The Use of Cell Culture Systems to Improve Bovine Embryo Viability in Vitro.

Stephen Harry Pool
Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://repository.lsu.edu/gradschool_disstheses

Recommended Citation

This Dissertation is brought to you for free and open access by the Graduate School at LSU Scholarly Repository. It has been accepted for inclusion in LSU Historical Dissertations and Theses by an authorized administrator of LSU Scholarly Repository. For more information, please contact gradetd@lsu.edu.
INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
The use of cell culture systems to improve bovine embryo viability in vitro

Pool, Stephen Harry, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1990
The Use of Cell Culture Systems to Improve Bovine Embryo Viability In Vitro

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Animal Science

by

Stephen H. Pool
B.S., West Virginia University, 1975
M.S., Louisiana State University, 1980
May, 1990
ACKNOWLEDGEMENTS

The author would like to express his greatest appreciation to Dr. Robert A. Godke for his guidance during his graduate studies and in the preparation of this dissertation. In addition, the author is indebted to the members of his graduate committee for their guidance and suggestions in the preparation of this manuscript.

The author would also like to extend special thanks to fellow graduate students and particularly to Rick and Rebecca Rorie for their assistance during the first year of the author's graduate studies and to Klaus Wiemer, Joseph Prichard and Griff Blakewood for their assistance in embryo collection for these and other research projects. A special thanks is extended to Klaus Wiemer for supplying the fetal uterine fibroblast cells for use in these studies.

Finally the author would like to extend his love and gratitude to his wife Wanda and two children Bradley and Jennifer for their understanding and unending love and support during the many hours spent in the laboratory during data collection and during the preparation of this manuscript.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>ix</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xi</td>
</tr>
<tr>
<td>CHAPTER I. LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>PART A. IN VITRO CULTURE OF MAMMALIAN EMBRYOS</td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Culture conditions necessary for mammalian embryo development in vitro</td>
<td>2</td>
</tr>
<tr>
<td>Media, pH, Osmolarity, Gaseous Atmosphere and Temperature</td>
<td>2</td>
</tr>
<tr>
<td>Media</td>
<td>2</td>
</tr>
<tr>
<td>pH</td>
<td>6</td>
</tr>
<tr>
<td>Osmolarity</td>
<td>8</td>
</tr>
<tr>
<td>Gaseous Atmosphere</td>
<td>8</td>
</tr>
<tr>
<td>Temperature</td>
<td>12</td>
</tr>
<tr>
<td>Medium Supplementation</td>
<td>12</td>
</tr>
<tr>
<td>Early Studies on In Vitro Development of Bovine Embryos</td>
<td>15</td>
</tr>
<tr>
<td>Bovine Embryo Morphology and Viability Assessment in Vitro</td>
<td>21</td>
</tr>
<tr>
<td>PART B: IN VITRO CO-CULTURE OF MAMMALIAN EMBRYOS.</td>
<td>26</td>
</tr>
<tr>
<td>Fibroblast Cell Co-culture</td>
<td>26</td>
</tr>
<tr>
<td>Oviductal Cell Co-culture</td>
<td>29</td>
</tr>
<tr>
<td>Trophoblastic Vesicle Co-culture</td>
<td>31</td>
</tr>
</tbody>
</table>
CHAPTER II. CULTURE MEDIUM FOR THE GROWTH OF
BOVINE FETAL UTERINE FIBROBLAST CELLS AND
BOVINE TROPHOBLASTIC VESICLES ................. 35

Introduction ..................................... 35
Materials and Methods .......................... 36
Part A. Fibroblast Cells Used For In Vitro Culture ................. 36
Cell Counting using the Hemocytometer ................. 38
Part B. Trophoblastic Vesicles For In Vitro Culture ................. 38
Results .......................................... 39
Part A. Cell Growth Characteristics ................. 39
Part B. Post-Thaw Trophoblastic Vesicle Integrit y ................. 41
Discussion ....................................... 44

CHAPTER III. THE USE OF TROPHOBLASTIC VESICLES
AND FETAL UTERINE MONOLAYER CELLS FOR THE
CULTURE OF PRECOMPAC TION-STAGE BOVINE EMBRYOS... 47

Introduction ..................................... 47
Materials and Methods ............................ 47
Embryo Donors .................................. 47
Experimental Design ............................ 48
Cell Monolayer Preparation ...................... 49
Trophoblastic Vesicle (bTV) Production .......... 51
Embryo Recovery ............................... 51
Statistical Analysis ............................. 52
Results .......................................... 52
Embryo Recovery and bTV Production .......... 52
In Vitro Culture of Embryos ...................... 52
<table>
<thead>
<tr>
<th>Chapter Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>(cont'd)</td>
<td></td>
</tr>
<tr>
<td>Discussion.</td>
<td>55</td>
</tr>
<tr>
<td>CHAPTER IV. THE USE OF A PEPTIDE FROM TROPHOBLASTIC VESICLES AND FETAL</td>
<td>61</td>
</tr>
<tr>
<td>UTERINE MONOLAYER CELLS FOR THE CULTURE OF PRECOMPACTION-STAGE BOVINE</td>
<td></td>
</tr>
<tr>
<td>EMBRYOS.</td>
<td></td>
</tr>
<tr>
<td>Introduction.</td>
<td>61</td>
</tr>
<tr>
<td>Materials and Methods.</td>
<td>62</td>
</tr>
<tr>
<td>Embryo Donors.</td>
<td>62</td>
</tr>
<tr>
<td>Experimental Design.</td>
<td>63</td>
</tr>
<tr>
<td>Cell Monolayer Production.</td>
<td>64</td>
</tr>
<tr>
<td>Peptide Isolation.</td>
<td>64</td>
</tr>
<tr>
<td>Embryo Recovery.</td>
<td>65</td>
</tr>
<tr>
<td>Statistical Analysis.</td>
<td>66</td>
</tr>
<tr>
<td>Results.</td>
<td>66</td>
</tr>
<tr>
<td>Embryo Recovery.</td>
<td>66</td>
</tr>
<tr>
<td>In Vitro culture of Embryos.</td>
<td>66</td>
</tr>
<tr>
<td>Discussion.</td>
<td>70</td>
</tr>
<tr>
<td>CHAPTER V. LIPID PRODUCTION BY VARIOUS FEEDER CELLS AND BOVINE TROPHOBLASTIC</td>
<td>72</td>
</tr>
<tr>
<td>VESICLES (bTV) IN VITRO.</td>
<td></td>
</tr>
<tr>
<td>Introduction.</td>
<td>72</td>
</tr>
<tr>
<td>Materials and Methods.</td>
<td>75</td>
</tr>
<tr>
<td>Experimental Procedure.</td>
<td>75</td>
</tr>
<tr>
<td>Cell Monolayer Production.</td>
<td>76</td>
</tr>
<tr>
<td>Extraction and Fractionation of Lipids.</td>
<td>78</td>
</tr>
<tr>
<td>Gas Chromatography.</td>
<td>79</td>
</tr>
<tr>
<td>Results.</td>
<td>80</td>
</tr>
<tr>
<td>Discussion.</td>
<td>83</td>
</tr>
<tr>
<td>TABLE</td>
<td>TABLE CONTENT</td>
</tr>
<tr>
<td>-------</td>
<td>--------------</td>
</tr>
<tr>
<td>1.</td>
<td>Viability of frozen-thawed bovine trophoblastic vesicles (bTV) over time in four culture media</td>
</tr>
<tr>
<td>2.</td>
<td>Viability of frozen-thawed caprine trophoblastic vesicles (gTV) over time in four culture media</td>
</tr>
<tr>
<td>3.</td>
<td>Embryo viability assessments during in vitro culture of bovine embryos</td>
</tr>
<tr>
<td>4.</td>
<td>Bovine embryo development after 72 and 96 hours of in vitro culture</td>
</tr>
<tr>
<td>5.</td>
<td>Viability assessments of bovine embryos during in vitro culture</td>
</tr>
<tr>
<td>6.</td>
<td>Bovine embryo developmental stages after 72 and 96 hours of in vitro culture</td>
</tr>
<tr>
<td>7.</td>
<td>Quantities of triglycerides and phospholipids produced by various cell types in vitro</td>
</tr>
<tr>
<td>8.</td>
<td>Relative percentages of the different fatty acids produced by various cell types in vitro</td>
</tr>
<tr>
<td>9.</td>
<td>Arachidonic acid metabolites produced by caprine embryos at various days post estrus</td>
</tr>
<tr>
<td>FIGURE</td>
<td>PAGE</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>1. Cell growth pattern of fetal bovine uterine fibroblast cells in different culture media across days of incubation.</td>
<td>40</td>
</tr>
<tr>
<td>2. Experimental design for culture treatments for bovine embryos in Experiment II.</td>
<td>50</td>
</tr>
<tr>
<td>3. Time course for the formation of 6-keto-PGF$_{1\alpha}$ and PGE$_2$ from the endogenous precursor by day-15 bovine embryos.</td>
<td>94</td>
</tr>
<tr>
<td>4. Morphological changes of typical bovine embryos collected during the early stages of embryonic development.</td>
<td>99</td>
</tr>
<tr>
<td>5. Radiochromatographic separation of prostaglandins from embryo or trophoblastic vesicle samples incubated with $^3$H-arachidonic acid.</td>
<td>102</td>
</tr>
<tr>
<td>6. Immunochromatographic analysis of day-9 embryos incubated with cold arachidonic acid.</td>
<td>103</td>
</tr>
<tr>
<td>7. Radiochromatographic separation of lipoxygenase-derived products from embryo or trophoblastic vesicle samples incubated with $^3$H-arachidonic acid.</td>
<td>105</td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbr.</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibiotic</td>
</tr>
<tr>
<td>Ab-Am</td>
<td>Antibiotic-antimycotic</td>
</tr>
<tr>
<td>B₂</td>
<td>Menezo's B₂ Medium</td>
</tr>
<tr>
<td>BMOC-3</td>
<td>Brinster's Medium for Ovum Culture-3</td>
</tr>
<tr>
<td>BOC</td>
<td>Bovine oviduct cells</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>bTV</td>
<td>Bovine trophoblastic vesicle</td>
</tr>
<tr>
<td>CS</td>
<td>Calf serum</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl-sulfoxide</td>
</tr>
<tr>
<td>EB</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FBUC</td>
<td>Fetal bovine uterine cells</td>
</tr>
<tr>
<td>FBUFC</td>
<td>Fetal bovine uterine fibroblast cell</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FDA</td>
<td>3'6' diacetyl-fluorescein</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>GOC</td>
<td>Goat oviduct cell</td>
</tr>
<tr>
<td>gTV</td>
<td>Goat trophoblastic vesicle</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>HM</td>
<td>Holding medium</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>mOsm</td>
<td>Milliosmoles</td>
</tr>
<tr>
<td>Abbr.</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MHF-10</td>
<td>Modified Ham's F-10 Medium</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>N</td>
<td>Normal</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>Prostaglandin F₂α</td>
</tr>
<tr>
<td>PGFM</td>
<td>Prostaglandin F metabolite</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Prostacyclin (6-keto-PGF₁α)</td>
</tr>
<tr>
<td>SOF</td>
<td>Synthetic Oviductal Fluid</td>
</tr>
<tr>
<td>TCM-199</td>
<td>Tissue Culture Medium 199</td>
</tr>
<tr>
<td>WM</td>
<td>Whitten's Medium</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
ABSTRACT

Growth characteristics for fetal bovine uterine fibroblast cells (FBUFC) and frozen-thawed bovine (b) and caprine (g) trophoblastic vesicles (TV) were similar in (A) Ham's F-10 (HF-10), (B) MEM (with Earle's salts), (C) RPMI-1640 and (D) Menezo's B2 (B2), each supplemented with 10% FCS and Ab-Am (Experiment (Exp) I). Cell numbers declined initially but increased linearly thereafter for 5 days. The bTV and gTV that survived freezing had similar viability across time and Treatments (Trt).

Exp II compared the (A) bTV, (B) FBUFC monolayer and (C) bTV-monolayer co-culture systems to (D) HF-10 alone for culture of 8- to 32-cell bovine embryos. The embryos (%) considered viable at 48, 72, and 96 hours were: 80, 80, 83 and 78, 80, 73, 51 and 46, 27, 51, 41 and 5 for Trt A, B, C and D, respectively. Trt A, B and C had more viable (P<.05) and grade 1 and 2 (P<.05) embryos at 96 hours than Trt D. The number of hatched blastocysts was greater (P<.05) in Trt B and C than in A and D.

Exp III Trt were identical to Exp II except that a peptide from trophoblastic tissue was substituted in Trt A and C. Viable embryos (%) at 48, 72 and 96 hours were 55, 67, 55 and 39, 33, 55, 55 and 33 and 11, 44, 50 and 33 for Trt A, B, C and D, respectively. Greater numbers of viable (P<.05) and transferable quality (P<.05) embryos were in Trt B and C than in A and D. More embryos hatched in Trt B and
C than in Trt A and D.

Lipid and free fatty acid (FFA) production by bTV, FBUFC, FBUC, (fetal bovine uterine cells), BOC and GOC (bovine and goat oviduct cells, respectively) compared with B2 medium were evaluated (Exp IV). Phospholipid and triglyceride production was similar to that found in B2. FFA identified were C14-C20 and four unknowns.

Arachidonic acid metabolites (prostaglandins; PGF) produced by early bovine and caprine embryos was determined (Exp V). Bovine and caprine embryos produced 6-keto-PGF1α, PGF2α, PGE2 and PGFM.
CHAPTER I
REVIEW OF LITERATURE

PART A: IN VITRO CULTURE OF MAMMALIAN EMBRYOS

Introduction

Development of non-surgical techniques for the collection and transfer of bovine embryos (see review by Brand and Drost, 1977) was a major factor in developing a commercial embryo transfer business in the United States. Prior to this (early 1970's), embryos were collected at slaughter or surgically, involving risk to the animal, and at a much greater expense (see advantages and disadvantages of each technique in Brand and Drost, 1977). Over the years, the embryo transfer industry has grown from the simple collection and transfer of embryos into a growing field of embryo biotechnology involving the use of such techniques as cryopreservation, micromanipulation, in vitro fertilization, gene injection and embryonic cloning. Along with the development of these new technologies, the need for the development of culture systems for embryos that would maintain in vitro viability, both short- and long-term, became a necessity.

Culture systems were initially developed for mammalian embryos to meet two criteria. The first systems were developed to maintain embryos for long periods of time (days) and involved the study of culture conditions (i.e. media, pH, osmolarity, gaseous atmosphere and temperature) designed to
mimic the in vivo system. In addition, embryo morphology and development in vitro could be compared with that of in vivo. The second was short-term culture (hours) where embryos could be held with little loss of viability prior to transfer to recipients. Initial studies on in vitro culture conditions for mammalian embryos were conducted in laboratory species (mouse; Brinster, 1965a-d; rabbit; Maurer et al., 1968; Kane and Foote, 1971; Ogawa et al., 1971; Kane, 1972; hamster; Bavister et al., 1983; ferret; Whittingham, 1975) and provided models for the culture of domestic animal embryos (see reviews by Seidel, 1977; Wright and Bondioli, 1981) from the early stages (2- to 4-cells) through to the hatched blastocyst stage. Due to the nature of this review, the literature concerning in vitro development of embryos of the large domestic species (sheep, cow, horse, pig and goat) will be the main emphasis of this discussion. The remainder of this review will discuss the current in vitro conditions and the recent development of co-culture systems that have greatly improved embryo survival in vitro.

Culture Conditions Necessary for Mammalian Embryo Development In Vitro.

Media, pH, Osmolarity, Gaseous Atmosphere and Temperature.

Media. Over the years, embryos have been cultured in a variety of chemically defined and undefined media. A chemically defined medium has been described as a liquid containing four basic components; inorganic salts, amino
acids, vitamins and an energy source at known concentrations. (For lists of ingredients of several common defined media see; Menezo, 1976; Wright and Bondioli, 1981; Freshney 1987; Wright and O'Fallon, 1987). In contrast, undefined medium is a liquid where the composition and the components can vary considerably. Early embryo studies utilized undefined medium such as whole blood, blood sera, follicular fluid and chick egg extracts for culture because they had been shown to support growth of various cancerous cells in vitro (see Wright and Bondioli, 1981 for references).

Little is known about the requirements of inorganic salts in embryo culture media. However, it has been generally accepted that they provide ions necessary for maintenance of pH, osmolarity and metabolic functions. The primary salts provided by defined culture media include: Na⁺, K⁺, Mg²⁺, Ca²⁺, Cl⁻, SO₄²⁻, PO₄³⁻ and HCO₃⁻ (Menezo, 1983; Freshney, 1987). Salt (NaCl), has the primary function of regulating osmolarity, while carbonate (HCO₃⁻) and phosphate (PO₄³⁻) the regulation of pH. Wales (1970) and Ducibella and Anderson (1975) reported little effect of the absence of SO₄²⁻ on the development of mouse embryos in vitro, however, development was inhibited in the absence of K⁺, Ca²⁺, PO₄³⁻, Mg²⁺. Likewise, Whitten, (1971) reported that Ca²⁺ was necessary for the cleavage and compaction of mouse morulae.

Synthetic media have been developed based on the secretions of the sheep oviduct (Restal and Wales, 1966) and the
oviduct and uterus of the cow (Menezo's B2) (Menezo, 1976). These media contain many of the inorganic salts found in earlier defined media (e.g. Medium-199, Morgan et al., 1950; Minimum Essential Medium (MEM), Eagle 1955, 1959; CMRL-1066, Parker et al., 1957, Freshney, 1987).

Essential amino acids are provided in defined media because they are not synthesized by the embryo. In addition, some nonessential amino acids (primarily glutamine and asparagine) are added because they may be lost or destroyed during culture due to heat or light inactivation (Menezo, 1983).

The role of vitamins and minerals in defined media has not been clearly demonstrated. The amounts found in defined media are related to concentrations found in blood sera. Simple media contain primarily the B vitamins. The number increases as the complexity of the media increases. In studies on secretions of the oviduct and the uterus in cattle, Menezo (1976) found the secretions to have a reducing redox potential of -.1 mV. Menezo (1976) has mimicked this redox potential by the addition of ascorbic acid to his B2 medium. This is the only commercially-available medium at present that has taken into consideration the redox potential in its formulation.

In a recent report, Kane et al. (1988) studied the role of several water-soluble vitamins found in Ham's F-10 medium on the in vitro development of rabbit and hamster blasto-
cysts. They noted that the lack of several vitamins resulted in a loss of development in rabbit and hamster morulae in Ham's F-10 medium. The lack of inositol, pantothenate and choline significantly reduced the number of hamster embryos undergoing hatching with the most severe reductions caused by the lack of inositol (Kane et al., 1988).

Kane et al. (1988) have hypothesized that inositol and choline, both essential in the biosynthesis of phospholipid components, may contribute to membrane development in the growing or dividing hamster embryo. Pantothenic acid, an essential component of coenzyme A, may be involved in energy metabolism within the tricarboxylic acid cycle.

In the rabbit, inositol, pyridoxine, riboflavin, niacinamide and thiamine stimulated rabbit blastocyst expansion in vitro (Kane et al., 1988). The optimal level of inositol was found to be 75 $\mu$M which is 25 times the normal concentration in commercially available Ham's F-10 medium. Unlike the hamster, supplemental choline was not necessary for blastocyst expansion in the rabbit. Perhaps choline may have been synthesized from the amino acid serine (Lehninger, 1982). To date there are no reports on the importance of vitamin supplementation on embryonic development in the large domestic species.

The role of minerals in in vitro culture systems has not been established. Kane (1978) has suggested that the inorganic salts included in defined medium may be
contaminated with enough trace elements to supply the needs of cultured embryos. Recently, Freshney (1987) has reported that trace elements of iron, copper, zinc and selenium may be bound to serum proteins, however, the role of these trace elements in embryonic development is only speculative.

Energy is usually provided in defined media by combinations of lactate, pyruvate and glucose. Davis and Day (1978) have suggested, however, that porcine embryos have a requirement for alpha-ketoglutarate and that lactate and pyruvate inhibit development *in vitro*. Glucose may be the key energy source since it can be metabolized by glycolysis to form lactic acid and via the Krebs cycle to form \( \text{CO}_2 \). The products of glucose metabolism can then be used for the synthesis of embryonic lipids, amino acids and nuclear material (Menezo, 1983).

Since the development of the first chemically defined medium, a whole new approach to cell growth and development *in vitro* has taken place. Modifications in the concentrations of various components have further defined the *in vitro* environment necessary for continued embryonic development.

**pH.** The role of pH of the culture medium has rarely been studied as separate treatments in *in vitro* embryo culture systems of large domestic species. Sreenan et al. (1975) evaluated TCM-199 with either Earle's salts and bicarbonate buffer or Hank's balanced salts (HBSS) with HEPES buffer for
the short-term culture (≤7.5 hours) of bovine 8-cell to blastocyst stage embryos. A considerable change in pH of the TCM-199 with Earle's salts and bicarbonate was noted as culture time increased. The pH increased from 7.0 initially to 8.0 by 1 hour, and then increased to 8.5 by 3 hours of culture. In contrast, the TCM-199 with HBSS and HEPES buffer had little variation (a range of only 7.0 to 7.3 after 8 hours). Thirty-five of 58 (60%) and 30 of 52 (58%) bovine embryos developed into fetuses following transfer to recipients suggesting that the increased pH did not effect embryo viability in this short-term culture study (Sreenan et al., 1975).

Studies in the rabbit (Kane, 1974) and mouse (Brinster, 1965a) have indicated that a slightly alkaline pH between 7.2 and 7.6 was optimal for in vitro development. Wright and O'Fallon (1987) reported that most culture systems for cattle, swine and sheep embryos that promoted development had a pH ranging between 7.1 and 7.4. These studies indicated that the pH of the optimal culture medium may be within these limits.

A recent report by Carney and Bavister (1987) on the role of pH in the hatching of hamster embryos in vitro may change this assumption. Their study reported that the optimal pH range for development to the blastocyst stage could be expanded from 6.5 to 7.4. Hatching did not occur, however, unless the pH ranged from 7.1 to 7.4. This finding will
likely lead to additional studies concerning the role of pH on in vitro development and hatching of farm animal embryos.

Osmolarity. Two- to 4-cell embryos in the mouse, (Brinster, 1965a), rabbit (Naglee et al., 1969) and hamster (Bavister et al., 1983) have been shown to develop over a wide range of osmolarities (200 to 354 mOsm) in vitro. In the cow, Bowen et al. (1975) have reported an increase in development of bovine 2- to 8-cell and morulae-stage embryos in Modified Ham's F-10 medium (MHF-10) and SOF (synthetic oviductal fluid) (Tervit et al., 1972) at 270 when compared with 300 mOsm. In this study, the number of 2- to 8-cell and morulae-stage embryos developing in MHF-10 and SOF between 270 to 300 mOsm were 75% and 89% and 23% and 53%, respectively.

Based on the reported for oviductal and uterine secretions, Menezo (1983) and Seidel (1977) are in agreement that the optimal osmolarity to support embryonic growth in vitro for mammalian embryos should be 280 ± 20 mOsm. The osmolarity values of several commonly defined media used for the in vitro culture of farm animal embryos have been recently reported by Wright and O'Fallon (1987).

Gaseous Atmosphere. The two most common gaseous atmospheres used for the culture of mammalian embryos are 5% CO₂ in air or a combination of 5% CO₂, 5% O₂ and 90% N₂ (see reviews by Wright and Bondioli, 1981; Menezo, 1983; Wright and O'Fallon, 1987). The CO₂ is reported to be necessary to
maintain pH (within the range of 7.2 to 7.4) in a bicarbonate-buffering system, which is most commonly used to culture mammalian embryos (Menezo, 1983). The N₂ portion is considered to be inert (Wright and O'Fallon, 1987). The use of 5% O₂ was first reported for the in vitro culture of mouse embryos by Quinn and Harlow (1978), when they reported that higher concentrations of O₂ (20% and 40%) resulted in a reduction of embryos developing to the blastocyst stage. Furthermore, the blastocysts that did develop at higher oxygen tensions had fewer blastomeres than those which developed under 5% O₂ (Quinn and Harlow, 1978). Menezo (1983) has also reported that 5% O₂ produces a P0₂ level that tends to mimic the environment in vivo.

As with studies involving osmolarity, very little information concerning the testing of gaseous atmospheres as separate treatments appears in the published literature for farm animal embryos. Seidel (1974) cultured 8-cell to morula-stage embryos in covered glass dishes in air up to 10 hours in TCM-199 with HEPES buffer and 5% serum. After transfer to recipients, 53% of the embryos developing into fetuses. This system was compared with that of placing embryos in MHF-10 medium covered with oil in a gaseous atmosphere of 5% CO₂ and 95% air. Although pregnancy rate following transfer was lower (43%), it was not different from those cultured in TCM-199.

Bowen et al. (1975) also reported no differences in the
number of 2- to 8-cell or morulae-stage bovine embryos that developed in atmospheres of 5% CO₂ in air compared with 5% CO₂, 5% O₂ and 90% N₂ in MHS-10, SOF supplemented with amino acids or SOF under paraffin oil.

In sheep, Tervit et al. (1972) tested varying the oxygen level in a 5% CO₂ gaseous atmosphere from 0% to 5%, 10% or 20% compared with the control (air) and noted an increase in the number of 8-cell embryos that developed into blastocysts in SOF incubated at 37°C. No embryos developed to the blastocyst stage in the gaseous atmospheres evaluated except for the 5% CO₂, 5% O₂ and 90% N₂ in which 21% of the embryos developed to blastocysts.

In contrast, Wright et al. (1976b) tested seven different culture media at two different gas atmospheres (5% CO₂ in air vs. 5% CO₂, 5% O₂, 90% N₂) for the development of 8-cell ovine embryos from both adult and prepuberal ewes. Although there were differences in the cleavage indices for the various media evaluated, development of embryos from both the adult and prepuberal ewes was not different in the two gas atmospheres, and there was no culture media by gas atmosphere interaction. In a recent report by Betterbed and Wright (1985), no differences were reported in the development of 1-cell ovine embryos in BPM (Brinster's Pyruvate Medium), with or without .1% glucose, in atmospheres of 5% CO₂ in air or 5% CO₂, 5% O₂ and 90% N₂.

These studies tend to indicate that the culture medium
rather than the gaseous atmosphere may play a critical role in the development of early-stage bovine and ovine embryos in vitro.

Swine embryos of various stages (2-cell to morulae) cultured in WM (Whitten's Medium) (Whitten and Biggers, 1968) or MHF-10 with two levels of bovine serum albumin (BSA) Fraction-V (1 or 15 mg/ml) at two different atmospheres (5% CO₂ in air or 5% CO₂, 5% O₂ and 90% N₂) develop similarly to the bovine and ovine embryos (Wright, 1977). In this study the culture medium influenced the number of embryos that underwent cleavage. However, unlike the bovine and ovine, the gaseous atmosphere did affect the number of porcine embryos undergoing cleavage in vitro. The reduced oxygen atmosphere of 5% CO₂, 5% O₂ and 90% N₂ was shown to be superior over the 5% CO₂ in air in promoting the development of swine embryos in vitro. One interesting finding in this study was that only those embryos in the reduced oxygen atmosphere underwent hatching. This appears to be the only report of the role of gaseous atmosphere on the in vitro hatching of embryos for the large domestic species.

Recently, Carney and Bavister, (1987) reported that a doubling of the concentration of CO₂ in the gas phase from 5% to 10% in a bicarbonate-buffered medium (TALP) (Bavister et al., 1983) doubled the percentage of 8-cell hamster embryos that developed to the blastocyst stage. It was proposed that the beneficial effect of high concentrations
of CO₂ on blastocyst development was due to the action of CO₂ as a weak acid in regulating intracellular pH (pHᵢ). A test of their hypothesis using media containing several concentrations of weak organic acids under 5% CO₂ supported their theory. This finding may lead to additional studies to evaluate the role of CO₂ in the regulation of intracellular pH in the farm animal species.

**Temperature.** Unfortunately, little information is available on comparison studies on the effect of temperature on mammalian embryo development in vitro. Two temperature ranges are usually cited; one for short-term storage, (hours) from collection to transfer or cyropreservation (20 to 25°C) (Willadsen et al., 1978) and one for long-term (days) in vitro (35 to 37°C) (see reviews by Seidel, 1977; Wright and Bondioli, 1981). Willadsen et al. (1978) have reported that late morula to early blastocyst stage embryos cultured in phosphate-buffered saline (PBS) with 20% serum up to 24 hours at room temperature (20°C) followed by transfer to 37.5°C for an additional 24 hours was not detrimental to embryo survival.

**Medium Supplementation.** By far the most common media supplement used in embryo culture is heat-inactivated serum (Chang, 1949). Many types of sera have been used in the culture of embryos including, newborn calf serum (i.e. fetal calf serum), fetal bovine serum, steer serum, ovine serum, human cord serum, porcine serum and homologous donor sera,
to name a few. However, the most widely used serum reported in the literature is of bovine origin (Wright and Bondioli, 1981; Freshney, 1987; Wright and O'Fallon, 1987). Serum contributes a wide variety of constituents including: amino acids, hormones and growth factors, lipids, inorganic compounds, sugars, vitamins and minerals (Menezo, 1983). For a listing of some stimulatory factors (hormones and proteins) found in serum see Barns and Sato (1980). Over the years it has been exceedingly difficult to analyze the role that each serum component has on embryo development largely due to the variation in serum from batch to batch. Recently a newly developed synthetic serum (Nu-Serum®) has reduced the potential need for natural serum. Early embryo culture of ≤4-cell embryos in serum-free media has resulted in few embryos in the farm animal species undergoing greater than one or two cleavage divisions in vitro (Wright and Bondioli, 1981).

No discussion on the role of serum on the basic culture medium of embryos can be complete without some knowledge of the complimentary role of the serum constituents. The roles of amino acids, inorganic salts, simple sugars, vitamins and minerals in culture media has previously been mentioned. Serum lipids (phopholipids, triglycerides, cholesterol and free fatty acids) are primarily utilized for the synthesis of cell membranes as the embryo undergoes cleavages. Without the addition of serum, as in serum-free medium, lipids
must be added (usually as phospholipids and/or cholesterol) (Menezo, 1983). Another primary source of lipids used in embryo culture media is bovine serum albumin.

Hormones and growth factors are important to stimulate cell proliferation and uptake of nutrients from the medium. For example, insulin and platelet-derived growth factor are considered to be two of the more important constituents found in serum. Insulin helps to promote the uptake of glucose from the medium and platelet-derived growth factor has mitogenic activity (Freshney, 1987). These, along with other hormones and growth factors, may stimulate metabolic processes necessary for the development of the embryo in vitro.

Not all components in serum are beneficial to the development of embryos in vitro (Menezo, 1983). Serum also contributes enzymes some of which are proteolytic and induce a degradation of the culture medium. Menezo (1983) has reported a drop in enzymatic activity in Medium B₂ + 15% serum compared with B₂ alone as culture time increased from 24 to 48 hours. In addition, there was an increase in the amount of urea present in the culture medium. It was suggested that the increase in enzymatic proteolysis could affect embryo surface components thus leading to a degradation in embryo quality.

A second major supplement to culture media is BSA. This macromolecule has generally been recognized as a protein
source in embryonic culture media, although its exact role in these studies has not been fully recognized. Kane (1978) has reported that commercially prepared BSA (Fraction V) is a relatively impure protein containing several low molecular weight proteins, fatty acids and steroids. In a more recent study, Kane (1985) reported the isolation of a low molecular weight (<10,000) extract of BSA that stimulated rabbit blastocyst cell division and expansion in \textit{vitro}. These results suggest that BSA contains a low molecular weight embryonic growth factor. Lindner et al. (1979) have also demonstrated a beneficial effect of the addition of BSA to the culture medium for ovine embryos.

**Early Studies on In Vitro Development of Bovine Embryos.**

Primarily due to the economic importance of embryo transfer to the seedstock cattle industry during the past two decades, research and development has concentrated efforts on gaining knowledge of developing embryos through the use of improved \textit{in vitro} culture techniques. Due to the high cost of research in cattle, and with the economic importance of sheep, swine and goats in Europe, Asia and Africa, development of culture procedures in these species has begun to flourish.

Prior to the late 1960's, bovine embryos collected either surgically or at slaughter were routinely cultured in egg-white or egg-yolk saline (Dowling, 1949), serum (Brock and Rowson, 1952; Wintenberger et al., 1953), serum-saline
(Hafez et al., 1963; Sreenan et al., 1968) or follicular fluid (Brock and Rowson, 1952; Thibault, 1966) and development in vitro was usually limited to one cleavage division. Transfers to recipients were rare and few pregnancies resulted.

In the late 1960's, however, there were several reports using chemically defined media (BMOC-3, Brinster, 1968; Ham's F-10 and Krebs-Ringer bicarbonate plus serum, Onuma and Foote, 1969; SOF, Sreenan et al., 1968; TCM-199, Rowson et al., 1969) for culturing of bovine embryos. Development, however, was limited to one or two cleavage divisions. Rowson et al. (1969) then reported the first acceptable pregnancy rate (65%) following transfer of short-term cultured bovine morulae to recipient females. In this study, embryos were collected surgically and cultured (.5 to 3 hours) in either TCM-199 or homologous serum. Transfer of 1 to 3 embryos per recipient resulted in pregnancy rates of 65% (13 of 20) and 0% (0 of 9), respectively. These encouraging results led scientists to continue to conduct research and begin evaluating the problems facing this new technology.

One of the first problems facing researchers was how to short-term culture embryos for subsequent transfer. In the years following the report of Rowson et al. (1969), other laboratories began to report varied success (as measured by the birth of live calves) with short-term culture followed
by transfer. Seidel (1974) reported 53% of the 8-cell to morula stage embryos (n = 88) cultured in air up to 10 hours in TCM-199 with HEPES buffer and 5% serum developed into fetuses after transfer compared with 43% of the embryos cultured in MHF-10 medium (covered with oil) in an atmosphere of 5% CO₂ and 95% air.

In the following year, Boland et al. (1975) reported that the number of embryos developing into fetuses (33%) following culture (0 to 3 hours) in PBS plus 15% serum increased compared with that of culturing with TCM-199 (17%). Embryos were cultured in air at 30°C in glass dishes and transferred non-surgically. In that same year, Sreenan et al. (1975) reported no differences in pregnancy rates (60.4% vs. 57.7%) for bovine morulae to blastocyst stage embryos cultured in air in TCM-199 with Earle's salts and bicarbonate buffer compared with that of TCM-199 with Hank's balanced salts (HBSS) with HEPES buffer. They did report, however, time in culture had a negative effect on pregnancy rates following transfer. Embryos cultured for ≤2 hours resulted in a higher pregnancy rate (72.6%) following transfer than those cultured for >2 hours and up to 8 hours (41.6%) prior to transfer, regardless of which medium was used (Sreenan et al., 1975). Gordon (1976), Trounson et al. (1976a,b) and Willadsen et al. (1978) have evaluated the use of a simple salt solution, Dulbecco's phosphate-buffered saline with serum for short-term culture of bovine embryos. Gordon
(1976) reported 60% of embryos (2-cell to morulae) cultured in PBS plus 15% fetal calf serum (FCS) continued development at 30°C in air compared with 5% in TCM-199 plus 15% FCS.

Trounson et al. (1976a) have reported a high rate of development (85 and 88%) for embryos (4- to 8-cell and morula stages) cultured in modified PBS in air for 1.5 to 7.5 hours at 18 to 21°C compared with that of TCM-199 (49 and 71%). In this study, viability was measured by development following transfer to rabbit oviducts.

In a second study, Trounson et al. (1976b) using morula stage embryos increased the temperature to 37°C, serum level to 20% and extended the culture interval to 48 hours. Following the transfer of cultured embryos to recipients a pregnancy rate of 61% was obtained as determined by the number of females carrying fetuses at slaughter 3 to 18 weeks post-transfer.

Willadsen et al. (1978) have reported that the culture of late morula to early blastocyst stage embryos up to 24 hours at 20°C in PBS plus 20% serum resulted in 100% embryo survival, however, culture at 20°C for 48 hours decreased embryo survival to 64%. These studies were the basis for room temperature (20 to 25°C) PBS plus 15 to 20% serum and antibiotics, being accepted for standard use by many commercial embryo transplant companies as a short term "holding medium" for embryos prior transfer.

One of the first successful long-term culture systems was
reported by Tervit et al. (1972) for the culture of sheep and cattle embryos. These researchers selected SOF, a medium with a pH 7.2 to 7.4 and an osmolarity of 270 mOsm, for bovine embryo studies. One-cell and 8-cell embryos were cultured in SOF in an atmosphere of 5% CO$_2$, 5% O$_2$ and 90% N$_2$ at 37°C for 144 and 96 hours, respectively. Fifty percent (3 of 6) of the 1-cell embryos developed to the 16-cell stage and 60% of the 8-cell embryos (3 of 5) developed to the blastocyst stage during the culture interval. Although the number was small in this study, the results were promising. This prompted other researchers (Shea et al., 1974; Bowen et al., 1975; Kanagawa et al., 1975) to further investigate the use of SOF in comparison with other defined media for the culture of bovine embryos.

Shea et al. (1974) cultured 8- to 12-cell bovine embryos in SOF supplemented with HEPES buffer under the same conditions as Tervit et al. (1972) and compared the results with that of BMOC-3 (Brinster, 1968). They reported 26% and 57% of the embryos developing into morulae in the SOF and BMOC-3 media, respectively. Two pregnancies resulted following the transfer of cultured embryos to 17 recipients. Similarly, Bowen et al. (1975) compared SOF and MHF-10 medium and reported results with 48% and 80% of 2- to 8-cell and morula stage embryos developing during the 48-hour culture interval. Correspondingly, Kanagawa et al. (1975) reported 65 to 80% of 8- to 32-cell embryos developing to the blastocyst
stage following 120 hours of \textit{in vitro} culture in both SOF and BMOC-3.

In 1976, several laboratories began comparing various media for the long-term culture of bovine embryos. These included PBS plus serum \textit{vs.} TCM-199 (Gordon, 1976); modified PBS \textit{vs.} TCM-199 (Trounson et al., 1976a,b); HF-10 with serum or BSA \textit{vs.} MEM, TCM-199, BMOC-3, SOF, Whitten's (Wright et al., 1976a,c) and BMOC-3 \textit{vs.} Menezo's $B_2$ medium (Renard et al., 1976). Embryos cultured in PBS plus serum in air at room temperature (Gordon, 1976; Trounson et al., 1976a,b) proved to be superior to TCM-199 with serum (60 to 85% development \textit{vs.} 5 to 50% development). Reduced development was thought to be due to the problem in maintaining pH between 7.0 to 7.3 in the bicarbonate-buffered medium.

The culture experiments by Wright et al. (1976a,c) at 37°C revealed that HF-10 plus 10% FCS in an atmosphere of either 5% $CO_2$, 5% $O_2$, 90% $N_2$ or 5% $CO_2$ in air was superior to all other media evaluated. These findings were very instrumental in helping to set the standards for \textit{in vitro} culture conditions of bovine embryos in the years that followed.

By the 1980's, the commercial embryo transfer industry had experienced considerable growth due to the increases in pregnancy rates following transfer of fresh and frozen-thawed embryos. Many cattleman, however, were unable to afford the costs of embryo transfer and the industry began
to search for ways to reduce procedural costs. Fetal calf serum, a component of collection, holding, freezing and transfer medium was in short supply, and very expensive. Research into the use of less expensive alternative sera, such as newborn calf serum (Allen et al., 1982) and steer serum (Allen et al., 1982; Rajamahendran et al., 1985; Smith et al., 1986) revealed that these sera could be substituted for the more expensive FCS without loss of embryo viability.

Great strides in the collection, transfer, cryopreservation and manipulation of bovine embryos have been made over the last 40 years. These would not have been possible without the knowledge of in vitro embryo development.

**Bovine Embryo Morphology and Viability Assessment In Vitro**

The success of any bovine embryo transfer program relies on the ability of the embryologist to differentiate between viable and nonviable embryos. A trained eye and knowledge of the developmental morphology associated with various stages of early embryo development is a necessity. It is difficult to determine viability by visual appraisal of embryos while viewing under a microscope. Several researchers have reported descriptive morphology of bovine embryos harvested at slaughter at different times post-mating (Hamilton and Laing, 1946; Chang, 1952; Betteridge and Flechon, 1988) and from superovulated donor females (Shea et al., 1976; Trounson et al., 1976b; Seidel, 1980; Shea, 1981; Lindner and Wright, 1983) following embryo
collection. These authors have provided a list of criteria for assessment of embryo stage (zygote, 2-cell, 4-cell through to the hatched blastocyst stage) and embryo quality (excellent, good, fair, poor). These criteria, including the mitotic index (Lindner and Wright, 1983), have been used to determine if embryos are at the proper stage at collection and during in vitro culture.

Generally, embryos are considered normal when the blastomeres are symmetrical and the embryonic stage corresponds to the expected stage based on the day of collection. Correspondingly, poor quality embryos have an asymmetrical cell mass, degenerate extruded cells and in the case of blastocysts, a collapsed blastocele. (Trounson et al., 1976b; Shea, 1976, 1981; Lindner and Wright, 1983). Other parameters based on appearance commonly used to evaluate embryo quality include: shape, color, number and compactness of cells, size of the perivitelline space, number of extruded and degenerated cells and the size of vesicles (Lindner and Wright, 1983).

One of the most widely accepted criterion to evaluate bovine embryos is whether the developmental stage corresponds to the expected stage at the time of collection (Elsden et al., 1978; Seidel, 1981; Shea, 1981). Elsden et al. (1978) have reported that pregnancy rates decreased sequentially following the transfer of excellent, good, fair and poor quality embryos (63%, 58%, 31% and 12%,
respectively). Quality was based on appearance as well as stage of development in relation to day of collection. Lindner and Wright (1983) reported similar but lower pregnancy rates of 45%, 44%, 27% and 20% for excellent, good, fair and poor quality embryos, respectively. It is interesting to note, however, that the pregnancy rate for poor quality embryos in both of these studies is >10%, indicating that subjective evaluation by trained embryologists is variable.

Over the last few years, development of the field of embryo biotechnology involving the use of techniques such as micromanipulation, cryopreservation, in vitro fertilization and embryonic cloning, often requires both short- and long-term in vitro cultures. This has created a need for a more positive efficient method of identifying viable embryos. Such a test must be rapid and relatively simple to perform, nontoxic to embryos and relate favorably with viability post-transfer.

Rotman and Papermaster (1966) introduced a test of viability of living cells based upon fluorochromatic principles. Using this approach, 3'6' diacetyl-fluorescein (FDA), being non-polar, readily crosses the cell membrane where it is hydrolyzed by esterases resulting in free fluorescein. The polar fluorescein cannot readily cross the cell membrane and therefore accumulates intracellularly where it can be visualized by fluorescence under UV light. The test is a measure
of both enzyme activity and membrane integrity. The use of the FDA viability test for mouse (Mohr and Trounson, 1980), bovine (Looney et al., 1982; Looney, 1984; Hoppe and Bavister, 1983), hamster (Hoppe and Bavister, 1983) and horse (Pruitt et al., 1988) embryos has been encouraging. Several reports have shown that FDA correlates favorably with standard subjective viability measurements for \textit{in vitro} cultured bovine embryos (Looney et al. 1982; Looney, 1984; Hoppe and Bavister, 1983) and pregnancy rates following transfer of FDA and control mouse (Mohr and Trounson, 1980), hamster (Hoppe and Bavister, 1983) and horse (Pruitt et al., 1988) embryos were not different. These studies indicate that FDA is not toxic to embryos of mice, hamsters and horses. The greatest drawback appears to be in the assessment of the degree of fluorescence. As with embryo grading the measure of fluorescence is subjective, and dependent upon the skill of the evaluator. The use of FDA as a sole test of embryo viability should be cautioned.

Recently, another fluorescent dye, ethidium bromide (EB), has been evaluated in mouse embryos in an attempt to predict embryo viability. Swanson et al. (1987) have reported that EB, which is capable of penetrating the cell membrane of dead or injured cells, intercalates into the DNA of the dead or injured cells and under UV light emits a red fluorescence. Correspondingly, in live cells no fluorescence is detected. Ethidium bromide was shown to be nontoxic to
mouse morulae and blastocysts cultured for 24-hours post-treatment in vitro. Viability was based on morphological development during the culture interval. Comparison of the viability of EB-treated and untreated control embryos following transfer to recipients was not reported. Additional studies are needed to further evaluate the EB fluorescence test as an indicator of viability for farm animal embryos.

Another method of measuring metabolic activity of embryos has been reported for mice (Gardner and Leese, 1987) and cattle (Renard et al., 1980, 1982) is to measure the in vitro uptake of glucose by embryos. Renard and du Mesnil du Buisson (1976) have reported that the ability of bovine blastocysts to hatch from the zona pellucida in vitro was glucose dependent (unpublished data as cited in Renard et al., 1980).

Attempts have been made to measure glucose uptake by embryos as a means of determining embryo viability (Renard et al., 1982). Unfortunately, the uptake of glucose by day-7 to day-8 blastocysts (embryos most often collected at commercial embryo transplant units) was not detected, however, glucose uptake by day-10 and day-11 blastocysts was monitored. There were no morphological differences between blastocysts that did or did not take up glucose. Eighty-one percent (48 of 59) of the embryos evaluated increased in size during the 20-hour culture interval, however, only 52.1% (25 of 48) had a significant uptake of glucose from
the culture medium. Development in vivo following recipient transfer of blastocysts resulted in higher pregnancy rates (at slaughter between days 45 and 52 post-transfer) for embryos that had taken up glucose (69.2%) than for those that had not (14.2%) (Renard et al., 1980).

In the mouse, glucose uptake has been correlated with embryo viability and sex (Gardner and Leese, 1987). Transfer of 50 blastocysts tested for glucose uptake prior to transfer, resulted in 25 (50%) male and 13 (26%) female offspring. Although not significant (P>.10), the glucose uptake of female embryos tended to be higher than male embryos.

PART B. IN VITRO CO-CULTURE OF MAMMALIAN EMBRYOS

Fibroblast Cell Co-Culture

Cole and Paul (1965) were the first to report the use of a feeder layer of irradiated uterine HeLa cells for the culture of early-stage (2-cell) murine embryos through to the blastocyst stage of development. These researchers noted that a higher portion of embryos hatched from the zona pellucida in the co-culture group than in medium (Waymouth's medium) alone. Following hatching, blastocysts attached to the feeder layer and initiated trophoblastic outgrowth. Similar results were reported in a later study when attachment and trophoblastic outgrowths of murine embryos were noted on monolayers of several transformed and non-transformed cell types (Glass et al., 1979).
Prior to the 1980's, the use of feeder layers for the in vitro culture of farm animal embryos had not been reported. Kuzan and Wright (1981) reported the first study for the culture of porcine embryos on to hatching. They compared a monolayer of bovine uterine or testicular fibroblasts to fibroblast conditioned medium and reported that 60% of the porcine embryos attached to the uterine fibroblast monolayer compared with only 4% on a testicular fibroblast monolayer. In addition, a higher percentage of porcine blastocysts hatched when placed on a monolayer of bovine fibroblasts than when placed in conditioned medium or fresh medium. The following year, Kuzan and Wright (1982a) reported that there were no differences between the number of porcine morulae that hatched from the zona pellucida when cultured on uterine and testicular monolayers, however, fewer expanded blastocysts were observed when embryos were co-cultured on the bovine testicular fibroblasts.

In the same year, Kuzan and Wright (1982b) evaluated the use of the bovine uterine and testicular fibroblast monolayers for the culture of bovine morulae. Contrary to the findings in the pig, bovine morulae developed equally well on the bovine uterine and testicular fibroblasts, however, better than those in conditioned or control medium. Furthermore, more embryos hatched when cultured on either monolayer than in medium alone. They concluded that the co-culture of bovine embryos with bovine fibroblasts was a
better system for promoting embryo hatching in vitro. Voelkel et al (1985) and Baker and Shea (1985) used feeder layers for the co-culture of bovine demi-embryos. The bovine uterine fibroblast monolayer (Voelkel et al., 1985) proved to be superior to that of a luteal cell monolayer (Baker and Shea, 1985) for the culture of demi-embryos. In fact, the luteal cells tended to have a negative effect on demi-embryo development compared with that of medium alone (Baker and Shea, 1985).

Recently, the use of fetal bovine uterine fibroblast cells (FBUFC) for the culture of bovine (Wiemer et al., 1987, 1988; Pool et al., 1988), equine (Wiemer et al., 1988) and human (Wiemer, 1989) embryos has proved superior over that of medium alone. Embryos cultured on the FBUFC have resulted in the live births of bovine, equine and human offspring (Wiemer, personal communication).

The source of the embryotropic properties exhibited by fibroblastic cells is unknown. Kuzan and Wright (1982b) have hypothesized that the helper cells may secrete an embryotropic substance(s) into the culture medium that is picked up by the embryos and/or the cells remove toxic products produced by embryonic metabolism. Correspondingly, Allen and Wright (1984) have suggested that cell to embryo contact is necessary for in vitro development of porcine embryos on uterine fibroblast cells. It was noted that porcine embryo development was retarded when embryos were
suspended by a thin membrane in conditioned medium above fibroblast cells compared with placement on fibroblast cells (Allen and Wright, 1984).

Oviductal Cell Co-Culture

Rexroad and Powell (1986) first evaluated the use of an ovine oviductal epithelial cell monolayer for the short-term culture of early-stage ovine embryos. In this study, embryos were transferred to recipients after 24 hours of culture and recovered 7 days later and compared with embryos transferred immediately following collection. The control and short-term co-cultured embryos had similar cleavage indices at recovery 7 days post-transfer. In follow-up studies, Rexroad and Powell (1988a,b) reported similar results for in vitro development of ovine embryos on oviductal cells. These studies prompted Rexroad and Powell (1988a,b) to suggest that the oviductal cells secrete embryotrophic substances important for early embryo development in the sheep.

Eyestone et al. (1987) introduced a similar oviductal epithelium cell culture system for early-stage bovine embryos. They reported that 5- to 8-cell bovine embryos cocultured on the bovine oviductal epithelium for 4 to 5 days resulted in 46% of the embryos developing to late morula or blastocyst stage embryos compared with none in Hams's F-10 medium alone (Eyestone et al., 1987). The results of this study were confirmed by a later report utilizing bovine
epithelial cell co-culture (Eyestone and First, 1988).

In a study designed to compare the oviductal cell monolayer to the uterine fibroblast monolayer, Gandolfi and Moor (1987) reported that sheep embryos could develop into blastocysts on fibroblast cells and oviduct cells. In this study, pronuclear-stage embryos were cultured for 3 or 6 days on cells or in medium alone. During the first 3 days of culture, all embryos on the monolayers (oviduct or fibroblast) cleaved similarly, however, only 13% cleaved in medium alone. After 6 days in culture 42% of embryos co-cultured with oviduct cells developed into expanded blastocysts compared with only 4.5% cultured on fibroblasts. Development in vivo following transfer to recipients revealed that ovine embryos cultured on fibroblasts were less likely to develop than embryos cultured on oviduct cells (Gandolfi and Moor, 1988).

Investigation into the secretory pattern of the in vitro cells of the oviduct compared with in vivo tubal secretions, revealed that both in vitro and in vivo cells of the oviduct secrete two proteins (a high and a low molecular weight). The secretion of the higher molecular weight protein declined sharply 96 hours post-ovulation while secretion of the low molecular weight protein increased. This change in secretory pattern corresponds to the passage of the embryo from the oviduct into the uterus. This secretory pattern was noted in both in vivo harvested cells (1 day post-ovula-
tion) and *in vitro* cultured oviduct cells. They have shown that the proteins can bind to the zona pellucida suggesting that these proteins have some role in the interaction of the sheep ovum with the oviduct.

**Trophoblastic Vesicle Co-Culture**

Another successful co-culture system for the *in vitro* culture of mammalian embryos was developed by Camous et al. (1984). This system utilizes embryonic tissue from the day-13 or day-14 bovine hatched blastocyst. Elongated embryos were dissected into pieces (embryonic disk discarded) and the trophectoderm allowed to develop into trophoblastic vesicles (bTV) for use in co-culture with precompaction-stage embryos. At the end of culture, 46% of the embryos (1- to 8-cell) in co-culture with bTV had developed into morulae compared with 18% in Menezo's B₂ medium (Menezo, 1976) alone. This was the first report of *in vitro* culture of bovine embryos through the 8- to 16-cell *in vitro* block described previously by Thibault (1966).

In that same year, Heyman et al. (1984) reported that the functional life of the corpus luteum could be extended by the ipsilateral noteral transfer of one or two bTV into the uterus of cyclic cattle. Examination of the uterine horns at slaughter (day-25 to day-28 post-estrus) revealed that the bTV had developed *in utero* (Heyman et al., 1984). Likewise, transfer of ovine trophoblastic vesicles to cyclic ewes increased estrous cycle length over that of the control
animals. These results indicated that early signals inhibiting luteolysis post-transfer of bovine and ovine embryos may be of trophoblastic origin. This finding confirms that after normal embryo transfer, increased cycle lengths in these species may be due to the development of trophoblastic tissue in utero.

Recent studies have shown that viability of co-cultured bovine embryos with bTV is greater than with medium alone (Heyman et al., 1987b) and that co-transfer of bovine embryos with frozen-thawed bTV also resulted in higher pregnancy rates post-transfer (Heyman et al., 1987a). In that same year, Rorie et al. (1987b) reported that co-culture of bovine demi-embryos with bTV improved in vitro survival over that of medium alone. Co-culture of ovine embryos with ovine trophoblastic vesicles (oTV) have revealed embryotropic responses in early-stage ovine embryos similar to those reported for the bovine embryo (Heyman et al., 1987b).

Trophoblastic vesicles have also been reported to develop from human trophectoderm (Menezo et al., 1987). Although there are no published reports of the co-culture of human embryos with human trophoblastic vesicles, their potential for improvement of human embryo survival in vitro could provide an alternative to culture in medium alone.

Unlike the fibroblast cell system previously described for porcine embryos (Allen and Wright, 1984), embryo to bTV
contact may not be necessary in order for bovine embryos to experience an embryotropic response. Heyman et al. (1987c) described that co-culture with "crossed medium" from bTV resulted in similar numbers of bovine embryos developing in vitro compared with those embryos co-cultured with bTV (38.6% vs. 41.8%, respectively).

With the aid of membrane ultrafiltration and Sephadex gel filtration of the ultrafiltrate, Heyman et al. (1987c) has been able to separate a high and a low molecular weight fraction from bTV conditioned medium. Supplementation of the culture medium with these fractions revealed that the low molecular weight fraction stimulated bovine embryonic growth over that of the high molecular weight fraction. It was concluded that bTV secrete low molecular weight compounds into the culture medium that are embryotropic to bovine embryos. Further inquiry into the nature of the embryotropic substance secreted by bTV has shown that bTV secrete three small peptides (Heyman and Menezo, 1987). No information on the embryotropic nature of these peptides was reported at that time.

Since bTV secrete embryotropic substances into the medium that are likely utilized by the developing embryo in vitro, Pool et al. (1988) conducted an experiment to evaluate if the concentration of these factors may be greater within the lumen of the vesicle. Early-stage bovine embryos were placed inside the lumen of bTV and development monitored
over a 96-hour culture interval. There were no differences detected between embryos co-cultured inside the bTV or in the medium surrounding the bTV suggesting that co-culture within the lumen of the bTV offered no advantage over co-culture with the bTV alone.

In summary, co-culture of early-stage bovine embryos on uterine fibroblasts (Kuzan and Wright, 1982b; Weimer et al., 1987), oviductal epithelial cells (Eyestone et al., 1987; Eyestone and First, 1988) and bTV (Camous et al., 1984; Heyman et al., 1987b,c; Heyman and Menezo, 1987) have resulted in improvement in embryo development over that of embryos cultured in medium alone. Research is currently underway in an attempt to identify the embryotropic properties of these various cell culture systems. However, the exact mechanisms and how they function remains unclear.

Comparative information is needed on the growth characteristics of the FBUFC monolayer (Wiener et al., 1987) and trophoblastic vesicles in various types of tissue culture media. In addition, direct comparisons of the FBUFC monolayer culture system and the bTV culture system for the in vitro culture of bovine embryos has not been evaluated. Finally, investigation into possible secretory products such as lipids and/or peptides may provide insight into how these culture systems exert their embryotropic effects on the development of bovine embryos in vitro.
CHAPTER II

EXPERIMENT I

VARIOUS CULTURE MEDIA FOR THE GROWTH OF BOVINE FETAL
UTERINE FIBROBLAST CELLS AND
TROPHOBLASTIC VESICLES

Introduction

Recent developments in the area of embryo culture have
shown that the use of feeder layers for the co-culture of
mammalian embryos offers an advantage over the culture of
embryos in medium alone (Cole and Paul, 1965; Kuzan and
Wright, 1982a,b; Allen and Wright, 1984; Voelkel et al.,
1985; Eyestone et al., 1987; Eyestone and First, 1988;
Gandolfi and Moor, 1987; 1988; Wiemer et al., 1987; Rexroad
and Powell, 1988a,b). In addition, the use of trophoblastic
vesicles in co-culture with bovine and ovine embryos has
offered an advantage over that of culturing in medium alone
(Camous et al., 1984; Heyman et al., 1987b,c; Heyman and
Menezo, 1987; Pool et al., 1988).

Although fetal bovine uterine fibroblast cell co-cul-
ture systems have been successfully used for culturing
embryos (Wiemer et al., 1987, 1988, 1989), little informa-
tion is available on the growth characteristics of these
fetal bovine uterine fibroblast cells (FBUFC) and tropho-
blastic vesicles in various tissue culture media. Therefore
a preliminary study was designed to evaluate the growth
characteristics of FBUFC and to evaluate viability of
frozen/thawed bovine (Rorie et al., 1987a) and caprine
(Pool, unpublished data) trophoblastic vesicles in four different commercial culture media.

Materials and Methods

Part A: Fibroblast Cells Used for In Vitro Culture

Fetal bovine uterine fibroblast cells have been previously established and supplied by Dr. Klaus Wiemer for this study. The cells had been originally established from explants from a bovine fetus (~270 days of gestation) as reported by Wiemer et al. (1988) using modified procedures previously described by Kuzan and Wright (1982b) and Voelkel et al. (1985). The FBUFC used in this study had been frozen in Ham's F-10 medium containing fetal calf serum (FCS) and 7% dimethyl-sulfoxide (DMSO), following seven subpassages. The 1 ml cryovials containing the cells were thawed in a 37°C water bath and the cryoprotectant (DMSO) removed by centrifugation following gradual addition of 10 ml of Ham's F-10 with 10% FCS and an antibiotic-antimycotic solution (Ab-Am) containing 100 units of penicillin, 100 μg of streptomycin and .25 μg of amphotericin-B per ml of medium (HF-10). The cells were then used to seed a 25 cm² tissue culture flask (Corning®: Corning, NY) and then allowed to form a monolayer while incubating (37°C) in a humidified atmosphere of 5% CO₂ in air.

Following the third post-thaw subpassage a 75 cm² flask containing a confluent monolayer of FBUFC was trypsinized (.025% trypsin; Sigma Chemical, St Louis, MO) and 1.2 X 10⁶
cells seeded into each of four 25 cm² flasks containing 50% of the respective treatment medium and 50% HF-10. The four treatment media were: (A) HF-10 (Ham, 1963); pH of 7.3, osmolarity = 290 mOsm; (B) RPMI-1640 (Moor et al., 1967); pH of 7.5, osmolarity = 283 mOsm; (C) MEM with Earle's salts (Eagle, 1955, 1959); pH of 7.6, osmolarity = 295 mOsm and (D) Menezo's B₂ medium (Menezo, 1976); (B₂) pH of 7.4, osmolarity = 282 mOsm each supplemented with 10% FCS and Ab-Am. After 48 hours, the media containing 50% of the treatment medium was replaced with 100% treatment medium. Following an additional 48 hours (96 hours of in vitro culture), the cells had monolayered and the flasks were trypsinized and the cells used to seed 15 wells of a 24-well tissue culture plate (Gibco, Grand Island, NY) at an initial seeding density of 1 X 10⁵ viable cells per well.

Cell growth characteristics were determined by allowing the cells to reattach and initiate growth for 24 hours prior to the first cell count. Twenty-four hours after seeding, three wells in each treatment group were trypsinized and the cells pooled to determine the mean number of cells/well. This procedure was duplicated every 24 hours up to 120 hours. The treatment media were replaced on all remaining treatment wells 72 hours after initial seeding. The experiment was replicated three times and the cell counts of each replicate averaged for an overall mean cell count at 24-hour intervals.
Cell Counting Using the Hemocytometer

Following trypsinization, the cells were resuspended in 500 µl of treatment medium and pooled in one well. A 100 µl aliquot was placed in 100 µl of a .04% Trypan Blue stain in Ca²⁺/Mg²⁺ free phosphate-buffered saline. Counts were then made using a hemocytometer (Richert, Buffalo, NY) at 100X magnification with an inverted Nikon Diaphot microscope. Details of the count determinations have been previously described by Freshney (1987). The equation used to determine the total number of viable cells was as follows:

\[
\text{Cells} = \frac{\text{(No. of viable cells)} \times (1 \times 10^5) \times \text{(dilution)}}{\text{No. of squares in hemocytometer}} \times \text{(Volume of the cell suspension)}
\]

The overall mean cell counts for the pooled well and replicates were plotted across time in culture resulting in a growth curve.

Part B: Trophoblastic Vesicles For In Vitro Culture

The bovine trophoblastic vesicles (bTV) used in this experiment (n = 116) had been previously frozen using the method reported by Rorie et al. (1987a), and stored in liquid nitrogen. The caprine trophoblastic vesicles (gTV) (n = 252) had been produced from day-15 caprine embryos (Pool et al. unpublished data) by a method similar to that previously described by Camous et al. (1984). The vesicles
were grown in MEM with 10% FCS and Ab-Am for 48 hours prior to loading into straws (5 to 7/straw) and frozen. After thawing and removal of the cryoprotectant (1.5 M glycerol), the viable bTV and gTV from each straw were equally distributed across four different treatment culture media and evaluated for their ability to re-expand and maintain their spherical shape and overall appearance during a 96-hour culture interval. The bTV and gTV were placed 3 to 4 per well per treatment in 24-well tissue culture plates and their viability assessed after 36, 60 and 96 hours of culture. The treatment media evaluated in this study were similar to those in Part A. The basel media included (A) HF-10 pH of 7.5, osmolarity = 275 mOsm; (B) RPMI-1640, pH of 7.7, osmolarity = 265 mOsm; (C) MEM; pH of 7.7, osmolarity = 288 mOsm and (D) B2, pH of 7.8, osmolarity = 287 mOsm.

Results

Part A: Cell Growth Characteristics

Cell growth was determined by counting the pooled sample of cells from three wells of a 24-well tissue culture plate over a period of 5 days. The cell counts/well were determined for each replicate, the means computed and then plotted across time in culture (Figure 1). A total of 1 X 10^5 cells had been initially seeded into each well. Cells, following a slight decline during the first 24-hour interval, showed a linear increase in cell number by treatment across time in culture. Mean cell counts by treatment
Figure 1. Cell growth pattern of fetal bovine uterine fibroblasts cells in different culture media across days of incubation. IPD = initial plating density, HF-10 = Ham's F-10, RPMI = RPMI-1640, MEM = minimum essential medium and B₂ = Menezo's B₂ medium.
(No. X 10^5 ± SE X 10^5) were: (A) .90 ± .10, .95 ± .11, 1.71 ± .50, 2.2 ± .57, 2.8 ± .51; (B) .72 ± .18, 1.55 ± .81, 2.35 ± 1.23, 2.76 ± 1.1, 3.69 ± .91; (C) .71 ± .48, 1.35 ± .80, 1.64 ± .78, 2.10 ± 1.01, and 2.37 ± .70; (D) .97 ± .44, 1.51 ± .19, 1.71 ± .49, 2.24 ± .46 and 2.98 ± .11 at 24, 48, 72, 96 and 120 hours of incubation, respectively.

Part B: Post-Thaw Trophoblastic Vesicle Integrity

The numbers (%) of viable appearing bTV and gTV placed in the four treatment media immediately post-thaw are presented in Tables 1 and 2, respectively. By 24 hours after thawing, the viable bTV and gTV had re-expanded and appeared spherical. The vesicles were pipetted up and down through a small-bore pipette to remove the dark rough-appearing dead cells in order to more readily identify the vesicles that were viable. After 36-hours in culture, the number of viable vesicles were easily identified each by their sphericity and lack of holes in the outer layers of the vesicle. There were no differences (P>.05) in the number of bTV that survived the freezing process and continued to develop in the four different culture media evaluated during the 96-hour culture interval (Table 1). Similarly, there were no differences (P>.05) in the number of gTV that survived freezing and developed in the different media evaluated (Table 2).
### TABLE 1. VIABILITY OF FROZEN-THAWED BOVINE TROPHOBLASTIC VESICLES (bTV) OVER TIME IN FOUR CULTURE MEDIA

<table>
<thead>
<tr>
<th>Medium</th>
<th>No./group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number(%) viable at 36 hours</th>
<th>Number(%) viable at 60 hours</th>
<th>Number(%) viable at 96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ham's F-10</td>
<td>29</td>
<td>10(34%)</td>
<td>10(34%)</td>
<td>10(34%)</td>
</tr>
<tr>
<td>MEM(Earle's)</td>
<td>29</td>
<td>9(31%)</td>
<td>9(31%)</td>
<td>9(31%)</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>29</td>
<td>9(31%)</td>
<td>9(31%)</td>
<td>9(31%)</td>
</tr>
<tr>
<td>Menezo's B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>29</td>
<td>5(17%)</td>
<td>5(17%)</td>
<td>5(17%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of bTV orginally placed in treatment medium.
TABLE 2. VIABILITY OF FROZEN-THAWED CAPRINE TROPHOBLASTIC VESICLES (gTV) OVER TIME IN FOUR CULTURE MEDIA

<table>
<thead>
<tr>
<th>Medium</th>
<th>No./group(^a)</th>
<th>Number(%) viable at 36 hours</th>
<th>Number(%) viable at 60 hours</th>
<th>Number(%) viable at 96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ham's F-10</td>
<td>63</td>
<td>23 (36%)</td>
<td>23 (36%)</td>
<td>23 (36%)</td>
</tr>
<tr>
<td>MEM(Earle's)</td>
<td>63</td>
<td>35 (55%)</td>
<td>24 (38%)</td>
<td>24 (38%)</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>63</td>
<td>14 (22%)</td>
<td>13 (21%)</td>
<td>11 (17%)</td>
</tr>
<tr>
<td>Menezo's B(_2)</td>
<td>63</td>
<td>21 (33%)</td>
<td>20 (32%)</td>
<td>19 (30%)</td>
</tr>
</tbody>
</table>

\(^a\)Number of gTV orginally place in treatment medium.
Discussion

The growth patterns of the FBUFC (Part A) in the four different culture media were similar. Cell growth showed a slight decline in cell number at the first cell count (24 hours after seeding) but increased linearly thereafter (Figure 1). This decrease in cell numbers may have been due to over trypsinization causing damage to the cell membrane leading to cell death. As cell density increased, the individual cells changed from a round flattened appearance to that of a thin elongated fibroblast cell. The more concentrated the cell number became (the closer to confluence) the cells appeared to line up in sheets with groups of cells clumping together leaving little space between cells.

Todaro and Green (1963) reported that cell numbers in culture will reach a plateau shortly after a confluent monolayer is achieved and then begin to decline when cells express "contact inhibition". Although the wells of cells across treatments appeared to monolayer (reach confluency) by 72 hours in culture, they did not exhibit contact inhibition as indicated by the linear increase in cell counts through the 120-hour culture interval. These cell counts suggest that FBUFC may require additional time to exhibit contact inhibition under the culture conditions of this study. In summary, FBUFC appeared to grow equally well in the four culture media evaluated.

The number of bTV (Part B) that survived freezing in
this study was considerably lower than that previously reported for bTV in CMRL-1066 medium by Rorie et al. (1987a). The reason for this is unknown, however, the culture time prior to freezing in this study was 48 hours, compared with 24 hours in the previous study. During this additional 24 hour period, some vesicles grew increased in diameter and had a greater vesicle surface area. The increased surface area provided a larger area exposed to the damaging effects of ice crystal formation during the freezing and thawing process (Mazur, 1979), perhaps leading to cell and consequently vesicle damage. This was evident when the vesicles were removed from the straws post-thaw. The surface of the vesicles was dark in color and had a rough appearance. This is not uncommon of trophoblastic vesicles following the freeze-thaw process due to the death of the outer layer of cells of the multilayered trophectoderm. In addition, there were fragments of vesicle tissue present in the cryoprotectant solution and still other vesicles had large surface rents. These vesicles and vesicle fragments (≈20%) were discarded and not used in the study.

The number of vesicles that were considered viable after 36-hours of culture remained relatively constant during the duration of the culture interval (Tables 1 and 2). These results are in agreement with preliminary study observations of the growth of bTV for up to 3 weeks (Pool, unpublished data) in CMRL-1066 medium with little loss of
vesicle integrity. The dark appearance and the loss of surface cells during the extended culture interval were not uncommon.

The gTV in this study performed similarly to the bTV. Following thawing, and removal of the cryoprotectant, inspection of the gTV revealed that they too were dark in color and exhibited rents in \( \approx 20\% \) of the vesicles. The gTV appeared to keep their dark appearance during the culture interval. Although, the reason for this is unknown, this may have been species specific. The gTV cultured in the RPMI-1640 medium did not appear to have the same desired degree of integrity displayed by the other three media evaluated. Although the number of viable gTV at 96 hours appeared to be greater in the MEM with Earle's salts than RPMI-1640, these means were not significantly different.

In summary, the results of this preliminary study suggest that FBUFC and trophoblastic vesicles (bTV and gTV) grew equally well in all media tested. The use of any of these media for the proliferation of FBUFC or trophoblastic vesicles are therefore recommended for future experiments.
CHAPTER III
EXPERIMENT II
THE USE OF TROPHOBLASTIC VESICLES AND FETAL UTERINE
MONOLAYER CELLS FOR THE CULTURE OF
PRECOMPACTION-STAGE BOVINE EMBRYOS

Introduction

A primary barrier to research involving the use of early cleavage-stage bovine embryos has been the inability to adequately support embryonic development in vitro. Recently, the development of culture systems that have used monolayers (Cole and Paul, 1965; Kuzan and Wright, 1982b; Voelkel et al., 1985; Eyestone et al., 1987; Wiemer et al., 1987) or bovine trophoblastic vesicles (bTV) (Camous et al., 1984; Heyman et al., 1987b,c; Heyman and Menezo, 1987; Pool et al., 1988) have been shown to improve development of mammalian embryos in vitro. With this information in mind, an experiment was conducted to compare the bTV culture system, the monolayer culture system and a combination of both systems for the culturing of early-stage bovine embryos through to blastocysts.

Materials and Methods

Embryo Donors

Forty mature crossbred beef cows (≥3 years of age) with a mean weight of 578 kg (range 458 to 660) were selected from a large donor pool and used as donor females in this study. Starting on days 8 to 15 of their respective estrous cycles (estrus = day 0), each donor female was placed on one
of two 4-day descending-dose superovulation treatment schedules using twice daily (i.m.) injections of follicle stimulating hormone (FSH) (FSH-P®: Schering Corporation, Kenilworth, NJ) resulting in a total dose of 28 mg or 36 mg of FSH per donor. At 48 hours after the initial FSH injection, a single 500 µg dose of cloprostenol (Estrumate®: Mobay Corporation, Shawnee, KS) was administered (i.m.) to induce luteolysis. Each donor was mated naturally at the onset of estrus with a fertile bull and then artificially inseminated (AI) with two units of frozen-thawed semen 24 hours later.

**Experimental Design**

Following collection, precompaction-stage embryos were evaluated under a stereomicroscope (100X) and equally assigned across treatments in a 2x2 factorial arrangement of treatments by donor, morphological developmental stage and embryo quality grade (1 = excellent, 2 = good, 3 = fair, 4 = degenerate) using the morphology and evaluation criteria previously reported for bovine embryos by Lindner and Wright (1983). Only those embryos with quality grades of 1 and 2 were used in this study.

Ham's F-10 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and an antibiotic-antimycotic solution (Ab-Am) containing 100 units of penicillin, 100 µg of streptomycin and .25 µg of amphotericin-B per ml of medium was used as the culture medium (HF-10) in this study. All embryos were equally and randomly assigned across treat-
ment wells of a 96-well tissue culture plate by embryo stage and quality grade and maintained in a CO₂ incubator at 37°C. The treatments were as follows: (A) embryos were cultured with a bTV (one bTV/well) in HF-10, (B) cultured on a fetal bovine uterine fibroblast cell (FBUFC) monolayer with HF-10 or (C) placed on a FBUFC monolayer in co-culture with a bTV in HF-10 and (D) HF-10 medium alone, which served as the control (Figure 2).

Cell Monolayer Preparation

Fetal bovine uterine fibroblast cells were prepared from explants from a 270-day fetal bovine uterus by a method similar to the one described for the collection of adult uterine endometrial cells by Voelkel et al. (1985). Following collection of a bovine fetus at a local abattoir, the uterus was extracted and placed into Hank's Balanced Salt Solution with five times Ab (HBSS) at 0°C for transport to the laboratory where the endometrial lining was aseptically removed and cut into 2 mm explants. The explants were placed into several 25 cm² tissue culture flasks (Corning, Corning, NY) with ≈2 ml of HF-10 medium per flask and the cells allowed to migrate from the explants to form a monolayer. Once the monolayer was confluent, the explants were discarded and the flask trypsinized with .025% trypsin (Sigma Chemical, St. Louis, MO). The cells were split (1:2) and used to start additional 25 cm² flasks as needed. Following two to three subpassages, and 24 hours prior to the
Figure 2. Experimental design for culture treatments for bovine embryos in Experiment II.
assignment of embryos or trophoblastic vesicles, cells were plated into wells of a 96-well culture plate. This corresponded to 0 or 12 hours prior to the addition of the bTV (one/well).

Trophoblastic Vesicle (bTV) Production

Thirty-six to 48-hours prior to collection of precompaction-stage embryos, elongated embryos were non-surgically collected from superovulated donors. The embryos were subsequently dissected with a razor blade held by a pair of hemostats (Rorie et al., 1985, 1987a) into embryonic tissue sections 1 to 2 mm² in size. The embryonic section containing the embryonic disk was discarded and the remaining tissue sections were placed into CMRL-1066 medium (Gibco, Grand Island, NY) supplemented with 10% (v/v) FCS and Ab-Am and placed in a 37°C humidified atmosphere of 5% CO₂ in air and allowed to develop into bTV 2 to 3 mm in diameter.

Embryo Recovery

Donors were collected non-surgically on days 4.5 to 5 post-estrus for precompaction-stage embryos or on days 13.5 to 14 post-estrus for trophoblastic vesicle (bTV) production using a modification of the closed-system flushing method previously described by Looney et al. (1981). The flushing medium consisted of Dulbecco's phosphate-buffered saline (PBS: Gibco, Grand Island, NY) supplemented with 2% heat-inactivated FCS and Ab-Am per milliliter of flushing medium. Following recovery, embryos were placed in a modified PBS
holding medium (HM) maintained at room temperature (20°C) until their allotment across treatment groups. The HM was composed of 10% (v/v) FCS, 4 gm/l of bovine serum albumin (BSA) Fraction-V, (Sigma Chemical, St. Louis, MO), 1 g/l of glucose, 0.036 g/l of pyruvic acid and 1% Ab-Am (Rorie et al., 1987c).

Statistical Analysis

Embryo viability (live vs. dead) and embryo quality grade scores (grades 1 and 2 vs. grades 3 and 4) were evaluated by Chi-square analysis (Steel and Torrie, 1980). The significance level was set at P<.05.

Results

Embryo Recovery and bTV Production

Elongated embryos from six donor females were successfully dissected into embryonic sections for bTV production with 95% of the sections developing into viable appearing bTV within 36 to 48 hours of in vitro culture. In addition, 164 excellent and good quality 12- to 32-cell embryos were collected from donors (n = 14) and randomly and sequentially assigned across each of the four treatment groups (41 embryos/treatment group). The remaining superovulated donors (n = 20) produced no embryos or embryos of poor quality (grades 3 and 4) and their embryos were not used in this study.

In Vitro Culture of Embryos

Following assignment to treatments, embryos were evalu-
ated at 12-hour intervals by two embryologists for morphological development and embryo quality (Lindner and Wright, 1983) during the 96-hour culture period. The number of embryos classified as viable and those of transferable quality (quality grades 1 and 2) after 48, 72 and 96 hours of in vitro culture are presented in Table 3. After 48 hours of in vitro culture, there were no differences in the number of embryos considered viable or having quality grades of 1 and 2 among the four treatment groups (P>.05). The number of embryos considered viable following 48 hours of in vitro culture were: 33(80%), 33(80%), 34(83%) and 32(78%) for Treatments A, B, C and D, respectively. The number(%) of viable appearing embryos that had quality grades of 1 (excellent) and 2 (good) were: 30(73%), 29(71), 29(71) and 28(68) and were not different between Treatments (P>.05), respectively.

After 72 hours of in vitro culture, there was a greater (P<.05) number of embryos considered viable in Treatments A (80%) and B (73%) than C (51%) and D (46%), respectively. Likewise, the number of excellent and good quality embryos in Treatment A (66%) was greater (P<.05) than those (34%) in Treatment D. In addition, Treatment B (68%) had a greater (P<.05) number of excellent and good quality embryos than Treatments C (46%) and D (34%), respectively.

At the end of the 96-hour culture interval, the number of viable appearing embryos and quality grade 1 and 2
### TABLE 3. EMBRYO VIABILITY ASSESSMENT DURING IN VITRO CULTURE OF BOVINE EMBRYOS

<table>
<thead>
<tr>
<th>Trt</th>
<th>0 hours</th>
<th>48 hours</th>
<th>72 hours</th>
<th>96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of grade 1&amp;2 embryos</td>
<td>Number(%) of viable embryos</td>
<td>Number(%) of viable embryos</td>
<td>Number(%) of viable embryos</td>
</tr>
<tr>
<td>A</td>
<td>41</td>
<td>33(80%)</td>
<td>30(73%)</td>
<td>33(80%)</td>
</tr>
<tr>
<td>B</td>
<td>41</td>
<td>33(80%)</td>
<td>29(71%)</td>
<td>30(73%)</td>
</tr>
<tr>
<td>C</td>
<td>41</td>
<td>34(83%)</td>
<td>29(71%)</td>
<td>21(51%)</td>
</tr>
<tr>
<td>D</td>
<td>41</td>
<td>32(78%)</td>
<td>28(68%)</td>
<td>19(46%)</td>
</tr>
</tbody>
</table>

*Treatments were as follows: (A) Embryos cultured with a bTV in HF-10, (B) embryos cultured on a fetal bovine uterine fibroblast cell (FBUFC) monolayer with HF-10, (C) embryos placed on a FBUFC monolayer in co-culture with a bTV in HF-10 and (D) embryos cultured in HF-10 medium alone.

*Significantly different at P<.05.*
embryos per treatment group had continued to decline to 27% and 17%, 51% and 34%, 41% and 24% and 5% and 5% for Treatments A, B, C and D, respectively (Table 3). All three co-culture groups (Treatments A, B and C) had a greater (P<.05) number of embryos considered viable than the medium alone (Treatment D) group. Likewise, embryos in Treatment D had the lowest number of grade 1 and 2 embryos.

The embryos that advanced in developmental stage from the initial stage of 12- to 32-cells to the blastocyst and hatched blastocyst stages are presented in Table 4. After 72-hours of in vitro culture a greater (P<.05) number of embryos in Treatments A (66%) and B (68%) had reached the blastocyst stage of development compared with Treatments C (49%) and D (37%), respectively. At the end of the 96-hour culture interval, (7%), (15%), (15%) and (2%) of the embryos in Treatments A, B, C and D had hatched, respectively. The number of hatched blastocysts being greater (P<.05) in Treatments B (15%) and C (15%) than A (7%) and D (2%), respectively (Table 4).

Discussion

The improved viability of culturing bovine embryos with bTV over that of the control medium alone in this experiment is in close agreement with the findings of others, who have similarly cultured early-stage bovine embryos (Camous et al., 1984; Heyman et al., 1987b,c; Heyman and Menezo, 1987; Pool et al., 1988). Heyman and Menezo (1987) have shown
TABLE 4. BOVINE EMBRYO DEVELOPMENT AFTER 72 AND 96 HOURS OF IN VITRO CULTURE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 hours</th>
<th>72 hours</th>
<th>96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of grade 1&amp;2 embryos</td>
<td>Embryo initial stage</td>
<td>Number(%) of embryos BLST</td>
</tr>
<tr>
<td>A</td>
<td>41</td>
<td>12-32 cell</td>
<td>27(66%)$^d$</td>
</tr>
<tr>
<td>B</td>
<td>41</td>
<td>12-32 cell</td>
<td>28(68%)$^d$</td>
</tr>
<tr>
<td>C</td>
<td>41</td>
<td>12-32 cell</td>
<td>20(49%)$^{d,e}$</td>
</tr>
<tr>
<td>D</td>
<td>41</td>
<td>12-32 cell</td>
<td>15(37%)$^e$</td>
</tr>
</tbody>
</table>

$^a$ Treatments were as follows: (A) Embryos cultured with a bTV in HF-10, (B) embryos cultured on a fetal bovine uterine fibroblast cell (FBUFC) monolayer with HF-10, (C) embryos placed on a FBUFC monolayer in co-culture with a bTV in HF-10 and (D) embryos cultured in HF-10 medium alone.

$^b$ BLST = blastocyst stage embryos.

$^c$ HBLT = hatched blastocyst stage embryos.

$^d,e$ Different superscripts in the same column are different at P<.05.
that the bTV secrete embryotropic factors into the medium by comparing the bTV culture system to that of conditioned medium from trophoblastic vesicles. Cleavage rates from beyond the 16-cell stage were similar between the trophoblastic vesicle co-culture (41.8%) and conditioned medium treatment groups (38.8%). Heyman and Menezo (1987) have reported that at least one embryotropic factor, a 180 to 2500 MW fraction, added to the culture medium (Menezo's B2) aided in the development of 1- to 2-cell bovine embryos through to morulae. The data from the present experiment supports the findings of Heyman and Menezo (1987) and further shows that some factor or substance from bTV may also be beneficial for later-stage embryos (12- to 32-cells) through to the hatched blastocyst stage.

The increase in the number of viable appearing embryos cultured on the fibroblast monolayer system is also in agreement with studies that have been conducted in the mouse (Cole and Paul, 1965), pig (Kuzan and Wright, 1982a; Allen and Wright, 1984) and cow (Kuzan and Wright, 1982b; Voelkel et al., 1985; Eyestone et al., 1987; Wiemer et al., 1987). The reason(s) for the advantage in embryonic development on fibroblast monolayers is unknown.

Kuzan and Wright (1982a,b) have suggested that the monolayer may secrete embryotropic factors that remove toxic by-products of metabolism secreted by the developing embryo. In addition, Allen and Wright (1984) noted that cell to
embryo contact was necessary for porcine embryos to benefit from monolayer co-culture and this contact may initiate secretion of an embryotropic product from the monolayer. These proposed mechanisms may account for the increased development of the embryos on the fetal uterine monolayer in this study. These cells may provide a more desirable environment similar to that of in vivo.

The use of both the bTV and the monolayer together in a co-culture system offered no advantage over that of either the bTV or monolayer used to culture bovine embryos alone in this study. However, as time in co-culture increased to 96-hours, the bTV-monolayer co-culture system did show an advantage (P<.05) over that of the control medium alone. This suggests that the embryotropic factor(s) of one and/or both the bTV or monolayer may be acting synergistically to promote development. This hypothesis is further supported by the fact that a greater (P<.05) number of embryos had undergone hatching at the end of the culture interval in the monolayer and bTV-monolayer co-culture groups (Treatments B and C) than in the bTV co-culture or control medium alone (Treatments A and D).

Although advancement in developmental stage is not a true indicator of viability, the results of this study are in agreement with others that have evaluated the monolayer (Kuzan and Wright, 1982a,b; Allan and Wright, 1984; Voelkel et al., 1985; Eyestone et al., 1987; Wiemer et al., 1987)
and bTV (Camous et al., 1984; Heyman et al., 1987b,c; Heyman and Menezo, 1987; Pool et al., 1988) culture systems. These researchers and others (see review by Wright and Bondioli, 1981) have indicated that advancement of embryo developmental stages in culture can serve as a realistic indicator of the effectiveness of the in vitro culture system.

Kuzan and Wright (1982b) reported that in vitro development of bovine morulae (collected on day 4 or 5 post-estrus) into hatched blastocysts (40%) on uterine fibroblast monolayers took 7 days, which corresponds to day 11 if the embryos had remained in vivo (Renard et al., 1976). In contrast, development of precompaction-stage embryos (12-to-32-cells) to the hatched blastocyst stage (15%) on the monolayer and bTV-monolayer co-culture groups in this study took only 4 days, which corresponded to day 8 if the embryos had remained in vivo.

The lower number of hatched blastocysts that resulted in the present study may have been because of a decreased culture interval (96 hours) compared with the one reported by Kuzan and Wright (1982b). However, 15% (6/41) does appear to be a higher figure than the 5% (25/499) of hatched blastocysts reported collected from superovulated donor cattle 8 days post estrus (Lindner and Wright, 1983). This may suggest that the embryos in this study were developing at an accelerated rate. Since the embryos in this study were not transferred to recipients to evaluate survival in
vivo, the question of viability cannot be answered at this time. Correspondingly, Wiemer (unpublished data) has recently had a live calf born following the transfer of an embryo co-cultured 48 hours on a FBUFC monolayer. Further research followed by recipient transfers is necessary to make further comparisons.
CHAPTER IV

EXPERIMENT III

THE USE OF A PEPTIDE ISOLATED FROM TROPHOBLASTIC TISSUE AND FETAL UTERINE MONOLAYER CELLS FOR THE CULTURE OF PRECOMPACTION-STAGE BOVINE EMBRYOS

Introduction

Culturing of bovine embryos on uterine (Voelkel et al., 1985), fetal uterine (Wiemer et al., 1987) and oviductal (Eyestone et al., 1987) monolayers has been shown to improve embryo development in vitro over that of medium alone. Kuzan and Wright (1982a,b) have suggested that the monolayer may secrete embryotropic factors or remove toxic byproducts of metabolism secreted by the developing embryo. In addition, Allen and Wright (1984) noted that cell-to-embryo contact was necessary for porcine embryos to benefit from monolayer co-culture, and that this contact may initiate secretion of an embryotropic substance from the monolayer. These proposed biochemical actions may account, in part, for the increased embryonic development noted while embryos were on the uterine monolayer in these studies.

Bovine trophoblastic vesicle co-culture systems have also been shown to increase bovine embryo development over that of medium alone (Camous et al., 1984; Heyman et al., 1987b,c; Heyman and Menezo, 1987; Pool et al., 1988). The advantage to embryo development in co-culture afforded by bTV tissue is not entirely understood. Heyman et al. (1987b,c) have shown that a small molecular weight compound
(180 to 2,500 daltons) isolated from trophoblastic vesicle conditioned medium will promote 1- to 2-cell bovine embryo development in vitro. In addition, Menezo and Khatchadourian (unpublished data) have isolated three small peptides released by bovine trophoblastic vesicles in the culture medium. Currently, no information on the role of these peptides on bovine embryonic development has been reported. An experiment was designed to evaluate the role of one of these three trophoblastic peptides on in vitro development of bovine precompaction-stage embryos.

Materials and Methods

Embryo Donors

Randomly cycling crossbred beef cows were selected from a large donor pool maintained at the St. Gabriel Research Station (St. Gabriel, LA). Donors females (n = 13) were ≥3 years-of-age and had a mean mature weight of 515 kg (range 392 to 590). Each donor was placed on a 4-day superovulation scheme beginning as the females reached days 8 to 15 of their respective estrous cycles. Follicle stimulating hormone (FSH) (FSH-P®: Schering Corporation, Kenilworth, NJ) was administered twice daily in descending doses of 5, 5, 4, 4, 3, 3 and 2, 2 mg per day resulting in a total dose of 28 mg FSH per donor female. Forty-eight and 60 hours after the initial FSH injection, a single 25 mg dose of prostaglandin F₂α (Lutalyse®: Upjohn Co. Kalamazoo, MI) was administered (i.m.) to induce luteolysis. Each donor was mated naturally
at the onset of estrus with a fertile bull and then artificially inseminated (AI) with two units of frozen-thawed semen 24 hours after mating.

**Experimental Design**

Following collection, precompaction-stage embryos were evaluated under a stereomicroscope (100X). Embryos that were retarded in development and not morphologically normal (see evaluation criteria by Lindner and Wright, 1983) were discarded. The remaining excellent (grade = 1) and good (grade = 2) quality embryos were randomly and sequentially assigned across treatments in a 2x2 factorial arrangement of treatments by donor female and embryo morphological development stage.

Ham's F-10 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and an antibiotic-antimycotic solution (Ab-Am) containing 100 units of penicillin, 100 μg of streptomycin and .25 μg of amphotericin-B per ml of medium was selected as the culture medium (HF-10) in this study. All embryos were assigned individually to each treatment well of a 24-well tissue culture plate (Gibco, Grand Island, NY) maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. Embryos in Treatment A were cultured in HF-10 containing 100 μg/ml of a trophoblastic peptide and embryos in Treatment B were cultured on a fetal bovine uterine fibroblast monolayer with HF-10. Those embryos assigned to Treatment C were placed on a monolayer
in co-culture with 100 μg/ml of peptide in HF-10. Embryos in Treatment D were placed in HF-10 medium alone and served as the control.

**Cell Monolayer Production**

Frozen-thawed bovine fetal uterine endometrial fibroblast monolayer cells (described in Experiment II) were used in this study. Following thawing and removal of the cryoprotectant, the cells were placed into 25 cm² tissue culture flasks (Corning™, Corning, NY) and allowed to monolayer (~4 days). The cells were then split (1:2) and used to start additional 25 cm² flasks as needed. Following three to five subpassages, a flask was trypsinized with .025% trypsin (Sigma Chemical, St. Louis, MO) 24 hours prior to the assignment of embryos and the cells plated into wells of a 24-well culture plate.

**Peptide Isolation**

The peptide used in this study was isolated and supplied by Dr. Y. Menezo (INSA, Laboratorire de Biologie, Lyon, France). Menezo and Khatchadourian (unpublished data) isolated this particular peptide, in addition to two other similar peptides (see Heyman and Menezo, 1987), from serum-free Menezo's B₂ medium in which day-14 trophoblastic vesicles had been cultured. The peptide was slightly acidic and exhibited a chemical migration pattern similar to that of serine. The peptide structure was identified by a similar procedure to that described by Menezo and Khatchadourian.
(1986) for amino acid peptides bound to albumin. Perchloric acid (.4 N) was used for peptide extraction. This was followed by ion exchange chromatography (resin AS-70 on Kontron Liquimat III with lithium citrate as the elution buffer) with the eluent collected under the serine peak. The extract was dried and treated with 50% ethanol to remove the remaining salts. The amino acid peptide analysis was performed after hydrolysis (6 N hydrochloric acid under nitrogen) and was found to be a hexapeptide with the following amino acid sequence: 'glycine-alanine-glutamine-glycine-glycine-serine' (Y. Menezo, personal communication).

Embryo Recovery

Donors were collected non-surgically on days 4.5 to 5 post-estrus for precompaction-stage embryos using a modification of the closed-system flushing method previously described in this laboratory (Looney et al., 1981). The flushing medium consisted of Dulbecco's phosphate-buffered saline (PBS: Gibco, Grand Island, NY) supplemented with 1% calf serum (CS) and Ab-Am per milliliter of flushing medium. Following recovery, embryos were placed in a modified PBS holding medium (HM) (Rorie et al., 1987c) maintained at room temperature (20°C) until their allotment across experimental treatment groups.
Statistical Analysis

Chi-square analysis (Steel and Torrie, 1980) was used to determine if there were differences in the number of viable and nonviable embryos and quality grade scores after 48, 72 and 96 hours of in vitro culture. The significance level was set at the P<.05 level for this experiment.

Results

Embryo Recovery

Seventy-two excellent and good quality 12- to 32-cell embryos were collected from superovulated donors (n = 11). The remaining two donors produced no embryos and were excluded from the study. The embryos were randomly and sequentially assigned across each of the four treatment groups (18 embryos per treatment group).

In Vitro Culture of Embryos

Following allotment to treatments, embryos were evaluated at 12-hour intervals by two embryologists for morphological development and embryo quality (Lindner and Wright, 1983) during a 96-hour culture interval. The number of embryos classified as viable and those designated as of transferable quality (quality grades 1 and 2) after 48, 72 and 96 hours of in vitro culture are presented in Table 5.

After 48 hours in culture, there were no differences in the number of embryos classified as viable. The number of embryos considered viable at 48 hours in culture were:

10(55%), 12(67%), 10(55%) and 7(39%) for Treatments A, B, C
<table>
<thead>
<tr>
<th>Trt&lt;sup&gt;a&lt;/sup&gt;</th>
<th>0 hours</th>
<th>48 hours</th>
<th>72 hours</th>
<th>96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18</td>
<td>10(55%)</td>
<td>4(22%)&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>6(33%)</td>
</tr>
<tr>
<td>B</td>
<td>18</td>
<td>12(67%)</td>
<td>8(44%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10(55%)</td>
</tr>
<tr>
<td>C</td>
<td>18</td>
<td>10(55%)</td>
<td>8(44%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10(55%)</td>
</tr>
<tr>
<td>D</td>
<td>18</td>
<td>7(39%)</td>
<td>3(17%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6(33%)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Treatments were as follows: (A) Embryos cultured in HF-10 containing 100 μg/ml of a trophoblastic peptide, (B) embryos cultured on a fetal bovine uterine fibroblast cell (FBUFC) monolayer with HF-10, (C) embryos placed on a FBUFC monolayer in co-culture with 100 μg/ml of peptide in HF-10 and (D) embryos cultured in HF-10 medium alone.

<sup>b,c</sup>Different superscripts in the same column are significantly different at P<.05.
and D, respectively. In addition, the number of viable embryos that had quality grades of 1 (excellent) and 2 (good) were: 22%, 44%, 44% and 17% for Treatments A, B, C and D, respectively. There were no differences (P>.05) in embryo quality grades among Treatments A, B and C. However, embryos in Treatment D had a lower quality grade (P<.05) than those in Treatments B and C.

After 72 hours of in vitro culture, there was no difference (P>.05) in the number of embryos considered viable: 6(33%), 10(55%), 10(55%) and 6(33%) in Treatments A, B, C and D, respectively (Table 5). Surprisingly, the number of excellent and good quality embryos in Treatments A through D had not changed from the previous 48-hour culture period.

At the end of the 96-hour culture interval, the number of viable embryos had declined to 11%, 44%, 50% and 33% for Treatments A, B, C and D, respectively. Correspondingly, the number of embryos with quality grades of 1 and 2 had declined to 11%, 44%, 44% and 17% for Treatments A, B, C and D, respectively. There was a greater number of embryos in the monolayer and peptide-monolayer co-culture groups (Treatments B and C) (P<.05) classified as viable than in the peptide alone (Treatment A) and medium alone (Treatment D) treatment groups.

The number of embryos that advanced in developmental stage from the initial stage (12- to 32-cells) to the blastocyst and hatched blastocyst stages are presented in Table
6. Following 72-hours of in vitro culture, there were 28%, 44%, 50% and 33% of the embryos in Treatments A through D, respectively, that had reached the blastocyst stage of development. These mean values were not different (P>.05).

At the end of the 96-hour culture interval, 0 (0%), 8 (44%), 8 (44%) and 2 (11%) of the embryos in Treatments A, B, C and D had hatched, with the number of hatched blastocysts being greater (P<.05) in Treatments B and C than in Treatments A and D (Table 6).

Discussion

The results of this experiment suggest that the hexapeptide isolated from trophoblastic-vesicle conditioned medium (Menezo and Khatchadourian, unpublished data) and used in this study is not a primary embryotropic factor secreted by bTV for promoting embryonic development in vitro. In Treatment A, the peptide was not able to stimulate development of precompaction-stage embryos when compared with that of the medium alone (Treatment D). This would suggest that this peptide (at the concentration/ml used in this study) was not effective in eliciting a positive embryotropic response on embryonic growth in vitro. Secondly, the peptide may act synergisticly with the two other peptides described by Menezo and Khatchadourian to elicit the embryotropic response. Furthermore, the low molecular weight compounds produced by bTV (Heyman 1987b,c)
TABLE 6. BOVINE EMBRYO DEVELOPMENTAL STAGES AFTER 72 AND 96 HOURS OF IN VITRO CULTURE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 hours</th>
<th>72 hours</th>
<th>96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of grade 1&amp;2 embryos</td>
<td>Embryo initial stage</td>
<td>Number(%) of embryos BLST</td>
</tr>
<tr>
<td>A</td>
<td>18</td>
<td>12-32 cell</td>
<td>5 (28%)</td>
</tr>
<tr>
<td>B</td>
<td>18</td>
<td>12-32 cell</td>
<td>8 (44%)</td>
</tr>
<tr>
<td>C</td>
<td>18</td>
<td>12-32 cell</td>
<td>9 (50%)</td>
</tr>
<tr>
<td>D</td>
<td>18</td>
<td>12-32 cell</td>
<td>6 (33%)</td>
</tr>
</tbody>
</table>

aTreatments were as follows: (A) embryos cultured in HF-10 containing 100 μg/ml of a trophoblastic peptide, (B) embryos cultured on a fetal bovine uterine fibroblast cell (FBUFC) monolayer with HF-10, (C) embryos placed on a FBUFC monolayer in co-culture with 100 μg/ml of peptide in HF-10 and (D) embryos cultured in HF-10 medium alone.

bBLST = blastocyst stage embryos.

cHBLT = hatched blastocyst stage embryos.

d Different superscripts in the same column are different at P<.05.
may promote embryonic development. Support of this hypothesis is evidenced by the fact that the bTV alone placed in co-culture with bovine embryos in Experiment II was able to elicit an embryotropic response.

Small peptides of embryonic origin are known to bind to serum proteins (Kent, 1978). Menezo and Khatchadourian (1986) have shown that bound peptides constitute 1 to 2% of the total weight of bovine serum albumin (BSA). Dissociation of the bound peptides is usually accomplished by competition with free fatty acids. The only source of free fatty acids in our peptide treatment group (Treatment A) was the Ham's F-10 culture medium. Ham's F-10, however, has been reported to be low in free fatty acids (Ham, 1963).

In this study it was noted that the Menezo and Khatchadourian peptide (at 100 μg/ml of medium) was not toxic to embryos as evidenced by the similarities in development between Treatments A and D and B and C. The addition of the peptide was the only difference between these treatment groups. The culture results suggest that this peptide, at the concentration evaluated, has no overt effect on embryo development. The present lack of information on the concentration of the embryonic peptides needed to elicit an in vitro response (positive or negative) by bovine embryos suggests that further research is needed to identify those peptides needed to induce a response in vitro.
CHAPTER V

EXPERIMENT IV

LIPID PRODUCTION BY VARIOUS FEEDER CELLS AND BOVINE TROPHOBLASTIC VESICLES IN VITRO

Introduction

Little information is available on the role of lipids in early-stage embryonic metabolism. Most research has focused on embryonic lipid content in mouse and rabbit embryos. Hensleigh and Weitlauf (1974) have reported that the lipid content of delayed implanting mouse blastocysts remained relatively constant until day 5 of gestation (the day of initiation of implantation) followed by a continual increase in lipids until day 10 and then remained stable. The role of the lipids in embryonic development was not evaluated in this experiment. Hensleigh and Weitlauf (1974) did propose, however, that the increase in the lipid content of the embryos may be related to the formation of "giant cells" by the trophectoderm of the developing embryo.

In other studies, radiolabeled precursors have been added to the culture medium (carbonate, pyruvate and glucose) and it has been shown that the majority of the labeled precursors were incorporated into the total lipid fraction of the developing mouse embryo (Wales et al., 1969; Wales and Whittingham, 1970; Wales, 1975). In addition, Flynn and Hillman (1980) have shown that exogenous fatty acids (in particular palmitic acid added to the culture medium) can be
incorporated directly into the lipid fraction of the 8-cell mouse embryo. It was also noted that the rate of fatty acid oxidation by the developing mouse embryo (from fertilization through to the blastocyst stage) was relatively constant.

Likewise, Kane (1979) has shown that the presence of pyruvate and certain long and short chain fatty acids can support growth of 1-cell rabbit ova to viable morulae in a simple salt solution containing defatted BSA. It was suggested that under these circumstances, the role of pyruvate and fatty acids was to act as an embryonic energy source.

Findings to date suggest that in the developing mouse and rabbit embryo, fatty acids and fatty acid precursors in the medium can be taken up by the developing embryo, incorporated into embryonic lipids and then utilized as an endogenous energy source.

Menezo et al. (1982) have evaluated the fatty acid composition of day-7 to day-14 bovine embryos. It was noted that the total amount of lipid per embryo did not change between days 7 and 10, but then sharply increased between days 11 and 13 of gestation. It was suggested that lipid content was related to volume and weight of the blastocysts. This increase in lipid concentration of the day-11 to day-13 bovine embryo corresponds to the period of rapid growth of the trophectoderm that is characterized by the elongation of the embryo. This finding is similar to the report by Hensleigh and Weitlauf (1974) who showed that there was an in-
crease in lipid content of developing mouse embryos beginning on day 5 and stabilizing by day 10 of gestation.

The lipid content of the bovine embryo between days 7 and 14 may be related to the uptake of glucose from the medium and its role in fatty acid synthesis (as suggested for the mouse by Flynn and Hillman, 1980) and possibly hatching. Loss of the zona pellucida is completely inhibited when glucose is replaced by pyruvate and lactate thus suggesting that glucose is necessary in order for hatching of the bovine embryo to occur in vitro (Renard et al., 1980).

Studies have shown that the use of monolayer feeder cells (Kuzan and Wright, 1982b; Eyestone et al., 1987; Weimer et al., 1987; Eyestone and First, 1988) as well as trophoblastic vesicles (Camous et al., 1984; Heyman et al., 1987b,c; Heyman and Menezo, 1987; Pool et al., 1988) for the co-culture of bovine embryos has led to a greater number of embryos developing and hatching in vitro over that of embryos cultured in medium alone. Several laboratories have suggested that the "helper" cells or trophoblastic vesicles promote embryo development by the secretion of embryotrophic factors into the medium that are subsequently used by the embryo (Kuzan and Wright, 1982b; Heyman et al., 1987b,c; Menezo, personal communication). At present, the function of these embryotropic factor(s) is not clear. There have been attempts, however, to identify these compounds in the
rabbit (Kane, 1985) and the cow (Heymen et al., 1987b,c; Hickey and Hansel, 1987). Information is needed on the production of lipids from "helper" cells. Based on the previously mentioned literature on lipid and fatty acid uptake by mouse and rabbit embryos from the culture medium, a study was undertaken to determine the lipid and free fatty acid (FFA) production of several cell types and trophoblastic vesicles used in our laboratory for the co-culture of bovine embryos.

Materials and Methods

Experimental Procedure

Bovine trophoblastic vesicles (bTV) and four different cell types including bovine oviduct cells (BOC), fetal bovine uterine fibroblast cells (FBUFC), fetal bovine uterine cells (FBUC) and goat oviduct cells (GOC) have been established for in vitro embryo culture in our laboratory. One or more 25 cm² tissue culture flasks containing a confluent monolayer of each cell type (fresh and/or frozen-thawed) on different subpassages was used in this study.

At the onset of the study, each monolayer was washed three times with serum-free growth medium (TCM-199 or Ham's F-10) in order to remove traces of serum. Serum has been shown to contribute a wide variety of constituents including: amino acids, hormones, growth factors, lipids, inorganic compounds, sugars, vitamins and minerals (Menezo, 1983). Thus, removal of the serum should result in the
removal of the lipid contaminants in the treatment medium. The treatment medium used was Menezo’s B₂ medium (B₂) (Menezo, 1976). This medium (aliquots from the same lot number) was selected because of the extensive quality control used in its production. After washing, 5 ml of B₂ was added to each flask of cells and 5 ml was added to an identical flask without any cells to serve as a control. The flasks were then placed into an incubator at 37°C in a humidified atmosphere of 5% CO₂ in air for 11 hours. The medium was then harvested, placed in plastic vials and frozen at -20°C until lipid extraction.

Fresh bovine trophoblastic vesicles (∼400) with a diameter ranging from 250 to 450 μm were washed through three 10 ml volumes of Dulbecco’s phosphate-buffered saline (PBS: Gibco, Grand Island, NY), allowing each bTV to settle in the PBS for 3 to 5 minutes. This was followed by gentle pipetting to remove loose or dying cells and to remove traces of serum attached to the outer cells of the trophectoderm. After washing, all bTV were placed in 10 ml of B₂ in a 150 mm plastic petri dish and incubated in a similar fashion to the various cell types with a control medium. Following incubation, medium (5 ml) was harvested and stored as previously described.

Cell Monolayer Production

Each cell type used was derived from tissues collected from a local slaughterhouse. The BOC and GOC cells were
extracted by a method similar to that described by Ouhibi et al. (1988). Ligated oviducts were inflated with .025% trypsin and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 45 minutes. The FBUC were obtained by trypsinizing the uterine horns of a 180- to 200-day-old bovine fetus. The incubation time was 60 minutes.

Following trypsinization of the bovine and caprine oviducts and fetal uterine horns, each cell type was suspended in 5 ml of tissue culture medium-199 (TCM-199) with 10% heat-inactivated fetal calf serum (FCS) and five times the antibiotic (100 µg penicillin, 100 µg streptomycin/ml) and washed twice by centrifugation. Following centrifugation, the supernatant was discarded and the pellet of cells resuspended in 3 ml of TCM-199 with 10% FCS and an antibiotic-antimycotic solution (Ab-Am) containing 100 units of penicillin, 100 µg of streptomycin and .25 µg of amphotericin-B per ml of medium by gentle, repeated pipetting that also aided in cell dissociation. The cell suspensions were then transferred to 25 cm² tissue culture flasks (Corning™, Corning, NY) and the volume of TCM-199 raised to 10 ml total. The flasks were placed in an incubator (37°C) with a humidified atmosphere of 5% CO₂ in air and allowed to form monolayers. Flasks containing confluent monolayers were split (1:2) using .025% trypsin and subcultured as needed. The FBUFC described in Experiment II were used in this experiment. These cells were maintained similar to the BOC,
GOC and FBUC previously described.

**Extraction and Fractionation of Lipids**

The total lipid fraction of each sample was extracted by placing a concentrated aliquot of each sample (1.6 ml) into a centrifuge tube and adding 6 ml of the solvent (1:2 v/v mixture of chloroform and methanol). The mixture was agitated followed by the addition of 4 ml of a chloroform:water (1:1 v/v) mixture. The tube containing the mixture was centrifuged for 8 minutes at 4,000 revolutions per minute. Following centrifugation, the upper aqueous phase was collected and washed with 2 ml of additional chloroform. The two organic phases were mixed and washed twice with 2 ml of distilled water. The aqueous phase was removed and discarded and the solvent (chloroform) evaporated under a stream of nitrogen. The dry residue (containing the free fatty acids, triglycerides and phospholipids) was stored at -80°C or diluted in chloroform (40 mg/ml) and stored at -20°C until further extraction by thin layer chromatography.

The dry residue from each sample was mixed with 50 µl of chloroform-methanol (2:1 v/v) containing .01% butylated-hydroxy-toluene (BHT) and 10 µl of each sample was placed on a silica gel plate (five samples/gel) along with a 10 µl sample of tritiated oleic acid (³H-C 18:1, 1 µCi/20 µl stock in chloroform) which served as the control. The gel plates were placed in the solvent solution (hexane-ether-alcohol at a ratio of 80:20:1) and then allowed to migrate for ≈ 30
minutes. The gel plates were removed from the solvent and allowed to dry at room temperature. The plates were sprayed with iodine and the fatty acids visualized under UV light. After the solvent fronts had been marked, the free fatty acids were removed from the gel and each were collected into a separate tube. Each sample was washed twice with 2 ml of ether, agitated and then allowed to settle for 5 minutes. Following centrifugation of the samples for 8 minutes at 3500 g, the organic phase was collected and concentrated by drying at room temperature.

The dry residue was methylated by the addition of diazomethane (500 μl) according to the method described by Schlenk and Gellerman (1960) to convert the FFA to methyl esters. After drying (under a nitrogen atmosphere), the residues containing the fatty acid methyl esters were dissolved in 150 μl of chloroform to prepare for gas chromatography.

**Gas Chromatography**

Methylated fatty acids were analyzed according to the procedures described by Menezo et al. (1982), with the aid of a Girdel 300 gas chromatograph, equipped with a hydrogen flame ionization detector coupled to a Hewlett-Packard 3380 integrator. Pyrex columns 2.5 and 4 m in length with an inside diameter of 2 mm filled with 10% DEGS were used. The three temperatures selected were 151°C, 171°C and 181°C. The injector temperature was 190°C and the detector tempera-
ture was 200°C. Internal and external standards were run to aid in monitoring recovery rates. Gas chromatography was coupled with mass spectrometry to aid in identifying unknown compounds.

Results

The triglycerides and phospholipids extracted from the treatment medium following 11 hours of in vitro culture are presented in Table 7. There was little or no difference in the triglyceride or phospholipid concentrations (production) compared with the control (medium alone). The cells produced between 0 and 0.02 g/l of triglycerides compared with the control during the culture period. Correspondingly, the cells produced between 0 and 0.01 mmols/l of phospholipids when compared with the controls.

With the aid of mass spectrometry, the chemical structures were confirmed and the free fatty acids identified as following: C14, C15, C16, C16:1n-7, C17, C18, C18:1n-9, C18:2n-6, C18:3n-6, C18:3n-3 and C20 (Table 8). In addition to the identified FFA produced by the cultured cells, there were several unknowns produced by the GOC cells. These unknowns had carbon condensation lower than C15, C16:1n-7, C18 and C18:1n-9. There were few differences in the relative percents of the fatty acids produced by the cells compared with that detected in B2 medium. There was no evidence of C14, C15 and C20 fatty acids produced by the bTV in this study.
Table 7. QUANTITIES OF TRIGLYCERIDES AND PHOSPHOLIPIDS PRODUCED BY VARIOUS CELL TYPES IN VITRO

<table>
<thead>
<tr>
<th>Cell type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of samples</th>
<th>Triglycerides g/l</th>
<th>Phospholipids mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOC&lt;sub&gt;0&lt;/sub&gt;</td>
<td>2</td>
<td>.14</td>
<td>.04</td>
</tr>
<tr>
<td>B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2</td>
<td>.12</td>
<td>.03</td>
</tr>
<tr>
<td>BOC&lt;sub&gt;3&lt;/sub&gt;</td>
<td>3</td>
<td>.13</td>
<td>.03</td>
</tr>
<tr>
<td>B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3</td>
<td>.13</td>
<td>.03</td>
</tr>
<tr>
<td>bTV</td>
<td>1</td>
<td>.12</td>
<td>.03</td>
</tr>
<tr>
<td>B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
<td>.13</td>
<td>.02</td>
</tr>
<tr>
<td>FBUFC&lt;sub&gt;7&lt;/sub&gt;</td>
<td>1</td>
<td>.15</td>
<td>.04</td>
</tr>
<tr>
<td>B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
<td>.14</td>
<td>.04</td>
</tr>
<tr>
<td>FBUC&lt;sub&gt;4&lt;/sub&gt;</td>
<td>1</td>
<td>.13</td>
<td>.03</td>
</tr>
<tr>
<td>B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
<td>.13</td>
<td>.03</td>
</tr>
<tr>
<td>FBUC&lt;sub&gt;8&lt;/sub&gt;</td>
<td>1</td>
<td>.13</td>
<td>.04</td>
</tr>
<tr>
<td>B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
<td>.13</td>
<td>.03</td>
</tr>
<tr>
<td>GOC&lt;sub&gt;0&lt;/sub&gt;</td>
<td>1</td>
<td>.16</td>
<td>.04</td>
</tr>
<tr>
<td>B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1</td>
<td>.13</td>
<td>.04</td>
</tr>
<tr>
<td>GOC&lt;sub&gt;5&lt;/sub&gt;</td>
<td>2</td>
<td>.13</td>
<td>.05</td>
</tr>
<tr>
<td>B&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2</td>
<td>.11</td>
<td>.05</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cell types with passage indicated by the subscript: BOC = bovine oviduct cells; bTV = bovine trophoblastic vesicles; FBUFC = fetal bovine uterine fibroblast cells; FBUC = fetal bovine uterine cells; GOC = goat oviduct cells; B<sub>2</sub> = Men-ezo's B<sub>2</sub> medium which was paired with each cell type and also served as the control.
Table 8. RELATIVE PERCENTAGES OF THE DIFFERENT FATTY ACIDS PRODUCED BY THE VARIOUS CELLTYPES IN VITRO

<table>
<thead>
<tr>
<th>Fatty acid carbons&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cell type vs. control&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BOC</th>
<th>B&lt;sub&gt;2&lt;/sub&gt;</th>
<th>bTV</th>
<th>B&lt;sub&gt;2&lt;/sub&gt;</th>
<th>FBUFC</th>
<th>B&lt;sub&gt;2&lt;/sub&gt;</th>
<th>GOC</th>
<th>B&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14 14.7</td>
<td></td>
<td>.3</td>
<td>.3</td>
<td>.0</td>
<td>.0</td>
<td>2.9</td>
<td>.2</td>
<td>2.7</td>
<td>2.4</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C15 14.5</td>
<td></td>
<td>.5</td>
<td>.5</td>
<td>.0</td>
<td>.6</td>
<td>.9</td>
<td>.5</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>C16 17.9</td>
<td></td>
<td>14.7</td>
<td>14.5</td>
<td>17.9</td>
<td>13.9</td>
<td>21.4</td>
<td>19.1</td>
<td>26.6</td>
<td>18.4</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1n-7 15.9</td>
<td></td>
<td>1.5</td>
<td>1.4</td>
<td>.0</td>
<td>1.2</td>
<td>1.8</td>
<td>1.7</td>
<td>1.4</td>
<td>3.2</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C17 18.5</td>
<td></td>
<td>.7</td>
<td>.9</td>
<td>1.2</td>
<td>1.4</td>
<td>1.1</td>
<td>1.3</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>C18 21.2</td>
<td></td>
<td>18.5</td>
<td>21.2</td>
<td>25.5</td>
<td>21.4</td>
<td>24.4</td>
<td>27.3</td>
<td>34.0</td>
<td>30.3</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27.7</td>
</tr>
<tr>
<td>C18:1n-9 21.3</td>
<td></td>
<td>6.3</td>
<td>7.5</td>
<td>11.3</td>
<td>9.7</td>
<td>8.0</td>
<td>8.6</td>
<td>9.6</td>
<td>10.1</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>C18:2n-6 21.5</td>
<td></td>
<td>.2</td>
<td>.5</td>
<td>.0</td>
<td>.0</td>
<td>.6</td>
<td>.4</td>
<td>.0</td>
<td>.0</td>
</tr>
<tr>
<td>C18:3n-6 21.6</td>
<td></td>
<td>.3</td>
<td>.5</td>
<td>.0</td>
<td>.0</td>
<td>.7</td>
<td>.7</td>
<td>.0</td>
<td>.0</td>
</tr>
<tr>
<td>C18:3n-3 21.7</td>
<td></td>
<td>.6</td>
<td>.4</td>
<td>.0</td>
<td>.0</td>
<td>.6</td>
<td>.5</td>
<td>.0</td>
<td>.0</td>
</tr>
<tr>
<td>C20 22.0</td>
<td></td>
<td>.4</td>
<td>.3</td>
<td>.0</td>
<td>.0</td>
<td>.0</td>
<td>.0</td>
<td>.0</td>
<td>.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of carbon atoms/fatty acid.

<sup>b</sup>Cell types: BOC = bovine oviduct cells; bTV = bovine trophoblastic vesicles; FBUFC = fetal bovine uterine fibroblast cells; GOC = goat oviduct cells; B<sub>2</sub> = Menezo's B<sub>2</sub> medium which was paired with each cell type and also served as the control.
Discussion

The concentrations of triglycerides and phospholipids produced by various feeder cell types in this study were similar to the quantities of these compounds in the medium (Table 8). These findings suggest that the cell types used in this study do not secrete large quantities of triglycerides and phospholipids into the medium. Furthermore, these compounds may have become bound to some component(s) in the medium. Menezo's B2 medium contains 10 mg/ml of bovine serum albumin, a macromolecule reported to have marked ligand-binding abilities (Kane, 1987) and perhaps binds triglycerides and phospholipids as well. Large quantities (as much as 99%) of free fatty acids bound to BSA (ranging from .18 to .49 mol/mol of protein) can be removed by charcoal treatment in a defatting process (Chen, 1967). Defatted BSA was not substituted for commercial grade BSA (Fraction V, Sigma Chemical, St Louis, MO) in the medium used in this study. This may explain the high concentrations of triglycerides and phospholipids in the control samples. Due to the limited information on the amount and type of triglycerides and phospholipids produced by these various cell types in vitro, interpretation of the findings in this study are only speculative.

The free fatty acids produced by the various cell types in this study were also similar to those found in the medium. It is interesting to note that palmitic (C16) and stearic
(C18) acids were the primary free fatty acids produced by the various cell types. These two fatty acids account for greater than 30% of the free fatty acids produced by the cells. Menezo et al. (1982) have reported that of the 1.58 mg of fatty acids bound to BSA, 16.7% and 27% were palmitic and stearic acids, respectively. In addition, these two fatty acids have been reported to account for 13.3%, 25.6%, 13.1%, 31.7% and 35.9% of the fatty acids in bovine embryos on days 7 to 8, 10, 11 to 12, 13 and 14, respectively (Menezo et al., 1982).

Pyruvate and the long chain fatty acids myristic (C14), palmitic (C16), stearic (C18), oleic (C18:1) and to a lesser extent the short chain acids, acetic (C4) and propionic (C3) have been reported to support growth of 1-cell rabbit embryos to viable morulae in a simple salt solution containing defatted BSA (Kane, 1979; Kane and Headon, 1980). Flynn and Hillman (1980) have also reported that palmitic acid from the culture medium is incorporated directly into the lipid fraction of the 8-cell mouse embryo. These studies have demonstrated that free fatty acids in the medium or added to the culture system are taken up and utilized by the developing embryo. The free fatty acids produced by the "feeder" cells may supply lipid precursors for embryos placed on the cell monolayers.

Results of Experiments II and III suggest that the FBUFC monolayer produce embryotrophic factors that stimulate
embryonic development. Likewise, trophoblastic vesicles also promoted embryonic development over that of medium alone (Experiment II). The free fatty acids produced by the FBUFC monolayer and bTV in these studies may be embryotropic in nature. These free fatty acids may be bound to BSA where they can be transported to the embryonic cells for use in membrane lipid metabolism. Pratt (1980) has proposed this as a physiological mechanism for the mouse embryo.

It is clear that further research in this area is needed to evaluate the role of lipids in embryonic metabolism and in vitro development.
CHAPTER VI
EXPERIMENT V

TRANSITIONAL CHANGES IN ARACHIDONIC ACID METABOLISM BY CAPRINE AND BOVINE EMBRYOS AT DIFFERENT DEVELOPMENTAL STAGES

Introduction

Maternal recognition of pregnancy is accompanied by the prevention of luteal regression in farm animal species. It has been demonstrated that the transfer of an embryo or the infusion of extracts of embryonic homogenates into the uterus of the sheep prevents cyclic regression of endogenous luteal tissue (Moor and Rowson, 1966a,b). The presence of an embryo-derived luteotropic substance has also been reported in swine (Niswender et al., 1970; Longnecker and Day, 1973; Segal and Baker, 1973), cattle (Betteridge et al., 1976; Northey and French, 1980), horses (Zavy et al., 1979), as well as in the sheep (Moor and Rowson, 1966c; Rowson and Moor, 1967; Moor, 1968). These findings suggest that the developing embryo may secrete a luteotropic substance or substances that is responsible for the events leading to luteal maintenance and maternal recognition of pregnancy. This embryo-derived substance(s), in some manner, may suppress or block the local effect of a luteolytic substance or substances originating from uterine tissues (Stabenfeldt et al., 1978).

In most farm animal species, prostaglandin $F_{2a}$ ($PGF_{2a}$) is now believed to be involved in cyclic regression of the
corpus luteum (CL) and considered to be the primary luteolytic substance in nonpregnant, cyclic cattle (Hafs et al., 1974; Thatcher and Chenault, 1976), sheep (McCracken et al., 1972; McCracken et al., 1984), goats (Bosu et al., 1978, Bretzlaff 1983) and swine (Gleeson et al., 1974; Moeljono et al., 1977). However, there is no clear evidence that uterine secretion of PGF$_{2\alpha}$ is diminished during early pregnancy in those species evaluated (Moeljono et al., 1977).

Both *in vitro* and *in vivo* studies suggest that PGE$_2$ has luteotropic properties. Spontaneous luteal regression and estradiol-induced luteal regression were delayed by uterine luminal infusion of PGE$_2$ in sheep (Magness et al., 1981; Pratt et al., 1984). Furthermore, PGE$_2$ attenuated the luteolytic action of PGF$_{2\alpha}$ when both were administered simultaneously to the ewe (Henderson et al., 1977; Mapletoft et al., 1977; Reynolds et al., 1981). In addition, it has been shown that the CL of early pregnancy is resistant to the luteolytic action of PGF$_{2\alpha}$ (Silva et al., 1984). Luteal tissue of pregnant sheep can be maintained in spite of a PGF$_{2\alpha}$ secretion pattern similar to that of the nonpregnant ewe (Silva et al., 1981; Lacroix and Kann, 1982).

Blastocyst or later-stage embryos of sheep (Lacroix and Kann, 1982), cattle (Lewis et al., 1982; Shemesh et al., 1984; Lewis et al., 1986) and pigs (Pakrasi and Dey, 1982; Racowsky and Biggers, 1983) have been shown to metabolize exogenous arachidonic acid (AA) to various prostaglandin
products. Lewis et al. (1982) have reported that the bovine conceptus at day 19 post-mating is capable of metabolizing AA to PGF\(_{2\alpha}\), PGE\(_2\) and PGF\(_{2\alpha}\) metabolite, with PGF\(_{2\alpha}\) being the major pathway product. Since the conceptus at that stage of development synthesizes more PGF\(_{2\alpha}\) than PGE\(_2\) it seems unlikely that PGE\(_2\) derived from the conceptus would exert luteotrophic action on the CL. The profile of AA metabolites produced by caprine and bovine embryos at the earlier stages prior to and during the period of maternal recognition is presently not known.

In addition, a preliminary experiment was conducted with caprine embryos to standardize procedures and provide insight into the AA metabolites produced by early stage embryos and to aid in constructing a physiological model for the cow. Thus, the primary objective of this study was to evaluate profiles of AA metabolites (prostaglandins and lipoxygenase-derived products) synthesized by bovine embryos collected at various stages of development (days 6 through 15 post-mating) from donor females. Such information could provide a clue to whether some embryo-derived prostaglandins were directly associated with the process of maternal recognition of pregnancy and embryo development.

Materials and Methods

**Embryo Donors**

**Caprine.** Thirty mixed breed dairy goats predominantly composed of Nubian, Toggenberg and Saanan breeds were used
in a preliminary study. The does were maintained on mixed pastures of native grass and bermudagrass during the day and fed .22 kg/head/day of a total mixed ration containing 12.5% crude protein, 3.1% fat, 12.7% fiber and 5.1% ash each evening. Each doe was synchronized using a 6 mg norgestomet implant (Syncromate-B®: CEVA Laboratories, Inc., Overland Park, KS) placed subcutaneously on the dorsal surface of the ear (as described by Pendleton et al., 1986) for 14 days. Two days prior to implant removal, follicle stimulating hormone (FSH) (FSH-P®: Schering Corporation, Kenilworth, NJ) was administered intramuscularly (i.m.) twice daily in descending doses (4, 3, 2, 1 mg FSH) to stimulate follicular growth for a total dose of 20 mg FSH per donor.

All does were checked for signs of behavioral estrus (estrus = day 0) twice daily with a fertile male beginning 12-hours after implant removal. Estrusl females were mated with two fertile males at 12-hour intervals until they would no longer stand to be mated. Donor does were then removed from feed and water 24 to 36 hours prior to surgical embryo recovery (days 6, 9, 12, 13 and 15 post-estrus).

**Bovine.** Thirty mature crossbred beef females (≥3 years of age) of Angus, Hereford and Brahman breeding were randomly selected from a larger embryo donor pool for the use in this study. These nonlactating, cyclic females were maintained on bermudagrass pasture and fed grass hay prior to and during the experimental interval.
Potential donor females between days 8 and 15 of their respective estrous cycles (estrus = day 0) were assigned to one of two standard gonadotropin treatment schedules to stimulate superovulation. Twice daily injections (i.m.) of follicle stimulating hormone (FSH) (FSH-P®: Schering, Omaha, NE) were administered to donors in descending doses over a 4-day interval. One injection schedule called for 12, 10, 8 and 6 mg FSH per day, resulting in a total dose of 36 mg FSH per donor female, and a second group of donors was similarly administered 10, 8, 6 and 4 mg FSH per day for a total dose of 28 mg of FSH per donor animal. Forty-eight hours after the initial FSH injection a single dose of 500 μg cloprostenol (Estrumate®: Miles Laboratory, Shawnee Mission, KS) was administered (i.m.) to each female to induce luteal regression. Each donor female was mated naturally at the beginning of standing estrus to a fertile Angus bull and then artificially inseminated with two units of frozen-thawed Angus semen 24 hours after the onset of estrus.

**Embryo Recovery**

*Caprine.* Does (on days 6, 9, 12, 13 and 15 post-mating) were anesthetized by administering .11 mg/kg of xylazine (i.m.) (Rompun®: Miles Laboratories, Inc., Shawnee, KS) followed 10 minutes later by 5.5 mg/kg of ketamine hydrochloride (i.v.) (Ketaset®: Bristol Laboratories, Syracuse, NY). Donors were then placed in dorsal recumbency and the reproductive tract exposed via a mid-ventral in-
cision immediately anterior to the udder.

A Foley catheter (10 French) was then inserted into the uterine lumen of the uterine horn near the posterior uterine bifurcation and the cuff inflated. An 18-gauge hypodermic needle attached to a 35 ml syringe containing flushing medium was then inserted into the uterine lumen near the utero-tubual junction (UTJ) and 70 to 80 ml of flushing medium flushed through the uterine horn out through the catheter and collected into an evaporating dish. The flushing medium was composed of Dulbecco's PBS supplemented with 2% heat-inactivated fetal calf serum (FCS) and an antibiotic-antimycotic solution (Ab-Am) containing 100 units of penicillin, 100 μg of streptomycin and .25 μg amphotericin-B per ml of medium (Rorie et al., 1987c). The procedure was then repeated on the opposite uterine horn. Following collection of the embryos, the evaporating dishes were searched under a stereomicroscope (20X) and harvested embryos transferred to a modified holding medium (HM) (Rorie et al., 1987c) until assignment to treatment.

Bovine. Donors were non-surgically collected on either days 6, 8, 10, 12, 13, 15 or 17 of their respective estrous cycles. Each uterine horn was flushed with a two-way Foley catheter (18 French) using a procedure previously described by Looney et al. (1981). Dulbecco's PBS with 2% (v/v) FCS and Ab-Am per ml was used as the flushing medium. Following collection, embryos were maintained in HM at 20°C.
Incubation of Embryos with Labeled Arachidonic Acid

Prior to incubation, caprine and bovine embryos were evaluated for morphological development using a stereomicroscope (100X) and assigned embryo quality grade scores. Embryos were classified as follows: grade 1 = excellent, grade 2 = good, grade 3 = poor and grade 4 = degenerate. The criteria used for assessing embryo quality grades were those previously outlined by Lindner and Wright (1983). Good quality embryos were washed with 1.2 ml of PBS four times to remove any FCS accumulated on the zona pellucida.

Tritiated [5, 6, 8, 9, 11, 12, 14, 15-3H(N)] arachidonic acid (specific activity 220.6 Ci/mmol) used in the embryo culture was obtained from DuPont NEN Research Products (Boston, MA). On arrival, the tritiated AA was stored in its original solvent (ethanol) at -20°C. Prior to the addition of the various stage embryos to culture, 10 μCi of 3H-arachidonic acid was placed in each well of a four-well sterile tissue culture plate (Nunclon: Nunc, Denmark) and the ethanol allowed to evaporate to near dryness. The ethanol was evaporated to reduce the chance of having a detrimental effect on the embryos during culture. A 250 μl volume of minimum essential medium (MEM) with Ab-Am was then added to each 3H-AA treated well of the culture plate. Embryos were then added to the culture plates and incubated for 15 hours at 37°C in a humidified atmosphere of 5% CO₂ in air.
To evaluate the time course of prostaglandin production by viable embryos from the endogenous precursor, embryos collected on day 15 post-mating were incubated for 24 hours in 5 ml of MEM and Ab-Am but without arachidonic acid. An aliquot of the medium (300 µl) was taken at various incubation times (0, 2, 4, 6, 8, 12, 15 and 24 hours) and assayed for PGE₂ and 6-keto-PGF₁α by RIA. The formation of PGE₂ and 6-keto-PGF₁α from the endogenous precursor by day-15 bovine embryos continued during a 24-hour incubation interval as shown in Figure 3. Prior experimentation with later stage bovine embryos (≥day 10) had shown that they were less viable when cultured for 24 hours in medium without serum. Thus, the incubation of embryos with ³H-AA in this study was carried out for 15 hours.

Good quality goat embryos were assigned to treatment on days 6, 9, 12, 13 and 15 post-mating (estrus = day 0). Correspondingly, embryos from superovulated donor cattle were assigned to treatment periods corresponding to days 6, 8, 10, 12, 13 and 15 post-mating. After equilibration at room temperature, 7 to 16 embryos of each species harvested prior to day 10 were designated as an embryo sample and incubated in the culture system. If the donor animal prior to day 10 produced good quality embryos less than the minimum number (n = 7) required for the incubation, embryos from other donors (collected at the same time period) were randomly selected and added to those of the original donor.
Figure 3. Time course for the formation of 6-keto-PGF$_{1\alpha}$ (●) and PGE$_2$ (○) from the endogenous precursor by the day-15 bovine embryos. 6-keto-PGF$_{1\alpha}$ and PGE$_2$ were measured by RIA.
to obtain a sufficient number of embryos for incubation. At least one caprine sample on days 9, 12, 13 and 15 did originate from a single donor female. Likewise, bovine embryo samples for days 6, 8 and 10 originated from embryos collected from a single donor female.

Because of rapid growth following the hatched blastocyst stage of development, less numbers of elongated embryos were assigned to culture wells for those collected at later stages (days 12 through 17). At least two donor animals were assigned to each of the treatment periods starting on day 6. In this study, a total of 169 caprine embryos and 104 bovine embryos were classified as viable and were used for incubation with tritiated arachidonic acid.

Following the culture period, embryos were evaluated for morphological development, assigned embryo quality grades and then harvested with the MEM culture medium and stored in 10 ml screw-top glass vials. A 250 μl volume of absolute ethanol was used to wash each well of the tissue culture plate three times (750 μl total per well). These washings were then added to the MEM containing the embryos and stored at -20°C until they were assayed by high performance liquid chromatographic (HPLC) analysis.

In another experiment bovine embryos (n = 9) collected on day 9 post-mating were incubated for 15 hours in 1 ml of MEM containing 10 μl of cold arachidonic acid for immuno-chromatographic analysis.
HPLC and RIA

Each ethanolic sample was evaporated under nitrogen gas to a volume of 700 to 800 µl. The sample was then diluted with 20 ml of HPLC-grade water to achieve an ethanol concentration of less than 5%. The aqueous solution was subjected to solid phase extraction using Sep-Pak C-18 cartridges (Waters, MA) as previously described by Powell (1982). The Sep-Pak was eluted with 4 ml of HPLC-grade methanol and subjected to HPLC analysis. The HPLC system (Beckman Model-342) consisted of two pumps (Model-112), a variable UV detector (Model-165) and a C-18 reversed phase column (Utasphere-ODS, 5 µm, 4.6 mm X 25 cm). A guard column (4.6 mm X 4 cm) was also packed with a C-18 reversed phase. Radioactivity from the eluents was monitored by a Flow-One Beta radioactivity detector (Radiomatic Instruments). The solvent system used for separation of prostaglandins from the embryo samples incubated with tritiated arachidonic acid consisted of a varying concentration of acetonitrile and water containing phosphoric acid (1 ml/l). A solution consisting of 25% of acetonitrile and 75% water were pumped for 20 minutes, followed by a gradient increase in acetonitrile to 35% over the next 20 minutes and thereafter, acetonitrile was increased to 100%. The flow rate in this system was 1.5 ml/minutes.

For immunochromatographic analysis of bovine embryo samples incubated with unlabeled arachidonic acid, the
sample was eluted with 25% acetonitrile (containing 10% water and 0.2% acetic acid) and 75% water for 21 minutes. Fractions were collected at 1-minute intervals, evaporated under nitrogen and then reconstituted 1 ml of PBS-gelatin (.1%) for RIA.

Radioimmunoassay for eicosanoids in bovine embryos was conducted as described in previous reports from this laboratory (Hwang and Kinsella, 1979; Bryant and Hwang, 1983; Hwang, 1985). Tritiated PGE₂ (100-200 Ci/mmol), PGF₁α (150-180 Ci/mmol) and 6-keto-PGF₁α 120-180 Ci/mmol) were obtained from DuPont NEN Research Products (Boston, MA).

Results

Embryo Recovery

Acceptable quality embryos (quality grades 1 and 2) were recovered from 24 of 30 (80%) donor goats and 22 of 30 (73%) superovulated donor cattle. Embryo stages ranged from early morulae collected on day 6 to elongated hatched blastocysts collected day 17 post-mating. Good quality embryos from at least two donor does and two donor cows were assigned to each treatment day.

In the preliminary study with goat embryos, it was found that as the embryos increased in size across collection days fewer embryos were needed in each culture well to produce detectable AA metabolites with the HPLC. Based on the preliminary results, the most bovine embryos cultured on any one treatment day was 23 for those collected on day 6
post-mating and the fewest number cultured was six embryos for a day-17 collection. The fewest number of day-6 bovine embryos producing detectable AA metabolites was seven per well and the fewest number of day-17 embryos producing detectable AA metabolites was three elongated embryos per culture well. The range in sizes (diameter without the zona pellucida or trophoblast length) of bovine embryos cultured was: 95-129, 108-155, 117-297, 288-495, 270-2,500, 1,000-4050 and 9,500-20,000 μm for treatment days 6, 8, 10, 12, 13, 15 and 17, respectively. The variability in conceptus size increased dramatically as the age of the embryo groups (days of gestation) advanced (Figure 4).

Arachidonic Acid Metabolites

Caprine All embryos (morulae to hatched blastocyst stages) from the does collected on both days 6 (n = 2) and 9 (n = 4) post-mating, metabolized arachidonic acid to a PGF$_{2\alpha}$ metabolite (PGFM) (Table 9). No other prostaglandin products were detected in the analysis by HPLC on these collection days. By day 12 of gestation, caprine embryos metabolized the radiated arachidonic acid precursor to several different prostaglandin products (6-keto PGF$_{1\alpha}$, PGE$_2$, and PGFM). Surprisingly, on day 13, no 6-keto PGF$_{1\alpha}$ was present in the three samples evaluated, however, PGF$_{2\alpha}$, PGE$_2$ and PGFM were detected. Perhaps, day 12 or 13 post-mating was a transitionary period because by day 15 6-keto PGF$_{1\alpha}$ in
Figure 4. Morphological changes of typical bovine embryos collected during the early stages of embryonic development. The mean diameters of embryos are given below each diagram.
<table>
<thead>
<tr>
<th>Days post-estrus&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of embryos/day</th>
<th>Arachidonic acid metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6-keto- F&lt;sub&gt;1α&lt;/sub&gt;</td>
</tr>
<tr>
<td>Day 6</td>
<td>23</td>
<td>neg.</td>
</tr>
<tr>
<td>% positive</td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>Day 9</td>
<td>20</td>
<td>neg.</td>
</tr>
<tr>
<td>% positive</td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>Day 12</td>
<td>78</td>
<td>pos.</td>
</tr>
<tr>
<td>% positive</td>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>Day 13</td>
<td>28</td>
<td>neg.</td>
</tr>
<tr>
<td>% positive</td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>Day 15</td>
<td>20</td>
<td>pos.</td>
</tr>
<tr>
<td>% positive</td>
<td></td>
<td>60%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Day of the estrous cycle does were collected (estrus = day 0).

neg. = negative for the AA metabolite.
pos. = positive for the AA metabolite.
addition to PGF$_{2\alpha}$ and PGE$_2$ was again detected in the samples and PGFM was no longer present (Table 9).

**Bovine.** Embryos collected on days 6 through 10 post-mating (morula to hatched blastocyst stages), in each case, metabolized arachidonic acid primarily to PGE$_2$ as shown in Figure 5. When embryo samples collected from individual or multiple donors before day 10 were analyzed none of the individual or pooled samples showed peaks other than that of PGE$_2$. Embryo samples collected after day 10 were all derived from embryos collected from individual donor females. The radioactive PGE$_2$ peak of the sample from the HPLC chromatogram was co-eluted with tritiated PGE$_2$ standard. To validate the culture system, embryos judged to be nonviable were similarly incubated with tritiated arachidonic acid for selected treatment days. However, no radioactive prosta-glandin peaks were detected from the nonviable embryos incubated with arachidonic acid.

Confirmation of the PGE$_2$ peak was assessed by immunochromatographic analysis of day-9 bovine embryos incubated with cold arachidonic acid. As shown in Figure 6, the immunoreactive PGE$_2$ peak appeared at the HPLC fractions corresponding to those of the peak for the radioactive PGE$_2$ standard. The cross-reactivity of the PGF$_{2\alpha}$ antibody with PGE$_2$ was 2.8%. No immunoreactive 6-keto-PGF$_{1\alpha}$ was detected from these embryo samples.

Elongating embryos on day 13 metabolized arachidonic
Figure 5. Radiochromatographic separation of prostaglandins from embryo or trophoblastic vesicle samples incubated with $^3$H-arachidonic acid. Full scale for each chromatogram was 20,000 dpm. See the text for the HPLC solvent system. The chromatogram for day-17 sample not shown here was similar to that of day-15 sample.
Figure 6. Immunochromatographic analysis of the embryo sample (day 9) incubated with cold arachidonic acid. HPLC solvent; 25% methanol and 75% water (containing 10% methanol and 0.2% acetic acid) for 21 min; thereafter methanol was increased to 35% during the next 40 min. Flow rate 1.5 ml.
acid not only to PGE₂ but also to PGF₂α. When either trophoblastic tissues or trimmed embryonic discs were separately incubated with tritiated arachidonic acid, each component synthesized both PGE₂ and PGF₂α (Figure 5). The appearance of PGF₂α peaks in other embryo samples occurred between days 12 and 15. Thus, enhancement of PGF₂α synthesis appears to occur between days 12 and 15 in bovine embryos. The enhanced ability of embryos to synthesize PGF₂α tended to coincide with the rapid development of trophoblastic tissue surrounding the embryonic disc (Figure 4). Embryos harvested after day 15 metabolized arachidonic acid to PGI₂ (as assessed by 6-keto-PGF₁α) in addition to PGE₂ and PGF₂α. These results indicate that there are distinct transitional changes in arachidonic acid metabolism during the early stages of development by bovine embryos.

Lipoxygenase products such as mono- and di-hydroxyeicosatetraenoic acid (HETE) of arachidonic acid were not detected from the bovine embryo samples regardless of the stage of the developing embryos as shown in Figure 7. Typically, elution times for 15-HETE, 12-HETE and 5-HETE were 21, 24 and 29 minutes, respectively. The elution time for 5(s), 12(s)-diHETE was 12 minutes under the chromatographic conditions used in this experiment.

Discussion

Prior to this study, the ability of caprine embryos to metabolize AA to prostaglandin units had not been reported.
Figure 7. Radiochromatographic separation of lipoxygenase-derived products from embryo or trophoblastic vesicle samples incubated with $^3\text{H}$-arachidonic acid. Full scale for each chromatogram was 20,000 dpm. See the text for the HPLC solvent system.
Other investigators have presented results indicating that bovine embryonic cells were able to metabolize AA to prostaglandins when embryonic tissues from embryos obtained after day 13 of pregnancy were evaluated (Lewis et al., 1982; Shemesh et al., 1984). Thus, this experiment was undertaken to identify transitional changes in arachidonic acid metabolism at earlier stages of embryo development in these species.

In an early study with sheep, Moor and Rowson (1966a) demonstrated that the transfer of an embryo to the uterus of a synchronized nonpregnant recipient ewe prevented cyclic regression of the corpus luteum. A similar effect was observed when extracts or homogenates of ovine embryos were infused into the uterus (Ellinwood et al., 1979; Moor, 1968). Prostaglandin $F_2\alpha$ originating from the uterus is known to be a luteolytic agent in a variety of domestic animal species. Thus, it is possible that a luteoprotective effect of the embryo may be at least, in part, due to inhibited secretion of PGF$_2\alpha$ from the uterus (McCracken et al., 1984). However, there is no evidence that secretion of PGF$_2\alpha$ from the uterus is suppressed by the presence of an embryo during early pregnancy (McCracken et al., 1984).

It has been suggested that PGE$_2$ may have a luteotrophic action. This suggestion was based on the observations that luteal regression was delayed by administration of PGE$_2$ in the ewe (Pratt et al., 1977; Magness et al., 1981), that the
luteolytic action of PGF$_{2\alpha}$ was attenuated by PGE$_2$ (Henderson et al., 1977; Mapletoft et al., 1977; Reynolds et al., 1981) and that PGE$_2$ stimulated secretion of progesterone by large lutein cells in the ewe (Silva et al., 1984). Furthermore, ovine corpora lutea of early pregnancy are resistant to the luteolytic action of PGF$_{2\alpha}$ (Silva et al., 1984). These observations also suggest the possibility that the embryo itself secretes a luteoprotective substance. Our findings that bovine embryos at early stages of development (prior to day 12) metabolized arachidonic acid primarily to PGE$_2$ offers a hypothesis that PGE$_2$ secreted by developing embryos may trigger the cascade of events leading to luteal maintenance and the maternal recognition of pregnancy.

Some studies suggest that prostaglandins are involved in implantation in laboratory animals. Such a suggestion is based on the observation that production of 6-keto-PGF$_{1\alpha}$ by the uterus increased significantly on day 5 of pseudopregnancy (Fenwick et al., 1977) and of pregnancy in rats (Phillips and Poyser, 1981). Correspondingly, implantation occurs on day 5 in rats. Furthermore, the concentrations of PGE$_2$ and 6-keto-PGF$_{1\alpha}$ have been shown to be greater at implantation sites than in surrounding tissue areas of the uterus in pregnant rats (Kennedy and Zamecnik, 1978). The differentiation of endometrial cells into decidual cells in response to the presence of blastocysts is known to be preceded by an increase in vascular permeability. It has
also been shown that indomethacin treatment delayed or inhibited the increased vascular permeability surrounding the blastocyst in the rabbit (Hoffman et al., 1978) and impaired implantation in mice (Lau et al., 1973; Saksana et al., 1976). Both PGE$_2$ and PGI$_2$ have been shown to be vasodilators.

In our study, PGI$_2$ (as assessed by 6-keto-PGF$_{1\alpha}$) became the major prostaglandin synthesized by developing bovine embryos harvested after day 15. Embryos harvested before day 9 gave no evidence of synthesizing PGI$_2$. Since implantation occurs after day 18 in domestic cattle (King et al., 1980; King et al., 1981), it is tempting to speculate that this programmed production of PGI$_2$ after day 15 may be involved in the physiological preparation for subsequent implantation.
SUMMARY AND CONCLUSIONS

The experiments described in this dissertation were conducted in an attempt to better understand the feeder cell culture systems, in particular the fetal bovine uterine fibroblast cells (FBUFC) described by Wiemer et al. (1987) and the trophoblastic vesicle culture system first described by Camous et al. (1984). These cell culture systems secrete embryotropic factors into the medium that can be picked up and utilized by developing mammalian embryos. Experiments were conducted in an attempt to identify certain products that may have been secreted by the monolayer, the trophoblastic vesicle or the embryo itself that may enhance development and viability in vitro.

Experiment I was conducted to evaluate the in vitro development of fetal bovine uterine fibroblast cells (FBUFC) and frozen-thawed trophoblastic vesicles (bovine and caprine) in four different commercially available tissue culture media (Ham's F-10, MEM with Earle's salts, RPMI-1640 and Menezo's B2). The growth patterns (as measured by direct cell counts) of the FBUFC in the four different culture media were similar. Cell growth showed a slight decline in cell number during the first 24 hours after the wells were seeded, followed by a linear increase during the remainder of the 120-hour culture interval.

The number of bovine trophoblastic vesicles (bTV) that survived freezing tended to be lower than previously
reported (Rorie et al., 1987a), however, it was not different than the number of caprine trophoblastic vesicles (gTV) that survived the freeze-thaw process. Both bTV and gTV showed signs of damage due to the freeze-thaw process such as rents in the surface of the vesicles and the loss of the outer cell layer. Culture of these vesicles in the four different treatment media did not affect the ability of the vesicles to re-expand and develop similarly in vitro. The gTV cultured in the RPMI-1640 medium appeared to have less integrity when compared with those in the other three media evaluated, however, the number of viable gTV over time in culture was not different among treatments.

Experiment II was conducted to compare the bTV culture system, the monolayer culture system and a combination of both systems for the in vitro culture of early-stage bovine embryos through to the blastocyst stage of development. Previous in vitro studies have shown that both the monolayer and the bTV are embryotrophic, however, they had not been compared against each other in the same experiment. In addition, if both systems are better than medium alone for the culture of bovine embryos perhaps co-culture (bTV and the monolayer) would further enhance the embryotrophic effects. The results of this study verified previous studies which have indicated that the culture of bovine embryos with bTV or monolayers is better than their culture in medium alone. Unfortunately, the bTV-monolayer co-
culture group did not promote development over that of either the bTV or the monolayer alone. These results suggest that bTV and FBUFC do not act synergistically to promote bovine embryonic development in vitro.

Experiment III was conducted to evaluate the embryotropic properties of a peptide isolated from trophoblastic-vesicle conditioned medium. The experimental design was identical to Experiment II except that the experimental peptide was substituted for the bTV. Unfortunately, the peptide did not exhibit any embryotropic properties at the concentration evaluated. Embryos cultured in medium containing the peptide developed similar to those in the control medium, whereas, embryos on the monolayer had better development and quality grades than embryos in the peptide and medium alone groups.

Experiment IV was conducted to evaluate the lipid and free fatty acid production by various cell types and bTV used for the in vitro culture of mammalian embryos. Free fatty acids added to the medium have been shown to be incorporated directly into the lipid portion of the developing mouse embryo (Flynn and Hillman (1980). Thus, the lipid production by various feeder cells and trophoblastic vesicles was evaluated. The results showed that the lipid and free fatty acid production of the various cell types in vitro was very similar to those lipids and free fatty acids found in Menezo's B₂ medium.
The production of arachidonic acid (AA) metabolites (particularly the prostaglandins; PGF$_{2\alpha}$, PGE$_2$, PGI$_2$ and PGFM) by caprine and bovine embryos at various stages of development was evaluated in the final study (Experiment V). Prostaglandin E$_2$ has been reported to have luteotropic properties in the sheep and PGI$_2$ has been shown to have vasodilatory properties at the time of implantation in the rat. In addition, prostaglandin production by later stage embryos ($\geq$16 days post-mating) has been reported in the sheep, cow and pig. Perhaps the early stage embryo may signal its presence via hormonal signals in the form of prostaglandins. Although cell quantities of prostaglandins were not monitored, a hormonal profile of the arachidonic acid metabolites produced by the developing caprine and bovine embryos in vitro was established. Early-stage caprine embryos (prior to day 12) metabolized arachidonic acid to a prostaglandin F metabolite (PGFm) while later stage ($\geq$day 15) caprine embryos produced PGI$_2$ (as measured by 6-keto-$F_1\alpha$), PGF$_{2\alpha}$, PGE$_2$ and PGFM. The transition period appeared to be at days 12 to 13 post-onset of estrus.

Embryos harvested before day 12 of the estrous cycle in the cow metabolized AA primarily to PGE$_2$, however, those harvested on day 13 of the cycle metabolized AA to both PGE$_2$ and PGF$_{2\alpha}$. Furthermore, embryos collected after day 15 of the cycle metabolized AA to PGI$_2$ in addition to PGE$_2$ and PGF$_{2\alpha}$. 
In conclusion, this series of experiments indicates that the embryotropic effects of feeder cells and trophoblastic vesicles in vitro are not complementary to each other when used in combination in a co-culture system. In addition, the experimental peptide used in this study was not embryotropic at the concentration evaluated. More research is needed to further evaluate this and other embryonic peptides in co-culture systems.

Lipid production by cell monolayers and trophoblastic vesicles is similar to those lipid and free fatty acids found in commercially-available media. Additional research is needed to determine the interactions of the products of metabolism (lipids and free fatty acids) produced by cells and trophoblastic vesicles in vitro.

Finally, in view of the luteotropic properties that have been attributed to PGE₂ and the vasodilatory effect of PGI₂, the transitional change in prostaglandin synthesis by the early-stage caprine and bovine embryos may be a part of the embryonic triggering mechanism leading to maternal recognition of pregnancy.
Literature Cited


114


Kennedy, T.G. and J. Zamecnik. 1978. The concentration of 6-prostaglandin-PGF$_{1a}$ is markedly elevated at the site of blastocyst implantation in the rat. Prostaglandins 16:599-605.


VITA

Stephen H. Pool was born January 22, 1953 in Woodbury, New Jersey. He was raised in the farming community of Woodstown, New Jersey. He graduated from Woodstown High School in June of 1971. Following his high school graduation, he enrolled in West Virginia University, Morgantown, West Virginia, where he received his Bachelor of Science degree in Agriculture in 1975.

He enrolled in graduate school at Louisiana State University, Baton Rouge, Louisiana, in August 1976 and received his Master of Science in Animal Science (Reproductive Physiology) in December, 1980.

He worked at several management jobs including two dairy farms, a beef ranch and a feed and animal health product store before returning to graduate school in June of 1985. He is currently a candidate for the degree of Doctor of Philosophy in Animal Science (Reproductive Physiology) at Louisiana State University, Baton Rouge, Louisiana.

In August of 1974 he married Wanda M. VandeLinde and has been blessed with two children, Bradley and Jennifer.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Stephen H. Pool

Major Field: Animal Science (Reproductive Physiology)

Title of Dissertation: The Use of Cell Culture Systems to Improve Bovine Embryo Viability In Vitro

Approved:

Robert A. Dodge
Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

August 24, 1989