ESTABLISHMENT OF A ROBUST CLONING TECHNOLOGY FOR MAMMALS

by

Manoel Paulo Menna Barreto Duarte

Department of Obstetrics and Gynaecology,
Medical School
The University of Adelaide

THIS THESIS HAS BEEN ACCEPTED FOR THE AWARD OF THE DEGREE
OF
MASTER OF MEDICAL SCIENCE

October 2001

TABLE OF CONTENTS

TABLES

FIGURES

ABSTRACT

This thesis is presented in two parts. The first part concerns production of sheep-goat hybrid embryos using oocyte *in vitro* maturation (IVM) and *in vitro* fertilization techniques.

Initial experiments were carried out to optimise procedures for harvesting and maturing sheep oocytes *in vitro* prior to *in vitro* fertilisation with sheep sperm. Comparisons were made between two procedures, viz. aspiration vs sectioning, for collecting oocytes from sheep ovaries obtained at a local abattoir. The aspiration procedure yielded less oocytes (average number of oocytes per ovary 1.09), compared with 5.18 for sectioning procedure ($p<0.001$). However the oocytes recovered by aspiration procedure were of better quality with more oocytes maturing *in vitro* to metaphase II (aspiration 95.5% vs sectioning 72.4%,$p<0.001$), more undergoing normal fertilisation (aspiration 71.8% vs sectioning 34.5%,$p<0.001$), and more showing a cleavage rate (aspiration 67.8% vs sectioning 23.3%,$p<0.001$).

Oocytes were matured *in vitro* in a medium supplemented with commercial fetal calf serum. Other protein supplements were investigated including serum from a geep and a sheep/geep hybrid, but the commercial fetal calf serum proved to be the most effective.

Hybrid embryos were then generated using *in vivo* matured goat or sheep x goat hybrid oocytes fertilised *in vitro* with sheep sperm.

With the goat oocytes fertilised with sheep sperm, zygotes developed from the *in vivo* matured oocytes yielded better cleavage rates than those which were matured *in vitro*, (86.8% vs 48.4%,$p<0.001$).

In contrast, in experiments with oocytes recovered from the sheep x goat hybrid and fertilised with sheep sperm, zygotes from *in vivo* matured oocytes produced similar cleavage rates (68.4%) to the *in vitro* matured oocytes (50.0%;$p>0.05$).

Blastocysts created by fertilizing goat oocytes with sheep semen were cultured *in vivo*. One blastocyst resulted from *in vitro* matured oocytes and 12 from *in vivo* matured oocytes ($p>0.05$). The recovery rate of the blastocysts following incubation *in vivo* for the *in vitro* matured oocytes was 29.9%, and for the *in vivo* matured oocytes, 32.6%($p>0.05$).

The second part of the thesis is aimed at developing efficient procedures for generating female chimeras for use as recipients for hybrid embryos and for exploring the use of the chimeric route as a means of re-establishing the genome of an embryonic stem cell line into the germ line. Initial experiments were carried out using a mouse model with the intention of subsequently applying the technology to sheep and goat embryos. This study presents only those studies undertaken with mouse embryos.

Comparison was made between various ways of creating chimeric mice, including injection of embryonic stem cells or inner cell mass cells into blastocysts and through embryo aggregation. The effects of mouse strain and time of transfer on the efficiency of production of mouse germline chimeras was investigated. For production of chimeras from embryo stem cells, host blastocysts were obtained from three strains of mice namely (Swiss albino, C57BL/6 and CBAC57/F1) which were injected with three lines of embryonic stem cells, namely (E14, EMBL-5 and E14 transfected). No chimeras were produced using blastocysts from C57BL/6 mice blastocysts as recipients, however chimeras were produced with blastocysts from the other two mouse strains. Altogether 925 blastocysts were injected with stem cells and transferred. These resulted in 105 offspring, of which 35 were chimeric. For production of chimeras by transferring inner cell mass cells, combinations of cells and blastocysts from Swiss albino and C57BL/6 strains were used. Only one chimera resulted from 64 embryos inoculated with inner cell mass cells and this chimera was produced from Swiss albino inner cell mass cells inserted into a C57BL/6 blastocyst. Swiss albino and C57BL/6 mice were also used for production of chimeras by embryo aggregation. In these experiments, 62 aggregated blastocysts were transferred, resulting in 13 offspring of which 3 were coat colour chimeras.

The creation of germline chimeric individuals is widely used as a means of materialising the karyoplast genome from embryonic stem cells or inner cell mass cultures. However, it is presently only successful at low efficiency. This inefficiency would be a major constraint in the application of this technology to farm animals, due to their generally low fecundity and long inter-generational interval. These limitations could be overcome through the more direct route of using nuclear transfer technology to re-establish the genome in a germline. However, this approach requires large numbers of host cytoplasts which are prepared by surgical enucleation of activated IVM oocytes. In anticipation of the requirement for simpler, more robust procedures for preparing cytoplasts, various techniques for enucleation of recipient oocytes (mouse) and activation of the cytoplasts (ovine) have been investigated.

The mouse oocytes were enucleated using centrifugation following incubation in cytochalasin B for 1 or 3 hours. All treatments were effective, however the best combination for enucleation was centrifugation following incubation in cytochalasin B for 3 hours. In respect to activation of sheep oocytes, four treatments were investigated. All were found capable of activating sheep oocytes, however electroactivation proved the best of the approaches used resulting in 64% of the oocytes being activated. This compared with only 35.4% for ethanol activation, using ethanol treatment.

The technologies explored in this thesis are part of a study aimed at developing the advanced reproductive technologies required to allow multiplication of rare mammalian genotypes such as animals threatened by extinction, endangered animals or novel commercially valuable genotypes, including transgenic animals through cloning procedures.

DECLARATION

The experimental work described in this thesis was conducted in the Department of Animal Science and Department of Scientific Industrial Research - DSIR at Massey University, Palmerston North, New Zealand during 1989-1991 and Department of Obstetrics and Gynaecology of Medical School, University of Adelaide, Adelaide Australia during 1991-1994.

The data presented are from original studies by the author. This thesis contains no material which has been submitted for the award of any other degree or diploma in any university wholly or in part.

I consent to this thesis being available for photocopying and loan if accepted for the award of the degree.

M.P.Menna Barreto Duarte

ACKNOWLEDGMENTS

I would like to express my acknowledgement of the enormous contribution of Dr. Robert F. Seamark for his supervision and advice regarding to this thesis and as well as the opportunity to carry out part of this study at the Department of Obstetrics and Gynaecology of at the Medical School at Adelaide University, Adelaide, Australia.

Also, I would like to acknowledge the administrative assistance of Professor Jeffrey Robinson and Dr. David Kennaway.

In the same way I would like to acknowledge Professor Maurice F McDonald and Dr. Robert Welch for supervision of this thesis and for the opportunity to carry out part of the experimental work at the Department of Animal Science at Massey University and Department of Scientific and Industrial Research in Palmerston North, New Zealand.

I am grateful to Mr Brian Sinclair (New Zealand Consulate in Brazil), Mr Geoffrey Powell (United Kingdom Consulate in Brazil) and Mr Kevin Lowe (Assistant to Vice-Chancellor, Massey University) for their administrative efforts, enabling me to undertake postgraduate studies at Massey University, New Zealand.

In New Zealand I am grateful to all staff at Department of Animal Science and Department of Scientific and Industrial Research for their assistance, cooperation and provision of facilities for the work consolidate in this thesis.

In particular to Dr. James William Catt, Ms Ann Pugh and Dr. Robin Tervit for their assistance and teaching in in vitro

maturation, *in vitro* fertilization and *in vitro* culture of mammal oocytes.

I would like to thank Mr Adrian King, for his assistance in the surgery and care with the animals, and Ms Sally Rhodes and Mr Bruce Sinclair for the assistance in the laboratory.

I am grateful to Massey University Computer Centre for their willingness to assist me with computer problems.

I would like to acknowledge the administrative assistance of Professor Robert Anderson, Dr. Phil Pearce, Dr. Tom Broad and Dr McCutcheon.

In Australia I am grateful to all staff at the Department of Obstetrics and Gynaecology for their assistance, cooperation and provision of facilities for the work consolidated in this thesis.

I would particularly like to thank Dr. Hiroshi Nagashima, Dr. Deirdre Warnes and Dr. Naomi Kashiwazaki for their assistance in the work developed in the micromanipulation of embryos and as well as embryo culture of mouse embryos. I would also like to thank Dr. Peter Kotaras and Dr. Peter Holm for their assistance in the development of the cloning procedures and the supply of sheep oocytes produced by *in vitro* maturation for the activation studies.

I would like to thank Mr Stephen McIlfatrick, Ms Vicki Tregenza, Ms Jane Lang and Ms Anna Seamark for their assistance in the preparation of media and supplying the material for work in the mouse surgery.

I am grateful to Dr. Brian Burke, of Animal Services and his group for the mouse supply and keeping the mice in good health.

Also I am grateful to Mr Don Bigham for his teaching and assistance with computer problems.

I would like to acknowledge the administrative assistance of Ms Glenys King and Mr Frank Carbone.

I also wish to acknowledge the assistance of Dr. Andrew French for the glucose phosphate isomerase analysis and discussion and Mr Phill Leppard, of the Statistics Department for the assistance in analysis of data.

I am indebted to the Department of Biochemistry, University of Adelaide, particularly the late Dr. Julian Wells, Ms Jacqueline Beall, Dr. Peter Rathjen and Dr. Peter Wigley and staff who provided mouse stem cells.

I would like to say thank to Dr. David Kennaway for reading and suggesting modifications in the text of this thesis.

Also I am indebted here in Brazil to the late Dr. Antonio Mies Filho and staff of the Veterinary Faculty of the Federal University of Rio Grande do Sul, Porto Alegre, who gave me encouragement to do postgraduate studies abroad. Providing recommendation letters to CNPq (Brazilian National Council of Research) for financial support and Dr. Robert W.S.P. Thomas who did the English review of this thesis.

Finally, financial support during this thesis was provided by CNPq -Brazilian National Council of Research, to whom I am grateful.

ABBREVIATIONS

CHEMICALS

BSA - BOVINE SERUM ALBUMIN

Ca^{2+} - CALCIUM

CO_2 - CARBON DIOXIDE

DNA - DEOXYRIBONUCLEIC ACID

EGTA - ETHYLENE GLYCOL-BIS (ß-AMINOETHYL ETHER) N,N,N'N'-TETRAACETIC ACID

EtOH - ETHANOL

Hepes - N-2-HYDROXYETHYLPIPERAZINE-N'-2-ETHANESULFONIC ACID

Mg^{2+} - MAGNESIUM

N_2 - NITROGEN

$NaHCO_3$ - SODIUM BICARBONATE

O_2 - OXYGEN

PBS - PHOSPHATE BUFFERED SALINE

PHE - PENICILLAMINE, HYPOTAURINE, EPINEPHRINE

RNA - RIBONUCLEIC ACID

DNA - DEOXYRIBONUCLEIC ACID

HORMONES

CSF - CYTOSTATIC FACTOR

FSH - FOLLICLE STIMULATING HORMONE

HCG - HUMAN CHORIONIC GONADOTROPHIN

LH - LUTEINIZING HORMONE

MPF - MATURATION PROMOTING FACTOR

PMSG\PMS - PREGNANT MARE SERUM GONADOTROPHIN

SYMBOLS AND UNITS

µg - MICROGRAM (S)

µl - MICROLITRE

µm - MICROMETRE

IU - INTERNATIONAL UNIT (S)

mg - MILLIGRAM (S)

ml - MILLILITRE (S)

mm - MILLIMETRE (S)

mOsm - MILLIOSMOLAR

w/v - WEIGHT PER VOLUME

ºC - DEGREES CENTIGRADE

MEDIA

HTF - HUMAN TUBAL FLUID

SOFM\SOF - SYNTHETIC OVIDUCT FLUID MEDIUM

TCM-199 - TISSUE CULTURE MEDIUM 199

OTHERS

2PN+T+PB - TWO PRONUCLEI + TAIL + POLAR BODY

AI - ANAPHASE I

AII - ANAPHASE II

BL - BLASTOCYST

CIDR - CONTROL INTERNAL DRUG RELEASING

DSIR - DEPARTMENT OF SCIENTIFIC AND INDUSTRIAL RESEARCH

ES - STEM CELL (S)

FCS - FETAL CALF SERUM

IVF - IN VITRO FERTILIZATION

IVM - IN VITRO MATURATION

IVMF - IN VITRO MATURED AND FERTILIZED

MI - METAPHASE I

MII - METAPHASE II

MII+T+HD+2PB - METAPHASE TWO, TAIL, HEAD, TWO POLAR BODIES

MQ - WATER ORIGINATING FROM A MILLI Q UF MACHINE

n - NUMBER OF SUBJECTS

n - NUMBER OF SUBJECTS

OF - OFFSPRING

PC - PERSONAL COMPUTER

r - NUMBER OF REPLICATES

r - NUMBER OF REPLICATES

SC - SCAR

TII - TELOPHASE II

UV - ULTRAVIOLET LIGHT

vs. - VERSUS

$\bar{x}$ - MEAN NUMBER

$\bar{x}$ - MEAN NUMBER

I - LITERATURE REVIEW

1.1 - INTRODUCTION

In the early 1990's the former Department of Scientific and Industrial Research of New Zealand, now AgResearch Grasslands, Palmerston North, was developing a project aimed at mapping the sheep genome to locate the genes responsible for economically important traits. One approach towards building such a map was based on the production of hybrid animals derived by mating a goat doe and ram to obtain a hybrid female, the geep, (Stewart-Scott et al., 1990).

The first experimental part of the thesis was developed in New Zealand and describes experiments carried out to generate sheep embryos. Geep and backcross (Geep x sheep) embryos between the female geep and ram using in vitro maturation, in vitro fertilisation and in vivo culture technology.

The second part of the thesis was developed in Australia, with the aim of extending the technology to include the production of chimeras as recipients of hybrid embryos. Because of scientific and economic constraints the preliminary stages were carried out in mice, with the initial experiments concentrating on developing more effective ways of creating chimeras. Embryonic stem cells and inner cell mass cells isolated by immunosurgery were used as the source of foreign cells.

In order to contribute to an alternative procedure for re-instating a foreign genotype into the germline, a study was initiated in two essential procedures for cloning embryos, the

activation and enucleation of oocytes.

The bibliographic review is not comprehensive but aims to select the pertinent published literature in the areas studied.

In this review the areas covered are: (one) hybrid animals, (two) chimeras, (three) stem cells, (four) embryo transfer in the mouse, (five) in vitro maturation and in vitro fertilisation of oocytes, in vivo/in vitro culture of embryos, (six) nuclear transfer/ oocyte activation and enucleation.

1.2 - HYBRID ANIMALS

1.2.1 - INTRODUCTION

The existence of hybrids have been noted in the literature since the middle to late 19th century, following identification by their odd looks or behaviour (reviewed by McGovern, 1969). As mating animals of different species exaggerates any differences which would normally exist between mother and foetus it has made them a focus of special scientific interest for reproductive immunologists and biologists.

Barriers to successful reproduction between species can result from failure of fertilisation, failure of gestation, failure of survival of the new born animal or sterility of the hybrid, (Hancock et al., 1968). These barriers to successful hybridisation between species thus play a major role in maintaining the integrity of species (Moore, 1983).

Only in a few cases have interspecific hybrids been systematically produced, usually for the purpose of propagating

an animal with the desirable characteristics of the two parental species, e.g., the hybrid between Bos taurus and Bos indicus is fertile and the hybrid calves present the heat tolerance and disease resistance of Asian domestic cattle, with the productivity of European breeds (Anderson, 1988).

1.2.2 - SHEEP AND GOAT HYBRIDS

Sheep x goat hybrids occur naturally as identified by cytogenetic studies (Roca and Rodero, 1971; Bunch et al., 1976; Eldridge et al., 1983; Denis et al., 1987 cited by Tucker et al., 1989; Pinheiro et al., 1989; Stewart-Scott et al., 1990). So far these natural hybrids have all been females with 57 chromosomes (Sheep, 2n=54; Goat, 2n=60) and all have proven to be fertile as assessed by successful conception when back-crossed with a ram.

Experimentally it has been demonstrated that conception commonly occurs when female goats are mated or inseminated by sheep (Alexander et al., 1967; Hancock et al., 1968; Dent et al., 1971; Eppleston and Moore, 1977; Moore, 1983), but the hybrid pregnancy rarely extends beyond the 3rd month, (Alexander et al., 1967; Dent et al., 1971; McGovern, 1973a; Moore, 1983).

1.2.3 - CAUSES OF HYBRID FETAL FAILURE

Hybrid animals generally fail to survive for one of two reasons; the intrinsic factors relating to defects in the hybrid embryo and extrinsic factors reflecting incompatibilities between

mother and fetus (McGovern 1969).

1.2.3.1 - INTRINSIC FACTORS

Cytological defects were noted by Berry in 1938 (cited by McGovern, 1969), but these researchers proposed that embryo loss was not due to the cytological abnormalities *per se* but to recessive lethal genes exposed in the absence of normal alleles in the hybrid.

There is evidence to suggest that hybridisation between sheep and goat is possible because the reproductive barrier between Capra and Ovis was not developed within Ovis (Bunch et al., 1976). Goats have maintained the basic karyotype, while in the sheep, the chromosomes have continued to be rearranged, resulting in populations of wild sheep with 2n values of 52 (*O. nivicola*); 54 (*O. aries*, *O. canadensis*, *O. dalli*, *O. musimon* and *O. orientalis*); 56 (*O. ammon*); and 58 (*O. vignei*) (Bunch et al., 1977). This diversity in chromosome number is comparable to the differences existing between goats and sheep. Recently, using reproductive technologies, Coonrod et al. (1994) obtained Armenian red sheep (O. orientalis) lambs from domestic sheep following interspecific embryo transfer, while Garde et al. (1995) reported a live birth of a hybrid (*O. musimon* X *O. aries*) using intrauterine insemination. These observations support the theory that cytological anomalies are not the main factor resulting in the death of sheep x goat fetal hybrids.

1.2.3.2 - EXTRINSIC FACTORS

a) Immunological responses occur in the doe in response to a hybrid conceptus (Hancock et al., 1968, Dent et al., 1971). Haemolytic antibodies against red blood cells of the sire have been found in the maternal goat sera shortly after cotyledonary attachment at about 30 days of gestation (Alexander et al., 1967). Attempts to improve the immunological tolerance by intravenous injections of sheep spermatozoa in goats, have generally proved unsuccessful in prolonging the survival of hybrid embryos (McGovern, 1973b).

b) Hormonal and Anatomical: Sheep and goats exhibit differences in oestrous cycle length and placental endocrine function but have similar placental anatomy and gestation period, although embryonic implantation begins 2-3 days earlier in the ewe (MacLaren et al., 1993). As observed by Moore (1983) the sheep placenta begins to assume a major role for the production of progesterone around the 5^{th} week of gestation, whereas in the goat the corpus luteum remains the major source of progesterone throughout pregnancy. It has been suggested (Benirschke, 1983) that corpus luteum failure is not the cause of fetal death because simultaneous homospecific embryo transfer did not save the hybrid embryo. In addition Hancock and McGovern (1968) transferred hybrid embryos to recipient goats previously mated to a buck, but no hybrid fetus developed to term, only kids were produced.

1.2.4 - FERTILITY OF GOATS MATED WITH RAMS

As noted previously, conception commonly occurs when female goats are mated to sheep. Alexander et al. (1967), for example, mated 29 does with rams and obtained 23 conceptions at the first mating while Hancock et al. (1968) inseminated 50 does with ram semen with only 10 returning to service within 25 days. Of twenty-three does examined at autopsy 28 to 42 days after insemination, 19 proved to be pregnant. Similarly McGovern (1973b) obtained four hybrid pregnancies from five does inseminated with sheep semen.

1.3 - CHIMERAS

1.3.1 - INTRODUCTION

A chimera, by definition is an organism resulting from the union of more than two gametes (McLaren, 1976 cited by Anderson, 1987).

Each chimera is unique and because of the degree of disparity between the two component genotypes (Rossant et al., 1983, Anderson, 1987), they provide a useful tool for investigating problems in both genetics and embryology. Chimeras can also provide a useful means of rescuing lethal phenotypes such as parthenogenetic and androgenetic stem cells (Stevens, 1978; Surani et al., 1987; Piedrahita et al., 1992), potential to the preservation of wild animals on the verge of extinction (Nagashima et al., 1991) as well as for propagating novel genotypes. Chimeras can also be created from different species as a means of helping to overcome problems relating to

incompatibility between the maternal and the trophoblast genotypes in experimental crosses between different species (Rossant et al., 1983).

1.3.2 - TECHNIQUES USED FOR PRODUCTION OF CHIMERAS

Production of embryo chimeras can be achieved by several different means including embryo aggregation, blastocyst injection and embryo co-culture.

1.3.2.1 - EMBRYO AGGREGATION

The embryo aggregation technique was developed by Tarkowski in 1961 who formed chimeras by mixing embryos of one or more different mice strains. Using this approach, trophoblast and inner cell mass cells result which are of mixed genotype and which develop into live offspring that consist of a random mixture of cells of the two parental strains (Buehr and Mclaren, 1974).

The chimeras produced through embryo aggregation can be generated with entire embryos (Tarkowski, 1961; Piedrahita et al., 1992) or bisected embryos (Kashiwazaki et al., 1988; Nagashima et al., 1991).

The successful production of an overt chimera through embryo aggregation depends on the synchrony of blastomere division of both strains involved in the process. Betteridge and Fléchon, (1988) and Prather and First (1987) observed that the time of blastomere division is asynchronous, while Warner et al. (1987)

observed that the rate at which the cleavage divisions progress depends upon environmental and genetic factors and even embryos of identical genetic make up can show some lack of synchrony of cleavage rate. This may be a normal physiological event as cells from the inner cell mass first appear at the 16-cell stage, (Somers et al., 1990). Studies with the re-aggregation of asynchronous embryos in the sheep have produced individuals that have a chimeric placenta but that are not chimeric themselves (Prather and First, 1987).

Successful production of chimeras by embryo aggregation has been reported for sheep and goats (Fehilly et al., 1984b; Meinecke-Tillmann and Meinecke 1984; Ruffing et al., 1993); mice (Petters and Mettus, 1984, Kashiwazaki et al., 1988); bovines (Brem et al., 1984) and hamster (Piedrahita et al., 1992) and sheep of mixed breed parentage (Fehilly et al., 1984a; Pugh et al., 1994).

The embryo aggregation technique is a tool which could give an indication of the compatible strains for optimal stem cell germline colonisation into host blastocysts (Kashiwazaki et al., 1988; Schwartzberg et al., 1989; Wilmut et al., 1991; Piedrahita et al., 1992).

1.3.2.2 - BLASTOCYST INJECTION

The blastocyst injection technique was developed by Gardner (1968). In this approach embryonic cells from disaggregated blastocysts are injected into the blastocoel of host blastocysts. This technique was initially developed for the mouse but

subsequently used to introduce inner cell mass cells into blastocysts to create chimeric sheep goats (Polzin et al., 1987), (Roth et al., 1989), different breeds of sheep, (Butler et al., 1985), cattle (First et al., 1994) and pigs (Kashiwazaki et al., 1992).

This approach has been adapted to teratocarcinoma embryonal carcinoma cells by Bradley et al. (1984), embryonic stem cells, (Gossler et al., 1986; Pease et al., 1990 ; Azuma and Toyoda, 1991) and primordial germ cell-derived cell lines, (Stokes et al., 1994). The mouse technology was followed by the production of a chimeric pig using swine embryonic stem cells (Wheeler, 1994).

1.3.2.3 - CO-CULTURE

The third technique, which is a simplification of the above procedures, involves simple co-culture of embryos with the pluripotent cell. This procedure was used by Picard et al. (1990) to combine day 8 inner cell mass cells with day 5 morula, by Wood et al. (1993) to obtain mice chimeras and by Stokes et al. (1994) to co-culture bovine morulae on a lawn of primordial germ cell-derived cells.

1.3.3 - INTERSPECIFIC PREGNANCY AND CHIMERAS

In addition to the use of chimeras as models for the study of maternal/fetal interactions, interspecific pregnancies may also play a role in the preservation of endangered mammals,

potentially allowing a means where embryos from endangered species can be carried to term in the uteri of females of a related non-endangered species (Anderson 1988; Coonrod et al., 1994).

As demonstrated by Fehilly et al. (1984b) and Meinecke-Tillmann and Meinecke (1984) a goat fetus can develop to term in a sheep and a sheep fetus can develop to term in a goat, providing the embryos are manipulated to influence the genotype of cells contributing to the trophectoderm versus the inner cell mass.

Subsequently Polzin et al. (1986, 1987) used immunosurgery to isolate inner cell mass from caprine embryos and injected them into ovine blastocysts that were then transferred to ovine recipients. Two sheep-goat chimeras were born. The degree to which trophoblast protects sheep-goat chimeric pregnancy appears to be complete.

More recently Gustafson et al. (1993) have produced six hybrid pregnancies: three in sheep-goat chimeras, one in a sheep-(sheep x goat) hybrid chimera and two in does. Chimeras carried hybrid pregnancies longer than previously reported for hybrid pregnancies in ewes and does, but none were carried to term. It was verified (MacLaren et al., 1992; 1993) that sheep-goat chimeras did not prove to be immunologically tolerant to interspecific pregnancy. The sheep-goat chimeras were capable of carrying ovine pregnancies to term and it is suggested that the uterine environments of the chimeras used were predominantly ovine and thus not favourable to caprine pregnancy.

1.4 - INNER CELL MASS CELLS AND TROPHOBLAST

As previously defined (Watson, 1992; Brison et al., 1993) the pre - implantation development encompasses the "free" living period of mammalian embryogenesis which culminates in the formation of the blastocyst. Morphologically, the blastocyst is a fluid-filled structure in which the trophectoderm surrounds the blastocoel cavity and encloses the inner cell mass cells. Betteridge and Fléchon (1988) histologically characterized the trophectoderm and inner cell mass cells. The trophectoderm contains cytoskeletal components that are typical of epithelial cells, whereas, the inner cell mass cells are not epithelial in nature. Functionally, those cells have distinct characteristics by McLaren, 1984. In nuclear transfer experiments a trophoblast, when transferred to recipient eggs, does not proceed beyond midcleavage, whereas, the inner cell mass cells, when transferred will develop to term. It was also demonstrated (Surani and Barton, 1977) that trophoblasts can evoke a decidual response, whereas, inner cell mass cells do not.

As defined by Wilmut et al. (1992), totipotent cells are able to contribute to all of the conceptus, including the placenta; but they are called pluripotent if they are able to contribute to all tissues of the fetus or multipotent if they contribute to several tissues. Although the general pattern of events is similar in all mammals studied, there are differences among species in the stage at which cells lose developmental potential. Cells of the inner cell mass of sheep embryos certainly contribute to all of the tissues of the fetus, but

studies of the placenta have apparently not been made.

Transfer of inner cell mass cells to produce chimeras or clones (Smith and Wilmut, 1984) is especially important for the multiplication of species threatened with extinction (Polge, 1990; Godke and Rorie, 1992) or individuals of unusual genotype.

1.5 - STEM CELLS

Embryonic stem cells have been isolated from embryos which had been treated by immunosurgery to remove trophoblastic cells (Martin, 1981) or following explanting of whole embryos into culture (Evans and Kaufman, 1981). Lately, new strategies have been used to obtain pluripotent cells such as embryonic stem (ES) cells lines, derived from (a) morula stage embryos in mice (Eistetter, 1989), in mink (Sukoyan et al., 1993) and bovines (Strelchenko and Stice, 1994); b) primordial germ cell derived (PGCs) cells in mice, (Matsui et al., 1992) and in bovines (Cherny and Merei, 1994; Cherny et al., 1994).

As reviewed by Seamark and Wells (1991) the establishment of such totipotent embryonal (ES) lines is feasible but their maintenance in vitro requires specialised conditions to avoid differentiation and loss of totipotency. If they are injected back into the blastocoel cavity of another embryo, such cells often retain the potential to contribute to all of the tissues of the fetus but not the placenta; that is they are pluripotent rather than totipotent. As there is recent evidence that the isolation of such embryonic stem cells depends upon a reversible

epigenetic change, it should not be assumed that cells in culture are exactly like the cells *in vivo* (Wilmut et al., 1992; Sleigh and Hannan, 1993; Talbot et al., 1993a; Seamark, 1994). However according to Seamark (1994), there are no further unanticipated phenomena such as the effect of tissue culture on imprinting, which may threaten the predictability of outcome of stem cell (ES) cell-derived pregnancies and further limit the potential usefulness of this technology to the livestock industry.

Stem cell technology is now routine in many laboratories, where it is used in gene knockout studies (Robertson, 1987) and as a potential vector of new DNA constructs for transgenic programmes (Seamark, 1989).

Stem cells have been defined for the *mouse* (Robertson 1987), *syrian golden hamster* (Doetschmann et al., 1988; Piedrahita et al., 1990c), *rabbit* (Giles et al., 1991, 1993; Graves and Moreadith, 1993; Niemann and Strelchenko, 1994), *mink* (Sukoyan et al., 1992, 1993), *pig* (Anderson et al., 1990; Evans et al., 1990; Fléchon et al., 1990; Notarianni et al., 1990; Piedrahita et al., 1990a,b; Strojek et al., 1990; Gerfen et al., 1991; Chen and Wu 1993; Onishi and Youngs, 1993a,b; Talbot et al., 1993a,b; Gerfen and Wheeler, 1994 (cited by Wheeler, 1994), *cattle* (Strelchenko et al., 1991; Stringfellow et al., 1991; Saito et al., 1992; Sims and First, 1993; Cherny and Merei, 1994; Stice et al., 1994; Strelchenko and Stice, 1994), and *sheep* (Handyside et al., 1987; Anderson et al., 1990; Fléchon et al., 1990; Piedrahita et al., 1990a; Notarianni et al., 1991; Tsuchiya et al., 1994).

1.5.1 - EFFICIENCY OF STEM CELL INTRODUCTION INTO HOST MOUSE BLASTOCYSTS

It is generally accepted that only stem cells with normal karyotype are able to contribute to the germline of chimeric mice, which is the most crucial test of the development potential (Pease et al., 1990).

Despite the widespread application of mouse stem cell (ES) cell technology most reports on the production of mouse germline chimeras for transgenic studies centre on only a few mouse strains, namely D3, E14, CCE, AB1 derived of the 129 mouse strain, (Tokunaga and Tsunoda 1992). In a recent study, Kawase et al. (1994) demonstrated that ES cell lines can be established for various mouse strains, but their characteristics are different among strains.

When the CCE transfected stem cell line was used with combination of C57BL\6, an inbred and two outbred (CD-1 and MF-1) strains of host blastocysts (Schwartzberg et al., 1989), none of the outbred strains transmitted stem cell marker to their offspring, although the three strains produced coat colour chimeras. These authors consider that the genotypes of the recipient blastocysts can profoundly influence the overall incorporation of stem cells into the developing concepts.

Pease and Williams (1990) similarly stressed that the correct choice of recipient blastocysts is crucial when using stem cells as a vector system. These authors obtained chimeric mice using D3 stem cells, and with mouse strain ICR host blastocysts obtained 21.43% chimeras, and with C57BL\6 host

blastocysts obtained 27.27% chimeras. The chimeras originating from the outbred ICR blastocysts did not form ES cell-derived germ cells.

Besides the genotype combination between the strain of stem cells and host blastocyst, the stem cell as such can influence the incorporation in the host blastocysts, as was demonstrated by Zijlstra et al. (1989) in a study where 2 clones of transfected stem cells were obtained. Each clone was injected into C57BL\6J host blastocysts; clone A resulted in a few animals with a low degree of chimerism, and clone B resulted in extensive and healthy chimeras. Nevertheless, Gossler et al. (1986) obtained 8.05% of coat colour chimeric mice and 2.1% of germline chimeras with normal stem cells; using transfected stem cells they obtained similar results in respect to coat colour and germline chimeras.

1.5.2 - TRANSGENESIS ACHIEVED BY GENE TRANSFER TO EMBRYOS

Transgenesis is recognised as a major developmental objective in animal biotechnology. Transgenesis is the technology which allows the transfer of functional gene rearrangements (promoter and coding sequences) between organisms, irrespective of species barriers. In animals, gene transfer can be achieved in a variety of ways; the new gene if incorporated is present in the cell(s) of the animal body, including the germline, and is passed on to subsequent generations (Seamark, 1994).

As reviewed by Seamark (1991b) and Simons et al. (1993), genetic improvement in livestock is still based on traditional

animal breeding practices and dependent on identifying and incorporating into breeding lines natural variants, often mutants, with enhanced productivity or disease resistance characteristics. However, introducing novel characteristics in this way is a lengthy and often unpredictable process due to co-transfer of other, often undesirable, genes. Transgenesis can avoid such constraints through allowing transfer of the specific desired genes to existing superior genotypes independent of other genes.

There are numerous potential applications of transgenic methodology to develop new or altered strains of livestock.

I - Practical applications of transgenics in livestock production are: increase feed utilization by modifying farm animal carcass composition in relation to production and quality (Seamark, 1991b; Griffin et al., 1992; Pursel and Rexroad Jr., 1993; Pursel and Solomon, 1993); by making changes in the composition of milk or in the production of entirely new products in milk (Krausslich, 1986; McWhir et al., 1990; Jimenez-Flores and Richardson, 1988; Gutiérrez et al., 1995; Kang et al., 1986; Wheeler et al., 1995); by increasing wool production (D'Andrea et al., 1988; Ward et al., 1988; Rogers, 1990); by enhancing prolificacy and reproductive performance (McNatty et al., 1985; Nottle et al., 1993); by making new approaches for disease resistance of the livestock (Blattman and Beh, 1993; Müller and Brem, 1994; Storb, 1987; Wheeler et al., 1995).

II - The medical field could yield benefit from transgenic animals by the production of entirely new products from animal milk. The mammary gland can be used as a bioreactor for

production, for instance, of human proteins in the milk (Simons et al., 1988; McWhir et al., 1990; Wall and Seidel, 1992). Pigs could be engineered in way that they could have human epitopes in the cellular membrane of their tissues, allowing xenotransplantation (i.e. between humans and pigs) (Galili, 1993) which could be an alternative solution to the problem of organ donor shortage. Also pigs could be engineered to have and express human hemoglobin in their red blood cells, the purified porcine derived human hemoglobin exhibit an oxygen affinity similar to that of human derived human hemoglobin, (Swanson et al., 1992).

III - Biology could benefit from transgenesis by the production of transgenic animal models for: endocrinological studies, such as endocrine hyperfunction and hypofunction (Seamark, 1991a); genetic imprinting studies through androgenetic mouse embryonic stem cells, (Mann et al., 1990); transgenic mouse models for human genetic diseases such Lesch-Nyhan syndrome (Hooper et al., 1987) and familial amyloidotic polyneuropathy (Yamamura et al., 1993).

1.5.3 - METHODS OF GENE TRANSFER TO EMBRYOS

Using the combination of embryo and gene technology, the first transgenic animals produced by gene transfer were made by microinjection of SV40 virus into the blastocoel cavity of 3.5 old day mouse embryos (Jaenisch and Mintz, 1974). Later transgenes were created by retroviral infection (Jaenisch, 1976), or by microinjection of purified DNA into the pronuclei of mouse zygotes (Gordon et al., 1980); or by "pricking", DNA uptake by

cells via mechanical poration of their cell membrane by a microneedle (Yamamoto et al., 1981; Kanoh and Nagashima, 1984; Sato et al., 1993), and most recently by DNA sperm-mediated transfer prior to in vitro fertilisation procedures, (Lavritano et al., 1989; 1992; Francolini et al., 1993).

The direct microinjection into the pronuclei of the zygote allows the DNA to integrate randomly into a chromosome and subsequently be expressed in somatic and germ tissues of the resultant individual. Unfortunately, these manipulations greatly reduce embryonic survival to approximately 25-35% (Wheeler et al., 1995). The efficiency of producing offspring carrying foreign genes by this technique ranges from 0.5-3% of injected eggs resulting in transgenic young in livestock breeds (Murray et al., 1988; Rexroad and Pursel, 1988; Eyestone, 1994; Seamark, 1994). This approach has been routinely applied to produce transgenic sheep, goats, pigs and cattle as reviewed by Eyestone (1994).

The use of retroviral vectors and liposome carriers to transfer foreign DNA into the embryos of domestic livestock have been less successful than pronuclear injection (Murray et al., 1988).

The viral vectors make use of the natural capacity of viral nucleic acids to catalyse their own integration into a single genomic site (McWhir et al., 1990). Vectors based on DNA and RNA virus have been used; the first remain infective and are potentially lethal while the second can co-exist with their host without causing any apparent sign of disease (Seamark and Wells, 1991).

One of the primary disadvantages of the pronuclear injection; "pricking" method and sperm mediated transfer are unpredictable sites of incorporation effects. Also, there is poor repeatability of experiments due to random incorporation. This lack of control over the site of integration could result in DNA re-arrangements or deletions which can cause developmental defects or sterility. In addition it is possible to add but not remove sequences (Palmiter and Brinster, 1986; McWhir et al., 1990; Seamark and Wells, 1991). However pronuclear injection has an advantage over the other methods because the integrations occur in the single cell embryo and are perpetuated in all tissues (McWhir et al., 1990). The viral vectors are potentially an efficient means of achieving gene transfer but have specific limitations in the size of the gene construct that can be incorporated and problems of regulation owing to their uncontrolled site of insertion. Moreover the viral techniques remain constrained by institutional regulatory bodies because of concerns with respect to the biohazard potential of the vectors (Seamark, 1994). Lately, as reviewed by Seamark (1994), gene technology may overcome positional effects using flanking injected transgenes with base sequences homologous to targeted insertion sites; the use of overlapping gene sequences; the use of dicistronic targeting vectors to couple position-independent and position-dependent transgene sequences.

1.5.4 - TRANSGENESIS ACHIEVED BY THE USE OF STEM CELL AS VECTOR OF GENES

Gene transfer based on stem cell methods have the potential to increase the number of transgenic animals and offer several advantages for the production of transgenic livestock that cannot be achieved using other DNA vectors.

Stem cells are of particular interest as they promise to allow both site-directed insertion of transgenes and genetic manipulation of resident genes *in situ* (Robertson et al., 1986; Smith et al., 1988; Capecchi, 1989; Seamark, 1994). However the critical feature of this system is that it involves a tissue culture intermediate. Introduction of DNA constructs to the ES cells rather than embryos offers the technical advantage that low frequency events can be selected, screened and subcloned before reintroducing the ES cells into the embryo (Gossler et al., 1986; McWhir et al., 1990).

The introduction of mutations into the germline of an organism is one of the most powerful genetic methods for determining the functions of a specific gene product (Schwartzberg et al., 1989). The resultant individual is a chimeric embryo composed of two distinct genotypes, and all tissues of the chimeric offspring that originate from the ES cells carry the transgene in an identical site in the genome (Wheeler et al., 1995).

So far, however, only transgenic mice have been produced by this method (Capecchi, 1989). The confirmation of the feasibility of using the chimeric route for reinstating an ES cell genome into the germline of the pig is a major advance (Wheeler, 1994).

1.5.5 - ANALYSIS OF THE CHIMERIC ROUTE USING STEM CELLS

Transgenic animals can be produced and multiplied using the chimeric route with stem cells. As noted by Seamark (1988), chimera formation, while technically simpler to achieve, has the limitation that the offspring is a mosaic and thus the germline is not chimeric, with the result that at least two matings are required to obtain homologous transgenic animals (Tsunoda and Kato, 1993). Thus the chimeric route has limitations in its application for livestock species, particularly those with long generation times and bearing single young (Seamark, 1994). In the face of this difficulty a more direct route is required, which is possible if stem cells could be viewed as a source of karyoplasts for nuclear transfer rather than as cells for chimeras. With the development of nuclear transfer it should be possible to shorten the time required for obtaining transgenic mice, moreover the individuals obtained would be clones with identical genes deriving from the ES cell line.

1.5.6 - STEM CELL AND SEX REVERSAL

1.5.6.1 - INTRODUCTION

Another potential limitation of the chimeric route is that the production of chimeras using XY stem cells can be expected to cause a distortion in the sex ratio of the chimeric population (85% male) due to the conversion of XX host embryos into phenotypic males (Iannaccone et al., 1985; Robertson et al., 1986).

1.5.6.2 - PHYSIOLOGY OF SEX REVERSAL

As reviewed by Anderson (1987) in non-mammalian species, primordial germ cells have been shown to develop from yolk sac endoderm and a similar origin has been assumed for mammalian primordial germ cells. However there are two lines of evidence indicating that in mammals the germ cells are derived from ectoderm in mammals rather than from endoderm. The first stems from studies by Falconer & Avery (1978), who showed that germ cell chimerism is positively correlated with chimerism of somatic tissues. It has been shown more conclusively that it is ectoderm but not endoderm injected into blastocysts which contributes to germ cell lineage of the chimeras Gardner and Rossant (1979).

It has been shown that (Jost et al., 1973; Wilkins, 1993) the urogenital system experiences parallel differentiation. Initially both sexes possess both kinds of urogenital structures, the female Mullerian ducts and the male Wolffian ducts, and in each sex one duct system develops while the other degenerates. The initial determination of the undifferentiated gonad is to become testes, the presence of a Y chromosome in a certain critical cell population leads to the secretion of testosterone by the gonad and this secretion in turn triggers further male genital development. In the absence of testosterone, the gonad develops sex characteristics that are female.

The initiation of male development in mammals requires the one or more genes on the Y chromosome be those genes (Sry - 14 kilobase genomic DNA) which are sufficient to induce differentiation of the testes and subsequent male development

when introduced into chromosomally female mouse embryos, (Koopman et al., 1991).

Surani et al. (1987) reported that the greatest efficiency in obtaining germ cell chimeras from pluripotential stem cells would be obtained by introducing cells with XY genotype into XX recipient embryos such that all sperm produced by the pseudomale is 100% derived from the injected male ES cell line.

1.6 - EMBRYO TRANSFER OF MOUSE EMBRYOS

The other potential constraint on chimera production is the transfer of micromanipulated embryos to the female uterine tract.

In non-micromanipulated mouse embryos (reviewed by Wilmut et al., 1986) the prenatal mortality is influenced by a number of factors including genotype. The existence of variation in embryo development in mice has been defined and related to prenatal loss. A thorough investigation of the influence of the gene on prenatal survival has not been carried out, but breeding records suggest an association of the slow allele with poor reproductive performance. L.Flaherty (cited by Warner et al., 1984) and Gates (1965) (cited by Wilmut, 1986) contrasted the survival of the slow cleaving and fast cleaving embryos after their transfer to the opposite horns of recipients. Cleavage rate did not influence the proportion of embryos able to implant, but significantly, the slow embryos died after implantation.

Among mouse inbred strains, Pomp et al. (1989) verified significant recipient genotype effects for embryo survival and

prenatal growth, while no donor genotype effects were observed.

Paria and Huet-Hudson (1993) reported that the mouse uterus became receptive on day 4 (day 1 = day of vaginal plug) and by day 5 the uterus became refractory. Blastocysts also fail to implant in the ovariectomised uterus primed only with progesterone, but an additional oestrogen injection induces the receptivity for implantation which presumably lasts for 24 hours.

Optimal embryo transfer should be performed when the transplanted eggs are more advanced in development than the eggs of the recipient or are at the same age (Tarkowski, 1959), and the female recipient is at day 2 or 3 of pseudopregnancy (Pomp et al., 1989).

In embryos submitted to in vitro embryo culture there is a delayed rate of development when compared with embryos in in vivo culture. As observed by various authors (e.g. Vanderhyden, 1987; Foxworth and Kraemer, 1993). The authors also explain that a female mouse recipient should have a post coital plug from a non-fertile mating 24 hours after the donor exhibits a post coital plug from a fertile mating because embryo survival can be improved by allowing time for "catch up" to the physiological status of the recipient.

Uterine asynchrony was studied by Wilmut et al. (1988), who verified that the sheep uterus has the capability of stimulating development of relatively retarded embryos and less effect in slowing the development of advanced embryos. Similar observations were made by Fischer (1989) on rabbit blastocysts which were influenced by the progestational stage of the recipient. In this case it was observed that 1 day after transfer into asynchronous

older uteri, blastocyst diameters were larger and cell proliferation was increased, but the development in asynchronous younger uteri was delayed. This suggests an attempt by the blastocyst to adjust to the more advanced maternal milieu.

In summary the requirement for synchrony is interpreted as reflecting the changes in uterine secretions that must occur at appropriate stages of embryo development; it is a consensus that mouse embryo transfers should be done with eggs more advanced in development than the eggs of the recipient or at least of the same age i.e. between day 2 and 3 of pseudopregnancy. However there are no bibliographic references in relation to what is the best procedure for producing microinjected blastocysts, moreover whether those micromanipulated embryos present the same capability for adaptation into the uterus as non-micromanipulated embryos.

1.7 - IN VITRO MATURATION, IN VITRO FERTILISATION AND IN VITRO CULTURE OF OOCYTES.

Efficient in vitro techniques as low cost sources of eggs and preimplantation embryos are of considerable value for research purposes and for the development of new biotechnologies of agricultural interest. Production of transgenic offspring requires large numbers of zygotes at the pronuclear stage, for gene injection as well as blastocysts for stem cell injection.

Multiplication of identical embryos in vitro can be achieved by using mature enucleated oocytes from any donor as recipients for nuclei from valuable multicellular stage embryos.

Producing mature oocytes and early embryos *in vivo* by superovulation and insemination is not optimal for research and commercial purposes. Considerable variations exist among ruminant females in their response to the superovulation treatments, and repeated stimulations of a given animal lead to variable results. Furthermore, the moment of ovulation and fertilisation cannot be precisely predicted and spreading of these events over a period of several hours is common in these species. Therefore at the time of recovery the eggs are not synchronous and the right stage cannot be ascertained (see reviews by Gordon, 1990 and Crozet, 1991).

1.7.1 - *IN VITRO* MATURATION OF OOCYTES

1.7.1.1 - INTRODUCTION

Driancourt and Fry (1988) concluded that differentiation of ovulatory follicles in sheep can be viewed as a two step process (i.e. recruitment of healthy gonadotropin-sensitive follicles, that are > 2mm in diameter and the selection at about 4mm in diameter of the ovulatory follicle that becomes dominant while the others undergo atresia). Recruitment, which coincides with luteolysis is triggered by increased LH pulsatility, after follicles have been primed with FSH.

Mammalian oocytes are arrested at the diplotene stage of the first meiotic division (Crozet, 1991). In response to the preovulatory surge of gonadotrophins the oocyte undergoes resumption of meiosis characterised by germinal vesicle

breakdown, chromosome condensation, formation of the first meiotic spindle, expulsion of the first polar body and arrest in metaphase of the second meiotic division (MII) until fertilisation activates them to complete meiosis (McGaughey et al., 1990).

As originally observed by Pincus and Enzmann (1935) and cited by McGaughey et al., (1990), rabbit oocytes matured spontaneously in simple culture medium once they were removed from the follicular environment. The early investigators concluded that mammalian ovarian follicles constituted an inhibitory environment in which oocytes would not mature until follicular exposure to luteinizing hormone. Mammalian oocytes removed from their follicular environment are able to undergo spontaneous nuclear maturation in vitro, however the oocytes that reach Metaphase II under these conditions are not competent to progress to normal fertilisation and further embryonic development (Crozet, 1991).

Once the oocyte completes parallel nuclear and cytoplasmic maturation, oogenesis is accomplished and the oocyte is ready to be fertilised and to sustain embryonic development (Gandolfi et al., 1990). In sheep, cattle, pigs and mice the first observations related to oocytes matured in vitro were by Edwards (1965) and Sreenan (1968) who reported nuclear maturation as occuring in culture. However, subsequent work made it clear that the concept of oocyte maturation had to be widened to include all those events (cytoplasmic maturation) that enable the egg to be fertilised and express its developmental potential (Moor and Trounson 1977; Staigmiller and Moor 1984; Thibault et al., 1987).

1.7.1.2 - FACTORS RELATED TO IN VITRO MATURATION

1.7.1.2.1 - OVARY STORAGE PRIOR TO OOCYTE RECOVERY

Yang et al. (1990) indicated that cattle ovaries can be stored at a temperature of about 25ºC for at least 11 hours without compromising the ability of the oocytes to be fertilised and to subsequently produce normal embryos. It was demonstrated by Moor and Crosby (1985) that temperature fluctuations caused chromosome abnormalities in maturing sheep oocytes by disrupting microtubule structures demonstrating that maturing sheep oocytes are very sensitive to a drop in temperature.

1.7.1.2.2 - OOCYTE COLLECTION

An important aim of an oocyte recovery method is to maximise the number of oocytes per ovary obtainable for use in in vitro maturation, in vitro fertilisation and in vitro culture studies (Carolan et al., 1994).

Dissection and subsequent rupture of the intact follicles has been one of the methods employed to collect oocyte cumulus complexes, (Lu et al., 1987; 1988). However aspiration of follicular oocytes, using either a pipette or syringe and needle, has been the most common method of recovering immature cattle eggs (Sreenan, 1968; Leibfried and First, 1979; Leibfried-Rutledge et al., 1986; Leibfried-Rutledge et al., 1989).

Assey et al. (1994) demonstrated structural differences between oocytes aspirated from dominant and subordinate

follicles, thus the fact that most oocytes are collected when immature from subordinate follicles may be another major cause for reduced developmental capacity following in vitro maturation.

Keskintepe et al. (1993) highlight that the method of oocyte recovery determines the success of in vitro procedures. Although mincing ovaries resulted in an increased yield of goat oocytes the final result was better if the oocytes were recovered by aspiration, most likely reflecting less disturbance to the integrity of the cumulus oocyte complexes. The importance of the integrity of the cumulus oocyte complexes was demonstrated by Yang and Lu (1990) who obtained a significantly higher yield of embryos from oocytes that had 4-5 layers of cumulus cells compared with those with 2-3 layers.

Tan and Lu (1990) could not find any evidence indicating that the stage of the bovine oestrous cycle is a significant factor influencing the outcome of oocyte maturation. The same authors did however verify that oocytes recovered from follicles less than 2mm in diameter gave significantly lower yields of embryos after maturation and fertilisation than larger follicles.

1.7.1.2.3 - THE IN VITRO MATURATION CULTURE SYSTEM OF OOCYTES

The first successful in vitro maturation systems were based on culture of the intact follicles supplemented with gonadotrophins and steroids in order to retain both the endocrine and local cellular regulatory mechanisms (Galli and Moor, 1991). Oocytes cultured within the follicle in hormone-free medium (Moor and Trounson, 1977) remained at the germinal vesicle stage. In

the presence of FSH and LH some oocytes reached the second meiotic metaphase in non-atretic and atretic cultured follicles.

In oocytes cultured outside the follicle (Galli and Moor, 1991) in the absence of exogenously added gonadotrophin fewer than 30% of oocytes reached metaphase II as compared with more than 80% in hormone supplemented medium. However, this large difference in the progression of the meiotic cycle was not reflected in the developmental capacity of oocytes from the two groups (gonadotrophin group, 34%; hormone-free group 20%).

1.7.1.2.3.1 - MEDIUM COMPOSITION, BUFFERING SYSTEM (pH), OSMOLARITY AND TEMPERATURE FOR IN VITRO MATURATION OF OOCYTES

Any culture medium employed in in vitro maturation has to give similar substrate conditions as the follicular fluid to promote the nuclear and cytoplasmic maturation of the oocyte. Various medium compositions have been used for in vitro maturation ranging from simple to complex physiological solutions with amino-acids, vitamins, purines and other supplements shown to be useful for general cell culture (Gordon, 1990).

The tissue culture medium 199 (TCM-199) has been widely employed in the maturation of ruminant oocytes, bovine (Catt, 1990; Gordon, 1990; Armstrong et al., 1992; Carolan et al., 1994), sheep (Holm et al., 1990; Pugh et al., 1990; Czlonkowska et al., 1991; Watson et al., 1994), and goats (Younis et al., 1991; Ling et al., 1992; Mogas et al., 1995).

The buffering system most widely used is the $NaHCO_3$ system, in conjuction with a 5% carbon dioxide in air gas atmosphere at

maximum humidity, resulting in a system with a pH which fluctuates between 7.1-7.4.

The osmolarity of the medium is close to that of body fluids (250-316 mOsm) but this could be expected to vary depending on the embryo species and type of medium used. However Walker et al. (1992) found that varying the osmolarity of SOFM between 250-300 mOsm did not significantly affect the development of sheep zygotes.

The incubation temperature for optimal in vitro maturation occurred at the average body temperature of the related species in sheep (Pugh et al., 1990) bovines, (Lenz et al., 1983; Gordon, 1990), and pigs (Eng et al., 1986).

1.7.1.2.3.2 - GRANULOSA CELLS AND MORPHOLOGICAL INTEGRITY OF OOCYTE-CUMULUS COMPLEXES.

The hypothesis that follicle cells play a central role in endowing the oocyte with developmental competence during maturation has been tested in co-culture studies by Staigmiller and Moor (1984). These researchers found, in sheep, that denuded and corona-enclosed oocytes resumed meiosis in culture but remained immature and developmentally incompetent. By contrast, 42% of oocytes supported by the cumulus and underlying granulosa (cumulus-oocyte complexes) underwent full maturation and normal subsequent embryonic development. Addition of supplementary follicle cells during culture ($5X10^6$ cell/ml of medium) was found to be without beneficial effect on the denuded oocytes. Galli and Moor (1991) demonstrated the importance of the junctional

coupling between sheep oocytes and somatic cells. Subsequently Galli and Lazzari (1995) showed that supplementary cells do confer competence on bovine cumulus-oocyte complexes.

In pigs there is some evidence that thecal cell support may favourably influence the developmental competence of oocytes (Mattioli et al., 1988) and a similar effect may apply in cattle (Gordon, 1990).

1.7.1.2.3.3 - PROTEIN SUPPLEMENTATION

Difference between protein supplements in in vitro maturation medium for gametes was demonstrated by Leibfried-Rutledge et al. (1986) who concluded that fetal calf serum was the superior protein supplement when compared to 6mg/ml bovine serum albumin.

Other researchers (Sanbuissho and Threlfall, 1988) obtained similar results and showed that when bovine serum albumin, estrous cow serum or fetal calf serum and their interaction with gonadotropins were examined, estrous cow serum or fetal calf serum was superior to bovine albumin serum in promoting the maturation of bovine oocytes.

In 1989 the same authors found no significant effect of serum obtained from a cow at the time of standing estrus, at ovulation, and at 24 hours after ovulation on the in vitro maturation of oocytes. An analogous conclusion was verified by Fukui and Ono (1989) who found no statistical difference between bovine oocytes in vitro when matured with fetal calf serum and oestrous cow serum.

In contrast, Saeki et al. (1991) showed that serum supplementation during bovine oocyte maturation was not required but hormonal supplementation with gonadotrophins and oestradiol enhanced the fertilisability and developmental ability of bovine oocytes matured in vitro. The authors highlighted that it is possible that hormones contained in the sera play an important role in nuclear and cytoplasmic maturation.

These effects were further investigated by Mogas et al. (1993) who showed no statistical difference among treatments in terms of maturation rate in the in vitro cleavage capacity of in vitro fertilised prepubertal goat oocytes matured with estrous goat serum with or without added hormone. However, at 48 hours post-insemination significantly higher cleavage rates were obtained in treatments which included hormones.

1.7.1.3 - OOCYTE CYTOPLASMIC MATURATION

Damiani et al. (1995) evaluated the cytoplasmic maturation of calf oocytes using transmission electron microscopy and microfluorometry and verified that calf oocytes after in vitro maturation showed an incomplete or delayed redistribution of cytoplasmic organelles.

1.7.1.4 - OOCYTES OBTAINED FROM STIMULATED FOLLICLES

Stimulation regimes are widely employed to increase the number of eggs harvested in order to improve the chances of

successful IVF (Testart et al., 1983). However, a number of studies have shown that following such treatment many follicles show an abnormal steroid content and abnormal cytology several hours before ovulation. Examination of oocytes collected just before the supposed time of ovulation following superovulation treatment has revealed a large proportion of immature gametes. Further, half of the oocytes collected were surrounded by an incompletely dissociated cumulus mass. Nevertheless Pugh et al. (1990) reported that in vitro matured oocytes obtained from FSH slaughtered primed ewes gave better cleavage rates than oocytes from non-primed ewes. The size of follicle from which an oocyte is derived before in vitro maturation influences its subsequent developmental potential when used for embryo cloning (Barnes et al., 1993). Earlier reports (Motlik and Fulka, 1986, cited by Barnes et al., 1993) described the RNA synthetic activity of oocytes derived from different follicle size categories, which led to the postulation that the follicle size from which an oocyte is derived may ultimately affect its developmental competence.

In summary, the complexity of the oocyte maturation (both nuclear and cytoplasmic) has led to a wide variety of experiments aimed at understanding and characterising the physiology and molecular events of oocyte maturation, while at the same time identifying suitable conditions which can be used in the in vitro maturation of mammals oocytes for commercial and research purposes.

The costs of producing oocytes and zygotes from in vivo sources are too expensive for their use in many advanced

reproductive technologies and transgenic programs, but their use is essential as reference control groups for in vitro maturation studies.

It is important to highlight that the conditions used for in vitro maturation of oocytes of different species is similar yet different.

1.7.2 - IN VITRO FERTILISATION

Fertilisation is the period of development commencing with the contact of the spermatozoa with the outermost investment of the animal egg and ending with the fusion of sperm and egg pronuclei within the ooplasmic mass. The in vitro fertilisation consists in the fertilisation of mammalian eggs outside the body as defined by Austin and Short (1990).

The essential conditions for in vitro fertilisation are sperm capacitation, sperm concentration, medium, buffering system (pH), osmolarity and temperature.

1.7.2.1 - SPERM CAPACITATION

The normal fertilisation and the healthy development of normal fertilised embryos can only happen when the oocyte has been through nuclear and cytoplasmic maturation with capacitated sperm.

Epididymal and ejaculated spermatozoa are actively motile but not able to fertilise oocytes.

Sperm capacitation physiologically takes place in the

genital tract of the oestrous female. Such a process initially consists of a biochemical alteration of the sperm plasma membrane (Gandolfi et al., 1990) followed by the acrosome reaction which is the fusion of the plasma membrane with the outer acrosomal membrane (Yanagimachi and Usui, 1974).

Capacitation is not site specific and can occur, for instance, in the peritoneal cavity or without the spermatozoa having passed the female tract (Yanagimachi, 1989), in the bovine follicular fluid (Fukui et al., 1983), in the bovine oviductal cells (Xu and King, 1990), or even in a variety of artificial medium like elevated pH (Cheng, 1985, cited by Gordon, 1990); ionophore A23187 (Hanada, 1985), heparin (Parrish et al., 1985;1988), percol gradient/hypotaurine (Utsumi et al., 1988) and caffeine (Niwa et al., 1988).

1.7.2.2 - SPERM CONCENTRATION

According to Cummings and Yanagimachi (1982) in vivo fertilisation is likely to occur in conditions where sperm and egg ratios are close to one, however in in vitro conditions such ratios are about 2000-200000:1 (Brackett et al., 1982; Lambert et al., 1986; Lu et al., 1987; Mattioli et al., 1989). The consequence of this high concentration is polyspermy, because in vitro matured oocytes are likely to develop defective polyspermy blocks (Gandolfi et al., 1990).

1.7.2.3 - MEDIUM COMPOSITION

The medium used for *in vitro* fertilisation has to give metabolism support to the cumulus oocyte complexes and allow efficient spermatic function.

In vitro fertilisation of oocytes has been achieved by the use of medium whith components that mimic the tubal oviductal fluid, Synthetic Oviduct Fluid Medium-SOFM (Tervit et al., 1972) and Human Tubal Fluid-HTF (Quinn et al., 1985).

In vitro fertilisation medium is composed of electrolytes (calcium, $NaHCO_3$); energy source (glucose, Na lactate and pyruvate); macromolecules (bovine serum albumin, polyvinyl alcohol); additives (caffeine, hypotaurine and catecholamines), as reviewed by Cox (1990).

Electrolytes have the function of assisting the transport through the membranes and maintaining the intracellular pH, and osmotic balance. Specifically, calcium is a important regulator of fertilisation by spermatic capacitation, acrosome reaction and flagellar activity (Fraser and Ahuja, 1989; Lindemann and Kaunus, 1989). It has been verified by Huneau and Crozet (1989) that raising the calcium concentration in the fertilisation medium to a value higher than that present in tubal fluids from oestrous ewes increased the fertilisation rate achievable *in vitro* by individual ejaculates from various rams.

The pH of the medium is generally controlled using $NaHCO_3$ which has the advantage that it is also known to stimulate spermatic metabolism (Cox, 1990).

The source of energy used in *in vitro* fertilisation medium is a combination of glucose, lactate and pyruvate. Physiologically these components are present in the oviduct fluid

(Leese, 1988).

The macromolecule source used in *in vitro* fertilisation medium is generally bovine serum albumin. The albumin is present in the secretions of the female genital tract (Leese, 1988) and in the follicular fluid (Wise, 1987). Macromolecules such as polyvinyl alcohol has also been used successfully in *in vitro* systems (Cox, 1990).

The additives used to stimulate the motility and fertility of mammalian spermatozoa include caffeine, hypotaurine, epinefrine and isoproterenol (Meizel, 1985; Cox, 1990).

1.7.2.4 - TEMPERATURE

The temperature for stable *in vitro* fertilisation is generally close to the average temperature of the related species. This follows the early studies by First and Parrish (1987) which showed that small variations in temperature severely affect the final success rate.

1.7.2.5 - *IN VITRO* FERTILISATION AND RUMINANTS

As reviewed by Crozet (1991), *in vitro* fertilisation has been achieved in ruminants, bovines, sheep and goats.

In bovines, Armstrong et al. (1992) obtained 70% *in vitro* fertilisation of oocytes matured *in vivo* following FSH and HCG treatment in calves, but this percentage was not significantly different from control oocytes recovered from adult cow ovaries matured *in vitro* (75%).

It has been observed by Fukui and Ono (1989) that the bovine *in vitro* fertilisation rate is significantly affected by serum type. In their experiments, fetal calf serum gave significantly higher rates (57-71%) than did oestrous cow serum (34-52%), but the proportions of polyspermic fertilisations were significantly higher with fetal calf serum (8-19%) than with oestrous cow serum (2-3%). However, cleavage and development was not affected by either type of serum.

In sheep, Cheng et al. (1986) obtained a penetration rate of 80% of ram spermatozoa in *in vitro* matured sheep oocytes. Over 97% of the fertilised oocytes were monospermic and showed normal pronuclear development at 16 hours post insemination *in vitro*. Using fresh semen and *in vitro* matured oocytes Pugh et al., (1990) obtained cleavage rates of between 51-71%, while Holm et al., (1990) obtained 50.1 - 50.5% *in vitro* fertilisation as determined by cytohistology.

With oocytes obtained from superovulated ewes and matured *in vivo*, Huneau and Crozet (1989) obtained rates of *in vitro* fertilisation between 50-75% and monospermic penetration of (41-58%). Cognié et al., (1991) obtained 115 zygotes from *in vitro* matured oocytes which were fixed 17 hours after *in vitro* fertilisation. Eighty-two percent (95 oocytes) were fertilised and 61% (71 oocytes) were monospermic, as attested to by the presence of both a male and female pronucleus and by the remnants of sperm flagellum in the ooplasm. Sixty-three percent of the oocytes underwent the first cleavage division.

In goats, Huang et al., (1989) flushed 48 *in vitro* matured oocytes from 6 donor female goats which were subsequently

cultured with capacitated spermatozoa and 33 were transferred to the oviducts of pseudopregnant rabbits after 4-6 days. Seven of these eggs developed to normal morulae. Ling et al. (1992) obtained 12 fertilised embryos from 17 in vitro oocytes.

In conclusion, in vitro fertilisation is a biotechnique now developed to such a stage that it offers reliable and repeatable procedures for the three ruminant livestock species. The source of in vitro and in vivo matured oocytes does not affect cleavage rates, but bovine serum presents a positive effect on in vitro fertilisation but not on cleavage rate and development of the fertilised oocytes.

This biotechnique (in vitro maturation IVM and in vitro fertilisation IVF) was not mentioned in the available literature as a viable method for production of preimplantation zygotes between goat oocytes and sheep sperm.

1.7.3 -IN VIVO\IN VITRO CULTURE OF EMBRYOS

Embryo culture is one of the fundamental steps for embryo production. The major interest in culture medium presently lies in the availability of reliable, stable culture medium preparations that are compatible with the viability of embryos during exposure for relatively brief periods of time. This involves the transfer of embryos that have been grown from an early stage in culture following some invasive procedure such as nuclear transfer, gene transfer, and/or in vitro fertilisation of in vitro matured oocytes (Bavister, 1988).

The early systems employed for embryo culture were in vivo

systems and consisted of surgical transfer of embryos to the fallopian tubes of a temporary host, usually a sheep (Willadsen, 1979) or a rabbit (Boland, 1984).

Tervit et al. (1972) was the first to report the benefit of in vitro culturing of ovine and bovine in vivo matured/fertilised zygotes in a simple defined salt solution (SOFM) under a reduced oxygen environment of 5% CO_2: 5%O_2: 90% N_2. From this work, many variants were developed to improve the culture of ruminant embryos, much triggered by the work of Gandolfi and Moor (1987) which demonstrated the ability of oviduct cell monolayers to support passage of sheep embryos through the critical 4^{th} cell cycle. The positive results of the sheep oviduct epithelial cell monolayer method on sheep embryos extended this system to several different species like cows, goats, mice, pigs, rabbits (Gandolfi et al., 1990).

Embryonic development can also be promoted by embryonic growth factor of low molecular weight contained in some samples of bovine serum albumin (Kane, 1985), co-culturing embryos with trophoblastic vesicles (Camous et al., 1984) or with granulosa cells (Goto et al., 1988).

Walker et al. (1992) studied an in vitro culture system for sheep preimplantation embryos that did not employ the use of somatic cell feeder layers; this system relied almost exclusively on the use of human serum as the nitrogen source. This system can generate relatively high numbers of blastocysts from zygotes cultured in vitro (up to 85%), the resulting cell numbers are about 60% of in vivo developed blastocysts. Thompson et al. (1992) reported that the inclusion of non-essential amino acids

in serum-free SOFM medium significantly increased blastocyst formation and resultant cell numbers compared to embryos developed in SOFM plus human serum albumin. However, embryo development in the presence of non-essential amino acids was still significantly less than in the presence of serum.

A study by Watson et al. (1994) compared the effectiveness of two distinct methods of supporting ovine preimplantation development *in vitro*. In the first system compared, the proportion of co-cultured IVMF (*in vitro* matured and fertilised) zygotes developing to the blastocyst stage was significantly higher (38% vs. 3.5%) than that of non co-cultured zygotes under a 5% CO_2 atmosphere. In the second approach the embryos were cultured under a reduced oxygen atmosphere (5%CO_2: 5%O_2: 90%N_2). Embryonic survival was significantly higher ($p<0.05$) in the non co-cultured treatment groups (21.9% vs. 0.4% for co-cultured zygotes). The authors highlight that co-cultured may exert a positive influence on early development by the secretion of embryotrophic factors into the culture medium and \ or by reducing the negative effects on preimplantation development of "toxic" components of the culture environment. Gardner et al. (1994) demonstrated that sheep embryo development in culture is impaired by ammonium. Sheep embryos produce a factor or factors that stimulates their development in culture. The culture of sheep zygotes in groups of 4 in the presence of Eagle's amino acids for 6 days, with the medium renewed every 48 hours, resulted in 95% blastocyst development, 79% hatching rate, and a blastocyst cell number of 173.

In vitro and *in vivo* culture systems were compared

simultaneously by Walker et al. (1991) in a study which examined the viability, to day 50 of pregnancy, of day 5 sheep embryos cultured in vitro from the zygote stage. The authors verified that fewer embryos cultured in vitro developed to day 50 of pregnancy compared with embryos cultured in vivo. The authors concluded that while zygotes can develop to blastocysts in vitro at rates comparable with that obtained in vivo, viability in the former group is reduced. In a similar study with ovine oocytes matured and fertilised in vitro which were submitted to in vitro and in vivo culture they produced 7.7% and 43% of lambs were produced respectively (Czlonkowska et al., 1991). In a further study, (Walker et al., 1992) obtained more zygotes developing to blastocyst in vitro compared with in vivo (91.1% vs. 78.4%), indicating that in vitro culture may protect embryos from selection, a physiological occurrence in the oviduct. As such, the viability of embryos (to day 50 of pregnancy) after 5 days of culture was reduced compared with embryos cultured in vivo (48.2% vs. 59.4%).

According to the literature reviewed the in vitro culture of ruminant embryos demonstrates that, at least morphologically, the preimplantation embryos are similar to the in vivo culture, however the (in vitro culture) does not completely mimic the fallopian tube environment (in vivo culture). This was demonstrated by studies which the end point was determined as the stage of advanced pregnancy or at delivery of offspring.

1.8 - NUCLEAR TRANSFER

1.8.1 - INTRODUCTION

As highlighted by Tsunoda and Kato (1993), if embryonic stem cells could develop directly into young through nuclear transfer rather than the chimera route, time required to obtaining homologous transgenic offspring would be dramatically shortened.

Cloning by nuclear transplantation entails the transfer of each nucleus of a multiple cell embryo into the cytoplasm of an enucleated metaphase II oocyte. The mature oocyte has the ability to reprogram the transplanted nucleus and to support development of a new embryo. After nuclear transfer and activation of the oocyte, the recipient oocyte initiates a remodelling of the donor nucleus, including swelling and growth of the nucleoli. These changes indicate a reprogramming of the nucleus. By the use of a metaphase II recipient cytoplasm, offspring have been produced in sheep, cattle, rabbits and pigs (Smith and Wilmut, 1989; Robl and Stice, 1989; Prather et al, 1989; McLaughlin et al., 1990; Collas and Robl, 1990). As pointed out by Smith (1992) the overall efficiency in producing "cloned embryos" remains low due to lack of knowledge of the many steps involved in the technique.

The following technical steps are necessary to achieve the cloning technology: a) choice of suitable donor embryos and or karyoplasts; b) choice of eggs for recipient cytoplasm; c) enucleation; d) fusion; e) activation; f) <u>in vitro</u> culture of the micromanipulated embryos. This review will focus on the activation and enucleation steps.

1.8.2 - ACTIVATION

Parthenogenetic embryos develop without any contribution from the male gamete (Beaty, 1957; Kaufman, 1983 (cited by Henery and Kaufman, 1993)). Their development can be initiated both spontaneously and experimentally by exposing the unfertilised oocyte to a variety of activating stimuli. Parthenogenetic embryos differ from gynogenetic embryos, as in the latter spermatozoa act as the activating stimulus, with the male genome being subsequently eliminated and taking no further part in the development of the embryo, (Henery and Kaufman, 1992).

Activation as defined by Rickords and White (1992) results in the formation of one pronucleus and one polar body, two or more pronuclei, or two cells each containing a nuclear structure.

The activation of the oocyte is essential to allow transformation of the nucleus of a penetrating spermatozoon into a pronucleus. Activation is also a critical requirement in nuclear transfer cloning, where a nucleus introduced into an enucleated oocyte is remodelled, enlarges, and becomes pronucleus-like, with subsequent DNA synthesis only if the oocyte is activated, as reviewed by Marcus (1990).

Mammalian oocytes may be activated by raising or lowering the temperature, by various chemical treatments externally applied or injected, by electric shock or pressure, and by mechanical or microsurgical stimulation of the egg (Markert, 1982). As reviewed by Fukui et al. (1992), parthenogenetic activation of mammalian oocytes can be induced by various stimuli, such as exposure to ethanol, calcium ionophore and

electric current.

Rickords and White (1992) have used a variety of treatments to alter calcium and induce artificial activation in mammalian oocytes. It has been demonstrated that in the mouse direct injection of calcium into the ooplasm results in parthenogenetic activation. Co-incubation with calcium ionophore A23187 causes release of calcium from intracellular stores and induces oocyte activation in several species. In addition, incubation in 7% ethanol is widely used as an effective activation procedure in the mouse and has been shown to be effective in activating bovine oocytes.

It has been established in various species that a transient increase in intracellular free calcium ion concentration is associated with fertilisation. An increase in calcium results in cortical granule exocytosis and initiates resumption of second meiosis, with subsequent second polar body extrusion (Marcus, 1990).

Henery and Kaufman (1992) showed that the lack of a paternal genome in parthenogenetic embryos clearly limits their postimplantation development, but apparently not their preimplantation development, since morphologically normal blastocysts can be formed.

Fulka et al. (1992) argued that maturation promoting factor (MPF) is the key cell cycle regulator in many cells, including oocytes. It is clear that MPF activity in mouse oocytes is high at MI, drops sharply as the oocyte enters anaphase, remains depressed throughout the anaphase-telophase transition, and increases again at the telophase-metaphase II boundary. Following

fertilisation or parthenogenetic activation, the biological MPF activity again falls precipitously from high MII levels to low levels during pronuclear formation.

Presicce and Yang (1994) have hypothesized that matured mammalian oocytes constantly synthesize a group of highly transient, labile proteins such as cytostatic factor (CSF) or the c-mos proteins, which maintain the function and the persistent high level of MPF.

The fertilising sperm normally initiates numerous, periodic, elevations of intracellular free calcium in the oocyte over a period of several hours depending on the species. It is believed that the function of these Ca^{2+} elevations is to destroy the existing and the nascent CSF. This in turn causes degradation of cyclin B and thus inactivation of MPF and resumption of meiosis. This probably is because a single Ca^{2+} elevation induced by these single artificial stimuli, including ethanol, can destroy only the existing CSF.

Because CSF is believed to be continuously synthesized in the young but not in aging oocytes, only aging oocytes can be readily activated by a single Ca^{2+} elevation stimulation. In contrast, the young oocytes may have both the presynthesized and the continuously newly synthesized CSF. The latter may not be affected by the single Ca^{2+} rise which could explain the poor response of young oocytes to ethanol treatment alone.

Presicce and Yang (1994) combined treatment regimen, ethanol-induced Ca^{2+} elevation would inactivate the existing CSF, and the subsequent cycloheximide exposure would prevent renewal of CSF synthesis in the oocyte.

Using ethanol and cytochalasin B treatments, Fukui et al. (1992) obtained 22-46% of parthenogenetic embryos cleaving to the 2- to 8-cell stage, while 5-11% developed to the blastocyst stage when oocytes were matured for 27 to 36 hours.

Nagai (1987) found that, in bovine oocytes cultured for 27-33 hours before ethanol treatment, 60-68% of the oocytes were activated, as demonstrated by a female pronucleus, whereas maturation for 24-26 hours resulted in a low activation rate (25-38%). King et al. (1988) reported a high level (46%) of spontaneous activation of bovine oocytes matured in vitro for 24-27 hours, when the cumulus cells were partially removed.

1.8.3 - ENUCLEATION

Ideally, an optimal enucleation procedure should be effective in eliminating the genomic material from the oocyte at all times, with little or no harm to the other non-genomic components. This step is necessary to completely eliminate the genetic contribution of the host cytoplasm and thus ensure maximal similarity between cloned animals and avoid ploidy abnormalities which detrimentally affect normal development (Smith 1992).

Smith (1992) reviewed four different approaches for enucleation:

The first consists of the bisection of the oocyte into two equal portions and the use of both halves for cloning. Half of the embryos produced will be triploid and therefore unable to develop normally to term.

The second approach is the physical or mechanical elimination of the chromosomes. This approach utilises the position of the first polar body to locate the metaphase plate followed by aspiration and elimination of a quarter to half of the adjacent oocyte cytoplasm. However this procedure has limitations. Considerable technical skill is necessary and the method probably removes important cytoplasmic components which may reduce cytoplast viability (Fulka and Moor, 1993). This procedure is complicated by the fact that in some oocytes the first polar body degenerates or migrates away from the chromosomes (Robl and Stice, 1989). Furthermore, as the oocyte ages the chromosomes have a tendency to migrate towards the centre of the oocyte causing an improper removal of chromosomes.

The third approach is the combination of physical enucleation and a chemical, using a DNA staining dye such as Hoechst 33342 with UV light in order to check for the removal of the chromosomes.

The last approach is regularly used for the enucleation of amphibian oocytes used for nuclear transplantation where chromosomes are destroyed with ultraviolet light-UV irradiation, which therefore eliminates the need for physical removal of the chromosomes. The use of UV irradiation has been considered as a potential enucleator of bovine cytoplasts for nuclear transplantation, however such irradiation clearly induces cytoplasmic changes in addition to effects upon the nuclear material (Fulka et al., 1993).

Fulka and Moor (1993) developed a chemical enucleation procedure which is not an invasive method and has the capacity

to enucleate large numbers of oocytes. At the same time other group of researchers (Tatham et al., 1993) demonstrated the use of centrifugation of oocytes in a Percoll gradient as a procedure to enucleate large numbers of bovine oocytes into fragments in such a way that the metaphase II plate breaks from the oocyte as it is stretched apart. Enucleation by centrifugation enables the rapid production of large numbers of enucleated oocyte fragments which can be used for the successful production of bovine nuclear transplantation embryos.

1.9 - SUMMARY

The present study explored a range of methods of interest to the application of recent advances in reproductive technology to the preservation of endangered mammals and to the multiplication of novel genotypes of transgenic animals.

The focus of research on interspecific pregnancies relate to their potential use as models for the study of maternal/fetal interactions. As previously discussed, the studies also offer an opportunity for the preservation of endangered mammals, particularly if embryos from endangered species can be generated in the laboratory and engineered in such a way that the uterine allogenic trophoblast can protect a xenogenic inner cell mass, (Fehilly et al., 1984b; Anderson, 1988).

The first specific objective of the study was to explore the use of <u>in vitro</u> techniques to generate hybrid preimplantation stage embryos between the sheep and goat. Chapter three describes experiments designed to evaluate conditions for <u>in vitro</u>

maturation and *in vitro* fertilisation of sheep oocytes in order to optimise this technique for the production of hybrid embryos.

In chapter four comparison is made of the efficiency of *in vitro* fertilisation procedures using sheep semen and goat oocytes which came from *in vitro* and *in vivo* sources.

In chapter five a study of *in vitro* fertilisation of the oocytes from hybrid female which have matured *in vitro* and *in vivo* is presented.

The second specific objective of the work was the production of sheep-goat chimeras as recipients of hybrid embryos. One possible strategy towards producing such hybrids (as demonstrated by Fehilly et al., 1984b; Meinecke-Tillmann and Meinecke 1984; Rossant et al., 1983) is to overcome the problem of the rejection of trophoblast genotype. It was thought initially that a chimeric uterus could help overcome this problem allowing a hybrid embryo (hybrid trophoblast) to develop, however since completing the present study Gustafson et al. (1993), have shown that while chimeras are capable of carrying hybrid pregnancies longer than normal ewes and does, none carried to term. In contrast, MacLaren et al. (1992; 1993) have shown that sheep-goat chimeras were capable of carrying ovine pregnancies to term, indicating that the uterine environment of the chimeras used were functionally ovine allowing lambs but not kids to survive.

Initial experiments have been carried out in mouse embryos in order to learn the basic micromanipulative procedures and to apply these skills to the creation of the sheep-goat chimeras. However due to the limitations of time and resources the last experiments were not executed and the studies focused on creation

of mouse chimeras.

Embryonic stem cell technology is important and proving of immense value in all aspects of cellular, molecular and developmental biology, and has high potential applications to the animal breeding industry for research and commercial purposes. Presently, its application is restricted to the mouse and its potential extension to livestock is still being explored.

According to Schwartzberg et al. (1989) and Pease and Williams (1990), the genotypes of recipient mouse blastocysts can profoundly influence the overall incorporation of stem cells in the developing conceptus, thus creating a potential constraint on the application of the stem cell and chimera technology to livestock species.

The experiments related to mice explore the feasibility of the use of other mouse strains as host blastocysts for stem cells and verify chimeric compatibility between inbred and outbred strains of mice and identify other possible limitations.

In chapter seven was study the relationship between the genotype of the blastocysts and stem cells and in chapter eight, the success of chimera production was explored in experiments in which the influence of mouse embryo transfer, using micromanipulated embryos performed at two different times of pseudopregnancy was evaluated.

In chapters nine and ten the compatibility of two strains of mice (C57BL\6 and SWISS albino) on chimera production was assessed. These two strains were used for the creation of chimeras by inner cell mass transfer and embryo aggregation.

As pointed out by Sleigh and Hannan (1993) and Seamark

(1994) the chimeric route has limitations when applied to livestock species, particularly those with long generation times and bearing single young. There is also no guarantee that the offspring will be germline chimeras. In light of these limitations, studies were initiated and reported in chapters 11 and 12, on the use of nuclear transfer as a more direct route towards restoring the ES cell genotype into the breeding line.

II - GENERAL MATERIAL AND METHODS PART I

The experiments were carried out in New Zealand with the participation of Massey University and the Department of Industrial Research (DSIR) in Palmerston North, during the period of March 1990 to October 1991.

The in vitro maturation and fertilisation systems used in this study were an adaptation of the in vitro bovine system used at DSIR and the protocols for in vitro sheep system were those used at Ruakura Research Centre in New Zealand.

2.1 - ANIMALS

The animals used in the experiments were five New Zealand Romney Marsh rams, a flock of feral goats and a single female geep, which is the hybrid animal resulting from a natural ♂ sheep and ♀ goat crossing (Stewart-Scott et al., 1990). The animals' health status was controlled by DSIR technical staff and the females were kept separated from the males in different paddocks with pasture and water *ad libitum*.

2.2 - SUPEROVULATION

2.2.1 - FERAL GOATS

The goats were superovulated in a cycle regulated with an intervaginal control internal drug releasing (CIDR-S) devices. The CIDR-S was introduced at an unknown phase of the oestrous cycle. Appendix 1 shows the different treatments imposed.

2.2.2 - HYBRID FEMALE

The geep was superovulated on three occasions. In the first treatment, a CIDR-S was inserted for 14 days prior to administration of 1200 IU of PMSG 48 hours before CIDR-S removal. In the second treatment CIDR-S was in place for 16 days prior to administration of 1500 IU of PMSG 48 hours before removal.
The third treatment 1500 IU PMSG was administered on removal of CIDR-S after 15 days of insertion at which time the CIDR-S was replaced by a new one for the remaining 2 days of treatment, (appendix 2).

2.3 - SOURCE OF OOCYTES

2.3.1 - SHEEP OOCYTES

I - Ovaries were recovered from ewes at slaughter at the local abattoir and transported to the laboratory within a period of 2.5 hours in a thermos flask which had been preheated to 39ºC.

At the laboratory the ovaries were washed 3 times with PBS at 38.5 ºC containing antibiotics prior to isolation of the oocytes.

2.3.2 - GOAT AND HYBRID FEMALE OOCYTES

The oocytes were obtained from ovaries of slaughtered female goats or by surgical procedure from live ones.

The female goat ovaries were collected at slaughter on site at DSIR.

For surgery, anaesthesia was induced with Thiopentone Sodium (Pentothal, Boehringer Ingelheim), and the animals maintained under Halothane (Fluothane, ICI) and oxygen using a McGill open circuit anaesthetic machine. The uterine tract was exposed and one of the following procedures executed:

2.3.2.1 - OVIDUCT FLUSHING

The oviducts were canulated with a 2 mm plastic cannula inserted into the infundibulum and the oocytes collected in a 90 mm petri dish (Falcon U.S.A.) to which was added 20 ml of (Hepes 199, BSA 0.4%) medium introduced with the aid of a 20 ml syringe fitted with a blunted 18 gauge needle into the uterine isthmus junction.

The cannula used for the oviduct flushing was flared and annealed at one end to help maintain the cannula inside of the oviduct during the flushing procedure.

2.3.2.2 - FOLLICULAR ASPIRATION

Follicles were aspirated with a 18 gauge needle bevelled to an angle of 45º and connected to a 5 ml syringe pre-loaded with 1 ml of Hepes 199, BSA 0.4% and Heparin 50 µg/ml. Following the aspiration the follicular fluid and medium mixture was poured in a 20 ml plastic container (Disposable Products, South Australia) maintained at 38.9ºC.

Immature oocytes were obtained using aspiration of non-developed follicles from the non-superovulated animals.

In vivo matured oocytes were obtained by aspiration of developed follicles and by oviduct flushing in superovulated animals 44 - 52 hours after CIDR-S removal.

The oocytes were recovered using a Gilson 10 µl pipette and transferred to 30 mm petri dish containing 1 ml of Hepes 199, BSA 0.4% at 38.9ºC.

Appendix 1 and 2 records the number of corpus luteum and in vivo matured oocytes obtained by follicle aspiration from the fallopian tubes of normal and hybrid female goats respectively.

2.4 - PREPARATION OF GRANULOSA CELLS FOR IN VITRO MATURATION

Granulosa cells were isolated from non-atretic follicles dissected from the abattoir-derived ovaries. The follicles (3-5 mm) were carefully recovered from the ovaries using watchmaker forceps, and the intact follicles placed in warm PBS and examined for clarity and good blood supply. The follicle was then burst to release the follicular fluid, everted and loose cells and

residual fluids removed by washing twice in dissection medium (Hepes 199, BSA 0.4%). Granulosa cells were then scraped from the inside surface of the follicle into a 30 mm petri dish containing 1 ml of maturation medium previously equilibrated in 5% CO_2. The concentration of granulosa cells was determined by haemocytometer and a portion of the medium containing 2 - 3 x 10^6 cells transferred to the oocyte maturation dishes. All manipulations were executed at room temperature.

2.5 - PREPARATION OF SERUM

Serum supplements were obtained from ewes in oestrus as identified by a teaser ram and from the geep during an unspecified phase of the oestrous cycle. The serum was heat-inactivated (30 minutes at 56-60ºC), filtered and stored in 1 ml aliquots at -20ºC.

2.6 - IN VITRO MATURATION

In vitro maturation (IVM) was carried out using a co-culture technique in a Bicarbonate buffered medium 199 supplemented with various additives for 24-25 hours in an incubator (Heraeus, Germany) in a 5% CO_2 atmosphere in air at 38.9ºC with gentle rocking.

For IVM, cumulus oocyte complexes were washed twice in Hepes 199 medium containing BSA 0.4% then twice in equilibrated maturation medium before being transferred to a 30 mm petri dish (Falcon, U.S.A.). The dish contained 2 ml of equilibrated

maturation medium supplemented with 10% FCS and 100 µl of additives (penicillamine, hypotaurine, epinephrine) and granulosa cells.

2.7 - IN VITRO FERTILISATION

2.7.1 - OOCYTE PREPARATION

Following maturation the oocyte complexes were recovered and transferred to a sperm wash medium supplemented with hyaluronidase (Sigma) (0.1 mg/ml) and 20% day 2 sheep serum, to remove excess granulosa cells. The oocytes were then washed twice in equilibrated IVF medium and transferred to 45-50 µl drops of IVF medium under oil which had been prepared and equilibrated at 38.9ºC at least 4 hours before in 5% CO_2 in air.

2.7.2 - SEMEN PREPARATION

2.7.2.1 - FRESH SEMEN

The semen was collected from rams with proven fertility with the aid of a short artificial vagina (Mies, 1987). The collected semen was transported to the laboratory in a insulated container and a portion diluted with warmed buffer (Hepes 199, BSA 04%) to check for sperm motility and progressive movement. The ejaculate was then maintained at 25ºC for 3 to 4 hours, and motile sperm recovered using a swim-up procedure. For this, ten 5 ml test tubes (Falcon, U.S.A.) containing 1 ml of sperm wash media was

equilibrated and held at ambient temperature sealed in a 50 ml tissue culture flask (Falcon, U.S.A.). Then a 100 µl sample of semen was carefully deposited in the bottom of each tube and the tubes incubated for 1 hour at 38.5ºC. Following incubation from each tube 600 µl of supernatant was careful removed and placed in two 10 ml test tubes (Falcon, U.S.A). The final volume was made up to 5 ml with sperm wash and both tubes were then centrifuged for 7 minutes at 50 x g. The supernatant was aspirated and 4 ml of sperm wash medium added. The centrifugation step was repeated and the supernatant discarded. The concentrated sperm was then resuspended in 250-400 µl of sperm wash medium supplemented with PHE and 20% of day 2 sheep serum and incubated for a further 45 minutes at 38.5ºC. For fertilisation, between 200-600 x 10^3 of sperm was added to each fertilisation drop (45-50 µl).

2.7.2.2 - FROZEN SEMEN

Frozen semen was obtained from a commercial source (Livestock Improvement, New Zealand). Three straws from the same sire (Coopworth) were thawed at 35ºC for 30 seconds and the semen collected in a test tube containing 1 ml of equilibrated sperm wash medium at 38.5ºC. A small sample of the diluted semen was taken for motility analysis and progressive movement assessment. The total volume of the sperm wash medium was then made up to 5 ml with more sperm wash and the tube centrifuged for 7 minutes at 50 x g. The supernatant was discarded and an additional 5 ml of sperm wash medium added and the centrifugation step repeated.

The sperm rich fraction of 100-200 µl was incubated for a further 45 minutes at 38.5ºC with PHE and 20% day 2 sheep serum. Each *in vitro* fertilisation drop (45-50 µl) was inseminated with 75-200 x 10^3 spermatozoa.

The *in vitro* fertilisation with fresh and frozen semen was carried out in 5% CO_2 in air atmosphere at 38.9 ºC.

2.8 - FIXING AND STAINING OOCYTES

Following *in vitro* fertilisation, oocytes were divested of cells using hyaluronidase (Sigma) (0.1 mg/ml). The cumulus free oocytes were fixed in acid alcohol during 24 hours (1 part glacial acetic acid : 3 parts EtOH) then stained with 1% aceto-orcein.

Oocytes were examined under phase contrast (Nikon Optiphot, Japan) for evaluation of nuclear events, specifically at the MII stage of development, normal fertilisation and polispermy.

2.9 - STATISTICAL ANALYSIS

Statistical analysis was carried out using the Minitab Statistical Software system, release 8 (PC version), 1991 package.

The Fisher's Exact Test was used as described by Ayres, M. and Ayres Junior, M. (1987).

III - IN VITRO MATURATION AND IN VITRO FERTILISATION OF SHEEP OOCYTES

3.1 - EXPERIMENTAL OUTLINE

The aim of these experiments was to compare different protein sources for in vitro maturation of sheep oocytes. The oocytes were obtained from the ovaries of slaughtered animals by two oocyte collection procedures.

The oocytes were submitted to IVF using fresh and frozen semen and the different treatments evaluated in relation to nuclear events and the efficiency of early cleavage divisions after in vitro fertilisation.

3.2 - MATERIAL AND METHODS

The experimental work was carried out at the DSIR in New Zealand, from 01\06 to 11\10 1990.

3.2.1 - COLLECTION OF OOCYTES

Oocyte cumulus complexes were recovered from the ovaries either by follicle aspiration, using a water vacuum pump connected to a test tube with 5 ml of Hepes 199 medium supplemented 0.4% BSA and Heparin (50 µg\ml) or slicing the ovaries with a surgical blade into a 90 mm petri dish (Falcon, U.S.A.) containing 15 ml Hepes buffer 199, 0.4% BSA and Heparin 100 µg/ml. During these procedures the tissues were maintained

on a warm stage at 38.9ºC.

3.2.2 - IN VITRO MATURATION: PROTEIN SUPPLEMENTATION

The supplements investigated included: I) 10% sheep serum, II) 10% Geep serum, III) 10% of a mixture of 50% sheep serum and 50% geep serum, IV) 10% fetal calf serum as a control.

3.2.3 - STATISTICAL ANALYSIS

The experiments were repeated 3 times, the first and second times with the aim of comparing the efficacy of aspiration versus slicing recovery procedures with fresh semen, and the third time with the aim of examining the efficiency of the method of recovery (slice) with frozen semen.

Comparisons were also made of the effects of serum supplementation and the success of IVM.

Oocytes were histologically examined 18-23 hours after IVF to assess nuclear changes or after 48 hours to determine cleavage rate.

Chi-Square tests were used for comparison of nuclear events and cleavage rate. As no statistical differences were detected in respect to nuclear events and cleavage rate, the groups were pooled and comparisons made between repeats.

A t-Test was used to compare the number of oocytes obtained by aspiration and slicing procedures.

3.2.4 - RECIPES

All composition of the various media used in this experiment are presented in appendix 3 A.

3.3 - RESULTS

Table 1 shows the results of experiments comparing two procedures used to collect sheep oocytes. The first method in which the oocytes were aspirated using a water vacuum pump, produced significantly less ($p<0.001$) oocytes than the second method which used a surgical blade to slice the ovary.

TABLE 1 - COMPARISON BETWEEN TWO PROCEDURES FOR COLLECTION OF OOCYTES FROM THE OVARIES OF SLAUGHTERED EWES.

PROCEDURE	NUMBER OF OVARIES	NUMBER OF COLLECTED OOCYTES	$\bar{x}$
ASPIRATION	728	792	1.09 [a]
SURGICAL	397	2056	5.18 [b]

DIFFERENT ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES STATISTICAL DIFFERENCE ($p<0.001$).

Table 2 presents the data related to the histology of sheep oocytes which were fixed between 18 and 23 hours after <u>in vitro</u> fertilisation with fresh sheep semen. These oocytes were

collected by the water vacuum pump method and submitted to *in vitro* maturation with different protein sources (sheep serum, geep serum, mixture of sheep/geep serum and fetal calf serum). The results in table 2 demonstrate that in all treatments the oocytes presented nuclear morphology more advanced than germinal vesicle breakdown. The different protein sources employed in the *in vitro* maturation systems were not statistically diferent for the groups compared ($P>0.05$) in relation to A - total normal fertilisation = total number of oocytes with normal fertilisation / total number of oocytes that achieved metaphase II; B - polyspermic penetrated oocytes = total number of oocytes with polyspermic penetration / total number of oocytes with normal fertilisation; C - total penetration = total number of oocytes that presented spermatozoa penetration / total number of oocytes that achieved metaphase II; D - total number of oocytes that achieved metaphase II = total number of oocytes that achieved metaphase II / total number of oocytes that were submitted to histology.

All oocytes which presented normal fertilisation displayed head of the spermatozoa decondensed indicating that the *in vitro* maturation system applied allowed cytoplasmic maturation irrespective of the serum sources used.

TABLE 2 - COMPARISON OF THE EFFECTS OF DIFFERENT SERUM SUPPLEMENTATION IN OOCYTE MATURATION MEDIUM. THE OOCYTES WERE COLLECTED BY ASPIRATION AND MATURED IN VITRO WITH THE SERUM SUPPLEMENT SHOWN, THEN SUBSEQUENTLY, FERTILISED IN VITRO WITH FRESH SHEEP SEMEN. FOR FURTHER DETAILS SEE TEXT.

IN VITRO MATURATION	NUMBER OF OOCYTES SUBMITTED HISTOLOGY	replicate	GERMINAL VESICLE	RESUMPTION OF MEIOSIS MI/AI	OOCYTES THAT ACHIEVED MII								TOTAL PENETRATION	TOTAL MII
					UNFERTILISED	FERTILISED								
						PENETRATION MII+T+HD+2PB	AII	TII	2PN+T+PB	SYNGAMY + TAIL	TOTAL NORMAL FERTILISATION	POLYSPERMIC		
											n (%)	n (%)	n (%)	n (%)
SHEEP SERUM	24	3	0	0	1	0	0	0	18	0	a 18 (75)	a 5 (27.8)	a 23 (95.8)	a 24 (100)
GEEP SERUM	20	3	0	2	3	0	0	0	9	0	a 9 (45)	a 6 (66.7)	a 15 (75)	a 18 (90)
SHEEP/GEEP SERUM	27	2	0	1	0	0	0	0	19	0	a 19 (70.4)	a 7 (36.8)	a 26 (96.3)	a 26 (96.3)
FETAL CALF SERUM	18	3	0	1	2	0	0	0	15	0	a 15 (83.3)	0 (0)	a 15 (83.3)	a 17 (94.4)

SAME ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE ($p>0.05$).

* SOME OF THE NUCLEAR EVENTS WHICH WERE PHOTOGRAPHED ARE IN FIGURES 1 AND 2.

Table 3 shows the same treatments as those applied to the oocytes in table 2, but in this case the results presented are concerned with the cleavage rate of the oocytes after *in vitro* fertilisation. The cleavage rate ranged between (76.8 - 62.3%) for all treatments. The statistical comparison among the percentages of cleavage rates of the oocytes submitted to the *in vitro* maturation system supplemented with different protein source was considered the same ($p>0.05$).

TABLE 3 - COMPARISON OF CLEAVAGE RATES OF OOCYTES WHICH WERE MATURED *IN VITRO* USING DIFFERENT PROTEIN SOURCES IN THE MATURATION MEDIUM AND FERTILISED *IN VITRO* WITH FRESH SHEEP SEMEN. OOCYTES WERE COLLECTED USING THE ASPIRATION PROCEDURE.

PROTEIN SOURCE	r	NUMBER OF OOCYTES	CLEAVAGE RATE ≥ 2	
			n	(%)
SHEEP SERUM	3	69	53	76.8 [a]
GEEP SERUM	3	72	48	66.7 [a]
SHEEP/GEEP SERUM	3	69	43	62.3 [a]
FETAL CALF SERUM	3	60	39	65.0 [a]

SAME ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE ($p>0.05$).
* SOME OF THE CLEAVED OOCYTES WHICH WERE PHOTOGRAPHED ARE IN FIGURE 2.

Table 4 describes the histology of sheep oocytes submitted to in vitro maturation with different protein sources (sheep serum, geep serum, a mixture of sheep/geep serum, fetal calf serum). The oocytes were collected by the sectioning procedure and fixed between 18 and 23 hours after in vitro fertilisation.

The majority of the oocytes of this group had advanced beyond the germinal vesicle breakdown stage. No differeces were seen between the protein sources used ($p>0.01$) in relation to A - total normal fertilisation = total number of oocytes with normal fertilisation / total number of oocytes that achieved metaphase II; B - polyspermic penetrated oocytes = total number of oocytes with polyspermic penetration / total number of oocytes with normal fertilisation; C total penetration = total number of oocytes that presented spermatozoa penetration / total number of oocytes that achieved metaphase II; D - total number of oocytes that achieved metaphase II = total number of oocytes that achieved metaphase II / total number of oocytes that were submitted to histology. The oocytes of all groups presented an indicative that had a suitable cytoplasmic maturation once the majority of the oocytes at morphological examination presented a spermatozoa head decondensed or at the syngamy stage. Few of the oocytes examined presented the head not decondensed at the anaphase II and telophase II stages. As the oocytes were fixed during the developmental stage it was not possible to tell whether the oocytes at anaphase II and telophase II presented a faulty cytoplasmic maturation or whether the oocytes were fertilised late with slow nuclear development in relation to the others.

TABLE 4 - COMPARISON OF THE EFFECTS OF DIFFERENT SERUM SUPPLEMENTATION IN OOCYTE MATURATION MEDIUM. THE OOCYTES WERE COLLECTED BY OVARY SLICE PROCEDURE, MATURED IN VITRO WITH THE SERUM SUPPLEMENT SHOWN, THEN SUBSEQUENTLY AND FERTILISED IN VITRO WITH FRESH SHEEP SEMEN.

IN VITRO MATURATION	NUMBER OF OOCYTES SUBMITTED HISTOLOGY	r	GERMINAL VESICLE	RESUMPTION OF MEIOSIS MI/AI	OOCYTES THAT ACHIEVED MII								TOTAL PENETRATION	TOTAL MII
					UNFERTILISED	FERTILISED								
						PENETRATION MII+T+HD+2PB	AII	TII	2PN+T+PB	SYNGAMY + TAIL	TOTAL NORMAL FERTILISATION	POLYSPERMIC		
											n (%)	n (%)	n (%)	n (%)
SHEEP SERUM	61	6	4	10	28	0	0	0	7	7	a 14 (22.9)	a 5 (35.7)	a 19 (31.1)	a 47 (77)
GEEP SERUM	31	4	0	8	7	0	0	2	7	1	a 10 (32.3)	a 6 (60)	a 16 (51.6)	a 23 (74.2)
SHEEP/GEEP SERUM	52	6	3	11	13	0	0	2	11	3	a 16 (30.8)	a 9 (56.3)	a 25 (48.1)	a 38 (73.1)
FETAL CALF SERUM	48	6	1	16	15	0	1	1	3	3	a 8 (16.7)	a 8 (100)	a 16 (33.3)	a 31 (64.6)

SAME ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE ($p>0.01$).

* SOME OF THE NUCLEAR EVENTS WHICH WERE PHOTOGRAPHED ARE IN FIGURES 1 AND 2.

Table 5 shows the cleavage rate of the oocytes after *in vitro* fertilisation following the same treatment as applied to the oocytes in table 4. The cleavage rate ranged between (25.8 - 19.4%) for all treatments. The statistical comparison among the percentages of cleavage rates of the oocytes submitted to the *in vitro* maturation system supplemented with different protein source was considered the same ($p>0.05$).

TABLE 5 - COMPARISON OF CLEAVAGE RATES OF OOCYTES WHICH WERE MATURED *IN VITRO* USING DIFFERENT PROTEIN SOURCES IN THE MATURATION MEDIUM AND FERTILISED *IN VITRO* WITH FRESH SHEEP SEMEN. OOCYTES WERE COLLECTED BY OVARY SLICING PROCEDURE.

PROTEIN SOURCE	r	NUMBER OF OOCYTES	CLEAVAGE RATE ≥ 2	
			n	(%)
SHEEP SERUM	7	119	30	25.2 a
GEEP SERUM	7	124	24	19.4 a
SHEEP/GEEP SERUM	7	124	32	25.8 a
FETAL CALF SERUM	7	117	27	23.1 a

SAME ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE ($p>0.05$).

* SOME OF THE CLEAVED OOCYTES WHICH WERE PHOTOGRAPHED ARE IN FIGURE 2.

Tables 6 and 7 show, respectively, the histology and cleavage rate of oocytes submitted to *in vitro* maturation with different protein sources (sheep serum, geep serum, mixture sheep/geep serum, fetal calf serum);the oocytes were collected by the sectioning procedure and fertilised *in vitro* with frozen

sheep semen.

As is shown by the data presented in table 6, the majority of the oocytes from all treatments were further from the nuclear morphology of germinal vesicle breakdown stage, the different protein supplements used in the in vitro maturation system were considered statistically the same, for the groups compared ($p>0.05$) in relation to A - total normal fertilisation = total number of oocytes with normal fertilisation / total number of oocytes that achieved metaphase II; B - polyspermic penetrated oocytes = total number of oocytes with polyspermic penetration / total number of oocytes with normal fertilisation; C - total penetration = total number of oocytes that presented spermatozoa penetration / total number of oocytes that achieved metaphase II; D - total number of oocytes that achieved metaphase II = total number of oocytes that achieved metaphase II / total number of oocytes that were submitted to histology.

TABLE 6 - COMPARISON OF THE EFFECTS OF DIFFERENT SERUM SUPPLEMENTATION IN OOCYTE MATURATION MEDIUM. THE OOCYTES WERE COLLECTED BY OVARY SLICE PROCEDURE, MATURED IN VITRO WITH THE SERUM SUPPLEMENT SHOWN, THEN SUBSEQUENTLY AND FERTILISED IN VITRO WITH FROZEN SHEEP SEMEN.

IN VITRO MATURATION	NUMBER OF OOCYTES SUBMITTED HISTOLOGY	r	GERMINAL VESICLE	RESUMPTION OF MEIOSIS MI/AI	OOCYTES THAT ACHIEVED MII								TOTAL PENETRATION	TOTAL MII
					UNFERTILISED	FERTILISED								
						PENETRATION MII+T+HD+2PB	AII	TII	2PN+T+PB	SYNGAMY + TAIL	TOTAL NORMAL FERTILISATION	POLYSPERMIC		
											n (%)	n (%)	n (%)	n (%)
SHEEP SERUM	86	7	2	11	56	0	0	1	10	3	a 14 (16.3)	a 3 (21.4)	a 17 (19.8)	a 73 (84.9)
GEEP SERUM	88	8	1	10	71	0	0	1	5	0	a 6 (6.8)	0 (0)	a 6 (6.8)	a 77 (87.5)
SHEEP/GEEP SERUM	89	8	1	12	61	0	0	1	8	3	a 12 (13.5)	a 3 (25)	a 15 (16.9)	a 76 (85.4)
FETAL CALF SERUM	86	8	2	7	65	0	1	1	9	1	a 12 (13.9)	0 (0)	a 12 (13.9)	a 77 (89.5)

SAME ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE ($p>0.05$).
SOME OF THE NUCLEAR EVENTS WHICH WERE PHOTOGRAPHED ARE IN FIGURES 1 AND 2.

Table 7 presents data of the oocytes which were collected by sectioning the ovaries with a surgical blade, submitted to *in vitro* maturation using different protein sources and then fertilised *in vitro* with frozen sheep sperm. The cleavage rates among the different treatments ranged between (25.9 - 20.7%), but the results were not statistically significant ($p>0.05$).

TABLE 7 - COMPARISON OF THE CLEAVAGE RATES OF OOCYTES COLLECTED BY A SLICING TECHNIQUE. THE OOCYTES WERE MATURED *IN VITRO* USING DIFFERENT PROTEIN SOURCES IN THE MATURATION MEDIUM AND FERTILISED *IN VITRO* WITH FROZEN SHEEP SEMEN.

PROTEIN SOURCE	r	NUMBER OF OOCYTES	CLEAVAGE RATE ≥ 2	
			n	(%)
SHEEP SERUM	7	96	22	22.9 [a]
GEEP SERUM	8	117	29	24.8 [a]
SHEEP/GEEP SERUM	8	116	24	20.7 [a]
FETAL CALF SERUM	8	104	27	25.9 [a]

SAME ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE ($p>0.05$).
* SOME OF THE CLEAVED OOCYTES WHICH WERE PHOTOGRAPHED ARE IN FIGURE 2.

Since there were no statistically significant differences among the different protein sources used in the *in vitro* maturation of the oocytes or the different collection procedures, the data was pooled in relation to histology and cleavage rate.

Table 8 compares the results of the two methods of oocyte collection with respect to oocyte histology. The collection

procedure influenced the percentage of oocytes that achieved metaphase II. Also in respect to normal fertilisation after in vitro fertilisation with fresh sheep semen ($p<0.001$). Either procedure used to collect oocytes not influenced the numbers of polyspermic penetrated oocytes ($p>0.05$).

TABLE 8 - POOLED DATA COMPARING THE EFFECTS OF DIFFERENT METHODS OF HARVESTING OOCYTES TO MATURATION RATES IN VITRO WITH DIFFERENT PROTEIN SOURCES IN THE MATURATION MEDIUM. THE OOCYTES WERE IN VITRO FERTILISED WITH FRESH SHEEP SEMEN.

METHOD OF OOCYTE COLLECTION	NUMBER OF OOCYTES SUBMITTED TO HISTOLOGY	TOTAL METAPHASE II		TOTAL NORMAL FERTILISATION		POLYSPERMIC	
	n	n	(%)	n	(%)	n	(%)
ASPIRATION	89	85[a]	(95.5)	61[a]	(71.8)	18[a]	(29.5)
SECTIONING	192	139[b]	(72.4)	48[b]	(34.5)	28[a]	(58.3)

DIFFERENT ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES STATISTICAL DIFFERENCE ($p<0.001$).

Table 9 shows the number of oocytes collected by the two methods and their cleavage rates. The procedure used to collect the oocytes influenced the quality of the oocytes; the oocytes collected by the water vacuum pump method had a statistical superior ($p<0.001$) cleavage rate (67.8%) compared to the oocytes

collected by slicing the ovaries surface with a surgical blade, which had a cleavage rate of 23.3%.

TABLE 9 - POOLED DATA COMPARING THE AMOUNT OF CLEAVED OOCYTES OBTAINED WHICH WERE MATURED IN VITRO WITH DIFFERENT PROTEIN SOURCES IN THE MATURATION MEDIUM. THE OOCYTES WERE COLLECTED BY THE ASPIRATION OR SLICING PROCEDURE AND IN VITRO FERTILISED WITH FRESH SHEEP SEMEN.

TREATMENT	r	NUMBER OF OOCYTES	CLEAVAGE RATE ≥ 2	
			n	%
ASPIRATION	12	270	183	67.8 [a]
SECTIONING	28	484	113	23.3 [b]

DIFFERENT ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES STATISTICAL DIFFERENCE ($p<0.001$).

There were no statistically significant differences among the protein sources used in in vitro maturation of the oocytes collected by slicing the ovaries with a surgical blade which were fertilised in vitro with fresh or frozen semen.

Table 10 compares the type of semen used (fresh or frozen) and the in vitro fertilisation of oocytes which were collected by slicing the ovaries with a surgical blade in respect to the their resulted histology.

The data demonstrates that fresh semen was statistically superior (see table 10) in terms of fertilisation of the oocytes,

monospermic and polyspermic penetrated, moreover the percentage of oocytes submitted to *in vitro* fertilisation with fresh semen presented less oocytes that achieved nuclear maturation, metaphase II.

TABLE 10 - POOLED DATA COMPARING FRESH AND FROZEN SHEEP SPERM AS FERTILISATION RATE OF OOCYTES MATURED *IN VITRO* WITH DIFFERENT PROTEIN SOURCES IN THE MATURATION MEDIUM. THE OOCYTES WERE HARVESTED BY SLICING PROCEDURE.

SEMEN NATURE	NUMBER OF OOCYTES SUBMITTED TO HISTOLOGY	TOTAL METAPHASE II	TOTAL NORMAL FERTILISATION	POLYSPERMIC
	n	n (%)	n (%)	n (%)
FRESH	192	139 (72.4) [a]	48 (34.5) [a]	28 (58.3) [a]
FROZEN	349	303 (86.8) [b]	44 (14.5) [b]	6 (13.6) [b]

DIFFERENT ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATE STATISTICAL DIFFERENCE ($p<0.001$).

Table 11, also compares the type of semen used (fresh or frozen) and the *in vitro* fertilisation of oocytes collected by slicing the ovaries with a surgical blade in relation to their cleavage rates.

This table shows that the oocytes which were fertilised *in vitro* with fresh and frozen semen had cleavage rates of 23.3% and

23.6% respectively and were not statistically different (p>0.05).

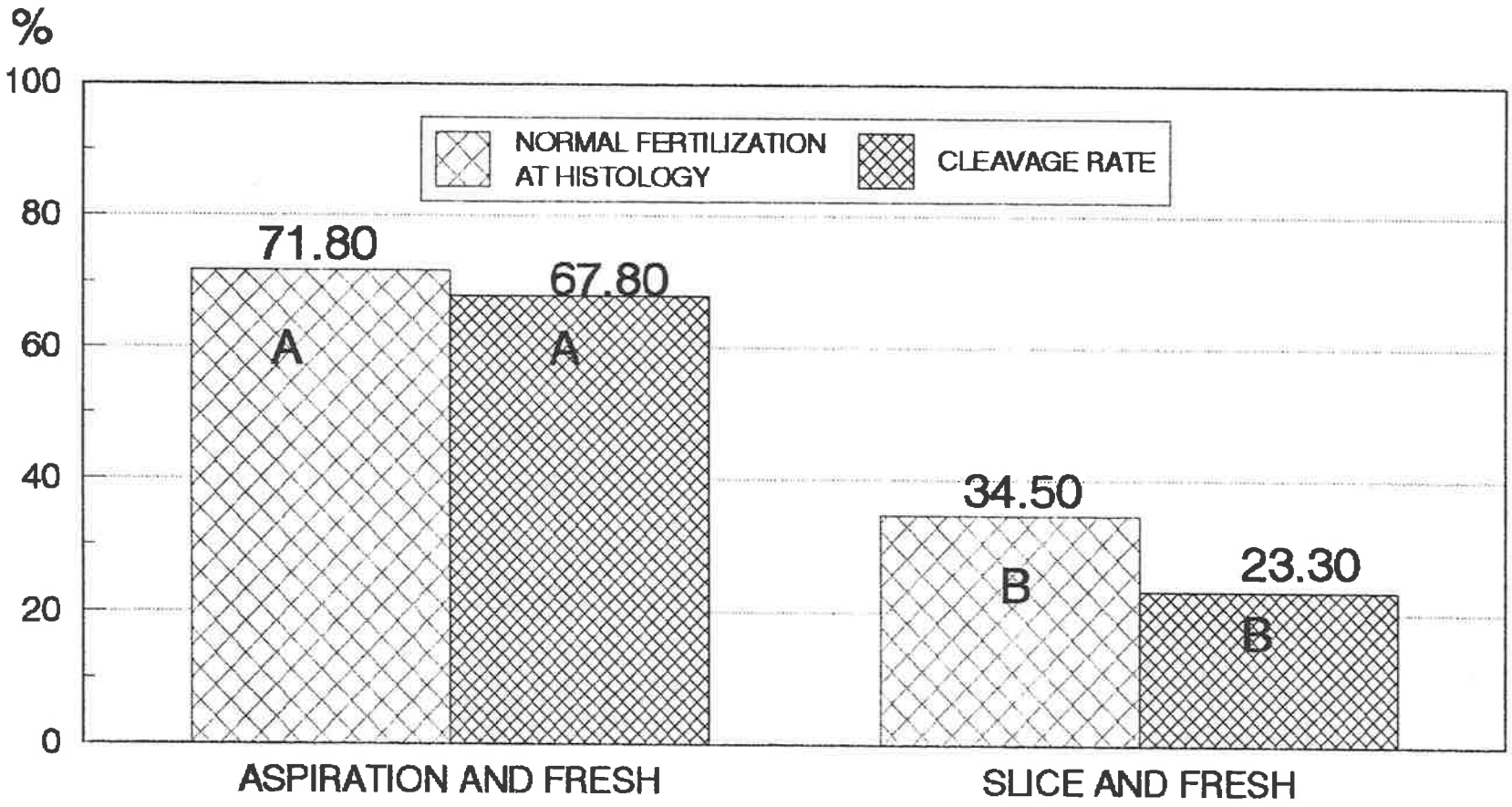
TABLE 11 - POOLED DATA COMPARING CLEAVAGE RATES OF OOCYTES MATURED IN VITRO WITH DIFFERENT PROTEIN SOURCES IN THE MATURATION MEDIUM. THE OOCYTES WERE COLLECTED BY SLICING PROCEDURE AND FERTILISED IN VITRO WITH FRESH OR FROZEN SHEEP SEMEN.

TREATMENT	r	NUMBER OF OOCYTES	CLEAVAGE RATE ≥ 2	
		n	n	%
FRESH SEMEN	28	484	113	23.3 [a]
FROZEN SEMEN	31	433	102	23.6 [a]

SAME ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE (p>0.05).

Figure 1 illustrates the comparison of the pooled data. Oocytes collected by aspiration with a water vacuum pump or by slicing the ovaries which were matured in vitro with different protein sources and in vitro fertilised with fresh semen, in relation to the oocytes that presented normal fertilisation at resulted histology and cleavage rate analysis.

FIGURE 1 - COMPARISON OF THE POOLED DATA OF THE OOCYTES IN VITRO MATURED IN RELATION TO NORMAL FERTILISATION AT HISTOLOGY AND CLEAVAGE RATE.



DIFFERENT ALPHABET LETTERS (CAPITAL LETTERS) IN EACH CLUSTER MEANS STATISTICAL DIFFERENCE ($p<0.001$).

3.4 - DISCUSSION

The results obtained with the in vitro maturation system of sheep oocytes supplemented with different protein sources (oestrous sheep serum; geep serum; mixture of 1/2 oestrous sheep serum and 1/2 geep serum; fetal calf serum) showed no statistical significances within each group (procedure of oocyte collection and type of semen nature, tables 2 and 3; 4 and 5; 6 and 7). Thus there was no evidence that the different serum additions under the conditions of this study had any influence on the number of oocytes achieving metaphase II, the fertilisation rate or the accomplishment of the first cleavage divisions. Similar data in respect to different serum supplementation were reported by Fukui and Ono (1989) and Sanbuissho and Threlfall (1989) for in vitro matured bovine oocytes. However, in respect to in vitro fertilisation, Fukui and Ono (1989) observed that the fertilisation rate was significantly affected by serum type; higher fertilisation rates were achieved with fetal calf serum than with oestrous cow serum. This observation is different to the data obtained in the present study within each group tested (procedure of oocyte collection and semen nature, tables 2 and 3; 4 and 5; 6 and 7), where no statistical differences were found in relation to the sheep oocytes submitted to the different protein sources employed in the in vitro maturation system in relation to normal fertilisation at histology examination and to the oocytes that achieved cleavage divisions.

By pooling the data of the in vitro matured oocytes with different protein sources and collection methods (water vacuum

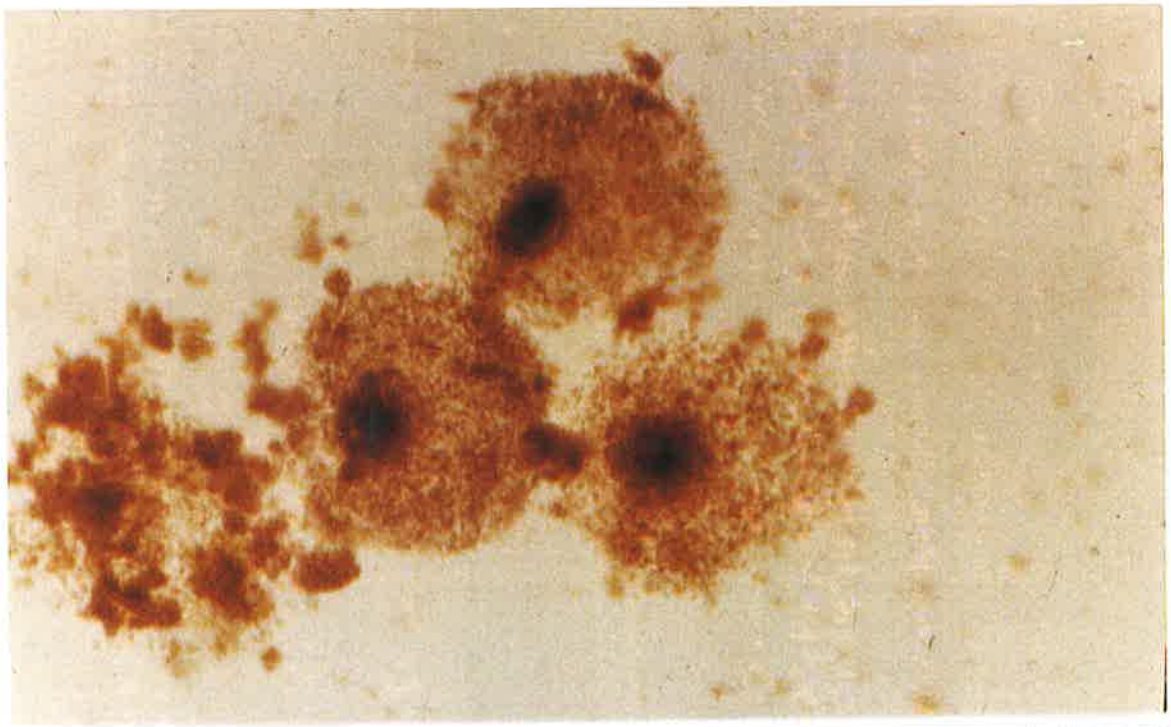
pump method or slicing with a surgical blade, tables 1, 8 and 9) and in vitro fertilised with fresh semen it was possible to show that the procedure used to collect the oocytes did have an influence, since the water vacuum pump method yielded less oocytes than slicing with a surgical blade. However the oocytes from the water vacuum pump method gave better results in relation to the number of oocytes that achieved metaphase II and normal fertilisation. Further, more of the oocytes collected by the water vacuum pump method presented cleavage than those collected by slicing the ovaries with a surgical blade.

Keskintepe et al. (1993) also found that the method of oocyte recovery could reflect in the success of the in vitro system. The superior results of the water vacuum pump oocyte collection method was related to the finding that the oocytes obtained by the slicing procedure were always contaminated with blood and other tissue debris.

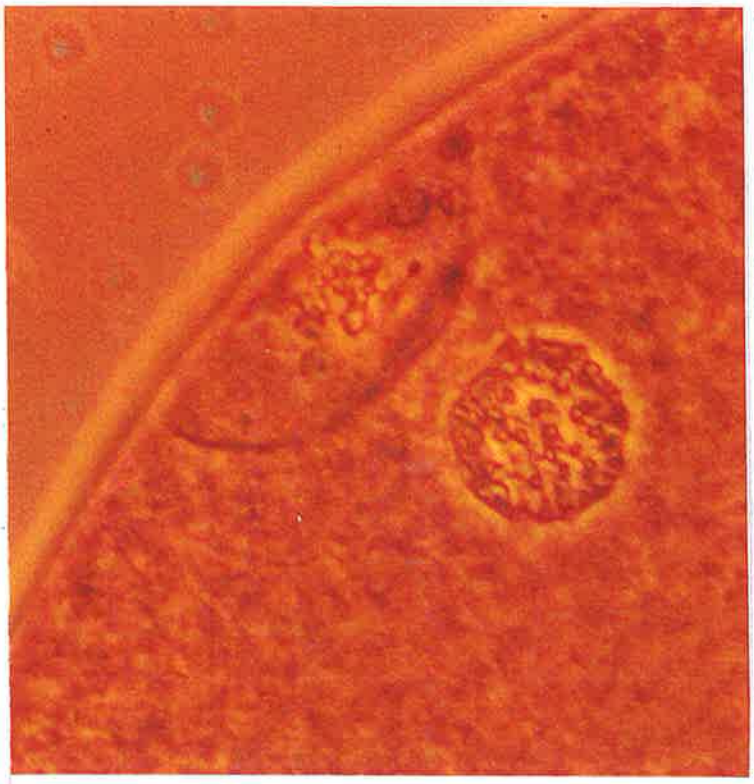
Another indication of the quality of the oocytes aspirated from the ovaries using the water vacuum pump method could be drawn from the data presented in tables 2, 4 and 6 which show that all oocytes collected using the water vacuum pump method had the head of the spermatozoa decondensed whereas in the other two groups the spermatozoa heads were not decondensed although assessed at the same time post-fertilisation.

The pooled data presented in table 10 show that oocytes in vitro fertilised with fresh semen produced better oocyte normal fertilisation and polyspermic fertilisation than those fertilised in vitro with frozen semen.

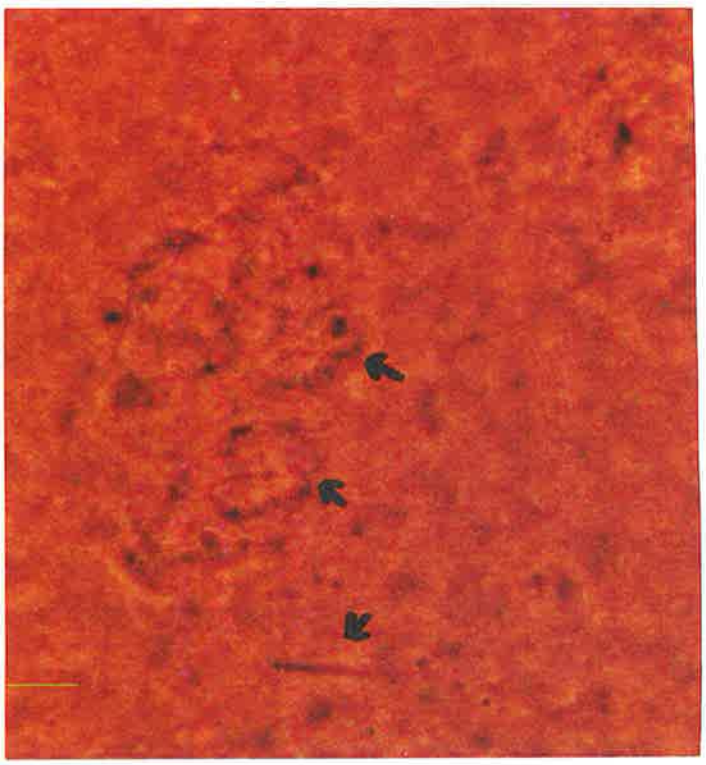
FIGURE 2



SHEEP OOCYTES AFTER IN VITRO MATURATION

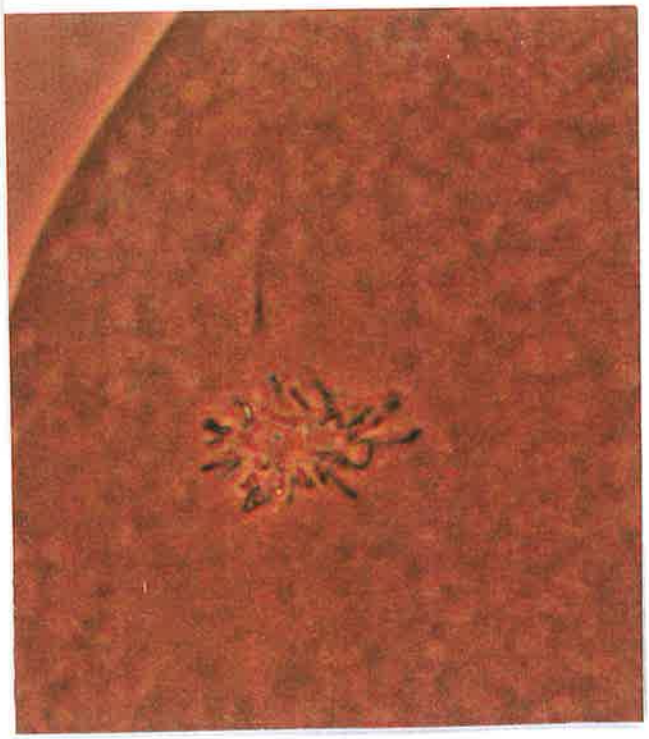


METAPHASE II
AND POLAR BODY

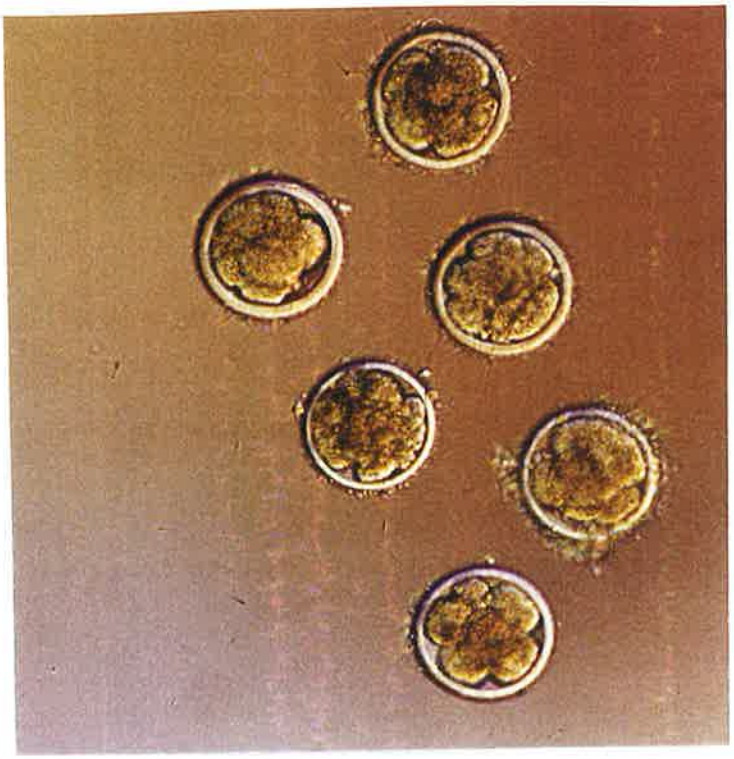


NORMAL FERTILISATION
2 PRONUCLEI + TAIL

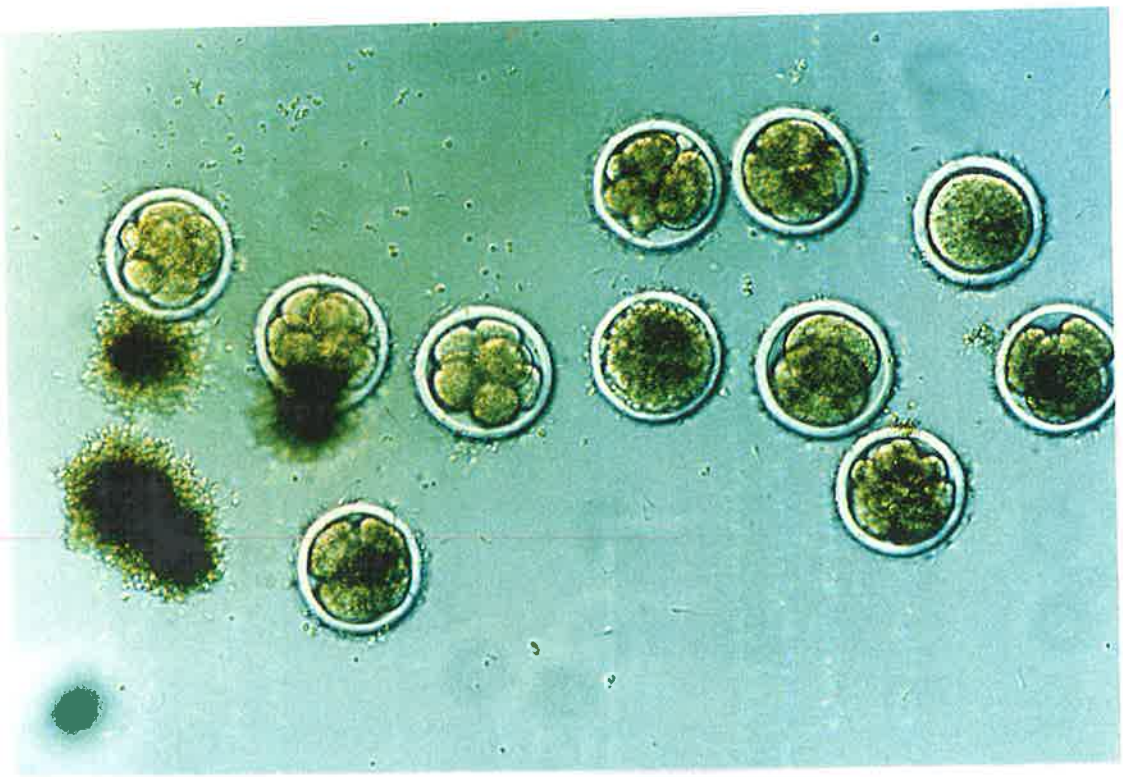
FIGURE 3



SYNGAMY PLATE + TAIL



CLEAVED OOCYTES AFTER IVMF



CLEAVED OOCYTES AFTER IVMF

IV - PRODUCTION OF HYBRID EMBRYOS THROUGH FERTILISATION OF IN VITRO AND IN VIVO MATURED GOAT OOCYTES WITH RAM SEMEN

4.1 - EXPERIMENTAL OUTLINE

The objective of these experiments were to produce (sheep X goat) hybrid embryos using in vitro techniques.

Comparisons were made between in vitro and in vivo matured goat oocytes subjected to in vitro fertilisation and the cleavage rate and number of blastocysts produced following in vivo culture in doe oviduct.

4.2 - MATERIAL AND METHODS

The experimental work was carried out at DSIR, New Zealand between 26\07\90 and 10\07\91.

4.2.1 - IN VITRO MATURATION

Ovaries were obtained from slaughtered animals or by surgery, the oocytes were recovered as described previously. The oocytes were in vitro matured using one of two procedures, the first being essentially the same as previously described but with the protein supplement varying between 10% or 20% hybrid serum without FCS. Co-culture was with granulosa cells.

Oocytes obtained using in vitro maturation were pooled to allow comparison between oocyte sources.

4.2.2 - IN VITRO FERTILISATION

In vitro fertilisation was carried out as described previously using hybrid serum as a protein supplement. In vivo matured oocytes obtained by follicular aspiration and oviduct flushing without cumulus cells were also submitted to in vitro fertilisation.

4.2.3 - SURGICAL TRANSFER OF EMBRYOS

Recipient animals were anaesthetised as described previously. Oviducts were ligated with silk at the uterine and isthmus junctions, and the embryos inserted into the ampulla using a Tom Cat Catheter (Sovereign, Sherwood, U.S.A.) attached to a 100 µl Hamilton syringe. The embryos were deposited in the ampulla of the recipient oviduct.

4.2.4 - IN VIVO CULTURE OF EMBRYOS IN LIGATED OVIDUCTS

The in vitro and in vivo matured embryos were transferred to the ligated oviducts between 20 and 48 hours after in vitro fertilisation and the recipient does slaughtered 6 days after embryo transfer (day 1 = day of transfer). After slaughter the tract was removed and transported to the laboratory for recovery of the embryos.

4.2.5 - STATISTICAL ANALYSIS

Comparisons were made between oocytes obtained from 4 different sources by assessing the cleavage rate achieved between 36 and 48 hours after *in vitro* fertilisation and the number of embryos cleaving to yield 4 or more blastomeres within 36 - 48 hours. Uncleaved oocytes were fixed and comparisons made between the four groups in relation to nuclear events.

The oocytes submitted to *in vivo* culture were analysed in relation to the stage of embryo development. Comparisons were also made between *in vitro* and *in vivo* matured oocytes, but no statistical difference was detected within each group (*in vitro* and *in vivo*) in relation to the source.

Chi-Square tests were used for comparisons of cleavage rate, development of embryos in *in vitro* culture and development of embryos *in vitro* as well as *in vivo* matured using *in vivo* culture.

4.2.6 - MEDIA

The composition of the mediums used in this experiment are shown in appendixes 3A and 3b.

4.3 - RESULTS

Table 12 shows the cleavage rates for *in vitro* and *in vivo* matured goat oocytes submitted to *in vitro* fertilisation with ram semen. The *in vitro* matured oocytes obtained from both sources were considered statistically the same in relation to the number of oocytes that achieved cleavage rate ($p>0.01$). A similar situation occurred with the other two groups of *in vivo* matured oocytes obtained from different sources, these two groups were also considered statistically equal in relation to the number of oocytes that reached cleavage rate ($p>0.01$).

TABLE 12 - COMPARISON OF CLEAVAGE RATES OF *IN VITRO* AND *IN VIVO* MATURED GOAT OOCYTES FERTILISED *IN VITRO* WITH RAM SEMEN.

MATURATION OF OOCYTES	OOCYTES SOURCE	r	NUMBER OF OOCYTES SUBMITTED TO *IN VITRO* FERTILISATION n	CLEAVAGE RATE n	CLEAVAGE RATE (%)
IN VITRO	ASPIRATED FROM IMMATURE FOLLICLES OF OVARIES FROM SLAUGHTERED ANIMALS	4	147	78	53.1 a
IN VITRO	ASPIRATED FROM IMMATURE FOLLICLES OF OVARIES USING SURGICAL PROCEDURE	4	43	14	32.6 a
IN VIVO	ASPIRATED FROM MATURE FOLLICLES OF OVARIES USING SURGICAL PROCEDURE(*)	23	77	61	79.2 b
IN VIVO	OVIDUCT FLUSHING FROM LIVE ANIMALS BY SURGICAL PROCEDURE(*)	25	264	235	89.0 b

(*) ANIMALS SUBMITTED TO A SUPEROVULATION TREATMENT
SAME ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE ($p>0.01$).

TABLE 13 - POOLED DATA COMPARISON OF CLEAVAGE RATES OF IN VITRO AND IN VIVO MATURED GOAT OOCYTES FERTILISED IN VITRO WITH RAM SEMEN.

MATURATION OF OOCYTES	r	NUMBER OF OOCYTES SUBMITTED TO IN VITRO FERTILISATION	CLEAVAGE RATE	
		n	n	(%)
IN VITRO	8	190	92	48.4 a
IN VIVO	48	341	296	86.8 b

DIFFERENT ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES STATISTICAL DIFFERENCE ($p<0.001$).

The pooled data of the oocytes in vitro and in vivo matured from table 12 is presented in table 13. The data indicate that the in vivo matured oocytes performed better than the in vitro matured in relation to the cleavage rate, ($p<0.001$).

Table 14 presents the data of the cleavage rate (data evaluation was made between 36-48 hours after in vitro fertilisation with sheep semen) from in vitro and in vivo matured goat oocytes. The oocytes within each group of maturation were considered statistically equal in relation to embryonic development.

TABLE 14 - COMPARISON OF CLEAVAGE RATES OF IN VITRO AND IN VIVO MATURED GOAT OOCYTES 36-48 HOURS AFTER IN VITRO FERTILISATION WITH SHEEP SEMEN.

SOURCE OF OOCYTES	n	r	2 CELLS		3 CELLS		≥4 CELLS	
			n	(%)	n	(%)	n	(%)
IN VITRO MATURED ASPIRATED FROM UNDEVELOPED FOLLICLES OF OVARIES FROM SLAUGHTERED ANIMALS	48	3	26	54.2^{a}	10	20.8^{a}	12	25.0^{A}
IN VITRO MATURED ASPIRATED FROM UNDEVELOPED FOLLICLES OF OVARIES FROM LIVE ANIMALS BY SURGICAL PROCEDURE	14	3	8	57.1^{a}	2	14.3^{a}	4	28.6^{A}
IN VIVO MATURED ASPIRATED FROM DEVELOPED FOLLICLES OF OVARIES FROM LIVE ANIMALS BY SURGICAL PROCEDURE(*)	63	20	6	9.5^{b}	3	4.8^{b}	54	85.7^{B}
IN VIVO MATURED OVIDUCT FLUSHING FROM LIVE ANIMALS BY SURGICAL PROCEDURE(*)	207	23	21	10.1^{b}	6	2.9^{b}	180	86.9^{B}

(*) ANIMALS SUBMITTED TO A SUPEROVULATION TREATMENT
SAME ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE ($p>0.05$).
SAME ALPHABET LETTER (CAPITAL LETTER) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE ($p>0.01$).

Table 15 shows the data pooled from table 14, in relation to the source of the oocytes. Embryos obtained from in vivo matured oocytes developed at a faster rate than the embryos produced from in vitro matured oocytes ($p<0.001$).

TABLE 15 - POOLED DATA COMPARISON OF CLEAVAGE RATES OF IN VITRO AND IN VIVO MATURED GOAT OOCYTES 36-48 HOURS AFTER IN VITRO FERTILISATION WITH SHEEP SEMEN.

SOURCE OF OOCYTES	n	r	2 CELLS		3 CELLS		≥4 CELLS	
			n	(%)	n	(%)	n	(%)
IN VITRO MATURED	62	6	34	54.8 a	12	19.4 a	16	25.8 a
IN VIVO MATURED	270	43	27	10.0 b	9	3.3 b	234	86.7 b

DIFFERENT ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN INDICATES STATISTICAL DIFFERENCE ($p<0.001$).

Table 16 shows the histology of goat oocytes matured in vitro and in vivo, obtained from different sources, after 48 hours of in vitro fertilisation with ram semen and which did not present cleavage division. The oocytes from all sources achieved nuclear maturation, metaphase II, ranging from 75.4 to 100% in the in vitro matured oocytes and 81.8 to 88.2% in the in vivo matured oocytes. Only the oocytes from the in vitro maturation group (obtained from the ovaries of slaughtered animals) were arrested at the germinal vesicle stage, the other groups were further advanced in the development of germinal vesicle breakdown. No differences were seen between the groups in relation to total sperm penetration and the number of polyspermic oocytes ($p>0.05$). The data also show that the oocytes from the three groups which presented oocytes with monospermic spermatozoa penetration did not present cleavage division.

TABLE 16 - HISTOLOGY OF NON-CLEAVED GOAT IN VITRO AND IN VIVO MATURED OOCYTES 48 HOURS FOLLOWING IN VITRO FERTILISATION WITH RAM SEMEN.

IN VITRO MATURATION	NUMBER OF OOCYTES SUBMITTED HISTOLOGY	r	GERMINAL VESICLE	UNIDENTIFIED	OOCYTES THAT ACHIEVED MII									TOTAL PENETRATION		TOTAL MII	
					UNFERTILISED	FERTILISED											
						PENETRATION MII+T+HD+2PB	AII	TII	2PN+T+PB	SYNGAMY + TAIL	TOTAL NORMAL FERTILISATION		POLYSPERMIC				
											n	(%)	n	(%)			
IN VITRO MATURED OBTAINED FROM OVARIES OF SLAUGHTERED ANIMALS	69	7	13	4	44	3	0	0	0	0	3	4.3	5	7.2[a]	8	11.6[a]	52	75.4[a]
IN VITRO MATURED ASPIRATED FROM UNDEVELOPED FOLLICLES OF OVARIES FROM LIVE ANIMALS BY SURGICAL PROCEDURE	12	2	0	0	12	0	0	0	0	0	0		0		0		12	100.0[a]
IN VIVO MATURED ASPIRATED FROM DEVELOPED FOLLICLES OF OVARIES FROM LIVE ANIMALS BY SURGICAL PROCEDURE(*)	11	9	0	2	6	1	0	0	0	0	1	9.1	2	18.2[a]	3	27.3[a]	9	81.8[a]
IN VIVO MATURED OVIDUCT FLUSHING FROM LIVE ANIMALS BY SURGICAL PROCEDURE(*)	17	5	0	2	13	0	0	0	0	0	0		2	11.8[a]	2	11.8[a]	15	88.2[a]

(*) ANIMALS SUBMITTED TO A SUPEROVULATION TREATMENT

SAME ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN MEANS NO STATISTICAL DIFFERENCE (P>0.05).

Table 17 shows that the embryo recovery rate from doe oviducts was the same for the in vitro and in vivo matured oocytes ($p>0.05$).

Both oocyte maturation groups presented all classes of embryos, although the in vivo matured oocytes had a better performance than the ones from in vitro maturation. Fewer in vitro matured oocytes were recorded as (>4-8 cell; >8-16 cell; blastocysts, $P>0.05$) than in vivo matured oocytes while more embryos from in vitro matured oocytes were present as 2-4 cell than the ones from in vivo maturation ($p>0.05$).

TABLE 17 - MATURATION OF GOAT OOCYTES CULTURED IN VIVO IN DOE OVIDUCTS AFTER IN VITRO FERTILISATION WITH RAM SEMEN.

SOURCE OF OOCYTES	NUMBER OF EMBRYOS TRANSFERRED	RECOVERY RATE		REPETITIONS	STRUCTURES YIELDED													
					2-4 CELL		>4-8 CELL		>8-16 CELL		MORULA		BLASTOCYST		DEGENERATE		ZONA PELLUCIDA	
	n	n	(%)	n	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
IN VITRO MATURED	164	49	29.9[a]	6	13	26.5[a]	3	6.1[a]	2	4.1[a]	1	2.0	1	2.0[a]	26	53.1	3	6.1
IN VIVO MATURED	175	57	32.6[a]	8	11	19.3[b]	13	22.8[b]	16	28.1[b]	2	3.5	12	21.1[b]	1	1.8	2	3.5

SAME ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN MEANS NO STATISTICAL DIFFERENCE (P>0.05).

4.4 - DISCUSSION

The *in vitro* fertilisation of goat oocytes with ram semen demonstrated that is possible to produce sheep X goat hybrid embryos using *in vitro* maturation and *in vitro* fertilisation technology.

Conception commonly occurs when female goats are mated or inseminated by rams. As reported by Alexander et al. (1967) and Hancock et al. (1968) the conception rate of goats mated or inseminated to rams were 79.3% and 80% and these results are an indication that the embryonic preimplantation development results in normal conditions.

The production of hybrid embryos using *in vitro* fertilisation in this work shows that by using *in vitro* and *in vivo* matured goat oocytes fertilised *in vitro* with ram semen it is possible to obtain 32.6 and 89% of cleavage rate respectively, with 21.1% of hybrid zygotes developing to the blastocyst stage by *in vivo* culture using doe oviducts. The hypothesis that recessive lethal genes are exposed in the absence of normal alleles in the hybrid embryos and cause early embryonic death is not supported by this or previous studies (Berry, 1938 cited by McGovern, 1969; McGovern, 1973b).

This study supports previous work by Bunch et al. (1976) and confirms that hybridization between sheep and goats is possible, probably because the reproductive barrier between Capra and Ovis is not developed within Ovis.

The *in vitro* environment might be improved the results, Walker et al. (1992) obtained more sheep zygotes developing to

blastocyst *in vitro* compared with *in vivo* indicating that the *in vitro* enviroment may protect the embryos.

During the development of the hybrid IVF experiments it was observed that normal cleavage rates of the *in vivo* matured goat oocytes did not start for all zygotes within 24 hours after *in vitro* fertilisation with ram semen, a situation which could also be attributed to the artificial nature of the environment. It was noted in *in vivo* conditions by Hancock and McGovern (1968) that hybrid eggs tended to be at a less advanced stage of development than goat oocytes fertilised by homologous spermatozoa.

The cleavage rates obtained in the present work with the *in vitro* fertilisation of goat oocytes with ram semen varied between 32.6 and 53.1% for the *in vitro* matured oocytes and 79.2 and 89% for the *in vivo* matured oocytes. The cleavage rates obtained from both sources of oocytes during the current work was superior to the cleavage rate obtained by Mogas et al. (1995) with *in vitro* matured and *in vitro* fertilised goat oocytes.

Comparisons between the development of embryos from goat oocytes matured *in vitro* and *in vivo* are shown in tables 13, 15 and 17. The *in vivo* matured oocytes showed higher cleavage rates in less time, ($p<0.001$) and more blastocysts ($p<0.05$) than the *in vitro* matured goat oocytes. This difference shows that the *in vitro* system of maturing goat oocytes is not allowing the same quality of maturation as is the case with oocytes matured inside the follicle. However, Armstrong et al. (1992) obtained with *in vitro* and *in vivo* matured bovine oocytes *in vitro* fertilisation which was not statistically significant between both maturation groups, indicating a reliable *in vitro* system for maturation of

bovine oocytes.

The data presented in table 16 is from goat oocytes which were submitted to in vitro fertilisation and did not present cleavage division after 48 hours of in vitro fertilisation. Some of the oocytes which were submitted to morphological examination presented some atypical nuclear morphology and were considered unidentified.

The in vitro and in vivo matured oocytes, table 16, did not differ statistically in relation to the presence of MII, ($p>0.05$) and this indicates that even the in vitro system is allowing nuclear maturation at the same level as the in vivo matured oocytes, although not at the ooplasm plane; the ability to decondense the fertilising sperm head has been used as an indication that cytoplasmic maturation has occurred (Bavister et al., 1992). The in vitro matured oocytes obtained from ovaries of slaughtered animals demonstrated that 3 oocytes with monospermic penetration of 64 submitted to in vitro maturation that achieved metaphase II and did not cleave presented the head of the spermatozoa without decondensation. Because of the small number of oocytes characterised with this morphology, this situation only can be seen as evidence that an improper ooplasm maturation occurred and, moreover, this situation illustrates that the success of fertilisation is an event which needs the active participation of both gametes. It is also important to take into consideration that this was hybrid fertilisation and the fact that there was no decondensation of the sheep spermatozoa in the goat oocyte could be ruled out as an incompatibility between both species.

Polyspermy was present in all groups but it was not statistically different ($p>0.05$). In their review Gandolfi et al. (1990) highlighted the fact that aged oocytes and *in vitro* matured oocytes present a reduced and a defective block to polyspermy, respectively. In the present study, the oocytes matured *in vivo* obtained by follicular aspiration presented a higher percentage of polyspermy table 16 than the other groups and this could be because they have been obtained from follicles that failed to ovulated and started to became atretic, thus speeding the oocyte aging process.

One of the limitations of the superovulation techniques for production of *in vivo* matured oocytes is that the ovulation is not synchronous (Gordon, 1990; Crozet, 1991). During the development of the present work it was observed that the start of ovulation of a superovulated goat was unpredictable and was linked to the individual animal. For instance, in many situations when hormonal treatments imposed at the time of surgery, appendix 1, it was obtained ovaries from females which presented very good superovulatory response translated by the presence in the ovaries surface with corpus rubrum, in other occasions ovaries which had corpus rubrum and developed follicles not ovulated with the size oscillating at 3 to 5mm diameter whilst in other circumstances the female goats only presented developed follicles (3 to 5mm) for the expected ovulation time (McDonald et al, unpublished manuscript). Because of this, improper reproducible condition, the ovulated oocytes were collected by oviduct flushing and the unovulated oocytes from developed follicles of stimulated animals by follicular aspiration. Tables 12 and 14 demonstrate that the

oocytes matured in vivo ovulated and not ovulated were considered statistically the same in relation to cleavage rates and the speed of development of the zygote cleavage ($p>0.01$). The results obtained from both sources of in vivo matured oocytes were compatible with the first cleavages of preimplantation development, demonstrating a procedure that could be used in those animals which respond to superovulation treatment but do not ovulate at the expected time.

Nevertheless it is necessary for a further evaluation of the developmental potential of oocytes aspirated from developed follicles, as the follicle size was not uniform and this could be one of the causes of a possible impairment of the development of potential embryos (Barnes et al., 1993 and Fair et al., 1995).

Cleaved hybrid embryos were submitted to in vivo culture in doe oviducts (see table 17). In both groups of oocyte maturation blastocysts were obtained, from the in vitro matured oocytes 2.0% and in vivo matured oocytes 21.1%, and thus the groups were considered statistically different ($p<0.05$). In the present experiments it was elected the in vivo culture system in the oviduct of live animals because at the time of the execution of this experiment the in vitro culture technology was incipient. Walker et al. (1992) demonstrated that in vitro culture may protect embryos from selection, a physiological occurrence in the oviduct. Because of this, the viability of sheep embryos (to day 50 of pregnancy) after 5 days of culture was reduced compared with embryos cultured in vivo. However it is necessary the development of a reliable in vitro culture system whith application for ruminant embryos, because one of the greatest

limitations of the *in vivo* culture of embryos is the loss of the potential embryos. In the present study, 339 hybrid embryos produced by *in vitro* fertilisation were transferred to a ligated oviduct and only 106 were recovered, giving a loss of 68.7% of the potential embryos transferred figure 4.

FIGURE 4 - PERCENTAGE OF RECOVERY OF HYBRID EMBRYOS CULTURED IN VIVO IN GOAT OVIDUCT.

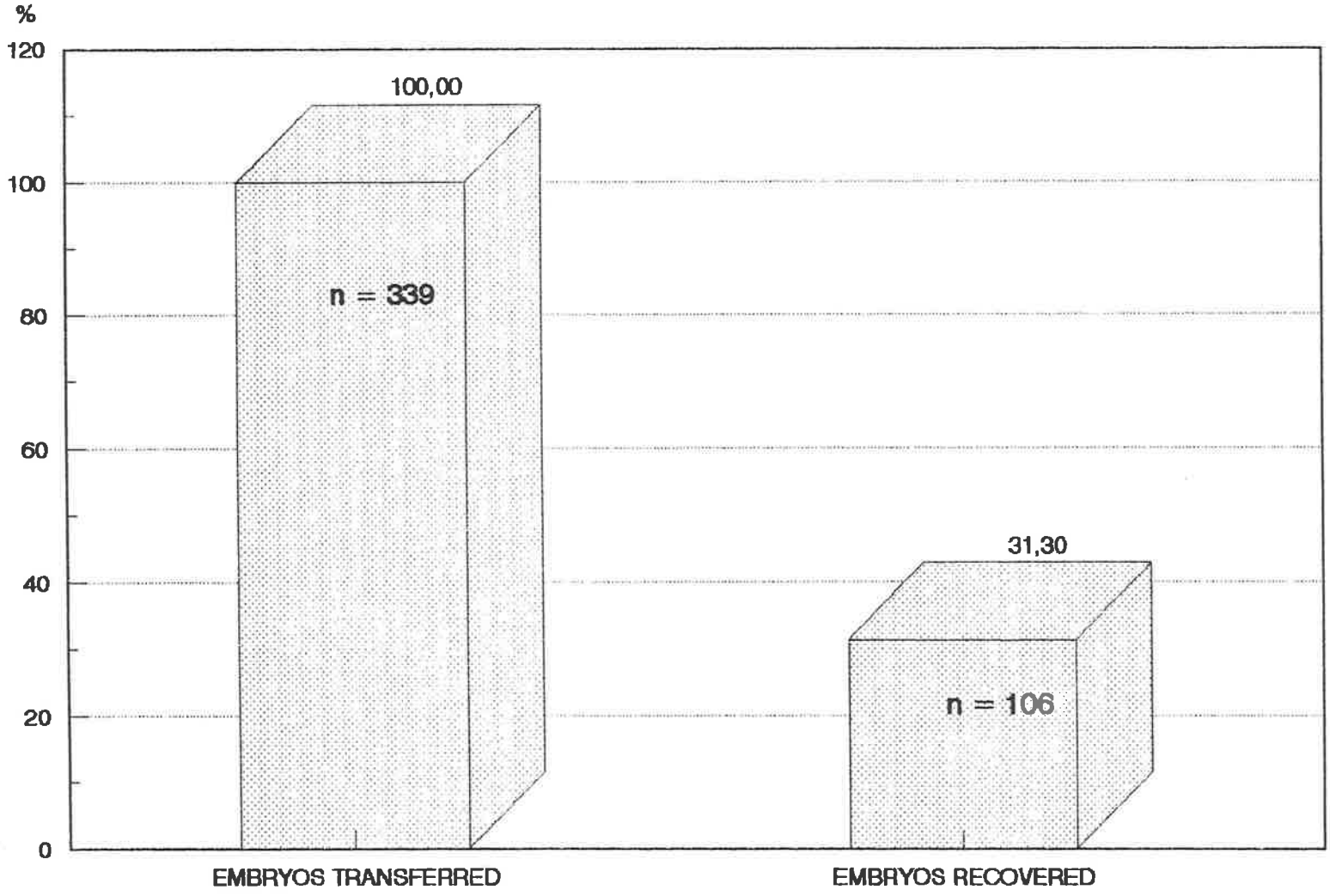


FIGURE 5

GROUP OF GOAT OOCYTE DONORS AND
THE FEMALE SHEEP x GOAT HYBRID

V - IN VITRO FERTILISATION WITH RAM SEMEN OF IN VITRO AND IN VIVO MATURED OOCYTES FROM A HYBRID FEMALE

5.1 - EXPERIMENTAL OUTLINE

The goal of the experiments was to produce hybrid embryos by using (sheep X goat) hybrid oocytes and ram semen and applying in vitro techniques. Oocytes from different sources were fertilised in vitro and their rate of early cleavage determined.

5.2 - MATERIAL AND METHODS

The experimental work was carried out at DSIR, on 21\08\90, 14\03\91 and 28\05\91.

5.2.1 - IN VITRO MATURATION AND IN VITRO FERTILISATION

In vitro maturation cumulus oocyte complexes were washed twice in Hepes 199 BSA 0.4% supplemented, prepared as described previously and matured in 10% hybrid serum in co-culture with 3 x 10^6 bovine granulosa cells. IVF was carried out as described previously.

5.2.2 - STATISTICAL ANALYSIS

Comparisons were made using Fisher's Exact Test between the source of oocytes in relation to cleavage rate.

5.2.3 - MEDIA

The compositions of media used in this experiment are shown in appendix 3 A and 3 B.

5.3 - RESULTS

Table 18 demonstrates the cleavage rates of *in vitro* and *in vivo* matured hybrid female oocytes which were submitted to *in vitro* fertilisation with ram semen. The two groups of *in vivo* matured oocytes which were compared statistically did not demonstrate difference in relation to cleavage rate ($p>0.05$).

TABLE 18 - CLEAVAGE RATES OF *IN VITRO* AND *IN VIVO* MATURED GEEP OOCYTES SUBMITTED TO *IN VITRO* FERTILISATION WITH RAM SEMEN.

MATURATION AND OOCYTES SOURCE	n	r	CLEAVAGE RATE	
			n	(%)
IN VITRO MATURED ASPIRATED FROM UNDEVELOPED FOLLICLES BY SURGICAL PROCEDURE(*)	2	1	1	50.0
IN VIVO MATURED ASPIRATED FROM DEVELOPED FOLLICLES BY SURGICAL PROCEDURE(*)	10	2	7	70.0[a]
IN VIVO MATURED OVIDUCT FLUSHING BY SURGICAL PROCEDURE(*)	9	1	6	66.7[a]

(*) ANIMALS SUBMITTED TO A SUPEROVULATION TREATMENT
SAME ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN MEANS NO STATISTICAL DIFFERENCE ($p>0.05$).

5.4 - DISCUSSION

Natural hybrids from sheep and goats are rare with only a few determined by cytogenetic studies (Roca and Rodero, 1971; Bunch et al., 1976; Eldridge et al., 1983; Denis et al., 1987 cited by Tucker et al., 1989; Pinheiro et al., 1989; Stewart-Scott et al., 1990). The hybrids were born by chance and were not outcomes from experimental studies. All were females with 57 chromosomes and all proved to be fertile when backcrossed with a ram.

In the present study, the results obtained with respect to the cleavage rates of oocytes from a hybrid female in vitro fertilised with ram semen demonstrate that this female was fertile in relation to the gamete side. It is important to emphasize that this statement is based only on the observation of the cleavage rates of the oocytes and not by histological interpretation. This observation stresses that the difficulty to obtain backcross pregnancies from a hybrid female is related to extrinsic factors which were discussed in the literature review section 1.2.3.2.

The female hybrid for this study was superovulated and submitted to surgery three times (Appendix 2). In the first attempt the hybrid was diagnosed pregnant at surgery and at that time only follicles were aspirated and obtained. After in vitro maturation (Figure 6) and subsequent in vitro fertilisation, one of these oocytes cleaved. As the female hybrid did not produced any offspring within the expected time, she underwent a second intervention. On this occasion, 10 oocytes were obtained: nine

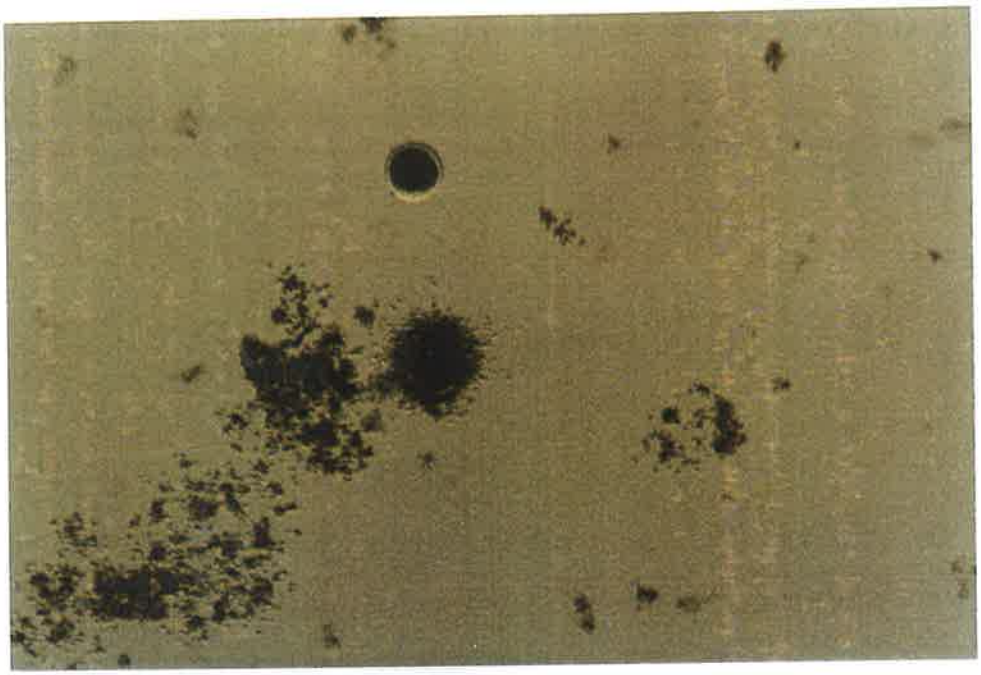
by oviduct flushing and 1 by follicular aspiration. All oocytes were then submitted to in vitro fertilisation and 7 cleaved (6 originated from oviduct flushing and 1 from developed follicles).

On the third attempt, both ovaries of the female hybrid had developed follicles by the time of collection, from which nine oocytes were obtained for in vitro fertilisation and six cleaved.

It is not possible to drawn any conclusions with respect to the oocytes that did not cleave, as to whether this was a situation due to a fault in the in vitro system or to hybrid incompatibility.

Table 18 shows that both groups of oocytes in vivo matured compared did not present statistical differences in terms of cleavage rates, ($p>0.05$) and a further study is needed in order to determine the development of those hybrid embryos originating from each source.

FIGURE 6



HYBRID OOCYTES BEFORE IN VITRO MATURATION

HYBRID FEMALE, GOAT AND SHEEP

* PICTURE REPRODUCED FROM INRA CATALOG OF ANIMALS AND PRODUCT QUALITY

VI - GENERAL MATERIALS AND METHODS PART II

This study was conducted at the Laboratory of Experimental Embryology of the Department of Obstetrics and Gynaecology, and at the Specific Pathogen Free (SPF) Animal House facility, both at the Medical School, Adelaide University, during the period of November 1991 to June 1994. The superovulation of mice, the mice uterus and oviduct embryo collection, the media preparation for embryo handling and in vitro culture and the embryo transfers were performed following protocols (appendix 4) in routine use at Department of Obstetrics and Gynaecology and approved by the Animal Ethics Committee of the University of Adelaide.

6.1 - ANIMALS

Four strains of mice were used, namely Swiss albino, C57BL\6, CBAC57\F1 and Balb C.

For embryo donors, 3 to 4 week old female Swiss albino, C57BL\6, and CBAC57\F1 were used and for embryo recipients 5 to 9 week old females of the CBAC57\F1, Swiss albino and Balb C strains were used.

The stud males (Swiss albino, C57BL\6 and CBAC57\F1) and vasectomized males (CBAC57\F1) were maintained apart from the females and individually housed in separate cages. All males used were a minimum of 8 weeks of age. Both the stud males and the vasectomized males were tested for fertility and sterility respectively before use. All animals were maintained with water

and food *ad libitum*, and held under temperature and photoperiod control (12 hours light : 12 hours dark).

6.2 - HORMONAL TREATMENT AND MATING OF THE EMBRYO DONOR MICE

The female mice were superovulated with a intraperitoneal injection of 5 IU of pregnant mare serum (Folligon, Intervet) followed by 5 IU of human Chorionic Gonadotrophin (Chorulon, Intervet) 44 and 48 hours later. After the HCG injection the female mice were caged individually with a male of the same strain. The next morning the presence or absence of a vaginal plug was determined and all plugged females were separated. The day of detection of vaginal plug following mating was referred to as day 0.5. This occurred on the first day after HCG injection.

6.3 - PREPARATION OF RECIPIENT MOUSE FOR EMBRYO TRANSFERS

Normally cycling recipient mice were individually caged with vasectomized males and examined each morning for the presence of vaginal plugs, and the mated females separated (day of plug identification = day 0.5)

6.4 - EMBRYO COLLECTION

Mice were killed by cervical dislocation, the abdominal skin torn to expose the abdomen and the reproductive tract removed and

transferred to a 30mm petri dish (Disposable Products, Australia) containing 2ml of HTF-Hepes/BSA medium, held at 37ºC.

6.4.1 - Uterine embryo collection

The uterus was flushed under a stereomicroscope (Olympus SZ 30, Japan) with 25 - 35 µl HTF-Hepes/BSA. An insulin syringe was introduced into the cervical end of the uterus and the flushings were collected from the tubal end into a 30 mm petri dish (Disposable Products, Australia). The bottom outside face of the dishes was marked into quadrangles to assist in the process of searching. The washings from the four uterine tracts were combined in each petri dish and examined for embryos under a stereomicroscope. The embryos collected were rinsed twice in HTF-Hepes/BSA and once with 1 ml of HTF-HCO_3/BSA, then transferred to a final <u>in vitro</u> culture drop of HTF-HCO_3/BSA under oil using a petri dish. The dish was then placed in an incubator (Steri-cult 200 Forma Scientific.INC, U.S.A) under 5% CO_2 in air at a temperature of 37.8ºC until all the other components for embryo micromanipulation were ready. During these manipulations all petri dishes and media were maintained at 37ºC on a warm stage (Ratek Instruments, Australia) in an air atmosphere.

6.4.2 - Oviduct embryo collection

Oviduct embryo collection was performed using a similar methodology as that used for uterine collection.

6.5 - EMBRYO CULTURE

For culture, embryos were transferred to 10 µl microdrops of HTF-HCO3-/BSA under oil (Paraffin liquid, BDH Laboratory Supplies, England). The culture media was prepared 24 hours prior to use and equilibrated in a incubator of 5% CO_2 in air at temperature of 37.8ºC.

Generally each petri dish was set up with 6 culture drops with a maximum 5 embryos per drop of 10 µl of HTF-HCO3/BSA media under oil. For all manipulations outside the incubator embryos were transferred to a HTF-Hepes/BSA media.

Embryo collection, handling and culture were carried out in 30 mm diameter petri dishes (Disposable Products, Australia).

6.6 - MEDIA RECIPES

All media recipes are presented in appendix 4.

6.7 - EMBRYO TRANSFER

Recipients for embryo transfer were anaesthetized with Avertin at a concentration of 0,015g per gram of body weight.

Following establishment of anaesthesia the uterine tract was exposed through a small incision and the tubal end of the uterus was perforated using an insulin syringe needle. The embryos were then carefully inserted into the uterine lumen with a fire-polished glass pipette connected to a mouth sucker pipette.

Following the embryo transfer the recipient was placed in a warmed cage (18ºC) with water and food (*ad libitum*) until the following morning.

6.7.1 - PREPARATION OF EMBRYO TRANSFER PIPETTES

The pipettes were prepared from Paster pipettes pulled in a gas flame and cut and fire polished to give an external diameter of about 200 µm and internal diameter of about 150 µm. The size of the pipettes was checked using a micrometric camera (Olympus, 0,01 mm, Tokyo) under a stereomicroscope.

6.8 - DETECTION OF PREGNANCY

The pregnancy status of the female mice recipient was assessed by recording the number of live or dead offspring and ascertaining the number of implantation scars following sacrifice of the female mice three weeks past the expected date of delivery.

6.9 - DETECTION OF CHIMERAS

A mouse was considered chimeric if the animal presented abnormal patterns in coat colour and, where cited, by performing a GPI (Glucose Phosphate Isomerase) analysis following cellulose acetate gel electrophoresis. The details of the technique are in Appendix V.

6.10 - DETECTION OF THE GERMLINE CHIMERAS

Male mice which exhibit coat colour chimerism were mated with Swiss albino females and the offspring were examined in order to detect the germline transmission of phenotype.

6.11 - MICROMANIPULATION

Micromanipulation procedures were required to introduce cells (stem cells and inner cell mass cells) into embryos collected at 2.5 and 3.5 days after vaginal plug detection. This was achieved using a Nikon inverted microscope (Diaphot-TMD) fitted with objectives 10X, 20X, 40X (Diascopic DIC, Normaski attachment TMD-NTZ), and a micromanipulator (Narashige Co., LTD, Japan). The blastocyst injection and holding pipettes were connected to two Gilmont microinjectors (Gilmont Instruments, Barnant Company, IL, U.S.A) via flexible tubing (Masterflex 6409-16, IL, U.S.A.) connected by a discofix (B.Braun, Belgium). This allowed controlled injection of a very small amount of media.

6.12 - PREPARATION OF MICROSURGICAL INSTRUMENTS

6.12.1 - EQUIPMENT

Microtools were made from a glass tube (Clark Electromedical

Instrument, Reading, UK, holding pipettes specification GC 100-15, blastocyst injection specification GC 100T-15), using an automatic horizontal pipette puller model (Sutter Instrument Co. California, P-77 Brown-Flaming Micropipette Puller) and microforge (CIT Alcatel, France). The blastocyst injection pipettes were bevelled on the end to facilitate penetration. This was achieved using a micropipette grinder (Bachofer, Germany).

Typically blastocyst injection pipettes were manufactured to have an internal diameter of between 20 and 25 µm and ends were bevelled at an angle of 45º degrees, with holding pipettes having an internal diameter of between 25 and 30 µm and an external diameter between 70 and 80 µm with fire polished ends.

6.13 - FLUORESCENCE MICROSCOPY

Fluorescence microscopy was done under on Olympus microscope (Olympus) fitted with differential interference contrast (BH2-WIC) and a fluorescence attachment (Model BHZ-RFL).

6.14 - MICROPHOTOGRAPHY AND PHOTOGRAPHY

The microscopes were fitted with an Olympus equipment model PM-10ADS and all the photography was done with a Nikon camera (F-

301 or EM).

6.15 - STATISTICAL ANALYSIS

Statistical analysis was carried out using the Minitab, Statistical Software, release 8 (PC version) 1991 package.

The Fisher's Exact Test was used as described by Ayres and Ayres Junior (1987). For the comparison between the percentages of two independent samples, the Z test was carried out as described by Jacques and Wagner (1985).

VII - PRODUCTION OF MOUSE CHIMERAS THROUGH INJECTION OF STEM CELLS INTO BLASTOCYSTS

7.1 - EXPERIMENTAL OUTLINE

The experiments presented in this chapter were aimed at evaluating the efficiency of chimera production by microinjection of stem cells into blastocysts. Comparisons were made between different strains of mice for both stem cells and host blastocysts. The micromanipulated uterine transferred blastocysts were assessed in relation to embryonic mortality.

7.2 - MATERIAL AND METHODS

7.2.1 - EMBRYO COLLECTION

Embryos were collected from Swiss albino, C57BL\6 CBAC57 F1 mice, 2.5 and 3.5 days after vaginal plug detection.

7.2.2 - PREPARATION OF STEM CELLS FOR INJECTION INTO BLASTOCYSTS

The stem cell lines utilised were designated EMBL-5, E14 and transfected E14, and were provided by the Biochemistry Department of Adelaide University and by Bresatec. The stem cell lines EMBL-5 cells were from Dr. Lindsay Williams, Melbourne, Australia and E14 cells were from Dr. Martin Hooper, Cambridge,U.K. The EMBL-5

and E14 stem cells were isolated from mouse strain 129.

The transfection of E14 stem cells was achieved by electroporation of a transgene cassette, containing PMCI-neo^R attached to a alpha 1 - 3 galactosyl transferase gene. The transfected cells were selected in g 418.

To prepare stem cells for blastocyst injection, the stem cell culture medium was replaced by PBS (Ca^{2+} Mg^{2+} free) medium containing EGTA and after five minutes incubation at room temperature, individual colonies of isolated stem cells were washed in PBS(Ca^{2+} Mg^{2+} free) and transferred to a 20 µl drop of medium containing trypsin DIFCO (1:250, 1 g in 100 ml of PBS Ca^{2+} Mg^{2+} free) in order to separate individual cells. Following the separation of the cells by pipetting 60 µl of HTF-Hepes/BSA was added to the 20 µl drop.

7.2.3 - BLASTOCYST INJECTION

Blastocysts classified on the basis of the blastocoel being at an early or middle stage of development were utilised as stem cell recipients. Manipulations were performed using a 90 mm petri dish (Falcon, U.S.A.) containing 20x 10 µl drops of the mixture (HTF-Hepes 9ml + HTF-Hepes/BSA 1ml) under oil. Blastocysts were individually placed in each drop. Other drops were used to contain the stem cells, the drops consisting of (20 µl Trypsin + 60 µl HTF-Hepes/BSA) and a DNase solution (100 µl drop of HTF-Hepes 100 µl containing DNase I 3000 IU/ml) was used for injection pipette cleaning.

Each blastocyst was injected with 15-25 stem cells directly into the blastocoel.

Successfully injected blastocysts were washed in HTF-Hepes/BSA and HTF-HCO3-/BSA and transferred to in vitro culture drops held in a incubator, where they were maintained for embryo transfer.

7.2.4 - EMBRYO TRANSFER

All embryo transfers were performed within 15 hours of blastocyst injection using recipients either 2.5 or 3.5 days after vaginal plug detection.

7.2.5 - STATISTICAL ANALYSIS

The success of embryo transfer was determined by relating the number of implantation scars found in the uterus to the number of blastocysts transferred (BLASTOCYSTS - SCAR OF IMPLANTATION) and the number of offspring obtained (SCAR OF IMPLANTATION - OFFSPRING) which gave respectively an indication of early and late embryonic mortality and the data obtained by difference of (BL-SC) and (SC-OF) of each strain of mice was statistically analised using paired t-tests.

Comparison between the different strains of mice for the same variables (BL-SC) (SC-OF) was made using the Kruskal-Wallis test.

The data from the blastocysts of each strain that were

inoculated with stem cells were pooled together because no statistical difference was found between the variables considered (BL-SC and SC-OF). The comparisons among the strains for each variable (BL-SC and SC-OF) was made using the Kruskal-Wallis, test.

Comparison between the development of embryos which were micromanipulated and those which were not micromanipulated embryos in in vitro culture was done using a Z Test. Analysis of coat colour chimeras and germline chimeras was made using Fishers' Exact Test.

7.3 - RESULTS

7.3.1 - PRODUCTION OF CHIMERAS BY INJECTION OF STEM CELLS INTO BLASTOCYSTS

Three kinds of stem cells (E14, EMBL-5 and E14 transfected) were injected into three different strains of mouse blastocysts: SWISS albino, C57BL\6 and CBAC57\F1.

7.3.2 - E14 STEM CELLS

The data is summarised in table 19. The total number of blastocysts injected with E14 stem cells and submitted to embryo transfer from the SWISS albino strain, the C57BL\6 strain and the CBAC57\F1 strain were 255, 185 and 85 respectively and the number of blastocysts which implanted in the uterus were 106, 66 and 29 respectively. The difference between the number of blastocysts transferred in each strain and the number of blastocysts that implanted into the uterus was statistically significant ($P<0.001$) indicating that early embryonic mortality was a common event for the three strains of mice.

The number of offspring recorded from each strain was respectively 52, 3, 17 respectively. The difference between the number of implanted blastocysts and offspring obtained was statistically significant for the SWISS albino strain and the C57BL\6 strain ($P<0.001$). However there was no statistically significant difference for the CBAC57\F1 strain ($P>0.05$) (figure

7).

No statistical difference (P>0.05) was detected when comparisons were made among the different strains of mice for each variable (number of blastocyst transferred / implanted) and (number of implanted / offspring) (figure 7).

The obtainment of overtly and germline chimeras only occurred with SWISS albino and CBAC57\F1 strains, and both strains were considered statistically equal (table 19).

The blastocysts from SWISS albino mice produced 18 chimeras of which 11 were males and seven females, while the CBAC57\F1 strain produced 6 chimeras: 4 males and 2 females. The females of both strains and the males from the CBAC57\F1 strain were fertile but the two males which originated from stem cell injection into the SWISS albino blastocysts were infertile (table 19).

The E14 stem cells which produced germline chimeras were between passages number 2 and 25.

TABLE 19 - EXPERIMENTS IN WHICH BLASTOCYSTS WERE INJECTED WITH E14 STEM CELLS. THE TABLE SHOWS NUMBER OF BLASTOCYSTS IMPLANTED AND THE NUMBER OF OFFSPRING BORN AND CHIMERAS PRODUCED.

MICE STRAIN	NUMBER OF BLASTOCYSTS TRANSFERRED	NUMBER OF RECIPIENTS	NUMBER OF BLASTOCYSTS IMPLANTED	NUMBER OF OFFSPRING BORN	CHIMERAS			FERTILITY *		GERMLINE CHIMERAS		
					n	n♂	n♀	♂	♀		n♂	n♀
	n	n	n(%)	n(%)	(%)			n (%)	n (%)	n (%)		
SWISS	255	26	106(41.6) a A	52(20.4) a A	18 (7.1)	11	7	9/11 (81.8)	7/7 (100.0)	4 (1.57)	4	-
C57BL\6	185	19	66(35.7) a A	3 (1.6) a A	0	-	-	-	-	-	-	-
CBAC57\F1	85	10	29(34.1) a B	17(20.0) a B	6 (7.1)	4	2	4/4 (100.0)	2/2 (100.0)	1 (1.18)	1	-

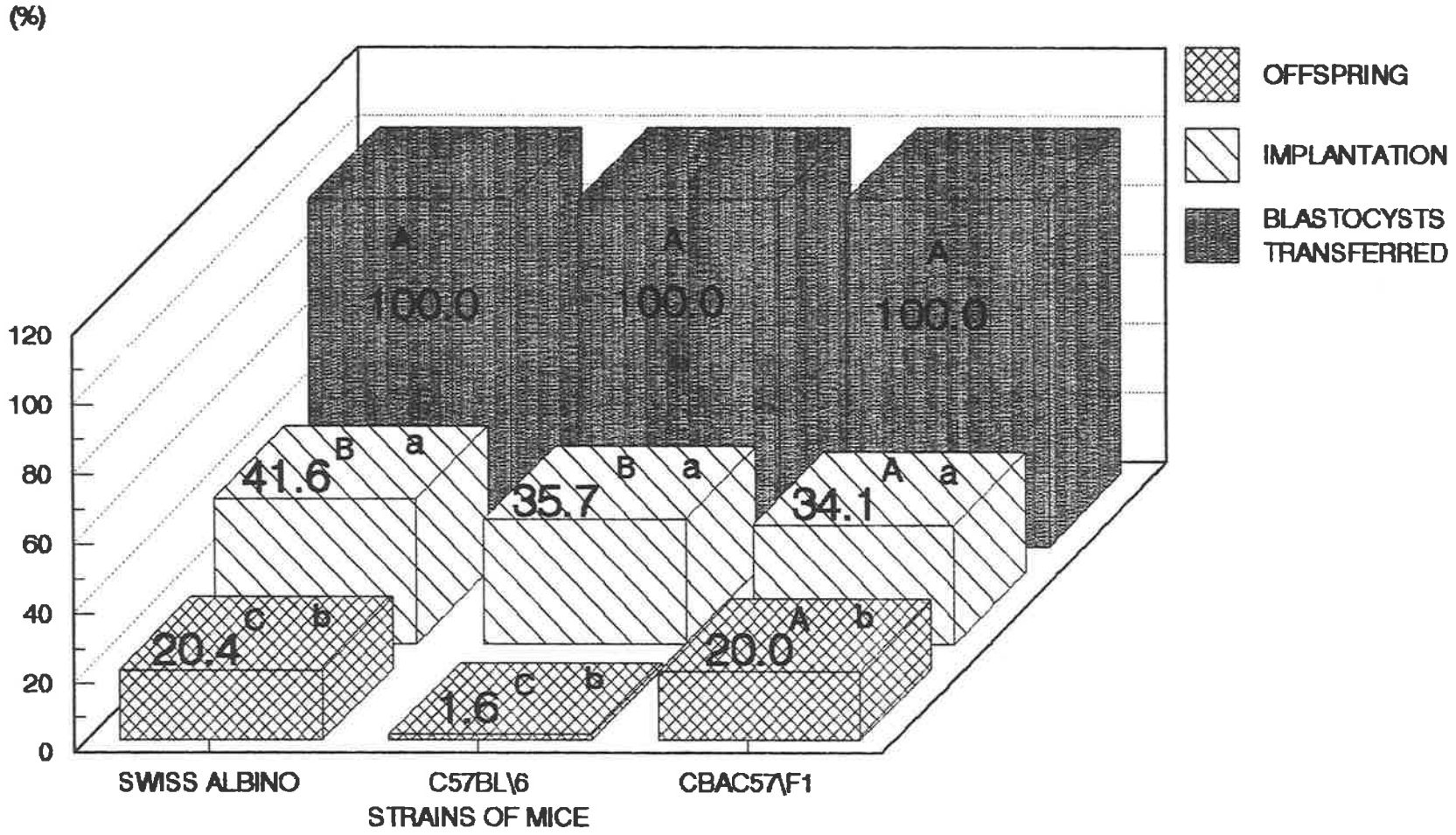
* FERTILITY = CAPACITY TO PRODUCE OFFSPRING.

SAME ALPHABET LETTERS (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE (P>0.05).

LETTER "A" (UPPER CASE) UNDERNEATH OF EACH VARIABLE INDICATES STATISTICAL DIFFERENCE (P<0.001).

LETTER "B" (UPPER CASE) UNDERNEATH OF EACH VARIABLE INDICATES NO STATISTICAL DIFFERENCE (P>0.05).

FIGURE 7 - UTERINE IMPLANTATION RATE AND NUMBER OF OFFSPRING RESULTING FROM EXPERIMENTS IN WHICH MOUSE BLASTOCYSTS WERE INJECTED WITH E14 STEM CELLS.



DIFFERENT ALPHABET LETTERS MEANS STATISTICAL DIFFERENCE, UPPER CASE IS RELATED TO EACH STRAIN OF MICE AND LOWER CASE TO VARIABLE.

FIGURE 8

MALE CHIMERA PRODUCED FROM SWISS albino BLASTOCYST + E14 STEM CELLS

FEMALE MICE MOSAIC ORIGINATED FROM SWISS albino BLASTOCYST + E14 STEM CELLS

GERMLINE MALE CHIMERA ORIGINATING FROM SWISS albino BLASTOCYST + E14 STEM CELLS CROSSED WITH TWO SWISS albino FEMALE MICE

FEMALE ORIGINATING FROM A MALE CHIMERA (SWISS albino BLASTOCYST + E14 STEM CELLS) AND SWISS albino FEMALE MOUSE.THE PICTURE ALSO SHOWS THE OFFSPRING OF THIS FEMALE WHICH WAS PRODUCED FROM A SWISS albino MALE MOUSE

7.3.3 - EMBL-5 STEM CELLS

The EMBL-5 stem cells were injected into blastocysts from SWISS albino and C57BL\6 strains.

The number of blastocysts transferred to the uterus of recipient mice were 214 and 125 and the numbers of blastocysts which implanted in the uterus were 85 and 51 from SWISS albino and C57BL\6 strains respectively (table 20).

Both strains presented a significant number of blastocysts that did not implant, which indicates a high incidence of early embryonic mortality ($P<0.001$) (figure 9).

The number of offspring obtained for each strain was 21 and 2 and the difference between the number of implanted blastocysts and offspring obtained was statistically significant for both strains ($P<0.01$) (figure 9).

No statistical difference for each variable (number of blastocyst transferred / implanted) and (number of implanted / offspring) was found between each strain figure 9.

Coat colour chimerism was only produced with the SWISS albino strain (table 20); 6 chimeras were obtained of which 4 were males and 2 females. Of all the chimeras, only one female did not produce any offspring and this female presented a hermaphrodite uterus at autopsy. The fertile chimeras were not germline.

The EMBL-5 stem cells which produced coat colour chimeras were between passages number 19 to 25.

TABLE 20 - SUMMARY OF EXPERIMENTS IN WHICH BLASTOCYSTS WERE INJECTED WITH EMBL-5 STEM CELLS. THE TABLE SHOWS THE NUMBER OF BLASTOCYSTS IMPLANTED AND THE NUMBER OF OFFSPRING BORN AND CHIMERAS PRODUCED.

MICE STRAIN	NUMBER OF BLASTOCYSTS TRANSFERRED	NUMBER OF RECIPIENTS	NUMBER OF BLASTOCYSTS IMPLANTED	NUMBER OF OFFSPRING BORN	CHIMERAS			FERTILITY *		GERMLINE CHIMERAS		
					n	n♂	n♀	♂	♀		n♂	n♀
	n	n	n(%)	n(%)	(%)			n (%)	n (%)	n (%)		
SWISS	214	17	85(39.7) a A	21(9.8) a B	6 (2.8)	4	2	4/4 (100.0)	1/2 (50.0)	0	-	-
C57BL\6	125	13	51(40.8) a A	2 (1.6) a B	0	-	-	-	-	-	-	-

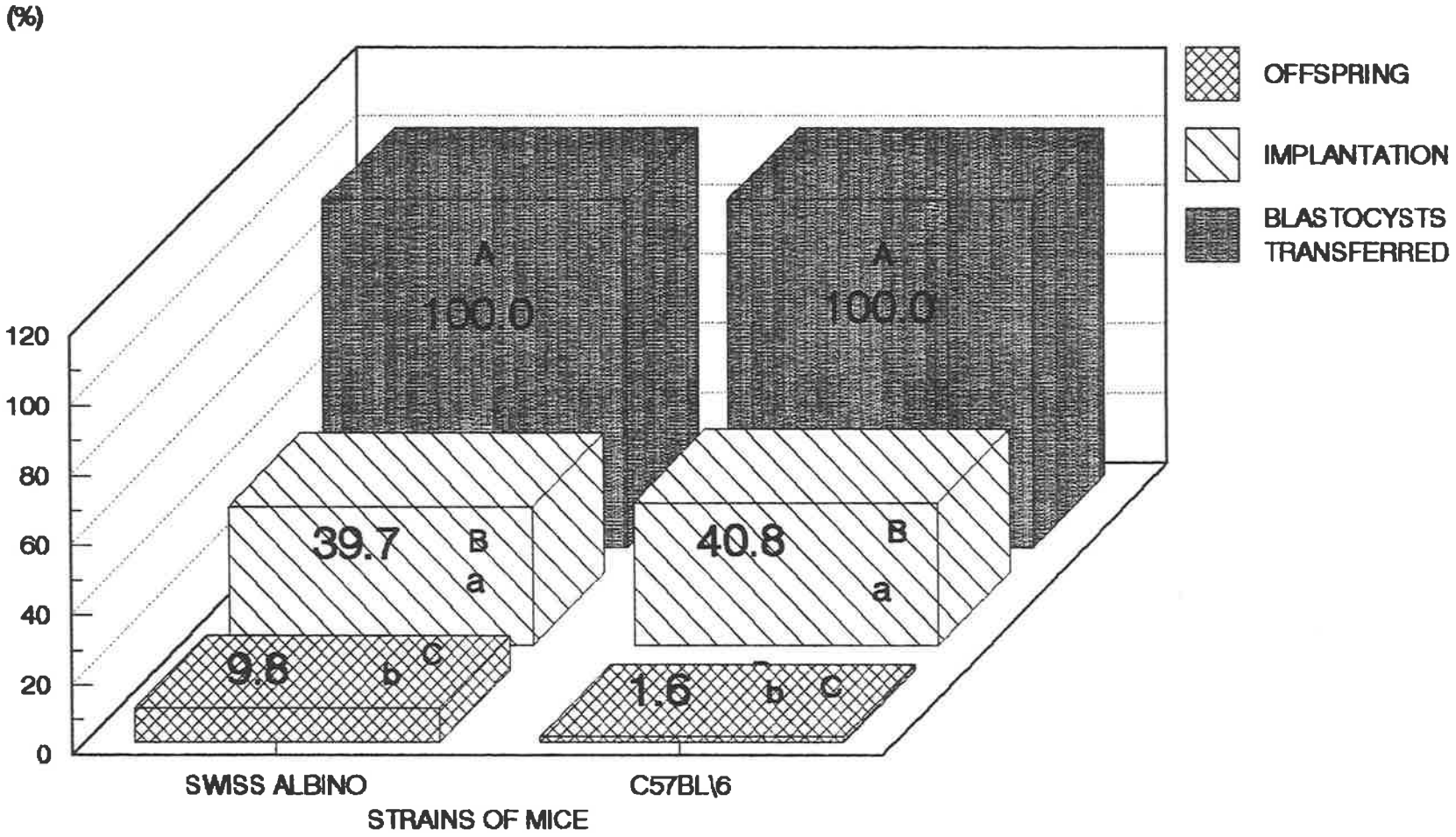
* FERTILITY = CAPACITY TO PRODUCE OFFSPRING.

SAME ALPHABET LETTERS (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE (P>0.05).

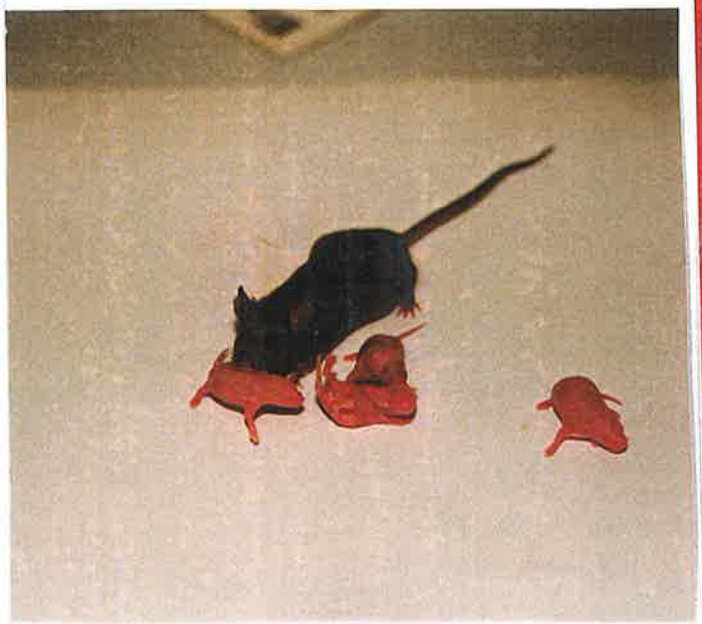
LETTER "A" (UPPER CASE) UNDERNEATH OF EACH VARIABLE INDICATES STATISTICAL DIFFERENCE (P<0.001).

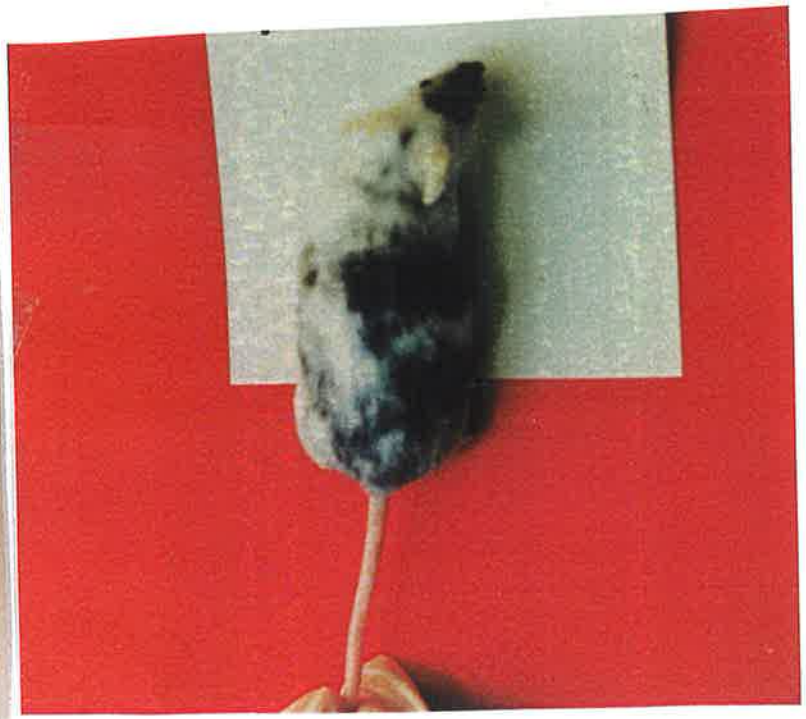
LETTER "B" (UPPER CASE) UNDERNEATH OF EACH VARIABLE INDICATES STATISTICAL DIFFERENCE (P<0.01).

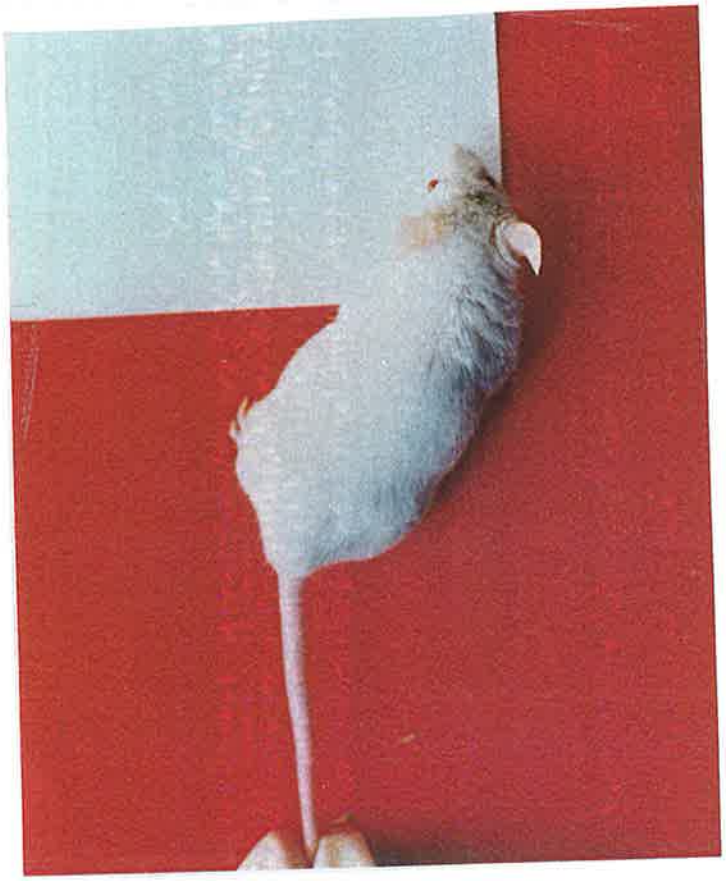
FIGURE 9 - UTERINE IMPLANTATION RATE AND NUMBER OF OFFSPRING RESULTING FROM EXPERIMENTS IN WHICH MOUSE BLASTOCYSTS WERE INJECTED WITH EMBL-5 STEM CELLS.



DIFFERENT ALPHABET LETTERS MEANS STATISTICAL DIFFERENCE, UPPER CASE IS RELATED TO EACH STRAIN OF MICE AND LOWER CASE TO VARIABLE.







7.3.4 - E14 transfected STEM CELLS

The E14 transfected stem cells were introduced into blastocysts of SWISS albino and CBAC57\F1 strains.

The numbers of blastocysts transferred to the uterus of the recipient mice were 32 and 29, the numbers of blastocysts which implanted into the uterus were 15 and 9 and the number of offspring produced were 9 and 1 respectively for the SWISS albino and CBAC57\F1 (table 21).

The loss of embryos due to early embryonic mortality for each strain were not statistically different ($P>0.05$) (figure 11).

Also both strains were considered statistically equal for each variable (number of blastocyst transferred / implanted) and (number of implanted / offspring) for SWISS albino only ($P>0.05$) (figure 11).

Coat colour chimerism was produced in both strains, (table 21) and all chimeras were fertile but a germline was not detected.

The E14 transfected stem cells which produced coat colour chimeras were from passages number 6 and 7.

TABLE 21 - AN OVERVIEW OF THE NUMBER OF BLASTOCYSTS IMPLANTED, OFFSPRING BORN AND CHIMERAS PRODUCED FROM BLASTOCYSTS INJECTED WITH E14 TRANSFECTED STEM CELLS.

MICE STRAIN	NUMBER OF BLASTOCYSTS TRANSFERRED	NUMBER OF RECIPIENTS	NUMBER OF BLASTOCYSTS IMPLANTED	NUMBER OF OFFSPRING BORN	CHIMERAS			FERTILITY *		GERMLINE CHIMERAS		
					n	n♂	n♀	♂	♀		n♂	n♀
	n	n	n(%)	n(%)	(%)			n (%)	n (%)	n (%)		
SWISS	32	2	15(46.9) a A	9(28.1) A	4 (9.4)	2	2	1/1[1] (100.0)	2/2 (100.0)	0	-	-
CBAC57\ F1	29	3	9(31.0) a A	1(3.4)	1 (3.4)	1	-	1/1 (100.0)	-	0	-	-

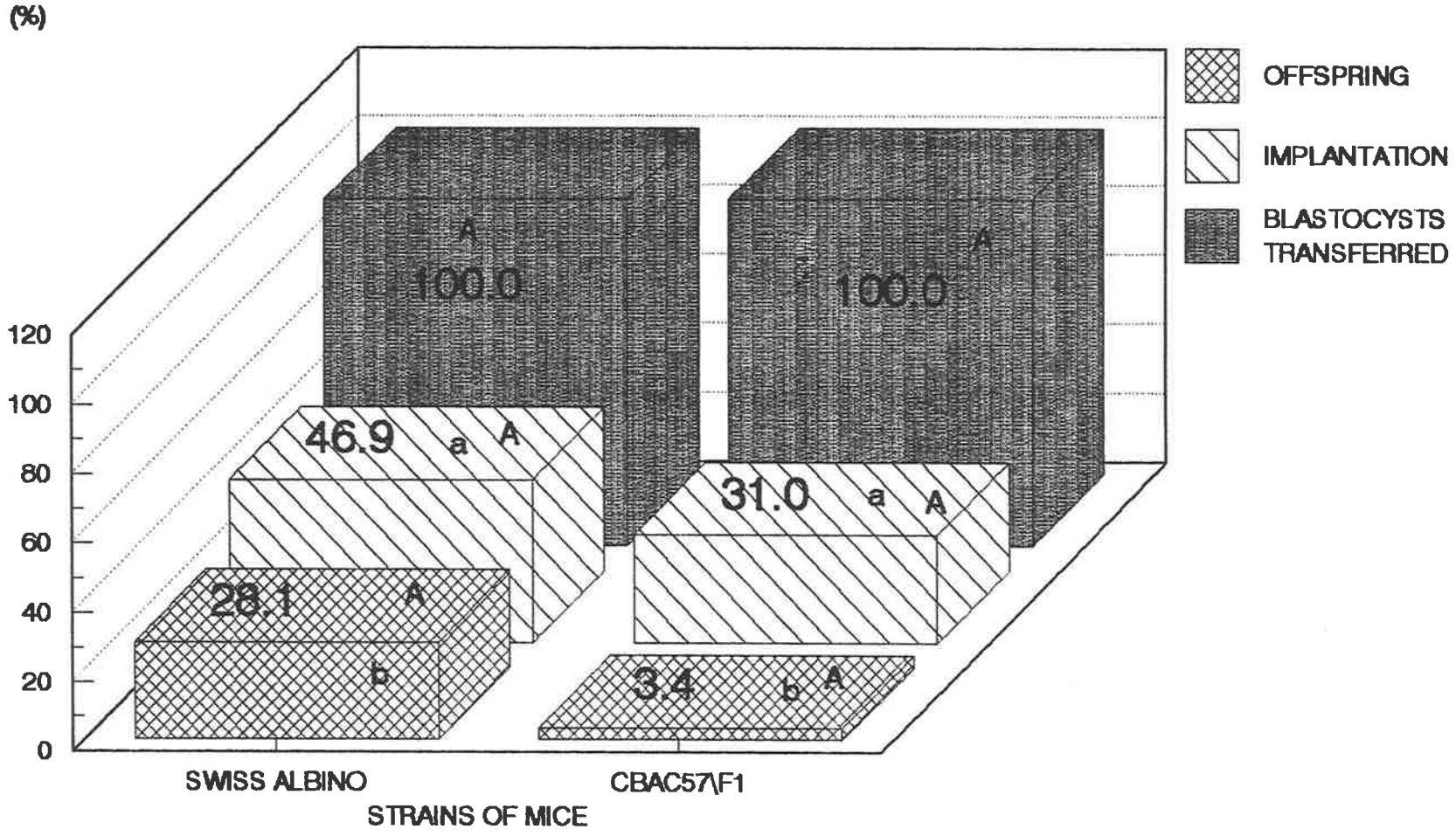
* FERTILITY = CAPACITY TO PRODUCE OFFSPRING.

SAME ALPHABET LETTERS (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE (P>0.05).

LETTER "A" (UPPER CASE) UNDERNEATH OF EACH VARIABLE INDICATES NO STATISTICAL DIFFERENCE (P>0.05).

[1] ONE CHIMAERA ♂ DIED BEFORE MATING

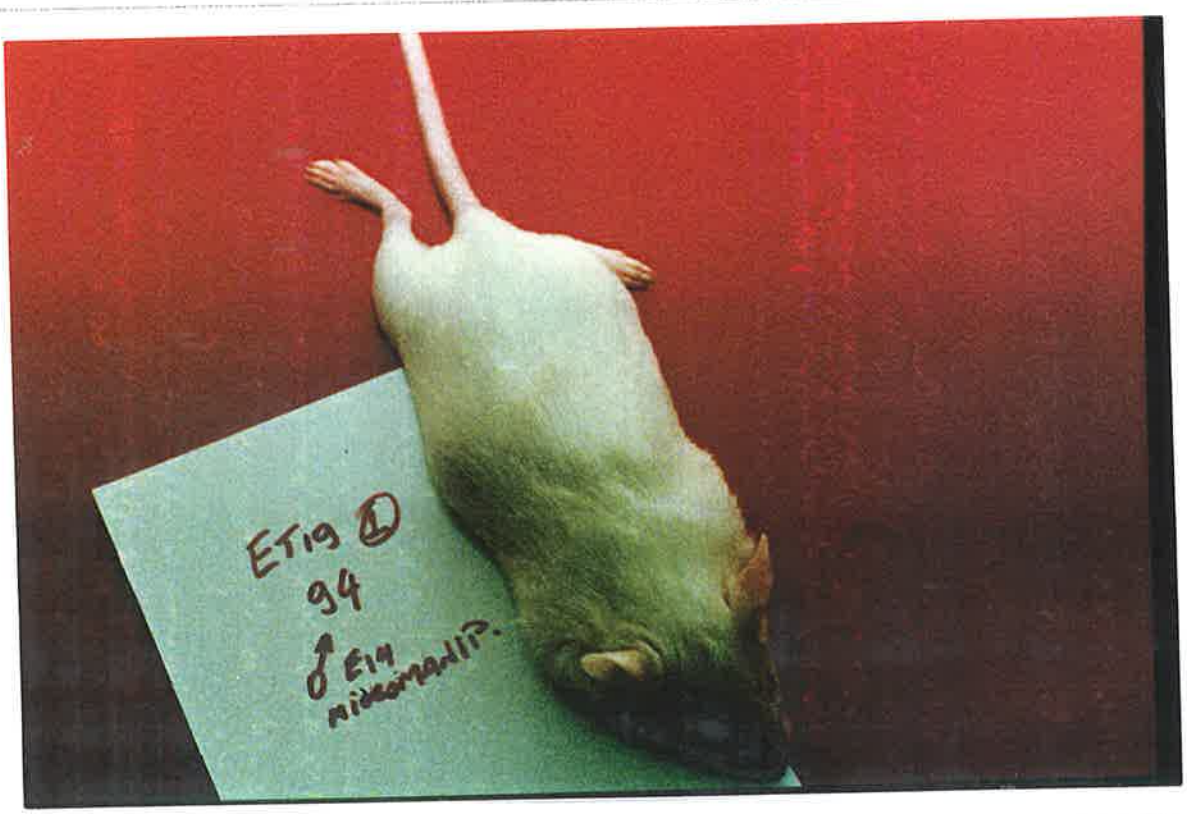
FIGURE 11 - UTERINE IMPLANTATION RATE AND NUMBER OF OFFSPRING RESULTING FROM EXPERIMENTS IN WHICH MOUSE BLASTOCYSTS WERE INJECTED WITH E14 TRANSFECTED STEM CELLS.



DIFFERENT ALPHABET LETTERS MEANS STATISTICAL DIFFERENCE, UPPER CASE IS RELATED TO EACH STRAIN OF MICE AND LOWER CASE TO VARIABLE.

FIGURE 12

MOSAIC PRODUCED WITH CBAC57\E1 BLASTOCYSTS AND E14 TRANSFECTED STEM CELLS



MOSAIC PRODUCED WITH SWISS albino BLASTOCYSTS AND E14 TRANSFECTED STEM CELLS

7.3.5 - COMPARISON BETWEEN CBAC57\F1 EMBRYOS COLLECTED AT DIFFERENT TIMES AFTER HCG INJECTION AND INOCULATED WITH E14 TRANSFECTED STEM CELLS

The number of embryos transferred to the uterus of recipient mice were 40 and 29, while the number of blastocysts which implanted in the uterus were 8 and 9 and the number of offspring born were 7 and 1 respectively for embryos collected 4 and 5 days after HCG (table 22).

There was a mean loss of early embryonic mortality which was statistically significant ($P<0.01$) for the embryos inoculated with stem cells at 4 days after HCG administration; the embryos collected 5 days after HCG were considered statistically equal ($P>0.05$), (figure 13). The embryos CBAC57\F1 collected 4 days after HCG for the variable (number of implanted / offspring) indicating that late embryonic mortality did not occur (figure 13). Both strains were considered statistically identical for each variable (number of blastocyst transferred / implanted) (figure 13).

Overt chimeras were produced with both kinds of embryos as shown in (table 22); all produced chimeras which were fertile but none were germline.

TABLE 22 - THE EFFECT OF EMBRYO DEVELOPMENT ON CHIMERA PRODUCTION. EMBRYOS WERE COLLECTED 4 OR 5 DAYS AFTER HCG ADMINISTRATION/MATING AND INJECTED WITH E14 transfected STEM CELLS. THE TABLE PRESENTS AN OVERVIEW OF NUMBERS OF BLASTOCYSTS IMPLANTED, OFFSPRING BORN AND CHIMERAS PRODUCED.

MICE STRAIN	NUMBER OF BLASTOCYSTS TRANSFERRED	NUMBER OF RECIPIENTS	NUMBER OF BLASTOCYSTS IMPLANTED	NUMBER OF OFFSPRING BORN	CHIMERAS			FERTILITY *		GERMLINE CHIMERAS		
					n	n♂	n♀	♂	♀		n♂	n♀
	n	n	n(%)	n(%)	(%)			n (%)	n (%)	n (%)		
CBAC57\ F1 4 DAYS AFTER HCG	40	4	8(20.0) a B	7(17.5) A	2 (5.0)	1	1	1/1 (100.0)	1/1 (100.0)	0	-	-
CBAC57\ F1 5 DAYS AFTER HCG	29	3	9(31.0) a A	1(3.4)	1 (3.4)	1	-	1/1 (100.0)	-	0	-	-

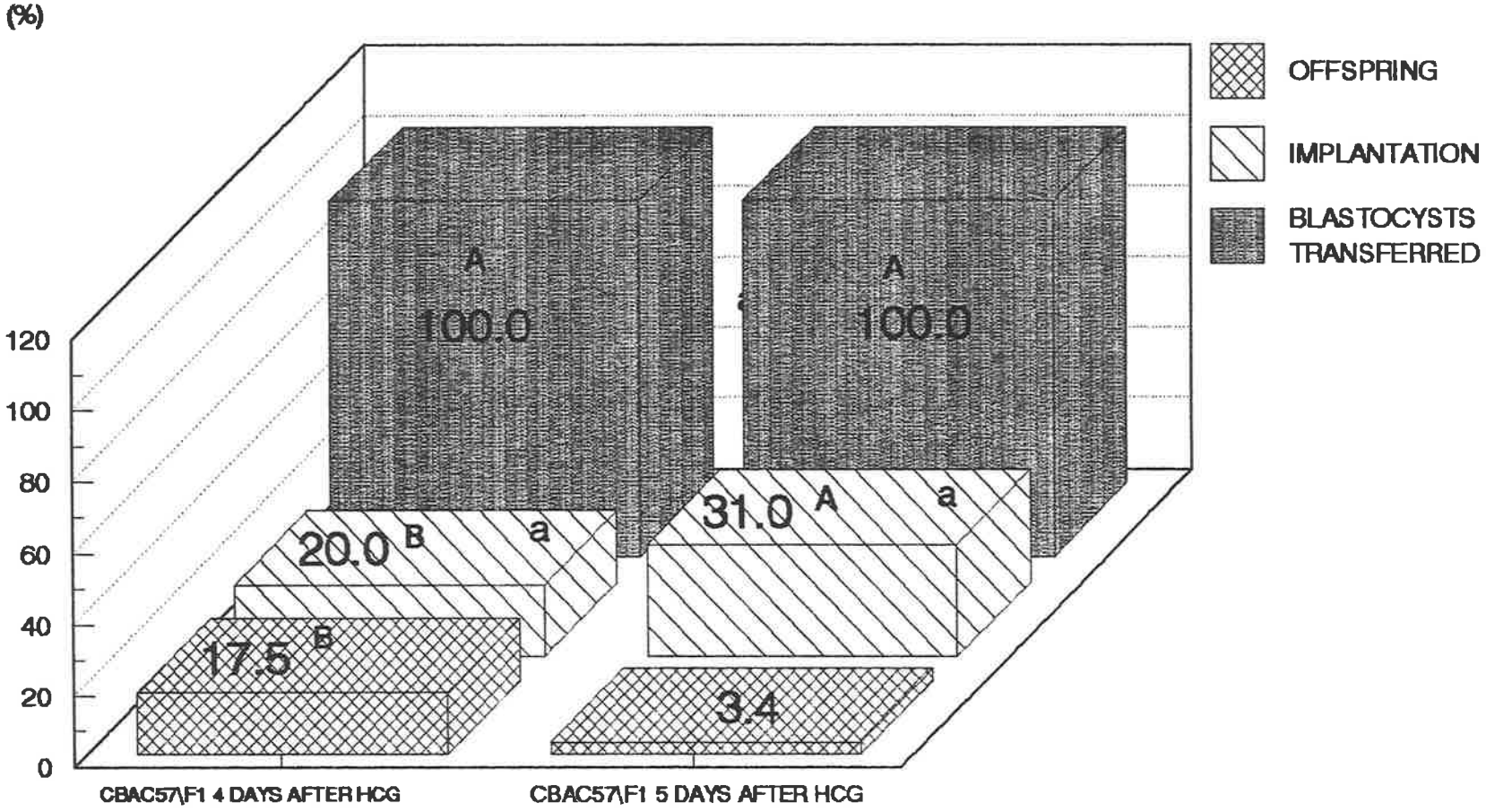
* FERTILITY = CAPACITY TO PRODUCE OFFSPRING.

SAME ALPHABET LETTERS (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE (P>0.05).

LETTER "A" (UPPER CASE) UNDERNEATH OF EACH VARIABLE INDICATES NO STATISTICAL DIFFERENCE (P>0.05).

LETTER "B" (UPPER CASE) UNDERNEATH OF EACH VARIABLE INDICATES STATISTICAL DIFFERENCE (P<0.01).

FIGURE 13 - UTERINE IMPLANTATION RATE AND NUMBER OF OFFSPRING RESULTING FROM EXPERIMENTS IN WHICH MOUSE BLASTOCYSTS OF CBAC57\F1 STRAIN WERE INJECTED WITH E14 TRANSFECTED STEM CELLS.



DIFFERENT ALPHABET LETTERS MEANS STATISTICAL DIFFERENCE, UPPER CASE IS RELATED TO EACH STRAIN OF MICE AND LOWER CASE TO VARIABLE.

7.3.6 - POOLED DATA FROM EXPERIMENTS IN WHICH BLASTOCYSTS FROM DIFFERENT STRAINS OF MICE WERE INJECTED WITH THREE KINDS OF STEM CELLS

Since no differences were found among the strains of mouse blastocysts injected with various stem cells the data were pooled in relation to the variables (number of blastocyst transferred / implanted) and (number of implanted / offspring), figure 14.

The blastocysts of SWISS albino strain mice were inoculated with 3 kinds of stem cells totalling 501 blastocysts injected, 206 (41,10%) and 82 (16,40%) implanted and resultant offspring respectively.

The C57BL\6 strain had 310 blastocysts, which received by micromanipulation 2 kinds of stem cells, with implantation occurring in 117 (37,70%) but only 5 (1,6%) offspring were born.

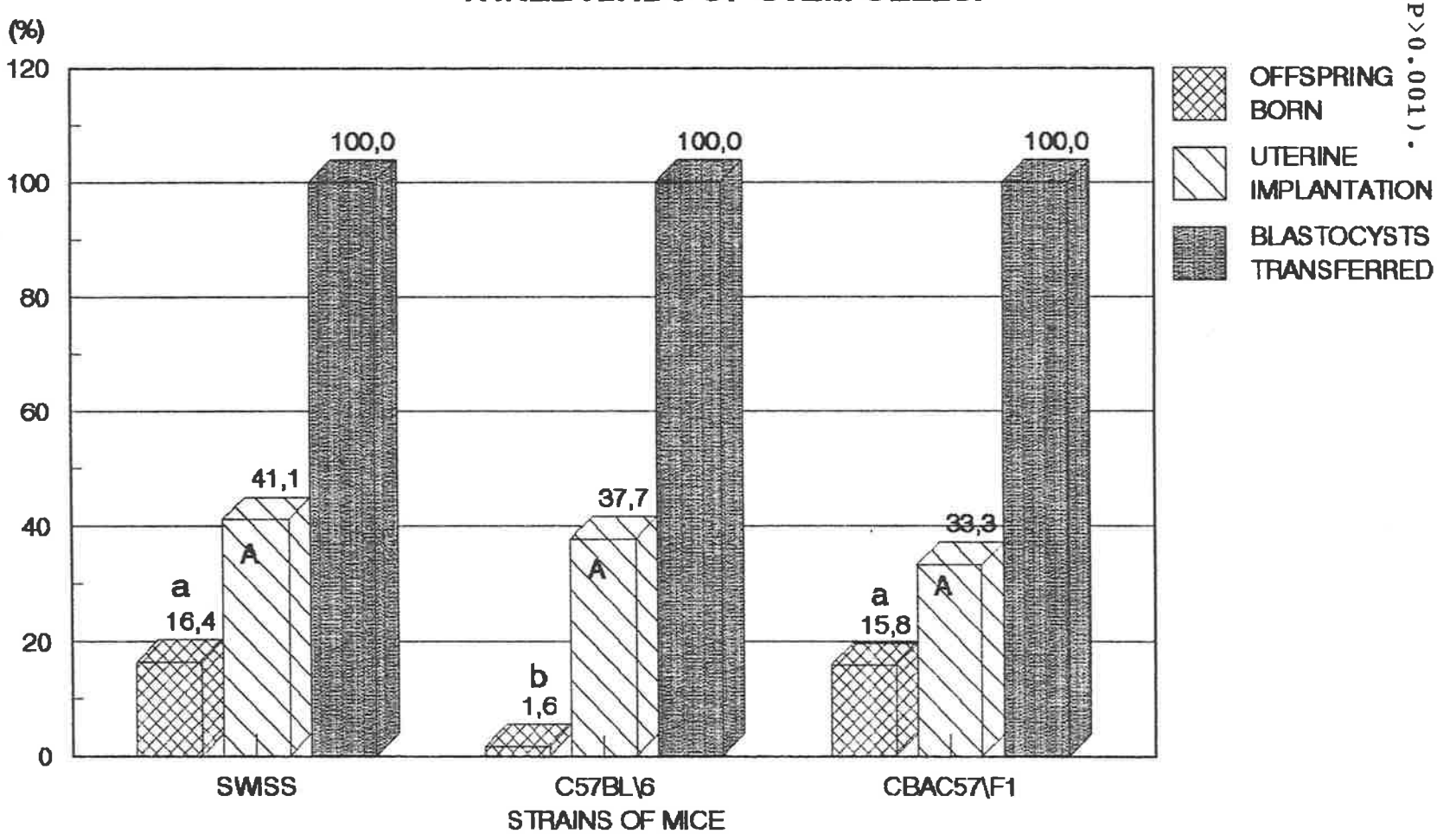
The CBAC57\F1 strain had 114 blastocysts inoculated with stem cells, of which 38 (33,30%) implanted and 18 (15,80%) generated offspring.

The three strains of mice had a similar pattern in respect to uterine implantation, but the number of offspring born was similar for the SWISS albino strain and the CBAC57\F1 strain while both strains differed from the C57BL\6 strain ($P<0.001$).

The only strain of mouse blastocyst which did not produce any chimeras was C57BL\6, the other two strains SWISS albino and CBAC57\F1 produced chimeras, (figure 15).

The number of chimeras produced in relation to the number of blastocysts injected with stem cells did not result in a

FIGURE 14 - POOLED DATA COMPARING RESULTS FROM THE THREE DIFFERENT MOUSE BLASTOCYST STRAINS INJECTED WITH THREE KINDS OF STEM CELLS.

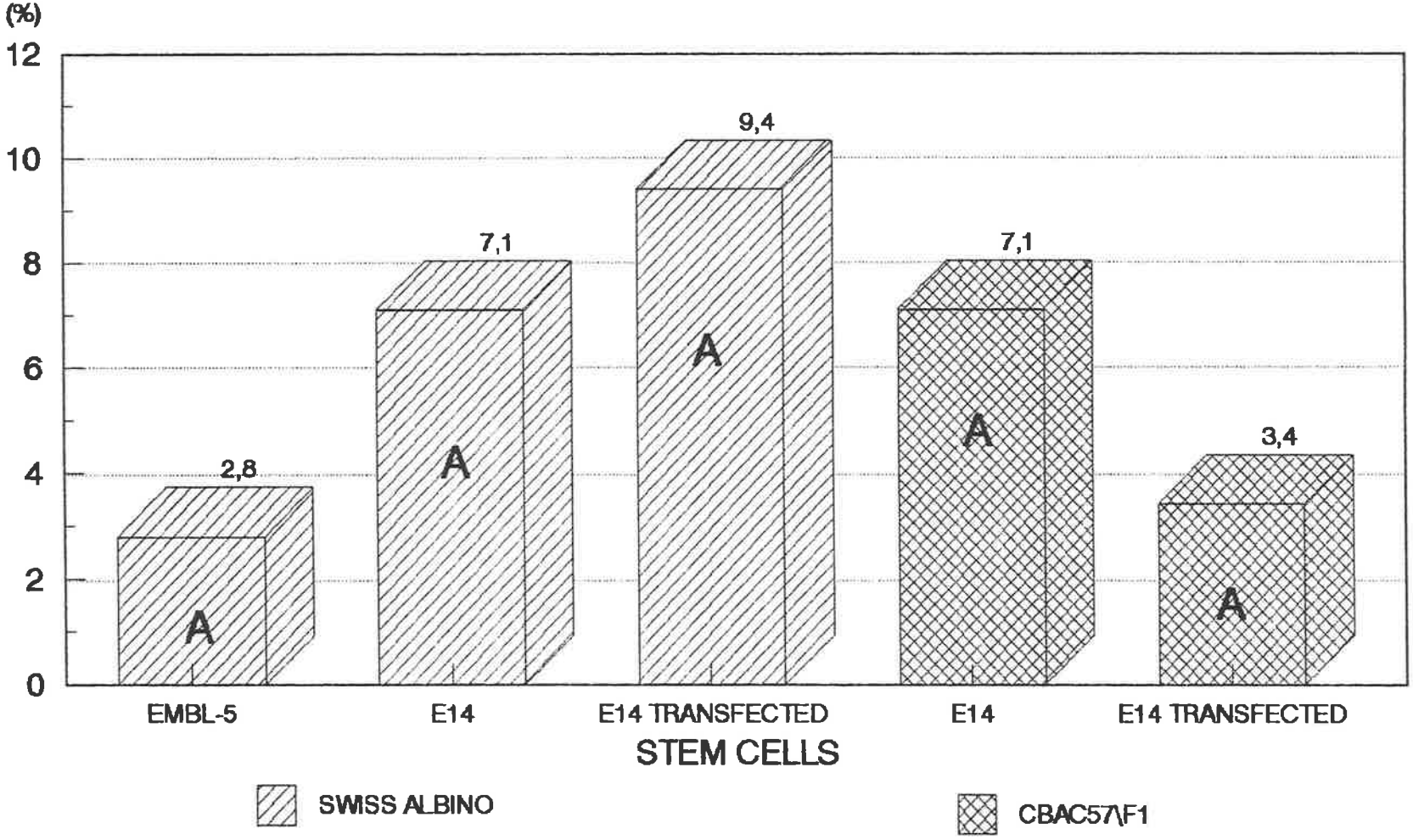


DIFFERENT ALPHABET LETTERS MEANS STATISTICAL DIFFERENCE, UPPER CASE IS RELATED TO IMPLANTATION AND LOWER CASE TO OFFSPRING.

used, ($P>0.001$).

statistical difference for all strains of mice and the stem cells

FIGURE 15 - EFFICIENCY OF PRODUCTION OF OVERT CHIMERAS USING DIFFERENT STRAINS OF MICE BLASTOCYSTS(*) AND STEM CELLS.



SAME ALPHABET LETTERS MEANS NO STATISTICAL DIFFERENCE.
(*) BLASTOCYSTS COLLECTED 5 DAYS AFTER HCG.

7.3.7 - RESULTS OF GLUCOSE PHOSPHATE ISOMERASE ANALYSIS FROM THE CHIMERAS PRODUCED WITH ALL STRAINS OF MICE AND STEM CELLS

A glucose phosphate isomerase (GPI) analysis was done to check the integration and manifestation of the stem cells (band characteristics of 129 mice) injected into host blastocysts (SWISS albino strain, C57BL\6 strain, CBAC57\F1 strain) in the blood of the coat colour chimeras produced, figure 16. The intensity of the bands were used as an indication of the amount of 129 contribution into the host blastocysts used.

7.3.7.1 - SWISS albino blastocysts and EMBL-5 stem cells

This combination of blastocysts and stem cells produced 6 chimeras of which 2 had the blood collected for GPI analysis. Both mice, a male and a female were overtly chimeric, but did not present a 129 band.

7.3.7.2 - SWISS albino blastocysts and E14 stem cells

The association of SWISS albino strain and E14 stem cells allowed the production of 18 chimeras, but the GPI analysis was done on only 13. The lowest amount of 129 detected was 50% and the maximum 100%.

7.3.7.3 - SWISS albino blastocysts and E14 transfected stem cells

From the 4 chimeras produced GPI analysis was done in 3, and the lowest 129 strain contribution was 60% while the maximum was 65%.

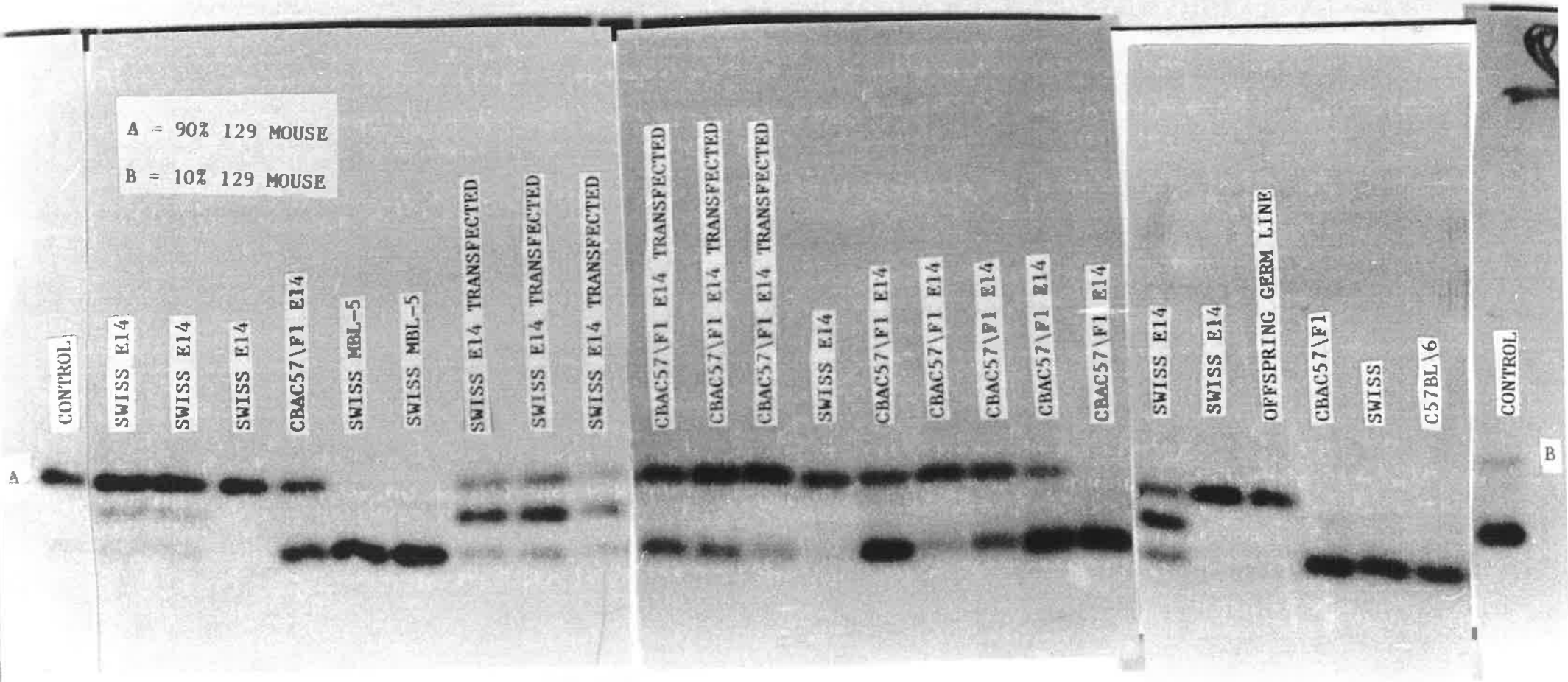
7.3.7.4 - CBAC57\F1 blastocysts and E14 stem cells

This combination of blastocysts and stem cells produced 6 chimeras, the 129 contribution ranging from 25% to 75%. One of the females overtly chimeric produced (with >50% coat colour contribution) had the GPI analysis repeated and on both occasions the 129 strain contribution was zero.

7.3.7.5 - CBAC57\F1 blastocysts and E14 transfected stem cells

The 3 chimeras produced had a minimum and maximum range of 129 contribution of 50% and 85%.

FIGURE 16 - GLUCOSE PHOSPHATE ISOMERASE ELECTROPHORESIS RESULTS OF THE CHIMERAS PRODUCED



7.4 - DISCUSSION

The analysis of the pooled data of different stem cells in relation to each blastocyst strain of mice (SWISS albino, C57BL\6 and CBAC57\F1) showed that loss of pre-implantation embryos was similar statistically between the three strains of mice (figure 14).

These losses could be due to many factors. The first possibility ruled out could be the inadequate combination of the blastocyst recipient strain of mice used and the stem cells injected.

The second reason for this embryo loss could be attributed to the micromanipulation process since the blastocysts were submitted to an invasive procedure by penetration of a microinjection pipette through the zona pellucida in order to introduce the stem cells into the blastocoel cavity, a situation which could have precipitated the hatching of the uterine transferred blastocysts. The early exposure of the trophectoderm cells to an inadequate uterine environment could be responsible for some embryo loss before implantation.

The third reason could be related to the asynchrony between the embryo development and the time of pseudopregnancy of the recipient females. This possibility is considered in the next chapter.

The fourth reason could be attributed to the amount of stem cells which attach to the inner cell mass cells of the host blastocyst, since there is a possibility that the excess of cells

and the imbalance between the modified inner cell mass cells and trophoblast of the host embryo along with the abrupt artificial increase of the inner cell mass could be responsible for embryo losses. As reviewed by Somers et al. (1990), embryos with a disturbed allocation of cells to the inner cell mass and trophectoderm have lower developmental potential in vitro and in vivo. Also the loss of embryos before implantation of all strains could be attributed to the embryo transfer process.

Late embryonic mortality was evident in all three strains of mice. The SWISS albino strain and CBAC57\F1 strain were similar statistically and both strains were statistically different from the C57BL\6 strain, (figure 14). The loss of embryos after implantation could be due to the same reasons enumerated for early embryonic mortality excluding the embryo transfer process.

The micromanipulated blastocysts of mouse C57BL\6 strain had the same implantation rate as the blastocysts of the SWISS albino strain and the CBAC57\F1 strain but showed the worst performance of the three strains of mice in relation to offspring production. During the development of those experiments it was observed that the C57BL\6 mouse embryos were always behind in development when compared with the other strains. The poor performance of the C57BL\6 strain could be explained by the work of (Gates 1965, cited by Wilmut, 1986), who showed that the cleavage rate did not influence the proportion of embryos able to implant, but did significantly influence the slow growing embryos, died after

implantation. A similar occurrence was observed in the experiments described here.

Cannibalism was observed in the C57BL\6 embryos which were micromanipulated and transferred to recipient female mice; all the offspring obtained were found dead in their cages. Piedrahita et al. (1992) observed incidences of cannibalism in hamsters in litters of 4 pups or less.

The data analysed for each blastocyst strain and stem cells demonstrated the presence of early and late embryonic mortality which was statistically significant for blastocysts of SWISS albino and C57BL\6 strain mice which were injected with E14 and EMBL-5 stem cells (tables 19 and 20; figures 7 and 9). However early and late embryonic mortality was not statistically significant in the transferred blastocysts of CBAC57\F1 strain mice combined with E14 normal (figure 7), and transfected stem cells (figure 11) and the blastocysts of SWISS albino with E14 transfected stem cells (figure 11). The diminished loss of embryos of the CBAC57\F1 mouse strain could be due to the fact that the embryo transfers were in the majority done in female mouse recipients of the same strain, and perhaps this close harmony between donor and recipient improved the results.

Coat colour chimeras were produced with SWISS albino mice, an outbred strain, and CBAC57\F1 mice, a hybrid strain. The pooled data of all stem cells used in relation to each strain of mice demonstrated that both strains had the same pattern in

relation to the production of chimeras (P>0.001) (figure 15). However germline chimeras were produced in both strains which received normal stem cells but none were obtained with transfected stem cells. This observation agrees with Zijlstra et al. (1989) who obtained contrasting results for chimera production with 2 clones of transfected stem cells. Gossler et al. (1986) report similar results in the production of germline chimeras with normal and transfected stem cells.

The blastocysts of strain C57BL\6, an inbred strain, were injected with E14 and EMBL-5 stem cells and did not produce any chimeras, mainly due to their low reproductive performance. This observation which is at variance with the work of Schwartzberg et al. (1989) and Pease and Williams (1990) who produced germline chimeras with C57BL\6 mice with CCE and D3 stem cells which were also generated from the 129 strain of mice.

The SWISS albino, an outbred strain, was injected with stem cells (E14, EMBL-5 and E14 transfected) and this strain of mice allowed the production of chimeras with all three stem cells lines injected, however, germline chimeras were only produced with normal E14 stem cells. Schwartzberg et al., (1989) found that different outbred albino strains of mice CD-1 and MF1 gave different results in relation to the production of chimeras with CCE stem cells and none of the strains produced germline chimeras. The same observation was published by Pease and Williams (1990) that the ICR outbred chimeras did not form ES-

derived germ cells.

The hybrid mouse strain CBAC57\F1 received E14 and E14 transfected stem cells and with both stem cells lines produced chimeras, however germline chimeras were produced with normal E14 stem cells.

Other researchers, for example Schwartzberg et al. (1989); Pease and Williams (1990); Tokunaga and Tsunoda (1992) highlighted the importance of the genotype of the recipient blastocysts which can profoundly influence the overall incorporation of stem cells in the developing conceptus, a decisive factor for germline transmission.

One of the possible explanations for the importance of the compatibility between two strains of mice when associated (stem cells and blastocysts) is presented in the work of Latham et al. (1993), who characterised the different patterns of protein synthesis in the different germ layers and the distinct behaviour of the individual layers in the mouse conceptus. During the differentiation process gene expression and proteins are expressed according to a strict time schedule. It is quite possible that this biological time of the gene and protein expression of each germ layer, perhaps distinct for each strain, could be responsible for the various results of the genotype combination of mice strains of stem cell and the host embryo used.

In the present work in three chimeras the integration and expression of stem cells was only in the ectoderm (coat colour

chimerism) and those chimeric mice did not present the characteristic (Glucose Phosphate Isomerase) band of the 129 strain mice in blood (mesoderm), (figure 16). Only one of these chimeras were male and the stem cells did not colonise the germline. It is suggested in the work of Iannaccone et al. (1985) and Mann et al., (1993) that coat chimerism is often used as an indirect guide to germline potential, although it does not indicate whether the primitive gonads have been colonised by the stem cells. In the present work with those three chimeras the stem cells inoculated did not contribute to the haematopoietic tissue and gonadal ridge.

Many strategies have been used to improve the colonisation of stem cells into host embryos. Peli and Schellander (1992) used tetraploid embryos and concluded that stem cells injected into a depressed embryo can support complete fetal development. Tokunaga and Tsunoda (1992) demonstrated that an unsatisfactory combination between the genotype of stem cells and blastocysts can be overcome by injection of stem cells into 8 cell embryos. The results of the present work demonstrated with CBAC57\F1 mouse embryos inoculated with E14 transfected stem cells at 4 days and 5 days after HCG demonstrated the possibility of production of overt chimeras but none of the produced chimeras were germline. The low number of chimeras obtained does not allow any solid conclusion that could show difference between the two kinds of embryos, however there is an indication that the stem cells used were limited in their ability to colonise the gonadal ridge of the chimeras obtained, and/or the host blastocysts were

incompatible to the stem cells (table 22, figure 13).

Blastocyst injection with stem cells is a system for the production of chimeras, those animals which have the ability to immortalise the genome transmitted by stem cells. The chimeras produced for this purpose present limitations. Robertson et al. (1983) demonstrated that XY stem cell lines are more stable than XX stem cells lines in culture. Because of this the majority of stem cell lines isolated and injected into blastocysts are from male embryos. As a consequence, the successful produced germline chimeras are mainly related to sperm production. Moreover, in the searched literature, a publication about a female germline overtly chimeric was not found.

In the present work it was observed with the XY stem cells injected into blastocysts the formation of female chimeras, which were mated and obviously not germline. The stem cells used were not capable of colonising the gonadal ridge of the host blastocyst or the host blastocysts did not offer conditions for this colonisation. This could be related to the genotype combination of stem cells and host blastocyst.

The distortion in the sex ratio among the chimeric population is caused by conversion of XX host embryos into phenotypic males, (Iannaccone et al., 1985). Surani et al. (1987) found the greatest efficiency in obtaining germline chimaeras from pluripotential stem cells by introducing cells with XY genotype into XX recipient embryos, such that all sperm produced by the pseudomale was 100% derived from the injected male stem cell line. Mann et al., (1993) observed that in some instances,

male chimeras transmitted the stem cell genotype to less than 3% of their offspring, indicating a low colonisation of a particular stem cell in the gonadal ridge.

In order to produce germline chimera females derived from stem cells it is necessary to improve our knowledge in order to isolate and keep in in vitro culture stable XX stem cell lines capable of overcoming or participating to a high degree in the genetics of the genetic gonadal ridge of XX or XY host blastocysts.

Cloning techniques could be used for the propagation of the genome of male and female stem cells.

Nuclear transfer promises to be one of the ways of achieving the propagation of the stem cell genome, however it is necessary to defeat many limitations of this procedure, not only for mouse, as well as for farm animals. At the present moment only the chimeras can be used as a way of transmitting the genome of the stem cells. Tsunoda and Kato (1993) claim that chromosome abnormality of the individual stem cells transferred to enucleated oocytes may explain the low ability of mouse embryos to develop into blastocysts in vitro, a situation which is overcome by construction of chimeras because in the blastocysts injection generally inoculates more than one stem cell.

Furthermore, some of the cloned generate offspring present health problems, possible due to the complexity of this technology which is being developed, (Seidel,Jr. 1992).

The problems related to the mouse chimeras produced during the course of the present work had problems related to

infertility in 2 normal phenotypic male chimeras and hermaphroditism in one female chimera.

Stem cell technology associated with the chimeric route offers a system which allow the multiplication and preservation of highly productive and rare breed animal genotypes. Moreover the transfected stem cells promise to be a suitable way for the creation of transgenic animals and thus novel genotypes.

As in the mouse experimental model, the knowledge about genotype combination between host embryo and stem cells for farm animals may reveal incompatibly among different breeds of animals in the same species.

VIII - EMBRYO TRANSFER OF BLASTOCYSTS MICROINJECTED WITH STEM CELLS TO RECIPIENT FEMALE MICE OF CBAC57\F1 STRAIN AT TWO TIMES OF PSEUDOPREGNANCY

8.1 - EXPERIMENTAL OUTLINE

The aim of this experiment was to compare the partial data of the blastocysts injected with stem cells and control blastocysts which where submitted to the embryo transfer to female mice recipients of CBAC57\F1 strain at two different times of pseudopregnancy.

8.2 - MATERIAL AND METHODS

8.2.1 - STATISTICAL ANALYSIS

The success of embryo transfer was determined by relating the number of implantation scars found in the uterus to the number of blastocysts transferred (BLASTOCYSTS TRANSFERRED - SCAR OF IMPLANTATION) and the number of offspring obtained (SCAR OF IMPLANTATION - OFFSPRING) which gave an indication of early and late embryonic mortality respectively.

The variables (BL-SC) and (SC-OF) were statistically tested:

a) to show the statistical similarity among the mouse strains at each pseudopregnant time ($P>0.05$);

b) to compare each time of pseudopregnancy;

c) between microinjected and control blastocysts.

The Kruskal - Wallis test was used for these comparisons.

8.3 - RESULTS

8.3.1 - EMBRYO TRANSFER OF BLASTOCYSTS INJECTED WITH KARYOPLASTS

Table 23 shows that no time effect was detected (P>0.05) for either variables (blastocyst transferred - scars of implantation into the uterus) and the (scar of implantation - number of offspring produced), although there is a trend towards the recipients which received the microinjected embryos at 2.5 days of pregnancy compared with those receiving embryos at 3.5 days.

TABLE 23 - BLASTOCYSTS INJECTED WITH KARYOPLASTS INTO THE BLASTOCOELE AND TRANSFERRED TO CBAC57\F1 MICE RECIPIENTS AT DIFFERENT DAYS OF PSEUDOPREGNANCY.

DAYS OF PSEUDOPREGNANCY	NUMBER OF EMBRYO TRANSFERS	NUMBER OF BLASTOCYSTS TRANSFERRED	NUMBER OF SCARS OF IMPLANTATION	NUMBER OF OFFSPRING PRODUCED
2.5	18	206	92 (44.7) [a]	44 (21.4) [a]
3.5	18	195	50 (25.6) [a]	3 (1.54) [a]

SAME LETTER "a" (LOWER CASE), IN THE SAME COLUMN, MEANS NO STATISTICAL DIFFERENCE (P>0.05).

8.3.2 - EMBRYO TRANSFER OF CONTROL BLASTOCYSTS

The two variables (blastocyst transferred - number of uterine scars) and (scar of implantation - offspring produced) were not different for either times (P>0.05) of pseudopregnancy (**table 24**) . However the control recipients which received embryo transfer at 2.5 days showed better performance than at 3.5 days.

TABLE 24 - CONTROL BLASTOCYSTS TRANSFERRED AT DIFFERENT TIMES OF PSEUDOPREGNANCY.

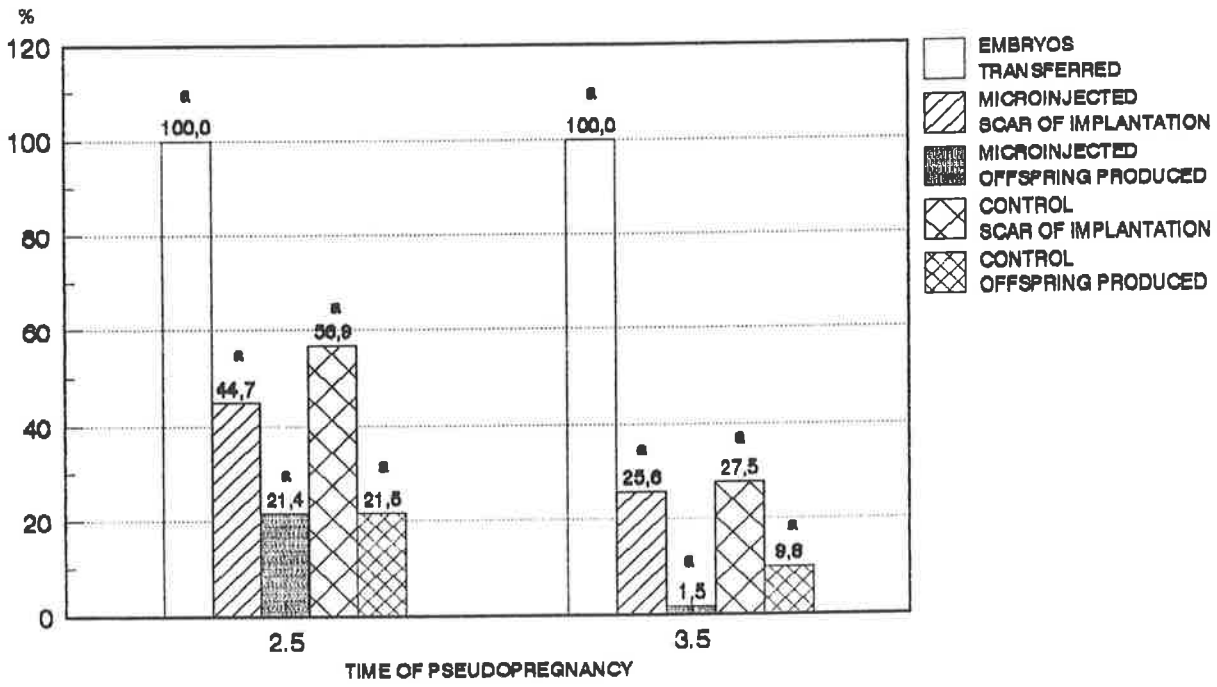
DAYS OF PSEUDOPREGNANCY	NUMBER OF EMBRYO TRANSFER	NUMBER OF BLASTOCYSTS TRANSFERRED	NUMBER OF SCARS OF IMPLANTATION	NUMBER OF OFFSPRING PRODUCED
2.5	6	79	45 (56.9) [a]	17 (21.5) [a]
3.5	9	102	28 (27.5) [a]	10 (9.8) [a]

SAME LETTER "a" (LOWER CASE), IN THE SAME COLUMN, MEANS NO STATISTICAL DIFFERENCE (P>0.05).

8.3.3 - COMPARISON OF MICROINJECTED AND CONTROL BLASTOCYSTS AT DIFFERENT TIMES OF PSEUDOPREGNANCY

The comparison between control and microinjected blastocysts failed to show any statistical differences for either variable (BL-SC) and (SC-OF) within and between the two times of pregnancy (P>0.05) (**figure 17**), but there is a clear trend for both classes of embryos towards better performance in relation to early and late embryonic loss at 2.5 days of pseudopregnancy.

FIGURE 17 - COMPARISON BETWEEN MICROINJECTED AND CONTROL BLASTOCYSTS AT TWO TIMES OF PSEUDOPREGNANCY



SAME ALPHABET LETTER (LOWER CASE) MEANS NO STATISTICAL DIFFERENCE.

8.4 - DISCUSSION

No differences were evident between the micromanipulated and control blastocysts submitted to embryo transfer for the three different strains of mice (CBAC57\F1, SWISS albino, C57BL\6) at 2.5 and 3.5 days in regard to the variables (BL-SC) and (SC-OF) ($P>0.05$). This observation is similar to that of Pomp et al. (1989) who failed to detect any effect of genotype on embryo survival as assessed by the number of young born and number of embryos transferred.

The recipients which were submitted to embryo transfer at 2.5 days of pseudopregnancy had better performance than at 3.5 days with reduced early and late embryonic loss. One possible explanation for this could be that all embryos (micromanipulated and control) were submitted to embryo collection, a period of in in vitro culture and finally to embryo transfer which together could cause the delay of the growth of the embryos. Foxworth and Kraemer (1993) observed that the development of *mus musculus* embryos was compromised when they were submitted to in vitro culture, and Vanderhyden (1987) showed that the survival of rat embryos from in vitro fertilisation could be improved by allowing time for the embryos to "catch up" to the physiological status of the recipient. In this way the uterine environment of the 3.5 days of the pseudopregnant recipients could be ahead of the embryos, and this asynchrony between a retarded embryo and advanced uterus could be responsible for early and late embryo loss.

The results obtained in relation to uterine receptivity at

2.5 days of pseudopregnancy in the present work are at variance with the studies of Paria and Huet-Hudson (1993), which indicated that the uterus becomes receptive on day 4, (day 1 being the day at which the vaginal plug was found). However, the present work is in agreement with (Foxworth and Kraemer 1993), who showed that optimal embryo transfer occurs when the recipient *mus musculus* exhibits a post coital plug 24 hours after the donor exhibits a post coital plug from a fertile mating. It was pointed out by Tarkowski (1959) that the best results of mouse embryo transfer is when the recipient uterus is retarded or at the same age as the transferred eggs.

No differences were evident between micromanipulated and control embryos ($P>0.05$) at either of the pseudopregnant times but the micromanipulated and control embryos which were transferred at 3.5 days had a poorer performance in relation to implantation and number of offspring produced, (**figure 17**). The uterine environment at 3.5 days. In practical terms, the uterine environment was obviously hostile to both categories of embryos transferred, but the micromanipulated and control embryos transferred at 2.5 days of pseudopregnancy showed better performance.

In order to perform adequate statistical analysis, it is necessary to have a higher number of experimental transfers to show that 2.5 day pseudopregnant female mice of strain CBAC57\F1 present a physiologically better uterine environment for embryo transfer than the pseudopregnant recipient at 3.5 days with the methodology used in the present work.

IX - EMBRYO TRANSFER OF AGGREGATED BLASTOCYSTS OF SWISS albino AND C57BL\6 MICE STRAINS

9.1 - EXPERIMENTAL OUTLINE

This experiment used the data of the **successful aggregated blastocysts** which were submitted to embryo transfer to female mice recipients of CBAC57\F1 strain mice at two different times of pseudopregnancy.

9.2 - MATERIAL AND METHODS

9.2.1 - STATISTICAL ANALYSIS

The structure of the statistical analysis used for aggregated blastocysts transferred at both pseudopregnant times was described previously.

The variables (BLASTOCYSTS TRANSFERRED - SCAR OF IMPLANTATION) and (SCAR OF IMPLANTATION - OFFSPRING) indicative of early and late embryonic mortality, were statistically tested:

a) to show the statistical similarity among the mouse strains in each pseudopregnant time ($P>0.05$);

b) to compare each time of pseudopregnancy.

The Kruskal - Wallis test was used for the comparisons.

9.3 - RESULTS

9.3.1 - EMBRYO TRANSFER OF AGGREGATED BLASTOCYSTS

Table 25 shows that the variable (blastocysts transferred - scar of implantation) was better in the recipients at 3.5 days of pseudopregnancy than at 2.5 days ($P<0.05$). The variable (scar of implantation -number of offspring produced) was statistically the same for the recipients at 3.5 and 2.5 days of pseudopregnancy, however in practical terms the recipients at 3.5 days produced more offspring ($P>0.05$).

TABLE 25 - BLASTOCYSTS PRODUCED FOLLOWING TRANSFER OF EMBRYO AGGREGATES ON DIFFERENT DAYS OF PSEUDOPREGNANCY.

DAYS OF PSEUDOPREGNANCY	NUMBER OF EMBRYO TRANSFERS	NUMBER OF BLASTOCYSTS TRANSFERRED	NUMBER OF SCARS OF IMPLANTATION	NUMBER OF OFFSPRING PRODUCED
2.5	7	86	37 (43.0)[a]	16 (18.6)[a]
3.5	3	26	20 (76.9)[b]	12 (46.2)[a]

DIFFERENT ALPHABET LETTER "a" (LOWER CASE), IN THE SAME COLUMN, MEANS STATISTICAL DIFFERENCE ($P<0.05$).

9.4 - DISCUSSION

No differences were evident for the three kinds of embryo aggregates (SWISS albino X SWISS albino; C57BL\6 X C57BL\6; SWISS albino X C57BL\6) transferred. Statistically the embryo aggregates were the same for the variables (BL-SC) and (SC-OF) ($P>0.05$) at every pseudopregnant time (2.5 or 3.5 days), being the data pooled in each time.

As is shown in (**table 25**), the embryo aggregates gave better performance on the uterine environment of 3.5 days of pseudopregnancy than 2.5 days of pseudopregnancy in relation to early embryonic mortality, ($P<0.05$). All embryo aggregates were transferred at **4.5 days** after the identification of the vaginal plug of the **embryo donors.** The better performance of the embryos at 3.5 days as compared to 2.5 days of pseudopregnancy agrees with Foxworth and Kraemer (1993) but differs from the work of Paria and Huet-Hudson (1993) (see previous discussion, item 8.4).

In the present experiment the uterine environment at 2.5 days was receptive to the transferred aggregated blastocysts and was possible the production of offspring from embryos 48 hours ahead of the uterus. Foxworth and Kraemer (1993) observed that the development of *mus musculus* embryos was compromised when they were submitted to <u>in vitro</u> culture and Vanderhyden (1987) made a similar observation with <u>in vitro</u> fertilised rat embryos.

In respect of 48 hour asynchronous implantation between a 2.5 day pseudopregnant uterus and a 4.5 day aggregated blastocyst. It could be that the <u>in vitro</u> micromanipulation procedures and specifically the re-organisation of the embryo

aggregates in order to form a successful blastocyst could intensify the retardation of the embryos in way that the chronological time age of the embryo did not correspond with intrinsic physiological embryo development. Also due to the flexibility of the blastocyst as demonstrated in rabbit by Fischer (1989) .

In respect to offspring production both pseudopregnant times were considered the same ($P>0.05$), however in practical terms, the pseudopregnant recipients at 3.5 days offered a better uterine environment and late embryonic mortality was minimised thus offering better offspring production.

For the statistical confirmation of the trend that the female mice of CBAC57\F1 strain at 3.5 days of pseudopregnancy present a more adequate uterine environment for offspring production of the transferred aggregated blastocysts, it would be necessary to increase the experimental number of embryo transfers.

X - PRODUCTION OF CHIMERAS THROUGH INJECTION OF ISOLATED INNER CELL MASS CELLS INTO MOUSE BLASTOCYSTS

10.1 - EXPERIMENTAL OUTLINE

The aim of these experiments was to examine the possibility of the production of mouse chimeras by inner cell mass cells injected into the blastocoele cavity of blastocysts of SWISS albino and C57BL\6 mice. The early and late embryonic mortality of the micromanipulated blastocysts which were submitted to embryo transfer was also examined.

10.2 - MATERIAL AND METHODS

10.2.1 - EMBRYO COLLECTION

Embryos were collected from mice of Swiss albino and C57BL\6 strains 3.5 days after vaginal plug detection.

10.2.2 - PREPARATION OF INNER CELL MASS CELLS

10.2.2.1 - IMMUNOSURGERY OF BLASTOCYSTS

The immunosurgery technique was performed following the technique of Solter and Knowles (1975), with minor modifications (Schiewe et al., 1992).

Blastocysts used for immunosurgery were obtained from Swiss

albino or C57BL\6 mice and were selected to be at the middle to fully expanded stages of development.

The blastocysts were incubated in a pronase (0.5%) solution, and checked periodically until the zona pellucida had dissolved completely. They were then washed twice, first with 1ml of HTF-Hepes/BSA then with 1ml of HTF-Hepes, prior to transfer to a 15% solution HTF-Hepes containing 10% of rabbit anti-sheep antiserum for 30 minutes. The blastocysts were then washed twice in HTF-Hepes/BSA and once in 1 ml of HTF-Hepes.

The blastocysts were then transferred to a 10% solution of Guinea Pig Serum in HTF-Hepes for 30 minutes and washed, as described later and then transferred to in vitro culture media for incubation in 5% CO_2 in air for 30 to 45 minutes. Lysed trophoblast cells were removed by pipetting and the inner cell mass cells transferred to 100 µl drop of Hepes buffered medium, under oil.

The inner cell masses were then washed twice in PBS (Ca^{2+} Mg^{2+} free) and transferred to an adjoining 30 µl drop of Trypsin-containing media held in the same petri dish. The cells were then dissociated by repeat pipetting using a pipette with an internal diameter of 25 µm and then added to a 30 µl drop of trypsin-containing media with 3 µl of FCS.

All the procedures were carried out with buffered media on a warm stage (Ratek Instruments, Australia) set at 37ºC.

10.2.2.2 - ANTIBODY PREPARATION

The rabbit antisheep antiserum used was a gift from Dr. Deirdre Warnes. It was developed by injection of sheep spleen cells into the ear vein of rabbits. The rabbit serum was collected and freeze-dried (Dynavac - Freeze Drier, Australia). The dried powder was reconstituted with 0.9% NaCl.

10.2.3 - DIFFERENTIAL STAINING

In order to check the efficiency of antibody/complement action on the blastocyst trophoblast cells, a sample of the blastocysts which were submitted to immunosurgery were stained (Hoescht stain and Propidium Iodide, Sigma U.S.A) and the complete lysis of the trophectoderm cells checked under the fluorescence microscope,(**figure 18**).

10.2.4 - BLASTOCYST INJECTION

This was carried out using a similar approach to that used for inoculation of stem cells. A minimum of 3 and the maximum of 7 inner cell mass cells were injected into each blastocoele.

10.2.5 - EMBRYO TRANSFER

Embryo transfer was done between 15 - 24 hours after blastocyst injection. The recipients were 2.5 or 3.5 days after vaginal plug detection.

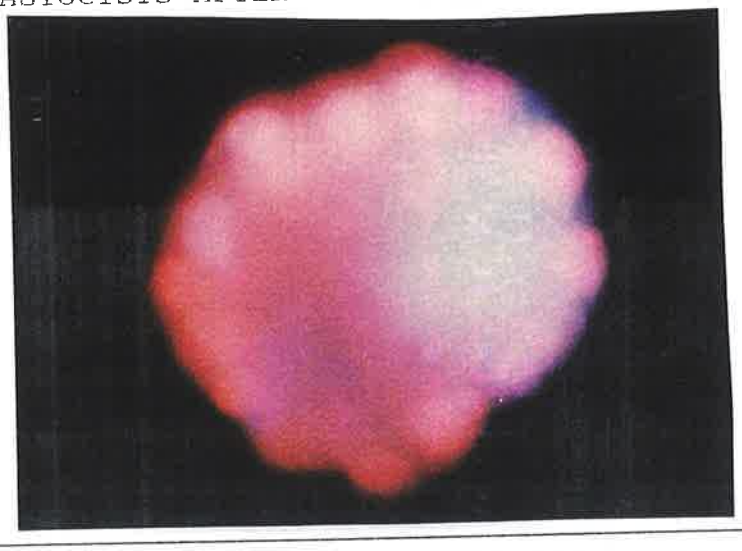
10.2.6 - STATISTICAL ANALYSIS

An analysis of the success of blastocyst transfer was done as described previously.

Comparison of the development of micromanipulated and non-micromanipulated embryos in in vitro culture was done using a Z Test.

FIGURE 18

MOUSE BLASTOCYSTS AFTER IMMUNOSURGERY



MOUSE BLASTOCYSTS SUBMITTED TO DIFFERENTIAL STAINING

10.3 - RESULTS

10.3.1 - PRODUCTION OF CHIMERAS USING BLASTOCYSTS INJECTED BY INNER CELL MASS CELLS ISOLATED BY IMMUNOSURGERY.

Table 26 shows the results relating to the host blastocysts of SWISS albino and C57BL\6 mouse strains which received inner cell mass cells isolated by immunosurgery.

The number of blastocysts submitted to successful micromanipulation and embryo transfer of each strain were 37 and 27, respectively for the SWISS albino strain and C57BL\6 strain host blastocysts. The number of blastocysts that implanted into the uterus (2 and 11) was statistically different for the SWISS albino ($P<0.05$) but the same for the C57BL\6 ($P>0.05$).

The number of offspring recorded from each host blastocyst strain was (0 and 4) for SWISS albino and C57BL\6 strains respectively. The comparisons between both strains in relation to the variable (number blastocysts transferred/implanted) was statistically significant ($P<0.05$).

Only one overtly male chimeric was obtained from the C57BL\6 host blastocyst injected with SWISS albino inner cell mass cells. This male chimera was fertile and transmitted the C57BL\6 germ line,(**figure 19**).

TABLE 26 - BLASTOCYSTS INJECTED INSIDE OF THE BLASTOCOELE WITH INNER CELL MASS CELLS ISOLATED BY IMMUNOSURGERY.

MICE STRAIN INNER CELL MASS	MICE STRAIN HOST BLASTOCYST	NUMBER OF BLASTOCYSTS TRANSFERRED	NUMBER OF RECIPIENTS	NUMBER OF BLASTOCYSTS IMPLANTED	NUMBER OF OFFSPRING BORNED	CHIMERAS			FERTILITY *		GERM LINE CHIMERAS			
											SWISS		C57BL\6	
						n	n♂	n♀	♂	♀	♂	♀	♂	♀
									n	n	n	n	n	n
		n	n	n (%)	n (%)	(%)			(%)	(%)	(%)	(%)	(%)	(%)
C57BL\6	SWISS	37	3	2 (5.4) a B	0	0			-	-	-	-	-	-
SWISS	C57BL\6	27	3	11 (40.7) b A	4 (14.8) A	1 (3.7)	1	-	1 (100.0)	-	-	-	1 (100.0)	-

* FERTILITY = CAPACITY TO PRODUCE OFFSPRING.
DIFFERENT ALPHABET LETTERS (LOWER CASE) IN THE SAME COLUMN INDICATES STATISTICAL DIFFERENCE (P<0.05).
LETTER "A" (UPPER CASE) UNDERNEATH OF EACH VARIABLE INDICATES NO STATISTICAL DIFFERENCE (P>0.05).
LETTER "B" (OPPER CASE) UNDERNEATH OF EACH VARIABLE INDICATES STATISTICAL DIFFERENCE (P<0.05).

FIGURE 19

MALE CHIMERA PRODUCED BY SWISS albino INNER CELL MASS TRANSFER INTO C57BL\6 HOST BLASTOCYST. THE CHIMERA WAS FERTILE AND PRODUCED OFFSPRING TYPICAL OF THE C57BL\6 GERMLINE

10.3.2 - IN VITRO CULTURE OF HOST BLASTOCYSTS FROM SWISS albino AND C57BL\6 MOUSE STRAINS WHICH WERE INJECTED WITH INNER CELL MASS CELLS.

The comparison between both strains of mice, **(table 27)**, demonstrated that the development of blastocysts of SWISS albino and C57BL\6 strains injected with inner cell mass cells had the same competent development in in vitro culture (P>0.05).

TABLE 27 - COMPARISON OF THE IN VITRO DEVELOPMENT OF BLASTOCYSTS FROM DIFFERENT MOUSE STRAINS FOLLOWING INJECTION OF INNER CELL MASS CELLS.

MICE STRAIN INNER CELL MASS	MICE STRAIN HOST BLASTOCYST	REPETITIONS n	DAY 5 n	DAY 6 n	(%)
C57BL\6	SWISS	3	37	32	(86.5) a
SWISS	C57BL\6	3	27	24	(88.9) a

SAME ALPHABET LETTERS (LOWER CASE) IN THE SAME COLUMN MEANS NO STATISTICAL DIFFERENCE (P>0.05).

10.3.3 - IN VITRO CULTURE OF BLASTOCYSTS FROM SWISS albino AND C57BL\6 MOUSE STRAINS COLLECTED 5 DAYS AFTER HCG IN MEDIUM SUPPLEMENTED WITH 10% HEAT INACTIVATED RABBIT ANTI-SHEEP SERUM

Table 28 illustrates that the blastocysts of SWISS albino and C57BL\6 mice strains submitted to in vitro culture in media supplemented with 10% of sheep antibody heat inactivated had the same pattern of development with both strains considered statistically equal (P>0.05).

TABLE 28 - IN VITRO CULTURE* OF EMBRYOS FROM SWISS albino AND C57BL\6 MICE COLLECTED ON THE 5TH DAY AFTER HCG\MATING.

MICE STRAIN	REPETITIONS	5 DAYS AFTER HCG LATE MORULA/EARLY BLASTOCYST	6 DAYS AFTER HCG EXPANDED BLASTOCYSTS	
	n	n	n	(%)
SWISS	1	20	20	(100.0) a
C57BL\6	1	12	12	(100.0) a

* IN VITRO CULTURE MEDIA = HUMAN TUBAL FLUID-NaHCO3 + 10% SHEEP ANTIBODY HEAT INACTIVATED.
SAME ALPHABET LETTERS (LOWER CASE) IN THE SAME COLUMN MEANS NO STATISTICAL DIFFERENCE (P>0.05).

10.4 - DISCUSSION

This experiment evaluated the production of chimeras through inner cell mass transfer isolated by immunosurgery and inoculation of those cells to blastocysts of SWISS albino and C57BL\6 strains.

The comparison between each host blastocyst strain showed evidence of early embryonic mortality which was only statistically significant for the SWISS albino mice which received inner cell mass cells from C57BL\6 strain ($P<0.05$). The comparison between both strains showed less embryonic loss in the C57BL\6 blastocysts strain submitted to embryo transfer ($P<0.05$).

Late embryonic mortality, which was the difference between the number of blastocysts implanted and offspring produced, was also present in both strains.

The embryo transfer as such could be one of the factors responsible for the early embryonic mortality. The combination of the strains of inner cell mass cells and host blastocysts could be ruled out as one of the causes of early and late embryonic mortality.

Micromanipulated embryos kept in in vitro culture before embryo transfer showed a satisfactory development, (**table 27**). Then the effect responsible for the embryo loss related to the combination of the strain of inner cell mass and host blastocyst should happen later in development.

The inner cell mass cells obtained from both blastocyst

sources were isolated by immunosurgery (Solter and Knowles, 1975; Schiewe et al., 1992). After the cells had been removed from the trophoblast cells they were submitted to a trypsin - containing media and pipetting in order to dissociate the clump of inner cell mass into individual cells. All the cells isolated and inoculated from both strains had different sizes and were considered to be of good morphology. However, the morphology of the inner cell mass cells and the functional potential as a karyoplast could not be related, and this situation could account for the low viability of chimera production.

For immunosurgery it was used sheep antibody (Schiewe et al., 1992) we do not believe that this source of interspecific antibody could be rule out as the cause of loss of the micromanipulated embryos before and after implantation because it was obtained one mouse chimera originated with cells of the inner cell mass isolated with this interspecific antibody. Moreover, the in vitro culture of blastocysts of both strains using sheep antibody was not harmful to the development of the embryos in in vitro culture but it was not done the embryo transfer of those embryos, **table 28.**

The low number of cells injected were due to the complex methodology. During the process of dissociating inner cell mass cells some cells were burst and others lost.

During the development of this work labelled stem cells were injected into blastocysts. Microinjected blastocysts were submitted to in vitro culture and the re-expanded ones were evaluated by fluorescence microscopy. It was observed that not

all the labelled stem cells were attached to the group of cells of the inner cell mass. Some formed other clumps like the inner cell mass while others cells, attached isolated to the trophoblast. This observation agrees with Beddington and Robertson (1989) who verified that the stem cells can contribute to the trophoblast in blastocyst injection chimeras, although at low frequency.

However remains the question if some of the cells isolated from the inner cell mass in this experiment could really re-differentiate to be part of the trophoblast and not contribute to all inner cell mass of the host blastocyst. Stem cells which are kept in in vitro conditions may lose some of the basic characteristics of the isolated inner cell mass cells (Parchment and Natarajan, 1992).

This observation can also justify that it is necessary for an optimal number of injected cells in order to attach the blastocysts' inner cell mass in order to participate and take over the inoculated foetus' gonadal tissue.

Only one (3.7%) chimera was obtained from the 27 micromanipulated blastocysts transfered. The chimera produced was a male of the C57BL\6 germline. The SWISS albino inner cell mass cells only contributed to tissues other than the gonad.

XI - PRODUCTION OF CHIMERAS BY EMBRYO AGGREGATION USING SWISS albino AND C57BL\6 MICE STRAINS

11.1 - EXPERIMENTAL OUTLINE

The aim of these experiments was to explore the production of mouse chimeras using the combination of SWISS albino and C57BL\6 mouse strains, evaluating early and late embryonic mortality as well as the predominant strain in the germline.

11.2 - MATERIAL AND METHODS

11.2.1 - EMBRYO COLLECTION

Eight cell embryos were collected from mice of the Swiss albino and C57BL\6 strains 2.5 days after vaginal plug detection.

11.2.2 - PREPARATION OF EMBRYOS FOR AGGREGATION

Aggregations were made between embryos of Swiss albino X C57BL\6, Swiss albino X Swiss albino, C57BL\6 X C57BL\6 using the following procedure.

The eight cell embryos were placed in pronase (0.5%) solution to remove the zona pellucida, washed twice in two different petri dishes with HTF-Hepes/BSA and transferred to a Terazaki plate (Disposable Products, Australia) with 60 drops of 10 µl each of HTF-HCO3-/BSA under oil and pre-equilibrated

overnight in a incubator with 5% CO_2 in air at 37.8ºC. Two embryos were placed in each drop and manipulated to make close contact with the aid of a fire polished glass pipette. The dishes were then incubated for 30 minutes and the embryos not in close contact after this period were again moved together and the process repeated. The embryos were then held in an incubator overnight for the in vitro culture period.

The embryos were re-evaluated during 24 and 48 hours after the initial co-culture period, and after 48 hours all blastocysts that had aggregated, (**figure 20**), were transferred to a recipient female mice.

11.2.3 - EMBRYO TRANSFER

All embryo transfers were done 4.5 days after donor vaginal plug detection, using recipients 2.5 or 3.5 days after vaginal plug detection.

11.2.4 - STATISTICAL ANALYSIS

The analysis of the three groups in relation to early and late embryonic mortality was carried out as described previously.

The assessment of development of the micromanipulated embryos was analyzed by the Chi-Square test and the non-micromanipulated embryos by the Z-test.

11.3 - RESULTS

11.3.1 - PRODUCTION OF CHIMERAS USING EMBRYO AGGREGATION

Table 29 shows that the number of aggregated blastocysts from SWISS albino X SWISS albino, C57BL\6 X C57BL\6 and SWISS albino X C57BL\6 mice that were submitted to the embryo transfer were (34, 16, 62 respectively). The difference between the number of blastocysts transferred in each strain and the number of blastocysts that implanted into the uterus (21, 12, 24) were considered statistically the same ($P>0.05$) for the SWISS albino X SWISS albino and C57BL\6 X C57BL\6. However it was considered statistically different for SWISS albino X C57BL\6 aggregates ($P<0.05$).

The number of offspring recorded from each embryo aggregate class was 15, 0 and 13 respectively, and the variable (number of scar of implantation - number of offspring produced) was considered statistically the same for all three classes of embryos ($P>0.05$).

When the comparisons were made among the three different classes of embryo aggregates for each variable (number of blastocyst transferred/implanted) and (number of implanted/offspring), there was no detected statistical difference ($P>0.05$).

The 15 offspring born as a consequence of the embryo aggregation of SWISS albino X SWISS albino were a product of the embryo transfer of blastocysts of very good morphology.

Nevertheless it was not possible to tell if the individuals produced had the contribution of the two embryos at inner cell mass and trophoblast layers.

The production of coat colour chimera was only expected with SWISS albino X C57BL\6. Blastocysts aggregates and three chimeras were obtained with this embryo combination, of which 2 were males and 1 female. The males and females were fertile. One of the males transmitted the SWISS albino germline, the other male and female transmitted the C57BL\6 germline.

TABLE 29 - PRODUCTION OF CHIMERAS BY EMBRYO AGGREGATION.

EMBRYO AGGREGATE		NUMBER OF BLASTOCYSTS TRANSFERRED	NUMBER OF RECIPIENTS	NUMBER OF BLASTOCYSTS IMPLANTED	NUMBER OF OFFSPRING BORN			COAT COLOUR CHIMERAS		FERTILITY OF* CHIMERAS		GERMLINE CHIMERAS			
					TOTAL	EMBRYO A	EMBRYO B					EMBRYO A		EMBRYO B	
A	B	n	n	n(%)	n(%)	n(%)	n(%)	n(%)		n♂	n♀	n♂	n♀	n♂	n♀
					n♂ n♀			n♂	n♀	(%)	(%)	(%)	(%)	(%)	(%)
SWISS	SWISS	34	3	21 (61.8) a	15(44.1) a	15(44.1)	-	-	-	-	-	-	-	-	-
				A	A										
					10 5										
C57BL\6	C57BL\6	16	2	12 (75.0) a	0 -		-	-	-	-	-	-	-	-	-
				A											
SWISS	C57BL\6	62	5	24 (38.7) a	13(20.9) a	10(16.1)	-	3 (4.8)		2/2	1/1	1	-	1	1
				B	A										
					9 4			2	1	(100)	(100)	(50)		(50)	(100)

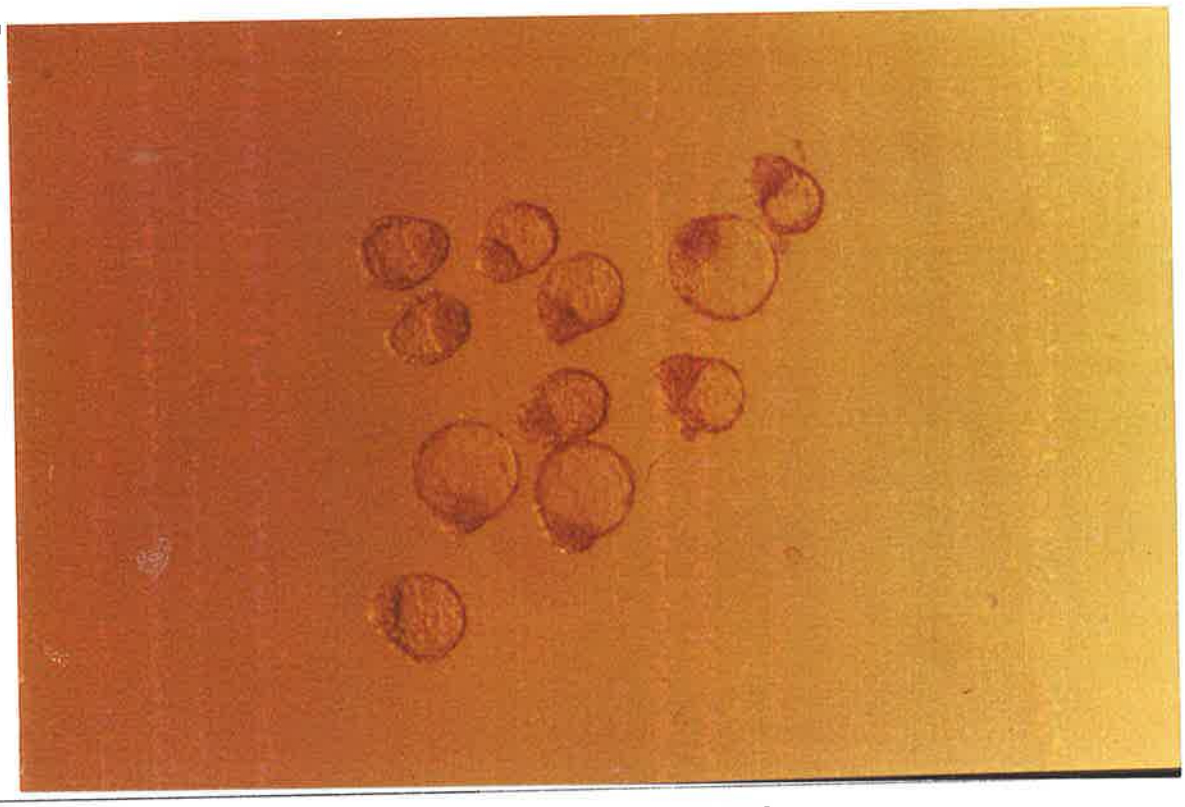
* FERTILITY = CAPACITY TO PRODUCE OFFSPRING.

SAME ALPHABET LETTERS (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE (P>0.05).

LETTER "A" (UPPER CASE) UNDERNEATH OF EACH VARIABLE INDICATES NO STATISTICAL DIFFERENCE (P>0.05).

LETTER "B" (UPPER CASE) UNDERNEATH OF EACH VARIABLE INDICATES STATISTICAL DIFFERENCE (P<0.05).

FIGURE 20



SINGLE AND AGGREGATED BLASTOCYSTS

FERTILE CHIMERAS PRODUCED BY AGGREGATION OF SWISS albino AND C57BL\6 EMBRYOS

11.3.2 - IN VITRO CULTURE OF EMBRYOS OF SWISS AND C57BL\6 MICE STRAINS COLLECTED ON THE 4TH DAY AFTER HCG + MATING

Comparison between the embryos submitted to in vitro culture of SWISS albino and C57BL\6 mouse strains indicate (table 30) that both embryo strains had the same pattern of development 5 and 6 days after HCG\mating, (P>0.05).

TABLE 30 - IN VITRO DEVELOPMENT OF EMBRYOS FROM SWISS albino AND C57BL\6 MICE COLLECTED ON THE 4TH DAY AFTER HCG/MATING.

		DAYS (POST HCG INJECTION)				
		4	5		6	
MICE STRAIN	REPETITIONS	8 CELL EMBRYOS	LATE MORULA EARLY BLASTOCYST		EXPANDED BLASTOCYST	
	n	n	n	(%)	n	(%)
SWISS	6	112	112	(100.0)[a]	110	(98.2)[a]
C57BL\6	5	63	62	(98.4)[a]	60	(95.2)[a]

SAME ALPHABET LETTERS (LOWER CASE) IN THE SAME COLUMN MEANS NO STATISTICAL DIFFERENCE (P>0.05).

11.3.3 - IN VITRO CULTURE OF EMBRYO AGGREGATES

Table 31 shows that the comparisons between all classes of embryo aggregates were considered the same 5 and 6 days after HCG\mating ($P>0.05$), this demonstrates that the in vitro culture system used was capable of achieving the blastocyst stage from embryo aggregates.

TABLE 31 - IN VITRO CULTURE OF EMBRYOS PRODUCED BY EMBRYO AGGREGATION.

MICE STRAIN AGGREGATES		REPETITIONS	DAYS (POST HCG INJECTION) 4 NUMBER OF PAIRS OF AGGREGATES		5 NUMBER OF AGGREGATED BLASTOCYSTS		6 NUMBER OF AGGREGATED BLASTOCYTS	
EMBRYO A	EMBRYO B	n	n	(%)	n	(%)	n	(%)
SWISS	SWISS	3	44	(100.0)	44	(100.0)[a]	43	(97.73)[a]
C57BL\6	C57BL\6	3	32	(100.0)	31	(96.88)[a]	27	(84.38)[a]
SWISS	C57BL\6	5	79	(100.0)	72	(91.14)[a]	71	(89.87)[a]

SAME ALPHABET LETTERS (LOWER CASE) IN THE SAME COLUMN INDICATES NO STATISTICAL DIFFERENCE (P>0.05).

11.4 - DISCUSSION

The present work evaluated the production of chimeras by embryo aggregation through three classes of embryo combination.

The comparison between the three classes of blastocysts transferred to the uterus of pseudopregnant female mice indicated that embryonic loss before embryo implantation was present in all classes of embryo aggregates and were considered statistically equal ($P>0.05$).

The comparison between each class of embryo aggregates for early embryonic loss only demonstrated statistical difference for the combination SWISS albino X C57BL\6, ($P<0.05$), **(table 29)**.

The presence of early embryonic loss for all three classes of embryos could be due to the effects of embryo transfer.

All three classes of embryos were submitted to in vitro culture, **(table 31)**, and the performance of the three classes of embryo aggregates had the same degree of competent development ($P>0.05$). As noted, all three strains had embryonic loss before implantation. However, only the combination SWISS albino X C57BL\6 proved to be statistically different and this could be due to the combination of the two strains, as binary cell-cell interaction often determines the fate of one or both cell partners (Singer, 1992).

The variable late embryonic mortality, the number of blastocysts implanted and offspring produced was not different for the classes of embryo aggregates compared ($P>0.05$).

Early and late embryonic mortality might have been caused by embryo transfer at an inappropriate stage of pseudopregnancy

of the female mouse recipient (see chapter 9).

The only class of embryos that did not produce any offspring was C57BL\6 X C57BL\6, the other two embryo aggregate combination produced offspring. The combination SWISS albino X C57BL\6 produced chimeric offspring and SWISS albino background.

Only aggregated blastocysts with very good morphology, were transferred (**figure 20**). All embryo aggregations were made between embryos collected 2.5 days after identification of the vaginal plug, (8 cell stage). It was verified that the embryo compaction starts at the 8 cell stage (Prather and First, 1993; Garrod, 1993). The obtaining of offspring of SWISS albino background from the embryo aggregates of SWISS albino X C57BL\6 certainly came from a chimeric embryo but the chimerism was restricted to trophoblast tissue. An explanation for this fact could be because the two embryos involved may have had different rate of development. Prather and First (1987) have previously shown that re-aggregating asynchronous embryos in the sheep has apparently produced individuals that have a chimeric placenta, but that tend not be chimeric themselves. A similar situation was described by Betteridge and Fléchon (1988) who showed that the chimerism of foetuses derived from embryo aggregation decreased when the degree of asynchrony between the constituent blastomeres increased. The embryo combination between SWISS albino X SWISS albino did not allow identification of chimerism through the coat colour and it is not possible to confirm if the offspring produced had the contribution of the two embryos.

Kashiwazaki et al. (1988) transferred aggregated mouse

blastocysts constructed from microsurgically-bisected morulae from two strains of mice and obtained chimeric and single type offspring.

Embryo aggregation between SWISS albino X C57BL\6 did not impede chimera production, but it is not possible to make a statement that those two strains are highly compatible. The superovulation treatments, embryo collection and micromanipulation were done at the same time for the two strains. It was observed that the embryos of those two strains presented different rates of development. An explanation for the success in the production of the three chimeras could find explication in the paper of Warner et al (1987), who observed that even embryos with identical make up show some lack of synchrony of cleavage rate and at any point in development there is a range in cell numbers and cell stages among the embryos. Tarkowski (1959) stated that the body weight of offspring originating from 1/2 blastomeres during the period from 2 to 20 days after birth was greater than the average weight of young ones from normal litters of similar size. The same author in 1961, verified that the blastocysts originated by embryo aggregation on return to the uterus of pseudopregnant recipients can regulate their size and produce normal offspring. In the present experiment was not observed any complications at the delivery indicative that the offspring produced by embryo aggregation were within the normal size. Tarkowski (1961) observed high postnatal mortality in the offspring produced by embryo aggregation, a situation which was not observed in the present work.

Three coat colour chimeric animals were produced, 4.8% of the total number of aggregated blastocysts submitted to the embryo transfer. This percentage yield of chimera was higher then the first set of experiments done in hamsters with embryo aggregation by Piedrahita et al (1992), but less than the numbers obtained in previous mouse studies by Kashiwazaki et al. (1988) and a second set of experiments with hamsters (Piedrahita et al., 1992).

As was shown by Wilmut et al. (1991) and Schwartzberg et al. (1989) the C57BL\6 strain behave differently in terms of what determines the chimeras' germlines in the different mouse strains. In the present work, the combination with SWISS albino and C57BL\6 mice embryos generated three fertile chimeras, of which 1 coat colour male with SWISS albino germline, and 1 male and 1 female with C57BL\6 germline.

It is not possible to draw term conclusions from these low numbers obtained with chimera production, however, these data suply the view that the slow embryo development of the C57BL\6 strain embryo is associated with a submissive strain to the SWISS albino in relation to contribution to the gonadal tissue.

Embryo aggregation, besides being a useful tool for embryological studies, has the practical potential to be a useful means of reproduction of animals in danger of extinction. It is, however, important to determine compatibility between two strains of mice, (or breeds) of farm animals with the objective to use the most appropriate association of blastocysts and stem cells.

XII - ACTIVATION OF SHEEP OOCYTES

12.1 - EXPERIMENTAL OUTLINE

In preparation for the use of sheep oocytes in cloning, experiments were carried out to compare physical agents and chemical agents or a combination of both for the activation of sheep oocytes produced by in vitro maturation.

12.2 - MATERIAL AND METHODS

12.2.1 - SOURCE OF OOCYTES AND IN VITRO MATURATION

For production of in vitro matured oocytes, ovaries were collected in the local abattoir and transported within 2 hours of slaughter in PBS at a temperature of 37ºC to the laboratory. In the laboratory all visible follicles were aspirated by vacuum (Thomas, U.S.A.) into collection tubes containing media. Oocytes were harvested and washed as described previously prior to IVM. For IVM, oocytes were transferred to 400 µl of media TCM-199-HCO_3 supplemented with hormones (FCS 2 ml, LH 50 µl, FSH 5 µl, 17 B-Estradiol 10 µl), (appendix 4) using 4-well dishes in an incubator with 5% CO_2 in air at 38.6ºC, during 24 - 25 hours. The in vitro matured oocytes used in the present experiment were a gift from Dr Peter Kotaras.

12.2.2 - ACTIVATION

The oocytes were hyaluronidase treated, 0.5mg\ml in SOFM-HEPES in order to remove the cumulus cells and were activated by one of three procedures namely a) - Ethanol 7% in SOFM-Hepes for 5 minutes, b) - Calcium ionophore 5 µM for 5 minutes followed by electroactivation using a Somatic Hybridiser, SHIMADZU using 1 pulse with 100 V\mm during 50 microseconds, c) - electroactivation using 1 pulse 100 V\mm during 50 microseconds. Some inactivated oocytes were left as a control group.

12.2.3 - IN VITRO CULTURE

After activation all oocytes were submitted to in vitro culture for 6 hours in SOFM-HCO_3^- + containing 2.5 µg/ml cytochalasin B and 10 µg/ml of cycloheximide. After this time the oocytes were transferred to SOFM-HCO_3^- containing 2.5 µg/ml of cytochalasin B for 24 hours of in vitro culture. After this time the oocytes were transferred to normal in vitro culture media which was SOFM-HCO_3^- supplemented with BSA.

12.2.4 - ASSESSMENT OF OOCYTES

The cleavage rate was assessed 48 hours after activation. Uncleaved oocytes were fixed (1 part glacial acetic acid : 3 parts EtOH) for further histological examination.

12.2.5 - STATISTICAL ANALYSIS

For oocytes that were submitted to a histological examination comparisons were made using the chi-square test between the four treatment groups, since statistical differences were a 2 X 2 chi-square (α=0.001) was used to identify the different groups.

For oocytes submitted to *in vitro* culture comparisons of cleavage rates were assessed by a 2 X 2 chi-square test (α=0.001).

12.3 - RESULTS AND DISCUSSION

Table 32 shows that calcium ionophore + electroactivation, calcium ionophore and electroactivation were considered statistically equal ($P<0.05$) in relation to the number of cleaved oocytes obtained. All of those treatments were considered statistically different from ethanol treatment.

TABLE 32 - COMPARISON OF DIFFERENT METHODS OF ACTIVATION FOR SHEEP OOCYTES MATURED IN VITRO.

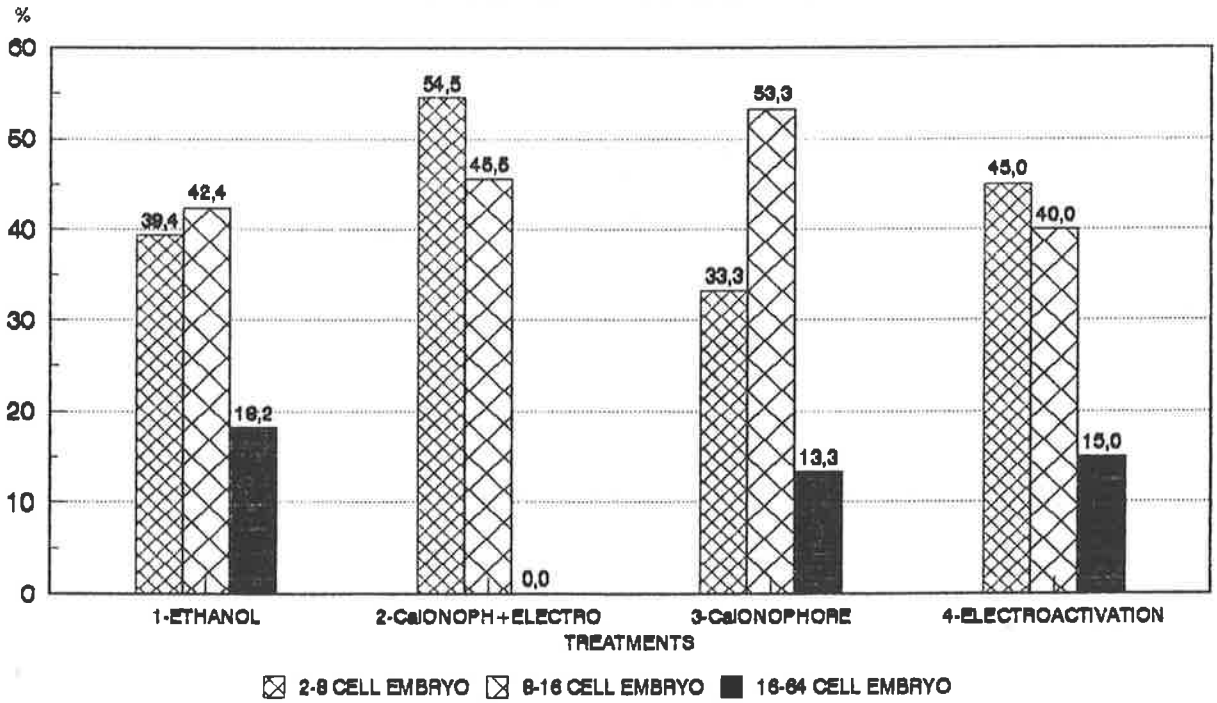
ACTIVATION TREATMENT	REPETITIONS	OOCYTES		
		ACTIVATED	CLEAVED	
	n	n	n	(%)
ETHANOL	5	175	62	35.4 b
Ca IONOPHORE + ELECTROACTIVATION	4	65	33	50.8 a
Ca IONOPHORE	3	45	24	53.3 a
ELECTROACTIVATION	3	50	32	64.0 a
CONTROL	1	26	5	19.2 b

DIFFERENT ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN MEANS STATISTICAL DIFFERENCE ($P<0.05$).

Ca ionophore + electroactivation was the only treatment which did not produced cleavage beyond the 16-cell stage, (**figure 21**). The failure of the oocytes to achieve this advanced cleavage could be explained because the oocytes had two stimulations in less than 15 minutes causing Ca^{2+} elevation, and these simultaneous waves could be responsible for the impairment of the oocytes.

The activation treatment with ethanol was the least effective in inducing oocyte cleavage ($P>0.05$) and this could be attributed to toxic effects of the ethanol to sheep oocytes.

FIGURE 21 - DEVELOPMENT OF CLEAVED SHEEP OOCYTES IN VITRO MATURED AND SUBMITTED TO DIFFERENT TREATMENTS FOR ACTIVATION.



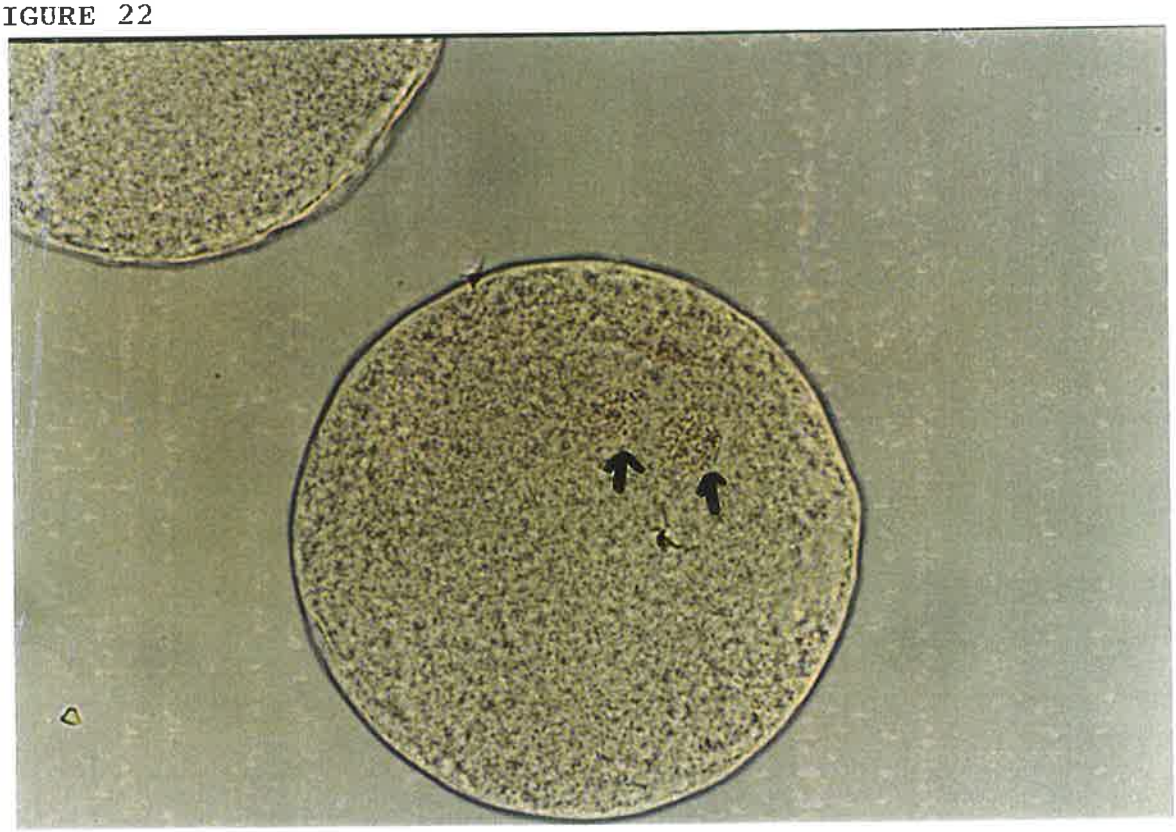
According to **table 33**, the oocytes not cleaved from all activation treatments presented the formation of a pronuclei, (**figure 22**). From the uncleaved oocytes the group which presented less oocytes which were not activated was the Ca ionophore + electroactivation, being statistically different ($P<0.05$) from the other treatment groups.

TABLE 33 - HISTOLOGY OF UNCLEAVED OOCYTES WHICH WERE SUBMITTED TO DIFFERENT ACTIVATION TREATMENTS.

TREATMENT	NUMBER OF OOCYTES NOT CLEAVED	NUMBER OF OOCYTES ACTIVATED	
	n	n	(%)
ETHANOL	59	30	50.8 a
Ca IONOPHORE + ELECTROACTIVATION	39	9	23.1 b
Ca IONOPHORE	30	19	63.3 a
ELECTROACTIVATION	19	11	57.9 a

DIFFERENT ALPHABET LETTER (LOWER CASE) IN THE SAME COLUMN MEANS STATISTICAL DIFFERENCE ($P<0.05$).

FIGURE 22



SHEEP OOCYTES AFTER ACTIVATION
TWO PRONUCLEI MAY BE NOTED

The failure of some oocytes to cleave following activation could be due to unsuitable conditions of the in vitro maturation system and/or oocytes originating from unstimulated follicles (Barnes et al., 1993).

The fact that the oocytes from the control group that showed parthenogenetic cleavage only could be attributed to the handling process, none of those cleaved oocytes overcame the 8-cell stage.

In summary it was demonstrated that all treatments were effective in order to take the oocytes out of the metaphase II arrest. However, this stimulus did not overcome the 64% with the electroactivation treatment. Further work is necessary in order to optimise the conditions to obtain blastocysts.

XIII - ENUCLEATION OF MICE OOCYTES

13.1 - EXPERIMENTAL OUTLINE

Several reports indicated that oocytes could be enucleated using a centrifugation procedure which needed to be adjusted to suit different species and breeds. The feasibility of using this procedure with mice was investigated.

13.2 - MATERIAL AND METHODS

13.2.1 - SOURCE OF OOCYTES

Oocytes were obtained from Swiss albino, C57BL\6 and CBAC57\F1 strains of mice between 11 and 14 hours after HCG injection.

13.2.2 - PREPARATION OF OOCYTES

The oocytes were denuded using HTF-Hepes\BSA containing hyaluronidase, and washed 3 times in HTF-Hepes\BSA and the zona pellucida removed by placing the denuded oocytes in a 30 mm petri dish (Disposable Products, Australia) with pronase solution (0.5%).

The oocytes without their zona pellucida were then cultured in the presence or absence of cytochalasin B 10 µg/ml for 1 or 3 hours and then subjected to centrifugation in a percol gradient

(7.5%, 30%, 40%) containing 3 layers of 1 ml each + 10 µg\ml of cytochalasin B. Centrifugation was at 8000 x g or 11000 x g (g=relative centrifugal field) for 1, 2 or 5 minutes.

13.2.3 - ASSESSMENT OF OOCYTES

After centrifugation the oocytes were identified under stereomicroscopy and fixed (1 part glacial acetic acid : 3 parts EtOH).

The fixed oocytes were checked for enucleation, chromosomal leftovers and the presence of metaphase II plate + a polar body.

13.2.4 - STATISTICAL ANALYSIS

The Chi-Square or Fisher's Exact Test was used to determine any differences between groups.

13.3 - RESULTS

The enucleation of oocytes was done by centrifugation using three procedures. Each procedure had variants in time and relative centrifugal field (g).

Table 34 shows that with an increase in time (1 minute to 2.5 and 5 minutes) using 8000 x g, more enucleated oocytes were obtained ($P<0.001$). However, when the (g) and time was increased to 11000 x g and 5 minutes, the percentile of oocytes enucleated obtained decrease, although this difference not statistically significant from the oocytes enucleated using 8000 x g and 2.5 and 5 minutes.

TABLE 34 - ENUCLEATION OF MOUSE OOCYTES BY CENTRIFUGATION IN A PERCOL GRADIENT FOR DIFFERENT LENGTHS OF TIME AND AT DIFFERENT RELATIVE SPEEDS (x g).

TREATMENT		n	ENUCLEATED	
(centrifugation, g)	(time, minutes)		n	(%)
8000	1	88	29	32.9[b]
8000	2.5	43	26	60.5[a]
8000	5	26	20	76.9[a]
11000	5	38	21	55.3[a]

DIFFERENT ALPHABET LETTER (LOWER CASE) MEANS STATISTICAL DIFFERENCE χ^2 ($P<0.001$).

Table 35 demonstrates that the preculture of oocytes in cytochalasin B for 1 hour was the same statistically (P>0.05) for the variants of time and (g) of the groups analysed.

TABLE 35 - MOUSE OOCYTES SUBMITTED TO AN ENUCLEATION PROCEDURE BY CULTURE OF THE OOCYTES IN CYTOCHALASIN B FOR 1 HOUR FOLLOWED BY CENTRIFUGATION FOR DIFFERENT TIMES AND AT DIFFERENT RELATIVE SPEEDS (x g) IN PERCOL GRADIENT.

TREATMENT		n	ENUCLEATED	
(centrifugation, g)	(time, minutes)		n	(%)
8000	1	99	38	38.3[a]
8000	2.5	4	3	75.0[**]
8000	5	37	16	43.2[a]
11000	5	35	9	25.7[a]

SAME ALPHABET LETTER (LOWER CASE) MEANS NO STATISTICAL DIFFERENCE χ^2 (P>0.05).

** THIS LINE WAS NOT CONSIDERED IN THE STATISTICAL ANALYSIS.

Table 36 also demonstrates that the previous culture of oocytes in cytochalasin B during 3 hours was the same statistically (P>0.05) for the variants of time and speed of all

groups.

TABLE 36 - MOUSE OOCYTES SUBMITTED TO AN ENUCLEATION PROCEDURE BY CULTURE OF THE OOCYTES IN CYTOCHALASIN B FOR 3 HOURS FOLLOWED BY CENTRIFUGATION FOR DIFFERENT TIMES AND AT RELATIVE SPEEDS (x g) IN PERCOL GRADIENT.

TREATMENT		n	ENUCLEATED	
(centrifugation, g)	(time, minutes)		n	(%)
8000	2.5	8	4	50.0[a]
8000	5	6	6	100.0[a]
11000	5	11	8	72.7[a]

SAME ALPHABET LETTER (LOWER CASE) MEANS NO STATISTICAL DIFFERENCE χ^2 ($P>0.05$).

Table 37 consists of the pooled data from the enucleation of each centrifugation procedure. Oocyte pre-incubation with cytochalasin B for 1 hour had the poorest performance in respect to enucleation of oocytes and was statistically different from the other procedures ($P<0.001$).

TABLE 37 - COMPARISON OF POOLED DATA FOR THE DIFFERENT PROCEDURES TO ENUCLEATE MOUSE OOCYTES.

TREATMENT	n	ENUCLEATED	
		n	(%)
PERCOL + CENTRIFUGATION *	107	67	62.6[a]
CYTOCHALASIN B 1 HOUR + PERCOL + CENTRIFUGATION *	171	63	36.8[b]
CYTOCHALASIN B 3 HOURS + PERCOL + CENTRIFUGATION *	25	1	72.0[a]

DIFFERENT ALPHABET LETTER (LOWER CASE) MEANS STATISTICAL DIFFERENCE χ^2 ($P<0.001$).

* POOLED DATA

13.4 - DISCUSSION

The objective of these experiments was to evaluate the role of centrifugation in enucleation of mouse oocytes.

All procedures and its variants allowed the enucleation of oocytes.

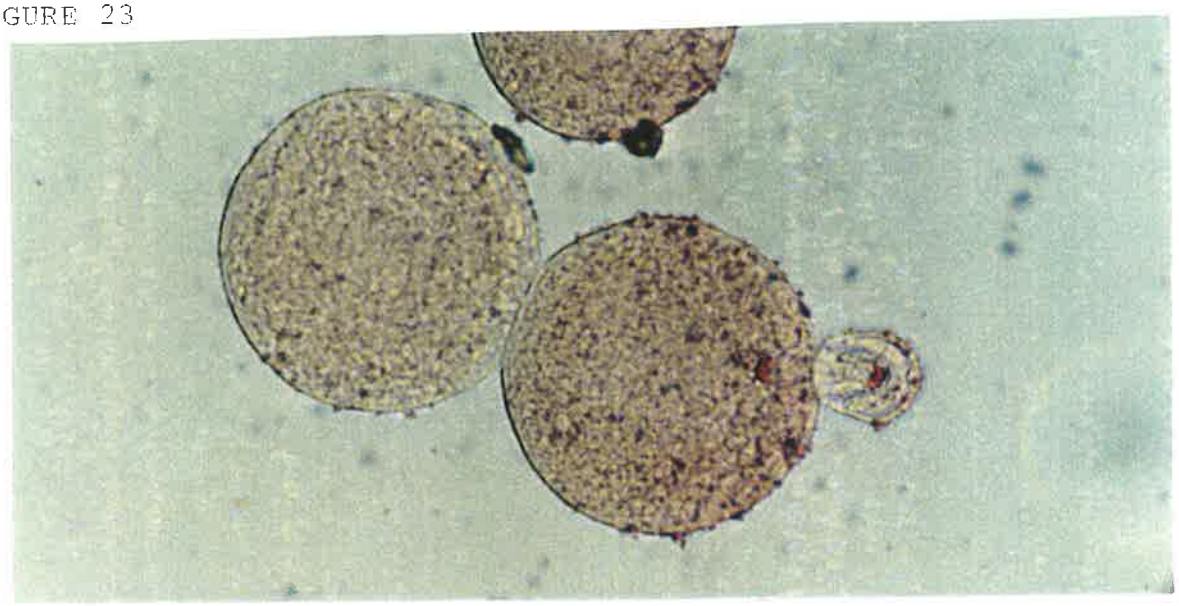
The comparison among the pooled data of different procedures demonstrates that the poorest procedure used for enucleation of oocytes was the one which used the preculture of the oocytes with cytochalasin B for 1 hour. In this group many oocytes after the centrifugation had leftover of chromosomes in the cytoplasm and were considered not to be enucleated. The increase of time to 3 hours of preculture in cytochalasin B was necessary to the enucleation procedure.

The oocytes submitted to enucleation which were not pre-incubated with cytochalasin B were similar in performance to oocytes submitted to 3 hours pre-incubation. However, the cytoplasts resulting from the latter treatment were of various sizes, which means that as a consequence of the centrifugal force other important cytoplasmic components as well as the nucleus could be withdrawn from the cytoplasts. The same problem was highlighted by Fulka and Moor (1993) who used mechanical enucleation with a micromanipulator. Nevertheless Tatham et al., (1993) obtained bovine morulae after nuclear transfer to enucleated oocyte fragments obtained by centrifugation.

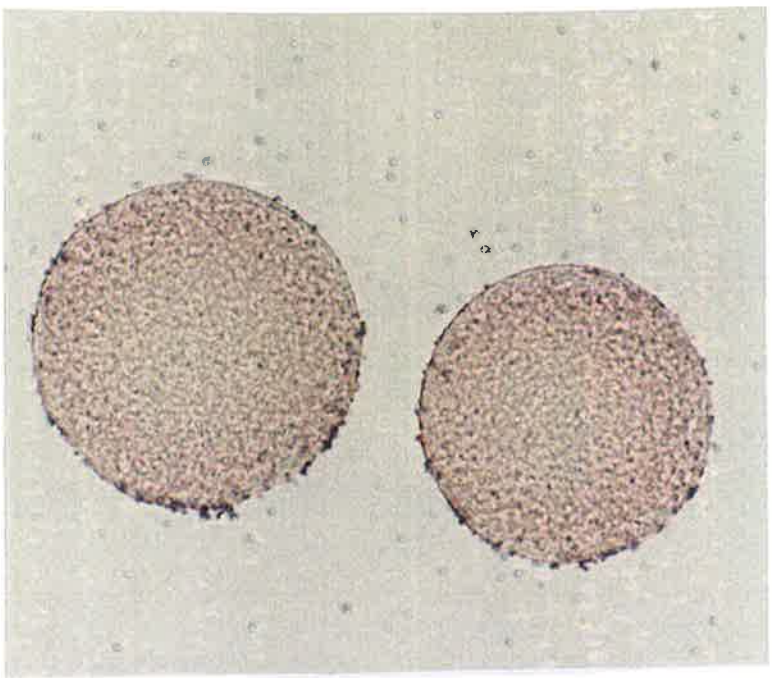
The centrifugation method has the advantage that it allows the enucleation of a unlimited number of oocytes without the need of accurate micromanipulation skills. However, like mechanical

enucleation using the micromanipulator, the technique lacks precision because possible important parts of the cytoplast can also be discharged together with the nucleus, resulting an unsuitable cytoplast for the use in nuclear transfer.

FIGURE 23



MOUSE OOCYTE WHICH WAS SUBMITTED TO THE CENTRIFUGATION PROCEDURE WHICH WAS NOT TOTALLY ENUCLEATED



MOUSE OOCYTES WHICH WERE SUBMITTED TO THE ENUCLEATION CENTRIFUGATION PROCEDURE AND WERE COMPLETELY ENUCLEATED

XIV - GENERAL DISCUSSION

The specific objective of this thesis was the production of sheep X goat hybrid animals for genetic studies. The advanced *in vitro* reproductive technology employed for this study can be more generally applied for the multiplication of mammalian genotypes.

From the work developed with sheep oocytes it is important to highlight that the *in vitro* maturation of sheep oocytes with different protein sources used in the *in vitro* maturation system was not different. However the procedure used to collect the oocytes from the ovaries affected nuclear maturation, fertilization and cleavage rates. The development of the sheep *in vitro* work was essential to optimise the technique for embryo hybrid production.

Hybrid fertilization (doe X ram; hybrid female X ram) was achieved using *in vitro* techniques which was the initial objective of this study. The problem however remains as to how to produce hybrids (sheep X goat) which survive beyond the post implantation stage. According to the literature very few hybrid animals have been produced and those animals were always born from a female goat. As chimeras used as recipients according to the literature are useless, perhaps another approach that could be used to produce such animals would be the identification, multiplication and study of those special female goats which delivered viable hybrids.

In respect to *in vitro* fertilization of *in vivo* matured goat oocytes, it was found that oocytes collected by oviduct flushing

or by aspiration from developed ovarian follicles at the time of surgery both had a similar pattern of development. It is important to emphasise this, because given the small number of ruminant female donors usually available and the varied response to the superovulation treatments (see discussion chapter 4), follicular aspiration could provide a valuable additional source of oocytes.

This supplementary source could be particularly valuable when working with a single donor animal carrying a rare or novel genotype, or when an individual female's response to a superovulation treatment has been poor.

Comparison of the development of *in vitro* and *in vivo* matured goat oocytes which had been fertilized *in vitro* showed that the *in vitro* matured oocytes developed less well than *in vivo* matured oocytes, indicating the need for further improvements in the *in vitro* maturation system.

No advantage was gained by supplementing the medium with homologous serum, and investigations of hormones, growth factors or other supplements may be warranted.

Stem cell technology presents an attractive route for the multiplication of rare genotypes, however this technology is still experimental and not easy to duplicate, even in well characterised species such as the mouse, and its potential extension to routine livestock breeding is still some way off. In the present study, mouse chimeras were produced from cultures of XY stem cells derived from different mouse strains injected into host blastocysts of unknown sex. As found by other

researchers,the stem cells failed to colonise the germline in many of the chimeras produced, and as a consequence, the genotype was not transmitted to the offspring. This situation could result from technical limitations, such as an inadequate incorporation of the stem cells into the host blastocyst. However, on at least 50% of occasions, the host blastocyst would be expected to be of the same sex as the stem cells, and the chances of germline chimeras would be reduced, due to competition between germ cells when invading the gonadal ridge.

The availability of host blastocyts of known sex could therefore be one useful means of improving the efficiency of this technology. This could be achieved by sexing blastocysts by biopsy, but this is a technically demanding and time-consuming procedure which risks decreasing the viability of the blastocysts. However, one possible solution to this problem may be the production of blastocysts by fertilising oocytes in vitro using sperm which has been sexed and sorted using cell separation procedure (FACS).

The experiments carried out in this study with inner cell mass (ICM) cells as the precursor cells for stem cell cultures, confirm that, in the absence of stem cell culture technology, it is possible to isolate individual cells from ICM of blastocysts by immunosurgery and to create chimeric embryos by injection of these cells into host blastocysts. Short term in vitro culture of ICM cells which allows some expansion in their numbers could be useful as an intermediate step in the creation of cloned livestock, until the creation of stem cell cultures is better

understood. More precise information on how many cells should be injected into a host blastocyst to produce a chimera would also be useful in allowing a better assessment of the value of this approach. The process is however technically demanding and not very practical.

However, using the chimera route to re-establish a stem cell genomic breeding time has the disadvantage that it does not offer any guarantees that the individuals produced will be germline transmitters. To achieve this requires a different technical approach, where individual stem cells are fused with enucleated oocytes to re-establish a fully competent zygote. Nuclear transfer technology is under active development in many laboratories, and when fully developed it has the potential to allow unlimited cloning.

The mouse with its high fecundity and short generation time provides a useful experimental model to explore and develop knowledge in this area.

The embryo aggregation procedure explored in this thesis also shows promise as a useful means of testing the compatibility between embryonic cells from two strains of mice or two breeds of farm animals.

The close affinity of distinct embryos could improve the possibility of production of germline chimeric individuals when generated by stem cell injection and this method can be used as an alternative to rescue some rare genotypes.

So far very few permanent cultures of totipotent stem cell lines have been produced, even in the mouse, and clearly there

is still much to be learned about the use of key growth factors such as LIF, which allow long term cell multiplication *in vitro* without cellular differentiation.

APPENDIX 1 (GOAT SUPEROVULATION)

SUPEROVULATION TREATMENT	TIME	r	CORPUS LUTEUM	IN VIVO MATURED OOCYTES	
				ASPIRATED FROM DEVELOPED FOLLICLES	OBTAINED BY OVIDUCT FLUSHING
			n	n	n
CIDR 14 DAYS PMSG 1000 IU BEFORE 48 hs CIDR WITHDRAWAL	27\07\ 1990	3	28	0	28
CIDR 21 DAYS PMSG 1000 IU BEFORE 48 hs CIDR WITHDRAWAL	18\12\ 1990	3	31	0	29
CIDR 21 DAYS PMSG 1000 IU + LUTALYSE 125µg BEFORE 48 hs CIDR WITHDRAWAL	15\01\ 1991	3	13	2	15
CIDR 14 DAYS PMSG 1000 IU BEFORE 48 hs CIDR WITHDRAWAL	26\02\ 1991	2	21	0	15
CIDR 21 DAYS CIDR 15 DAYS + NEW CIDR 6 DAYS PMSG 1000 IU BEFORE 48 hs CIDR WITHDRAWAL	17\04\ 1991	3	47	2	35
CIDR 13 DAYS PMSG 1000 IU BEFORE 48 hs CIDR WITHDRAWAL	23\04\ 1991	3	17	2	15
CIDR 12 DAYS PMSG 1000 IU BEFORE 48 hs CIDR WITHDRAWAL	30\04\ 1991	3	8	5	7
CIDR 14 DAYS PMSG 1000 IU BEFORE 48 hs CIDR WITHDRAWAL	07\05\ 1991	3	23	11	17
CIDR 17 DAYS PMSG 1000 IU BEFORE 48 hs CIDR WITHDRAWAL	04\06\ 1991	4	31	3	25

SUPEROVULATION TREATMENT	TIME	r	CORPUS LUTEUM	IN VIVO MATURED OOCYTES	
				ASPIRATED FROM DEVELOPED FOLLICLES	OBTAINED BY OVIDUCT FLUSHING
			n	n	n
CIDR 17 DAYS PMSG 1000 IU BEFORE 48 hs CIDR WITHDRAWAL	18\06\ 1991	6	44	8	29
CIDR 14 DAYS PMSG 1000 IU BEFORE 48 hs CIDR WITHDRAWAL	25\06\ 1991	4	36	18	27
CIDR 12 DAYS PMSG 1000 IU BEFORE 48 hs CIDR WITHDRAWAL + 0.3mg CLOPROSTENOL AT CIDR REMOVAL	09\07\ 1991	5	15	14	3
CIDR 13 DAYS PMSG 1000 IU BEFORE 48 hs CIDR WITHDRAWAL + 0.3mg CLOPROSTENOL AT CIDR REMOVAL	10\07\ 1991	5	49	11	46

SURGERY DONE 44-52 HOURS AFTER CIDR REMOVAL.

APPENDIX 2 (GEEP SUPEROVULATION)

SUPEROVULATION TREATMENT	REPETITIONS	NUMBER OF CORPUS LUTEUM	TIME	IN VITRO MATURED	IN VIVO MATURED	
				ASPIRATED FROM UNDEVELOPED POLLICLES	ASPIRATED FROM DEVELOPED POLLICLES	OBTAINED BY OVIDUCT FLUSHING
		n		n	n	n
CIDR 14 DAYS PMSG 1200 IU BEFORE 48 hs CIDR WITHDRAWAL(*)	1	0	21\08\90	2[1]	0	0
CIDR 16 DAYS PMSG 1500 IU BEFORE 48 hs CIDR WITHDRAWAL(*)	1	15	14\03\91	0	1	9
CIDR 17 DAYS PMSG 1500 IU BEFORE 48 hs CIDR WITHDRAWAL + NEW CIDR AT THE TIME OF PMSG INJECTION(*)	1	1	28\05\91	0	9	0

(*) SURGERY DONE 44 - 50 HOURS AFTER CIDR REMOVAL

[1] HYBRID FEMALE WAS FOUND PREGNANT

APPENDIX 3 A

1 - BICARBONATE 199

25 ml - 1 LITRE SACHET M199(MODIFIED) WITH EARL'S SALTS, WITH GLUTAMINE, WITHOUT SODIUM BICARBONATE; FLOW LABORATORIES, INC., DILUTED IN 500 ml OF ELGASTAT WATER WITH 200 UI/ml OF PENICILLIN PLUS 100 µg/ml OF STREPTOMYCIN SO_4.

12.5 mM - $NaHCO_3$

0.025% w/v - PHENOL RED

25 ml - ELGASTAT WATER

2 - HEPES 199

100 ml - 1 LITRE SACHET M199(MODIFIED) WITH EARL'S SALTS, WITH GLUTAMINE, WITHOUT SODIUM BICARBONATE; FLOW LABORATORIES, INC. DILUTED IN 500 ml OF ELGASTAT WATER WITH 200 UI/ml OF PENICILLIN PLUS 100 µg/ml OF STREPTOMYCIN SO_4.

42 mM - HEPES (FREE ACID)

7.9 mM - $NaHCO_3$

0.4% w/v - BSA

0.1% w/v - PHENOL RED

80 ml - ELGASTAT WATER

3 - SPERM WASH

112.08 mM - NaCl

4.02 mM - KCl

2.24 mM - $CaCl_2 2H_2O$

0.52 mM - $MgCl_2 6H_2O$

0.83 mM - $NaH_2PO_4 2H_2O$

13.87 mM - $C_6H_{12}O_6$

0.63 mM - $C_3H_3O_3$

21.00 mM - HEPES (FREE ACID)

3.99 mM - $NaHCO_3$

0.05% w/v - PHENOL RED

0.4% w/v - BSA (FATTY ACID FREE)

100 UI/ml - PENICILLIN G

4 - IN VITRO FERTILIZATION MEDIA

112.08 mM - NaCl

4.02 mM - KCl

2.24 mM - $CaCl_2 2H_2O$

0.52 mM - $MgCl_2 6H_2O$

0.83 mM - $NaH_2PO_4 2H_2O$

13.87 mM - $C_6H_{12}O_6$

0.63 mM - $C_3H_3O_3$

18.75 mM - $NaHCO_3$

13.90 mM - DL-LACTIC ACID

0.04% w/v - PHENOL RED

0.4% w/v - BSA (FATTY ACID FREE)

100 UI/ml - PENICILLIN G

5 - ADDITIVES

0.015 mM - VITAMIN B_{12}

0.069 mM - INSULIN

0.014 mM - POLYVINYL ALCOHOL

13.690 mM - GLUTAMINE

8.520 mM - ASCORBIC ACID

0.555 mM - INOSITOL

24.380 mM - SODIUM ACETATE

20.000 mM - Na PYRUVATE

18.553 mM - GLUCOSAMINE

5 - PHE

0.025 mM - PENICILLAMINE

0.01 mM - HYPOTAURINE

0.001 mM - EPINEPHRINE

7 - PBS

136.893 mM - NaCl

2.683 mM - KCl

1.469 mM - KH_2PO_4

15.211 mM - Na_2HPO_4

8 - ANTIBIOTICS (100 X)

50 UI/ml - PENICILLIN

50 µg/ml - STREPTOMYCIN

100 µg/ml - NEOMYCIN

9 - STAIN

1 g - ORCEIN, OBTAINED FROM SIGMA (MADE BY GURR).

40 ml - ACETIC ACID

60 ml - ELGASTAT WATER

OSMOLARITY OF ALL WORKING MEDIAS ADJUSTED BETWEEN 271 TO 285.

APPENDIX 3 B

2 - IN VITRO FERTILIZATION MEDIA

112.08 mM - NaCl

4.02 mM - KCl

2.24 mM - $CaCl_2 2H_2O$

0.52 mM - $MgCl_2 6H_2O$

0.83 mM - $NaH_2PO_4 2H_2O$

0.69 mM - $C_3H_3O_3$

24.99 mM - $NaHCO_3$

6.95 mM - DL-LACTIC ACID

0.001% w/v - PHENOL RED

0.4% w/v - BSA (FATTY ACID FREE)

100 UI/ml - PENICILLIN G

1.066 mM - GLUTAMINE

OSMOLARITY OF ALL WORKING MEDIAS ADJUSTED BETWEEN 271 TO 285.

APPENDIX 4

1 - HUMAN TUBAL FLUID (HTF-HCO_3)

50.74 mM - NaCl

2.35 mM - KCl

0.10 mM - $MgSO_4 7H_2O$

0.18 mM - KH_2PO_4

1.39 mM - $C_6H_{12}O_6$

12.85 mM - Na LACTATE

12.50 mM - $NaHCO_3$

0.17 mM - $C_3H_3O_3$

1.02 mM - $CaCl_2 2H_2O$

0.0005% w/V - PHENOL RED

50 UI/ml - PENICILLIN

2 - HUMAN TUBAL FLUID (HTF-HEPES)

50.74 mM - NaCl

2.35 mM - KCl

0.10 mM - $MgSO_4 7H_2O$

0.18 mM - KH_2PO_4

1.39 mM - $C_6H_{12}O_6$

12.85 mM - Na LACTATE

2.00 mM - $NaHCO_3$

0.17 mM - $C_3H_3O_3$

1.02 mM - $CaCl_2 2H_2O$

10.05 mM - HEPES Na SALT

0.0005% w/v - PHENOL RED

50 UI/ml - PENICILLIN

For HTF-HCO_3 and HEPES, 50 mg Bovine Serum Albumin (BSA - Fraction V, Sigma Co.) was added for each 10 ml of media prepared.

The pH of the HTF-Hepes media was adjusted to 7.4-7.5, and the osmolarity of both medias between 275 - 280.

3 - SYNTHETIC OVIDUCT FLUID MEDIA (SOFM-HCO_3)

53.81 mM - NaCl

3.58 mM - KCl

0.59 mM - KH_2PO_4

0.37 mM - $MgSO_4.7H_2O$

2.09 mM - Na LACTATE

12.49 mM - $NaHCO_3$

0.33 mM - $C_3H_3O_3$

2.25 mM - $CaCl_2 2H_2O$

0.001% w/v - PHENOL RED

100 UI/ml - PENICILLIN G

4 - SYNTHETIC OVIDUCT FLUID MEDIA (SOFM-HEPES)

53.81 mM - NaCl

3.58 mM - KCl

0.59 mM - KH_2PO_4

0.37 mM - $MgSO_4.7H_2O$

2.09 mM - Na LACTATE

12.49 mM - $NaHCO_3$

0.33 mM - $C_3H_3O_3$

2.25 mM - $CaCl_2 2H_2O$

10.05 mM - HEPES Na SALT

0.001% w/v - PHENOL RED

100 UI/ml - PENICILLIN G

THE pH OF THE SOFM-HEPES MEDIA WAS ADJUSTED TO 7.4-7.5, AND THE OSMOLARITY OF BOTH MEDIAS BETWEEN 270 - 280.

5 - FOLLICLE ASPIRATION MEDIA

TCM-199-EBSS SUPPLEMENTED WITH HEPES IN 900 MQ WATER

2 mM - $NaHCO_3$

2 mM - Na PYRUVATE

25 mM - HEPES Na SALT

100 UI/ml PENICILLIN G

2% - FETAL CALF SERUM

50 UI/ml - HEPARIN

6 - TCM-199-HCO_3-20% FCS

25 ml - TCM 199 VIA OF 9.9g DILUTED IN 500 ml OF MQ WATER.

24.99 mM - $NaHCO_3$

1.16 mM - Na PYRUVATE

0.5 ml - ANTIBIOTIC POOL

25 ml - MQ WATER

7 - ANTIBIOTIC POOL

10000 UI/ml - PENICILLIN

10000 UI/ml - STREPTOMYCIN SULPHATE

8 - PBS (DULBECCO'S)

0.90 mM - $CaCl_2 2H_2O$*

2.68 mM - KCl

1.47 mM - KH_2PO_4

0.49 mM - $MgCl_26H_2O$*

136.89 mM - NaCl

8.09 mM - Na_2HPO_4 ANHYD

* OMITTED THESE REAGENTS FOR Ca^{2+} AND Mg^{2+} FREE PBS. ADJUSTED pH TO 7.4 AND OSMOLARITY 280.

9 - EGTA

50 mM STOCK SOLUTION DILUTED 1:100 INTO PBS $Ca^{2+}Mg^{2+}$ FREE

10 - TRYPSIN

1 g - TRYPSIN DIFCO 1:250

100 ml - PBS $Ca^{2+}Mg^{2+}$ FREE

11 - DNase I

3000 UI/ml

12 - HYALURONIDASE

150 - 300 UI/ml + 0.5% BSA

13 - PRONASE (ROBERTSON 1987)

0.5% - PROTEASE FROM STREPTOMYCES GRISEUS.

0.5% - POLYVINYLPYRROLIDONE.

14 - ANTIBODY PRODUCTION

- RABBIT WAS IMMUNISED WITH SPLEEN CELLS PRODUCED FROM SHEEP.

- RABBIT RECEIVED INTRAVENOUS INJECTION IN THE EAR VEIN, 2

x 10^8 PURIFIED SPLEEN CELLS IN 1.6 ml OF PHOSPHATE BUFFERED SALINE ($Ca^{2+}Mg^{2+}$ FREE) EVERY 7 DAYS FOR 5 WEEKS.

- AT THE END OF THE IMMUNISATION PERIOD, THE RABBITS WERE EXSANGUINATED AND SERUM COLLECTED.

- THE SERA COLLECTED WAS FROZEN AND DRIED (FREEZE DRIER DYNAVAC, AUSTRALIA)

- THE SERUM WAS HEAT INACTIVATED 56ºC FOR 30 MINUTES BEFORE USE.

15 - ORCEIN

ORCEIN ACETIC SOLUTION (LA COUR) "GURR".

45% ACETIC ACID

16 - HOECHST (BISBENZIMIDE)

1 mg/ml

17 - PROPIDIUM IODIDE

10 mg/ml

18 - HORMONES

A - LUTROPIN (VETREPHARM) 1 mg/ml

B - FOLTROPIN (VETREPHARM) 40 mg / 4 ml

C - 17 ß-ESTRADIOL 1 mg/ml

19 - FILTER STERILIZATION

Sterilisation of the media filter was performed using Millipore millex-gs and Sterivex-gs (millipore Corporation, Ma, USA), discarding the initial filtrate (Harrison et al 1990).

APPENDIX 5 (PROTOCOL FOR GPI ELECTROPHORESIS USING ACETATE GEL)

-EQUIPMENT (SOURCE:HELENA LABORATORIES, 1530 LINDBERGH DRIVE P.O. BOX 752, BEAUMONT, TEXAS 77704).

- SUPER Z APPLICATOR KIT (CAT.Nº4093)

  CONTAINS:

  (a) SUPER Z APPLICATOR (12 SAMPLES)
  (b) SUPER Z ALIGNING BASE
  (c) SUPER Z WELL PLATE

- TITAN III CELLULOSE ACETATE PLATES (CAT.Nº3024)

- DISPOSABLE WICKS (CAT.Nº5081)

- ZIP ZONE CHAMBER (CAT.Nº1283)

BUFFERS:

- SAMPLE BUFFER: 50mM TRIS HCL (pH 8.0) + 0.1% TRITON X 100 ADDED
- RUNNING BUFFER: SUPRE-HEME BUFFER (HELENA LABOARTORIES, CAT.Nº 5802) OR 3g TRIS + 14.4g GLYCINE (SIGMA CAT.NºG7126) MADE UP 1 LITRE WITH DISTILLED WATER

STOCK SOLUTIONS FOR STAINING:

	STOCK CONCENTRATION
1 - MAGNESIUM ACETATE 0.25M	5.41g/100ml
2 - FRUCTOSE-6-PHOSPHATE(SIGMA CAT.Nºf3627)	75mg/ml
3 - TETRAZOLIUM-MTT(SIGMA CAT.NºM2128)	10mg/ml
4 - PHENAZINE METHOSULPHATE-PMS(SIGMA CAT.NºP9625)STORE IN LIGHT PROOF CONTAINER	1.8mg/ml
5 - NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE-NADP(SIGMA CAT.NºN0505)	10mg/ml
6 - GLUCOSE-6-PHOSPHATE DEHYDROGENASE-G6PDH(SIGMA CAT.NºG8878)	50U/ml
7 - 7.1% AGAROSE (1 g/100ml) IN 0.2 m TRIS-HCl pH 8.0	2.42g/100ml

* STORE STOCKS 1 - 6 IN 200 µl ALIQUOTS AT -20ºC

PREPARATION OF SAMPLES

01 - FREEZE-THAW THE SAMPLES AT LEAST TWICE IN THE APPROPRIATE VOLUME(1/20)OF SAMPLE BUFFER. IF THE TISSUE VOLUME IS LARGE ENOUGH, STANDARD HOMOGENIZATION TECHNIQUES CAN BE USED. FOR SMALL SAMPLES SIMPLY PRESS THE TISSUE AGAINST THE WALL OF THE TUBE WITH THE TIP OF A PIPETTE.
02 - SPIN THE SAMPLES AND TRANSFER 8µl OF SUPERNATANT TO THE WELLS OF THE SUPER Z WELL PLATE.

ELECTROPHORESIS

03 - PREPARE THE CHAMBER BY FILLING THE BUFFER COMPARTMENTS WITH SUPREHEME BUFFER HALF WAY AND FOLDING THE WICKS OVER THE INNER WALLS TO ELECTRICALLY CONNECT THEM TO THE BUFFER COMPARTMENTS.
04 - SOAK THE CELLULOSE-ACETATE GEL IN SUPREHEME BUFFER 15 MIN PRIOR TO APPLYING THE SAMPLE BY LOWERING THE GEL SLOWLY INTO THE BUFFER TO AVOID BUBBLE FORMATION ON THE SURFACE.
05 - NEED TO RUN GPI A CONTROL ONE END OF GEL AND GPI B CONTROL AT OTHER END OF GEL.
06 - TAKE THE GEL OUT AND REMOVE EXCESS BUFFER BY BLOTTING BETWEEN PAPER TOWELS. MARK THE PLASTIC BACKING WITH THE NECESSARY INFORMATION e.g. GEL NUMBER, ANODE.
07 - PRESS DOWN THE APPLICATOR INTO THE SAMPLES IN THE SUPER Z WELLS AND THEN BLOT IT ON TISSUE PAPER. RETURN THE APPLICATOR TO THE SAMPLE WELLS AND HOLD IT DOWN IN SAMPLES FOR A FEW SECONDS ON THE GEL PLACED ON THE ALIGNING BASE.
08 - TRANSFER THE GEL(GEL SURFACE DOWN) TO THE CHAMBER AND WEIGHT IT DOWN WITH A COIN IN THE CENTER. RUN AT 300 V (FROM ANODES TO CATHODE) FOR 30 MINUTES.

STAINING

09 - WHILE THE GEL IS RUNNING BOIL 1% AGAROSE IN BUFFER AND PUT IT INTO A 55ºC WATERBATH.
10 - JUST BEFORE THE ELECTROPHORESIS IS COMPLETE, PLACE 200µl OF EACH STOCK SOLUTION OF STAIN COMPONENTS 1-5 INTO A TEST TUBE(>12 ml) SHIELDED FROM LIGHT WITH ALUMINIUM FOIL. WARM THE MIXTURE IN THE 55ºC WATERBATH, THEN ADD 10 ML OF 55ºC AGAROSE SOLUTION. MIX THOROUGHLY.
12 - TERMINATE GEL RUN, BLOT THE GEL ON TISSUE AND PLACE THE GEL (GEL SIDE UP) ON A LEVEL SURFACE.
13 - ADD 200µl OF STAINING STOCK 6 (G6PDH) TO THE STAIN MIXTURE FROM (9). MIX QUICKLY AND POUR IT EVENLY OVER THE GEL. TO DECREASE BACKGROUND, WORK IN DIM LIGHT, SINCE THE STAIN IS LIGHT SENSITIVE.
14 - STAIN FOR 10 - 15 MINUTES(DEPENDING ON THE INTENSITY OF THE REACTION) AT 37ºC, THEN FIX THE GEL IN 1:3 ACETIC ACID:GLYCEROL FOR 10 MINUTES.
15 - READ THE GEL AND MAKE A PHOTOGRAPHIC RECORD.

XV - BIBLIOGRAPHY

Alexander,G., Williams,D., Bailey,L. (1967) Natural immunization in pregnant goats against red blood cells of their sheep x goat hybrid foetuses. Aust. J. Biol. Sci.,20:1217-26.

Anderson,G.B. (1987) Use of chimeras to study development. J. Reprod. Fert., Suppl. 34:251-259.

Anderson,G.B. (1988) Interspecific Pregnancy: Barriers and Prospects. Biology of Reproduction38:1-15.

Anderson,G.B., Piedrahita,J.A., Bondurant,R.H. (1990) On the isolation of embryonic stem (ES) cells: comparative behavior of murine, porcine, and ovine embryos. Biology of Reproduction (Suppl. 1)42:175. [Abstr 403]

Armstrong,D.T., Holm,P., Irvine,B., Petersen,B.A., Stubbings,R.B., McLean,D., Stevens,G., Seamark,R.F. (1992) Pregnancies and live birth from in vitro fertilization of calf oocytes collected by laparoscopic follicular aspiration. Theriogenology 38:575-580.

Assey,R.J., Hyttel,P., Greve,T., Purwantara,B. (1994) Oocyte morphology in dominant and subordinate follicles. Molecular Reproduction and Development 37:335-344.

Austin,C.R. and Short,R.V. (1990) Reproduction in mammals, 1 - Germ cells and fertilization, 2º edition, Cambridge University Press.

Ayres,M. and Ayres Júnior,M. (1987) Aplicações estatísticas em basic. Editora Santuário, McGraw-Hill, São Paulo, pp.157-166.

Azuma,S. and Toyoda,Y. (1991) Production of a germ-line chimeric mouse derived from newly established embryonic stem cells. Jpn. J. Anim. Reprod. 37:37-43.

Barnes,F., Endebrock,M., Looney,C., Powell,R., Westhusin,M., Bondioli,K. (1993) Embryo cloning in cattle: the use of in vitro matured oocytes. Journal of Reproduction and Fertility 97:317-320.

Bavister,B.D. (1988) Role of oviductal secretions in embryonic growth in vivo and in vitro. Theriogenology 29(1):143-154.

Bavister,B.D., Rose-Hellekant,T.A., Pinyopummintr,T. (1992) Development of in vitro matured/ in vitro fertilized bovine embryos into morulae and blastocysts in defined culture media. Theriogenology 37(1):127-146.

Beddington,R.S.P. and Robertson,E.J. (1989) An assessment of the developmental potential of embryonic stem cells in the midgestation mouse embryo. Development 105 (4):733-737.

Benirschke,K. (1983) Placentation. The Journal of Experimental Zoology228:385-389.

Betteridge,K.J. and Fléchon,J.E. (1988) The anatomy and physiology of pre-attachment bovine embryos. Theriogenology 29(1):155-187.

Blattman,A. and Beh,K. (1993) Haemonchus contortus resistance genes in sheep. Fourth Australasian Gene Mapping Workshop, University of Adelaide 10-13th, July.

Boland,M. (1984) Use of rabbit oviduct as a screening tool for the viability of mammalian eggs. Theriogenology 21(1):126-137.

Brackett,B.G., Bousquet,D., Boice,M.L., Donawick,W.J., Evans,J.F., Dressel,M.A. (1982) Normal development following in vitro fertilization in the cow. Biol. Reprod. 27:147-158.

Bradley,A., Evans,M., Kaufman,M.H., Robertson,E. (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. Nature 309:255-256.

Brem,G., Tenhumberg,H., Kräublich,H. (1984) Chimerism in cattle through microsurgical aggregation of morulae. Theriogenology 22(5):609-613.

Brison,D.R., Hewitson,L.C., Leese,H.J. (1993) Glucose, pyruvate and lactate concentrations in the blastocoel cavity of rat and mouse embryos. Molecular Reproduction and Development 35:227-232.

Buehr,M. and Mclaren,A. (1974) Size regulation in chimaeric mouse embryos. J. Embryol. Exp. Morphol. 31(1):229-234.

Bunch,T.D., Foote,W.C., Spillett,J.J. (1976) Sheep-goat hybrid karyotypes. Theriogenology 6(1):379-385.

Bunch,T.D., Rogers,A., Foote,W.C. (1977) G-band and transferrin analysis of aoudad-goat hybrids. The Journal of Heredity 68:210-212.

Butler,J.E., Anderson,G.B., BonDurant,R.H., Pashen,R.L. (1985) Production of ovine chimeras. Theriogenology 23(1):183.

Camous,S., Heyman,Y., Méziou,W., Ménézo,Y. (1984) Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles. J. Reprod. Fert. 72:479-485.

Capecchi,M.R. (1989) Altering the genome by homologous recombination. Science 244:1288-1292.

Carolan,C., Monaghan,P., Gallagher,M., Gordon,I. (1994) Effect of recovery method on yield of bovine oocytes per ovary and their developmental competence after maturation, fertilization and culture in vitro. Theriogenology 41:1061-1068.

Catt,J.W. (1990) Operation of a bovine IVF centre. In: Manipulating the mammalian embryo, Proceedings of the third symposium on advanced topics in animal reproduction, Jaboticabal, São Paulo, Brazil, August, 6-10 pp 89-100.

Chen,L.R. and Wu,M.C. (1993) Establishment of porcine-blastocyst-derived embryonic stem cells and production of chimeras by blastocyst injection. In 'Control of Reproduction in the Pig IV. (Eds G.R. Foxcroft, M.G. Hunter and C. Doberska) J. Reprod. Fertil. (Suppl.) 48:94.

Cheng,W.T.K., Moor,R.M., Polge,C. (1986) In vitro fertilization of pig and sheep oocytes matured in vivo and in vitro. Theriogenology 25(1):146.

Cherny,R.A. and Merei,J. (1994) Evidence for pluripotency of bovine primordial germ cell-derived cell lines maintained in long term culture. Theriogenology 41(1):175.

Cherny,R.A., Stokes,T.M., Merei,J., Lom,L., Brandon,M.R., Williams,R.L. (1994) Strategies for the isolation and characterization of bovine embryonic stem cells. Reprod. Fertil. Dev.6:569-75.

Cognié,Y., Guerin,Y., Guyader,C., Poulin,N., Crozet,N. (1991) In vitro fertilization of sheep oocytes matured in vivo. Theriogenology 35(2):393-400.

Collas,P. and Robl,J.M. (1990) Factors affecting the efficiency of nuclear transplantation in the rabbit embryo. Biology of Reproduction 43:877-884.

Coonrod,S.A., Flores-Foxworth,G., Moreno,J.F., Westhusin,M., Byrd,S.R., Kraemer,D.C. (1994) Birth of armenian red sheep (Ovis orientalis) lambs to domestic sheep (Ovis aries) following interspecific transfer of IVM-IVF derived embryos. Theriogenology 41(1):182.

Cox,J.F. (1990) Capacitacion espermatica para fertilizacion in vitro en ruminantes aspectos basicos y aplicados. In: Manipulating the mammalian embryo, Proceedings of the third symposium on advanced topics in animal reproduction, Jaboticabal, São Paulo, Brazil, August, 6-10 pp 27-62.

Crozet,N. (1991) Manipulation of oocytes and in vitro fertilization. J. Reprod. Fert., Suppl.43:235-243.

Cummins,J.M. and Yanagimachi,R. (1982) Sperm-egg ratios and the site of acrosome reaction during in vivo fertilization in the hamster. Gamete Res. 5:239-256.

Czlonkowska,M., Eysymont,U., Guszkiewicz,A., Kossakowski,M., Dziak,J. (1991) Birth of lambs after in vitro maturation, fertilization, and coculture with oviductal cells. Molecular Reproduction and Development 30:34-38.

D'Andrea,R.J., Sivaprasad,A.V., Bawden,S., Kuczek,E.S., Whitbread,L.A., Rogers,G.E. (1988) Isolation of microbial genes for cysteine synthesis and prospects for their use in increasing wool growth. In (G.E. Rogers et al eds) The Biology of wool and hair (Chapman & Hall London & New York) pp447-463.

Damiani,P., Fissore,R.A., Cibelli,J.B., Robl,J.M., Duby,R.T. (1995) Evaluation of cytoplasmic maturation of calf oocytes. Theriogenology 43(1):191.

Dent,J., McGovern,P.T., Hancock,J.L. (1971) Immunological implications of ultrastructural studies of goat x sheep hybrid placentae. Nature231:116-7.

Doetschman, T., Williams, P., Maeda, N. (1988) Establishment of hamster blastocyst-derived embryonic stem (ES) cells. Develop. Biol. 127:224-227.

Driancourt,M.A. and Fry,R.C. (1988) Differentiation of ovulatory follicles in sheep. J. Anim. Sci 66(Suppl.2):9-20.

Edwards,R.G. (1965) Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey & human ovarian oocytes. Nature 208:349-351.

Eistetter,H.R. (1989) Pluripotent embryonal stem cell lines can be established from disaggregated mouse morulae. Dev. Growth & Differ. v.31 (3):275-282.

Eldridge,F., Leipold,W.H., Harris,N. (1983) Goat x sheep hybrid: An additional case. J. Dairy Sci. Supp.66:253.

Eng,L.A., Kornegay,E.T., Huntigton,J., Wellman,T. (1986) Effects of incubation temperature and bicarbonate on maturation of pig oocytes in vitro. J.Reprod.Fert.76:657-662.

Eppleston,J. and Moore,N.W. (1977) Fertilization between sheep and goats and survival of hybrid embryos. Theriogenology8(4):165.

Evans,M.J. and Kaufman,M.H. (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154-156.

Evans,M.J., Notarianni,E., Laurie,S., Moor,R.M. (1990) Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocysts. Theriogenology 33(1):125-8.

Eyestone,W.H. (1994) Challenges and progress in the production of transgenic cattle. Reprod. Fertil. Dev. 6(5):647-52.

Fair,T., Hyttel,P., Greve,T. (1995) Bovine oocyte size in relationship to follicular diameter, maturational competence and rna synthesis. Theriogenology 43(1):209.

Falconer,D.S. and Avery,P.J. (1978) Variability of chimaeras and mosaics. J. Embryol. exp. Morph. 43:195-219.

Fehilly,C.B., Willadsen,S.M., Tucker,E.M. (1984a) Experimental chimaerism in sheep. J. Reprod. Fert.70:347-351.

Fehilly,C.B., Willadsen,S.M., Tucker,E.M. (1984b) Interspecific chimaerism between sheep and goat. Nature 307:634-36.

First,N.L. and Parrish,J.J. (1987) In vitro fertilization of ruminants. J. Reprod. Fert. Suppl. 34:151-165.

First,N.L., Sims,M.M., Park,S.P., Kent-First,M.J. (1994) Systems for the production of calves from cultured embryonic cells. Reprod. Fertil. Dev.6(5):553-562.

Fischer,B. (1989) Effects of asynchrony on rabbit blastocyst development. J. Reprod. Fert. 86:479-491.

Fléchon,J.E., Notarianni,E., Laurie,S., Galli,C., Evans,M.J Moor,R.M., (1990) Characterization of ovine and porcine embryonic stem cells. J. Reprod. Fertil. Abstract Series 6:25, [Abstr 40].

Foxworth,B. and Kraemer,D. (1993) Comparison of the implantation rates with subsequent placental development of tetraploid Mus musculus embryos tranferred to Mus musculus recipients during differing stages of pseudopregnancy. Biol. Reprod. 48(supplement 1):78, [Abstr 80].

Francolini,M., Lavitrano,M.L., Lamia,C.L., French,D., Frati,L., Cotelli,F., Spadafora,C. (1993) Evidence for nuclear internalization of exogenous DNA into mammalian sperm cells. Molecular Reproduction and Development 34:133-139.

Fraser,L.R. and Ahuja,K.K. (1988) Metabolic and surface events in fertilization. Gamete Res.20:491-519.

Fukui,Y., Fukushima,M., Ono,H. (1983) Fertilization in vitro of bovine oocytes after various sperm procedures. Theriogenology 20(6):651-660.

Fukui,Y. and Ono,H. (1989) Effects of sera, hormones and granulosa cells added to culture medium for in-vitro maturation, fertilization, cleavage and development of bovine oocytes. J. Reprod. Fert. 86:501-506.

Fukui,Y., Sawai,K., Furudate,M., Sato,N., Iwazumi,Y., Ohsaki,K. (1992) Parthenogenetic development of bovine oocytes treated with ethanol and cytochalasin B after in vitro maturation. Molecular Reproduction and Development 33:357-362.

FulkaJR,J., Bradshaw,J., Jung,T., Antalikova,L., Moor,R.M. (1993) UV irradiation of metaphase II bovine oocytes. J. Reprod. Fert. abstract series nº11, July, Cambridge:29 [Abst 48].

FulkaJR,J., Jung,T., Moor,R.M. (1992) The fall of biological maturation promoting factor (MPF) and histone H1 kinase activity during anaphase and telophase in mouse oocytes. Molecular Reproduction and Development 32:378-382.

Fulka,JR,J. and Moor,R.M. (1993) Noninvasive chemical enucleation of mouse oocytes. Molecular Reproduction and Development 34:427-430.

Galili,U. (1993) Interaction of the natural anti-gal antibody with α-galactosyl epitopes: a major obstacle for xenotransplantation in humans. Immunology Today 14(10):480-482.

Galli,C. and Moor,R.M. (1991) Gonadotrophin requirements for the in vitro maturation of sheep oocytes and their subsequent embryonic development. Theriogenology 35(6):1083-1093.

Galli,C. and Lazzari,G. (1995) Granulosa cell supplementation during oocyte maturation improves the development of IVM-IVF bovine oocytes cultured in the sheep oviduct. Theriogenology 43(1):216.

Gandolfi,F. and Moor,R.M. (1987) Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. J. Reprod. Fert. 81:23-28.

Gandolfi,F., Brevini,T.A.L., Modina,S., Lauria,A. (1990) Embryogenesis in vitro: role of culture media and co-culture. In: Manipulating the mammalian embryo, Proceedings of the third symposium on advanced topics in animal reproduction, Jaboticabal, São Paulo, Brazil, August, 6-10 pp 13-25.

Garde,J., Perez,S., Aguado,M.J., Ayllon,E., Garrido,D., Montoro,V. (1995) Live birth of Hybrid (O.musimon X O. aries) lambs following intrauterine insemination in domestic sheep with mouflon semen obtained 40 hours postmorten. Theriogenology43(1):218.

Gardner,R.L. (1968) Mouse chimaeras obtained by the injection of cells into the blastocyst. Nature 220:596-597.

Gardner,D.K., Lane,M., Spitzer,A., Batt,P.A. (1994) Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. Biology of Reproduction 50:390-400.

Gardner,R.L. and Rossant,J. (1979) Investigation of the fate of 4.5 day post-coitum mouse inner cell mass cells by blastocyst injection. J. Embryol. exp. Morph. 52:141-152.

Garrod,D.R. (1993) Cell to cell and cell to matrix adhesion. British Medical Journal 306:703-705.

Gerfen,R.W., Fajfar-Whetstone,C.J., Wheeler,M.B. (1991) Isolation of embryonic cell lines from porcine blastocysts. In 'Preimplantation Embryo Development'. (Ed.B. Bavister.) p. II-16. [Abstr.] (Springer-Verlag: New York.)

Giles,J.R., Foote,R.H., Mark,W. (1991) Development of embryo stem cell technology for production of chimeric rabbits. Biol. Reprod. (Suppl. 1)44:57.

Giles,J.R., Yang,X., Mark,W., Foote,R.H. (1993) Pluripotency of cultured rabbit inner cell mass cells detected by isozyme analysis and eye pigmentation of fetuses following injection into blastocysts or morulae. Mol. Reprod. Dev.36:130-8.

Godke,R.A. and Rorie,R.W. (1992) The use of new embryo engineering technologies in farm animals. In: Abstracts & Proceedings of International symposium of biotechnology on animal reproduction, the Urumuqi Scientific Hall Xinjiang China. pp 93-96.

Gordon,I. (1990) Laboratory production of cattle embryos. In: Manipulating the mammalian embryo, Proceedings of the third symposium on advanced topics in animal reproduction, Jaboticabal, São Paulo, Brazil, August, 6-10 pp 63-87.

Gordon,J.W., Scangos,G.A., Plotkin,D.J., Barbosa,J.A., Ruddle,F.H. (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. Proc. Natl. Acad. Sci. USA 77:7380-7384.

Gossler,A., Doetschman,T., Korn,R., Serfling,E., Kemler,R. (1986) Transgenesis by means of blastocyst-derived embryonic stem cell lines. Proc. Natl. Acad. Sci USA 83:9065-9069.

Goto,K., Kajihara,Y., Kosaka,S., Koba,M., Nakanishi,Y., Ogawa,K. (1988) Pregnancies after co-culture of cumulus cells with bovine embryos derived from in vitro fertilization of in vitro matured follicular oocytes. J. Reprod. Fert. 83:753-758.

Graves,K.H. and Moreadith,R.W. (1993) Derivation and characterization of putative pluripotential embryonic stem cells from preimplantation rabbit embryos. Molecular Reproduction and Development 36:424-433.

Griffin,H.D., Cameron,N.D., Bulfield,G. (1992) Breeding and transgenesis as means of decreasing adiposity in farm animal species: practice and promise. Proceedings of the Nutrition Society 51:441-446.

Gustafson,R.A., Anderson,G.B., BonDurant,R.H., Sasser,G.R. (1993) Failure of sheep-goat hybrid conceptuses to develop to term in sheep-goat chimaeras. J. Reprod. Fert. 99:267-273.

Gutiérrez,A., Maga,E.A., Meade,H.M., Medrano,J.F., Anderson,G.B., Murray,J.D. (1995) Alteration in micelle size of milk from transgenic mice expressing a bovine κ-casein gene. Theriogenology 43(1):228.

Hanada,A. (1985) In vitro fertilization in cattle, with particular reference to sperm capacitation by Ionophore A23187. Japan.J.Anim.Reprod.31:56-61.

Hancock,J.L. and McGovern,P.T. (1968) Transfer of goat x sheep hybrid eggs to sheep and reciprocal transfer of eggs between sheep and goats. Res. Vet. Sci9:411-5.

Hancock,J.L., McGovern,P.T., Stamp,J.T. (1968) Failure of gestation of goat x sheep hybrids in goats and sheep. J. Reprod. Fert., Suppl.3:29-36.

Handyside,A., Hooper,M.L., Kaufman,M.H., Wilmut,I. (1987) Towards the isolation of embryonal stem cell lines from sheep. Roux's Arch. Dev. Biol. 196:185-90.

Harrison,K.L., Sherrin,D.A., Hawthorne,T.A., Breen,T.M., West,G.A., Wilson,L.M. (1990) Embryotoxicity of micropore filters used in liquid sterilization. Journal of in Vitro Fertilization and Embryo Transfer 7(6):347-50.

Henery,C.C. and Kaufman,M.H. (1992) Cleavage rate of haploid and diploid parthenogenetic mouse embryos during the preimplantation period. Molecular Reproduction and Development. 31:258-263.

Henery,C.C. and Kaufman,M.H. (1993) The incidence of aneuploidy after single pulse electroactivation of mouse oocytes. Molecular Reproduction and development. 34:299-307.

Holm,P., Irvine,B., Armstrong,D.T., Seamark,R.F. (1990) In vitro system for developing preimplantation sheep embryos from immature slaughterhouse oocytes. Australian Society for Reproductive Biology. Proceedings of the twenty second annual meeting. University of Western Australia, September 24 - 26,p92.

Hooper,M., Hardy,K., Handyside,A., Hunter,S., Monk,M. (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. Nature 326:292-295.

Huang,J.C., Wen,S.H., Shieh,R.C., Cheng,W.T.K. (1989) In vitro fertilization and early development of caprine oocytes and embryos. Journal of Taiwan Livestock Research. 22(1):39-46.

Huneau,D. and Crozet,N. (1989) In vitro fertilization in the sheep: effect of elevated calcium concentration at insemination. Gamete Research 23:119-125.

Iannaccone,P.M., Evans,E.P., Burtenshaw,M.D. (1985) Chromosomal sex and distribution of functional germ cells in a series of chimeric mice. Experimental Cell Research 156:471-477.

Jacques,S.M.C. and Wagner,E.M. (1985) Notas de aula de bioestatística. Departamento de Estatística, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Jaenisch,R. (1976) Germline integration and mendelian transmission of the exogenous moloney leukemia virus. Proc. Natl. Acad. Sci. USA 73(4):1260-1264.

Jaenisch,R. and Mintz, B. (1974) Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. Proc. Natl. Acad. Sci. USA 71(4):1250-1254.

Jiminez-Flores,R. and Richardson,T. (1988) Genetic engineering of the caseins to modify the behaviour of milk during processing: A review. J. Dairy Sci. 71:2640-2654.

Jost,A., Vigier,B., Prépin,J., Perchellet,J.P. (1973) Studies on sex differentiation in mammals. Recent Prog. Horm. Res. 29:1-41.

Kane,M.T. (1985) A low molecular weight extract of bovine serum albumin stimulates rabbit blastocyst cells division and expansion in vitro. J. Reprod. Fert. 73:147-150.

Kang,Y., Jiminez-Flores,R., Richardson,T. (1986) Casein genes and genetic engineering of the caseins. In: Genetic Engineering of Animals, Eds.J.W. Evans and A. Hollaender. Plenum Press. New York. pp 95-111.

Kanoh,Y. and Nagashima,H. (1984) Introduction of plasmid DNA (pBR322) into mouse blastocyst by pricking method. Jpn J Anim Reprod 30:49-53.

Kashiwazaki,N., Mizuno,J., Nagashima,H., Mizuno,A., Yamakawa,H., Yamanoi,J., Ogawa,S. (1988) Sexing of half-embryos produced by microsurgical bisection of mouse morulae and production of chimeric mouse of defined sex composition by aggregating two sexed half-embryos. Exp. Anim. 37 (2):127-135.

Kashiwazaki,N., Nakao,H., Ohtani,S., Nakatsuji,N. (1992) Production of chimeric pigs by the blastocyst injection method. Vet. Rec. 130:186-187.

Kawase,E., Suemori,H., Takahashi,N., Okazaki,K., Hashimoto,K., Nakatsuji,N. (1994) Strain difference in establishment of mouse embryonic stem (ES) cell line. Int. J. Dev. Biol. 38(2):385-90.

Keskintepe,L., Darwish,G.M., Paramio,M.T., Brackett,B.G. (1993) Caprine oocyte fertilization and development in vitro. Biol. Reprod. 48(suplement 1):171.

King,W.A., Xu,K.P., Sirard,M.A., Greve,T., Leclerc,P., Lambert,R.D., Jacques,P. (1988) Cytogenetic study of parthenogenetically activated bovine oocytes matured in vivo and in vitro. Gamete Res. 20:265-274.

Koopman,P., Gubbay,J., Vivian,N., Goodfellow,P., Lovell-Badge,R. (1991) Male development of chromosomally female mice transgenic for Sry. Nature 351:117-121.

Krausslich,H. (1986) Criteria for useful gene transfer. In: 3rd world congress on genetics applied to livestock production XII. Biotechnology, Selection Experiments, Parameter Estimation, Design of Breeding Systems, Management of Genetic Resources. Lincoln Nebraska, July 16-22. pp. 22-31.

Lambert,R.D., Sirard,M.A., Bernard,C., Béland,R., Rioux,J.E., Leclerc,P., Ménard,D.P., Bedoya,M. (1986) In vitro fertilization of bovine oocytes matured in vivo and collected at laparoscopy. Theriogenology 25(1):117-133.

Latham,K.E., Beddington,R.S.P., Solter,D., Garrels,J.I. (1993) Quantitative analysys of protein synthesis in mouse embryos.II: Differentiation of endoderm, mesoderm and ectoderm. Molecular Reproduction and Development 35:140-150.

Lavritano,M., Camaioni,A., Fazio,V.M., Dolic,S., Farace,M.G., Spadofara,C. (1989) Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. Cell 57:717-23.

Lavritano,M., French,D., Zani,M., Frati,L., Spadafora,C. (1992) The interaction between exogenous DNA and sperm cells. Molecular Reproduction and Development 31:161-169.

Leese,H.J. (1988) The formation and function of oviduct fluid. J. Reprod. Fert. 82:843-856.

Leibfried,L. and First,N.L. (1979) Characterization of bovine follicular oocytes and their ability to mature in vitro. J.Anim.Sci. 48(1):76-86.

Leibfried-Rutledge,M.L., Critser,E.S., First,N.L. (1986) Effects of fetal calf serum and bovine serum albumin on in vitro maturation and fertilization of bovine and hamster cumulus-oocyte complexes. Biol. Reprod. 35:850-7.

Leibfried-Rutledge,M.L., Critser,E.S., Parrish,J.J., First,N.L. (1989) In vitro maturation and fertilization of bovine oocytes. Theriogenology 31(1):61-74.

Lenz,R.W., Ball,G.D., Leibfried,M.L., Ax,R.L., First,N.L. (1983) In vitro maturation and fertilization of bovine oocytes are temperature dependent processes. Biol.Reprod. 29:173-179.

Lindemann,C.B. and Kaunus,K.S. (1989) Regulation of mammalian sperm motility. Arch. Androl. 23:1-22.

Ling,L., Jufen,Q., Yong,Z. (1992) In vitro fertilization of goat ovarian oocytes. In: Abstracts & Proceedings of International symposium of biotechnology on animal reproduction, the Urumuqi Scientific Hall Xinjiang China. pp 25.

Lu,K.H., Gordon,I., Gallagher,M., McGovern,H. (1987) Pregnancy established in cattle by transfer of embryos derived from in vitro fertilisation of oocytes matured in vitro. Vet. Rec. 121:259-260.

Lu,K.H., Gordon,I., McGovern,H., Gallagher,M. (1988) Production of cattle embryos by in vitro maturation and fertilization of follicular oocytes and their subsequent culture in vivo in sheep. Theriogenology 29(1):272.

MacLaren,L.A., Anderson,G.B., BonDurant,R,H., Edmondson,A.J., Bernoco,D. (1992) Maternal serum reactivity to species-specific antigens in sheep-goat interspecific pregnancy. Biol Reprod. 46:1-9.

MacLaren,L.A., Anderson,G.B., BonDurant,R,H., Edmondson,A.J. (1993) Reproductive cycles and pregnancy in interspecific sheep-goat chimaeras. Reprod. Fertil. Dev.5:261-70.

Mann,G.B., Fowler,K.J., Grail,D., Dunn,A.R. (1993) Identification of germ-line chimaeras by polymerase chain reaction and isoenzyme analysis of mouse spermatozoa. J. Reprod. Fert. 99 :505-512.

Mann,J.R., Gadi,I., Harbison,M.L., Abbondanzo,S.J., Stewart,C.L. (1990) Androgenetic mouse embryonic stem cells are pluripotent and cause skeletal defects in chimeras: Implications for genetic imprinting. Cell 62:251-260.

Marcus,G.J. (1990) Activation of cumulus-free mouse oocytes. Molecular Reproduction and Development 26:159-162.

Markert,C.L. (1982) Parthenogenesis, homozygosity and cloning animals. The Journal of Heredity 73:390-397.

Martin,G.R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA. 78:7634-7638.

Matsui,Y., Zsebo,K., Hogan,B.L.M. (1992) Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70:841-847.

Mattioli,M., Bacci,M.L., Galeati,G., Seren,E. (1989) Developmental competence of pig oocytes matured and fertilized in vitro. Theriogenology 31(6):1201-7.

Mattioli,M., Galeati,G., Seren,E. (1988) Effect of follicle somatic cells during pig oocyte maturation on egg penetratability and male pronucleus formation. Gamete Research 20 :177-183.

McGaughey,R.W., Racowsky,C., Rider,V., Baldwin,K., DeMarais,A.A., Webster,S.D. (1990) Ultrastructural correlates of meiotic maturation im mammalian oocytes. Journal of Electron Microscopy Technique 16:257-280.

McGovern,P.T. (1969) Goat and sheep hybrids. Animal Breeding Abstracts 37(1):1-11.

McGovern,P.T. (1973a) The effect of maternal immunity on the survival of goat x sheep hybrid embryos. J. Reprod. Fert.34:215-220.

McGovern,P.T. (1973b) The fate of goat x sheep hybrid embryos in goats treated parenterally with sheep semen. J. Reprod. Fert.34:221-225.

McLaren,A. (1984) Methods and success of nuclear transplantations in mammals. Nature 309:671-2.

McLaughlin,K.J., Davies,L., Seamark,R.F. (1990) In vitro embryo culture in the production of identical merino lambs by nuclear transplantation. Reprod. Fertil. Dev. 2:619-622.

McNatty,K.P., Henderson,K.M., Lun,S., Heath,D.A., Ball,K., Hudson,N.L., Fannin, J., Gibb,M., Kieboom,L.E., Smith,P. (1985) Ovarian activity in Booroola X Romney ewes which have a major gene influencing their ovulation rate. J. Reprod. Fertil. 73 :109-120.

McWhir,J., O'Neill,G.T., Wilmut,I. (1990) Transgenesis in farm animals. In: Manipulating the mammalian embryo, Proceedings of the third symposium on advanced topics in animal reproduction, Jaboticabal, São Paulo, Brazil, August, 6-10 pp 101-117.

Meinecke-Tillmann,S. and Meinecke,B. (1984) Experimental chimaeras-removal of reproductive barrier between sheep and goat. Nature 307:637-38.

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

Mies,A.F. (1987) Reprodução dos animais. 1º e 2º volume 6º ed, Sulina, Porto Alegre, Brasil.

Mogas,T., Izquierdo,M.D., Palomo,M.J., Paramio,M.T. (1995) Effect of hormones, serum source and culture system on the IVM and IVF of prepubertal goat oocytes and subsquent embryo development. Theriogenology 43(1):284.

Mogas,T., Palomo,M.J., Izquierdo,D., Martino,A., Paramio,M.T. (1993) Effect of hormones on the in vitro maturation, fertilization and early cleavage of prepubertal goat oocytes. J. Reprod. Fert. abstract series nº11, July, Cambridge:67 [Abst 124].

Moore,N.W. (1983) Survival of hybrid sheep X goat embryos. Proceedings of the fifteenth annual conference, Australian Society for Reproductive Biology, Canberra, Australia, September 4-7, 1983.

Moor,R.M. and Crosby,I.M. (1985) Temperature-induced abnormalities in sheep oocytes during maturation. J. Reprod. Fert. 75:467-473.

Moor,R.M. and Trounson,A.O. (1977) Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. J. Reprod. Fert. 49:101-109.

Müller,M. and Brem,G. (1994) Transgenic strategies to increase disease resistance in livestock. Reprod. Fertil. Dev.6:605-13.

Murray,J.D., Nancarrow,C.D., Ward,K.A. (1988) Techniques for the transfer of foreign genes into animals. 11th International Congress on Animal Reproduction and Artificial Insemination 5:20-27.

Nagai,T. (1987) Parthenogenetic activation of cattle follicular oocytes in vitro with ethanol. Gamete Res. 16:243-249.

Nagai,T. (1987) Parthenogenetic activation of cattle follicular oocytes in vitro with ethanol. Gamete Research 16:243-249.

Nagashima,H., Kobayashi,K., Yamakawa,H., Ogawa,S. (1991) Cryopreservation of mouse half-morulae and chimeric embryos by vitrification. Molecular Reproduction and Development 30:220-225.

Niemann,H. and Strelchenko,N. (1994) Isolation and maintenance of rabbit embryonic stem (ES) cell like cells. Theriogenology 41:265.

Niwa,K., Ohgoda,O., Yuhara,M. (1988) Effects of caffeine in media for pretreatment of frozen-thawed sperm on in vitro penetration of cattle oocytes. Proc.11th.Int.Congr.Anim.Reprod.&A.I.(Dublin) 3:346.

Notarianni,E., Laurie,S. Moor,R.M., Evans,M.J. (1990) Maintenance and differentiation in culture of pluripotential embryonic cell lines from pig blastocysts. J. Reprod. Fert. (Suppl.) 41:51-6.

Notarianni,E., Galli, Laurie,S., Moor,R.M., Evans,M.J. (1991) Derivation of pluripotent, embryonic cell lines from the pig and sheep. J. Reprod. Fert. (Suppl.) 43:255-60.

Nottle,M.B., French,A.J., Seamark,R.F. (1993) The transgenic pig. Proceedings from the Genetics and pig breeding workshop, Brisbane, February:1-4.

Onishi,A. and Youngs,C.R. (1993a) Comparison of immunosurgery with calcium ionophore treatment for isolation of inner cell masses of porcine blastocysts. Theriogenology 39:274.

Onishi,A. and Youngs,C.R. (1993b) Isolation of inner cell mass (ICM) derived cell colonies from intact porcine blastocysts. J. Anim. Sci. 71 (Suppl. 1):74.

Palmiter,R.D. and Brinster,R.L. (1986) Germline transformation of mice. Ann. Rev. Genet. 20:465-499.

Parchment,R.E. and Natarajan,K. (1992) A free-radical hypothesis for the instability and evolution of genotype and phenotype in vitro. Cytotechnology 10:93-124.

Paria,B.C. and Huet-Hudson,Y.M. (1993) Blastocyst's state of activity determines the "window" of implantation. Biol. Reprod. 48 (supplement 1):76,[Abstr 72].

Parrish,J.J., Susko-Parrish,J.L., First,N.L. (1985) In vitro fertilization of bovine oocytes using heparin treated and swim-up separated frozen-thawed bovine semen is repeatable and results in high frequencies of fertilization. Theriogenology 23(1):216.

Parrish,J.J., Susko-Parrish,J., Winer,M.A., First,N.L. (1988) Capacitation of bovine sperm by heparin. Biology of Reproduction 38:1171-1180.

Pease,S., Braghetta,P., Gearing,D., Grail,D., Williams,R.L. (1990) Isolation of embryonic stem (ES) cells in media supplemented with recombinant leukemia inhibitory factor (LIF). Developmental Biology 141:344-352.

Pease,S., and Williams,L. (1990) Formation of germ-line chimeras from embryonic stem cells maintained with recombinant leukemia inhibitory factor. Experimental Cell Research 190:209-211.

Peli,J. and Schellander,K. (1992) Capacity of pluripotent embryonic stem cells in producing chimeric embryos. Proc. 12th International Congress of Animal Reproduction. The Hague, The Netherlands 2:730.

Petters,R.M. and Mettus,R.V. (1984) Survival rate to term of chimeric morulae produced by aggregation of five to nine embryos in the mouse, mus musculus. Theriogenology 22(2):167-174.

Picard,L., Chartrain,I., King,W.A., Betteridge,K.J.(1990) Production of chimaeric bovine embryos and calves by aggregation of inner cell masses with morulae. Molecular Reproduction and Development 27:295-304.

Piedrahita,J.A., Anderson,G.B., BonDurant,R.H. (1990a) On the isolation of embryonic stem cells: comparative behaviour of murine, porcine, and ovine embryos. Theriogenology 34(5):879-901.

Piedrahita,J.A., Anderson,G.B., BonDurant,R.H. (1990b) Influence of feeder layer type on the efficiency of isolation of porcine embryo-derived cell lines. Theriogenology 34(5):865-77.

Piedrahita,J.A., Guillespie,L., Maeda,N. (1990c) Establishing an embryonic stem (ES) cell system utilizing hamster embryos. Biol. Reprod. (Suppl. 1)42:175. [Abstr 405]

Piedrahita,J.A., Gillespie,L., Maeda,N. (1992) Production of chimeric hamsters by aggregation of eight cell embryos. Biology of Reproduction 47:347-354.

Pinheiro,L.E.L., Guimaraes,S.E.F., Almeida Jr,I.L., Mikich,A.B. (1989) The natural occurrence of sheep x goat hybrids. Theriogenology 32(6):987-94.

Polge,C. (1990) Potential impact of advanced biotechnology on genetic conservation programmes. In: Genetic Conservation of Domestic Livestock, Chapter 23, Ed Lawrence Alderson, CAB Internatitional, UK.

Polzin,V.J., Anderson,D.L., Anderson,G.B., BonDurant,R.H., Butler,J.E., Pashen,R.L., Penedo,M.C.T. Rowe,J.D. (1986) Production of sheep-goat chimeras by blastocyst injection. Theriogenology 25(1):183.

Polzin,V.J., Anderson,D.L., Anderson,G.B., BonDurant,R.H., Butler, J.E., Pashen,R.L., Penedo,M.C.T., Rowe,J.D. (1987) Production of sheep-goat chimeras by inner cell mass transplantation. J. Anim. Sci. 65:325-330.

Pomp,D., Cowley,D.E., Eisen,E.J., Atchley,W.R., Hawkins-Brown,D. (1989) Donor and recipient genotype and heterosis effects on survival and prenatal growth of transferred mouse embryos. J. Reprod. Fert.86:493-500.

Prather,R.S. and First,N.L. (1993) Cell-to-cell coupling in early stage bovine embryos: a preliminary report. Theriogenology 39:561-567.

Prather,R.S. and First,N.L. (1987) Developmental fate in chimeras derived from highly asynchronous murine blastomeres. The Journal of Experimental Zoology 242:27-33.

Prather,R.S., Sims,M.M., First,N.L. (1989) Nuclear transplantation in early pig embryos. Biol. Reprod. 41:414-418.

Presicce,G.A. and Yang,X. (1994) Nuclear dynamics of parthenogenesis of bovine oocytes matured in vitro for 20 and 40 hours and activated with combined ethanol and cycloheximide treatment. Molecular Reproduction and Development 37:61-68.

Pugh,P.A., Fukui,Y., Tervit,H.R., Thompson,J.G. (1990) Successful use of frozen ram semen for in vitro fertilisation of in vitro matured sheep oocytes. Autralian Society for Reproductive Biology. Proceedings of the twenty second annual meeting. University of Western Australia, September 24 - 26,p88.

Pugh,P.A., Scobie,D., Tervit,H.R. (1994) Micromanipulation of early cleavage stage sheep embryos to produce chimeric embryos of mixed breed parentage. Theriogenology 41(1):279.

Pursel,V.G. and Rexroad,Jr.C.E. (1993) Recent progress in the transgenic modification of swine and sheep. Molecular Reproduction and Development 36:251-254.

Pursel,V.G. and Solomon,M.B. (1993) Alteration of carcass composition in transgenic swine. Food Reviews International 9(3):423-439.

Quinn,P., Kerin,J.F., Warnes,G.M. (1985) Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertility and Sterility 44(4):493-498.

Rickords,L.F. and White,K.L. (1992) Effect of electrofusion pulse in either electrolyte or nonelectrolyte fusion medium on subsequent murine embryonic development. Molecular Reproduction and Development. 32:259-264.

Rexroad,C.E. and Pursel,V.G. (1988) Status of gene transfer in domestic animals. 11th International Congress on Animal Reproduction and Artificial Insemination 5:29-35.

Robertson, E.J. (1987) Embryo derived stem cells. In: E.J. Robertson (Editor), Teratocarcinomas and embryonic stem cells: a practical approach. IRL Press, Oxford, pp. 71-112.

Robertson, E., Bradley, A., Kuehn, M., Evans, M. (1986) Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. Nature 323:445-448.

Robertson,E.J., Evans,M.J., Kaufman,M.H. (1983) X-chromosome instability in pluripotential stem cell lines derived from parthenogenetic embryos. J. Embryol. exp. Morph. 74 :297-309.

Robl,J.M. and Stice,S.L. (1989) Prospects for the commercial cloning of animals by nuclear transplantation. Theriogenology 31(1):75-84.

Roca,R. and Rodero,A. (1971) The chromosomes of a sheep x goat hybrid. Archivos de zootecnia 20(79):235.

Rogers,G.E. (1990) Improvement of wool production through genetic engineering. Trends-Biotechnol.8(1):6-11.

Rossant,J. (1993) Immortal germ cells? Current Biology 3(1):47-49.

Rossant,J., Croy,B.A., Clark,D.A., Chapman,V.M. (1983) Intespecific hybrids and chimeras in mice. The Journal of Experimental Zoology 228:223-233.

Roth,T.L., Anderson,G.B., Bon Durant,R.H., Pashen,R.L. (1989) Survival of sheep x goat hybrid inner cell masses after injection into ovine embryos. Biol. Reprod. 41:675-682.

Ruffing,N.A., Anderson,G.B., Bondurant,R.H., Currie,W.B., Pashen,R.L. (1993) Effects of chimerism in sheep-goat concepti that developed from blastomere-aggregation embryos. Bilogy of Reproduction 48:889-904.

Saeki,K., Hoshi,M., Leibfried-Rutledge,M.L., First,N.L. (1991) In vitro fertilization and development of bovine oocytes matured in serum-free medium. Biology of Reproduction 44:256-260.

Saito,S., Strelchenko,N., Neimann,H. (1992) Bovine embryonic stem cell-like cells lines cultured over several passages. Roux's Arch. Dev. Biol. 201:134-41.

Sanbuissho,A. and Threlfall,W.R. (1988) The influence of serum and gonadotropins on bovine oocyte maturation in vitro. Theriogenology 29(1):301.

Sanbuissho,A. and Threlfall,W.R. (1989) The effects of estrous cow serum on the in vitro maturation and fertilization of the bovine follicular oocyte. Theriogenology 31(3):693.

Sato,M., Tada,N., Iwase,R., Amann,E. (1993) Gene introduction into mouse blastocysts via "pricking". Molecular Reproduction and Development 34:349-356.

Schiewe,M.C., Hansen,C.T., Schmidt,P.M. (1992) Lack of antibody specificity by mouse trophectoderm during immunosurgery. Theriogenology 38:21-32.

Schwartzberg,P.L., Goff,S.P., Robertson,E.J. (1989) Germ-line transmission of a c-abl mutation produced by targeted gene disruption in ES cells. Science 246:799-803.

Seamark.R.F. (1988) Efficient creation of transgenic sheep: the challenge for the cell biologist. In (G.E. Rogers et al., eds) The Biology of wool and hair (Chapman & Hall London & New York) pp479-488.

Seamark,R.F. (1989) Transgenesis: The chalange for the reproductive Biologist. Reprod. Fertil. Dev.1:391-5.

Seamark,R.F. (1991b) Possibilities of using transgenic animals to improve the human health value of food. Assoc. Anim. Breeding Genetics 9.

Seamark,R.F. (1991a) The use of transgenic animals to study the role of growth factors in endocrinology. Baillière's Clinical Endocrinology and Metabolism 5(4):833-845.

Seamark,R.F. (1994) Progress and emerging problems in livestock transgenesis: A summary perspective. Reprod. Fertil. Dev.,6:653-657.

Seamark,R.F. and Wells,J.R.E. (1991) Biotechnology and Reproduction. In: World Animal Science Series B, Vol 14, Chapter 12 pp 345 - 362. Ed. Elsevier.

Seidel,Jr.G.E. (1992) Overview of cloning mammals by nuclear transplantation. In: Proceedings symposium on cloning mammals by nuclear transplantation, Ed G.E.Seidel,Jr. Fort Collins, Colorado, U.S.A. pp 1-4.

Sims,M.M. and First,N.L. (1993) Production of fetuses from totipotent cultured bovine inner cell mass cells. Theriogenology 39:313.

Simons,J.P., Ali,S., Archibald,A.L., Brown,P., Harris,S., McClenaghan,M., Whitelaw,C.B.A., Wilmut,I., Clark,A.J. (1993) Genetic manipulation in livestock improvement. In: Animal Health and Production for the 21st century, Ed K.J.Beh. Melbourne, Australia. pp 15-25.

Simons,J.P., Wilmut,I., Clark,A.J., Archibald, A.L., Bishop,J.O., Lathe,R. (1988) Gene transfer into sheep. Biotechnology 6:179-183.

Singer,S.J. (1992) Intercellular communication and cell-cell adhesion. Science 255:1671-1677.

Sleigh,M.J. and Hannan,G.N. (1993) Embryonic stem cells in animal breeding.Genetic manipulation in livestock improvement. In: Animal Health and Production for the 21st century, Ed K.J.Beh. Melbourne, Australia. pp 35-40.

Smith,A.G., Heath,J.K., Donaldson,D.D., Wong,G.G., Moreau,J., Stahl,M., Rogers,D. (1988) Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 336:688-690.

Smith,L.C. (1992) Removal or destruction of meiotic chromosomes of oocytes. Proceedings Symposium on Cloning Mammals by Nuclear Transplantation. January 15, Fort Collins, Colorado.pp.21-23.

Smith,L.C. and Wilmut,I. (1989) Influence of nuclear and cytoplasmic activity on the development in vivo of sheep embryos after nuclear transplantation. Biol. Reprod. 40:1027-1035.

Solter,D. and Knowles,B. (1975) Immunosurgery of mouse blastocyst. Proc. Nat. Acad. Sci. USA 72(12):5099-5102.

Somers,G.R., Trounson,A.O., Wilton,L.J. (1990) Allocation of cells to the inner cell mass and trophectoderm of 3/4 mouse embryos. Reprod. Fertil. Dev. 2:51-9.

Sreenan,J. (1968) In vivo and in vitro culture of cattle eggs. Proc.6th.Int.Congr.Anim.Reprod.& A.I. (Paris),1,577-580.

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

Stevens,L.C. (1978) Totipotent cells of parthenogenetic origin in a chimaeric mouse. Nature 276:266-267.

Stewart-Scott,I.A., Pearce,P.D., Dewes,H.F., Thompson,J.W.L. (1990) A case of sheep-goat hybryd in New Zealand. New Zealand Veterinary Journal 38:7-9.

Stice,S., Strelchenko,N., Betthauser,J., Scott,B., Jurgella,G., Jackson,J., David,V., Keefer,C., Matthews,L. (1994) Bovine pluripotent embryonic cells contribute to nuclear transfer and chimeric fetuses. Theriogenology 41:301.

Stokes,T.M., Cherny,R.A., Brandon,M.R. (1994) Production of chimaeric bovine embryos. Theriogenology 41:303.

Storb,U. (1987) Transgenic mice with immunoglobin genes. Annu. Rev. Immunol. 5:151-174.

Strelchenko,N., Saito,S., Niemann,H. (1991) Towards the establishment of bovine embryonic stem cells. Theriogenology 35(1):274.

Strelchenko,N. and Stice,S. (1994) Bovine embryonic pluripotent cell lines derived from morula stage embryos. Theriogenology 41:304.

Stringfellow,D.A., Gray,B.W., Toivio-Kinnucan,M., Galik,P., Riddell,K.P., Brock,K.V., Kemppainen,R.J. (1991) A continous cell line established from a preimplantation bovine embryo. Theriogenology 35(1):275.

Strojek,R.M., Reed,M.A., Hoover,J.L., Wagner,T.E. (1990) A method for cultivating morphologically undifferentiated embryonic stem cells from porcine blastocysts. Theriogenology 33(4):901-13.

Sukoyan,M.A., Golubitsa,A.N., Zhelezova,A.I., Shilov,A.G., Vatolin,S.Y., Maximovsky,L.P., Andreeva,L.E., McWhir,J., Pack,S.D., Bayborodin,S.I., Kerkis,A.Y., Mizilova,H.I., Serov,O.L. (1992) Isolation and cultivation of blastocyst-derived stem cell lines from American Mink (Mustela vison). Mol. Reprod. Dev.33:418-31.

Sukoyan,M.A., Vatolin,S.Y., Golubitsa,A.N., Zhelezova,A.I., Zemenova,L.A., Serov,O.L. (1993) Embryonic stem cells derived from morulae, inner cell mass, and blastocysts of mink: comparisons of their pluripotencies. Mol. Reprod. Dev. 36:148-58.

Surani,M.A.H. and Barton,S.C. (1977) Trophoblastic vesicles of preimplantation blastocysts can enter into quiescence in the absence of inner cell mass. J. Embryol. exp. Morph. 39:273-277.

Surani,M.A.H., Barton,S.C., Norris,M.L. (1987) Experimental reconstruction of mouse eggs and embryos: an analysis of mammalian development. Biology of Reproduction 36:1-16.

Swanson,M.E., Martin,M.J., O'Donnell,J.K., Hoover,K., Lago,W., Huntress,V., Parsons,C.T., Pinkert,C.A., Pilder,S., Logan,J.S. (1992) Production of functional human hemoglobin in transgenic swine. Bio/technology 10:557-559.

Talbot,N.C., Rexroad,Jr.C.E., Pursel,V.G., Powell,A.M., Nel,N.D. (1993a) Culturing the epiblast cells of the pig blastocyst. In Vitro Cell. Dev. Biol. 29a:543-554.

Talbot,N.C., Rexroad,Jr.C.E., Pursel,V.G., Powell,A.M. (1993b) Alkaline phosphatase staining of pig and sheep epiblast cells in culture. Mol. Reprod. Dev. 36:139-47.

Tan,S.J. and Lu,K.H. (1990) Effects of different oestrous stages of ovaries and sizes of follicles on generation of bovine embryos in vitro. Theriogenology 33(1):335.

Tarkowski,A.K. (1959) Experimental studies on regulation in the development of isolated blastomeres of mouse eggs. Acta Theriologica 3:191-267.

Tarkowski,A.K. (1961) Mouse chimaeras developed from fused eggs. Nature 190:857-860.

Tatham,B.G., Dowsing,A.T., Trounson,A.O. (1993) Enucleation by centrifugation for bovine nuclear transplantation. Autralian Society for Reproductive Biology Inc. Proceedings of the twenty fifth annual conference. University of Otago, Dunedin, New Zealand, August 23-25,p-31.

Tervit,H.R., Whittingham,D.G., Rowson,L.E.A. (1972) Successful culture in vitro of sheep and cattle ova. J. Reprod. Fert. 30:493-497.

Testart,J., Lassalle,B., Frydman,R. Belaisch,J.C. (1983) A study of factors affecting the success of human fertilization in vitro. II. influence of semen quality and oocyte maturity on fertilization and cleavage. Bilogy of Reproduction 28:425-431.

Thibault,C., Szollosi,D., Gerard,M. (1987) Mammalian oocyte maturation. Reprod.Nutr.Develop. 27(5):865-896.

Thompson,J.G., Simpson,A.C., Pugh,P.A., Tervit,H.R. (1992) Requirement for glucose during in vitro culture of sheep preimplantation embryos. Mol Reprod Devel 31:253-257.

Tokunaga,T. and Tsunoda,Y. (1992) Efficacious production of viable germ-line chimeras between embryonic stem (ES) cells and 8-cell stage embryos. Develop. Growth & Differ. 34:561-566.

Tsuchiya,Y.,Raasch,G.A., Brandes,T.L., Mizoshita,K., Youngs,C.R. (1994) Isolation of ICM-derived cell colonies from sheep blastocysts. Theriogenology 41(1):321.

Tsunoda,Y. and Kato,Y. (1993) Nuclear transplantation of embryonic stem cells in mice. Journal of Reproduction and Fertility 98:537-540.

Tucker,E.M., Denis,B., Kilgour,L. (1989) Blood genetic marker studies of a sheep-goat hybrid and its back-cross offspring. Animal Genetics20:179-186.

Utsumi,K., Katoh,H., Iritani,A. (1988) Developmental ability of bovine follicular oocytes matured in culture and fertilized in vitro. Theriogenology 29(1):320.

Vanderhyden,B.C. (1987) Increased embryonic survival of in vitro fertilized oocytes given additional time for pre-implantation development. Biol. Reprod. 36 Suppl. 1, Abstr. 95.

Walker,S.K., Heard,T.M., Ashman,R.J., Seamark,R.F. (1991) Viability of day-5 sheep embryos cultured in vitro from the zygote stage. Australian Society for Reproductive Biology. Proceedings of the twenty third annual conference, University of Sydney, Septemper 30 - October 2,p65.

Walker,S.K., Heard,T.M., Seamark,R.F. (1992) In vitro culture of sheep embryos without co-culture: successes and perspectives. Theriogenology 37(1):111-126.

Wall,R.J. and Seidel,Jr.,G.E. (1992) Transgenic farm animals - a critical analysis. Theriogenology 38:337-357.

Ward,K.A., Murray,J.D., Shanahan,C.M., Rigby,N.W., Nancarrow,C.D. (1988) The creation of transgenic sheep for increased wool productivity. In (G.E. Rogers et al eds) The Biology of wool and hair (Chapman & Hall London & New York) pp465-477.

Warner,C.M., Gollnick,S.O., Goldbard,S.B. (1984) Genetic control of early embryonic development by gene(s) in the MHC (H-2 complex). Proc. 10th Int. Cong. Anim. Reprod. & A.I., Urbana4,XIII,10-15.

Warner,C.M., Gollnick,S.O., Goldbard,S.B. (1987) Linkage of the preimplantation-embryo-development (Ped) gene to the mouse major histocompatibility complex (MHC). Biology of Reproduction 36:606-610.

Watson,A.J. (1992) The cell biology of blastocyst development. Molecular Reproduction and Development 33:492-504.

Watson,A.J., Watson,P.H., Warnes,D., Walker,S.K., Armstrong,D.T., Seamark,R.F. (1994) Preimplantaion development of in vitro-matured and in vitro-fertilized ovine zygotes: Comparison between coculture on oviduct epithelial cell monolayers and culture under low oxygen atmosphere. Biology of Reproduction 50:715-724.

Wheeler,M.B. (1994) Development and validation of swine embryonic stem cells:a review. Reproduction, Fertility and Development 6 (5):563-568.

Wheeler,M.B., Rund,L.A., Bleck,G.T. (1995) The use of embryonic stem cells in the production of transgenic livestock, In: International Embryo Transfer Society, Embryo transfer Newsletter 13(1):20-25.

Willadsen,S.M. (1979) A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. Nature 277:298-300.

Wilmut,I., Ashworth,C.J., Springbett,A.J., Sales,D.I. (1988) Effect of variation in embryo stage on the establishment of pregnancy, and embryo survival and growth in ewes with two embryos. J. Reprod. Fert.83:233-237.

Wilmut,I., Campbell,K.H.S., O'neill,G.T. (1992) Sources of totipotent nuclei including embryonic stem cells. In: Proceedings symposium on cloning mammals by nuclear transplantation, Ed G.E.Seidel,Jr. Fort Collins, Colorado, U.S.A. pp 8-16.

Wilmut,I., Hooper,M.L., Simons,J.P. (1991) Genetic manipulation of mammals and its application in reproductive biology. J. Reprod. Fert.92:245-279.

Wilmut,I., Sales,D.I., Ashworth,C.J. (1986) Maternal and embryonic factors associated with prenatal loss in mammals. J. Reprod. Fert. 76:851-864.

Wise,T. (1987) Biochemical analysis of bovine follicular fluid: albumin, total protein, lysosomal enzymes, ions, steroids and ascorbic acid content in relation to follicular size, rank, atresia classification and day of estrous cycle. J. Anim. Sci. 64:1153-1169.

Wilkins,A.S. (1993) Mouse postimplantation development. In: Genetic Analysis of Animal Development, pp 379-432. Publisher Wiley-Liss, Inc., U.S.A.

Wood,S.A., Allen,N.D., Rossant,J., Auerbach,A., Nagy,A. (1993) Non-injection methods for the production of embryonic stem cell-embryo chimaeras. Nature 365:87-89.

Xu,K.P. and King,W.A. (1990) Effects of oviductal cells and heparin on bovine sperm capacitation in vitro. Society for the Study of Reproduction (Suppl1), Biology of Reproduction 42, July 15-18, Knoxville, [Abstr 145].

Yamamoto,F., Furusawa,M., Takamatsu,K., Miura,N., Uchida,T. (1981) Intracellular introduction of a fixed quantity of substances by pricking cells using a modified microscope. Exp. Cell Res. 135:341-345.

Yamamura,K-I, Tashiro,F., Yi,S., Wakasugi,S., Araki,S., Maeda,S., Shimada,K. (1993) Transgenic mouse model for human genetic diseases. Molecular Reproduction and Development 36:248-250.

Yanagimachi,R. (1989) Sperm capacitation and gamete interaction. J. Reprod. Fert. Suppl. 38:27-33.

Yanagimachi,R. and Usui,N. (1974) Calcium dependence of the acrosome reaction and activation of guinea pig spermatozoa. Expl. Cell Res. 89:161-174.

Yang,Y.B. and Lu,K.H. (1990) The influence of bovine oocyte type on in vitro fertilization and subsequent development in vitro. Theriogenology 33(1):355.

Yang,N.S., Lu,K.H., Gordon,I. (1990) In vitro fertilization (IVF) and culture (IVC) of bovine oocytes from stored ovaries. Theriogenology 33(1):352.

Younis,A.I., Zuelke,K.A., Harper,K.M., Oliveira,M.A., Brackett,B.G. (1991) In vitro fertilization of goat oocytes. Biol. Reprod. 44(6):1177-82.

Zijlstra,M., Li,E., Sajjadi,F., Subramani,S., Jaenisch,R. (1989) Germ-line transmission of a disrupted B2-microglobulin gene produced by homologous recombination in embryonic stem cells. Nature 342:435-438.