BOVINE EMBRYO MICROINJECTION, CULTURE, MICRO SURGERY, AND DNA ANALYSIS BY THE POLYMERASE CHAIN REACTION TECHNIQUE

by

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(ABSTRACT)

The first experiment was conducted to determine the optimum in vitro culture system for one-cell bovine embryos. Subsequent experiments compared bisection and biopsy for acquisition of cellular material from bovine morulae for DNA amplification by the polymerase chain reaction technique (PCR), and evaluated the use of DNA microinjection, in vitro culture, morula bisection, and PCR for production and selection of transgenic bovine preimplantation embryos. In vivo fertilized one-cell bovine embryos were cultured in TCM-199 (n=46), co-cultured with bovine oviductal epithelial cells (OEC; n=38), or in explanted immature mouse oviducts (n=54). Of the embryos that cleaved once, 52.6, and 30.4 and 0.0% developed to morulae/blastocysts after culture in OEC, TCM-199, and explanted mouse oviducts. Mean cell number for embryos cultured in OEC (24.5) was higher than for embryos cultured in TCM-199 (12.8) or in explanted mouse oviducts (5.9; P<.05).
Bovine morulae were subjected to bisection (n=44; 20 to 30 cells) or biopsy (n=50; 8 to 12 cells) to assess embryo development in vitro and compare the efficiency of PCR amplification of an endogenous 18S rRNA. Mean development scores (1=degenerate, 2=morula, 3=blastocyst) and mean cell number after microsurgery and 48 h of culture did not differ between treatments (P>.05; 2.4 ± 1 and 41.8 ± 2.5 versus 2.3 ± 1 and 48.8 ± 2.9, respectively). Frequency of the 18S rRNA amplification was similar (P>.05) for demi-morulae (78%; 32/41) and biopsies (81%; 39/48).

In the third experiment, in vivo fertilized one- (n=155), two- (n=57) and four-cell (n=62) bovine embryos were collected for pronuclear and nuclear DNA microinjection. Approximately 70% of the embryos were injected with DNA and 30% served as controls. Injection did not affect (P>.05) mean development scores after 144 h of cultured in TCM-199 with OEC. Sixty-five (34%) of the DNA injected embryos developed to morulae and were bisected. Injected DNA was amplified by PCR in 29% (19/65) of the demi-morulae. Frequency of DNA detection was more frequent (P<.01) in morulae injected at the 1-cell stage (50%; 16/32) than at the 2-cell (10%; 1/10) or 4-cell (9%; 2/23) stage. Production and selection of transgenic bovine morulae was most successful when one-cell bovine embryos were microinjected.
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CHAPTER I.
INTRODUCTION

Advances in gene transfer technology, isolation and characterization of genes encoding for medically valuable proteins, and mammalian embryo micromanipulation have generated the potential to produce transgenic mammals. Numerous investigators have selected the mammary gland for expression of complex human proteins (Wright et al., 1991; Denman et al., 1991; Clark et al., 1989; Gordon et al., 1987; Hennighausen et al., 1990). Expression of integrated genes at a concentration of milligrams per milliliter of milk in the mouse (Simons and Land, 1987) has encouraged the use of mammals with higher milk production in gene transfer research.

During the past decade the mouse has served as the model system for studying transgene integration, expression, and regulation. Factors such as low animal cost, high embryo production, easily visualized pronuclei for gene microinjection, and ease of surgical transfer into recipient females, made the mouse an excellent mammalian candidate for early gene transfer research.

Limited access to a large pool of donors, embryos and recipients has hindered the progress of genetic engineering of
Costly surgical embryo recovery and transfer, reduced embryo viability following micromanipulation, and lack of adequate in vitro embryo culture systems also have impeded production of transgenic livestock. Genes encoding for therapeutic proteins have been successfully integrated and expressed in mammary glands of sheep (Clark et al., 1989; Wright et al., 1991), goats (Denman et al., 1991) and pigs (Velande, unpublished), but gene integration and expression rates were low. Thus, production costs are far from being economically feasible.

The objectives of these experiments were to 1) identify the best in vitro culture system for development of one-cell bovine embryos to the blastocyst stage, 2) examine the effectiveness of bovine embryo splitting versus biopsy for amplifying genomic RNA by the polymerase chain reaction (PCR) technique, and 3) utilize in vitro culture, microsurgery, and the PCR technique to facilitate selection of transgenic bovine embryos.
CHAPTER II.
LITERATURE REVIEW

The following is a review of research in the areas of bovine embryo microinjection, transgene integration and expression in bovine embryos, embryo culture systems, and bovine embryo microsurgery.

Pronuclear Visualization for Microinjection

Efficiency of gene transfer via microinjection into bovine zygotes has been hampered by the inability to visualize pronuclei in the pronuclear stage embryos. Zygotes of domestic animals have a relatively opaque cytoplasm compared to those of mice, and efforts have been made to stratify vitelline constituents of the cytoplasm by centrifugation to facilitate visualization of the pronuclei (Wall et al., 1985). Nancarrow et al. (1984) examined the effect of centrifugation on ovine nuclear visibility and embryo viability. Centrifugation at 12,000 g for 1 min failed to improve visualization of nuclei in either one- or two-cell embryos and did not significantly affect embryo viability.

Pronuclei or nuclei in 1- and 2-cell porcine embryos cannot be seen with bright-field or interference contrast optics. Wall
et al. (1985) centrifuged 715 one-cell and 359 two-cell porcine embryos at approximately 15,600 g for 3 min. Pronuclei or nuclei were observed in 85% (914) of the embryos following centrifugation. Centrifuged (n = 117) embryos were transferred into one oviduct and uncentrifuged (n = 116) were transferred into the other oviduct of a synchronized recipient gilt. Recipients were sacrificed and embryos were recovered from uteri 4 d following transfer. Embryo development was evaluated by staining with Hoechst 33342 to assess cell number. Centrifugation did not appear to have any effect on subsequent embryo development.

Nakamura et al. (1986) evaluated the effect of centrifugation speed and duration on pronuclear visualization and subsequent embryo development in murine embryos. Pronuclear stage embryos were centrifuged at 5,000 g, 10,000 g, 15,000 g, and 20,000 g for 3 or 10 min or at 25,000 g for 10 min. The degree of pronuclear visualization was positively correlated with centrifugation speed and duration. Centrifugation at 15,000 g or 20,000 g for 3 or 10 min and 25,000 g for 10 min yielded optimal pronuclear clarity. The proportion of centrifuged embryos that developed to blastocysts in vitro and to term following transfer did not differ from uncentrifuged embryos.
Centrifugation also has proven useful for revealing pronuclei and nuclei in bovine embryos. Loskutoff et al. (1986) reported that centrifugation at 15,000 g for 3 min facilitated pronuclear and nuclear visualization for gene microinjection. Wall and Hawk (1988) found that centrifugation at 15,600 g for 3 min allowed visualization of pronuclei in 73% (n = 106) of the bovine embryos which were subsequently confirmed as pronuclear stage embryos by nuclear staining. They reported that bovine embryo development did not differ between centrifuged and uncentrifuged embryos following 5 or 7 d of culture in ligated rabbit oviducts. Additional studies have reported visualization of pronuclei in 82% (n=84; IVF embryos; Gagne et al., 1990), 68% (n=119; McEvoy and Sreenan, 1990), 62% (n = 1325; Biery et al., 1988), and 60% (n=802; Roschlau et al., 1989) of all pronuclear stage bovine embryos following centrifugation.

**Microinjection of Bovine Embryos**

Early studies involving microinjection of domestic livestock embryos demonstrated the need for a screening tool to assess development of embryos after micromanipulation and prior to transfer. Kraemer et al. (1985) microinjected in vivo fertilized, one-cell bovine embryos. Eight of fifteen embryos (40%) cleaved in Ham’s F-10 + 20% fetal calf serum (FCS) in
vitro, one developing to the morula stage. Twenty-three embryos were transferred to temporary bovine recipients and recovered 48 to 72 h post-transfer. A total of 9 (39%) embryos were recovered, 7 (78%) of which had developed. The remaining 41 embryos were surgically transferred to 11 synchronized recipients in groups of 2 to 5 per transfer. Two recipients carried the pregnancies to term, producing a total of three calves. These results show the need for improved in vitro culture systems and handling procedures that would yield increased pregnancy rates following DNA microinjection.

In a more recent study, McEvoy and Sreenan (1990) surgically transferred 66 centrifuged and DNA-injected bovine embryos to 26 heifers (1-4 embryos/recipient). Fourteen (54%) heifers were confirmed pregnant with 26 (39%) fetuses on d 55. A total of 21 calves were carried to term by 11 heifers.

Roschlau et al. (1989) transferred 598 gene-injected one-cell bovine embryos to 42 recipient heifers. Embryos were recovered 7 or 14 d post-transfer. Recovery rate (39%) and proportion of those developing (39%) did not differ between times of recovery. These results revealed that embryo viability following microinjection was reduced and assessment of embryo viability prior to transfer would reduce the need for a large recipient pool.
Oviducts of sheep and rabbits have been used for short term culture of microinjected bovine embryos. Biery et al. (1988) transferred 819 microinjected bovine embryos into ligated ovine oviducts. Embryos were recovered 6 d following transfer with a recovery rate of 84%. A total of 175 (21% of those injected) developed into morulae or blastocysts. Nonsurgical transfer of these embryos yielded 79 (45% of those transferred) 60 d fetuses. The ovine and rabbit culture systems appear to allow greater bovine embryo recovery rates than the bovine oviduct. The disadvantage of these systems is that embryos must be surgically recovered, regardless of the length of culture.

Rabbit oviducts have served as the site of incubation for comparison of the effects of centrifugation and microinjection on bovine embryo development. Gagne et al. (1990) transferred 226 centrifuged and 206 centrifuged and microinjected in vitro matured/ in vitro fertilized (IVM/IVF) bovine embryos to rabbit oviducts. Recovery rates on d 5 post-transfer ranged from 45% (n=95) for microinjected embryos to 54% (n=110) for centrifuged, control embryos. Microinjection significantly reduced the proportion of embryos reaching the morula/blastocyst stage (5%) compared to non-injected, centrifuged embryos (25%). The proportion of non-injected, centrifuged embryos developing to the morula/blastocyst stage
was similar to a previous study using the rabbit oviduct for culture of IVM/IVF bovine embryos (Sirard et al., 1985).

In a similar study, Hawk et al. (1989) cultured injected and non-injected, centrifuged, in vivo fertilized, bovine embryos in ligated rabbit oviducts. Embryos were cultured for 7 to 9 d. Length of culture time did not significantly affect embryo recovery rates of 38\%, 63\%, and 68\% on d 7, 8, and 9, respectively. Contrary to results from IVM/IVF embryo culture, microinjection did not significantly reduce the proportion of recovered embryos developing to the morula/blastocyst stage (55\% non-injected, 52\% injected).

An additional study conducted by Hawk et al. (1989) evaluated development of centrifuged and DNA-injected bovine embryos in the bovine uterus following 8 d of incubation in the rabbit oviduct. Embryos were either non-manipulated and transferred directly from donor to recipient, or microinjected and/or centrifuged, cultured in rabbit oviducts, and transferred to bovine recipients. Forty-five percent of the embryos were recovered nonsurgically from the bovine recipients. All embryos recovered from cows on d 15 had embryonic discs and appeared to be viable with mean embryonic lengths of 12.5 + 5.1 (controls), 11.1 + 6.9 (centrifuged), and 10.5 + 4.5 mm (centrifuged and microinjected). Results suggest that
microinjection has no effect on embryo development in rabbit oviducts or cow uteri. This is in contrast to conclusions made by Gagne' et al. (1989). It is difficult to predict if the discrepancy is solely due to the differences between embryo source (in vivo fertilized versus IVM/IVF). Duration of culture and microinjection techniques could also contribute to the results that led to these contrasting conclusions.

Krimpenfort et al. (1991) cultured microinjected IVM/IVF bovine embryos with bovine oviductal epithelial cells (OEC) in vitro. The authors reported that microinjected embryos tended to develop more slowly than non-injected embryos. Additionally, 19% of the microinjected embryos and 20 to 25% of the non-injected embryos developed to the morula/blastocyst stage. Transfer of 69 single blastocysts and 30 twin blastocysts resulted in 7 and 14 pregnancies, respectively.

Two conclusions can be drawn from these reports. First, microinjection reduced embryo viability. Secondly, the use of IVM/IVF bovine embryos for gene transfer studies may compromise embryo development and thus, increase the embryo population required for such experiments.
Gene Transfer in Cattle

There are a limited number of successful gene transfer reports in bovine embryo research. Current gene integration frequencies in cattle require a tremendous number of embryos and recipients to which few investigators have access.

Church (1987) submitted the first report of successful gene transfer in cattle. Transfer of 1161 microinjected embryos yielded 126 calves (10.8% of those injected) with 7 (0.6%) of the calves revealing integration of the transgene. One calf (0.08%) was reported to express the gene. The nature of the gene was not mentioned.

The relatively long bovine gestation period (282 days) led Biery et al. (1988) to use day 60 fetuses for assessments of gene integration frequency. A linear construct of chloramphenicol acetyltransferase structural gene fused to a Rous sarcoma viral promoter (RSV-\text{CAT}) was microinjected into embryos. Embryos that developed to the morula/blastocyst stage in ligated ovine oviducts (n=175) were nonsurgically transferred to synchronized bovine recipients. In Experiment I, 459 embryos were injected with 2 ng/\mu l of RSV-\text{CAT}. A total of 93 embryos reached the morula/blastocyst stage and 39 (8.2% of those injected) were recovered as day 60 fetuses.
Incorporation of the RSVCAT gene was evident in one fetus and placenta (0.22%). A titration study was conducted in Experiment II with 360 (120/treatment) embryos injected with 1, 2, or 4 ng/μl of RSV CAT. Transfer of these embryos led to 10%, 11%, and 10% pregnancy rates. Incorporation was detected in 0.83%, 1.67%, and 0% of the embryos in the respective treatment groups.

Roschlau et al. (1989) measured integration frequencies of four gene constructs in either day 14 embryos or calves. A total of 802 1-cell pronucleus stage embryos were injected with bovine papilloma virus DNA (bPV; n=156), alcohol dehydrogenase gene of Drosophila melanogaster (pAM; n=130), human growth hormone (hGH; n=62), or bovine growth hormone (bGH; n=250). Embryos were surgically transferred to temporary recipients and were recovered either on day 7 for assessment of embryo development prior to transfer into a permanent recipient (hGH and bGH), or day 14 for analysis of gene integration (bPV and pAM). Fifteen percent of all injected embryos were judged viable upon recovery. Embryos injected with hGH (n=4) and transferred into permanent recipients failed to develop into pregnancies. Fourteen calves and one fetal monster were produced following transfer of 43 embryos injected with bGH. Integration was detected by dot-blot hybridization in one calf and one fetal monster (0.8%
of those injected with bGH), 5 embryos injected with bPV (3.2%), and 9 embryos injected with pAM (6.9%).

Krimpenfort et al. (1991) recently reported the birth of a transgenic calf that had been generated by microinjection of an IVM/IVF embryo. A total of nineteen calves were produced from transfer of 129 microinjected IVM/IVF embryos that developed to the morula/blasto cyst stage in vitro. One calf had evidence of transgene integration in the placenta, while another had the transgene integrated in the placenta, blood and ear tissue.

The low integration rates reported in these studies represent the challenges that biotechnologists face as they attempt to produce genetically engineered livestock. Current lack of information regarding the mechanism of integration has forced investigators to direct their efforts towards early screening for incorporation of transgenes rather than increasing integration frequencies.

Development of the polymerase chain reaction (PCR) technique (Saiki et al., 1985) has provided researchers with a tool to detect specific DNA sequences in minute amounts of cellular material. The PCR allows amplification of discrete regions of DNA which are selected based on specific sequences rather than
the presence of restriction endonuclease cleavage sites or flanking sequences with specific functional characteristics. Amplification is accomplished by repeated cycles of annealing, synthesis, and denaturation of sequence-specific primers that flank the gene of interest, in the presence of a thermostable DNA polymerase (Thermus aquaticus DNA polymerase). The amplified DNA region can then be identified when placed on an agarose gel and subjected to electrophoresis.

This technology has been used to amplify endogenous and exogenous single copy genes in bovine and murine blastocysts (King and Wall, 1988) and murine demi-morulae (Ninomiya et al., 1989). King and Wall (1988) attempted to determine the limits of the PCR sensitivity by using B chain bovine luteinizing hormone (bLHB) oligonucleotides to amplify sequences from bLHB. The bLHB gene is a single copy gene in cows. Bovine genomic DNA samples representative of 5 to 250 diploid cells were subjected to 30 rounds of PCR amplification. Southern blot hybridization of the amplified samples revealed that the bLHB gene could be detected in samples representing as few as 25 cells. The bLHB gene also was detected in all bovine blastocysts (approx. 50 cells) subjected to the PCR technique. A prokaryotic neo transgene in murine embryos fertilized by transgenic males also was detected by the PCR technique (King and Wall, 1988).
Blastocysts were recovered from females that had been mated to either a 15-copy or single copy transgenic male. Positive signals for the neo gene were detected in 55% (n=11) of the single copy embryos and 55% (n=20) of the 15 gene copy embryos. These frequencies were indicative of Mendelian inheritance. Dot-blot analysis of tail samples of progeny from the founder mice had previously confirmed Mendelian inheritance.

Ninomiya et al. (1989) evaluated the PCR technique's ability to select transgenic embryos by analysis of demi-morulae. Morulae were collected from female mice which had been mated to transgenic males. Morulae were bisected and one demi-embryo was subjected to PCR while the other was transferred. Five of the 41 implanted embryos judged as positive by the PCR did not contain the transgene. Additionally, one of the 28 PCR-negative implanted embryos was transgenic. The PCR technique also was used to detect transgenes in microinjected preimplantation embryos prior to transfer. Following microinjection, embryos were cultured in vitro through the morula stage. The injected DNA was detected in 30 of the 84 morulae. Seven implanted embryos developed from PCR-negative demi-morulae and all were negative for the transgene. One of two implanted embryos from PCR-positive morulae was
transgenic. The authors suggested that the false positive was due to the presence of free-floating injected DNA.

Embryo Culture

Despite considerable progress, successful in vitro culture of preimplantation mammalian embryos from the zygote to blastocyst is limited to a few species. Development of embryos that do advance from the one-cell to blastocyst stage in vitro is typically retarded compared to development in vivo (Bowman and McLaren, 1970). Embryos recovered from domestic livestock readily develop from the morula to blastocyst stage in various media (Wright and Bondioli, 1981), but earlier stage embryos experience a developmental block in culture media. Bovine embryos experience "block stage" or "critical stage" at the 8- to 16-cell stage (Thibault, 1966), porcine embryos at the four-cell stage (Davis and Day, 1978), hamster embryos at the two- and four-cell stages (Whittingham and Bavister, 1974), and outbred stains of mouse embryos at the two-cell stage (Cross and Brinster, 1973; Goddard and Pratt, 1983). The goal of current research in mammalian embryo culture is to better define the requirements for development of early embryos of domestic animals. Biologically active culture systems such as co-culture with other cell types, explanted oviducts, inter- and intra-species oviductal
transfer and a limited number of defined media have provided scientists with environments that support development of mammalian embryos from the one-cell stage, through the block stage, to blastocyst.

Cellular Aspects of the Block Stage
The block stage is a species specific and strain specific event. The developmental block appears to occur concurrently with the switch from maternal to embryonic genome control of development. This occurs at the two-cell stage in the mouse (Braude et al., 1979), between the four- and eight-cell stages in human embryos (Braude et al., 1988), and between the 8- to 16-cell stage in ovine and bovine embryos (Crosby et al., 1988; Frei et al., 1989). During this transition the embryo appears to be very sensitive to in vitro environments.

The mouse has served as a unique model for study of the block stage because in vitro development from the one-cell to blastocyst stage is restricted to certain inbred strains and F1 hybrids (Whitten and Biggers, 1968; Goddard and Pratt, 1983). In most random bred strains the one-cell embryo becomes blocked at the two-cell stage and will only continue development if transferred to the oviduct (Whittingham and Biggers, 1967).
In vitro culture of embryos obtained from reciprocal crosses between randomly bred and F1 eggs and sperm revealed that the genotype of the egg determined whether the embryo experiences the 2-cell block or continues to develop to the blastocyst stage (Goddard and Pratt, 1983). Activation of the embryonic genome was assessed by detection of synthesis of alpha-amanitin-sensitive polypeptides and it was concluded that the embryonic genome of "blocked two-cell" embryos was activated prior to the halt in development. Examination of numbers of cell cycles and nuclear divisions, the extent and method of methionine transport, and qualitative and quantitative polypeptide synthesis suggest that the blocked two-cell embryo develops to the late two-cell stage. The authors suggested that the two-cell block was due to a culture-induced cytoplasmic defect which prevented further development.

Muggleton-Harris et al. (1982) demonstrated that the two-cell block can be overcome by injection of cytoplasm obtained from two-cell F1 hybrid embryos into one blastomere of a randomly bred two-cell embryo. Additionally, transfer of F1 hybrid cytoplasm from one-cell embryos to randomly bred one-cell embryos did not promote development of randomly bred embryos beyond the two-cell stage in vitro. Development of both the injected and non-injected blastomeres in the two-cell embryos led the authors to suggest that the cytoplasmic bridge was
probably still present, thus allowing material to pass between the two blastomeres. Pratt and Muggleton-Harris (1988) later reported that the cytoplasmic factors that overcome the block stage peak in activity during transition between G2 and M phase. Injection of factors present in G2-phase cytoplasm early (0-1 h post-cleavage, G1) or late (6-14 h post-cleavage, G2) in the cell cycle will promote development through the second cell cycle. It is doubtful that this factor is an intermediary factor or macromolecule due to the fact that a small volume (approx. 8 pl) of cytoplasm can overcome the developmental block. The authors suggested that there is some type of amplification of the foreign cytoplasmic factors following transfer.

Limited information regarding the nature of the developmental block has restricted identification of factors that are required by developing embryos and are present in vivo but appear to be lacking in current in vitro culture media. It should be noted that in most species, except in rodents, the block stage occurs at the stage of development when the embryo would move from the oviduct to uterus in vivo. Continued analysis of environments provided by oviducts in vivo, explanted oviducts maintained in organ culture, and various co-culture systems should give researchers greater insight into the environmental requirements for development of early
mammalian embryos. Review of embryo culture media that support development of embryos that do not exhibit the block stage in vitro, as well as media that fail to promote development of blocking embryos beyond the block stage, is necessary for understanding early embryonic development requirements. This review will address the use of defined and semi-defined media for bovine embryo culture as well as the use of oviducts and co-culture systems for general embryo culture.

**Bovine Embryo Culture in Semi-Defined or Defined Media**

Successful in vitro culture of bovine embryos from the one-cell to blastocyst stage in semi-defined or defined media has been limited. An early study by Onuma and Foote (1969) evaluated bovine 1- to 16-cell embryo development in rabbit or bovine serum, bovine follicular fluid, Krebs-Ringer-bicarbonate solution supplemented with 10% serum, or Ham’s F-10. None of these culture media supported development beyond the 16-cell stage. In a later study, Seidel et al. (1971) compared one- to eight-cell bovine embryo development in Ham’s F-10 and 1.5% BSA to Tissue Culture Medium 199 (TCM-199) and 1.5% bovine serum albumin (BSA). Embryos failed to cleave beyond the 12-cell stage.
The first report of bovine embryo development from the two- to eight-cell stage to the expanded blastocyst stage was submitted by Wright et al. (1976a). Embryos were cultured in Minimal Essential Medium with 10% or 50% heat-treated fetal calf serum (HTFCS), TCM 199 and 10% or 50% HTFCS, Ham’s F-10 and 10% or 50% HTFCS, Brinster’s Mouse Ova Culture Medium (BMOC-3), Synthetic Oviduct Fluid (SOF), or Whitten’s Medium and 1, 2, or 4 mg/ml BSA. The effect of two gas atmospheres, 90% \( N_2 \), 5% \( O_2 \), 5% \( CO_2 \) or 5% \( CO_2 \) in air, also was evaluated. Ham’s F-10 supplemented with 10% or 50% HTFCS under an atmosphere of 90% \( N_2 \), 5% \( O_2 \), 5% \( CO_2 \) promoted development of 7 of 20 and 2 of 23 eight-cell embryos to expanded blastocyst. Embryos failed to develop beyond the early blastocyst stage in any of the other treatments.

Wright et al. (1976b) also compared BSA and HTFCS as supplements to Ham’s F-10 for one- and eight-cell bovine embryo development in vitro. Supplementation with 10% HTFCS promoted development of 21% (\( n = 14 \)) of one- and two-cell embryos and 55% (\( n = 20 \)) of eight-cell embryos to the expanded blastocyst stage. Ham’s F-10 supplemented with 1.5% BSA failed to support development past the early blastocyst stage.
Completely defined media have been developed for culture of one-cell mouse (Chatot et al., 1989) and hamster (Schini and Bavister, 1988) embryos through their two-cell block to the blastocyst stage. Both media were evaluated for their ability to promote development of one-cell bovine embryos through the 8-cell block. Ellington et al. (1989) reported that CZB (mouse embryo culture medium) failed to promote one-cell bovine embryo development beyond the 16-cell stage (0/23). Limited development from the one-cell to morula/blastocyst stage was achieved in hamster embryo culture medium (HECM1) supplemented with 6 mg/ml BSA (3.9%; Rose, 1990).

Addition of glutamine appears to be the most significant modification of the CZB medium (Chatot et al., 1990). The failure of CZB medium to support development of bovine embryos through the eight-cell block led Moore and Bondioli (1991) to evaluate the effect of amino acid supplementation on IVM/IVF bovine embryo development in vitro. Addition of glycine (G), alanine, or glycine + alanine to BMOC/BSA supported development of 45% (134/297), 33% (98/297) and 42% (126/297) of the one-cell IVM/IVF embryos to the morula/blastocyst stage. These results indicate that glycine and alanine, when used independently, directly enhance embryo development in vitro.
The Role of Growth Factors in Embryo Development In Vitro

Efforts to develop a one-cell bovine embryo culture medium have recently focused on the addition of factors that enhance cell proliferation and initiate gene activation. Extracellular matrix factors affect cellular proliferation by binding to specific transmembrane receptors that affect cell morphology, cytoplasm structure, and gene expression. Larson et al. (1989) hypothesized that addition of soluble extracellular matrix factors to bovine embryo culture medium would initiate events necessary for development through the eight-cell block. In vitro fertilized (IVF) embryos were cultured in glucose- and cholesterol-free B2 medium supplemented with fibronectin (50 ug/ml), heparin (10 ug/ml), or both. Embryos cultured in un-supplemented medium did not develop beyond the eight-cell stage (n = 12). Fibronectin, heparin, and heparin + fibronectin supported development of 33% (n = 13), 14% (n = 15), and 36% (n = 25) to the 16-cell stage or beyond.

Supplementation of HECM1/BSA with heparin (10 ug/ml) or estradiol-17β (E2; 1 ug/ml) increased the percentage of one-cell IVF embryos developing to the morula/blastocyst stage (7.8% and 20.9%, respectively) compared to HECM1/BSA alone (3.9%; Rose, 1990). Supplementing HECM1 with both heparin and E2 revealed an additive effect with 29.2% of the embryos
advancing to the morula/blastocyst stage. Embryo development in oviductal cell conditioned TCM 199 + 10% BCS was not different from culture in HECM1 supplemented with heparin and E2 (32.6%). These results suggest that the presence of extracellular matrix factors in completely defined medium allow bovine embryo development through the 8-cell block.

A report of growth factor expression in preimplantation mouse embryos (Rappolee et al., 1988) has stimulated numerous investigations involving addition of growth factors to embryo culture media. Paria and Dey (1990) demonstrated individual and synergistic effects of various growth factors on in vitro preimplantation mouse embryo development. Addition of epidermal growth factor (EGF; 10 ng/ml), or transforming growth factor α (TGFα; 10 ng/ml) or β (TGFβ; 2 ng/ml) to culture medium promoted development of 86% (18/21), 89% (33/37) and 89% (32/36) individually cultured, one-cell murine embryos to blastocyst compared to 49% (89/191) cultured in unsupplemented medium when embryos were cultured in 25 μl microdrops. Culturing the embryos in 50 μl microdrops decreased the proportion of embryos developing to blastocyst in control medium to 28% (24/87). The authors hypothesized that a two-fold increase in the medium volume:embryo ratio diluted the autocrine-acting growth factors released by the embryo. Addition of EGF, or EGF + TGFβ significantly enhanced
the proportion of embryos developing to the blastocyst stage (51% and 87%; P<.05), but the effect of EGF supplementation was impaired by the additional medium.

To further explore the hypothesis that growth factors produced by preimplantation embryos act in an autocrine fashion, Paria and Dey (1990) cultured murine embryos individually or in groups with and without EGF supplementation. A greater proportion of the embryos cultured in groups of 10 (83%; 33/40) or 5 (82%; 37/45) in 25 μl of medium developed to the blastocyst stage than embryos cultured singly (49%; 89/181). Addition of EGF (10 ng/ml) increased the proportion of blastocysts hatching in vitro for singly cultured embryos (65% vs. 11% control) and embryos cultured in a group of 5-14 (64% vs. 52% control). The authors concluded that growth factors of embryonic and/or reproductive tract origin play a role in embryo development.

Transforming growth factor β promotes synthesis of fibronectin that had been shown to enhance embryo development (Larson et al. 1989). Basic fibroblast growth factor (bFGF) is a mitogen that increases protein synthesis, specifically, that of proto-oncogene products involved in control of the cell cycle. Larson et al. (1990) tested the effects of TGFβ (1 ng/ml) and bFGF (50 pg/ml) supplementation on development of 1-cell
bovine embryos in cholesterol-free B2 medium adjusted to 3 mM glucose. No oocytes matured beyond the 16-cell stage in unsupplemented medium (0/154), whereas the proportion of embryos cleaving beyond the 16-cell stage was increased by TGFβ (1.1%; n = 89), bFGF (15.7%; n = 72), and TGFβ + bFGF (38.8%; n = 314).

Gondolfi et al. (1991) incubated bovine oviducts and collected secreted proteins for a 24 h period. In an attempt to characterize a mitogenic factor secreted by the oviduct the proteins were exposed to antibodies for EGF, acidic fibroblast growth factor, bFGF, and platelet derived growth factor (PDGF). A 70,000 dalton protein was recognized by the PDGF antibody and a 50% reduction of mitotic activity was observed after addition of the antibody to culture medium. The authors concluded the PDGF present in the bovine oviduct fluid may be associated with a carrier protein and another factor may be responsible for the residual mitogenic activity.

Larson et al. (1991) hypothesized that addition of PDGF would trigger embryonic gene expression, thus promoting completion of the fourth cell cycle and supporting development through the bovine embryo eight-cell block stage. Two-cell bovine embryos were cultured in glucose- and cholesterol-free B2 medium supplemented with PDGF alone or in combination with
TGFβ and bFGF. Equal proportions of two-cell embryos developed through the fourth cell cycle with PDGF (39%; 40/94) and PDGF + TGFβ + bFGF (26%; 48/117). However, embryos cultured with PDGF alone completed the fourth cell cycle faster (26 h; \( n=12 \)) than those cultured in TGFβ + bFGF (44 h; \( n=13 \)). Embryos completing the fourth cell cycle in medium supplemented with PDGF were placed in culture medium supplemented with TGFα or bFGF. Addition of TGFα to the culture medium increased the proportion of 16-cell embryos developing to blastocysts from 8% (5/58) to 40% (28/70).

**Inter-specific Embryo Culture in Oviducts**

Early work by Tarkowski (1962) demonstrated that the mouse oviduct could support development of two-, four-, and eight-cell rat embryos to the blastocyst stage while two- and eight-cell mouse embryos developed to blastocysts in the rat oviduct. These results demonstrated that the factors required by "blocking embryos" to overcome the block stage in vitro are provided by the oviduct and are not species specific. Additionally, transport to the uterus was not necessary for development of the embryo to the blastocyst stage.

Lawson et al. (1972a) reported successful development of two-, four-, eight-, and sixteen-cell ovine embryos to the blastocyst stage when transferred to the ligated oviducts of
pseudopregnant or estrous rabbits. After recovery from the rabbit oviduct embryo viability was confirmed by retransfer of embryos to recipient ewes. The highest survival rate (69%) was achieved with embryos that had been transferred to the rabbit at the two- to four-cell stage. Lawson et al. (1972b) repeated this study using bovine embryos and evaluated the embryos’ viability by retransfer to recipient heifers. One- to eight-cell bovine embryos were transferred to pseudopregnant or estrous rabbits. Eighty-three percent (34/41) of the embryos recovered appeared normal in development. Transfer of 15 embryos yielded 11 calves. Both of these studies demonstrate that development of the embryos was not affected by cleavage stage at the time of transfer or by the endocrine status of the rabbit.

Advances in bovine in vitro fertilization (IVF) work have increased the demand for culture systems that support development of early bovine embryos to the blastocyst stage at which time the embryos can be nonsurgically transferred to recipients. Sirard et al. (1985) transferred one- to eight-cell bovine embryos that had been fertilized in vitro to the ligated oviduct of pseudopregnant rabbits. Forty percent (27/67) of the embryos recovered from the rabbit oviduct had development to the ≥ 16-cell stage (56% vs 23%). However, the longer culture time reduced embryo recovery rate from 77% to
50%. Nevertheless, these results suggest that the rabbit oviduct can be used to culture bovine embryos to an embryonic stage sufficient for nonsurgical transfer to a synchronized recipient.

Recent investigations have shown that the environment of the immature mouse oviduct can support mouse embryo development through the blastocyst stage. Papaioannou and Ebert (1986) reported that the development of one-cell mouse embryos transferred to oviducts of immature mice or pseudopregnant mature mice did not differ. The advantage of using the immature rather than pseudopregnant mouse oviducts is that synchrony of the embryo with the temporary host is not required.

Ebert and Papaioannou (1989) later tested the immature mouse oviducts's ability to support development of early stage rabbit and porcine embryos. The immature oviduct was able to support development of one-cell rabbit embryos to the compact morula stage, however, development was not superior to development in vitro. One-cell porcine embryos failed to develop beyond the block stage (four-cell) in the mouse oviduct. Porcine embryos transferred to the mouse oviduct at the early morula stage developed to the blastocyst stage after 2 d in the oviduct and had a significantly higher cell number
that embryos cultured in vitro. The value of the immature mouse oviduct as a culture system for porcine morulae was not completely assessed as viability of embryos cultured in the mouse oviduct was only measured morphologically.

Inter- and intra-species embryo transfer is a useful tool for culturing early stage embryos that display a developmental block in vitro. However, some of the disadvantages of culturing embryos in "foster" oviducts compared to in vitro culture include a) the need for additional animals, b) increased technical skill for transfer, c) recovery rates of less that 100% of the embryos transferred and d) embryo development that cannot be assessed during culture.

**Embryo Culture in Explanted Oviducts**

The use of explanted oviducts for in vitro culture of embryos past the developmental block was first practiced by Biggers et al. (1962). Oviducts containing fertilized one-cell embryos were placed on a raft that was in contact with culture medium BGJb that contained glucose and lactate. The raft consisted of a disc of tea-bag paper supported by a stainless steel grid. This system allowed the oviduct to utilize nutrients provided by the culture medium while having access to an adequate supply of oxygen. Forty-three oviducts (80%) contained developing embryos and 74% (159/215) of the embryos
had developed to the morula stage after 72 h of culture. Transfer of blastocysts recovered from the organ culture to synchronized recipients produced live pups which suggested that the culture system did not adversely affect the embryos.

Whittingham and Biggers (1967) demonstrated that mouse embryos that develop in vitro from the one-cell stage to the two-cell stage are capable of further development only if they are transferred to explanted oviducts maintained in organ culture. The oviduct was explanted on Ham’s F-10 and two-cell embryos were transferred to the oviduct. Blastocysts were recovered from the oviducts after 72 h of culture.

Whittingham (1968a) reported that the explanted mouse oviduct’s ability to support mouse embryo development was restricted to the ampulla. Zygotes degenerated when transferred to the explanted isthmus or anterior portion of the uterine horn. Removal of the cumulus from the embryos prior to transfer to the explanted oviduct did not adversely affect embryo development within the oviduct when cultured in Ham’s F-10. Additionally, ampullae recovered from mice at metestrus I supported greatest (63% blastocyst) embryo development, while only 26% of the embryos cultured in ampullae recovered at diestrus II developed to the blastocyst stage (Whittingham, 1968b). Oviducts recovered from
ovariectomized mice treated with E2 promoted greater embryo
development than oviducts removed from untreated
ovariectomized mice, progesterone/estrogen pre-treated or
progesterone pre-treated ovariectomized mice. These
observations suggested that the presence of estrogens enhances
the oviduct’s ability to support mouse embryo development.

Similar to the oviduct maintained in vivo, the ability of the
explanted mouse oviduct to support development of embryos
through the block stage is apparently not species specific.
Minami et al. (1988) successfully cultured hamster embryos
from the one-cell to blastocyst stage in flushed oviducts
obtained from immature mice. Mice were treated with either 5
IU hCG or 5 IU PMSG and oviducts were recovered at 0, 14 to
16, 30 to 32, and 70 to 72 h post-hCG or 30 to 32 h post-PMSG.
Ampullae isolated at 14 to 16, 30 to 32 h post-hCG and 30 to
32 h post-PMSG best supported embryo development with 12, 21,
and 13% developing to morulae, respectively, compared to
ampullae isolated 0 or 70 to 72 h post-hCG supporting 0 and 3%
to blastocyst.

Krisher et al. (1989a) cultured one-cell porcine embryos in
explanted oviducts recovered from superovulated immature mice.
Oviducts were explanted on Krebs Ringer bicarbonate medium
modified by deleting lactate and pyruvate and increasing NaCl.
After 6 d of culture in the mouse oviduct 78.1% of the one-cell porcine embryos reached the morula or blastocyst stage compared to 35.7% reaching the morula or blastocyst stage when cultured in medium alone. Cell number for embryos that reached the morula or blastocyst stage did not differ between treatments.

The effect of oviductal condition on the development of one-cell porcine embryos in the mouse oviducts also has been assessed (Krisher et al., 1989b). Oviducts were recovered from superovulated immature mice that were mated to fertile or infertile males and rat oviducts were obtained from superovulated rats that were mated to fertile males. Upon recovery, half of the mouse oviducts recovered from fertile matings were flushed to remove the cumulus-oocyte complex. Porcine embryos were transferred to the three types of mouse oviducts as well as explanted rat oviducts and culture medium alone. Oviducts containing embryos or unfertilized ova supported 77 and 74.5% of the one-cell porcine embryos to the blastocyst stage. These results demonstrated that the presence of developing mouse embryos did not affect porcine embryo development in explanted oviducts. More embryos reached blastocyst stage when cultured in flushed mouse oviducts (58.7%) compared to rat oviducts (17.8%) or culture medium (17.3%). Flushing the explanted mouse oviduct
significantly reduced the percentage of embryos developing to the blastocyst stage compared to unflushed oviducts. Removal of the cumulus complex, factors from the male, or damage to the oviductal epithelium due to flushing may have altered the oviduct’s ability to support porcine embryo development. Gardner and Leese (1990) observed lower pyruvate and higher glucose levels in mouse oviducts void of cumulus cells compared to oviducts containing cumulus. Flushing the oviduct did not appear to adversely affect the organ’s ability to support mouse or hamster embryo development (Whittingham and Biggers, 1967; Minami et al., 1988). However, the medium used in those studies contained pyruvate, lactate, and glucose, while medium used for explanted oviducts containing porcine embryos (Krisher et al., 1989b) did not contain pyruvate or lactate. The cumulus may provide a low concentration of pyruvate that may be necessary for successful culture of porcine embryos in explanted oviducts.

Co-culture of Embryos

Co-culture of embryos with various cell types has been shown to support greater embryo development than culture medium alone. Two co-culture systems that seem to support substantial embryo development are co-culture with trophoblastic and oviductal cells. Our discussion will be
limited to these two co-culture systems and their use for selected species of embryos.

Camous et al. (1984) were the first to use trophoblastic vesicles for co-culture with embryos. Trophoblastic vesicles were obtained from trophoblasts that were nonsurgically collected from superovulated heifers. Trophoblasts were cut into several pieces (up to 25 for 40 mm trophoblasts) and those that had transformed into trophoblastic spheres or vesicles after 24 h of culture were used for co-culture. Forty-six percent of the one- to eight-cell bovine embryos co-cultured with trophoblastic vesicles in Medium B2 for 3 to 4 d reached the morula stage compared to 18% cultured in medium alone. These results suggest that the trophoblastic vesicles may provide metabolic compounds that are required for embryonic development or they may produce a signal that either induces or regulates cleavage.

The role of trophoblastic vesicles and serum in the co-culture system was later assessed by Heyman et al. (1987). One-cell bovine embryos were assigned to one of the four following culture conditions: 1) B2 alone, 2) B2 + 15% fetal calf serum (FCS), 3) B2 + trophoblastic vesicles (TV), 4) B2 + TV + 15% FCS. The combination of B2 + TV + 15% FCS supported significantly more embryo development (69%) past the eight-
cell stage than B2 + TV (12.7%), B2 + 15% FCS (23.8%), or B2 alone (4.5%). Co-culture with ovine TV in B2 + 15% FCS or B2 + TV significantly increased the number of one-cell ovine embryos developing to the morula stage (75% and 68%, respectively) compared to culture in B2 + 15% FCS or in B2 alone (35% and 29%, respectively). It appears that serum enhances the effect of trophoblastic vesicles on embryo development either by stimulating the trophoblastic vesicle, embryo, or both.

Embryos cultured in spent medium revealed that the positive effect of trophoblastic vesicles on early embryo development was due to factors released by trophoblastic vesicles into the medium. One- and two-cell bovine embryos were cultured in conditioned medium that had previously supported development of trophoblastic vesicles. The medium was diluted by adding fresh medium at a 1:1 ratio. Thirty-nine percent of the embryos cultured in the conditioned medium developed to the morula stage. This percentage was close to that previously achieved in co-culture (41.8%). The ability of conditioned medium to support embryo development similar to co-culture demonstrates that cell-embryo contact is not necessary for enhanced embryo development. In an attempt to characterize the factors released by trophoblastic vesicles, a low molecular weight (MW 180-2500) and a high molecular weight (MW
> 10,000) fraction were separated from conditioned medium. Supplementation of B2 + FCS with the low molecular weight fraction supported development of 24% of one- to two-cell embryos to the morula stage while none of the embryos cultured in the high molecular weight fraction developed beyond the eight-cell stage. The authors did not state the ratio of each molecular weight fraction to fresh culture medium that was used for embryo culture. While it appears that the embryotrophic factors released by the trophoblastic vesicles were contained in the low molecular weight fraction, fewer embryos reached the morula stage in the low molecular weight fraction-supplemented medium than when cultured in conditioned medium or in co-culture. This may be due to a greater dilution of the active compounds in the fraction-supplemented medium than in the conditioned medium (1:1) or synergistic action of the combination.

It may be expected that cells from the reproductive tract would support embryo development in vitro as well as in vivo. Rexroad and Powell (1988a) co-cultured 1- and 2-cell ovine embryos with oviductal cell, uterine cell, or kidney cell monolayers. Ovine embryos co-cultured for 3 d on oviductal cells had greater development following transfer to recipients that embryos co-cultured on uterine or kidney cell monolayers. Oviductal cells recovered from ewes either 2 d postestrus or
luteal-phase ewes appeared to support the same degree of embryo development in co-culture.

Gandolfi and Moor (1987) compared ovine embryo development in co-culture with oviductal epithelial cells and fibroblasts. In vitro development of the embryos did not differ between treatments during the first 3 d of culture. However, co-culture with oviductal epithelial cells supported significantly greater embryo development beyond the 8- to 16-cell block stage (45.8%; n=72) compared to co-culture with fibroblasts (4.5%; n=67). Additionally, embryos co-cultured with oviductal cells for 3 d yielded more fetuses after transfer to recipients (80%; n=44) than those co-cultured with fibroblasts (33%; n=68). These results support the hypothesis that the factors required by the embryo are found only in cells specific to the reproductive tract.

Co-culture of one-cell ovine embryos on oviductal cell monolayers has been shown to support greater embryo development than co-culture with trophoblastic vesicles (Rexroad and Powell, 1988b). Co-culture on an oviductal epithelial cell monolayer with Ham’s F-10 + 10% FCS for 72 h promoted a significantly higher cleavage index (2.0) compared to co-culture with trophoblastic vesicles (1.53) or culture in medium alone (1.6). The authors suggested that failure to
obtain results similar to previous reports (Camous et al., 1984; Heyman et al., 1987) may be due to differences such as species or medium.

Aoyagi et al. (1989) compared the effects of co-culture with oviductal epithelial cells, cumulus cells, trophoblastic vesicles, amniotic sac cells, or culture in ligated rabbit oviducts on development of eight-cell bovine embryos derived from IVF. Co-culture with trophoblastic vesicles in TCM-199 + 10% FCS supported the greatest proportion of embryos developing to the blastocyst stage (50.7%; n=69). However, this percentage was not significantly different from those obtained in co-culture with oviductal epithelial cells (39%; n=59), cumulus cells (19.5%; n=82), amniotic sac cells (48.6%; n=74), or culture in the ligated rabbit oviduct (29.3%; n=58). As mentioned previously, the discrepancies between results of this study and other experiments may be due to differences in media and species.

Recent research has focused on the use of various media and supplements in co-culture systems using oviductal epithelial cells. Fukui (1989) evaluated the effect of sera and steroid hormone supplementation on bovine embryo development in co-culture with bovine oviductal epithelial cells. Addition of estradiol (1 or 10 ug) and/or progesterone (1 or 10 ug) to
TCM-199 supplemented with either 10% FCS or 10% estrus cow serum (ECS) did not enhance bovine embryo development compared to media and sera alone. Embryo development to the blastocyst stage did not differ between 10% FCS and 10% ECS supplemented media.

The use of various media in an early bovine embryo and oviductal cell co-culture system was evaluated by Ellington et al. (1989). Medium CZB (Chatot et al., 1989), a simple, defined, glucose- and serum-free medium promoted development of 85% (n=13) of one- or two-cell bovine embryos co-cultured with bovine oviductal epithelial cells. This culture system supported greater embryo development beyond the 16-cell stage than media alone (CZB = 0/23; Ham's F-10 + 10% FCS = 1/8; Connaught Medical Research Laboratory medium (CMRL-1066) + 10% FCS = 0/6) but did not differ from co-culture with Ham's F-10 + 10% FCS (79%, n=14) or CMRL-1066 + 10% FCS (73%, n=15). Addition of insulin, transferrin, and epidermal growth factor (EGF) to the CMRL-1066 + 10% FCS and CZB after 36 h in co-culture supported development of 68% (n=44) and 88% (n=40) of the embryos to the morula stage, respectively. These results demonstrated that co-culture in a simple, defined, and serum-free medium (CZB) supported significantly greater embryo development compared to a complex, semi-defined medium.
Development of bovine one- to two-cell embryos in co-culture with oviductal epithelial cells (OEC) in CZB was later compared to development in ligated rabbit oviducts (RO; Ellington et al., 1990). Embryo development was assessed 5 d after the initiation of culture. Mean cell count (RO: 52; OEC: 52), percentage of mitotic cells per embryo (RO: .5%; OEC: .5%), and percentage of embryos reaching the morula or blastocyst stage by d 5 (RO: 83%; OEC: 84%) did not differ between treatments.

In an additional experiment, Ellington and Farrell (1990) compared one-cell bovine embryo development in co-culture with oviductal epithelial cells in CZB (CC) with in vivo development (IV). In vivo developed embryos were recovered on d 6. Percent embryos greater than 16 cells (CC: 83%; IV: 81%), mean cell number + SE (CC: 61; IV: 76), mean percent poor quality nuclei + SE (CC: 15; IV: 9), and mean percent cells in mitosis + SE (CC: 1; IV: 2) were not different between treatments.

Eyestone and First (1989) reported that co-culture of in vitro matured/in vitro fertilized bovine embryos on oviductal epithelial cell monolayers supported development comparable to culture of in vitro matured/in vitro fertilized embryos in rabbit oviducts. Conditioned medium supported development to
the same degree as co-culture (23% and 25%, respectively). Additionally, results from co-culture of embryos on monolayers or in oviductal cell suspensions indicated that the formation of monolayers is not necessary for co-culture systems.

It must be noted that co-culture systems’ ability to support embryonic development is not limited to bovine and ovine embryos. Co-culture of rabbit embryos with rabbit oviductal epithelial cells (Carney and Foote, 1988) has been shown to significantly increase the percentage of 2-cell embryos developing to the blastocyst stage. Porcine embryo development was enhanced by co-culture with either porcine oviductal epithelial cells or porcine oviductal epithelial cells and porcine fetal fibroblast monolayers whereas co-culture with porcine fetal fibroblasts was not different from culture medium alone (White et al., 1989). Equine (Weber et al., 1990; Ball et al., 1990), caprine (Prichard et al., 1990), and rhesus monkey (Goodeaux et al., 1990) embryos also have been reported to benefit from co-culture with genital tract epithelial cells.

In vitro cleavage rates of mouse embryos are typically retarded compared to embryos in vivo. Sakkas et al. (1989) reported that co-culture of two-cell mouse embryos with mouse
oviductal epithelial cells allowed embryos to maintain cleavage rates comparable to those in vivo.

While these reports appear to support the hypothesis that embryonic development can be enhanced only by cells recovered from the genital tract, there have been reports of successful co-culture with cells from other sources. Menezo et al. (1990) reported improved development of human one- to eight-cell embryos in co-culture with Vero cells (Green monkey kidney epithelial cells; 61% blastocysts) compared to culture in medium alone (3% blastocysts). This was the first report of drastically improved embryo development in co-culture with epithelial cells from a source other than the genital tract. These results suggest that the beneficial effect of co-culture may be epithelium dependent.

Blakewood et al. (1988) described a co-culture system that used the amniotic cavity of a 96 h chicken embryo as a host environment for embryos that experience a block stage in vitro. The developmental block of one-cell mouse embryos (Blakewood et al., 1988) and two- to eight-cell caprine embryos (Blakewood et al., 1989) has been overcome by this co-culture system. Additionally, live births have resulted from four-cell caprine embryos cultured for 72 h in chicken embryos and transferred to recipient does (Blakewood et al., 1990).
Co-culture with oviductal epithelial cells was a highly successful method of culturing early stage mammalian embryos. Oviductal cell suspensions and confluent monolayers serve as an attractive alternative to in vivo culture in ligated oviducts of sheep or rabbits as well as in vitro culture in explanted mouse oviducts. Evidence of enhanced human embryo development in co-culture with commercially available epithelial cell lines suggests that the need for oviductal cell sources may be eliminated and embryonic co-culture systems could be easily replicated in any laboratory (Menezo et al., 1990).

**Embryo Development in Oviductal Fluid**

If the environment of the oviduct does promote embryo development and medium conditioned by oviductal cells is able to support embryo development in vitro, then supplementation of culture medium with oviduct secretions should also support development of early stage embryos. Additionally, analysis of oviductal secretions should provide information that could be used to formulate more effective culture media.

In an early study Kille and Hamner (1973) reported that optimal rabbit embryo development was attained when cultured in oviductal fluid recovered from d 8 of pseudopregnancy. The
embryotrophic factors were heat liable at 60° C, but were stable for up to 9 d at 4° C.

More recently, Archibong et al. (1989) examined the effect of early luteal phase oviductal fluid (OVF) or culture medium supplemented with OVF (CM-OVF, 25% OVF v/v in CM) with or without transfer to fresh culture medium on one- and two-cell porcine embryo development. Development to the blastocyst stage was lowest among embryos cultured in OVF and highest among those cultured in CM-OVF. When embryos exposed to OVF and CM-OVF for 24 h (two-cell) or 48 h (one-cell) were transferred to fresh culture medium, development to the blastocyst stage was increased. The authors suggested that porcine OVF contains embryotrophic factor(s). However, the effect of the factor(s) is masked by embryotoxic factors in vitro. Dilution of the medium with OVF may have reduced the concentration of embryotoxic factors in the medium, whereas embryotrophic factors in the OVF increased the percentage of embryos developing to the blastocyst stage.

Current embryo culture media are mostly modifications of somatic cell culture media and are based on the composition of blood sera rather than oviductal fluid. Modifications of some culture media have been shown to either fully or partially
overcome the block stage in vitro. These modifications include: a) elimination of glucose (hamster eight-cell block; Seshagiri and Bavister, 1989), b) addition of amino acids and vitamins (rabbit morula block; Kane, 1987), c) deletion of pyruvate and lactate (porcine four-cell block; Davis and Day, 1978), d) deletion of glucose and addition of glutamine (mouse two-cell block; Chatot et al., 1989) and e) addition of glycine or alanine (bovine, Moore and Bondioli, 1991).

Gardner and Leese (1990) used an ultramicrofluorometric technique to measure the nutrient composition of mouse oviduct fluid. The concentration of pyruvate within the oviduct was significantly reduced in the absence of the cumulus mass. Glucose concentration was higher in oviducts that were void of the cumulus compared to oviducts with cumulus. As mentioned earlier, these differences may explain results reported in studies that used explanted oviducts in organ culture as a system to culture embryos past the block stage.

Following analysis of mouse oviduct fluid components, Gardner and Leese (1990) created a medium referred to as Mouse Tubal Fluid (MTF) that contained .37 mM pyruvate, 3.40 mM glucose and 4.79 mM lactate. There was no difference in the ability of M16 and MTF to support one-cell mouse embryo development to
the blastocyst stage. The main difference between M16 and MTF is the concentration of lactate (23.3 mM in M16).

It is evident that researchers have yet to fully mimic the oviductal environment in vitro. Analysis of oviductal secretions has produced limited advances in embryo culture. While we may understand the chemical environment of the oviduct, the physical environment deserves more attention (i.e. pH and gas tension). It is only then, when the entire physico-chemical environment is understood, that substantial advances in embryo culture will be achieved.

**Embryo Splitting and Biopsy**

Cell samples for the PCR technique can be obtained from microinjected embryos by two methods. Embryos can be split and one demi-embryo could be transferred while the other is subjected to PCR. The other option would be to biopsy the embryo and use the biopsy for analysis by PCR. The following is a brief review of bovine embryo splitting and biopsy.

Takeda et al. (1986) evaluated the effect of splitting on excellent and good quality bovine embryos compared to intact bovine morulae. Embryo viability was assessed by pregnancy rates resulting from nonsurgical transfer of demi-embryos
placed in surrogate zona pellucidae or whole embryos. The pregnancy rates for intact, excellent and good quality morulae were 70% (n=69) and 64% (n=25), respectively. The percent of demi-embryos that developed into pregnancies was 63% (n=88) for excellent quality embryos and 57% (n=28) for good quality embryos.

Placement of demi-embryos in the original or surrogate zona pellucida is a time consuming process. McEvoy and Sreenan (1987) assessed the development of zona-free, bisected, bovine embryos in vitro. A total of 94 good quality morulae and blastocysts were bisected and 184 demi-embryos (196%) were cultured in PBS + 20% FCS. Blastocoele formation was observed in 158 (86%) embryos after 48 h in culture.

Williams and Moore (1988) transferred either demi, zona-free or whole bovine blastocysts into synchronized recipients. Pregnancy rates on d 70 were 38% (n=13) and 45% (n=20) for recipients receiving either twin or single demi-embryos. These rates did not differ from the proportion of whole embryos that developed to d 70 fetuses (45%; n=28). These results confirm that demi-embryos obtained from morulae or blastocysts do not need zonae pellucidae for normal development in vitro or in vivo.
Cell loss during murine, ovine, and bovine embryo bisection was assessed by Skrzyszowska and Smorag (1989). Number of cells present in intact and demi-embryos was determined by staining with acridine orange. Early, mid- and expanded murine blastocysts had an average of 39.4, 54.1, and 71.9 cells. Average cell loss due to bisection was 6.4, 7.6, and 9.9, respectively. The ovine and bovine expanded blastocysts had an average of 115.3 and 138.5 cells, with 13.2% and 12.7% of the cells damaged by bisection.

Heyman and Chesne (1988) evaluated 1) the effect of embryonic stage before splitting and freezing and 2) the effect of biopsy and freezing on embryo survival. In experiment 1, 59 early and 47 hatching bovine blastocysts were split and frozen. Transfer of thawed pairs of demi-embryos developed into pregnancies in 43.7% (7/16) and 10% (2/20) of the recipients receiving early and hatched demi-blastocysts. The development of in vitro cultured demi-embryos did not reflect development in vivo. Blastocoele expansion was observed in 55.8% and 50% of the demi-embryos from the early blastocyst and hatching blastocysts in vitro. In this situation the in vitro development was an unreliable criterion for assessment of embryo survival.
In experiment 2, 72 bovine expanded blastocysts were biopsied, frozen, thawed, and either cultured in vitro or transferred to a synchronized recipient. The proportion of biopsied embryos developing in vitro was slightly lower than intact, frozen-thawed, whole embryos (56% versus 67%). Pregnancy rate for biopsied embryos was 34.5% (n=29) while transfer of control embryos yielded a pregnancy rate of 48.5% (n=33). Embryo viability was reduced by biopsy and subsequent cryopreservation. One cannot conclude that biopsy alone reduced embryo viability because the interactive effect between biopsy and freezing cannot be accounted for.
CHAPTER III.

CULTURE OF ONE-CELL BOVINE EMBRYOS IN EXPLANTED MOUSE OVIDUCT AND BOVINE OVIDUCTAL EPITHELIAL CELLS

ABSTRACT

One-cell bovine embryos fertilized in vivo were cultured in TCM-199 and bovine oviductal epithelial cells, TCM-199, or explanted immature mouse oviducts supported by TCM-199 to compare development to the blastocyst stage. Morphological stage of development and cell number were determined following 144 h of culture. Of the embryos that cleaved at least once, 52.6, 30.4 and 0.0% developed to the morula/blastocyst stage after culture in oviductal epithelial cells, TCM-199 alone, or explanted mouse oviducts, respectively. Mean total cell number for embryos cultured in oviductal epithelial cells (24.5) was higher than for embryos cultured in TCM-199 (12.8) or in explanted mouse oviducts (5.9; P<0.05). The mean cell number of embryos cultured in TCM-199 or in explanted mouse oviducts did not differ. Explanted immature mouse oviduct supported by TCM-199 did not provide an environment adequate for development of one-cell bovine embryos to the blastocyst stage. Development of one-cell bovine embryos was best supported by co-culture with oviductal epithelial cells in TCM-199 medium.
INTRODUCTION

Failure to fully define the requirements for development of bovine embryos from the one-cell to the blastocyst stage has prevented the development of a defined culture medium for bovine embryos. The lack of adequate in vitro culture systems for bovine embryos has hindered progress in the application of micromanipulation techniques such as nuclear transfer and gene transfer.

Embryos recovered from domestic livestock at the morula stage readily develop to the expanded blastocyst stage in various media (Wright and Bondioli, 1981), but embryos recovered at earlier stages experience a developmental block in vitro. Bovine (Thibault, 1966) and ovine embryos experienced the "block stage" at the 8- to 16-cell stage of development, and porcine embryos have demonstrated a developmental block at the four-cell stage in vitro (Davis and Day, 1978). Culture systems such as in vitro co-culture with other cell types (Camous et al., 1984; Rexroad and Powell, 1988; Aoyagi et al., 1989; Fukui, 1989) were shown to support greater one-cell ovine and bovine embryo development than culture medium alone. McCaffrey et al. (1991) reported that co-culture with oviductal epithelial cells supported development of 81% of one- to four-cell bovine embryos to the morula or blastocyst
stage. Explanted mouse oviducts in organ culture supported development of one-cell mouse, rat, hamster and porcine embryos (Biggers et al., 1962; Whittingham and Biggers, 1967; Minami et al., 1988; Krisher et al., 1989a; Krisher et al., 1989b) through the blastocyst stage. Krisher et al. (1989b) demonstrated that optimal porcine embryo development was achieved in the ampullary region of the mouse oviduct. The presence of the mouse embryos and cumulus within the ampulla provided the best environment for embryo development. The mechanism by which the oviductal epithelial cells and mouse oviduct are able to support development of intra- and inter-specific embryos is not known.

The objective of this study was to determine if the explanted mouse oviduct supports development of one-cell bovine embryos to the blastocyst stage more efficiently than co-culture with bovine oviductal epithelial cells.

**MATERIALS AND METHODS**

**Experimental Design**

One-cell embryos (n=286) from 24 donor cows were randomly assigned to one of the following culture systems: 1) co-culture with oviductal epithelial cells in TCM-199 and 10%
newborn calf serum (NBCS); 2) TCM-199 and 10% NBCS; or 3) explanted mouse oviduct supported by TCM-199 and 10% NBCS.

Superovulation
Twenty-four nonlactating, reproductively normal Holstein cows were superovulated for the collection of one-cell embryos. Superovulation was initiated between d 10 and 14 of the estrous cycle with follicle stimulating hormone (FSH; 35 mg; Shering Corp., Kenilworth, NJ) administered at 12 h intervals in 5-mg doses over 3.5 consecutive d. Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$; Lutalyse, Upjohn, Kalamazoo, MI) was administered at 48 and 60 h (12.5 mg) after the initial FSH injection to induce estrus. Cows received human chorionic gonadotropin (hCG; 1500 IU; Sigma, St. Louis, MO) 48 h after the first PGF$_{2\alpha}$ injection (estrus) to facilitate ovulation. Animals were artificially inseminated at 0, 12 and 24 h after the initial observation of estrus.

Embryo Collection
Ovaries and oviducts were collected at slaughter 36 to 48 h after the initial observation of estrus. Tissue was maintained at 37.5° C prior to embryo collection. The number of corpora hemorrhagica and unovulated follicles and the size of the unovulated follicles were recorded. Oviducts were
flushed with TCM-199 (Gibco, Grand Island, NY) supplemented with 5% (v/v) newborn calf serum (NBCS; Gibco) and 1.3% (v/v) antibiotic/antimycotic (Gibco). The oviducts were flushed until the number of embryos recovered equalled the number of corpora hemorrhagica on the corresponding ovary or the volume of flushing media had reached 100 ml.

The fluid was collected in 100 x 15-mm sterile, plastic petri dishes (25 ml per dish; Falcon, Oxnard, CA) and examined under 40x microscopy. Embryos were held in TCM-199 supplemented with 10% NBCS and 1.3% antibiotic/antimycotic in an atmosphere of 5% CO₂ in air at 37.5°C prior to treatment assignments.

Oviductal Epithelial Cells
Oviductal epithelial cells recovered in the oviduct flushes were pooled prior to washing. The cells were held in a conical centrifuge tube with 10 ml of TCM-199 and 10% NBCS and were washed with six changes of the medium. Following washing, the cells were resuspended in TCM-199 and 10% NBCS and placed in 25-μl microdrop aliquots, which were covered with silicone oil (Aldrich Chemical Co., Milwaukee, WI) in 35 x 10-mm sterile petri dishes (Falcon). The medium was replaced 24 h after the initiation of culture.
Mouse Oviducts

The mouse oviduct organ culture system was adapted from Krisher et al. (1989). Immature female CD1 mice (21 to 28 d old) were superovulated with 5 IU pregnant mare serum gonadotropin (PMSG; Ayerst Laboratories Inc., New York, NY) followed 48 h later by 5 IU hCG and mated with mature CD1 males. Females with vaginal plugs 20 h after hCG administration were euthanized by cervical dislocation and oviducts and ovaries were removed and held in TCM-199 and 10% NBCS. Ovaries were separated from the oviducts with forceps under 40x microscopy. The oviducts were then uncoiled by tearing the mesosalpinx with forceps.

Only oviducts that had ampullae swollen with embryos and cumulus cells were selected for the organ culture. Bovine embryos assigned to the mouse oviduct treatment were transferred via mouth pipette immediately after the oviduct was uncoiled. The hand-drawn pipette was placed in the ostium of the oviduct and three to five embryos were transferred to the ampullary region of each oviduct.

Oviducts were transferred to organ culture chamber immediately following embryo transfer. Organ culture chambers consisted of one 35 x 10-mm petri dish containing a 10 x 10-mm piece of 0.2-μm filter paper (Gelman, Ann Arbor, MI) supported by 2 ml
of TCM-199 and 10% NBCS. The culture chamber was placed in a 100 x 15-mm petri dish that contained 0.5 ml of sterile water to maintain humidity within the chamber.

The organ culture was placed in an atmosphere of 5% CO₂ in air at 37.5°C for 144 h. Embryos were removed from the mouse oviducts at the end of the culture period. Morphological stage of both bovine and murine embryo development was scored visually and recorded. Mouse embryo development was indicative of the viability of the organ culture. At recovery all mouse embryos were hatching or hatched blastocysts.

**In Vitro Culture**

Embryos assigned to either TCM-199 and 10% NBCS or oviductal epithelial cell co-culture were cultured in 25-μl microdrops of their respective treatments. All microdrops were covered with silicone oil to minimize evaporation and buffer gas exchange. In vitro culture systems were placed in an atmosphere of 5% CO₂ in air at 37.5°C for 144 h. Morphological stage of development was scored visually and recorded every 24 h. Stages of development were scored as 1=one-cell, 2=two-cell, 3=four-cell, 4=eight-cell, 5=16-cell, 6=morula, 7=early blastocyst, 8=blastocyst, 9=expanded blastocyst. Following final morphological scoring nuclei of all bovine embryos were
stained using the procedure of Pursel et al. (1985), and the cell numbers were determined.

The final development scores and mean cell number for each treatment were calculated and analyzed by analysis of variance. Treatment and donor cow served as independent variables in the model. The means were compared by Tukey’s procedure.

RESULTS

The 24 embryo donors used in this study averaged $15.3 \pm 2.1$ (mean $\pm$ SEM) corpora hemorrhagica at the time of slaughter with a mean of $11.9 \pm 1.8$ embryos recovered. The embryo recovery rate, as determined by the number of corpora hemorrhagica and embryos recovered, was 78%. Of the 286 embryos recovered, 3% were at the two-cell stage and 49.6% failed to develop past the one-cell stage in vitro. For embryos that cleaved at least once in vitro, 52.6% of those cultured in oviductal epithelial cells developed to the morula or blastocyst stage compared with 30.4 and 0% of the embryos cultured in TCM-199 and in explanted mouse oviducts, respectively (Table 1).
Table 1. Mean final development score, cell number, and percentage developing to the morula or blastocyst stage for one-cell bovine embryos cultured in TCM-199 and oviductal epithelial cells (OEC), in TCM-199 alone, or in explanted immature mouse oviducts for 144 h.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>%</th>
<th>( \bar{x} )</th>
<th>SEM</th>
<th>n</th>
<th>( \bar{x} )</th>
<th>SEM</th>
</tr>
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<tbody>
<tr>
<td>OEC</td>
<td>38</td>
<td>52.6(^a)</td>
<td>5.0(^a)</td>
<td>.3</td>
<td>27</td>
<td>24.5(^a)</td>
<td>3.5</td>
</tr>
<tr>
<td>TCM-199</td>
<td>46</td>
<td>30.4(^b)</td>
<td>4.4(^a)</td>
<td>.3</td>
<td>36</td>
<td>12.8(^b)</td>
<td>1.9</td>
</tr>
<tr>
<td>Explanted mouse oviduct</td>
<td>54</td>
<td>0.0(^c)</td>
<td>3.2(^b)</td>
<td>.1</td>
<td>50</td>
<td>5.9(^b)</td>
<td>.5</td>
</tr>
</tbody>
</table>

\(^a,b,c\) Means with different superscripts within the same column differ at (P<0.05).

\(^1\) Development was scored as: 1=one-cell, 2=two-cell, 3=four-cell, 4=eight-cell, 5=16-cell, 6=morula, 7=early blastocyst, 8=blastocyst, 9=expanded blastocyst.
There were significant donor (P<0.01) and donor-by-treatment interaction (P<0.01) effects on the final development score. Partitioning mean final development scores from donors that had 10 or more embryos assigned across treatments into a "blocked" (≤4; 4 = 8-cell stage) or "non-blocked" (>4) classification resulted in mean final development scores of 1.7 (n=9), 1.8 (n=11) and 1.9 (n=14) for donors with "blocked" mean final development scores and 5.8 (n=5), 5.3 (n=3) and none for donors with "non-blocked" mean final development scores for oviductal epithelial cells, TCM-199, and explanted mouse oviduct treatments, respectively. Treatment significantly affected the final development score (P=0.07). Mean final development scores of embryos cultured in oviductal epithelial cells or TCM-199 were significantly higher than embryos cultured in explanted mouse oviducts (Table 1). Final development scores for embryos in oviductal epithelial cells and TCM-199 were not different (P>0.05). The total number of cells was affected by the donor (P<0.01), donor-by-treatment interaction (P<0.01), and treatment (P<0.05). Embryos cultured in oviductal epithelial cells had a significantly higher total number of cells (P<0.025) than those cultured in TCM-199 or in explanted mouse oviducts (Table 1). The mean total number of cells did not differ between embryos cultured in TCM-199 or in explanted mouse oviducts (P>0.05).
DISCUSSION

This is believed to be the first report of culturing in vivo fertilized, one-cell bovine embryos in explanted mouse oviducts. Failure to support development beyond the eight-cell stage indicates the explanted mouse oviduct does not provide an adequate environment for development of one-cell bovine embryos.

The proportion of embryos developing beyond the 16-cell stage with oviductal epithelial cells was similar to that of previous reports using the same culture system (Eyestone and First, 1989; McCaffrey et al., 1991). A greater proportion of embryos cultured in TCM-199 developed to the morula or blastocyst stage (14/46; 30.4%) than expected. The substantial development was due to the donor effect since all of the embryos (n=14) that developed to the morula or blastocyst stage in TCM-199 were from two donors.

Results with the explanted mouse oviduct system are similar to those of Sharif et al. (1991), who evaluated development of three-to four-cell, in vitro fertilized bovine embryos in explanted mouse oviducts. Oviducts from the report of Sharif et al. (1991) were cultured on TCM-199, TCM-199 and 20% estrous cow serum, Krebs Ringer bicarbonate medium, modified
Krebs Ringer bicarbonate medium (Davis and Day, 1978), or CZB medium (Chatot et al., 1989). Embryos also were cultured in CZB medium alone. Bovine embryo development beyond the eight-cell stage was only achieved in explanted mouse oviduct cultured on serum-free CZB medium. All other culture systems failed to support bovine embryo development beyond the eight-cell stage. Ellington et al. (1990) showed that development of one-cell in vivo fertilized bovine embryos in co-culture with bovine oviductal epithelial cells in CZB medium was comparable to that achieved with incubation in ligated rabbit oviducts. Bovine embryos developed in oviductal epithelial cell co-culture systems supported by either CZB medium or TCM-199 and 10% NBCS, but only CZB medium supported bovine embryo development in explanted mouse oviducts.

It is difficult to explain why TCM-199 in oviductal epithelial cell co-culture supported bovine embryo development, while explanted mouse oviduct supported by TCM-199 could not. Gardner and Leese (1990) measured the nutrient composition of mouse oviduct fluid in superovulated mice 21 h post hCG treatment. Pyruvate and glucose levels were 0.37 and 3.40 mM in the presence of cumulus and 0.14 and 5.19 mM, respectively, in the absence of cumulus. Decreased glucose and increased pyruvate were likely due to the cumulus cells. Leese and Barton (1985) demonstrated that cumulus masses and isolated
cumulus cells readily form pyruvate from glucose in vitro. Krisher et al. (1989) demonstrated that explanted mouse oviducts containing cumulus supported greater porcine embryo development than oviducts from which cumulus had been removed. The ability of the cumulus cells to metabolize glucose may contribute to the ability of explanted mouse oviduct to support porcine, hamster, murine and bovine embryo development. However, in the presence of TCM-199, the mouse oviduct may not metabolize enough glucose to provide a low glucose environment for bovine embryos, whereas the mouse embryos can develop to the blastocyst stage.

In conclusion, the explanted mouse oviduct on TCM-199 and 10% NBCS does not support development of one-cell bovine embryos beyond the eight-cell stage. Co-culture with bovine oviductal epithelial cells in TCM-199 and 10% NBCS was the best culture system supporting bovine embryo development from the one-cell to blastocyst stage.

REFERENCES


CHAPTER IV.
EVALUATION OF BOVINE MORULA BISECTION AND BIOPSY AS CELL
ACQUISITION TECHNIQUES FOR DNA AMPLIFICATION BY THE
POLYMERASE CHAIN REACTION

ABSTRACT

Bovine morulae (n=94; d 6-6.5) were collected nonsurgically from superovulated dairy cows (n=22) to assess embryo viability in vitro after bisection or biopsy and compare the efficiency of polymerase chain reaction (PCR) amplification of 18S rRNA genes from embryo biopsies and demi-morulae. Biopsies and one of each demi-morulae pair were subjected to PCR while the biopsied embryo and remaining demi-morula were cultured in vitro for 48 h to assess embryo development after microsurgery. Development scores of 1 (degenerate), 2 (morula), and 3 (blastocyst) were assigned to embryos at 24 and 48 h of culture. Mean development scores for biopsied embryos (n=50) at 24 and 48 h were 2.4 ± .1 and 2.3 ± .1 (x ± s.e.) and did not differ from 24 h (2.3 ± .1) and 48 h (2.4 ± .1) mean development scores of bisected embryos (n=44). Mean cell number after 48 h of culture did not differ between treatments with 41.8 ± 2.5 (n=22) for bisected embryos and 48.8 ± 2.9 (n=29) for biopsied embryos. The endogenous 18S
rRNA gene was detected in 78% (32/41) of the demi embryos and 81% (39/48) of the biopsies. These results indicate that recovery of a biopsy has no advantage over removal of half of the embryo with regard to subsequent embryo development and DNA amplification using PCR.

INTRODUCTION

Production of transgenic cattle via pronuclear microinjection has traditionally required large embryo recipient pools (McEvoy and Sreenan, 1990). The demand for a large number of recipients is primarily driven by extremely low transgene integration frequencies (McEvoy and Sreenan, 1990; Hill et al., 1992; Biery et al., 1988; Hawk et al., 1989). Current lack of information regarding mechanisms of transgene integration has forced researchers to direct their efforts towards developing methods to select embryos for transfer. Several investigators have used various embryo culture systems to reduce the total number of embryos transferred by selecting only those embryos that develop to the morula stage and eliminate the need for surgical transfer of oviductal stage embryos (Biery et al., 1988; Hawk et al., 1989; Roschläu et al., 1989; Krimpenfort et al., 1991).
Development of the polymerase chain reaction (PCR; Saiki et al., 1985) technique has provided researchers with a powerful tool to detect specific DNA sequences in minute quantities of cellular material. Endogenous genes have been detected in bovine and murine blastocysts (King and Wall, 1988), murine demi-morulae (Ninomiya et al., 1990), bovine demi-blastocysts (Puera et al., 1991), and single human blastomeres (Verlinsky et al., 1991) following DNA amplification by PCR. Ninomiya et al. (1990) demonstrated the ability of PCR to facilitate selection of transgenic murine embryos by amplification of a transgene in F1 murine demi-morulae.

Cell samples for the PCR technique can be obtained from microinjected bovine embryos by two methods. Takeda et al. (1986) showed that bisection of bovine morulae had minimal adverse effects on subsequent development with pregnancy rates of 70 and 63% following nonsurgical transfer of intact or split, excellent quality bovine morulae. Peura et al. (1991) reported successful amplification of Y chromosome-specific DNA sequences from 20- to 30-cell biopsies taken from bovine expanded blastocysts.

The objectives of this study were to assess bovine embryo viability in vitro following bisection or biopsy, and to
compare the efficiency of PCR amplification of endogenous 18S rRNA genes from bovine embryo biopsies or demi-morulae.

MATERIALS AND METHODS

Embryo Recovery
Bovine morulae were recovered from 22 superovulated, non-lactating, reproductively sound, dairy cows. Cows were superovulated with either 2500 IU pregnant mare serum gonadotropin (PMSG; Diosynth, Inc., Chicago, IL) administered in a single dose between day 10 to 12 of the estrous cycle or 32 mg of follicle stimulating hormone (FSH; Schering Corporation, Kenilworth, NJ) using a decreasing dose regime over 4 consecutive days initiated between day 9 to 13 of the estrous cycle. Prostaglandin F2α (25 mg; Lutalyse; Upjohn, Kalamazoo, MI) was administered 48 h after PMSG or 72 (30 mg) and 84 h (10 mg) after the initial FSH injection to induce luteal regression. Cows were bred naturally at 0 h and artificially 12 and 24 h after visual detection of estrus. Embryos were recovered nonsurgically with 1,000 ml of Dulbecco’s phosphate-buffered saline (DPBS; Gibco, Grand Island, NY) supplemented with 1% newborn calf serum (NBCS; Gibco) and 1.3% antibiotic/antimycotic (Gibco) on day 6 after the first breeding.
Upon recovery, embryos were transferred to phosphate-buffered saline (PBS; embryo transfer freezing medium; Gibco) and held at 37.5°C until microsurgery. Embryos were scored as excellent, good, fair or poor morulae according to morphological criteria described by Renard and Heyman (1979) and randomly assigned to one of two microsurgical treatments.

**Embryo Splitting and Biopsy**

All manipulations occurred in a 100 X 15 mm sterile, plastic petri dish lid which contained a 60 µl microdrop of PBS covered by silicone oil. Microsurgeries were conducted at room temperature on an inverted microscope under Hoffman modulation optics at 100X with a Leitz micromanipulator (Eastern Microscopes, Raleigh, NC).

Embryos assigned to the biopsy treatment were held with a fire polished (30 µm i.d.) holding pipette positioned parallel with the microsurgery blade (microblades, model MTB-05, The Micromanipulator Microscope Co., Inc., Escondido, CA), perpendicular to the bottom of the chamber. The blade was passed through the zona pellucida and approximately 1/4 of the embryonic tissue was cut against the holding pipette with an up and down cutting motion of the blade (Figure 1a). Embryo
Figure 1. Bovine morula biopsy (a) and bisection (b).
bisection was accomplished by passing a microsurgical blade (Special Blade S, Klein, Heidelberg, Germany) through the zonapellucida and cell mass in a vertical fashion and cutting against the lid surface (Figure 1b).

Polymerase Chain Reaction RNA Amplification

Biopsies and one of each pair of demi-embryos were placed into 5 µl of filtered sterilized (0.22 µM) deionized water in sterile, 250 µl plastic vials with less than 2 µl of PBS. Vials were stored at -86°C and thawed just prior to PCR. The PCR was performed in a Genetic Thermal Cycler-1 (Precision Scientific, Chicago, IL) using primers (5’-CTACACTGACTGGCTACGC-3’ and 5’-TGATCCTTCGCCAGGTTCACC-3’) which are complimentary to the 18S gene and Taq polymerase (Promega, Madison, WI) as described by Saiki et al. (1988). Biopsies and demi-morulae were subjected to PCR in a volume of 25 µl that contained 50 mM KCl; 10 mM Tris (pH 8.8); 1.5 mM MgCl₂; 0.1% Triton X-100; 0.2 µM each dATP, dTTP, dCTP, and dGTP; 0.5 µM of each oligonucleotide primer; and 0.625 units of Taq polymerase. The entire reaction mixture was overlaid with 25 µl of paraffin oil (Fisher Scientific, Pittsburg, PA). The PCR consisted of an initial denaturation of 60 sec at 96°C, followed by 40 cycles of denaturation at 96°C for 15 sec, annealing at 55°C for 120 sec, and extension at 77°C for 30
sec. Reaction products were visualized by adding 10 μl of 6X loading dye (15% Ficoll-400, 0.25% bromophenol blue) to each tube followed by electrophoresis of 15 μl of this mixture in a 1% agarose gel containing 0.5 μg/ml ethidium bromide. Amplified DNA was subjected to electrophoresis in 40 mM Tris-HCl (pH 8.1), 40 mM acetate, 2 mM EDTA, and 0.5 μg/ml ethidium bromide for 1 h at 50 V. Amplified PCR fragments were visualized by UV transillumination and photography. The entire procedure from embryo manipulation to agarose gel electrophoresis required 7 h.

**Embryo Culture**

One of each pair of demi-embryos and all biopsied embryos were individually cultured in 25 μl microdrops of Ham's F-10 supplemented with 10% NBCS and 1.3% antibiotic/antimycotic. All cultures were conducted in 5% CO₂ in air at 37.5°C for 48 h. Morphological stage of development was assessed at 24 and 48 h of culture. Each embryo was assigned a development score of 1 to 3 with 1=degenerate, 2=morula, and 3=blastocyst at each observation. Nuclei of embryos that did not show signs of degeneration were stained at 48 h with Hoechst 33342 DNA stain (Sigma, St. Louis, MO) for determination of cell number. No attempt was made to estimate cell number in degenerate embryos.
Final development scores of embryos and cell number for each treatment were subjected to the general linear models procedure of the Statistical Analysis System (1988). Treatment, embryo donor, embryo grade, and observation time served as independent variables in the model. Treatment by embryo donor interaction served as the error term for testing treatment effects. Differences of gene amplification and detection frequency by manipulation treatment were tested using Chi-square analysis.

RESULTS

Number of corpora lutea detected by rectal palpation day 6 post-estrus averaged 12.8 ± 2.7 (X ± SE). Each nonsurgical embryo collection yielded a mean of 7.6 ± 1.6 embryos with 4.5 ± .6 of these embryos classified as morulae. Embryo recovery rate, as determined by the number of corpora lutea palpated and embryos recovered was 59%. Of the morulae collected, 34, 27, 19, and 19 were of excellent, good, fair, and poor quality, respectively. Two good and three poor quality morulae were destroyed during microsurgery.

Mean development scores assessed at 24 and 48 h of culture are shown in Table 2. The scores did not differ between treatments or time of evaluation (P>0.05). The
Table 2. Mean development score (± SE; 1=degenerate, 2= morula, 3= blastocyst) for split and biopsied embryos after 24 and 48 h of culture in Ham's F-10 supplemented with 10% new born calf serum.

<table>
<thead>
<tr>
<th>Microsurgical Treatment</th>
<th>n</th>
<th>24 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>split</td>
<td>44</td>
<td>2.3 ± .1</td>
<td>2.4 ± .1</td>
</tr>
<tr>
<td>biopsy</td>
<td>50</td>
<td>2.4 ± .1</td>
<td>2.3 ± .1</td>
</tr>
</tbody>
</table>
Figure 2. Percent of demi- and biopsied embryos classified as degenerate (1), morula (2), or blastocyst (3) after 24 and 48 h of culture.
Table 3. Mean cell number (± SE) and transgene detection in bovine embryos after splitting or biopsy and 48 h of in vitro culture.

<table>
<thead>
<tr>
<th>Microsurgical Treatment</th>
<th>n</th>
<th>Cell Number ± SE</th>
<th>n</th>
<th>Transgene Detection %</th>
</tr>
</thead>
<tbody>
<tr>
<td>split</td>
<td>22</td>
<td>41.8 ± 2.5</td>
<td>41</td>
<td>78</td>
</tr>
<tr>
<td>biopsy</td>
<td>29</td>
<td>48.8 ± 3.9</td>
<td>48</td>
<td>81</td>
</tr>
</tbody>
</table>
distribution of developmental scores among each treatment population for each observation is shown in Figure 2. Mean cell number after 48 h of culture did not differ between treatments (Table 3). Embryo donor and embryo grade did not significantly affect (P>0.05) mean development scores or cell number.

The 18S ribosomal RNA gene detection frequency did not differ (P>0.05) between cell acquisition techniques with successful DNA amplification and gene detection in 32/41 (78%) of the demi-embryos and 39/48 (81%) of the biopsies (Table 3).

DISCUSSION

Morphological assessment of development in vitro and estimation of cell numbers 48 h after culture show that embryo bisection and biopsy are equally appropriate methods for acquisition of embryonic cells for DNA amplification by PCR. Lucas-Hahn and Niemann (1991) reported a decrease in the proportion of day 7 demi-embryos that had developed to the blastocyst stage in Ham’s F-10 from 24 (63%) to 48 h (26%) after microsurgery. Similar results were reported by Voelkel et al. (1985) with 41 and 28% of day 6-7 demi-morulae cultured in Ham’s F-10 developing to blastocyst after 24 and 48 h, respectively. Addition of endometrial fibroblasts to in vitro
culture in Ham's F-10 supported development of 69% and 67% demi-embryos to the blastocyst stage after 24 and 48 h (Voelkel et al., 1985). The co-culture results were similar to our observation of 41 and 52% and 68 and 62% development to blastocyst for demi- and biopsied embryos after 24 and 48 h of culture, respectively. Our increase in percentage of demi-embryos reaching the blastocyst stage after 48 h may be due to the use of day 6 morulae while other researchers have used day 6-7 (late morula-blastocyst) embryos (Takeda et al., 1986; Lucas-Hahn and Niemann, 1991; Voelkel et al., 1985).

Earlier studies have used bovine embryo biopsy and splitting techniques to acquire embryonic cell samples for DNA analysis, but none have compared the two techniques. Bondioli et al. (1989) biopsied 101 day 6-7 bovine embryos and subjected the biopsies to DNA hybridization to detect male-specific DNA sequences. Embryos were frozen immediately after the biopsy to await the DNA hybridization results that were available 6 days later. Ninety-one (90%) biopsies were sexed and sex determination had an accuracy of 97% when confirmed by evaluation of fetuses from 34 of the sexed embryos.

Peura et al. (1991) bisected bovine morula and blastocysts and subjected one of each demi-embryo pair to PCR for DNA amplification and subsequent sex determination. The lysates
from the demi-embryo undergoing PCR were divided into two aliquots. Bovine Y-chromosome-specific primers were used in each aliquot and bovine DNA-specific primers were added to one aliquot to confirm the presence of embryonic material. Sex was successfully determined in 79% (23/29) of the embryos bisected. In a second experiment Peura et al. (10) successfully sexed 80% of 50 demi-embryos utilizing two sets of primers. These gene amplification and detection rates are similar to our 78% (demi-embryos) and 81% (biopsies) detection frequency of 18S ribosomal RNA gene detection using one aliquot and one set of primers.

In conclusion, embryo bisection or biopsy are both appropriate means of acquiring cellular material from bovine morulae for subsequent DNA amplification by PCR. Use of the PCR technique provides the user with a rapid assay of the embryonic DNA and an opportunity to transfer embryos without cryopreservation.

REFERENCES


CHAPTER V.

EVALUATION OF IN VITRO CO-CULTURE, MICROSURGERY, AND THE POLYMERASE CHAIN REACTION TECHNIQUE FOR SELECTION OF TRANSGENIC BOVINE PREIMPLANTATION EMBRYOS

ABSTRACT

In vivo fertilized one- (155), two- (57), and four-cell (62) bovine embryos were surgically collected from superovulated dairy cows to utilize in vitro culture, embryo bisection, and the polymerase chain reaction (PCR) technique for selection of transgenic bovine embryos. Seventy percent of the embryos with visible pronuclei or nuclei (192/274) were microinjected with DNA (WAP-PC: mouse Whey Acid Protein promoter and human Protein C cDNA) and the remaining 30% served as non-injected controls. All embryos were co-cultured with bovine oviductal epithelial cells for 48 h at which time four- to eight-cell embryos were placed in co-culture supported by transforming growth factor-α supplemented medium or non-supplemented medium. Mean final development scores (4=8-cell, 5=16-cell, 6=morula, 7=early blastocyst, 8=blastocyst) of embryos within the same initial cell stage at the time of collection were not affected by microinjection (P>.05). Non-injected four-cell and two-cell embryos had higher mean final development scores (5.6 ± .4, 5.9 ± .6; x ± s.e.) than one-cell embryos (4.4 ± 8.2)
3; P < .05). However, mean final development score of microinjected four-cell (5.6 ± 0.3) embryos did not differ from that of microinjected one-cell (5.1 ± 0.2) embryos but was greater than the mean final development score of microinjected two-cell embryos (P < .05). After 144 h of culture, 45% (87) of the microinjected embryos developed to the morula or blastocyst stage and 34% (65) were classified as acceptable for bisection. The WAP-PC cDNA was detected in 50% (16/32), 10% (1/10), and 9% (2/23) of demi-morulae from embryos microinjected at the one-, two-, and four-cell stage. Frequency of transgene detection was higher in morulae from one-cell embryos (P < .01) than in morulae from two- and four-cell embryos. Use of in vitro co-culture, embryo bisection, and PCR facilitated selection of bovine embryos that carried the transgene.

INTRODUCTION

Several investigators interested in production of therapeutic human proteins in genetically engineered domestic animals have chosen the lactating mammary gland for transgene expression (Wright et al., 1991; Denman et al., 1991; Clark et al., 1989; Gordon et al., 1987; Hennighausen et al., 1990). Transgene expression levels of mg/ml in mouse milk (Simons and Land, 1987) have encouraged use of mammals with high milk production
to serve as pharmaceutical bioreactors. Genes coding for therapeutic proteins have been successfully integrated and expressed in mammary glands of sheep (Clark, et al., 1989; Wright, et al., 1991), goats (Denman et al., 1991) and pigs (Velander, unpublished data). Whereas milk production from these animals is greater than that of the mouse, the dairy cow’s milk production is superior to all of these species. Thus, the cow would be the ultimate animal for large-scale production of foreign proteins in the mammary gland.

Production of transgenic dairy cattle by pronuclear microinjection has been hampered by a need for large numbers of embryo recipients (McEvoy and Sreenan, 1990), increased embryo mortality rate following microinjection (Roschlau et al., 1989; Krimpenfort et al., 1991) and low transgene integration and expression frequencies (McEvoy and Sreenan, 1990; Hill et al., 1992; Biery et al., 1988; Roschlau et al., 1989). Efforts to reduce the number of embryo recipients required have included temporary placement of the microinjected embryos into rabbit, ovine or bovine oviducts (Biery et al., 1988; Hawk et al., 1989; Roschlau et al., 1989) or culturing the embryos in vitro (Krimpenfort et al., 1991) to facilitate selection of only those embryos that develop to the morula stage and eliminate the need for surgical transfer of oviductal stage embryos. Drawbacks associated with use of
surrogate oviducts for short term culture include the need for additional animals, surgical transfer and collection of the embryos and the potential for embryo loss (Hawk et al., 1989; Gagne et al., 1990). While in vitro culture does not require additional surrogates or surgery, the early bovine embryo’s development requirements have not been completely defined. As a result bovine embryo development in vitro is typically retarded or blocked (Wright and Bondioli, 1981).

Numerous attempts have been made to improve bovine embryo development in vitro (Larson et al., 1990; Larson et al., 1991; Eyestone et al., 1989; Fukui 1989; Aoyagi et al., 1989; Ellington et al., 1990a; Ellington et al., 1990b; Goto et al., 1992; Bavister et al., 1992). Supplementation of culture medium with transforming growth factor β (TGF-β), basic fibroblastic growth factor (bFGF) and platelet-derived growth factor (PDGF) was shown to enhance bovine embryo development in vitro (Larson et al., 1990; Larson et al., 1991). Reports of growth factor expression in preimplantation mouse embryos (Rappolee et al., 1988) and enhancement of mouse embryo development in vitro by addition of epidermal growth factor (EGF), TGF-α, or TGF-β (Paria and Dey, 1990), or insulin-like growth factor (IGF-1; Harvey and Kaye, 1991) to the culture medium have encouraged further investigation of
the role of growth factors in early mammalian embryo development.

Addition of bovine oviductal epithelial cells (OEC) to a simple, defined, glucose- and serum-free medium supported bovine embryo development from the one-cell to morula/blastocyst stage, equivalent to culture in rabbit oviducts or in vivo development (Ellington et al., 1990a; Ellington et al., 1990b). The hypothesized mechanisms by which the oviductal cells are able to support development of bovine embryos can be generalized into two categories: 1) secretion of embryotrophic factors and 2) removal of embryotoxic factors. The information regarding the effects of growth factor supplementation on embryo development favor the "secretion" hypothesis, but the role of growth factors in co-culture systems has not yet been defined. A recent report of growth factor ligand and receptor gene expression in early bovine embryos (Watson et al., 1992) further supports the hypothesis that growth factors are involved in preimplantation embryo development. Addition of a mitogenic growth factor that acts in an autocrine/paracrine manner to a co-culture system may enhance embryo development and reduce mortality in vitro.

Increasing transgene integration frequencies would be one method to enhance production of transgenic cattle. However,
current lack of information regarding mechanisms of transgene integration requires researchers to use other techniques to increase the number of transgenic calves born per transfer. Ninomiya et al. (1990) used the polymerase chain reaction technique (PCR; Saiki et al., 1985) for detection of a transgene in murine demi-morulae produced from matings with transgenic males that carried 2 copies of the transgene. King and Wall (1988) reported that a single copy gene encoding the 6 chain of bovine luteinizing hormone could be detected in purified bovine genomic DNA representing 25 cells.

Cell samples for DNA analysis can be acquired by either blastomere biopsy or embryo bisection. We recently reported that either technique is suitable with regard to subsequent embryo development in vitro and DNA amplification (Sparks et al., 1991).

The objectives of this study were to evaluate the effect of TGF-α supplementation on microinjected and non-microinjected bovine embryo development in co-culture and to utilize in vitro culture, embryo bisection, and the PCR technique to facilitate selection of transgenic bovine embryos for subsequent transfer.
MATERIALS AND METHODS

Superovulation

Twenty-four reproductively sound, non-lactating dairy cows were superovulated with 32 mg of follicle stimulating hormone (FSH; Shering Corporation, Kenilworth, NJ) using a decreasing dose regime over 4 consecutive days initiated between day 9 and 13 of the estrous cycle. Prostaglandin F2α (Lutalyse; Upjohn, Kalamazoo, MI) was administered 72 (30 mg) and 84 h (10 mg) after the initial FSH injection to induce luteal regression. Cattle displayed estrus 36-48 h after the first prostaglandin injection and were bred naturally at 0 h and artificially 12 and 24 h after the first observation of estrus.

Embryo Recovery

One- to four-cell bovine embryos were surgically collected 36-64 h after the first observation of estrus. Oviducts were exteriorized via the caudal flank approach described by Wolfe et al. (1990). Anesthesia was initiated with guaifenesin and thiopental and maintained with halothane. After initiation of anesthesia the animals were positioned in lateral recumbency. The rear limb of the exposed side was pulled caudally, and the flank of the same side was prepared and draped for aseptic surgery.
The proximal ovary, oviduct and uterine horn were exteriorized by gentle traction on the broad ligament. Number of ovulations and follicles were recorded. A polyethylene cannula (polyethylene tubing, I.D. 1.4 mm, O.D. 1.57 mm; Intramedic, Clay Adams, Parsippany, NJ) was threaded through the ostium of the infundibulum and 4 to 5 cm into the ampulla of the oviduct to facilitate fluid collection. Approximately 20-30 ml of phosphate-buffered saline (PBS; embryo transfer freezing medium, Gibco, Grand Island, NY) were flushed through the oviduct by introducing a 20 g blunt-ended needle into the uterine lumen 1 cm from the utero-tubal junction. The fluid was collected into sterile test tubes and transported to the laboratory at 37°C. This procedure was repeated with the contralateral oviduct when it could be exteriorized through the same incision site. The reproductive tract was irrigated with 3% (v/v) glycerol in sterile saline to reduce adhesion formation before it was returned to the abdominal cavity (Bondurant, 1986). When the distal ovary could not be exteriorized through the first incision site the cow was rotated and the entire procedure described above was repeated to flush the remaining oviduct.

Recovered fluid was searched for both embryos and OEC. The OEC were washed with tissue culture medium 199 (M199; Gibco)
supplemented with 10% newborn calf serum (NBCS; Gibco), pyruvate (1 mg/ml), penicillin (1 mg/ml) and streptomycin (1 mg/ml) 4-6 times in a conical tube. After washing, OEC were placed in 25 µl drops of M199 and 10% NBCS and maintained at 39℃ in an atmosphere of 5% CO₂ in air.

**Embryo Microinjection**

Recovered embryos were washed 3 times in PBS and transferred in 500 µl of medium to sterile plastic vials for centrifugation. Embryos were centrifuged at 16,000 g for 6 min to facilitate visualization of pronuclei. Following centrifugation, embryos were observed at 200X under Hoffman modulation optics. Embryos with visible pronuclei or nuclei were used in this study. Approximately seventy percent of the embryos with visible nuclei or pronuclei were microinjected while remaining embryos were assigned to control treatments. A greater proportion of embryos were assigned to the microinjection treatments to optimize the number of potentially transgenic embryos.

Microinjection was performed by holding the embryo by suction with a holding pipette (30 µm inner diameter) controlled by one arm of the micromanipulator. The other arm controlled the injection pipette which was connected to an Eppendorf
automatic microinjector (model 5242, Eastern Microscopes, Raleigh, NC).

The DNA used for microinjection was a 5.7 kb fragment consisting of the mouse Whey Acid Protein (WAP) promoter and 3’ elements with a human Protein C (PC) cDNA inserted at the WAP coding region. The DNA was diluted in 10 μM Tris–HCl–1 8 mM EDTA buffer (pH 8.0) to make a final DNA concentration of 250 copies/pl. The most visible or largest pronucleus or nucleus was injected with 1–2 pl of the DNA solution. Visualization of swelling of the pronucleus or nucleus indicated a successful injection.

**Embryo Culture**

Microinjected embryos were cultured for approximately 144 h in 25 μl drops of M199 and 10% NBCS, pyruvate, penicillin, streptomycin and OEC in an atmosphere of 5% CO₂ in air at 39°C. Morphological stage of development was assessed 48 h after the initiation of culture. All OEC and four- and eight-cell embryos were washed after 48 h of culture and randomly assigned to one of two culture treatments. Treatments consisted of M199 supplemented with 10% NBCS, pyruvate, penicillin, streptomycin and OEC (control) or M199 supplemented with transforming growth factor alpha (TGFα, 10
ng/ml; Sigma, St. Louis, MO), 10% NBCS, pyruvate, penicillin, streptomycin, and OEC. Morphological development was assessed at the termination of culture.

Amplification of the Transgene

Microinjected embryos developing to the morula stage in culture were bisected to obtain cellular material for amplification of the transgene by the PCR technique. Bissection occurred in a chamber consisting of a 100 X 15 mm sterile, plastic petri dish lid that contained a 100 μl microdrop of PBS covered with silicone oil. A microsurgical blade (Special Blade S, Storz, Heidelberg, Germany) was passed through the zona pellucida and cell mass in a vertical fashion.

One demi-embryo was transferred with less than 2 μl of PBS to 5 μl of filtered sterilized (0.22 μM) deionized water in a sterile, 250 μl plastic vial with less than 2 μl of PBS. Vials were held at -86°C and thawed immediately prior to PCR.

The PCR was performed in a Genetic Thermal Cycler-1 (Precision Scientific, Chicago, IL) using primers 5′-CTCCTGCAGTGTCACCCCGCAGT-3′ and 5′-CACCATGGTGCATGCAGCTCGCTG-3′ which were complimentary to the PC gene and Taq polymerase (Promega, Madison, WI) as described by Saiki et al. (1988).
Demi-embryos were subjected to PCR in a volume of 25 μl that contained 50 mM KCl; 10 mM Tris (pH 8.8); 1.5 mM MgCl₂; 0.1% Triton X-100; 0.2 μM each dATP, dTTP, dCTP, and dGTP; 0.5 μM of each oligonucleotide primer; and .625 units of Taq polymerase. The entire reaction mixture was overlaid with 25 μl of paraffin oil (Fisher Scientific, Pittsburg, PA). Each cycle consisted of denaturation at 96 °C for 15 sec, annealing at 55°C for 120 sec, and extension of primers for at 77°C 30 sec. The initial cycle had denatured DNA by heating the samples to 96 °C for 1 min. Reaction products were visualized by adding 10 μl of 6X loading dye (15% Ficoll-400, 0.25% bromophenol blue) to each tube followed by electrophoresis of 25 μl of this mixture in a 1% agarose gel (SeaKem; FMC BioProducts, Rockland, ME) containing 0.5 μg/ml ethidium bromide. Amplified DNA was subjected to electrophoresis in 40 mM Tris-HCl (pH 8.1), 40 mM acetate, 2 mM EDTA, and 0.5 μg/ml ethidium bromide for 1 h at 50 V. Amplified PCR fragments were visualized by UV transillumination and photography.

The remaining demi-embryos were returned to the co-culture system immediately after microsurgery. The entire procedure from embryo manipulation to DNA amplification and identification was completed in approximately 7.5 h.
Embryo Transfer

Embryos identified as positive for the transgene were transferred to recipient heifers within 10 h after microsurgery. Heifers were synchronized with 35 mg of PGF$_2$$_\alpha$ 12-24 h after PGF$_2$$_\alpha$ was administered to the superovulated donor.

Embryos were loaded into a 1/4 cc straw and transferred nonsurgically to the uterus with a 1/4 cc, 21 inch Cuozzo Gun. Following transfer, heifers were observed for signs of repeat estrus and pregnancies were confirmed by rectal palpation at approximately 35 days of gestation.

Statistical Analysis

Mean final development scores of embryos for each treatment were calculated and analysis was by analysis of variance. Initial cell stage, microinjection and TGF-α treatment served as independent variables in the model. Differences in percentage of embryos developing to the morula stage for microinjection, initial cell stage, culture treatment, and transgene detection frequency were tested by Chi-square analysis.
RESULTS

Twenty-two of the 24 superovulated cows had an average of 18.5 ± 3.9 (\(\bar{x} \pm\) s.e.) corpora hemorrhagica with a mean of 14.9 ± 2.7 embryos recovered. Two cows failed to ovulate prior to the time of surgery. Embryo recovery rate, as determined by the total number of embryos recovered and corpora hemorrhagica observed, was 80.4% (329/409). Of the 329 embryos recovered, 62 (19%) were at the four-cell stage, 57 (17%) were classified as two-cell, 208 (63%) were one-cell, and 2 (1%) were degenerate. Fifty-three (25%) of the 208 one-cell ova were classified as unfertilized due to lack of visible pronuclei and failure to cleave in culture. No attempt to stain pronuclei in the uncleaved one-cell ova was made.

Stage of embryo development at the time of recovery was affected by time between the first observation of estrus and surgery (\(P<.01\); Figure 3). Two donors collected at 48 and 46 h post-estrus had only unfertilized ova and were not included in Figure 3. Ninety-four percent (123) of the 131 embryos collected at 44 to 53 h after first observation of estrus were at the one-cell stage with 6 (5%) at the two-cell stage and 2 (1%) unfertilized ova. Thirty-six hours after first observation of estrus was the earliest recovery time for collection of fertilized one-cell embryos. When the interval
between first observation of estrus and surgery was increased to 60 h or more, the recovered embryo population was primarily at the 2- to 4-cell stage.

A total of 105 one-cell, 49 two-cell, and 43 4-cell embryos were microinjected with WAPPC while 50 one-cell, 8 two-cell, and 19 four-cell embryos remained non-injected. Five (3%) one-cell embryos were destroyed by DNA microinjection. Percent of embryos with at least one cleavage after the initial 48 h of culture and percent developing to the morula stage was not affected by initial cell stage or microinjection (P>.05; Table 4.).

Mean final development score of embryos that cleaved at least once in culture was affected (P<.05) by initial cell stage and injection by initial cell stage interaction (Table 5). Non-injected four- and two-cell embryos had higher (P<.05) mean development scores than non-injected one-cell embryos, but mean development scores for injected four-cell and two-cell embryos did not differ from that of injected one-cell embryos. Mean development scores for injected one-, two-, and four-cell embryos were not different (P>.05) from those of non-injected one-, two-, and four-cell embryos.
Figure 3. Mean cell number in embryos collected between 36 and 63 h after first observation of estrus.
Supplementation of culture media with TGF-α when embryos reached the four-cell stage did not affect the mean final development score, regardless of initial cell stage or microinjection. The mean final development score for embryos was 5.6 ± .2 in TGF-α supplemented medium and 5.2 ± .2 for non-supplemented medium.

A total of 87 microinjected one-, two-, and four-cell embryos developed to the morula stage. Sixty-five (75%) of these morulae were acceptable for bisection (Table 6). Morulae that had numerous blastomeres that degenerated or failed to compact were considered unacceptable for bisection. Frequency of transgene detection was greater (P<.001) in morulae that developed from embryos microinjected at the one-cell stage than embryos microinjected at the two- and four-cell stage.

Thirteen of the 19 demi-embryos containing WAP-PC were transferred to six synchronized recipients. Three recipients returned to estrus with no evidence of an extended estrous cycle. One recipient had a 28 day estrous cycle. A membranous sac was expelled from one recipient 36 days after embryo transfer. We did detect the WAP-PC gene in DNA extracted from the recovered membrane. The remaining recipient was confirmed pregnant 45 days after embryo
<table>
<thead>
<tr>
<th>Initial Cell Stage</th>
<th>Treatment</th>
<th>n</th>
<th>Number (%) cleaved after 48 h</th>
<th>Number (%) developing to morula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-cell</td>
<td>injected</td>
<td>100</td>
<td>88 (88)</td>
<td>42 (42)</td>
</tr>
<tr>
<td></td>
<td>non-injected</td>
<td>50</td>
<td>50 (100)</td>
<td>15 (30)</td>
</tr>
<tr>
<td>2-cell</td>
<td>injected</td>
<td>49</td>
<td>45 (92)</td>
<td>18 (37)</td>
</tr>
<tr>
<td></td>
<td>non-injected</td>
<td>8</td>
<td>8 (100)</td>
<td>5 (63)</td>
</tr>
<tr>
<td>4-cell</td>
<td>injected</td>
<td>43</td>
<td>35 (81)</td>
<td>27 (63)</td>
</tr>
<tr>
<td></td>
<td>non-injected</td>
<td>19</td>
<td>19 (100)</td>
<td>8 (42)</td>
</tr>
</tbody>
</table>
Table 5. Least squares mean final development scores for injected and non-injected one-, two-, and four-cell embryos cleaving at least once in vitro.

<table>
<thead>
<tr>
<th>Initial Cell Stage</th>
<th>Injected embryos</th>
<th>Non-injected embryos</th>
<th>All embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-cell</td>
<td>$5.1 \pm .2 \ a^{,b}$</td>
<td>$4.4 \pm .3 \ b$</td>
<td>$4.8 \pm .2 \ a$</td>
</tr>
<tr>
<td></td>
<td>(88)</td>
<td>(50)</td>
<td>(138)</td>
</tr>
<tr>
<td>2-cell</td>
<td>$4.6 \pm .3 \ b$</td>
<td>$5.9 \pm .6 \ a$</td>
<td>$5.2 \pm .3 \ a^{,b}$</td>
</tr>
<tr>
<td></td>
<td>(45)</td>
<td>(8)</td>
<td>(53)</td>
</tr>
<tr>
<td>4-cell</td>
<td>$5.6 \pm .3 \ a$</td>
<td>$5.6 \pm .4 \ a$</td>
<td>$5.6 \pm .2 \ b$</td>
</tr>
<tr>
<td></td>
<td>(35)</td>
<td>(19)</td>
<td>(54)</td>
</tr>
</tbody>
</table>

1 Development was scored as: 1=1-cell, 2=2-cell, 3=4-cell, 4=8-cell, 5=16-cell, 6=morula, 7=early blastocyst, 8=blastocyst, and 9=expanded blastocyst.

2 $\bar{x} \pm$ s.e.

$a,b$ Development scores with different superscripts within the same column differ at (P<.05).
Table 6. Transgene detection in demi-morulae derived from microinjected 1-, 2-, and 4-cell bovine embryos.

<table>
<thead>
<tr>
<th>Initial cell stage</th>
<th>Number developing to morula</th>
<th>Number of morulae acceptable for bisection (%)</th>
<th>Number transgenic (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-cell</td>
<td>42</td>
<td>32 (76)</td>
<td>16 (50) a</td>
</tr>
<tr>
<td>2-cell</td>
<td>18</td>
<td>10 (56)</td>
<td>1 (10) b</td>
</tr>
<tr>
<td>4-cell</td>
<td>27</td>
<td>23 (85)</td>
<td>2 (9) b</td>
</tr>
<tr>
<td>Total</td>
<td>87</td>
<td>65 (75)</td>
<td>19 (29)</td>
</tr>
</tbody>
</table>

a, b Chi square P<.01 between initial cell stages.
transfer, but was observed in estrus 51 days post-transfer. The fetus was not recovered from this recipient.

**DISCUSSION**

The objectives of this study did not initially include comparisons of the effects of microinjection on embryo development and gene integration frequency in embryo of different initial cell stages, but recovery of two- and four-cell embryos in our study led us to include these comparisons in our report. Our recovery of two- and four-cell embryos while attempting to collect in vivo fertilized one-cell embryos represents one of the many problems encountered when attempting to produce transgenic cattle by pronuclear DNA injection. Brinster et al. (1985) reported that pronuclear microinjection was a more efficient method of producing transgenic mice than DNA microinjection into nuclei of more advanced embryos. These results have driven researchers to enhance production of bovine one-cell embryos by either determining the optimum time between estrus and collection (Roschla et al., 1989; McEvoy and Sreenan, 1990) or producing one-cell embryos by in vitro fertilization (Krimpenfort et al., 1991). Embryo recovery between 44 and 53 h after the onset of estrus was the optimum time for collection of one-cell fertilized embryos (Figure 1). Embryo collection at 36
to 48 h post-estrus in an earlier study yielded a higher proportion of unfertilized ova (50%; Sparks et al., 1992) than we observed in this study (17%).

Pursel et al. (1988) reported a higher percent of two-cell microinjected porcine embryos developing beyond the 16-cell stage than one-cell microinjected embryos when cultured in oviducts of synchronized recipient gilts. Similarly, the proportion of microinjected one-cell ovine embryos (75%) that developed to the 8-cell stage after three days of culture on a monolayer of ovine oviductal cells was lower than non-injected embryos (96%; Rexroad et al., 1990). No difference between the percentage of microinjected one-, two-, and four-cell bovine embryos developing to the morula stage in vitro was observed in this study (Table 4). Hawk et al. (1989) reported that 55% of 120 bovine microinjected embryos recovered from rabbit oviducts had developed to the morula stage. The proportion of microinjected one-cell embryos developing to the morula stage in vitro (42%) is comparable to results of Hawk et al. (1989) and higher than the 21% developing to the morula stage in the sheep oviduct reported by Biery et al. (1988). The proportion of one-, two-, and four-cell embryos developing to the morula stage in vitro was not affected by microinjection (Table 4). Hawk et al. (1989) had similar results with 52% in vivo fertilized, microinjected
one-cell bovine embryos and 55% non-injected embryos developing to the morula stage in the rabbit oviduct. Culture of in vitro fertilized, microinjected bovine embryos resulted in a decrease in percentage of embryos developing to the morula stage following microinjection (Gagne et al., 1990; Krimpenfort et al., 1991). Gagne et al. (1990) cultured in vitro fertilized, microinjected and non-injected bovine embryos in rabbit oviducts and found that microinjection reduced the proportion of embryos reaching the morula stage (5% versus 25%). More recently, Krimpenfort et al. (1991) reported development to morula at rates of 19% and 20-25% for in vitro fertilized, microinjected and non-injected bovine embryos cultured in vitro. The results in this report and those reported by Hawk et al. (1989) suggest that in vivo fertilized embryos are better candidates for DNA microinjection and culture than in vitro fertilized embryos.

Mean final development score of the one-, two-, and four-cell bovine embryos was not affected by microinjection, but did differ between initial cell stage (Table 5). Mean final development score for all four-cell embryos was higher than for one-cell embryos, but was not different from two-cell embryos. This was not the same for microinjected one- and four-cell embryos that had similar mean final development scores. These results are contrary to an earlier study by
Brinster et al. (1985), where microinjection of one- and two-cell mouse embryos led to lower post-microinjection survival rates and integration for the embryos injected at the two-cell stage. Data presented by Pursel et al. (1988) and in our study suggest that developmentally advanced livestock embryos (>1-cell) are more resilient to microinjection than advanced mouse embryos.

Addition of TGF-α to the co-culture system did not increase the mean final development score of injected or non-injected embryos regardless of initial cell stage. Larson et al. (1991) reported that addition of TGF-α at a concentration of 1 ng/ml to serum- and cholesterol-free B2 medium increased the proportion of 16-cell bovine embryos forming blastocysts from 8.6% (5/58) to 40% (28/70). Watson et al. (1992) recently evaluated expression of several growth factor ligand and receptor genes in preimplantation bovine embryos. While they could detect expression of the TGF-α gene, they did not detect expression of the TGF-α receptor gene. This may explain the lack of enhanced embryo development in medium supplemented with TGF-α in our study. Another possible explanation may be that the oviductal epithelial cells are providing the embryo with sufficient amounts of TGF-α and other growth factors to maintain development in vitro. The study by Larson et al. (1991) used defined medium which most likely lacked sufficient
amounts of growth factors necessary to support bovine embryo development.

Sixty-five (75%) of the 87 morulae that developed from microinjected one- two- and four-cell embryos were judged acceptable for bisection. There was no difference in the proportion of acceptable versus non-acceptable morulae based on initial cell stage (Table 6). We did observe a higher transgene detection rate in morulae that developed from embryos injected at the one-cell stage (16/32) than embryos injected at the two-cell (1/10) or four-cell (2/23) stage. We are not aware of any other studies that have evaluated gene integration rates in embryos that developed from bovine embryos microinjected at different cell stages. Transgene integration in piglets developing from embryos microinjected at the two-cell stage was similar to pigs produced from one-cell microinjected embryos (1.03 versus 0.48%; Pursel et al., 1988). In contrast, Brinster et al. (1985) reported a lower transgene integration frequency in mouse embryos microinjected at the two-cell stage (14%) than embryos microinjected at the one-cell stage (30%).

Similar mean final development scores were found for embryos microinjected at the one- and four-cell stage. Comparable percentages of microinjected one-, two-, and four-cell embryos
developed to the morula stage in vitro. Higher frequency of transgene detection was found in morulae that developed from microinjected one-cell embryos. These results show that the pronucleus is the preferred target for transgene microinjection. While low numbers of transferable transgene-positive demi-embryos did not allow us to establish pregnancies, we conclude that the combination of in vitro culture, embryo bisection, and DNA amplification by the PCR technique can be utilized for selecting potentially transgenic bovine embryos for non-surgical transfer to synchronized recipients.

REFERENCES


MA p. 59 (Abstr.).


CHAPTER VI.
COMPARISON OF SUPEROVULATORY RESPONSE TO FSH OR PMSG IN DAIRY CATTLE: A RETROSPECTIVE STUDY

ABSTRACT

Response to 110 superovulations was examined in retrospect to assess the effects of different doses of FSH (32 to 40 mg) and PMSG (2500 IU) on superovulatory responses. All superovulations were initiated between d 9 and 14. Oviductal stage embryos (1- to 8-cell) were recovered at slaughter from cows superovulated with 40 mg (FSH-1; n=12) or 35 mg (FSH-2; n=25) of FSH administered in 5 mg doses twice daily for 3.5 to 4.0 d. Embryos were surgically collected from 23 cows superovulated with 32 mg of FSH administered over a four d period in a decreasing dose regime (FSH-3). Uterine stage embryos were recovered from 23 cows superovulated with FSH-3 and 19 cows superovulated with 2500 IU PMSG (PMSG) d 6 post-estrus. Mean intervals between first PGF$_{2a}$ injection and first observation of estrus ranged from 33.3 to 41.8 h and the proportion of cows responding to PGF$_{2a}$ (80-92%) was not affected by superovulation regime or superovulation initiation day. Total number of d one to three stage embryos recovered
was higher for FSH-1 (19 ± 2.3; \( \bar{x} \pm SE \)) than FSH-2 (9.0 ± 4.0), but was not different from FSH-3 (13.8 ± 3.3). Total number of embryos recovered 6 d post-estrus did not differ between superovulation treatments with 7.1 ± 1.1 for FSH-3 and 5.1 ± 1.2 for PMSG. More (P<.05) poor quality morulae were recovered from FSH-3 superovulated cows (1.0 ± 0.3) than PMSG superovulated cows (0.2 ± 0.3). Superovulations with PMSG yielded more (P<.05) unfertilized ova (2.1 ± 0.5) than FSH-3 (0.7 ± 0.5). Superovulation with FSH-1 or FSH-3 yielded the most oviductal stage embryos, while number of embryos recovered 6 d post-estrus was not affected by superovulation treatment.

**INTRODUCTION**

Administration of exogenous gonadotropins has been the most common method of inducing superovulation in cattle. The two gonadotropins most commonly used to superovulate cows are pregnant mare serum gonadotropin (PMSG) and follicle stimulating hormone (FSH). The gonadotropic effect of PMSG is similar to FSH and luteinizing hormone (Cole and Hart, 1930; Laster et al., 1970; Vincent and Mills, 1972). Both gonadotropins are glycoproteins. However, the higher carbohydrate content in PMSG renders a relatively longer half-
life than FSH (Papkoff, 1977; Schams et al., 1977; Laster, 1972).

Despite the longer half-life of PMSG, Elsden et al. (1978) reported that administration of a single 32 mg dose of FSH resulted in more corpora lutea, recovered embryos, transferable embryos, and pregnancies than a single 1800 IU dose of PMSG. The relatively short half-life of FSH has led investigators to evaluate superovulatory regimes with multiple doses of FSH. Bellows et al. (1969) found that administration of multiple doses of FSH over a 5 d period stimulated more follicular development and ovulations than FSH administered in a single dose. Garcia et al. (1982) compared administration of FSH (32 mg) twice daily over 3, 4, and 5 d periods in crossbred cattle. Number of corpora lutea, embryos recovered, and number of transferable embryos recovered did not differ among the three superovulation regimes.

Use of FSH by commercial embryo transfer units, the need for administration of FSH over a number of days, and the desire to economically and physiologically optimize superovulation, has stimulated evaluation of the number of doses administered per day as well as the dose-response relationship. After comparing number of FSH injections per day and administration of a constant dose or decreasing doses, Chupin and Procureur
(1982) concluded that a total dose of 32 mg of FSH administered twice daily over a period of 4 d in decreasing doses yielded the most corpora lutea, total embryos, and transferable embryos. In contrast, Looney et al. (1981) reported that superovulatory responses to single, constant dose injections of FSH administered over a 4 d period were equal to responses of FSH administered in constant doses, twice daily over a 4 d period.

Pawlyshyn et al. (1986) compared superovulatory response to FSH administered over a 4 d period in a decreasing dose regime using total doses of 15, 30, 45, and 60 mg. Total number of corpora lutea and ova/embryos did not differ between doses of 30, 45, and 60 mg, but were significantly greater than 15 mg. A dose-response curve to FSH was produced from research conducted by Donaldson (1984). Number of transferable and total embryos was highest (5.9) using 28 mg of FSH and decreased with doses of 38, 40, 50, and 60 mg. Donaldson (1984) also found no advantage in using a 5 d treatment over a 4 d treatment regime or in using a constant over a decreasing dose regime.

Three FSH superovulation regimes were used for the three experiments described in this dissertation. Additionally, some of the morulae used in the splitting versus bisection
study were collected from cows that were stimulated with PMSG. The following is a retrospective analysis of superovulatory response to the four superovulation regimes.

MATERIALS AND METHODS

One hundred seven superovulations were performed over a 30 month period. Cows were observed for estrus twice daily and those showing estrus were selected for superovulation between d 9 and 13 of the cycle. Cows were rectally palpated to confirm presence of a corpus luteum before initiation of superovulation. The total FSH dose ranged from 32 to 40 mg (Schering Corporation, Kenilworth, NJ) and was administered over a 3.5 or 4.0 d period in 7 or 8 injections (i.m.) at 12 h intervals. Doses of 35 and 40 mg of FSH were administered in a consistent dose of 5 mg, while cows received the 32 mg dose in a decreasing dose regime (Table 7). Pregnant mare’s serum gonadotropin (Diosynth, Inc., Chicago, IL) was administered in a single 2500 IU dose (i.m.).

Heat mount devices (Kamar*; Steamboat Springs, CO) were placed on the cows according to the manufacturer’s recommendations to assist visual observation of estrus. Embryo donors that were superovulated with FSH-3 and underwent surgical embryo
collection were fitted with heat mount devices (Livestock Management Systems; Kernersville, NC) that were a combination of a physical pressure sensor, computer chip, circuit board, liquid crystal digital display (LCD), and 3 rechargeable cadmium batteries. The pressure sensor (16.5 cm in length) was encased in polyvinyl tubing and the LCD, computer chip, circuit board, and batteries were encased with fiberglass (8.5 x 12.8 x 34 cm, weighing 196 g). The pressure sensor was mounted over the sacral vertebrae in a position that would allow the brisket of the mounting cow to contact the sensor with the fiberglass portion of the device lying in the concave area between the tuber sacral and caudal vertebrae. The device could be charged for up to 220 h and recorded and displayed the time of the first mount, last mount, and the total number of mounts. One also could retrieve the number of mounts for each hour after the initiation of mounting activity. The device recorded as one mount pressure that was exerted for at least 2.5 sec.

Prostaglandin F$_{2\alpha}$ (Lutalyse; Upjohn, Kalamazoo, MI) was administered in two 12.5 mg injections with the last two FSH injections or 48 and 60 h after PMSG. All cows were observed for estrus at least twice daily. Cows superovulated with the
Table 7. Superovulation regimes.

<table>
<thead>
<tr>
<th>Regime</th>
<th>n(^1)</th>
<th>Total Dose</th>
<th>Day of Superovulation(^2,3)</th>
<th>cost(^6) ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH-1</td>
<td>12</td>
<td>40(^4)</td>
<td>5:5 5:5 5:5 5:5</td>
<td>49.65</td>
</tr>
<tr>
<td>FSH-2</td>
<td>25</td>
<td>35(^4)</td>
<td>0:5 5:5 5:5 5:5</td>
<td>43.85</td>
</tr>
<tr>
<td>FSH-3</td>
<td>49</td>
<td>32(^4)</td>
<td>6:6 5:5 3:3 2:2</td>
<td>40.37</td>
</tr>
<tr>
<td>PMSG</td>
<td>24</td>
<td>2500(^5)</td>
<td>2500(^5) - - -</td>
<td>12.63</td>
</tr>
</tbody>
</table>

\(^1\) n=number of cows superovulated.

\(^2\) 1,2,3, or 4 refer to d 9 to 13 of the estrous on which superovulation was initiated.

\(^3\) Superovulation started AM for FSH-1, FSH-3 and PMSG treatments and PM for FSH-2.

\(^4\) mg

\(^5\) IU

\(^6\) 1992 prices: $1.16/ mg FSH, $3.25/25 mg PGF\(_{2\alpha}\), and $9.38/2500 IU PMSG.
FSH-2 (35 mg) treatment were artificially inseminated at 0, 12, and 24 h after the first observation of estrus. Cows superovulated by the FSH-1 (40 mg), FSH-3 (32 mg), and PMSG treatments were bred naturally at 0 h post-estrus and artificially at 12 and 24 h after the first observation of estrus.

Embryos were recovered 24 to 72 h post-estrus from cows superovulated with FSH-1, FSH-2 and FSH-3 superovulation regimes. Ovaries and oviducts were collected at slaughter from cows receiving FSH-1 or FSH-2 superovulation treatments. Embryo recovery was as described in Chapter 3. Approximately half of the cows receiving the FSH-3 treatment underwent surgical embryo collection 24 to 63 h post-estrus as described in Chapter 5. Morulae were recovered from the remaining FSH-3 cows and all cows superovulated with PMSG by nonsurgical uterine flush 6 d post-estrus as described in Chapter 4.

Response to PGF₂α, time from PGF₂α to first observation of estrus, total number of ova/embryos recovered, and morula quality were analyzed by analysis of variance with superovulation treatment and day of the estrous cycle that superovulation was initiated (initiation day) serving as
independent variables. Frequency of response to PGF$_{2\alpha}$ by superovulation treatment was tested using Chi-square analysis.

RESULTS AND DISCUSSION

The mean interval between the first PGF$_{2\alpha}$ injection and first observation estrus ranged from 33.3 to 41.8 h and did not differ between superovulation regimes (Table 8) nor was it affected by initiation day. These intervals to estrus were shorter than the 42 to 45 h interval reported by Donaldson (1983) and 50 to 56 h intervals to estrus observed by Rajamahendran et al. (1987). More recently, Ellington et al. (1990) reported that superovulated cows prepared for collection of one-cell embryos had a first PGF$_{2\alpha}$ injection to estrus interval of 36-48 h. Shorter intervals between the first PGF$_{2\alpha}$ injection and estrus may be due to the fact that the window for collection of one-cell fertilized embryos appears to be smaller (1 d) than the window for collection of morulae (1.5 d) and the frequency and intensity of observation for estrus may be greater when collecting one-cell fertilized embryos. We also had precise mounting activity information from nine animals that were fitted with the digital heat mount detectors (Table 9).
Superovulation initiation day had no effect (P > 0.05) on the total number of 1 to 3 or 6 d embryos recovered or on the number of good and excellent quality embryos recovered on d 6. Donaldson (1984) and Hasler et al. (1983) reported similar results with superovulation initiation between d 9 and 13 of the estrous cycle. Superovulation treatment and initiation day had no effect (P > 0.05) on the proportion of cows responding to PGF$_{2\alpha}$ (Table 8). Donaldson (1983) reported a response rate of 86.6% to two injections of PGF$_{2\alpha}$ (50 mg total) in cows superovulated with FSH.

Total number of embryos collected 1 to 3 d post-estrus was significantly higher (P < 0.05) after superovulation with FSH-1 (19.0; 40 mg) than FSH-2 (9.0; 35 mg), but neither treatment was different from superovulation with FSH-3 (13.8; 32 mg; Table 10). Total number of embryos collected was not affected by superovulation initiation day. These results are similar to previous reports (Ellington et al., 1990; McEvoy and Sreenan, 1990). Ellington et al. (1990) surgically recovered an average of 17 embryos from cows superovulated with 20 mg of highly purified FSH (Folitropin; Vetrepharm, London, Ontario, Canada) which was comparable to 35 mg of NIH-FSH-S-1 standard. McEvoy and Sreenan (1990) superovulated embryo donors with 2000 IU PMSG and surgically recovered 10.8 embryos per donor.
Table 8. Hours to estrus in superovulated cows after the initial prostaglandin $F_2\alpha$ injection.

<table>
<thead>
<tr>
<th>Superovulation</th>
<th>Time (h)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>$\bar{x}$</td>
<td>SE</td>
<td>$n^1$ (%)</td>
</tr>
<tr>
<td>FSH-1</td>
<td>38.0</td>
<td>4.1</td>
<td>11 (92)</td>
</tr>
<tr>
<td>FSH-2</td>
<td>41.8</td>
<td>3.2</td>
<td>23 (92)</td>
</tr>
<tr>
<td>FSH-3</td>
<td>40.4</td>
<td>2.4</td>
<td>43 (88)</td>
</tr>
<tr>
<td>PMSG</td>
<td>33.3</td>
<td>4.3</td>
<td>16 (80)</td>
</tr>
</tbody>
</table>

$^1$ Number of cows observed in estrus.
Table 9. Time between the first injection of \( \text{PGF}_{2\alpha} \) and the first mount triggering a digital heat mount device for cows superovulated with FSH-3.

<table>
<thead>
<tr>
<th>Cow Number</th>
<th>Hours between 1st ( \text{PGF}_{2\alpha} ) Injection and first recorded mount</th>
</tr>
</thead>
<tbody>
<tr>
<td>2248</td>
<td>48</td>
</tr>
<tr>
<td>2267</td>
<td>38</td>
</tr>
<tr>
<td>2329</td>
<td>39</td>
</tr>
<tr>
<td>2509</td>
<td>44</td>
</tr>
<tr>
<td>2052</td>
<td>36</td>
</tr>
<tr>
<td>2749</td>
<td>36</td>
</tr>
<tr>
<td>2618</td>
<td>45</td>
</tr>
<tr>
<td>2560</td>
<td>36</td>
</tr>
<tr>
<td>2487</td>
<td>48</td>
</tr>
</tbody>
</table>
In contrast, Kraemer et al. (1985) superovulated crossbred donor heifers with 50 mg of FSH administered in a constant dose of 5 mg twice daily for 5 d and recovered only 6.9 embryos per donor.

Embryo cell number was affected by the time between estrus and embryo recovery ($P<.001$; Figure 4). The best time to recover fertilized one-cell embryos was between 44 and 53 h post-estrus, with recoveries prior to 44 h post-estrus having more a greater proportion of unfertilized ova (Chapter 3 and 5).

Superovulation with FSH-3 yielded 7.1 d 6 ova/embryos compared to 5.1 with PMSG-induced superovulation (Table 11). The PMSG superovulated cows had more unfertilized ova ($2.1; P<.05$) than cows superovulated with FSH-3 (0.7). However, more poor quality morulae were recovered from FSH-3 superovulated cows ($1.0; P<.05$) than PMSG superovulated cows (0.2). Almeida (1987) collected an average of 4.8 d 7 embryos from cows superovulated with 3,000 IU PMSG. Twenty-one percent of the ova was unfertilized and 20% was classified as degenerate. Approximately 41% of the ova recovered from PMSG superovulated cows in our study was unfertilized and 14% was classified as degenerate.
The number of embryos recovered from FSH-3 was similar to a superovulation study conducted in our laboratory (Rajamahendran et al., 1987), where 5.5 embryos were recovered from dairy cows superovulated with 32 mg FSH administered in a decreasing dose regime. These results are lower than the 13.4 d 6 embryos recovery rate reported by Chupin and Procureur (1982) and higher than the 3.7 d 6 embryos recovered by Looney et al. (1981). Ten percent (0.7) of the embryos recovered from FSH-3 superovulated cows was classified as unfertilized and 40% (2.9) had degenerated. Fertilization rate was higher than the 60 to 70% fertilization rate reported by a commercial embryo transfer unit (Hasler et al., 1983), but was lower than the 98% fertilization rate reported by Rajamahendran et al. (1987). The relatively high fertilization rate (90%) in our study may be attributed to use of natural service for the first insemination rather than artificial insemination (Saacke, personal communication).

Addition of the mean number of good and excellent quality morulae in Table 11 shows that 1.5 (29%) and 2.7 (38%) of the 5.1 and 7.1 embryos recovered from PMSG and FSH-3 superovulated cows would be classified as transferable (Renard and Heyman, 1979), which was lower than the 2.9 and 6.7
Table 10. Least squares mean (± SE) number of embryos per superovulation treatment for oviductal stage embryos collected surgically or at slaughter 1 to 3 d post-estrous and uterine stage embryos collected nonsurgically 6 d post-estrous.

<table>
<thead>
<tr>
<th>Superovulation Treatment</th>
<th>Interval Between Estrus and Embryo Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>---- 1-3 d</td>
</tr>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>FSH-1</td>
<td>12</td>
</tr>
<tr>
<td>FSH-2</td>
<td>25</td>
</tr>
<tr>
<td>FSH-3</td>
<td>26</td>
</tr>
<tr>
<td>PMSG</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> means with different superscripts within column differ (p<.05).
Figure 4. Mean cell number in embryos collected between 24 and 72 h after first observation of estrus.
Table 11. Least squares mean (± SE) number of excellent, good, fair, or poor quality morulae and unfertilized and degenerate embryos for superovulations using FSH and PMSG.

<table>
<thead>
<tr>
<th>Embryo Grade</th>
<th>Superovulation Treatment</th>
<th>-- PMSG --</th>
<th>-- FSH-3 --</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X</td>
<td>SE</td>
<td>X</td>
</tr>
<tr>
<td>Excellent</td>
<td>0.8</td>
<td>0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Good</td>
<td>0.7</td>
<td>0.4</td>
<td>1.1</td>
</tr>
<tr>
<td>Fair</td>
<td>0.5</td>
<td>0.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Poor</td>
<td>0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.3</td>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Degenerate</td>
<td>0.7</td>
<td>0.9</td>
<td>2.9</td>
</tr>
<tr>
<td>Unfertilized</td>
<td>2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5</td>
<td>0.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>5.1</td>
<td>1.2</td>
<td>7.1</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> means in the same row with different superscripts differ (P<.05).
transferable embryos recovered from cows superovulated with a single injection of PMSG or FSH, respectively (Eldsen et al., 1978). However, the number of transferable embryos recovered from cows superovulated with FSH-3 was similar to the 3.8 transferable embryos recovered by Rajamahendran et al. (1987).

In conclusion, superovulation regime and initiation day did not affect time to observation of estrus after first injection of PGF$_{2a}$ or percent of cattle responding to PGF$_{2a}$. All four regimes stimulated growth and ovulation of multiple follicles. However, FSH-1 and FSH-3 were optimal for collection of oviductal stage embryos and FSH-3 yielded the most embryos collected on d 6.

REFERENCES


GENERAL CONCLUSIONS

The first experiment demonstrated that the explanted mouse oviduct cultured on tissue culture medium 199 (TCM-199) and 10% serum did not support development of one-cell bovine embryos beyond the 8-cell stage. Co-culture with oviductal epithelial cells (OEC) in TCM-199 and 10% serum was the best culture system for development one-cell bovine embryos to the morula/blastocyst stage. These results suggest that the mechanism by which the bovine OEC support ovum development is different from that of the explanted mouse oviduct.

Results of the second experiment suggested that morula biopsy and bisection are equally appropriate methods for acquisition of bovine embryonic cells for gene amplification. Mean final development score and mean cell number of bovine morulae that were subjected to microsurgery and subsequently cultured in vitro for 48 h were not affected by microsurgical treatment. Frequency of endogenous 18S rRNA amplification did not differ between demi-morulae and biopsies. Our inability to amplify the DNA in all cell samples may have been due to inadequate cell lysing procedures.

We opted to use bisection for acquiring cells from potentially transgenic bovine morulae in our third study. This decision
was based on the results from the second experiment and on our need for a relatively quick microsurgical method. Bisection was much faster than biopsy because it did not require a holding pipette and separation of the cell sample from the embryo was much easier.

The third experiment demonstrated that microinjection did not affect development of bovine embryos microinjected at the one-, two-, or four-cell stage. Stage of development at initiation of culture did affect the final development score with four-cell embryos reaching a higher mean final development score that one-cell embryos. Supplementation of culture medium with TGF-α when embryos reached the four-cell stage did not affect mean final development score, regardless of initial stage or microinjection.

Transgene detection frequency was higher in demi-morulae that developed from embryo microinjected at the one-cell stage than in embryos injected at the two- or four-cell stage. These results give support that the pronucleus is the preferred target for transgene microinjection. Low numbers of positive demi-embryos (n=19) did not allow us to establish successful pregnancies. We conclude that the combination of pronuclear microinjection, co-culture with OEC in vitro, embryo bisection, and the PCR technique can be used to facilitate
production and selection of transgenic bovine embryos for transfer to surrogate dams.

Retrospective analysis of superovulatory responses to various FSH regimes and PMSG revealed that superovulation protocol affected the number of embryos recovered 1 to 3 d post-estrus. Embryo recovery was best when cows were superovulated with 40 mg FSH administered in 5 mg doses twice daily for 4 d or 32 mg FSH administered in a decreasing dose regime twice daily for 4 d. Embryo recovery between 44 and 53 h after initial visualization of estrus was best for collection of fertilized, one-cell embryos. Number of embryos recovered by non-surgical uterine flush d 6 post-estrus was not different between cows superovulated with 32 mg of FSH or 2500 IU PMSG.
REFERENCES


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ABSTRACTS


