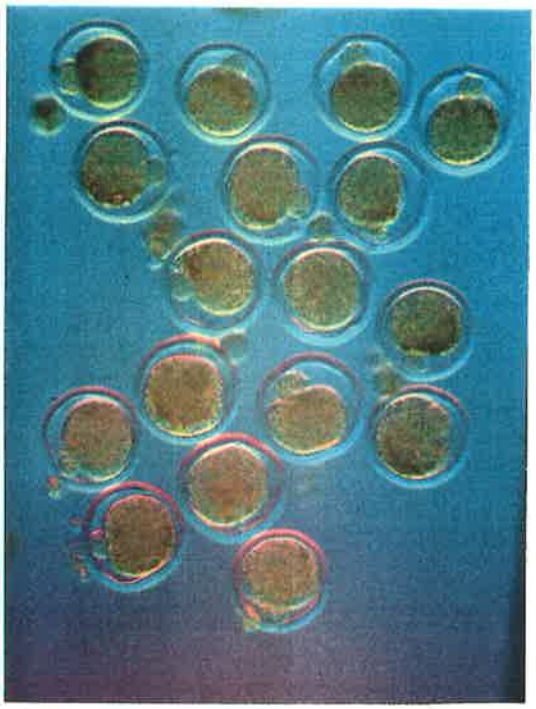
Prefusion ovine nuclear transfer embryos.

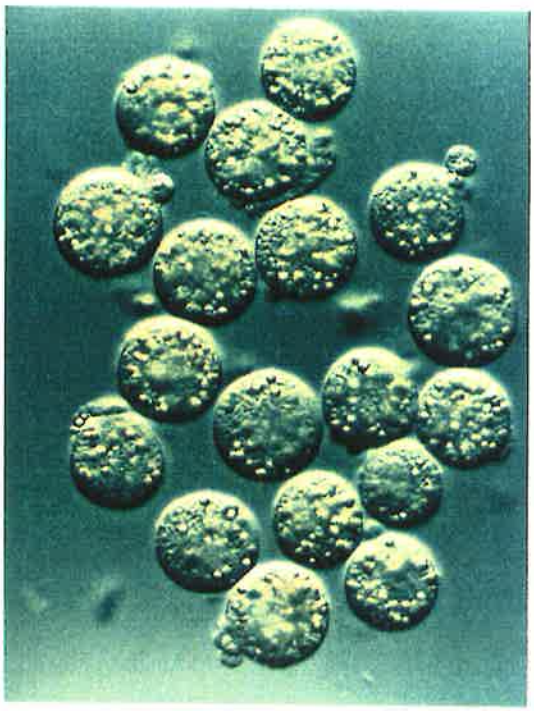
(Optics: DIC 200x)

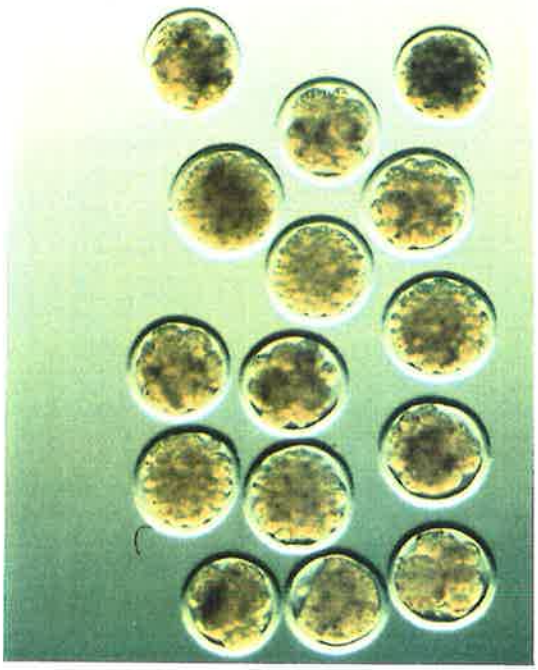
Photograph 4

Nuclear transfer embryos after 72 hours *in vitro* culture. Typical morphological phenotypes include (1) normal (top row second from left), (2) severely fragmented (second row, second from left), (3) unfused (second row, far right) (4) limited cleavage (top row, far right)

(Optics: DIC 200x







2.4. Discussion

The proportion of stained oocytes with the metaphase plates proximal to the polar body and the enucleation rate indicate that the enucleation method was efficient for the region of cytoplasm removed. The enucleation rate of *in vitro* matured oocytes was not different to *in vivo* matured oocytes, however the proportion of *in vivo* matured oocytes initially containing metaphase chromosomes is unknown. Previously, the *in vitro* maturation of ovine oocytes has resulted in 90% (Holm *et al* 1990) reaching metaphase II which corresponds closely with the frequency observed in the sample group tested (87%).

The production of nuclear transfer embryos demonstrated that the *in vitro* culture system developed by Walker *et al.* (1988) can be used to support the early development of nuclear transplantation embryos. The pregnancy rate achieved in Trial 2 (22%) is similar to that obtained using *in vivo* culture of nuclear transfer embryos from sheep (17%; Smith and Wilmut 1989) and cattle embryos (22.5%; Bondioli *et al.* 1990) but much lower than the pregnancy rate of control embryos (70%). Unmanipulated ovine embryos cultured for a similar period *in vitro* in the same system prior to transfer produce high numbers of elongated conceptuses (94%) at day 14 (Walker *et al.* 1988). Thus the losses experienced with micromanipulated embryos are more likely to relate to the manipulation procedures or the inability of the donor nuclei to be reprogrammed sufficiently to support development rather than deficiencies in the culture system.

In the mouse, variation in the cell cycle stage of donor nuclei can influence development following transfer to the enucleated zygote (Smith *et al.* 1988), and this may account for the large variation seen in the rate of development of manipulated sheep embryos in culture (Trial 2). This variation may result in post transfer losses due to asynchrony between the developmental stage of the embryos and the physiological status of the recipient reproductive tract (Rowson and Moor 1966).

The morphological criteria used to screen embryos after 66 hours *in vitro* culture identified that the majority of embryos were suitable for transfer with only 7/84 (8%) of the remaining embryos undergoing further but retarded development as assessed by the low cell numbers. This was supported from the observation of a higher proportion of embryos developing to the blastocyst stage from nuclear transfer embryos (25%) used as *in vitro* controls. Further extension of the culture period for the manipulated embryos prior to transfer was not attempted as experience with unmanipulated embryos (Walker *et al* 1988; Gandolfi and Moor 1988) indicates that this would have probably resulted in a further reduction in the pregnancy rate.

The in vivo culture of the nuclear transfer embryos for 66 hours post fusion resulted in major embryo losses compared with the *in vitro* cultured embryos. It was expected that the relatively minor damage inflicted upon the zona (see Plate 2: photograph 2), and the relative integrity of the embryos subsequent to manipulation (Plate 2: photograph 3) would not have justified agar embedding. Cloned nuclear transfer embryos created by the method described by Willadsen (1986) involve creating a large hemispherical fissure directly exposing the embryo and dramatically reducing the overall strength of the zona. However the results from this study are very similar to that observed when ovine embryos were transferred with partially removed zonae (Trounson & Moore 1974). Damage to the zona may have increased susceptibility to antibody mediated lysis (Trounson *et al* 1976) or entry by hostile lymphocytes. Alternatively, the oviductal transit could facilitate deterioration of an embryo with a damaged zona as observed with denuded embryos (Bronson & McLaren 1970; Modlinski, 1970). The transfer of mouse eggs with similar zona damage to mouse oviducts also results in significant embryos losses for early cleavage stages but not thereafter or with interim culture (Nichols & Gardner 1989).

Whilst agar embedding would provide a control with an alternative culture system it does not provide a strict *in vivo* control due to the presence

of agar. This may be overcome with a method of partially covering the embryo to repair the site of puncture. Alternatively, culture to 120 hours post fusion would increase selection of embryos and avoid embryo losses as a result of zona damage.

The use of nuclear transfer embryos as a source of donor nuclei donor and hence serial cloning has been reported for ovine (Willadsen 1986) and for bovine (Bondioli *et al* 1990) embryos. However the efficiency of the procedure is unknown. In demonstrating the possibilites of serial nuclear transplantation using *in vitro* culture, the totipotency of the nuclei was also established. Development of second generation nuclear transplant embryos was found to be no different from those from the first generation. Larger experiments would be required to adequately ascertain viability subsequent to transfer to recipients. The donor embryo source was limited to one embryo in this experiment and the results may have been largely biased by the inherent viability of the chosen embryo. Furthermore, repeats of serial cloning may result in an increased productivity per donor embryo but may risk an increased probability of chromosomal abnormalities as observed with lower vertebrates (King & Briggs 1956, DiBerardino 1979).

The results of this Section (3.2) indicate that the simple culture system described may provide a viable alternative to *in vivo* culture systems previously employed for short term culture of manipulated zygotes. If as the present data suggests, the *in vitro* culture system is as efficacious as the *in vivo* system, it can be recommended as the method of choice. It is not only simpler to manage, but has the advantage that it will permit direct observation of embryos thus enabling experimentation aimed at improving understanding of factors limiting the early critical stages of their development. These advantages can immediately be amplified with the ability to serially clone embryos *in vitro*. Further studies aimed at providing direct comparison of the efficacy of the *in vitro* and the *in vivo* systems are warranted.

3. ASSESSMENT OF OOCYTE SOURCE FOR NUCLEAR TRANSFER

3.1 INTRODUCTION

The use of *in vitro* matured ovine oocytes for nuclear transfer has not been described previously. *In vitro* matured bovine oocytes have been utilised for nuclear transfer with low development to the morula and blastocyst stage (Prather *et al.* 1987) and low fusion rates (Kinis *et al* 1989). The proportion of *in vitro* matured and fertilised bovine oocytes that develop to the morula and blastocyst stage is between 20-28% when cultured in sheep oviducts (Sirard *et al* 1988)) and 22% in oviductal epithelial coculture (Eyestone and First 1989). This is lower than observed for *in vivo* matured and fertilised oocytes cultured *in vitro* with 43% developing to the morula and blastocyst stage (Eyestone and First, 1989).

In vitro matured ovine oocytes have a lower developmental capacity than oocytes matured *in vivo* (Crozet *et al* 1987). This may be due to either nuclear or cytoplasmic factors. If the function of the nucleus is compromised in *in vitro* matured eggs, the cytoplasm may be able to better support development with an introduced *in vivo* matured nucleus. Alternatively an inadequate cytoplasm might not only be unable to support development of its own nucleus, but not contain the factors necessary to reprogram and advanced introduced nucleus.

The following experiments investigate the viability of *in vitro* matured ovine oocytes in nuclear transfer programs. Firstly, various aspects of producing nuclear transfer embryos with *in vivo* and *in vitro* derived oocytes are compared. Secondly, fertilised oocytes from both sources, fertilised *in vivo* are subject to nuclear transfer to determine the effect of the procedure on *in vitro* development. Thirdly the effect of site of transfer and zona damage on embryo viability is examined.

3.2 MATERIALS AND METHODS

3.2.1 **Oocyte and embryo collection**

Superovulation was induced in New Zealand Coopworth ewes treated with progesterone CIDR pessaries (CIDR-G, Carter Holt Harvey, N.Z) for 13 days (insertion = day 0) with replacement at day 9. Ewes for providing oocytes were placed with teaser rams while ewes for providing embryos were run with entire rams. At approx 1600 hours on day 10 of CIDR treatment ewes were injected with an initial injection of 800 i.u. PMS (Pharmaco, N.Z.) and 2 mg FSH (Ovagen, Immunochemical Products, N.Z.). For embryo donors this was followed with 12 mg of FSH over 4 subsequent injections until 1600 hours on day 13 of CIDR treatment. Embryo donors were injected with 150 i.u. of GnRH (Fertagyl, Intervet, Holland) at 0900 hours on day 14. Embryos for nuclear transplantation were collected at 0900 hours on day 18.

Oocyte donors were injected with a total of 12 mg of FSH at twelve hour intervals with the last injection at 0800 hours on day 13. followed by an injection of 150 m.g. of GnRh at 2400 hours on day 13. The oocytes were collected by mid-ventral laparotomy at 1000 hours on day 15.

SOFM-T supplemented with 2% HIHS was used to flush the oviducts of donor animals for oocyte and embryo collection.

3.2.2 ***In vitro* matured oocytes**

Oocytes were aspirated from the ovaries of slaughtered ewes. They were matured for 27 hours at 39°C under 5% CO_2 in air in TCM 199 supplemented with 10% FCS, 10 μg/ml FSH and LH, and 1 μg/ml estradiol and 1 x 10^6. Oocytes were then denuded of any cells attached to the zona by manual pipetting in 0.01% hyaluronidase. Under 100x magnification on a dissecting microscope, oocytes were selected as suitable if they had a perivitelline space and/or a polar body.

3.2.3 **Culture**

Embryos and oocytes were immediately isolated from the flushing fluid (SOFM-H with 2% HIHS) or *in vitro* maturation media and placed into SOFM-T with 10% HISS. The media was previously equilibrated in an atmosphere of 5% CO_2 in air at 37°C. The time between collection and initiation of manipulations was 30 minutes to 2 hours. After the manipulations all embryos were placed in microdrops of SOFM-C with 20% HIHS.

3.2.4 **Manipulation procedures.**

Manipulations were carried out at room temperature (25°C). The manipulation medium for donor embryos and oocytes was SOFM-M with 7.5 µg/ml cytochalasin B (Sigma, USA) and 10% heat inactivated sheep serum, (SOFM-CB). Donor nuclei embryos and oocytes were placed in SOFM-CB for 15 minutes prior to manipulation. Isolation of donor nuclei blastomeres, enucleation of oocytes and blastomere transfer was essentially as described by Prather *et al.* (1987).

Electrofusion was performed using both the multiple and single fusion chamber (Section 2.5). The multiple fusion chamber parameters were a single 1.5 kV/cm D.C. pulse with a 10 second 500 V/cm AC prealignment. Single fusion chamber parameters consisted of an aligning AC field of 150 V/cm, 500 kHz followed by a 675 V/cm DC pulse of 100 µsec. duration followed by a reduction of the AC field strength to zero volts over 15 secs. The embryos were then cultured in SOFM-CB for 1 hour before being placed in microdrop culture.

3.2.5 **Embryo Transfers.**

Recipient ewes were treated with progesterone CIDR pessaries for 13 days with 400 i.u. PMS injection and CIDR removal at 0800 hours. Recipients were starved for 24 hours prior to surgery which was performed 120 to 144 hours after CIDR removal. Manipulated embryos were selected from culture at 72-74 hours post fusion on the basis of morphological

appearance and cleavage regularity during culture. One to three embryos were transferred per ewe according to recipient availability, condition of embryos and number of clones derived from each donor embryo. Recipient animals were tested for pregnancy by ultrasound on day 60 of pregnancy to determine the number of fetuses in utero.

3.2.6 **Gamete recipients**

Animals used as recipients for oocytes and sperm were prepared as per recipients for embryo transfer. At 24 hours after CIDR removal, up to 50 oocytes were transferred per oviduct with 0.25 ml of fresh or frozen thawed ram semen was deposited into each uterine horn. Oocytes and zygotes were recovered 17 hours post transfer by retrograde flushing of the oviduct.

3.2.7 **Statistical analysis**

Data were analysed using a group t-test, χ^2 analysis or stepwise logistic regression.

3.3. EXPERIMENTAL

2.3.1 **Visualisation of metaphase chromosomes**

3.3.1.1. *Experimental outline*

This experiment aimed to determine the proportion of oocytes with visible metaphase plates in both *in vivo* and *in vitro* matured oocytes. Oocytes were utilised at the same time as for nuclear transfer. Oocytes with obvious morphological abnormalities such as lack of perivitelline space and fragmentation were not used. The oocytes were recorded for observation of the metaphase plate during the enucleation procedure.

3.3.1.2. *Results*

The IVM oocytes were variable in quality both between and within collections. This resulted in 34% not being selected for enucleation (Table 3.9). Visualisation of metaphase plates in the clear patch

PLATE 3

Photograph 1

A metaphase II ovine oocyte with the clear patch of cytoplasm and metaphase chromosome at approximately 11 o'clock with the remnants of the polar body at 12 o'clock

(Optics: DIC 400x)

Photograph 2

A karyoplast from an MII ovine oocyte containing visualised chromosomes surrounded by clear cytoplasmic area.

(Pipette diameter 35µm)
(Optics: 400x DIC)

Photograph 3

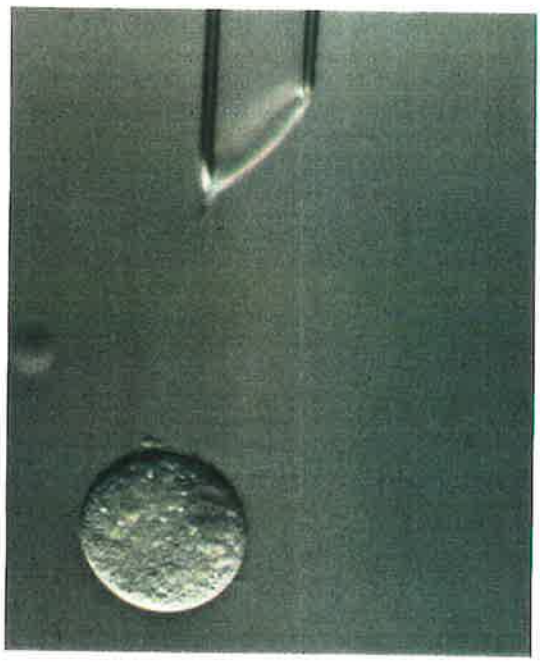
A karyoplast from an MII ovine oocyte still inside the enucleation pipette with the visualised chromosomes.

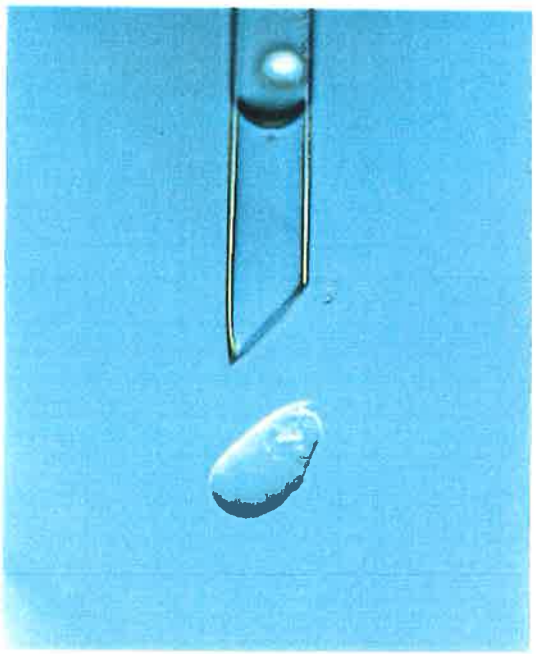
(Pipette diameter 35µm)
(Optics: 400x DIC)

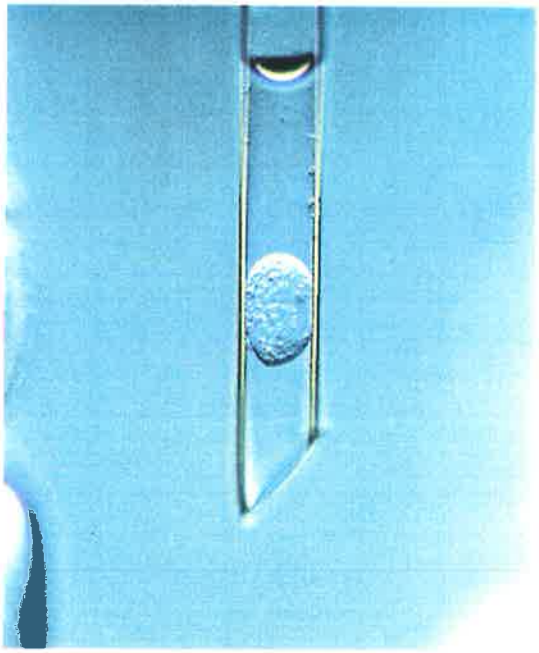
Photograph 4

A karyoplast from an MII ovine oocyte with visualised chromosomes isolated in a totally clear patch of cytoplasm.

(Pipette diameter 35µm)
(Optics: 400x DIC)







of cytoplasm was dependent on the size and clarity of the clear cytoplasmic patch associated with the polar body. The optimal operation of the DIC optics was essential for visualisation. The visualisation of the chromosomes was often faciliated by rotation of the oocyte to maximise the perspective of the clear cytoplasm hence enabling the metaphase plate more visible when the orientation of the spindle was perpendicular to the viewing angle. Where the metaphase plate was only partially visible, visualisation was usually verified during removal or subsequently in the enucleation pipette. A membrane type structure was often visible around the chromosomes (Plate 3: photographs 1 to 4).

The visualisation of metaphase plates in the *in vivo* matured group (64% of manipulated oocytes) only represented 43% of the total oocytes collected. The time required to perform positive enucleation was approximately 50 to 100% longer than for blind enucleation, however this was reduced with practice.

Table 3.9 Metaphase chromosome visualisation during attempted enucleation of *in vivo* and *in vitro* matured oocytes.

Oocyte maturation	Trials	N	Manipulated (% N)	Visualised MII plate
in vivo	2	89	76 (85)	61 (80)[a]
in vitro	2	110	73 (66)	47 (64)[b]

[a,b] Values differ ($P<0.05$)

3.3.2 **Assessment of oocyte source for the production of lambs using nuclear transfer**

3.3.2.1 *Experimental outline*

The viability of *in vitro* matured oocytes used as a recipient cytoplasm for nuclear transplantation was assessed. Either *in vitro* and *in vivo* matured oocytes were used for nuclear transfer with *in vivo* matured embryos. Fused embryos were cultured 72 hours culture in SOFM-C and transferred to recipients. The manipulated embryos were fused using the

multiple fusion chamber (Section 2.5.2.1) in the first three trials (Group 1) and single fusion chamber (Section 2.5.2.2) in the remaining three trials (Group 2). The *in vitro* development of the embryos was recorded 24 and 72 hours post fusion. Subsequent pregnancies were monitored by ultrasonography.

3.3.2.2 *Results*

Due to the availability of *in vitro* matured oocytes, there was more nuclear transfer embryos produced per donor embryo (16.7) than from *in vivo* derived oocytes (11.1). The highest productivity per donor embryo was achieved for the *in vitro* matured oocytes in Group 2 (23.8) with a maximum of 47 nuclear transfer embryos produced from an early blastocyst.

Fusion of *in vivo* matured oocytes was lower in Group 1 but not different in Group 2. Overall fusion was higher in Group 2 (Table 3.10). The *in vitro* development of embryos at 24 and 72 hours post fusion was greater for nuclear transfer embryos from the *in vivo* matured oocytes with the exception of 72 hour development in Group 1.

Table 3.10 *In vitro* development of nuclear transfer embryos produced with *in vivo* and *in vitro* matured oocytes.

Source of oocytes	Donor embryos	Pre-Fusion embryos	Fused embryos (%)	Cleavage at 24 hrs (% fused)	8/16 cell at 72 hrs (% fused)
Group 1*					
in vitro	12	144	97[a](67)	5[a] (5)	30[a](31)
iv vivo	11	84	40[b](48)	15[b](38)	17[a](43)
Group 2#					
in vitro	8	190	150[a](79)	32[a](21)	68[a](45)
in vivo	7	116	102[a](88)	44[b](43)	67[b](66)

Values in same column with different superscripts differ (P<0.05)
*Multiple fusion method
#Single fusion method

There was no difference in the subsequent *in vivo* development (Table 3.11). Nuclear transfer embryos derived from both oocyte sources resulted in a total of 7 fetuses from 5 pregnancies being detected from a total of 182 embryos transferred to 65 recipients.

Table 3.11 Post transfer development of nuclear transfer embryos produced from *in vivo* and *in vitro* matured oocytes

Source	Embryos	Recips	Pregnancies (% of recips)	Fetuses (% embryos)
in vivo	84	30	3 (10)	4 (5)
in vitro	98	35	2 (6)	3 (3)

3.3.3 **Preimplantation development of pronuclear embryos subsequent to nuclear transfer.**

3.3.3.1 *Experimental outline*

The aim of this experiment was to assess the effect of nuclear transfer procedures on preimplantation development. To evaluate the effect of the nuclear transfer procedure on preimplantation development, oocytes were matured either *in vitro* or *in vivo* and fertilised to produce pronuclear embryos which were then either placed directly into culture or subject to the removal of both pronuclei in a karyoplast and subsequent refusion (Plate 4: photographs 1 and 2). Embryos were cultured for 72 hours post fusion and scored for development to the 8/16-cell stage. At 144 hours post fusion, the number of blastocysts was recorded and the nuclei number determined using DNA specific fluorescent staining with Hoechst 33342.

3.3.3.2 *Results*

The visualisation of two pronuclei for *in vitro* matured oocytes (31/86; 36%) was lower than for *in vivo* derived oocytes 34/50 (68%; $P<0.05$). The cytoplasm of zygotes following *in vitro* maturation was denser than *in vivo* matured zygotes and thus hindered visualisation in the former group. There was also more irregularity in the size and shape of the pronuclei derived from *in vitro* matured oocytes (Plate 4: photographs 3 and 4). During manipulations, the *in vitro* matured cytoplasm was more fragile and susceptible to lysis during the nuclear transfer procedure.

The *in vitro* development of manipulated and control embryos is presented in Table 3.12. There was no effect of oocytes source or nuclear transfer on development up to the 8 to 16 cell stage after 72 hours culture. However the proportion of embryos that developed to the blastocyst stage and the number of nuclei per embryo were significantly lower for *in vivo* derived oocytes.

Table 3.12 Preimplantation development of *in vivo* and *in vitro* matured pronuclear embryos subsequent to nuclear transfer manipulations.

Source	N/T@	N	8/16 cell (% N)	Blasto-cysts (% N)	Cell nos. ± S.D.
in vivo	-	32	31 (97)	27 (84)[a]	97.1±51[a]
in vivo	+	27	26 (96)	14 (52)[b]	46.4±56.4[b]
in vitro	-	19	17 (89)	3 (16)[c]	13.6±20.6[c]
in vitro	+	13	9 (69)	8 (62)[ab]	79.4±42.2[ab]

Values in same column with different superscripts differ (P<0.05)
@Nuclear transfer treatment

3.3.4 **Effect of zona puncture, oocyte source and transfer site on embryo elongation**.

3.3.4.1 *Experimental outline*

The aim of this experiment was to evaluate the effect of zona puncture, transfer site and oocyte source for embryos transferred at the 8 to 16-cell stage. *In vivo* and *in vitro* matured oocytes were fertilised in sheep oviducts, recovered and placed into *in vitro* culture for 72 hours, after which they were divided and punctured with a nuclear transfer pipette. They were then transferred into either the oviduct or uteri of synchronised recipients. The recipient reproductive tracts were flushed 11 days after transfer and the contents examined for elongated blastocysts.

3.3.4.2 *Results*

Puncture damage, oocyte source and site of transfer were independent in their effect on development of elongated blastocysts. The *in vivo* maturation of oocytes resulted in a higher proportion of embryo elongation (21/41; 51%) than *in vitro* matured oocytes (7/37; 19%).

PLATE 4

Photograph 1

An *in vivo* matured ovine pronuclear zygote with clearly visualised pronuclei.

(Optics: DIC 400x)

Photograph 2

An enucleated pair of pronuclei from an *in vivo* matured ovine zygote

(Optics: DIC 400x)

Photograph 3

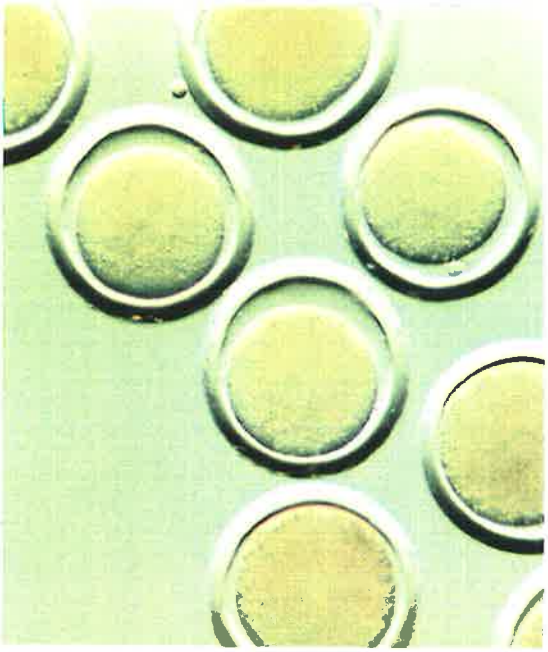
In vivo matured ovine pronuclear zygotes

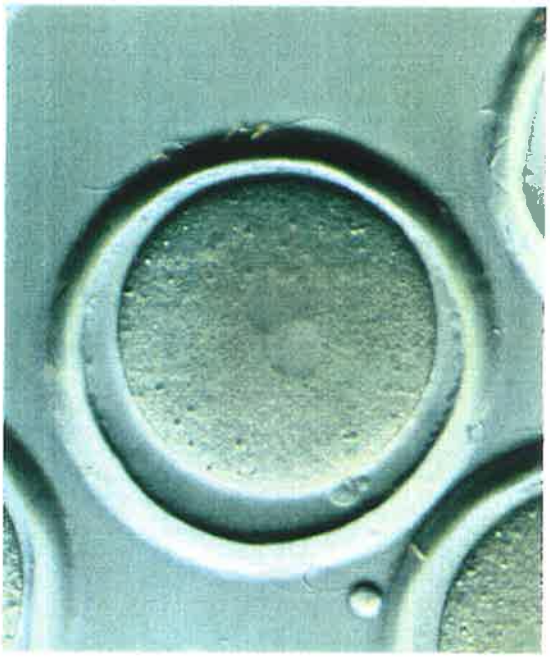
(Optics: DIC 200x)

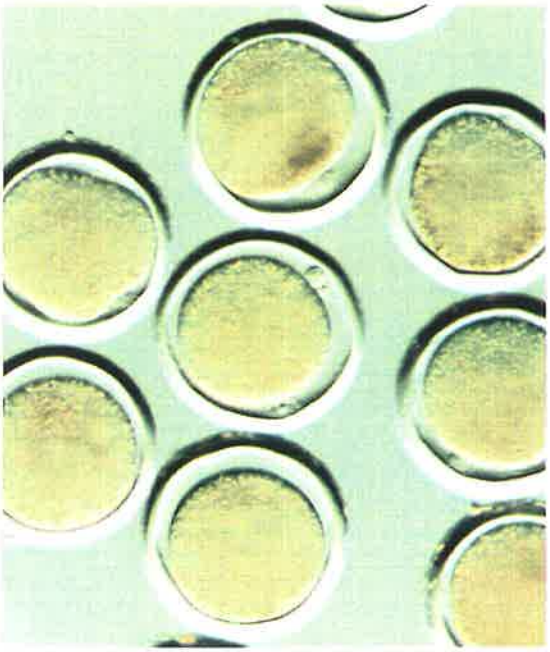
Photograph 4

In vitro matured ovine pronuclear zygotes

(Optics: DIC 200x







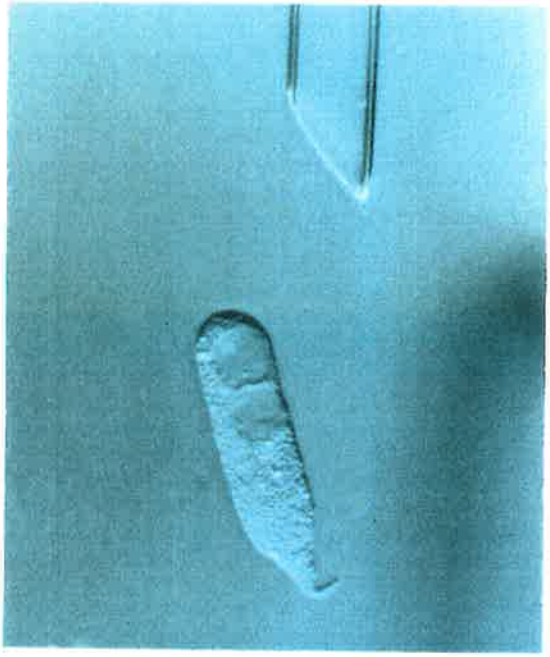
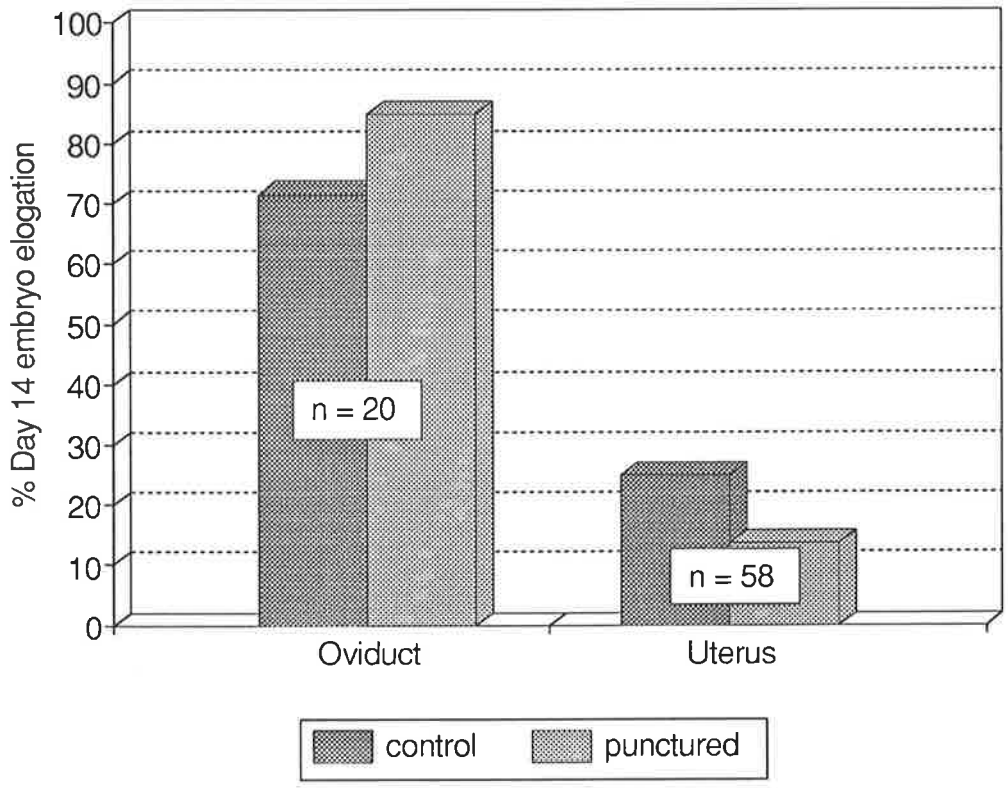


Figure 3.1 Embryo elongation subsequent to 72 hours *in vitro* culture with or without zona puncture, transferred to either the uterus or oviduct (includes both *in vitro* and *in vivo* matured oocytes)



There was no synergistic effect of oocyte maturation on either zona puncture or site of transfer and the combined results for both oocyte sources is presented in Figure 3.1. There was no individual effect of puncture on the site of transfer. In contrast, the site of transfer had a significant effect with a reduction in number of elongated blastocysts when embryos were transferred to the uterus (12/58; 21%) compared with the oviduct (16/20; 80%).

3.4 DISCUSSION

3.4.1 **Visualisation of metaphase plates**

The rate of visualisation of the chromosomes in ovine oocytes during chromosome enucleation has not been reported previously and has only been used to assist the enucleation of rabbit oocytes for nuclear

transplantation (Stice and Robl 1988). This is partly due to variation in the optical density of the cytoplasm between species. The percentage of enucleation observed in this Experiment for both *in vitro* and *in vivo* oocytes (64 and 80%) sources indicates that enucleation by visualisation may be useful as a standard methodology for enucleating ovine oocytes. To further evaluate the efficiency, the unselected and blindly enucleated oocytes could be stained to determine what percentage contained metaphase chromosomes.

With the ability to identify chromosomes during enucleation, it will be possible to produce experimental groups of oocytes to compare development with oocytes enucleated by previously described methods (Willadsen 1986). Potentially it should eliminate the occurence of triploid and parthenogenetic development and improve the current survival rate of nuclear transfer embryos. In real terms, if the current rate of enucleation is approximately 67% (Smith and WIlmut 1989) to 77% (Section 3.2), then there will be a 25 to 50% increase in the number of potentially viable post fusion embryos.

3.4.2 **Development of nuclear transfer embryos using *in vitro* matured cytoplasm**

Whilst attempts have been made to use *in vitro* matured oocytes as the cytoplasmic component for nuclear transfer in bovine embryos (Prather *et al* 1987, Kinis *et al* 1989) successful development has only been reported for preimplantation stages. The *in vitro* matured oocyte in this study provided a useful alternative to the *in vivo* matured oocyte in most respects, but had a lower rate of *in vitro* development up to 72 hours post fusion. The availability of *in vitro* matured oocytes compared with *in vivo* matured oocytes was reflected by the greater number of fused embryos produced per nuclei donor embryo.

The *in vivo* development of the *in vitro* matured derived embryos was lower than reported in other nuclear transplantation studies using *in vivo* derived material (Willadsen 1986, Smith and Wilmut 1989), and in Section

3.2, but not less than the *in vivo* derived oocytes transferred simultaneously. The fact that several fetuses developed from nuclear transfer embryos derived from *in vitro* matured cytoplasm establishes their future prospect as a replacement for *in vivo* matured oocytes. However as embryos derived from *in vivo* matured cytoplasm showed better elongation rates at day 14 than *in vitro* matured oocytes, the postimplantation viability of nuclear transfer embryos derived from in vitro matured oocytes may also be lower. However this may be a consequence of nuclear rather than a cytoplasmic disfunction, hence not applicable to nuclear transplant embryos.

3.4.3 **Effect of nuclear transfer manipulations on embryo survival**

Previously the development to term of porcine and bovine zygotes subjected to pronuclear transfer has been achieved but with a substantial loss in viability compared to unmanipulated, transferred embryos (Robl *et al* 1987, Prather *et al* 1987). The effect of the nuclear transplantation manipulations on the development of both *in vitro* and *in vivo* matured oocytes was tested by performing nuclear transfer on oocytes subsequent to *in vivo* fertilisation. Although zygotes were able to survive manipulations, there was a significant loss in viability compared to unmanipulated zygotes, with the exception of *in vitro* matured zygotes where manipulations resulted in the selection of more viable embryos. The nuclei numbers observed with the nuclear transfer of *in vivo* matured oocytes (46±56) were lower than control embryos, and could therefore explain the low cell numbers in nuclear transfer blastocysts observed in Section 3.2. This may indicate that the lower cell numbers were largely attributed to manipulation induced factors rather than a reprogramming inability.

The loss in viability at preimplantation stages was apparent as a result of manipulation of cytoplasm and nuclei, but there was also a postimplantation embryo loss subsequent to transfer. In retrospect of the results in Section 3.2, where nuclear transfer embryos were severely damaged in the oviduct, the site of transfer and the effect of zona damage

was tested. It was anticipated that transfer of nuclear transfer embryos to the uterus would improve the development of fetuses, however this was not observed. The simultaneous comparison of transfer site demonstrated that the uterus was more detrimental to the development of both zona damaged and control embryos when transferred at the 8 to 16 cell stage. If nuclear transfer reduces the number of nuclei per embryo due to retarded development, then transfer to the uterus is likely to result in losses due to the endocrine/physiological reasons (i.e. asynchronous transfer).

SECTION 4.

CULTURE AND NUCLEAR TRANSFER OF BOVINE EMBRYOS

1. VIABILITY OF ONE-CELL BOVINE EMBRYOS CULTURED IN SYNTHETIC OVIDUCT FLUID MEDIUM

1.1. INTRODUCTION

The lack of procedures allowing successful *in vitro* culture of bovine embryos from one-cell to the blastocyst stage has been a major constraint to preimplantation manipulation. Until recently, successful culture of one-cell stage bovine embryos has been largely dependent on transfer to xenogenous oviducts (Eyestone *et al* 1987, Lambert *et al* 1986, Parrish *et al* 1986), but successful embryo culture has now been achieved through co-culture with various cell types (Eyestone *et al* 1987, Camous *et al* 1984, Goto *et al* 1988, Kuzan *et al* 1982, Heyman *et al* 1987) implying that cell derived factors contribute to development (Camous *et al* 1984, Bavister 1988). Co-culture is now increasingly being employed, particularly with embryos derived from *in vitro* maturation and fertilization procedures (Goto *et al* 1988, Eyestone and First 1989, Fukui *et al* 1989, Kajihara *et al* 1987). In the bovine, it has been shown that 22% of *in vitro* derived embryos and 43% of in vivo derived embryos will develop to the morula or blastocyst stages in co-culture (Eyestone and First 1989), but attempts to develop a simple cell-free media have met with limited success (reviewed by Wright and Bondioli 1981). Recently, high yields of ovine blastocysts have been reported using a modification of synthetic oviduct fluid (SOF; Tervit *et al* 1972) which contains heat-inactivated human serum as a protein supplement (SOFM-C; Walker *et al* 1988).

This study assessed whether SOFM-C could provide the necessary conditions to support early development and maintain the viability of bovine embryos in culture. Firstly, this was tested by culturing zygotes to the blastocyst stage and comparing the post transfer viability with *in vivo* cultured, and freshly flushed blastocysts. Secondly, single blastocysts were

transferred to assess the efficacy of collecting zygotes surgically versus post mortem.

1.2 METHODS

1.2.1. **Animals**

One to 2 year old Friesian or Friesian cross beef heifers were used as embryo donors and recipients. Treatment of donor animals commenced on Days 8-12 of the estrus cycle (estrus = Day 0) with administration of follicle stimulating hormone (FSH-P,Schering Corporation, U.S.A., 28 mg administered in decreasing doses at 12-hour intervals over 4 days) or Folltropin-V (Vetrepharm, Canada, 18 mg administered in equal doses at 12-hour intervals over 4 days), or pregnant mares serum gonadotrophin (Pregnecol, Heriot Agvet Pty. Ltd.;2000 i.u. administered as a single injection) or FSH-P. Estrus was induced in donors with 750 μg cloprostenol (Estrumate, Coopers Animal Health Aust. Ltd.) administered 48 hours after the initial superovulation injection followed by 250 μg cloprostenol 12 hours later. Synthetic gonadotropin releasing hormone (GnRH, 250 μg; Fertagyl, Intervet Aust, Pty. Ltd.) was administered 96 hours after the initial FSH injection to regulate the time of ovulation in recipients. Donors were tailpainted and observed for estrus twice daily. Those observed in estrus were inseminated three times with frozen-thawed semen, at the time of GnRH injection and at 12 and 24 hours thereafter.

Recipient heifers were synchronized by administering 500 μg cloprostenol 12 hours prior to the first cloprostenol injection in the donors and observed for estrus as for donor animals.

1.2.2. **Embryo Collection and Transport**

Fertilised one-cell stage embryos were collected either post mortem (30 to 60 minutes post exsanguination) or surgically by midventral laparotomy (10 minutes). Day-7 embryos were also collected surgically. Oviducts and

uteri were flushed with phosphate buffered saline supplemented with 1% fetal calf serum. After collection embryos were immediately placed into tubes containing SOFM-T with 20% human serum. Media in tubes was previously equilibrated in an atmosphere of 5% O_2, 5% CO_2 ,90% N_2 at 38.5°C and were transported from the collection site to the laboratory at 37°C. The time between abattoir collection of embryos and return to the laboratory was 60 min, and it was 25 min for laparotomy collections.

1.2.3. **Culture**

Embryos were cultured in 10 µl drops of SOFM-C with 20% human serum under paraffin oil in 35 mm plastic dishes in an atmosphere of 5% CO_2, 5% O_2, 90% N_2. The microdrop culture was equilibrated in the culture environment 24 hours prior to addition of embryos. Incubation was maintained at 38.5°C with 95% relative humidity. Morphology was examined every 24 hours after the commencement of culture and the developmental stages were recorded. The number of nuclei per embryo was determined by staining with Hoechst 33342 and examination with fluorescence microscopy (200x).

1.2.4. **Embryo Transfers**

One-cell embryos were transferred to synchronized recipients within 3 hours of collection by laparotomy. Embryos cultured *in vitro* for 120 to 144 hours and embryos collected at Day 7 were transferred via flank incision to the uterine horn ipsilateral to a palpated corpus luteum of synchronized recipients. Pregnancy was determined by blood progesterone levels (Stone and Seamark 1985) at Day 23, with levels greater than 8 nMol/l being considered indicative of pregnancy. Recipients were also examined by palpation per rectum between Day 70 and 90. The number of fetuses was counted following slaughter of the pregnant recipients that had more than one embryo transferred.

1.2.5. **Statistical Analysis**

Data were analyzed by one-way analysis of variance and Fisher's exact test.

1.3. EXPERIMENTAL

1.3.1. **Viability of blastocysts cultured in SOFM-C.**

1.3.1.1. *Experimental outline*

The initial objective was to determine the viability of blastocysts developing from one-cell embryos cultured in SOFM-C. One-cell embryos were collected surgically on Day 2 and either transferred directly to synchronized recipients (Treatment 1) or placed in culture medium for 120 hours and then transferred to Day-7 synchronized recipients (Treatment 2). Comparison was also made with embryos collected from Day-7 donors and transferred directly to Day-7 recipients (Treatment 3). Three embryos were transferred per recipient in all treatment groups.

1.3.1.2. *Results*

When cultured in SOFM-C, 75.4% (46/61) of all one-cell embryos developed to the 16-cell stage and 63.9% (39/61) formed blastocysts. No evidence of any stage-specific developmental block was seen in culture. Various stages of development of cultured embryos are presented in Plate 5. There were no significant differences in the pregnancy rates between treatments as assessed on either Day 23 or 70 (Table 4.1). However, the mean number of fetuses per pregnant recipient from cultured embryos (1.3) was lower than one-cell embryos transferred immediately (1.6) but did not differ from Day 7 embryos transferred immediately (1.3).

Table 4.1 Pregnancy rates following transfer of bovine blastocysts cultured from Day 2 to Day 7 in SOFM-C compared to those obtained following direct transfer of Day 7 blastocysts and Day 2 one-cell embryos.

Treatment group	Recipient	Embryos	Pregnancy (%)		Fetuses/ Pregnancy
			Day 23[a]	Day 70[b]	
1	Day 2	Day 2	6/8 (75)	5/8 (63)	1.6[c]
2	Day 7	Culture	11/13(84)	8/13(62)	1.3[d]
3	Day 7	Day 7	8/9 (88)	6/9 (67)	1.3[c,d]

[a] Progesterone > 8 nMol/l

[b] Rectal palpation

[c,d] Values differ ($P<0.05$)

1.3.2. **Viability of one-cell embryos collected surgically and post mortem with culture in SOFM-C.**

1.3.2.1. *Experimental outline*

The aim was to evaluate the effect of SOFM-HS culture on the *in vitro* and *in vivo* development of embryos collected post mortem from abattoir slaughtered cows with those obtained surgically. Donor heifers were paired on the basis of breed, liveweight and condition score before allocation to either abattoir or laparotomy collection. One-cell embryos were collected, cultured and examined for development over 144 h. Embryos forming blastocysts were transferred to synchronized recipients (one per recipient) or stained and the number of nuclei counted.

1.3.2.2. *Results*

Development of embryos collected post mortem was lower than of embryos collected surgically in terms of the proportion developing past the 16-cell stage and the proportion reaching the blastocyst stage after 4, 5 and 6 days of culture (Table 4.2). The mean number of nuclei in blastocysts developed in culture was 72.4 and 67.3 for surgical and abattoir collections, respectively. Surgical embryo collections also contained two- to eight-cell stage embryos, of which 78.2% (61/78) developed to the blastocyst stage in culture.

PLATE 5

Photograph 1

Two cell stage bovine embryo cultured *in vitro* from the one-cell stage.

(Optics: DIC 400x)

Photograph 2

A 12-cell bovine embryo cultured *in vitro* from the one-cell stage.

(Optics: DIC 400x)

Photograph 3

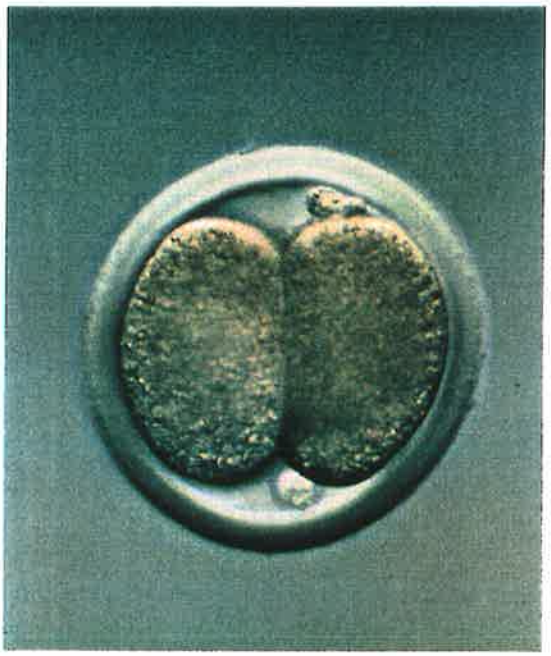
Bovine blastocyst cultured *in vitro* from the one-cell stage. Blastocyst is rotated to demonstrate well developed inner cell mass

(Optics: DIC 200x)

Photograph 4

A selection of bovine blastocyst cultured for 120 hours *in vitro* from the one-cell stage

(Optics: DIC 200x)



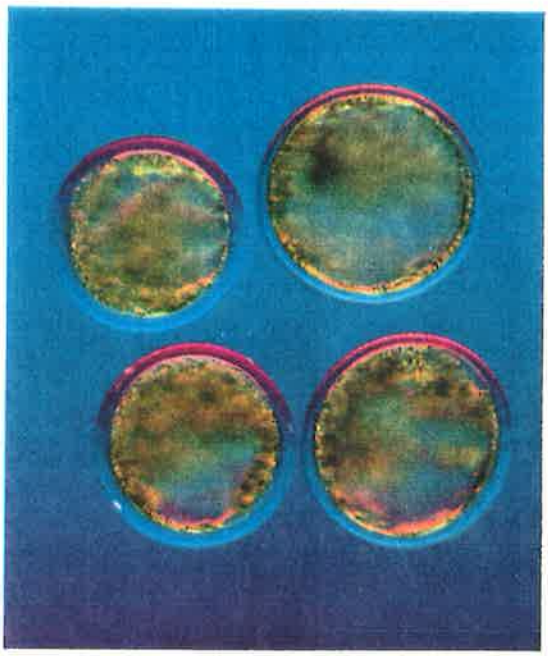


Table 4.2 *In vitro* development of one-cell embryos derived surgically or by post mortem collection.

Source of embryos	n	>16cell(%)	Blastocyst formation(%) Day 4	Day 5	Day 6	Nuclei#
Surgical	41	83[a]	37[a]	61[a]	76[a]	72.4
Post mortem	106	68[b]	1[b]	24[b]	54[b]	67.3

Values in same column with different superscripts differ (P<0.05)
#Number of nuclei in blastocysts after 144 hours in culture.

There was no difference in pregnancy rates between collection procedures as assessed by blood progesterone levels at Day 23 or by palpation per rectum at Day 70 (Table 4.3). Fewer pregnancies were recorded at Day 70 compared with Day 23 but the difference was only significant for post mortem derived embryos. The combined pregnancy rate for both treatments was 55% as measured by blood progesterone (Day 23) and 28% by manual palpation (Day 70).

Table 4.3. Pregnancy rates after transfer of single blastocysts cultured from one-cell stage embryos collected surgically or post mortem.

Source of embryos	Pregnancy (%) Day 23[a]	Day 70[b]	Pregnancies Lost between Days 23 and 70
Surgical	7/15 (47)	5/15 (33)	2/7 (29)
Post mortem	9/14 (64)[c]	3/14 (21)[d]	6/9 (67)

a Progesterone > 8 nMol/l.
b Rectal palpation.
[c,d] Values differ (P<0.05)

1.4 DISCUSSION

The outcome of these experiments demonstrate that one-cell bovine embryos can be reliably cultured in a simple cell-free medium, with up to 76% forming blastocysts. The viability of blastocysts developed in culture proved similar to that of blastocysts developed *in vivo* when transferred on Day 7. However, fewer embryos developed following transfer as blastocysts than as one-cell embryos.

The pregnancy rate at day 70 (62%) achieved following transfer of three blastocysts per recipient was similar to that reported in other studies using blastocysts derived by co-culture with oviductal cells (55%; Eyestone and First 1989) and ovine oviductal tissue explants (50%; Eyestone *et al* 1987). Although these pregnancy rates are comparable to commercial embryo transfer rates (53%; Massey and Oden 1984), the individual embryo survival is only about 30%, which compares closely with that obtained in this study with the transfer of single embryos cultured in SOFM-C (33%). The lower embryo survival experienced with the transfer of three embryos per recipient may be due to effects of multiple embryo transfer (Rowson *et al* 1971). However, similar rates of survival were found following the transfer of single ovine embryos after 5 days of culture *in vitro* with either SOFM-C (45%;Walker *et al* 1988) or co-culture with oviductal cells (Gandolfi and Moor 1987).

Culture of ovine embryos with SOFM-C, reduces viability of ovine embryos cultured more than 3 days (Walker *et al* 1988), indicating that a culture medium based on the composition of oviductal fluid may only be suitable for the period when the embryo is normally in the oviduct *in vivo.* Changes in gene transcription after activation of the embryonic genome, which occur at the time when the conceptus would normally enter the uterus, may incur additional requirements for development not available in the oviduct or culture systems based on oviductal environments (Bavister

1988). Also the lack of oviductal factors during initial cell divisions could influence development past the 16-cell stage (Gandolfi & Moor 1987).

Embryos derived from the abattoir collection showed a delay in blastocyst formation compared with those from the surgical collection. No loss of post mortem derived embryos was evident in the early stages of pregnancy, but there was a dramatic decline in the pregnancy rate between Days 23 and 70 (67% vs 29%). The practice of collecting bovine embryos following slaughter is now used extensively for various purposes (Eyestone *et al* 1987, Camous *et al* 1984, Tervit and Rowson 1972, and Leibfried-Rutledge *et al* 1987). However, in view of this potential loss, adoption of this procedure must be carefully considered. Similar embryonic losses were reported by Tervit and Rowson (1972) following the direct transfer of early cleavage-stage bovine embryos collected at slaughter.

Although some embryos collected after slaughter have been cultured to yield viable offspring following transfer, the dramatic changes in the tubal environment which begin at slaughter, such as a decrease in temperature, increase in lactic acid, and change in gas tensions (Ward and Buttery 1979), would contribute to a decreased embryo viability. Because the time elapsing between death and collection is a major determinant of the extent of these adverse changes, collection after slaughter may still be a viable option if this period can be significantly reduced.

The ability of one-cell embryos to form blastocysts without any apparent developmental block in SOFM-C supports the initial observation of Tervit *et al.* (1972) using SOF supplemented with BSA and of others who have utilized various culture media based on SOF (Wright and Bondioli 1981). Media other than SOF have also been successfully used for this purpose (Wright *et al* 1976), indicating that it is not a factor unique to the composition of SOFM. Essential factors derived from protein supplements or other cells in media probably contribute to provide the necessary culture conditions in specific media. In comparison with homologous fetal serum or BSA, human serum has not been used extensively as a supplement in

bovine embryo culture systems. It should be noted that it has proven to be superior to homologous serum, as a protein supplement in the culture of porcine (Stone *et al* 1984) and ovine (Walker *et al* 1986) embryos, and its use in the culture of bovine and other embryos warrants further study.

2. NUCLEAR TRANSFER OF BOVINE EMBRYOS

2.1 INTRODUCTION

Production of identical cattle by nuclear transfer has been attempted using methodology adapted from murine nuclear transplantation (McGrath & Solter, 1983a) to produce live offspring albeit at a low efficiency (Robl *et al* 1987, Prather at al 1987). As for ovine nuclear transplantation bovine nuclear transfer has utilised ovine oviducts as a temporary culture environment resulting in a low rate of development to the morula or blastocyst stage (5-20 %; Prather *et al* 1987). The use of *in vitro* culture for bovine nuclear transfer embryos has only been utilised for 72 hours post fusion and with no development past 6 cells (Prather *et al* 1987). No attempt has been made to culture bovine nuclear transfer embryos *in vitro* to the morula or blastocyst stage. To achieve this with subsequent viability would eliminate transfer procedures to interim recipients and avoid losses associated with recovery.

The results of the first part of this Section demonstrated that 76 % of fertilized zygotes can develop to the blastocyst stage in a simple medium and are viable subsequent to transfer. This following experiments attempt to produce bovine embryos using the nuclear transfer procedures as described by Prather *et al* (1987) but utilising *in vitro* culture with SOFM-C.

2.2 METHODS

2.2.1. **Animals**

Embryo donors were treated as described previously (Section 4.1). Embryo transfers were also similar except that embryos treated were transferred both surgically and non-surgically (Newcomb 1979).

2.2.2 **Embryo Collection and Transport**

Oviductal oocytes were collected from uninseminated superovulated donors 32 to 36 hours post GnRH by salpingectomy. Oviducts were immediately flushed with PBS-FCS. Embryos were collected 5.5 days after estrus either by non surgical flushing of the uterus or by flushing the top 5 to 10 cm of the uterine horn after a salpingectomy which included the anterior third of the uterine horn. Isolated embryos and oocytes were placed immediately into SOFM-T at 37°C. Oocytes were maintained in SOFM-T for approximately 2 hours prior to manipulations. Embryos were either used within 3-4 hours of collection or cultured overnight in SOFM-C to increase the cell number.

2.2.3. **Culture and manipulation.**

Nuclear transplantation manipulations and culture were essentially as described for nuclear transfer of ovine embryos (Section 3.2). Oocyte cytoplasm was generally much darker than for ovine embryos so that enucleation was directed more by the position of the polar body than by an area of clear cytoplasm. Fusion was performed using the single fusion chamber method (Section 2.5). Embryos in non transfer trials were stained with Hoechst 33342 (Section 2.3) after 144 hours of culture and the number of nuclei determined by flourescence microscopy.

2.2.4 **Statistical analysis**

Data was analysed using χ^2 analysis and group t-test.

2.3 EXPERIMENTAL

2.3.1 **Cell numbers of *in vitro* cultured nuclear transfer embryos**

2.3.1.1 *Experimental outline*

Several embryos were treated as nuclei donors and the resultant nuclear transfer embryos cultured *in vitro* in SOFM-C for 144 hours post fusion. Embryos were then stained and the number of nuclei determined.

2.3.1.2. *Results*

For the purpose of determining the *in vitro* development of nuclear transfer embryos, three 16-cell stage embryos and 29 *in vivo* derived oocytes were manipulated to produce 24 manipulated embryos. Placing of the embryos in electrofusion medium often resulted in an osmotic reaction with the embryos often forcing themselves through the fissure in the zonae. This occurred despite the osmolarity of the fusion medium being similar to the culture medium (280 vs. 270 mOsm). Consequently embryos were exposed to the fusion medium gradually with two five minute washes in a 25% and 50 % dilution of fusion medium in SOFM-T. The electrofusion of the manipulated embryos resulted in 19 fusing, resulting in development of three blastocysts with cell numbers of 20,23 and 32 (Table 4.4). One non blastocyst embryo (morula) also developed to 18 cells.

Table 4.4 Cell numbers of cloned embryos cultured 144 hours post fusion *in vitro*

Donor embryos	Embryo cell numbers: Fused embryos	2-16 cell	>16 cell	Blastocyst	Blastocyst cell numbers
3	19	11	4	3	25±6.2*

*Standard deviation

2.3.2 **Viability of *in vitro* cultured nuclear transfer embryos**

2.3.2.1 *Experimental outline*

Embryos were produced as in experiment 2.3.1. Morphology was assessed by recording cleavage at 24 hours, 8/16 cell stage at 72 hours and the blastocyst stage at 116 and 140 hours post fusion. Embryos forming morula or blastocysts were then transferred to recipients and the pregnancy monitored after implantation .

Table 4.6. Development of cloned embryos with transfer to recipients after 120 to 144 hours culture

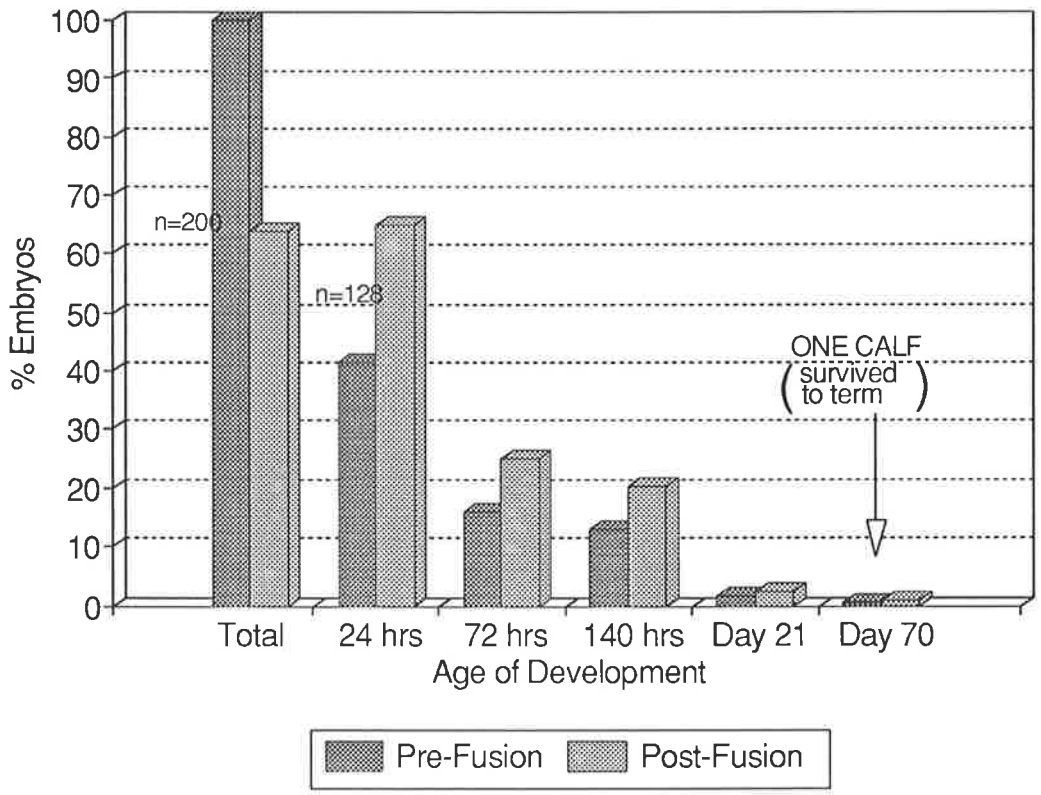
Donor embryo stage	Pre-fusion embryos	Fused embryos	Cleav-age at 24 hrs	8/16-cell at 72 hrs	Stage* at 140 hrs	Proges-terone^ day 23	Fetuses at day 70@
M[a]	6	5	0	0	_		
M	7	5	3	3	BL[d] HBL[e]	31.4 3.1	-
M	11	10	7	6	HBL BL M	1.2 3.9 0.5	
CM[b]	10	6	2	0	_		
CM	17	14	11	0	_		
CM	10	7	7	7	_		
CM	24	18	12	3	M M,M M	0.5 3.7 0.5	
CM	20	18	11	5	M,M M,M M M,M,M	0.5 3.9 0.5 0.5	
CM	11	6	1	0	_		
CM	18	8	7	3	M,BL M,BL	33.5 0.8	+[#]
CM	22	10	6	1	BL	0.5	
EB[c]	44	21	16	4	BL,BL BL,BL	13.9 5.6	-

*Each line represents the embryos and stages thereof transferred to each recipient.
[a]Morula
[b]Compact Morula
[c]Early blastocyst
[d]Blastocyst
[e]Hatched blastocyst
[#]Bull calf born 281 days, 45 kg.
^ Progesterone > 8 nMol/l
@ Rectal palpation.

2.3.2.2 *Results*

To produce offspring from embryo nuclei twelve embryos ranging from the 16 to 64 cell stage were manipulated to produce 200 (16.7 per embryo) reconstructed embryos. The productivity of manipulated embryos was directly proportional to the cell stage of the donor embryo but was also constrained by temporal availability and quality of oocytes. A maximum of 44 pre-fusion embryos were derived from a single embryo at the early blastocyst stage. The blastomeres were separated by manual dissaggregation and hence not sorted by morphological origin in the case of early blastocysts.

Figure 4.1 Development of pre and post fusion nuclear transfer bovine embryos



A summary of the *in vitro* cleavage and subsequent *in vivo* development of nuclear transplant embryos is presented in Figure 4.1 and is detailed in Table 4.5. Fusion occurred successfully in 64% of embryos with

25% of these developing to 8/16 cell stage after 72 hours in culture. After 140 hours culture there were 11 (8.6%) embryos that developed to the blastocyst stage and 15 (11.7%) morulae. Transfer to 16 recipients with 1 to 3 embryos resulted in three animals with pregnant levels of progesterone at day 23 and one animal pregnant at day 70 resulting in a term calf.

2.4 DISCUSSION

In vitro development in SOFM-C of nuclear transfer embryos compared to fertilised one-cell embryos (4.1) was lower in the proportion forming blastocysts and the blastocyst cell numbers (25.0 vs 72.4). Furthermore, of the developing morulae and blastocysts produced for transfer to recipients, only 11/26 were either blastocysts or hatching blastocysts. The viability of the morulae and blastocysts was established with transfer and establishment of three pregnancies of which one produced a term delivery. The nucleus for the surviving embryo was derived from a 32-cell stage embryo but blastocysts were also derived from nuclei isolated from 16, 32 and 64 cell stage embryos.

The *in vitro* development of the nuclear transfer embryos was not compared simultaneously with an *in vivo* culture as used previously for bovine nuclear transplantation (Robl et al 1987, Prather *et al* 1987) and therefore direct assessment of the *in vitro* culture system is not possible. However, the development of nuclear transfer embryos in SOFM-C compared with fertilised zygotes (Section 4.1) demonstrates that the drop in viability is more likely due either to the manipulations or inability of nuclei to support development in the oocyte cytoplasm rather than *in vitro* culture. This is supported by the low rate of morulae and blastocyst formation observed previously with *in vivo* culture (6 to 20% ;Prather *et al* 1987) and more recently published experiments where oviductal cell cocultures were used (13% to morulae or blastocyst from attempted fusions; Bondioli *et al* 1990). The nuclei numbers observed in blastocysts gives cause to believe that the

embryos forming blastocysts after nuclear transfer were either not fully reprogrammed or were retarded in development, however there is no previous data of cell numbers in nuclear transfer bovine embryos with which to assess this result.

The survival of transferred nuclear transfer blastocysts and morulae to day 70 after *in vitro* culture in SOFM-C (1/26; 1 to 3 per recipient) was lower than observed previously for unmanipulated single blastocysts (5/15). The recent study by Bondioli *et al* (1990) reported a higher pregnancy rate than this study for nuclear transfer embryos (22.5%; 104/463) using *in vivo* culture and 3/11 for embryos cultured with oviductal cells. The low survival rate observed for nuclear transfer embryos cultured in SOFM-C is similar to the result of Prather *et al* (1987) using *in vivo* culture where only 2/19 embryos survived to term. A major difference exists between the nuclear transfer method used by Prather *et al* (1987) and that of Bondioli *et al* (1990). The method described by Bondioli *et al* (1990) which was developed by Willadsen (1986) utilises the recipient oocyte by halving the cytoplasm to provide two recipient cytoplasms rather than the whole oocyte cytoplasm. This has two implications the first being that 50% of the oocyte-nuclei complexes contain a triploid set of chromosomes which could facilitate a proportion of oocytes developing initially through parthenogenetic control. This would expectantly result in a subsequent decrease in post transfer viability as a result of an excess set of chromosomes (Surani and Barton 1983, Szulman and Surti 1984). Secondly, halving of the oocyte dramatically reduces the nucleocytoplasmic ratio which has been demonstrated to be beneficial to transplanted advanced stage nuclei (Howlett *et al* 1987) and haploid parthenogenotes in mice (McGrath and Solter 1986). Whether the reduction in cytoplasmic volume has played a significant role in the survival of bovine nuclear transplant embryos remains to be resolved in an extensive study utilising both methods.

A range of cell stages from 16 to 64 were tested in this experiment and there was no difference in subsequent *in vitro* or post transfer development.

Previously Prather *et al* (1987) achieved pregnancies with nuclear transfer embryos generated from 9-15 cell stage nuclei. Marek *et al* (1990) demonstrated that there was no loss in viability with later stage embryos and actually an increase in the *in vitro* development of nuclei derived for day 6 compared with day 5 embryos. This however does not necessarily demonstrate the totipotency of all nuclei from the embryo but only a group of cells as shown with the nuclear transfer of ovine ICM and trophoblast nuclei to ovine oocytes (Smith and Wilmut 1989).

In summary, the need to use interim recipients to culture nuclear transfer embryos may be alleviated, particularly using SOFM-C. The major problem associated with nuclear transfer with bovine embryos pertains to the low rate of preimplantation development and viability subsequent to transfer. Further research in bovine nuclear transplant embryos will probably utilise in vitro culture more extensively than has been to date. However the main advantage may not be in eliminating interim recipients, but as a system in which to determine the reason for embryo losses as a result of early nuclear cytoplasmic interactions.

SECTION V

NUCLEOCYTOPLASMIC RATIO AND THE PREIMPLANTATION DEVELOPMENT OF MOUSE EMBRYOS

1. NUCLEOCYTOPLASMIC RATIO AND THE PREIMPLANTATION DEVELOPMENT OF MOUSE EMBRYOS.

1.1 INTRODUCTION

In the production of identical animals, nuclei are transferred to the earlier stage cytoplasm of the enucleated oocyte (Willadsen 1986, Bondioli et al 1990, Section 3.2). Studies evaluating the outcome of transferring nuclei to earlier stage cytoplasm have been predominantly with mouse embryos. Primarily, the capacity of mouse nuclei to develop upon transfer to earlier stage cytoplasm is inversely proportional to the stage of the donor nuclei (Surani et al 1987; Prather and First 1989). The reason for this trend may simply be due to the age and differentiation state of the nucleus. However differences between the donor nuclei and recipient cell stage cytoplasm may also limit the development (Howlett et al 1987, Barnes et al 1987, Surani et al 1987). In particular there may be differences due to changes in the content of the cytoplasm, or overall differences in the nucleocytoplasmic ratio.

To determine the effect of transplanting a nucleus into different cytoplasms the effect of increasing the cytoplasmic volume must be simultaneously examined. The following experiments determine this by transferring nuclei to earlier stage cytoplasms whilst observing an equivalent volume increase in the recipient cell stage.

1.2. METHODS

1.2.1 **Animals and embryo collection**

Laboratory mice were obtained from Gilles Plains Animal Resource Centre, Gilles Plains , S.A. They were housed at 20°C, with light for 12 hours of each day. Food and water were available ad libitum. For mating, sexually

mature mice were used with isolation for two weeks prior to first mating and abstinence two days prior to actual mating. Immature female F1 hybrid CBA x C57BL/6 cross were induced to superovulate by intraperitoneal injections of 10 i.u. PMSG (Folligon, Intervet, Holland) followed 48 hours later by 10 i.u. HCG (Chorulon, Intervet, Holland). At the time of HCG injection each female was placed with one male. On the following morning the females were checked for vaginal plugs. For the collection of zygotes, the oviducts were flushed at 20 to 24 hours post HCG with HEPES-HTF with 300 i.u. hyaluronidase/ml. Two-cell stage embryos were flushed from oviducts with HEPES-HTF at 48 hours post HCG. Four-cell stage embryos were derived from flushed two-cell stage embryos cultured for 6 to 12 hours .

Recipient animals were prepared by placing mature females (10-15 weeks) with vasectomised males. Females were monitored daily for the presence of a copulatory plug and were separated when plugs were present. The morning of detection of the copulatory plug was considered day $^1/_2$ of pregnancy.

1.2.2 **Micromanipulation**

Embryos were incubated in HEPES-HTF supplemented with 0.3 μg/ml nocodazole (Sigam, USA) and 5 μg/ml cytochalasin B for 20 minutes prior to manipulation. Enucleation and nuclear transfer were performed with a 20 to 25 μm O.D. pipette bevelled to 45° (Plate 6: photographs 1,2 and 3). Blastomeres of two and four cell stage embryos were fused using a single 1.3 kV/cm DC pulse at 90° to the cleavage plane. Karyoplasts were fused using either beta-propiolactone inactivated sendai virus, a 1.3 kV/cm DC pulse, with a simultaneous 1 kV/cm AC field, or a 1.3 kV/cm DC pulse subsequent to treatment of the karyoplast with 35 μg/ml PHA in HEPES-HTF.

1.2.3 **Culture**

Embryos were cultured in 10 μl microdrops in HTF with 5 mg/ml BSA, at 37°C.

1.2.4 **Statistical analysis**

Statistical analysis was done using a group t-test and χ^2 analysis.

1.3 EXPERIMENTAL

1.3.1 **Evaluation of fusion methods and subsequent embryo viability**

1.3.1.1. Experimental outline

Pronuclear embryos were used as a preliminary model to test fusion methods efficiency and post fusion viability. Embryos were subject to the removal and replacement of both pronuclei within the same embryo. Fusion was tested using both viral and electrically mediated fusion. Fused embryos were cultured for 96 hours in vitro and transferred to day $3^1/_2$ recipients. The uteri were recovered at day 13 and the number of fetuses recorded.

1.3.1.2. *Results*

The fusion rates of embryos and subsequent development is presented in Table 5.1. Investigation of different fusion methods indicated that the PHA assisted DC fusion was not as efficient as the viral or AC/DC fusion ($P<0.05$). Subsequent development to two-cells, blastocysts and day 13 fetuses did not differ between treatments with the exception of lower blastocyst development in the AC/DC fusion group. Manipulations with viral fusion were efficient provided the virus batch was an effective fusogen and embryos were not over-exposed to viral solution. PHA assisted DC fusion was difficult after exposure to the PHA as it induced a course surface on the nuclear transfer pipette hence increasing manipulation lysis of karyoplasts.

Table 5.1 Efficiency of fusion method on pronuclear mouse embryos

Fusion	Number	Fused (%)	2-cell (%)	Blastocysts (%)	Fetuses*
Viral	71	97[a]	86	79[ab]	59 (4)
PHA electro	42	71[b]	100	96[a]	75 (1)
AC-electro	94	99[a]	99	56[b]	48 (5)

*percentage of fetuses from embryos in pregnant recipients
Values in the same column with different superscripts differ ($P<0.05$)

1.3.2 **Nucleocytoplasmic ratio, cytoplasmic content and nuclei development.**

1.3.2.1 *Experimental outline*

The effect of doubling the nucleocytoplasmic ratio was determined by fusing the two blastomeres of a two-cell embryo. Embryos were manipulated at 48 hours post HCG. To simultaneously examine the effect of cytoplasmic content, nuclei were also transferred to one-cell stage zygotes at 26 hours post HCG. Unmanipulated one-cell stage embryos were cultured simultaneously. All embryos were observed for in vitro development for 96 hours.

To further examine the influence of an increased nucleocytoplasmic ratio, the blastomeres of a four cell embryo were fused into single cell and three of the four nuclei removed. Four cell stage nuclei were also transferred to a fused enucleated two cell cytoplasm and an enucleated one-cell cytoplasm. Unmanipulated one- and four-cells were culture simultaneously. The subsequent cleavage and development was recorded at 18 and 96 hours post fusion. Blastocysts forming from the two and four cell stage cytoplasm were transferred to the uteri of synchronized day $2^1/_2$ recipients.

1.3.2.2. *Results*

Fusion between two cell blastomeres together using a single DC pulse resulted in 91% fusion. Treatment of four cell stage embryos with three DC pulses resulted in 55% fusing into a single cytoplasm and the remainder

fusing into three phenotypes (Plate 6; photograph 4) at frequencies presented in Table 5.2.

Table 5.2 Fusion phenotype frequency for four cell stage embryos pulsed with 3 x 1.3 kV/cm D.C. pulses in three different intercellular planes.

	Three cell	Two-cell	Uneven two-cell	One-cell
n	15	25	19	71
%	12	19	15	55

When two cell stage nuclei were exposed to an increased cytoplasm cleavage to the 2-cell and blastocyst stage was reduced compared with unmanipulated one-cell stage embryos (Table 5.3). When the nuclcocytoplasmic ratio was halved for the two-cell nucleus, the recipient cell cytoplasm only resulted in reduced cleavage compared to unmanipulated one-cells. However cleavage of the two cell nuclei to the blastocyst stage was also reduced in the one-cell cytoplasm ($P<0.05$). The time of blastocyst formation in either cytoplasm was generally in advance of unmanipulated one-cell embryos (6 to 12 hours) but retarded compared with two-cell stage embryos (12 hours).

Table 5.3 Development of two-cell stage nuclei in cytoplasm with volume equivalent to the one-cell stage.

Recipient cytoplasm	Number	2-cell stage (%)	Blastocyst stage (%)
one-cell	191	152[a](80)	57[a](30)
two-cell	233	185[a](79)	147[b](63)
control-one-cells	181	177[b](98)	169[c](95)

Values in the same column with different superscripts differ ($P<0.05$)

Table 5.4 Development of four-cell stage nuclei in cytoplasm with volume equivalent to the one-cell stage.

Recipient cytoplasm	Number	2-cell stage (%)	Blastocyst stage (%)
one-cell	23	12[a](52)	0[a]
2-cell	31	14[a](45)	3[a](10)
4-cell	75	45[a](60)	13[a](17)
control-four cells	82	-	79[b](96)
one-cell to-one-cell	41	37[b](90)	27[c](66)

Values in the same column with different superscripts differ ($P<0.05$)

When four cell stage nuclei were exposed to three different stage cytoplasms of similar volume, development up to the two cell stage did not differ (Table 5.4). The nuclei in one-cell stage cytoplasm were subsequently unable to develop to the blastocyst stage. The development to the blastocyst stage for nuclei in the four-cell stage cytoplasm was higher (but not significantly) than in two-cell and one-cell stage cytoplasm. The formation of blastocysts occurred approximately 60 hours post fusion which was much slower than control four-cell stage embryos (48 hours) but in advance of the nuclei in the two-cell stage cytoplasm (72 hours). Embryo transfers of blastocysts from four-cell stage cytoplasm (four embryo transfers) and two cell stage cytoplasm (one embryo transfer) resulted in no offspring.

PLATE 6

Photograph 1

Pronuclear mouse embryo (19 hours post HCG) containing two visible pronuclei and two polar bodies

(Optics: DIC 400x)

Photograph 2

Enucleation of a pronuclear mouse embryo showing the separation of the karyoplast and remaining cytoplast with the pinching of the plasma membrane.

(Pipette diameter 25μm)
(Optics: 400x DIC)

Photograph 3

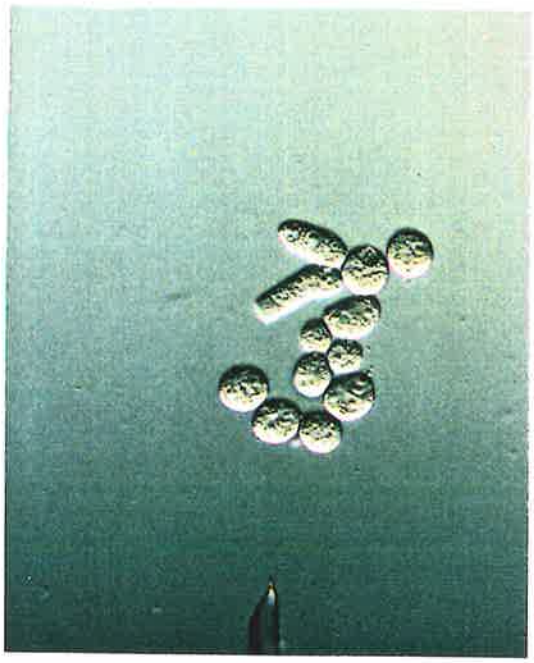
Karyoplasts isolated from fused four cell stage mouse embryos. The karyoplasts contain up to three nuclei.

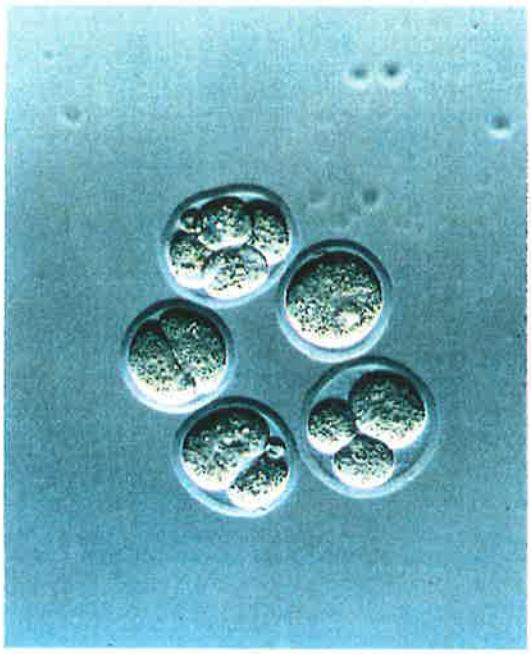
(Pipette diameter 35mm)
(Optics: 400x DIC)

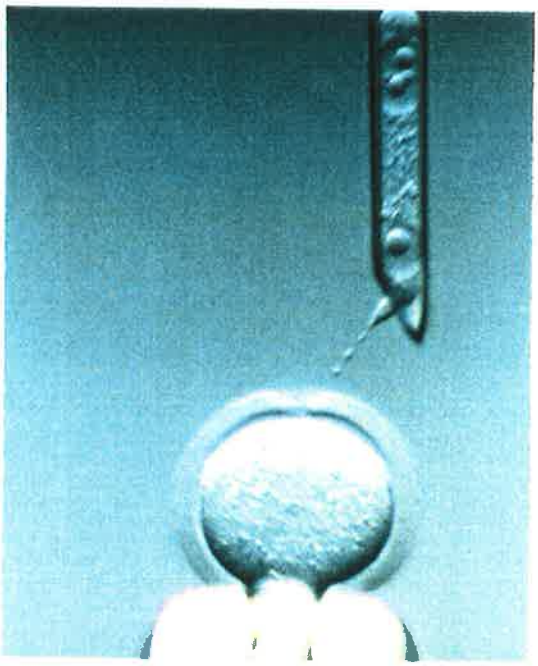
Photograph 4

Different morphologies of four cell embryos exposed to three fusion pulses and an untreated four cell. The successful fusion appears as a one cell, with the unsuccessful fusions as an even two cell, uneven two cell, and an uneven three cell.

(Pipette diameter 35μm)
(Optics: 200x DIC)







4. DISCUSSION

Transferring two- and four-cell stage nuclei into earlier stage cytoplasm with equivalent volume resulted in a reduction in the number of blastocysts forming. Decreasing the nucleocytoplasmic ratio of two and four cell nuclei in homologous cytoplasm also resulted in poorer cleavage than control embryos. This also resulted in slower blastocoel formation, but not the same as the nuclei stages normally in the same nucleocytoplasmic ratio.

Nuclei from the late 2-cell stage onwards transferred into the zygote cytoplasm only have the capacity for cleavage to the two-cell stage (McGrath and Solter 1984; Howlett et al 1987, Robl et al 1986, Tsunoda et al 1987). The suggested cause has been incompatibility of pre-genomic activation cytoplasm with post-genomic stage nuclei, which has been supported by the fact that the later stage nuclei (four- and eight-cell) can support term development in the late two-cell cytoplasm (Tsunoda et al 1987). However as this study has shown, retarded development of single nuclei from the late-two or four-cell stage was autonomous of the stage of recipient cytoplasm. In the case of the four cell nucleus with a quadrupled volume, blastocyst development was only 17% in four cell cytoplasm, 10% in late two-cell cytoplasm, with no development in the enucleated zygote. Therefore it cannot be ruled out that a transferred nucleus in the zygote cytoplasm is incompatible partly because of its larger volume.

Studies testing the viability of four and eight-cell stage nuclei in the two-cell cytoplasm have used two nuclei each fused to an enucleated blastomere (Robl et al 1986) with live offspring resulting from eight-cell nuclei (Tsunoda et al 1987). Cleavage of the resulting embryos has been superior to single nuclei in the enucleated zygote, however the number of nuclei per reconstructed embryo may have contributed to this difference. This is supported by the failure to obtain live offspring from single eight-cell nuclei in an enucleated two-cell blastomere (Kono and Tsunoda 1989). A parallel observation of nuclei in a two-cell blastomere in this study, may

have been useful to further quantify the reduced development observed with the four-cell nucleus in the fused/enucleated two-cell. However, the blastocyst development of single four-cell nuclei in enlarged two-cell cytoplasm (10%) was much lower than observed previously for normal sized two-cell blastomeres (74%; Kono and Tsunoda). Therefore reduced viability of transplanted nuclei to the earlier stage cytoplasm was probably associated with the increase in cytoplasmic volume (decreased nucleocytoplasmic ratio).

Regulation of morphogenetic events in the preimplantation development of mouse embryos have been shown not to be controlled by the number of DNA cycles (Smith and McLaren 1977, Dean and Rossant 1984). This is also supported from observation of lower cell numbers in blastocysts after nuclear transfer of eight cell nuclei to two-cell blastomeres (Kono and Tsunoda 1989). This study did not count the cell numbers and using cleavage and blastocoel formation to assess nuclei reprogramming of nuclei may have been inadequate. The delays observed in the blastocyst formation are not necessarily indicative of reprogramming as there was no verification of increased cell numbers. They could be explained due to the inability of the transplanted nucleus to synchronise with the recipient cytoplasm and hence result in a rate of development that was a composite rate of development as observed with asynchronous blastomere chimaeras (Prather and First 1987,1988).

The relevance of the nucleocytoplasmic ratio to nuclear reprogramming may already be apparent from existing nuclear transfer studies in non murine species . The highest rate of survival for bovine and ovine nuclei have been observed with the nuclear transfer technique described by Willadsen (1986) for sheep and used on bovine embryos (Bondioli et al 1990) where the oocyte is split and both halves used as cytoplasm recipients. In these results the survival to blastocyst and term are similar or greater than seen with the approach used by Robl et al (1987) and Prather et al (1987) where only the region of the nuclear material is removed which is approximately 5 to 20% of the cytoplasm. When one considers that

the former approach, 50 % of nuclear transfer embryos contain both the donor nuclei and oocyte metaphase nuclei, it is surprising that a similar number survive. One explanation could be that when the oocyte is halved to produce two recipient cytoplasms (Willadsen 1986) the nucleocytoplasmic ratio is double that in the whole oocyte. This may be improving either the ability of the nuclei to be reprogrammed, or reducing the effect of a decreased nucleocytoplasmic ratio as was observed with mouse embryos in this study.

Several inferences can be made about the effect of nucleocytoplasmic ratio on nuclear reprogramming and survival of transplanted nuclei. However when applying this information to oocyte cytoplasm, the differences between the fertilised egg and oocyte cytoplasm should be considered. Furthermore, the reprogramming of mouse nuclei is temporally constrained by the early time of genomic activation. In comparison, the ovine and bovine embryo undergo three cell divisions prior to genomic activation which may be a major difference if the extent of reprogramming is temporally dependent. However from the results of these experiments, it can be argued that development will be retarded due to an increase in the cytoplasmic volume. Whether the reprogramming capacity of the oocyte can overcome this remains to be elucidated.

SECTION VI

SUMMARY

Recognition of the potential for cloning embryos in commercial livestock provides motivation for research into the development of efficient and reliable techniques. This thesis explores the application of *in vitro* culture in the development and assessment of such cloning procedures.

The culture system which is based on a synthetic oviduct fluid medium was initially proven by its capacity to support the development of one-cell caprine, bovine and split eight-cell caprine embryos to the blastocyst stage. As the bovine embryos represented a species of major interest, the postimplantation viability of cultured bovine blastocysts was verified by direct comparison with in vivo cultured embryos

When the culture system was applied to ovine nuclear transfer embryos, approximately 25% of fused embryos developed to the blastocyst stage. However when cultured embryos were transferred to sheep oviducts three days after fusion only 4% of the original isolated blastomeres resulted in lambs. Although it represents a low overall efficiency, it is similar to that previously reported in studies using in vivo culture. Application of system to nuclear transfer bovine embryos resulted in 20% of the fused embryos developing into either morulae or blastocysts, with one term pregnancy.

The major loss of transferred embryos in both the sheep and cow was found to occur during the pre- and postimplantation periods of development. A potential cause for this may have been the low cell numbers of cultured blastocysts. The fact that low cell numbers were also found in pronuclear embryos simply subject to the manipulation procedures of removal and refusion of the nuclei, suggests that the procedures themselves play a major part in overall embryo attrition.

Specific loss of embryos occurred due to transfer of blastomere nuclei into enucleated oocytes. As demonstrated in studies with the mouse, one factor in this loss was the decreased nucleocytoplasmic ratio established in the reconstructed embryo.

Development aimed at improving the overall efficiency of the nuclear transfer technology included (1) improvements in the manipulation

procedures, (2) the use of serial cloning and (3) the use of in vitro matured oocytes.

In the ovine, improvement of the manipulation procedures was achieved using differential interference contrast optics to improve enucleation accuracy following better visualisation of the metaphase chromosomes. However similar improvement could not be achieved in the bovine due to the darker cytoplasm.

In a study of the feasibility of serial nuclear transfer, it was found possible to produce second generation nuclear transfer embryos capable of developing up to the sixteen cell stage. There was no obvious attrition in the *in vitro* development of the second generation embryos, however further evaluation of the in vivo viability of embryos produced by this means, needs to be carried out.

Previous studies on investigating the use of *in vitro* matured oocytes as cytoplasm recipients in nuclear transfer experiments have been carried out in the bovine. In the major trial reported in this thesis, *in vitro* matured ovine oocytes were shown to provide an effective option to *in vivo* matured oocytes in supporting embryo development for up to three days in culture with maintained viability as proven by transfer to recipients. However the low pregnancy rates observed (4-5%) for both oocyte sources was lower than obtained in earlier trials reported in this thesis (14%), probably related to the decision to transfer the embryos to the uterus rather than the oviduct.

The viability of in vitro procedures aimed at developing cloning technology have been established. As demonstrated in this thesis, its application has provided useful insight into factors likely to influence the production of identical animals using nuclear transfer.

SECTION VII

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