The Production, Culture and Cryopreservation of Bovine Demi-Early Embryos.

Rickey Wayne Rorie

Louisiana State University and Agricultural & Mechanical College

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The production, culture and cryopreservation of bovine demi-embryos

Rorie, Rickey Wayne, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1987
The Production, Culture and Cryopreservation of Bovine Demi-Embryos

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

Rickey W. Rorie
B.S., University of Arkansas, 1977
M.S., University of Arkansas, 1979

August, 1987
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TABLE OF CONTENTS

ACKNOWLEDGEMENTS................................................................. ii
LIST OF TABLES......................................................................... vii
LIST OF FIGURES................................................................. viii
LIST OF APPENDIX TABLES......................................................... ix
ABSTRACT............................................................................... xi

CHAPTER I. LITERATURE REVIEW

PART A. BISECTION OF BOVINE EMBRYOS................................. 1
Introduction.............................................................................. 1
Early Studies in Embryo Micromanipulation.............................. 2
Bisection of Precompaction-Stage Embryos.............................. 4
Bisection of Postcompaction-Stage Embryos.............................. 6
Factors Affecting Pregnancy Rates for Bisected Embryos........... 9
Cryopreservation of Demi-Embryos......................................... 12

PART B. IN VITRO CO-CULTURE OF BOVINE EMBRYOS............. 14
Introduction.............................................................................. 14
Co-Culture Systems.................................................................. 16
Fibroblast Monolayer Co-Culture.............................................. 16
Oviductal Epithelium Co-Culture.............................................. 20
Trophoblastic Vesicle Co-Culture.............................................. 21

CHAPTER II. BISECTING BOVINE EMBRYOS
WITHOUT THE USE OF A COMMERCIAL
MICROMANIPULATION UNIT....................................................... 28
Introduction.............................................................................. 28
# Table of Contents (cont'd)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials and Methods</td>
<td>29</td>
</tr>
<tr>
<td>Embryo Donor Preparation</td>
<td>29</td>
</tr>
<tr>
<td>Embryo Collection Procedure</td>
<td>29</td>
</tr>
<tr>
<td>Experimental Treatments</td>
<td>30</td>
</tr>
<tr>
<td>Enzymatic Zona Pellucida Removal</td>
<td>32</td>
</tr>
<tr>
<td>Construction and Use of Micromanipulators</td>
<td>32</td>
</tr>
<tr>
<td>Microscope Slide Micromanipulators</td>
<td>32</td>
</tr>
<tr>
<td>Razor Blade Method</td>
<td>34</td>
</tr>
<tr>
<td><strong>In Vitro Culture</strong></td>
<td>35</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>35</td>
</tr>
<tr>
<td>Results</td>
<td>37</td>
</tr>
<tr>
<td>Discussion</td>
<td>41</td>
</tr>
</tbody>
</table>

## CHAPTER III. CULTURE OF EARLY-STAGE BOVINE EMBRYOS INSIDE OR IN CO-CULTURE WITH DAY-13 TO 14 TROPHOBLASTIC VESICLES

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>45</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>48</td>
</tr>
<tr>
<td>Embryo Donors</td>
<td>48</td>
</tr>
<tr>
<td>Embryo Recovery</td>
<td>48</td>
</tr>
<tr>
<td>Experimental Treatments</td>
<td>49</td>
</tr>
<tr>
<td>Trophoblastic Vesicle Production and <strong>In Vitro</strong> Culture**</td>
<td>49</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>52</td>
</tr>
<tr>
<td>Results</td>
<td>52</td>
</tr>
<tr>
<td>Embryo Recovery and bTV Production</td>
<td>52</td>
</tr>
<tr>
<td><strong>In Vitro</strong> Culture of Embryos</td>
<td>53</td>
</tr>
<tr>
<td>Discussion</td>
<td>57</td>
</tr>
<tr>
<td>Chapter IV. THE CO-CULTURE OF INTACT AND BISECTED PRECOMPACTION-STAGE BOVINE EMBRYOS WITH BOVINE TROPHOBLASTIC VESICLES</td>
<td>Page</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Introduction</td>
<td>61</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>64</td>
</tr>
<tr>
<td>Embryo Donors</td>
<td>64</td>
</tr>
<tr>
<td>Embryo Recovery</td>
<td>65</td>
</tr>
<tr>
<td>Experimental Treatments</td>
<td>65</td>
</tr>
<tr>
<td>Embryo Bisection</td>
<td>66</td>
</tr>
<tr>
<td>Trophoblastic Vesicle Production</td>
<td>68</td>
</tr>
<tr>
<td>In Vitro Culture</td>
<td>68</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>69</td>
</tr>
<tr>
<td>Results</td>
<td>69</td>
</tr>
<tr>
<td>Embryo Recovery and Micromanipulation</td>
<td>69</td>
</tr>
<tr>
<td>In Vitro Culture</td>
<td>70</td>
</tr>
<tr>
<td>Discussion</td>
<td>73</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter V. THE VIABILITY OF BOVINE &quot;HALF&quot; EMBRYOS PRODUCED BEFORE OR AFTER LIQUID NITROGEN FREEZING</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>79</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>81</td>
</tr>
<tr>
<td>Donor Preparation</td>
<td>81</td>
</tr>
<tr>
<td>Embryo Collection</td>
<td>82</td>
</tr>
<tr>
<td>Experimental Treatments - Trial I</td>
<td>83</td>
</tr>
<tr>
<td>Experimental Treatments - Trial II</td>
<td>83</td>
</tr>
<tr>
<td>Micromanipulation Procedure</td>
<td>86</td>
</tr>
</tbody>
</table>
Table of Contents (cont'd) Page

Agar Embedding.............................. 88
Embryo Cryopreservation.................. 89
**In Vitro** Culture............................ 90
Statistical Analysis - Trials I and II........ 90
Results........................................ 91
Micromanipulation and Embryo Recovery - Trial I........ 91
Micromanipulation and Embryo Recovery - Trial II........ 91
**In Vitro** Survival - Trial I.............. 94
**In Vitro** Survival - Trial II.............. 96
Discussion.................................... 99
Micromanipulation........................... 99
**In Vitro** Survival.......................... 101
LITERATURE CITED................................... 107
VITA........................................... 123
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. EXPERIMENTAL DESIGN FOR EMBRYO ALLOTMENT TO TREATMENTS</td>
<td>31</td>
</tr>
<tr>
<td>2. BISECTION OF BOVINE MORULAE AND DEVELOPMENT OF DEMI-EMBRYOS</td>
<td>38</td>
</tr>
<tr>
<td>3. TOTAL NUMBER OF DEMI-EMBRYOS (DE) FORMED AND DEVELOPMENT OF DE TO BLASTOCYSTS IN VITRO</td>
<td>39</td>
</tr>
<tr>
<td>4. VIABILITY RATINGS AND EMBRYO QUALITY GRADES DURING IN VITRO CULTURE</td>
<td>54</td>
</tr>
<tr>
<td>5. DEVELOPMENTAL STAGE OF EMBRYOS AFTER 96 HOURS OF CULTURE</td>
<td>56</td>
</tr>
<tr>
<td>6. VIABILITY AND QUALITY OF BISECTED AND INTACT EMBRYOS CO-CULTURED WITH BOVINE TROPHOBLASTIC VESICLES</td>
<td>71</td>
</tr>
<tr>
<td>7. FINAL DEVELOPMENTAL STAGES OF INTACT AND BISECTED BOVINE EMBRYOS CULTURED WITH OR WITHOUT BOVINE TROPHOBLASTIC VESICLES</td>
<td>74</td>
</tr>
<tr>
<td>8. DISTRIBUTION OF EMBRYOS BY INITIAL QUALITY GRADE AND DEVELOPMENTAL STAGE IN TRIAL I</td>
<td>92</td>
</tr>
<tr>
<td>9. DISTRIBUTION OF EMBRYOS BY INITIAL QUALITY GRADE AND DEVELOPMENTAL STAGE IN TRIAL II</td>
<td>93</td>
</tr>
<tr>
<td>10. DEMI-EMBRYO QUALITY GRADES AT 12 HOURS OF IN VITRO CULTURE IN TRIAL I</td>
<td>95</td>
</tr>
<tr>
<td>11. EMBRYO/DE VIABILITY AND QUALITY IN TRIAL I AT 12 HOURS POST-THAW</td>
<td>97</td>
</tr>
<tr>
<td>12. DEMI-EMBRYO QUALITY GRADES AT 12 HOURS OF IN VITRO CULTURE IN TRIAL II</td>
<td>98</td>
</tr>
<tr>
<td>13. EMBRYO/DE VIABILITY AND QUALITY IN TRIAL II AT 12 HOURS POST-THAW</td>
<td>100</td>
</tr>
</tbody>
</table>
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Bisection of bovine morulae using the hand-held razor blade method</td>
<td>36</td>
</tr>
<tr>
<td>2.</td>
<td>Illustration of the treatments in experiment II</td>
<td>50</td>
</tr>
<tr>
<td>3.</td>
<td>Illustration of the treatments in experiment III</td>
<td>67</td>
</tr>
<tr>
<td>4.</td>
<td>Illustration of the treatments in trial I of experiment IV</td>
<td>84</td>
</tr>
<tr>
<td>5.</td>
<td>Illustration of the treatments in trial II of experiment IV</td>
<td>85</td>
</tr>
</tbody>
</table>
# LIST OF APPENDIX TABLES

<table>
<thead>
<tr>
<th>TABLES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ANALYSIS OF VARIANCE FOR THE NUMBER OF DEMI-EMBRYOS FORMED IN EACH TREATMENT IN EXPERIMENT I</td>
<td>116</td>
</tr>
<tr>
<td>2. ANALYSIS OF VARIANCE FOR THE NUMBER OF BLASTOCYSTS FORMING FROM DEMI-EMBRYOS IN EXPERIMENT I</td>
<td>116</td>
</tr>
<tr>
<td>3. ANALYSIS OF VARIANCE FOR THE NUMBER OF VIABLE EMBRYOS AT 60 HOURS IN VITRO IN EXPERIMENT II</td>
<td>117</td>
</tr>
<tr>
<td>4. ANALYSIS OF VARIANCE FOR THE NUMBER OF QUALITY GRADE 1 OR 2 EMBRYOS AT 60 HOURS OF CULTURE IN EXPERIMENT II</td>
<td>117</td>
</tr>
<tr>
<td>5. ANALYSIS OF VARIANCE FOR THE NUMBER OF VIABLE EMBRYOS AT 96 HOURS IN VITRO IN EXPERIMENT II</td>
<td>118</td>
</tr>
<tr>
<td>6. ANALYSIS OF VARIANCE FOR THE NUMBER OF QUALITY GRADE 1 OR 2 EMBRYOS AT 96 HOURS OF CULTURE IN EXPERIMENT II</td>
<td>118</td>
</tr>
<tr>
<td>7. ANALYSIS OF VARIANCE FOR THE NUMBER OF VIABLE EMBRYOS AT 48 HOURS OF IN VITRO CULTURE IN EXPERIMENT III</td>
<td>119</td>
</tr>
<tr>
<td>8. ANALYSIS OF VARIANCE FOR THE NUMBER OF QUALITY GRADE 1 OR 2 EMBRYOS AT 48 HOURS OF IN VITRO CULTURE IN EXPERIMENT III</td>
<td>119</td>
</tr>
<tr>
<td>9. ANALYSIS OF VARIANCE FOR THE NUMBER OF VIABLE EMBRYOS IN EXPERIMENT III AT 96 HOURS OF IN VITRO CULTURE</td>
<td>120</td>
</tr>
<tr>
<td>10. ANALYSIS OF VARIANCE FOR THE NUMBER OF QUALITY GRADE 1 OR 2 EMBRYOS AT 96 HOURS OF IN VITRO CULTURE IN EXPERIMENT III</td>
<td>120</td>
</tr>
<tr>
<td>11. ANALYSIS OF VARIANCE FOR THE NUMBER OF VIABLE FROZEN-THAWED EMBRYOS/DE IN TRIAL I OF EXPERIMENT IV</td>
<td>121</td>
</tr>
</tbody>
</table>
List of Appendix Tables (cont'd)

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.</td>
<td>ANALYSIS OF VARIANCE FOR THE NUMBER OF TRANSFERABLE QUALITY EMBRYOS/DE IN TRIAL I OF EXPERIMENT IV</td>
<td>121</td>
</tr>
<tr>
<td>13.</td>
<td>ANALYSIS OF VARIANCE FOR THE NUMBER OF Viable EMBRYOS/DE POST-THAW IN TRIAL II OF EXPERIMENT IV</td>
<td>122</td>
</tr>
<tr>
<td>14.</td>
<td>ANALYSIS OF VARIANCE FOR THE NUMBER OF TRANSFERABLE EMBRYOS/DE IN TRIAL II OF EXPERIMENT IV</td>
<td>122</td>
</tr>
</tbody>
</table>
The objective of experiment (Exp) I was to develop and evaluate a simplified method for producing bovine demi-embryos (DE). Treatment (Trt) A and B were the bisection zona pellucida intact (ZI) and free (ZF) embryos, respectively, using a microscope slide micromanipulator. In Trt C and D, ZI and ZF embryos, respectively, were bisected with a hand-held razor blade method. More DE were produced by the razor blade method (Trt C and D) than by the microscope slide micromanipulator method (Trt A and B). More DE were produced from ZF embryos (Trt B and D) than from ZI embryos (Trt A and C). A greater number of DE developed to blastocysts in Trt C (82%) than in Trt A, B, or D.

In Exp II, the in vitro development of bovine embryos cultured inside (Trt A) bovine trophoblastic vesicles (bTV) were compared with co-culture with bTV (Trt B) and with medium alone (Trt C). After 96 h of culture, 58%, 72% and 44% of the embryos in Trt A, B and C, respectively, were considered viable. The number of grade 1 or 2 embryos in Trt B after 96 h of culture was greater (50%) than the number in Trt C (17%), but similar to the number in Trt A (36%).

In Exp III intact and bisected bovine embryos (n=33/Trt) were cultured 96 h with bTV or in medium alone. The Trt were: (A) DE cultured in medium alone, (B) DE co-cultured with a bTV, (C) intact embryos cultured in medium alone, (D) intact embryos cultured with a bTV.
Fewer DE (Trt A and B) were viable or of grade 1 or 2 than intact embryos (Trt C and D). More embryos co-cultured with bTV (Trt B and D) were viable and quality grade 1 or 2 at the end of 96 h culture than were embryos cultured in medium alone (Trt A and C).

In Exp IV, the viability of DE produced before or after freezing were compared in two trials. The Trt in Trial I were: (A) DE formed before freezing, (B) DE formed after freezing-thawing, (C) intact control embryos, (D) intact control embryos. Embryos in Trt A, B and C were agar embedded before freezing. The Trt in Trial II were: (A) DE formed before freezing, (B) DE formed after freezing-thawing, (C) intact control embryos. One DE of each pair in Trt A was agar embedded to produce a ZI embryo (Trt A-I) and the other was frozen ZF (Trt A-II). In Trial I, more embryos were viable and transferrable in Trt C and D post-thaw than in Trt A or B. The number of viable and transferable embryos in Trt A and B were similar. In Trial II, the number of viable and transferable embryos in A-I and A-II of Trt A were similar. Also, the number of viable and transferable embryos in Trt A and B were similar. More embryos were viable and of transferable quality in Trt C than in either Trt A or B.
CHAPTER I

REVIEW OF LITERATURE

PART A. BISECTION OF BOVINE EMBRYOS

Introduction

Monozygotic twins, offspring originating from a single fertilized ovum, have proven their value as experimental animals in a number of physiological investigations (Hancock, 1954; Stormont, 1954; Christian et al., 1965; Biggers, 1986). Unfortunately, the incidence of natural monozygotic twinning is low. Monozygotic twins in cattle occur in about one of every one-thousand births (Hafez, 1974). In addition, the natural monozygotic twins available to researchers may not be of the sex or the parentage of interest.

A number of studies have been conducted during the last 10 years to develop embryo microsurgical techniques for artificially producing monozygotic twin offspring. In the 1970's, it was thought that later-stage mammalian embryos would not survive following microsurgery so micromanipulation studies were directed toward the use of early-stage embryos. Studies in the early 1980's, however, showed that morula and blastocyst stage embryos of farm animals were able to develop following bisection and could produce viable offspring.

Embryo bisection has generated a great deal of interest among commercial embryo transplant stations. As procedures for splitting embryos have become more efficient, commercial transplant units began incorporating this new methodology into their offerings to livestock producers.
Early Studies in Embryo Micromanipulation

The early studies in mammalian embryo micromanipulation involved evaluation of the developmental potential of single blastomeres isolated from early-stage embryos. Pincus (1936) transplanted single blastomeres of 2-cell rabbit embryos to the oviduct of a pseudopregnant doe and observed the development of normal, but small size blastocysts. In a similar study, Nicholas and Hall (1942) obtained pregnancies from the isolated blastomeres of 2-cell rat embryos. However, none of the embryos survived beyond day 10 of gestation.

Seidel (1952) was the first to report on the birth of live offspring from micromanipulated embryos. In this study, a glass needle was used to puncture and destroy one of the blastomeres of 2-cell rabbit embryos. These single blastomere embryos were transplanted to recipient does resulting in the birth of two normal offspring. In a later study, Tarkowski (1959) used a glass needle to puncture and destroy one blastomere of 2-cell mouse embryos. The resulting single blastomere embryos were transferred to recipients and a portion of the females were sacrificed on days 5 through 15 of gestation to monitor fetal development. Examination of the fetuses on days 5 to 10 revealed that fetuses of "half" embryos were about one-half the size of the fetuses produced from intact embryos. By day 11 to 15 of gestation, however, the fetuses produced from "half" and intact embryos were similar in size. Three recipient mice carrying "half" embryo fetuses were allowed to go to term resulting in the birth of six normal offspring.

Daniel and Takahashi (1965) further evaluated the in vitro developmental potential of single blastomeres of rabbit embryos by destroying all but one blastomere of 2-, 4- and 8-cell embryos. The
"half" and "quarter" embryos were observed to regularly develop in vitro. Very few of the "eighth" rabbit embryos developed in vitro indicating there was a limit to the totipotency of individual blastomeres of the more advanced 8-cell embryos. In a later study, Moore et al. (1968) transplanted isolated blastomeres of 2-, 4- and 8-cell rabbit embryos and reported that 11 to 30% could survive in vivo to produce viable offspring. It was concluded that survival of the micromanipulated rabbit embryos in vivo was dependent on the presence of a near intact zona pellucida.

The studies mentioned above provided evidence that the individual blastomeres of early-stage mammalian embryos were totipotent and the potential existed for the production of multiple offspring from a single embryo. The technique of selective destruction of blastomeres used in these studies, however, had the limitation that all of the blastomeres were not available to produce offspring. The production of multiple offspring from a single embryo would require the separation of blastomeres into two or more groups to produce two or more embryos.

Moore et al. (1969) separated the blastomeres of 4- and 6-cell porcine embryos and injected these individual blastomeres into evacuated zonae pellucidae. However, none of the micromanipulated embryos survived in vivo. In the same study, the developmental potential of isolated blastomeres were also evaluated by destroying all but one blastomere of 4- and 6-cell porcine embryos. Thirty-five percent of these embryos did survive in vivo. It was noted, as with rabbit embryos, that porcine embryos only survived in vivo when the zonae pellucidae were near intact. The injection of individual blastomeres
into separate zonae pellucidae likely resulted in too much damage to the zona pellucida for the embryos to survive.

Other studies showed that the in vivo survival of precompaction-stage mouse embryos (Bronson and McLaren, 1970; Modlinski, 1970) and sheep embryos (Trounson and Moore, 1974) was dependent on the presence of a near intact zona pellucida. The poor survival in vivo of micromanipulated precompaction-stage embryos (without an intact zona pellucida) has been attributed to disaggregation of the embryonic cells (Bronson and McLaren, 1970; Willadsen and Fehilly, 1983), invasion and destruction of the blastomeres by leucocytes (Moore et al., 1968) and detrimental effects due to direct contact of the embryonic cells with the uterine endometrium (Modlinski, 1970).

The inability of precompaction-stage embryos to survive in vivo without a near intact zona pellucida became a major obstacle to micromanipulation studies with early-stage embryos. This obstacle was not overcome in farm animals until Willadsen (1979) developed an agar embedding technique to produce an "intact" zona pellucida following embryo micromanipulation.

**Bisection of Precompaction Stage Embryos**

Willadsen (1979) first reported the use of the embryo agar embedding technique for producing monozygotic twin offspring in sheep. In this study, the blastomeres of 2-cell sheep embryos were microsurgically separated and placed into evacuated zonae pellucidae. The single blastomere embryos were then placed into a liquid solution of 1% agar and aspirated with the agar solution into a pipette with a diameter slightly larger than the zona pellucida of the embryo. After the agar
solution solidified, the agar embedded embryos were expelled into a liquid solution of 1.2% agar. The agar embedded embryos were again aspirated into a larger bore pipette and expelled after the agar solidified. This procedure produced a double cylinder of agar surrounding the experimental embryos. The double agar embedded embryos were then transferred to the ligated oviduct of an anestrous ewe for 3.5 to 4.5 days of in situ culture. After the in vivo culture period, the embryos were recovered from the temporary recipient, removed from the agar and examined. Forty embryos were recovered from the recipient and 35 (including 16 monozygotic pairs) had developed normally to the late morula and blastocyst stages of development. The 16 monozygotic embryo pairs were transferred to 16 recipient ewes resulting in the birth of five single and five monozygotic twin lambs.

In a second study, Willadsen (1980) microsurgically separated the blastomeres of 2-, 4- and 8-cell sheep embryos to produce "half" embryos of one, two and four blastomeres, respectively. The "half" embryos were agar embedded and cultured in vivo, as was done in the first study. Following the in vivo culture, 30 "half" embryos were selected for transfer to recipients and 12 embryos (monozygotic twins to 12 of the embryos transferred) were cryopreserved and stored in liquid nitrogen. After 1 to 2 months of storage, the cryopreserved embryos were thawed and nine of these embryos were transferred to recipients resulting in the birth of three lambs which were monozygotic twins to three lambs born previously. This was the first report of the birth of monozygotic twins of differing age.

In a subsequent study, Willadsen and Polge (1981) separated the blastomeres of 8-cell bovine embryos into "quarter" embryos of two
blastomeres each. Following agar embedding and in vivo culture, 34 (77%) of the "quarter" embryos had developed to form blastocysts. Twenty-six "quarter" blastocysts were transferred to recipient heifers resulting in the birth of two sets of monozygotic twins, one set of monozygotic triplets and a single calf. Also, Willadsen et al. (1981) used the blastomere separation and agar embedding techniques to produce twin calves from non-surgically collected day-5 to day-6 bovine embryos. Willadsen (1982) later used his embryo micromanipulation techniques to produce twin foals from single blastomeres of 4-cell embryos and twin piglets from blastomere separated 2- and 4-cell embryos.

Although the agar-embedding technique has been shown to be an excellent method for the micromanipulation and culture of early-stage embryos, this procedure is generally considered by most not to be practical enough for routine use. The procedure is time consuming, labor intensive and usually results in some embryo loss when the embryos are recovered following in vivo culture. In order to be more practical, procedures for bisecting embryos were required which would allow for efficient bisection of non-surgically collected day-6 to day-8 embryos and which would allow immediate transfer into recipient animals. In 1982, three independent research groups reported on micromanipulation techniques for non-surgically collected later-stage bovine embryos (Ozil et al., 1982; Williams et al., 1982; Lambeth et al., 1982).

**Bisection of Postcompaction Stage Embryos**

Ozil et al. (1982) described a technique using six microinstruments to bisect bovine embryos. Two glass microneedles were used to
open the zona pellucida, and a glass transfer pipette was used to remove the embryonic cell mass from the zona pellucida. The zona pellucida-free embryo was bisected on a vertical plane with a microscalpel while resting on the floor of the micromanipulation vessel. The halves were replaced into evacuated zonae pellucidae with the transfer pipette. Using these micromanipulation procedures, 14 early blastocysts were bisected and transferred non-surgically to 14 recipients, resulting in a pregnancy rate of 64.2% and a twinning rate of 66.6%.

In the same year, Williams et al. (1982) described a procedure for splitting the embryo while in the zona pellucida. This method used a razor blade chip mounted with glue (Super Glue) on a glass capillary tube to bisect the intact embryo. A glass pipette was then used to transfer each of the "half" embryos (demi-embryos) to evacuated zonae pellucidae. In this study, 20 bovine embryos (morula to early blastocyst stage) were bisected and transferred as demi-embryo pairs either surgically or non-surgically to 20 recipient cattle. Fourteen surgical and six non-surgical transfers resulted in pregnancy rates of 64% and 17%, respectively.

Also in 1982, Lambeth et al. (1982) described an embryo bisection procedure for non-surgically collected bovine embryos. With this method, a fine glass needle was used to make a rent in the zona pellucida. The embryonic cell mass was removed with the same flexible glass needle and bisected on a vertical plane while resting on the bottom of the petri dish. A glass transfer pipette was used to transfer each demi-embryo to a separate evacuated zona pellucida. In this study, eight demi-embryo pairs and six individual demi-embryos were transferred non-surgically to 14 beef recipients. The twin demi-embryo
transfers resulted in a pregnancy rate of 62.5% and the single demi-embryo transfers resulted in a pregnancy rate of 16.6%.

At present, the more simplified method described by Williams et al. (1982) has become more widely adapted for use by researchers and embryo transplant units than the other procedures. However, embryo bisection using the microsurgical blade technique on zona pellucida intact (Chesne et al., 1987) or on zona pellucida-free bovine embryos (Picard et al., 1986) results in destruction of 15% or more of the embryonic cells. In a recent comparative study, Mertes and Bondioli (1985) have suggested that the bisection technique using a fine glass needle (Lambeth et al., 1982) may cause less embryonic cell damage than the microsurgical blade technique (Williams et al., 1982).

Until recently, the embryo bisection technique of Ozil et al. (1982) has had the disadvantage of requiring six microinstruments to bisect an embryo. Massip et al. (1986) has reported a modification of the procedure which only requires two microinstruments. With this procedure, the zona pellucida-intact embryo is positioned on the bottom of the micromanipulation vessel with a suction pipette while a microsurgical blade is lowered vertically to bisect the embryo. In the latter study, 80% of the demi-embryos produced using this bisection method showed further development in vitro. The transfer of 12 demi-embryo pairs to 12 bovine recipients resulted in four twin and two single pregnancies.

A simplified method for bisecting morula to hatched blastocyst-stage ovine embryos has been reported (Willadsen and Godke, 1984), which is readily adaptable for bovine embryos. This procedure uses a fine glass needle to penetrate and to bisect zona pellucida-intact
morulae or blastocysts and zona pellucida-free hatched blastocysts. The overall transfer pregnancy rate in sheep using this procedure was 89%. More recently, a simplified technique for bisecting later-stage farm animal embryos was reported by Rorie et al. (1985). This technique allows for rapid bisection of intact embryos without the use of a commercial micromanipulation unit. With this method, an intact embryo is placed in a small drop of holding medium on a microscope slide, and a razor blade held by a pair of hemostats is used to bisect the embryo. In this study, 98% of the intact bovine embryos were successfully bisected using this razor blade method. This new method is simple to execute, inexpensive and easy to learn.

Factors Affecting Pregnancy Rates for Bisected Embryos.

Recently, research efforts have been directed toward identifying factors that contribute to optimal pregnancy rates from bisected embryos. Experience has shown that, with proper technique, excellent and good quality embryos (grades 1 and 2) are suitable for bisection. The transfer of demi-embryos produced from either excellent or good quality embryos has been shown to result in pregnancy rates similar to that obtained with intact embryos of the same quality (Voelkel et al., 1984b,c; Baker et al., 1984; Takeda et al., 1986). Pregnancy rates expected from single demi-embryos non-surgically transplanted to individual recipient females usually range from 45 to 65% (Baker and Shea, 1985; Takeda et al., 1986; Leibo and Rall, 1987).

Studies have shown that when embryos are bisected into "quarter" embryos from precompaction (Willadsen et al., 1981) or postcompaction-stage embryos (Voelkel et al., 1985b; 1986) lower uterine survival
occurs. It has been suggested that a single "quarter" embryo is less likely to produce a sufficient luteotrophic signal to prevent luteal regression in the recipient female (Willadsen et al., 1981). Correspondingly, the reduced size of the developing blastocyst from a "quarter" embryo may lack sufficient embryonic cells in the inner cell mass to form a viable embryo. Recently, Voelkel et al. (1986) bisected cultured blastocysts that had been derived from bovine demi-embryos. In this case, only 17.6% of the "quarter" embryos derived from cultured demi-embryos produced a second blastocyst in culture.

Efforts have been made to evaluate the optimal stage of morphological development to bisect bovine embryos for transfer. Similar pregnancy rates have been reported for bovine demi-embryos produced from late morula and blastocyst stage embryos (Williams et al., 1984; Takeda et al., 1986). However, pregnancy rates from demi-embryos produced from early morulae have been reported to be significantly lower than pregnancy rates resulting from demi-embryos produced from early blastocysts (Williams et al., 1984). These results suggest that early morulae are less suitable for bisection.

The zona pellucida appears to have little effect on the in vivo survival of demi-embryos produced from late morula to blastocyst-stage bovine embryos. Studies have shown that the number of pregnancies produced from bovine demi-embryos placed into the original zona pellucida were similar to those placed in foreign zona pellucida (Williams et al., 1984; Takeda et al., 1986).

In contrast, Baker and Shea (1985) have noted a lower pregnancy rate from demi-embryos placed into surrogate zona pellucida than for demi-embryos left in the original zona pellucida (58% vs. 74%). Baker
and Shea (1985) suggested that the lower pregnancy rate was likely due to injury to the "half" embryos during their transfer into the foreign zona pellucida. Furthermore, it was mentioned that in 19 of 23 cases where the demi-embryos were recorded as proportionately uneven, the smaller half was transferred to the foreign zona pellucida. However, the disproportionate size may not explain the lower pregnancy rates since pregnancy rates have been reported to be similar for demi-embryos which are slightly different in size (Picard et al., 1986).

It is important to note that other studies have shown that later-stage zona pellucida-free demi-embryos survive in vivo as well as demi-embryos encased in a zona pellucida prior to transfer (Voelkel et al., 1984b; Warfield et al., 1986). Although the zona pellucida may not be required for in vivo survival, there is a practical reason for replacing the demi-embryos into a zona pellucida prior to transfer. The zona pellucida makes the demi-embryo readily identifiable to the technician loading the embryos into plastic straws for transfer to recipients and may protect the embryo during the actual transfer process (S. Leibo, personal communication).

The highest pregnancy and twinning rates have been obtained when demi-embryo pairs are transferred to a recipient rather than a single demi-embryo (Ozil, 1983; Lambeth et al., 1983; Baker and Shea, 1985). Pregnancy rates have been reported to be similar when demi-embryo pairs are transferred to the same uterine horn or when one demi-embryo is transferred to each uterine horn (Baker and Shea, 1985).
Cryopreservation of Demi-Embryos

Embryos are routinely bisected as soon as possible after collection and then transferred to recipients. Efforts have been made to evaluate the possibility of bisecting frozen-thawed embryos and/or frozen storage of bisected embryos. Willadsen (1980) was the first to successfully cryopreserve demi-embryos of farm animals. Twelve sheep blastocysts produced by blastomere separation and in vivo culture of 2- to 8-cell sheep embryos were cryopreserved for 30 to 60 days. Nine demi-embryos (75%) survived cryopreservation and were transferred to five recipient ewes, resulting in two ewes lambing to produce three frozen-thawed transplant lambs. These lambs were monozygotic twins to three lambs born 30 to 60 days previously.

In cattle, Lehn-Jensen and Willadsen (1983) attempted to cryopreserve "half" and "quarter" bovine embryos produced by blastomere separation, agar embedding and in vivo culture in the ligated oviducts of ewes prior to freezing. The transfer of six monozygotic pairs of "half" embryos to recipients resulted in the birth of four pairs of monozygotic calves. Eight of 16 monozygotic "half" embryo pairs (50%) and three of five monozygotic "quarter" embryo pairs frozen were considered viable post-thaw. Overall, 16 monozygotic pairs were cryopreserved, but only four pairs of monozygotic calves were produced. Four frozen-thawed "quarter" embryos were transferred but none of these resulted in a pregnancy. The results from this preliminary study suggested that frozen-thawed "half" embryos were less viable than fresh "half" embryos, and that it may not be feasible to produce offspring from frozen-thawed "quarter" embryos.
Heyman (1985) bisected frozen-thawed day-8 bovine blasotcysts and reported pregnancy rates of 20% at 90 days of gestation for 20 demi-embryos as compared with a 36% pregnancy rate for 11 demi-embryos produced from fresh blastocysts. It was indicated that the lower pregnancy rate for the demi-embryos produced from frozen-thawed embryos could possibly be attributed to a reduction in the luteotrophic signal of the bisected frozen-thawed embryos.

In a very recent study, Massip et al. (1987) compared the viability of bovine demi-embryos frozen by a conventional method or the vitrification method of Rall and Fahy, (1985). Six monozygotic pairs of demi-embryos were cryopreserved using phosphate-buffered saline with 10% glycerol and 20 monozygotic pairs of demi-embryos were cryopreserved in a vitrification solution of 25% glycerol and 25% propanediol in phosphate-buffered saline. The six pairs of demi-embryos cryopreserved by conventional methods were transferred to six recipients, resulting in the birth of a single calf. The 20 demi-embryo pairs cryopreserved by vitrification were transferred as pairs to 20 recipients resulting in the birth of eight calves (including two monozygotic pairs).

Embryo bisection has potential uses other than increasing the number of offspring from a single embryo. An example of this is embryo sexing, where half of the embryo is sexed and the remaining half is transferred to a recipient (Nakagowa et al., 1985; Picard et al., 1985). In a recent study, White et al. (1987) bisected bovine embryos and sexed one demi-embryo of the pair using an H-Y antibody procedure. Then each demi-embryo of the pair was transferred to a different recipient animal. The success rate for sexing was 90%, and there was no
significant difference in pregnancy rates between the sexed and control demi-embryos (47% vs. 44%).

The bisection methods described by Ozil et al., (1982), Lambeth et al. (1982) and Williams et al. (1982) have been successfully adapted for use by researchers and the commercial embryo transplant industry. Research has shown that excellent-to-good quality late morula to blastocyst stage bovine embryos can be successfully bisected to produce split-embryo offspring. Under optimal conditions, bisected embryos can produce pregnancy rates comparable to those achieved with intact embryos from the same collection.

Research has shown that replacement of the bisected embryo into a zona pellucida prior to transfer is not necessary for later-stage bovine embryos. Optimal pregnancy and twinning rates have been achieved thus far by transfer of both halves of a bisected embryo to the same recipient. In this case, there remains a potential for problems during gestation and at the time of calving. Research is needed to develop a procedure for producing transplant offspring from demi-embryos which have been bisected either before or after freezing.

PART B. IN VITRO CO-CULTURE OF BOVINE EMBRYOS

Introduction

Early studies into the in vitro culture requirements of mammalian embryos focused on culture of embryos of the laboratory animal species. Whitten (1957) reported that 2-cell mouse embryos could be cultured to the blastocyst stage of development in a simple physiological saline
medium containing bovine serum albumin and lactate. This study led to a number of other studies into the in vitro culture requirements of mammalian embryos. A multitude of media and culture systems have been evaluated for the in vitro culture of farm animal embryos (see reviews by Betteridge, 1977; Kane, 1978, 1987; Wright and Bondioli, 1981). The most successful in vitro culture systems to date have been co-culture systems using "helper" cells derived from biological tissue.

There are two periods of embryonic development in which in vitro culture is of practical importance in farm animals. The development of new embryo technologies in farm animals such as in vitro fertilization (Brackett et al., 1982), gene injection (Hammer et al., 1985) and embryonic cloning (Willadsen, 1986) require the culture of ova and(or) very early cleavage-stage embryos prior to transfer to surrogate females. The inability to adequately maintain the viability of early-stage embryos in vitro has been a major obstacle to the further development of these new technologies.

The second period of embryonic development which is of practical importance is the morula to blastocyst stage of development. Embryo transfer, bisection and cryopreservation are routinely performed with morulae and blastocysts. A short-term culture period is required for the evaluation of embryo viability before transfer to recipients and before and(or) after micromanipulation and cryopreservation. More success has been achieved in the in vitro culture of later-stage embryos than for the culture of the early cleavage-stage embryos.
Fibroblast Monolayer Co-Culture

Co-culture systems were first developed as an in vitro model for the study of embryonic development during the preimplantation and implantation stages. Cole and Paul (1965) first reported on the successful use of an irradiated feeder layer of HeLa cells for the culture of 2-cell murine embryos to the blastocyst stage of development. It was noted in this study that a higher percentage of murine embryos co-cultured with HeLa cells hatched from the zona pellucida than when cultured in the control medium alone (Waymouth's medium). The hatched embryos attached to the HeLa cells and initiated trophoblastic outgrowth. Later studies reported the attachment and subsequent trophoblastic outgrowth of murine hatched blastocysts was not limited to any specific cell type, but occurred with a variety of monolayers of transformed and non-transformed cell types (Saloman and Sherman, 1975; Glass et al., 1979).

The monolayer co-culture system was first evaluated for use in the in vitro culture of farm animal embryos in the early 1980's. Kuzan and Wright (1981) evaluated the use of monolayers of bovine uterine or testicular fibroblasts for the culture of hatched porcine embryos. Twenty-one (60%) of the hatched porcine embryos attached to the uterine fibroblast monolayer compared with one attaching (4%) for hatched porcine embryos cultured on the testicular fibroblast monolayer. The porcine embryos cultured on the uterine fibroblast monolayer increased in size and continued to proliferate for 20 days in vitro.
In a subsequent study, Kuzan and Wright (1982a) conducted two experiments to evaluate the effect of bovine uterine or testicular fibroblasts on the *in vitro* development of porcine morulae and blastocysts. In the first experiment, porcine morulae were cultured with uterine fibroblasts, testicular fibroblasts, uterine fibroblast conditioned medium or the control medium alone (MEM medium). No differences were noted between the number of porcine morulae developing to the expanded blastocysts in the uterine fibroblast, MEM-conditioned medium or MEM medium treatments. In addition, the culture of porcine morulae with a monolayer of testicular fibroblasts was found to suppress embryonic development.

In the second experiment, porcine blastocysts were cultured with either bovine uterine fibroblasts, uterine fibroblast conditioned medium or the MEM control medium alone. In this experiment, a greater portion of the porcine blastocysts (56%) developed to hatched blastocysts when cultured on a uterine fibroblast monolayer than when cultured in fibroblast conditioned medium (18%) or the control medium alone (20%). The reason for the uterine monolayer co-culture system being superior for blastocyst-stage porcine embryos, but not porcine morulae was not evident. The authors suggested that porcine morulae and blastocysts differ in their *in vitro* culture requirements and that the uterine fibroblast monolayer provided a superior *in vitro* environment for porcine blastocysts.

In the same year, Kuzan and Wright (1982b) evaluated the use of a bovine fibroblast monolayer of uterine or testicular origin for the culture of bovine morulae. In this study, both the uterine and testicular fibroblast monolayer culture systems showed a marked improvement
over the fibroblast-conditioned medium or MEM control medium alone. Twelve (40%) and 11 (41%) bovine morulae cultured on the uterine and testicular fibroblast monolayer, respectively, hatched \textit{in vitro}. In contrast, only one morulae (3%) cultured in MEM alone and one (3%) cultured in fibroblast conditioned medium hatched \textit{in vitro}.

Studies have been conducted to evaluate the monolayer co-culture system with bisected bovine morulae and blastocysts. Voelkel et al. (1985a) bisected 40 day-7 bovine embryos and placed one demi-embryo of each demi-embryo pair on a monolayer of bovine uterine fibroblasts or in Ham's F-10 control medium. At the end of the 72-hour culture period, 66% of the demi-embryos co-cultured on the uterine fibroblast monolayer were viable compared with 30% for the demi-embryos cultured in Ham's F-10 medium alone.

Baker and Shea (1985) evaluated the use of a monolayer of bovine luteal cells for the culture of bovine demi-embryos. Eighteen day-7 bovine embryos were bisected and one demi-embryo of each pair was cultured in Ham's F-12 medium (control) or on a luteal cell monolayer. After 18 hours of \textit{in vitro} culture, three demi-embryos (17%) cultured on the luteal cell monolayer were viable while seven demi-embryos (39%) cultured in Ham's F-12 medium were considered viable. These findings suggested the luteal cells had a negative rather than positive effect on demi-embryo viability.

Uterine endometrium obtained from mature cows has routinely been used to establish uterine fibroblast monolayers for the \textit{in vitro} co-culture of embryos (Kuzan and Wright 1981, 1982a,b; Allen and Wright, 1984; Voelkel et al., 1984a,1985a). Recently, Wiemer et al. (1987) evaluated the use of a fetal uterine fibroblast monolayer, pre-
pared from the uterus of a near-term fetus. In this study, an estrogen and progesterone pre-treated fetal uterine fibroblast monolayer was compared with an untreated fetal uterine fibroblast monolayer and with Ham's F-10 medium alone for the culture of day 5.5 to 6.5 bovine morulae. After 60 hours of in vitro culture, 85% and 82% of the embryos cultured on the fetal fibroblast monolayer and hormone pre-treated fibroblast monolayer, respectively, were considered viable compared with 26% for the embryos cultured in Ham's F-10 medium alone.

The results of the latter study show that the fetal fibroblast monolayer co-culture system is more effective than medium alone for the culture of bovine morulae. To date, fetal uterine fibroblasts have not been compared with adult uterine fibroblasts to determine which fibroblast population would be more advantageous for use in a co-culture system.

The mechanism by which the monolayer co-culture system enhances embryo viability in vitro has not been determined. Fibroblast-conditioned medium, collected from fibroblast cell cultures, has not been shown to be superior to control medium alone for the culture of bovine (Kuzan and Wright, 1982b) or porcine embryos (Kuzan and Wright, 1981, 1982a, Allen and Wright, 1984). This finding would suggest the beneficial effects of the fibroblast monolayer co-culture system on co-cultured embryos are not due to the secretion of embryotrophic substances into the medium, or that any embryonic growth promoting substances secreted by fibroblast monolayers are extremely labile. Kuzan and Wright (1982b) have hypothesized that fibroblast cells might chelate and thus remove embryotoxic substances from the culture medium.
Allen and Wright (1984) have demonstrated that fibroblast cell to embryo contact was necessary for enhancement of porcine embryonic development in vitro. In this study, 4-cell to morula-stage porcine embryos were cultured on a monolayer of porcine uterine or ovarian fibroblasts or cultured on a membrane (.22 μm pore diameter) that was placed directly over the fibroblast monolayer. The membrane allowed free diffusion of cell products without direct fibroblast cell to embryo contact. The mean cleavage indices of the porcine embryos cultured in direct contact with either the ovarian or uterine fibroblasts were found to be three times greater than the cleavage indices of embryos separated from the fibroblast monolayer.

Oviductal Epithelium Co-Culture

The co-culture systems discussed to this point have used monolayers of uterine or testicular fibroblast cells. Rexroad and Powell (1986) evaluated the use of a monolayer of sheep oviductal epithelial cells for the short-term culture of 1- to 8-cell ovine embryos. The embryos were recovered from donor ewes and cultured for 24 hours in vitro in either Ham's F-10 medium (5 ml), a Ham's F-10 medium microdrop (50 μl), oviductal epithelial cell-conditioned medium or on the oviductal fibroblast monolayer. After the 24-hour culture period the embryos in each of the treatments were transferred to recipient ewes for 7 days of in vivo culture. At the time embryos were allotted to the culture treatments, another group of 1- to 8-cell embryos were transferred to recipient ewes for in vivo culture. These embryos were also recovered 7 days later for comparison with the in vitro cultured embryos. The mean cleavage index (3.07) of the embryos cultured on the
oviductal monolayer for 24 hours was found to be similar to the mean cleavage index (3.68) of the embryos transferred immediately to recipient ewes following recovery from the donor animals. These results indicate that the oviductal cell monolayer is adequate for short-term (24 hour) culture of early cleavage-stage ovine embryos. The mean cleavage index of the embryos cultures in Ham's F-10 medium, a Ham's F-10 microdrop and oviductal cell-conditioned medium were 0.51, 2.03 and 1.02, respectively. These results suggest there is no advantage to the use of oviductal fibroblast cell-conditioned medium over Ham's F-10 medium alone for the culture of 1- to 8-cell ovine embryos.

A co-culture system using dispersed oviductal epithelium has also been shown to be effective for the culture of early stage bovine embryos (Eyestone et al., 1987). In this study, 5- to 8-cell bovine embryos were co-cultured with bovine oviductal epithelium or Ham's F-10 medium alone for 4 to 5 days. By the end of culture, 38 of the co-cultured embryos (46%) had developed to the late morula or blastocyst stage while none of the embryos cultured in Ham's F-10 medium alone developed beyond 8 to 16 cells.

Trophoblastic Vesicle Co-Culture

A successful co-culture system has been developed with bovine trophoblastic vesicles for the co-culture of precompaction-stage embryos (Camous et al., 1984a). In this study, 1- to 8-cell bovine embryos were assigned to in vitro culture in B$_2$ medium (Menezo, 1976) or in B$_2$ medium with a trophoblastic vesicle formed from a section of a day-14 elongated bovine embryo. The number and percentage of the 1-cell, 2-cell, 4-cell and 8-cell embryos cultured in B$_2$ medium which
developed to morulae *in vitro* were: 9(18%), 2(8%), 2(17%) and 6(30%), respectively. In the trophoblastic vesicle co-culture treatment, 23(42%), 30(38%), 11(50%) and 23(70%) of the 1-cell, 2-cell, 4-cell and 8-cell embryos, respectively, developed to morulae. More of the embryos co-cultured with trophoblastic vesicles developed to morulae, regardless of the initial cell stage at the onset of the study.

Overall, 87 of the 1- to 8-cell bovine embryos (46%) developed to morulae when co-cultured with trophoblastic vesicles compared with 19 embryos (18%) when cultured in B2 medium alone. The embryos successfully co-cultured to morulae were transferred to recipients for further culture *in vivo*. The embryos were recovered from the recipients 2 to 3 days later and those which had developed to blastocysts were frozen and later thawed for evaluation. Ten of these frozen-thawed embryos were then individually transferred to recipients, resulting in four pregnancies.

It has been reported by Camous et al. (1984b) that 1- to 8-cell bovine embryos cultured in B2 medium without serum supplementation did not develop beyond the 8- to 12-cell developmental block stage (Thibault, 1966), whereas 35% of the 1- to 8-cell bovine embryos cultured in B2 medium with 15% fetal calf serum developed to the 9- to 16-cell stages of development. In the first of a series of experiments, Heyman et al. (1987b) cultured 1- to 8-cell bovine embryos in B2 medium with 15% fetal calf serum, B2 medium with a trophoblastic vesicle or B2 medium with 15% fetal calf serum and a trophoblastic vesicle to determine the respective roles of trophoblastic vesicles and serum during co-culture. When both trophoblastic vesicles and serum were added to B2 medium in the co-culture system, 38 of the 1-cell bovine embryos
(69%) developed beyond the developmental block stage and 23 (42%) developed to morulae. Only 1(5%), 7(10%) and 5(11%) of the bovine embryos cultured in B2 medium alone, B2 medium with serum and B2 medium with a trophoblastic vesicle, respectively, developed beyond the developmental block stage. Nine (13%) and 1 (2%) of the embryos developing beyond the developmental block stage in B2 medium with serum and B2 medium with a trophoblastic vesicle, respectively, continued development to morulae.

In the second experiment, 1-cell ovine embryos were cultured using the same treatments as were used in the first experiment. Ovine trophoblastic vesicles produced from day-12 ovine embryos were used in this experiment rather than bovine trophoblastic vesicles. At the end of the 3-day culture period, 14(29%), 15(35%), 19(68%) and 18(75%) of the 1-cell ovine embryos cultured in B2 medium alone, B2 medium with serum, B2 medium with a trophoblastic vesicle and B2 medium with serum and a trophoblastic vesicle, respectively, cleaved to the morula stage of development.

The results of the first experiment suggests there is a synergistic relationship between trophoblastic vesicles and serum in the bovine co-culture system. A previous study has shown that serum albumin stimulates the uptake of amino acids by bovine trophoblastic vesicles (Heyman and Menezo, 1986). The results of the second experiment would indicate that the addition of serum to the ovine trophoblastic vesicle co-culture system is not necessary nor beneficial, since there were no differences in the number of 1-cell ovine embryos developing to morulae when serum was included or omitted from the culture system.
Unlike the fibroblast monolayer co-culture system (Allen and Wright, 1984), direct contact between the embryo and trophoblastic vesicle is not necessary for the embryotrophic effects of the trophoblastic vesicle co-culture system (Heyman et al., 1987b). In a third experiment, Heyman et al. (1987b) compared trophoblastic vesicle conditioned medium with trophoblastic vesicle co-culture to determine if trophoblastic vesicles released embryotrophic substances into the medium. In this experiment, 1- to 2-cell bovine embryos were cultured with bovine trophoblastic vesicles in serum supplemented B₂ medium or in medium containing 1:1 trophoblastic vesicle conditioned medium and serum supplemented B₂ medium. After 3 to 4 days of culture, 23(42%) and 14(39%) of the 1- to 2-cell bovine embryos cultured with trophoblastic vesicles and in trophoblastic vesicle conditioned medium, respectively, developed to the morula stage of development. These results demonstrate that trophoblastic vesicles secrete embryo growth promoting factors into the medium.

A fourth experiment was conducted by Heyman et al. (1987b) in order to characterize the growth promoting factors released by trophoblastic vesicles. Medium collected from cultures of bovine trophoblastic vesicles was separated into molecular weight (MW) fractions of 180 to 1,500 and greater than 10,000. Each fraction was added to fresh B₂ medium and used for the culture of 1- to 2-cell bovine embryos. Thirteen embryos (62%) cultured in medium containing the 180 to 1,500 MW fraction developed beyond the 8- to 12-cell block stage and 5 embryos (23.8%) continued development to morulae. Six of the embryos (21.4%) cultured in medium containing the greater than 10,000 MW fraction developed beyond the block stage, but none continued development to the
morula stage in vitro. Further analysis has revealed that bovine trophoblastic vesicles secrete at least three peptides (Heyman and Menezo, 1987). The embryotrophic substance secreted by bovine trophoblastic vesicles has been shown to be a peptide with the amino acid sequence of Gly-Ala-Glu-Gly-Gly-Ser (Y. Menezo, personal communication).

The trophoblastic vesicle co-culture system is the only co-culture system which has been shown to be adequate for the culture of bovine embryos from the 1-cell stage through the 8- to 12-cell developmental block stage to morulae. The co-culture system utilizing oviductal epithelium (Eyestone et al., 1987) has been successfully used to culture 5- to 8-cell embryos to late morulae or blastocysts, but has not been evaluated on embryos earlier than the 5-cell stage of development. Trophoblastic vesicle conditioned medium also has been shown to be as effective as trophoblastic vesicle co-culture. This eliminates the requirement of having cells or tissue available for culture studies, as is required for other co-culture systems.

Uterine fibroblast monolayers established from bovine uterine endometrium have been successfully used for the culture of both porcine embryos (Kuzan and Wright, 1981, 1982a) and bovine embryos (Kuzan and Wright, 1982b). This would suggest that there may not be a species specificity in the use of the fibroblast monolayer co-culture system. Species specificity has not been evaluated for the trophoblastic vesicle co-culture system. However, species specificity has been evaluated by uterine transfer of trophoblastic vesicle to cows and ewes (Martal et al., 1984). In this study, Martal et al. (1984) performed reciprocal transfers of bovine and ovine trophoblastic vesicles to cows.
and ewes. One or two trophoblastic vesicles were transferred surgically to ewes and cows on day 12 of the estrous cycle and the recipients were observed daily for return to estrus. Two of 11 ewes receiving bovine trophoblastic vesicles had not returned to estrus by day 38, when the ewes were slaughtered. Two of 10 cows receiving ovine trophoblastic vesicles had their estrous cycles extended by 10 and 15 days, respectively. Thus, the transfer of ovine and bovine trophoblastic vesicles to cows and ewes, respectively, resulted in luteal tissue maintenance in 20% of the females.

In another study, it was found that the transfer of ovine trophoblastic vesicles to ewes and bovine trophoblastic vesicles to cows resulted in corpora lutea maintenance in 67% of the recipient cows and 58% of the recipient ewes (Heyman et al., 1984). The cows had extended estrous cycles of 25 to 37 days and the ewes had extended estrous cycles of 20 to 54 days. The results of the studies by Martal et al. (1984) and Heyman et al. (1984) indicate that some species specificity exists with the luteotrophic factors secreted by trophoblastic vesicles and that both ovine and bovine trophoblastic vesicles secrete some type of a luteotrophic factor(s).

Since trophoblastic vesicles have been shown to secrete a luteotrophic factor, they should be of value for co-transfer with embryos as well as for co-culture. In a recent report, Heyman et al. (1987a) evaluated the use of bovine trophoblastic vesicles with frozen-thawed bovine embryos in a co-transfer study. Forty-nine recipients received a frozen-thawed blastocyst and two frozen-thawed trophoblastic vesicles while 53 recipients were transplanted with a frozen-thawed blastocyst. At 90 days of gestation, 28 of the recipients (57%) co-transferred with
trophoblastic vesicles were diagnosed as pregnant compared with 21 pregnancies (40%) in the control group receiving only a frozen-thawed blastocyst. These results demonstrate the potential usefulness of trophoblastic vesicles as a means of increasing transfer pregnancy rates in embryo recipients.

The in vitro co-culture systems which have been evaluated for use with farm animal embryos have been more effective than medium alone. The trophoblastic vesicle co-culture system has the advantage over the other co-culture systems in that trophoblastic vesicles actively secrete embryotrophic and luteotrophic factors. Trophoblastic vesicles have not been used for the culture of embryos beyond the morula stage of development. Further studies are needed to evaluate the effectiveness of the trophoblastic vesicle co-culture system with later-stage embryos. The trophoblastic vesicle co-culture system might also be beneficial for the culture of bisected embryos following micromanipulation or for the evaluation of embryo viability after freezing-thawing of intact or bisected embryos.

Also, studies are needed to evaluate further the in vivo survival of embryos following co-culture. Very few culture studies have reported on the in vivo survival of embryos cultured longer than one day. Pregnancy rates achieved with embryos cultured for extended periods of time have been shown to be lower than those achieved by direct transfer of "fresh" embryos (Renard et al., 1980).
CHAPTER II

EXPERIMENT I

BISECTING BOVINE EMBRYOS WITHOUT THE USE OF A COMMERCIAL MICROMANIPULATION UNIT

Introduction

The production of multiple offspring from single, micromanipulated farm animal embryos was first reported for sheep (Willadsen, 1979, 1980) and cattle (Willadsen and Polge, 1981; Willadsen et al., 1981). Present micromanipulation techniques range from blastomere separation of precompaction-stage embryos and in vivo culture in an intermediate recipient prior to transfer (Willadsen, 1979) to micromanipulation of later-stage embryos and direct non-surgical transfer to recipients (Ozil et al., 1982; Lambeth et al., 1982; Williams et al., 1982; Willadsen and Godke, 1984).

Embryo bisection procedures presently employed require the use of a commercially available micromanipulation unit. Previously, attempts have been made to construct a simplified, inexpensive micromanipulator to remove the zona pellucida of mammalian embryos (Goldacre, 1954; Hoppe and Bavister, 1983). However, practice and abundant patience were required with these approaches to avoid embryonic cell damage when removing the zona pellucida. Furthermore, embryo bisection was not attempted with either of these methods.

One of the more difficult steps to achieve with embryo microsurgery is the removal of the zona pellucida (ZP) prior to bisection of the embryonic cell mass. McFarland et al. (1985) have reported in
this laboratory that exposure of embryos to a 2.5% pronase solution for 5 or more minutes effectively removes the zona pellucida from bovine embryos without a detrimental effect to embryo viability. The use of 2.5% pronase and a simplified micromanipulator apparatus could provide an inexpensive method of bisecting morulae and blastocysts under field as well as laboratory conditions. The objective of this study was to develop a simplified method for producing bisected bovine embryos.

Materials and Methods

Embryo Donor Preparation

Twenty mature crossbred beef cows (weight range = 468 to 623 kg) of Hereford, Angus and Brahman breeding were used as embryo donors for this study. The donors were between 3 and 10 years of age and in good to excellent body condition. The donor cows were superovulated with twice daily (12 hours apart) injections (im) of 5 mg of follicle stimulating hormone (FSH; FSH-P: Burns-Biotec, Oakland, California) starting on days 12 through 15 of the estrous cycle (day 0 = estrus). A 25 mg dose of prostaglandin F₂α (Lutalyse: Upjohn Co., Kalamazoo, Michigan) was injected (im) 48 hours and 60 hours, respectively, after the first FSH injection to induce luteolysis. Donor females were mated to a fertile Angus bull at estrus or artificially inseminated with one unit of frozen-thawed semen at 12 hours and again at 24 hours after the onset of standing estrus.

Embryo Collection Procedure

At 6.5 to 7.0 days after the onset of estrus, embryos were non-surgically collected using Dulbecco's phosphate-buffered saline (PBS)
medium (Gibco, Grand Island, New York) supplemented with 2% (v/v) fetal calf serum (FCS) and an antibiotic-antimycotic (Ab-Am) solution of 100 units of penicillin, 100 μg of streptomycin, .25 μg of amphotericin-B per ml of medium. Uterine horns were flushed using a 16 to 18 French two-way Foley catheter attached to a sterile 60 ml syringe (Looney et al., 1981). Immediately after collection, embryos were evaluated for morphological stage of development and assigned an embryo quality grade (1 = excellent, 2 = good, 3 = fair, 4 = degenerate) using a Wild M-8 stereomicroscope (100X). The embryos were evaluated for developmental stage and embryo quality using the basic criteria reported by Lindner and Wright (1983).

Experimental Treatments

Twenty-eight late morula-stage (LM) embryos of excellent or good quality (embryo quality grades of 1 and 2) were randomly assigned by donor across each of the four treatments (table 1) in the 2 X 2 factorial treatment arrangement. The treatments were designated A and B for bisection of zona pellucida intact (ZI) and zona pellucida free (ZF) embryos, respectively, using simple microscope slide manipulators constructed in the laboratory. The remaining two treatments were designated C and D for ZI and ZF embryos, respectively, bisected by a hand-held razor blade method.

The embryos were held at room temperature (20°C) in a modified PBS holding medium (HM) prior to and during micromanipulation. The HM consisted of PBS with 10% FCS (v/v), 1 mg/ml glucose, 4 mg/ml bovine serum albumin, 36 μg/ml sodium pyruvate and Ab-Am.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of embryos</th>
<th>Embryo status</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscope Slide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (ZI)</td>
<td>28</td>
<td>X</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B (ZF)</td>
<td>28</td>
<td>-</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>Razor Blade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C (ZI)</td>
<td>28</td>
<td>X</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D (ZF)</td>
<td>28</td>
<td>-</td>
<td>X</td>
<td>-</td>
</tr>
</tbody>
</table>
Enzymatic Zona Pellucida Removal

The ZP were removed from embryos in Treatments B and D by exposing the embryos to 2.5% pronase prepared from Streptomyces griseus protease (Protease P5147: Sigma Chemical Co., St. Louis, Missouri). The 2.5% pronase solution was prepared by adding 137.5 activity units of fresh protease per ml of PBS. The pronase solution was filtered through a .45 μm filter (Acrodisc: Gelman Sciences, Ann Arbor, Michigan) and placed into the wells of a 24-well culture plate (Falcon: Becton, Dickinson and Co., Oxnard, California) at a volume of 1 ml per well. The embryos in the ZF treatments were rinsed through three washes of PBS to remove any residual FCS and then placed into the pronase solution at room temperature (20°C).

The embryos were then observed with a Wild M-8 microscope using phase contrast microscopy (100X) and were removed from the pronase solution when the ZP was observed to have been dissolved from the embryo. If the ZP was not totally removed from an embryo after a 15-minute exposure to pronase, the embryo was removed from the pronase solution and noted accordingly, but included in the treatment. After pronase treatment, embryos were rinsed with two washes of PBS with 20% FCS (v/v) to inactivate the pronase. The ZF embryos were then maintained in HM at room temperature until bisection.

Construction and Use of Micromanipulators

Microscope Slide Manipulator. Two simplified micromanipulators were constructed in the laboratory for evaluation on ZI and ZF late morula stage bovine embryos (Treatments A and B). The glass microscope slide micromanipulator were patterned after the glass microscope
slide apparatus used to remove the ZP from hamster and bovine embryos (Hoppe and Bavister, 1983). The micromanipulator unit used for holding the fine glass needle for ZP removal and embryo bisection was constructed by gluing (Krazy Glue) a 50 x 75 mm microscope slide vertically to the 50 x 75 mm base microscope slide. Another 50 x 75 mm microscope slide, on which the fine glass needle was later mounted, was affixed to the vertical microscope slide with a standard stopcock grease. The base of the microscope slide micromanipulator was also affixed to a 20 x 40 cm glass stand with stopcock grease. The stopcock grease on the glass surfaces between the base of the microscope slide micromanipulator and the glass stand and between the vertical portions of the micromanipulator unit facilitated smooth controlled movements of the micromanipulation instrument.

The fine glass needle used for the bisection and ZP removal procedures was attached to the micromanipulator by inserting the needle into a 2 cm segment of silastic tubing (.65 mm O.D.), glued to the vertical slide of the micromanipulator unit. The section of the unit constructed for the suction pipette was slightly modified from the unit constructed for embryo bisection and ZP removal. The portion of the micromanipulator on which the suction pipette was mounted, was constructed by gluing a 25 x 50 mm piece of microscope slide between the ends of two adjacent 50 x 75 mm microscope slides. This was affixed to the vertical portion of the micromanipulator with stopcock grease so that the vertical microscope slide was between the two adjacent microscope slides. This design doubled the contact surface area, allowing for more precise, controlled positioning of the suction pipette. The suction pipette was attached to the micromanipulator by
inserting it into silastic tubing glued to the micromanipulator. A 1-ml syringe was attached to the opposite end of the silastic tubing to provide suction for holding embryos during the manipulation procedure. The same approach to ZP removal and embryo bisection, using a Leitz micromanipulator (Lambeth et al., 1983) was used with the glass microscope micromanipulator.

The embryos were bisected in an uncovered sterile Lux petri dish (100 mm) containing 30 ml of HM. The suction pipette was used to position the ZI embryo while the fine glass needle was used to make a rent in the ZP. The rent was continued until the ZP was cut into halves. The ZF embryo was bisected by positioning the glass needle above the embryo resting on the floor of the petri dish and lowering the glass needle on a vertical plane. If the glass needle did not quite bisect the embryo, the bisection process was completed by repositioning the embryo between the holding pipette and the glass needle and using a gentle sawing motion with the needle.

**Razor Blade Method.** The second bisection approach used for Treatments C and D was developed in the laboratory. This method required a 50 mm X 75 mm microscope slide, a standard double-edged razor blade (Gillette) and a pair of small surgical hemostats. The microscope slide was prepared by scouring the upper surface with 3 μm diamond compound (Metadi: WMR Scientific, Houston, Texas) and fine steel wool. This scouring produced a slightly abrasive surface on the slide, which helped to stabilize the embryo during bisection. The ZI or ZF embryo to be bisected was placed on the microscope slide in a small drop (20 μl) of holding medium. The embryo was viewed through a Nikon Diaphot inverted microscope (200X) while a quarter section of
razor blade, secured by a pair of hand-held hemostats, was carefully lowered to vertically bisect the embryo (figure 1). During the bisection procedure, the handles of the hemostats were rested on the stage of the microscope to provide stability to the razor blade and to maintain the vertical position of the razor blade.

In Vitro Culture

After the embryos in all treatments were bisected, they were placed in 24-well plastic culture plates with 1 ml per well of Ham's F-10 medium with 10% FCS (v/v) and Ab-Am. The demi-embryos were then placed in culture at 37°C in a humidified atmosphere of 5% CO₂ in air. Demi-embryos (DE) were evaluated every 12 hours for in vitro development during the 48-hour culture period. Any DE that developed to a blastocyst with a discernible inner cell mass during in vitro culture was classified as a viable embryo for this study.

Statistical Analysis

Differences between bisection methods, ZP treatments and the interaction of bisection and ZP treatment were analyzed by the analysis of variance procedure using the SAS General Linear Model option (SAS, 1982). A score of "1" was assigned to successfully formed DE and a score of "0" was assigned when viable DE were not formed by the bisection treatment. Similarly, any DE which formed a blastocyst in vitro was assigned a score of "1" and DE which failed to form a blastocyst was assigned a score of "0". The treatment means for the number of DE formed and the number of blastocysts developing in vitro were compared using Tukey's significant difference test (Steel and Torie, 1980).
FIGURE 1. BISECTION OF BOVINE MORULAE USING THE HAND-HELD RAZOR BLADE METHOD
Results

Twelve ova were recovered on the average from 20 superovulated beef cows, resulting in 8.8 embryos (quality grades 1 to 3) per donor. Only embryos classified as grade 1 (excellent) or 2 (good) late morula stage were assigned to the four treatment groups. This resulted in a total of 21 grade 1 late morulae and 7 grade 2 late morulae being allotted to each treatment. After bisection, there was the potential for 56 demi-embryos in each of the treatments. No differences (P>.05) were found in the number of DE formed or in the number of blastocysts forming between grade 1 and 2 embryos (table 2), so the results for grade 1 and 2 embryos were combined in each treatment (table 3).

The total number of demi-embryos produced by each bisection method (treatment) is presented in table 3. Forty-six (82%), 55 (98%), 55 (98%) and 56 (100%) demi-embryos were produced by Treatments A, B, C and D, respectively. More demi-embryos were produced by Treatments B, C and D than by Treatment A (P<.01). Overall, more DE (P<.01) were produced by the razor blade method (Treatments C and D) than by the microscope slide micromanipulator method (Treatments A and B). Also, more DE (P<.01) were produced from ZF embryos (Treatments B and D) than from ZI embryos (Treatments A and C).

Thirty-eight (67.9%), 34 (60.7%), 46 (82.1%) and 37 (66.1%) demi-embryos in Treatments A, B, C and D, respectively, developed to blastocysts in vitro (table 3). Any DE which achieved the blastocyst stage of development, did so by 12 to 24 hours of culture. More demi-embryos developed to blastocysts in vitro in the Treatment C (P<.05) than in Treatments A, B and D. The number of DE produced by the razor blade method (Treatments C and D) which developed to blastocysts in vitro was
**TABLE 2. BISECTION OF BOVINE MORULAE AND DEVELOPMENT OF DEMI-EMBRYOS**

<table>
<thead>
<tr>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of embryos</th>
<th>Embryo grade/stage</th>
<th>Number of demi-embryos formed</th>
<th>Number of demi-embryos forming BLAST&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>21</td>
<td>1 LM</td>
<td>34/42</td>
<td>28/34</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2 LM</td>
<td>12/14</td>
<td>10/12</td>
</tr>
<tr>
<td>B</td>
<td>21</td>
<td>1 LM</td>
<td>41/42</td>
<td>23/41</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2 LM</td>
<td>14/14</td>
<td>11/14</td>
</tr>
<tr>
<td>C</td>
<td>21</td>
<td>1 LM</td>
<td>42/42</td>
<td>38/42</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2 LM</td>
<td>13/14</td>
<td>8/13</td>
</tr>
<tr>
<td>D</td>
<td>21</td>
<td>1 LM</td>
<td>42/42</td>
<td>31/42</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2 LM</td>
<td>14/14</td>
<td>6/14</td>
</tr>
</tbody>
</table>

<sup>a</sup>Treatment A and B were the bisection of ZI and ZF embryos, respectively, using the simple microscope micromanipulator method. Treatment C and D were the bisection of ZI and ZF embryos, respectively, using the hand-held razor blade method.

<sup>b</sup>BLAST=blastocysts
### TABLE 3. TOTAL NUMBER OF DEMI-EMBRYOS (DE) FORMED AND DEVELOPMENT OF DE TO BLASTOCYSTS IN VITRO.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of embryos</th>
<th>Number(%) of DE formed</th>
<th>Number(%) of DE forming blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>28</td>
<td>46 (82%)(^b)</td>
<td>38 (68%)(^b)</td>
</tr>
<tr>
<td>B</td>
<td>28</td>
<td>55 (98%)(^c)</td>
<td>34 (61%)(^b)</td>
</tr>
<tr>
<td>C</td>
<td>28</td>
<td>55 (98%)(^c)</td>
<td>46 (82%)(^c)</td>
</tr>
<tr>
<td>D</td>
<td>28</td>
<td>56 (100%)(^c)</td>
<td>37 (66%)(^b)</td>
</tr>
<tr>
<td>Total</td>
<td>112</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Treatment A and B were the bisection of ZI and ZF embryos, respectively, using the simple microscope micromanipulator method. Treatment C and D were the bisection of ZI and ZF embryos, respectively, using the hand-held razor blade method.

\(^b,c\) Numbers in the same columns with different superscripts differ (P<.05)
similar (P<.05) to the number of DE produced by the microscope slide micromanipulator method (Treatments A and B) which developed to blastocysts. A greater number (P<.05) of DE formed from ZI embryos (Treatments A and C) developed to blastocysts in vitro than did those formed from ZF embryos (Treatments B and D).

The ZP was successfully removed from the embryos of 14 donor cows by 2.5% pronase in less than 5 minutes. The ZP was not totally removed from the embryos of the six other donors after 15 minutes exposure to 2.5% pronase. These embryos were removed from pronase after 15 minutes, but were included in the ZP treatments. The ZP was not removed from 11 of the 28 embryos (39%) allotted to Treatment B after 15 minutes of exposure to pronase. The 11 embryos in which the ZP was not removed by pronase treatment, however, were successfully bisected to produce 22 DE. Only 6 (27.3%) of these DE formed blastocysts in vitro. The 17 embryos in Treatment B, in which the ZP was successfully removed by pronase treatment in less than 5 minutes were bisected to produce 34 DE, and 28 (82.4%) of these DE developed into blastocysts in culture.

Similarly, treatments with pronase failed to remove the ZP of 12 of the 28 embryos (43%) allotted to Treatment D. These 12 embryos were bisected to produce 24 DE, of which 11 (45.8%) DE formed blastocysts in vitro. The remaining 16 embryos in Treatment D, in which the ZP was successfully removed by pronase, were successfully bisected to produce 32 DE. Twenty-six (81.2%) of these DE formed blastocysts in vitro.
Discussion

The lower number of DE produced from ZI embryos, using the microscope slide manipulators (Treatment A) reflects the difficulty encountered in removing the ZP from embryos using this method. Hoppe and Bavister (1983) have reported they totally destroyed 5% of the bovine morulae and 10 to 15% of the bovine blastocysts during the ZP removal procedure, using microscope slide micromanipulators. The microscope slide manipulator used in this study took a great deal of practice before the ZP could be removed without excessively damaging the embryonic cells. After practice however, ZI embryos could be bisected in approximately 10 minutes and ZF embryos could be bisected in less than 2 minutes. Even though the microscope manipulators are inexpensive to construct, suction pipettes and bisection micro-instruments must be either purchased or expensive special equipment must be purchased to manufacture them.

The razor blade method used for Treatments C and D required less practice to become proficient at bisection of late morula bovine embryos. Both ZI and ZF embryos could be bisected in less than 1 minute. Previous micromanipulation experience was not necessary with the razor blade method; whereas, it was very beneficial for using the glass microscope slide micromanipulators. The razor blade method also resulted in more successful bisections and a similar number of DE which developed to blastocysts in vitro.

Fewer DE formed blastocysts during the in vitro culture in Treatment A than in C. The lower number of blastocysts forming DE in Treatment A is likely due to injury to the embryos during the ZP removal and bisection process. In this study, more DE were formed from ZF embryos
(Treatments B and D) than from ZI embryos (Treatments A and C). However, fewer DE produced from ZF embryos formed blasocysts in vitro.

The trend for lower demi-embryo viability noted in both Treatments B and D (ZF embryos) compared to Treatments A and C (ZI embryos) could be partially explained by the time of exposure of embryos to pronase. As was previously reported, 27 to 46% of the DE formed from embryos exposed to pronase for greater than 5 minutes developed to blastocysts in culture compared with 81 to 82% of the DE formed from embryos exposed to pronase for 5 minutes or less.

The results of this study demonstrated that a 2.5% solution of pronase is not effective for removal of the ZP from embryos of some donor cows. Embryos from 6 of 20 donors did not have their ZP removed after 15 minutes of exposure to 2.5% pronase. This variability can not be explained by variations in pronase lots or solutions, since the ZP was removed from the embryos of some donors exposed to the same solution of pronase in which the ZP of other donor's embryos were only softened or not affected at all.

Summers et al. (1983) previously reported that donor variation exists in the ability of a .5% pronase solution to remove the ZP of bovine embryos. In their study, the ZP of day-6 bovine morulae from 13 Friesian, Angus and Friesian-Angus crossbred cows were exposed to pronase. The ZP of embryos from five donors were totally removed by exposure to a .5% pronase solution for 6 to 8 minutes. However, the ZP of embryos from four other donors were only softened or partially dissolved by exposure to pronase for 10 minutes while the ZP was neither removed nor softened from embryos obtained from four donors after greater than 10 minutes exposure to .5% pronase. Embryos were recover-
ed from some of the donors for three consecutive collections, with the same pattern of pronase digestion occurring within donor each time.

The same donor variation in the ability of pronase to remove the ZP has also been reported for ovine embryos. Moor and Crangle (1971) reported that the ZP of embryos from approximately one-third of the donor ewes studied were not removed by exposure to .5% pronase treatment. Hoppe and Bavister (1983) reported exposure of bovine embryos to .5% pronase for 7 to 10 minutes softened the ZP, but failed to totally remove the ZP. Massey et al. (1982) used .5% pronase to remove the ZP of 1-cell to expanded blastocyst stage bovine embryos. The time required to remove the ZP varied with embryo developmental stage and ranged from 7 to 246 minutes. The only pronase treated embryos to survive transfer to recipients were blastocysts treated with pronase for 7 minutes. In this study, only 4 blastocysts (22%) survived in vivo.

The results of the present study and the findings of Summers et al. (1983) suggest that resistance to digestion by pronase of the bovine ZP may be genetically determined. Even at a higher pronase concentration in this study (2.5% vs .5%), the donor variation was still evident. These findings are in contrast to those of McFarland et al. (1985) who reported that a 2.5% pronase solution was effective for removing the ZP from day 6.5 to 7.5 bovine embryos of 22 donor cows without any detrimental affects. Summers et al. (1983) reported the exposure of ZP intact embryos to a .5% pronase solution for greater than 10 minutes resulted in partial disaggregation of the blastomeres. It is likely that this type of injury occurred to the pronase-treated embryos in the present study.
Based on the high rate of success of the simplified methods for embryo bisection used in this study, both can be considered viable alternatives to the use of commercial micromanipulators for bisecting ZI and ZF late morula stage bovine embryos. The success achieved with both simplified embryo bisection methods is similar to that reported for using commercial micromanipulators and a microsurgical blade (Williams et al., 1982) or a fine glass needle (Lambeth et al., 1983; Voelkel et al., 1985a).

The DE produced using the simplified bisection methods in this study were not transferred to recipients for evaluation of in vivo survival. The hand-held razor blade method developed in this laboratory has now been used by others to bisect bovine morulae and blastocysts. The transfer of the DE produced by this method has resulted in pregnancy rates similar to those achieved with bisection methods using commercial micromanipulators (G. Lindner, personal communication).
CHAPTER III

EXPERIMENT II

CULTURE OF EARLY-STAGE BOVINE EMBRYOS INSIDE OR IN CO-CULTURE WITH DAY-13 TO 14 TROPHOBLASTIC VESICLES

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. Various recipes for media and a multitude of different culture systems have been evaluated for the culture of early-stage bovine embryos over the years; however, these efforts have only resulted in limited success (see reviews by Betteridge, 1977; Wright and Bondioli, 1981). In vivo culture of precompaction-stage embryos of farm animals in the ligated oviducts of non-pregnant sheep prior to embryo transfer has achieved noteworthy results (Willadsen, 1979). This approach however, is labor intensive, time consuming and often results in some embryo loss during the recovery process.

One of the most successful in vitro culture systems to date involves the use of "helper" cells to co-culture mammalian embryos. Cole and Paul (1965) first reported on the successful use of a feeder layer of irradiated HeLa cells for the culture of 2- to 4-cell stage murine embryos to the blastocyst stage of development. Subsequently, Kuzan and Wright (1982b) reported encouraging results when a monolayer of bovine fibroblast cells of either uterine or testicular origin were used in a co-culture system for morula-stage bovine embryos. Voelkel et al. (1984a;1985a) evaluated the use of bovine uterine fibroblast
monolayer co-culture system and concluded that the co-culture system was superior to that of Ham's F-10 medium when used alone for the culture of bisected day-7 bovine embryos. In a recent study, Wiemer et al. (1987) evaluated a fetal uterine fibroblast monolayer co-culture system, produced from near term fetal bovine uterine endometrial cells, for the culture of morula-stage bovine embryos. The percent viability of embryos cultured on the fetal uterine fibroblast monolayer was reported to be 85%, compared with 26% for the control embryos cultured in medium alone.

Kuzan and Wright (1982b) reported that conditioned medium collected from the uterine fibroblast cultures had no advantage over the control medium alone for in vitro culture of later-stage bovine embryos. Allen and Wright (1984) postulated that the method by which the uterine fibroblast co-culture system improves the in vitro survival of porcine embryos is by fibroblast cell to embryo contact rather than by secretion of growth factors or other substances into the culture medium. To date, the uterine fibroblast monolayer culture system has not been shown to be effective for the culture of pre compaction-stage bovine embryos.

A recent report has suggested that oviductal epithelium integrated into an in vitro co-culture system holds promise for successful culture of early-stage cattle embryos (Eyestone et al., 1987). In this study, 5- to 8-cell bovine embryos were cultured for 4 or 5 days with Ham's F-10 and oviductal epithelium, resulting in 46% of the embryos developing to late morulae or blastocysts. In comparison, none of the 5- to 8-cell embryos cultured in Ham's F-10 alone developed beyond the
characteristic bovine 8- to 12-cell developmental block (Thibault, 1966) stage.

Using a different approach to in vitro culture of embryos, Camous et al. (1984a,c) first reported that trophoblastic vesicles, produced from sections of trophectoderm from a day-14 bovine conceptus, enhanced embryo viability in vitrō when used as a co-culture system. In this initial report, bovine trophoblastic vesicles (bTV) were used for the successful in vitrō culture of 1-, 2-, 4- and 8-cell bovine embryos to the morula stage of development. During the culture interval 42, 38, 50 and 70% of the 1-, 2-, 4- and 8-cell embryos, respectively, developed into morulae when co-cultured with bTV compared with 30% or less for embryos in the control group cultured in medium alone.

More recently, Heyman et al. (1987a) evaluated the bTV co-culture system using 1- to 8-cell bovine embryos and found that the bTV secrete embryo growth promoting factors into the medium during culture. It has been previously reported that bTV may also produce a luteotrophic substance in cattle (Plante et al., 1985). At present, the bTV co-culture system is the only co-culture system that has been reported to be effective for the in vitrō culture of farm animal embryos from the 1-cell stage through the 8- to 12-cell developmental block stage to morulae (Camous et al., 1984a,b,c).

Since trophoblastic tissue may secrete one or more embryotrophic factors, it is possible that the concentration of these factors may be greater within the lumen of the vesicle than that dispersed in the culture medium supporting the vesicles. The objective of the present experiment was to evaluate the in vitrō development of bovine embryos
cultured inside individual bTV and to assess this approach as a method for the culture of bovine embryos.

Materials and Methods

Embryo Donors

Twenty-two mature crossbred Angus, Hereford and Brahman beef cows were selected as embryo donors for this study. These non-lactating, cyclic females ranged in weight from 538 to 661 kg. The donors had been maintained on bermudagrass pasture and grass hays during the preceding months and were in good to excellent body condition at the time of treatment. Starting on days 8 to 15 of the estrous cycle (day 0 = onset of estrus), the potential donor females were placed on a 4-day descending-dose gonadotropin treatment schedule using twice daily intramuscular injections of follicle stimulating hormone (FSH-P: Schering Corp., Omaha, NE) which resulted in a total dose of 28 mg of FSH per donor animal. At 48 hours after the initial FSH injection, a single 500 μg dose of cloprostenol (Estrumate: Miles Laboratories, Shawnee, KS) was administered intramuscularly to each female to induce luteolysis. Each donor cow was mated naturally at estrus with a fertile Angus bull and artificially inseminated with two units of frozen semen 24 hours after the onset of estrus.

Embryo Recovery

Donors were non-surgically collected on day 4.5 to 5 post-estrus for precompaction-stage embryos or on days 13.5 to 14 post-estrus for bovine trophoblastic vesicle production. Each uterine horn was flushed using an 18 to 20 French Foley catheter in a manner similar to that previously described by Looney et al. (1981). Dulbecco's phosphate-
buffered saline (PBS) medium (Gibco, Grand Island, New York) with 2% (v/v) fetal calf serum (FCS) and an antibiotic-antimycotic solution (Ab-Am) containing 100 units of penicillin, 100 μg of streptomycin and 0.25 μg of amphotericin-B per ml was used as the flushing medium. After recovery, embryos were maintained at room temperature (20°C) in a modified PBS holding medium (HM) until their allotment across treatment groups. The HM was composed of PBS with 10% (v/v) FCS, 4.0 gm/l of bovine serum albumin (BSA fraction V), 1.0 gm/l of glucose, .036 gm/l of pyruvic acid and Ab-Am.

**Experimental Treatments**

Embryos from each donor were evaluated under a stereomicroscope (100X) for morphological development and assigned a quality grade score (1 = excellent; 2 = good; 3 = fair; 4 = degenerate) using the criteria previously reported for bovine embryos by Lindner and Wright (1983). Quality grade 1 embryos were randomly assigned within donor and developmental stage across each of three treatments (n=36/treatment). The embryos in Treatment A were individually placed inside a bTV and co-cultured while embryos in Treatment B were individually co-cultured with a single bTV. Fresh bTV of 2 to 3 mm in diameter were randomly selected from a pre-cultured population for use in Treatments A and B (figure 2). Embryos in Treatment C were cultured in medium without bTV tissue, and served as the control.

**Trophoblastic Vesicle Production and In Vitro Culture**

Thirty-six to 48 hours prior to collection of the precompaction-stage embryos, bTV were prepared from day 13.5 to 14 elongated embryos. The elongated embryos were placed into a plastic petri dish with .1 to
FIGURE 2. ILLUSTRATION OF THE TREATMENTS IN EXPERIMENT II

Treatment A
Embryos co-cultured inside a bTV (n=36)

Treatment B
Embryos co-cultured outside of a bTV (n=36)

Treatment C
Embryos cultured in medium alone (n=36)
0.2 ml of HM and dissected into embryonic sections 1 to 2 mm² with a razor blade held by a pair of hemostats (Rorie et al., 1987a). The embryonic tissue containing the embryonic disc was discarded. The bTV which had expanded to a diameter of 2 to 3 mm after 36 to 48 hours in culture were selected for co-culture with embryos in Treatments A and B. Only bTV of similar size and morphology were assigned across treatment groups.

Embryos in Treatment A were placed into the lumen of the bTV by opening a small rent in the vesicles with two 30 gauge hypodermic needles and inserting the embryo inside the bTV with the aid of a 150 μm diameter fire polished glass suction pipette. After inserting the embryo into the bTV, the rent was closed by pressing each side of the opening together using the fire-polished end of the glass suction pipette.

The embryonic sections for bTV production were cultured in four-well culture plates containing 1 ml/well of CMRL-1066 medium (Sanyal and Naftolin, 1983) with 10% (v/v) FCS and Ab-Am. The embryos in Treatments A, B and C were individually cultured in 96-well culture plates with 0.3 ml/well of CMRL 1066 medium (Gibco, Grand Island, NY) with 10% (v/v) FCS and Ab-Am. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. One-half of the medium in each well was replaced after 3 days of culture. The embryos were evaluated at 12-hour intervals for morphological development and embryo quality during the 96-hour culture period, using the criteria as established by Lindner and Wright (1983).
Statistical Analysis

Embryo viability and quality grade scores were compared across time in culture using one-way analysis of variance (Steel and Torie, 1980). In order to statistically analyze the viability and embryo quality scores, these data were converted to a "0" or "1" scale. Embryo viability was analyzed by first assigning a score of "1" to viable embryos and a score of "0" to non-viable embryos. The analysis of embryo quality was performed by assigning "1" to quality grade 1 or 2 embryos and "0" to the remaining embryos in each treatment. Treatment means were compared using Tukey's significant difference test (Steel and Torie, 1980).

Results

Embryo Recovery and bTV Production

Sixty-four day 13.5 to day 14.0 elongating embryos were nonsurgically recovered from 10 donor cows. All of these embryos were successfully dissected into sections for bTV production. After a period of 36 to 48 hours of in vitro culture in CMRL 1066 medium, over 95% of the embryonic sections developed into viable appearing bTV, as described by Camous et al. (1984c).

A total of 108 excellent quality day 4.5 to day 5 embryos were recovered from 12 donor cows, giving an average of 9.0 potentially viable embryos per donor. Embryos allotted to treatments contained between 12 and 32 embryonic cells at the time of collection. Thirty-six quality grade 1 embryos were assigned across each of the three treatment groups.
All 36 embryos in Treatment A were successfully placed inside the pre-cultured bTV. Twenty-two of these 36 bTV (61%) re-expanded during the 96-hour culture interval. The remaining 14 bTV (39%) only partially re-expanded or failed to re-expand during the same culture period. One bTV (3%) in Treatment B collapsed during culture and failed to re-expand during the 96 hours of culture. At the end of the 96 hour culture period, all 36 embryos in Treatment A were recovered from the bTV for examination.

**In Vitro Culture of Embryos**

The embryo viability ratings and quality grades during in vitro culture are presented in Table 4. After 60 hours of culture inside bTV, 23 embryos (64%) in Treatment A were considered viable embryos (grades 1, 2 and 3 = excellent to fair embryo quality) and 13 embryos (36%) were classified as quality grades 1 (excellent) or 2 (good). At the end of the 96-hour in vitro culture period, 21 embryos (58%) in Treatment A were assessed as viable and 13 embryos (36%) were classified as having embryo quality grades of 1 or 2.

In Treatment B, 29 embryos (81%) were judged viable after 60 hours of co-culture with bTV tissue. Twenty-five of these embryos (69%) were graded as excellent or good quality embryos (grades 1 and 2). At the end of the 96-hour culture period, 26 embryos (72%) were classified as viable. Eighteen embryos (50%) considered to be of excellent or good embryo quality (grades 1 and 2).

Of the 36 embryos cultured in the control medium alone in Treatment C, 24 (67%) were considered viable at 60 hours of in vitro
<table>
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<tr>
<th>Treatment</th>
<th>Number of embryos</th>
<th>0 Hours</th>
<th>60 Hours</th>
<th>96 Hours</th>
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<tr>
<td></td>
<td>Number of viable embryos</td>
<td>Number(%) of viable embryos</td>
<td>Number(%) of grade 1&amp;2 embryos</td>
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</tr>
<tr>
<td>A</td>
<td>36</td>
<td>23(64%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13(36%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21(58%)&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>B</td>
<td>36</td>
<td>29(81%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25(69%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26(72%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>36</td>
<td>24(67%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8(22%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16(44%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
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<sup>a,b</sup>Numbers with different superscripts in the same column are different at the P<.01 level.
culture. Eight embryos (22%) were judged to be excellent or good quality embryos. When the embryos were re-evaluated after 96 hours *in vitro*, 16 embryos (44%) were considered to be viable and 6 embryos (17%) were classified as excellent or good embryo quality.

There were no differences in the number of viable embryos among the treatments after 60 or 96 hours of *in vitro* culture (P>.05). However, the number of quality grade 1 and 2 embryos were higher (P<.01) at 60 hours of culture for embryos co-cultured with bTV (Treatment B) than those for embryos co-cultured inside bTV (Treatment A) or in the control medium alone (Treatment C). After 96 hours of culture, there were more quality grade 1 and 2 embryos in Treatment B than in the control treatment (Treatment C), but not more than in Treatment A (P>.05).

The final morphological developmental stages for embryos after 96 hours of culture are presented in table 5. At the end of the culture period, the embryos assessed as viable in Treatment A ranged from late morulae to expanded blastocysts. Fourteen embryos (39%) in Treatment A had developed to or beyond the full blastocyst stage of development. In Treatment B, the embryos ranged from late morulae to hatched blastocysts at the end of the culture period, with 21 embryos (58%) developing to or beyond the full blastocyst developmental stage.

The control embryos in Treatment C ranged from the late morula to expanded blastocyst stage at the end of the culture interval. Only 9 of the control embryos (25%) had achieved or developed beyond the full blastocyst stage of development following 96 hours in culture. At the completion of the experiment (96 hours), 42% of the embryos cultured
<table>
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<tr>
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</tr>
<tr>
<td>Late morula</td>
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<tr>
<td>Early blastocyst</td>
<td>1</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>8</td>
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<td>6</td>
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<td>0</td>
</tr>
<tr>
<td>Degenerated</td>
<td>15</td>
</tr>
<tr>
<td>Total embryos/treatment</td>
<td>36</td>
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</tbody>
</table>
inside bTV (Treatment A) and 50% of the embryos in the control medium (Treatment C) had degenerated. Only 28% of the embryos co-cultured with bTV (Treatment B) had degenerated at the end of the 96-hour culture period.

Discussion

The mechanism by which trophoblastic vesicles improve embryonic development in vitro has been shown to be through the secretion of embryotrophic factors (Heyman et al., 1987). This factor(s) has been found in the 180 to 2500 MW fraction of medium ("conditioned" medium) collected from cultures of trophoblastic vesicles. Heyman et al. (1987) compared the in vitro development of 1- to 2-cell bovine embryos cultured in B2 medium (Menezo, 1976) with and without this 180 to 2500 MW fraction. Sixty-two percent of the 1- to 2-cell bovine embryos cultured in medium supplemented with this low MW fraction developed into morulae in vitro compared with 21% cultured in B2 medium alone.

It is possible that the embryotrophic factors secreted by trophoblastic vesicles might be at a greater concentration within the lumen of the bTV than in the surrounding medium. Therefore, the present study was conducted to evaluate the co-culture of bovine embryos inside bTV.

The results of this study suggest that re-expansion of the bTV is necessary for secretion of embryotrophic factors by the bTV. It was noted that when the bTV used in Treatment A were opened for insertion of an embryo the bTV would contract. In 14 instances (39%), the bTV failed to re-expand during the culture period. The embryos contained in 13 of 14 bTV (93%) which failed to re-expand, were either degenerate
or of poor quality at the end of the culture period. The close proximity of the bTV trophoblastic cells may have inhibited adequate biochemical exchange between the embryo and the bTV or the embryo/bTV and the culture medium outside the bTV. It has been noted that the fluid inside the bTV has different concentrations of medium components than the medium outside the bTV and it has been proposed that the close proximity of the collapsed bTV to the embryo may have caused the concentrations of some medium components within the bTV to reach a toxic level (Y. Menezo, personal communication). The failure of the bTV to re-expand may have contributed to the failure of the embryo to continue its development.

Although the number of viable appearing embryos (grades 1, 2 and 3) between treatments in this study did not differ, the results tend to suggest that either of the bTV co-culture systems (Treatments A and B) would maintain embryo quality longer in vitro than the culture of embryos in the CMRL medium alone. Embryo viability decreased with time in culture in all treatments of this study. Other studies have shown that the in vitro culture of embryos for longer than 24 hours decreases embryo viability (Tervit et al., 1972,; Willadsen et al., 1978).

The percentage of viable embryos (81%) co-cultured with bTV (Treatment B) for 60 hours in this study, compares favorably to the percentage of day 5.5 to 6.5 bovine embryos (85%) cultured for 60 hours on a fetal uterine fibroblast monolayer (Wiemer et al., 1987). At 60 hours in vitro, the bTV co-culture method (Treatment B) resulted in more good or excellent quality embryos than Treatments B and C. Also, at the end of the 96-hour culture period, the embryos co-cultured with bTV (Treatment B) were assessed as having higher embryo quality grades.
than the embryos cultured in medium alone (Treatment C). As previously mentioned, the decline in embryo quality with increasing culture time in Treatment A could be caused by the failure of 39% of the bTV to re-expand in culture.

The final developmental stage of the embryos in the treatments in this study were compared to determine if the advancement in embryonic development was normal. Previous reports have indicated that embryos cultured several days in vitro will lag in development by one or more days compared to embryonic development in vivo (Camous et al., 1984b,c). The embryos allotted to this study were recovered from the donor on day 4.5 to 5 and cultured for 4 days. If normal development had occurred, the embryos (day 8.5 to 9) should have been expanded blastocysts or hatched blastocysts at the end of the 96-hour culture period (Lindner and Wright, 1983). In Treatments A, B and C, 29%, 50% and 6% of the viable embryos, respectively, had developed to the expanded blastocyst or hatched blastocyst stages after 96 hours of culture. After the 96-hour culture period, the only hatching or hatched blastocysts (n=6) were in Treatment B. The remaining potentially viable embryos in the treatment groups ranged from late morulae to blastocysts.

Even though the embryos lagged in development in all of the treatments, the co-culture treatments (A and B) were superior to the control treatment (C). As previously mentioned, 29% and 50% of the viable embryos in Treatments A and B, respectively, had advanced to or beyond the expanded blastocyst stage of development compared with 6% for the control group (Treatment C).
These results further indicate that a culture system utilizing bTV could be advantageous to \textit{in vitro} embryo development. This conclusion is in agreement with those of others using the monolayer culture system (Kuzan and Wright, 1982a,b; Allen and Wright, 1984) who have concluded that advancement of embryo developmental stages in a culture system is an indicator that the culture system is adequate for embryo development \textit{in vitro}.

The embryos in this study were not transferred to recipient females to evaluate survival, so the question of \textit{in vivo} viability cannot be answered at this time. Further research using this culture system in conjunction with transfer to recipients will indicate its true usefulness as a culture system for precompaction-stage bovine embryos. Other studies, using the bTV co-culture system have shown that pregnancies can be established from bovine embryos co-cultured with bTV for 3 to 4 days (Camous et al., 1984b).
CHAPTER IV

EXPERIMENT III

THE CO-CULTURE OF INTACT AND BISECTED PRECOMPACTION-STAGE BOVINE EMBRYOS WITH BOVINE TROPHOBLASTIC VESICLES

Introduction

In recent years several embryo micromanipulation techniques have been successfully developed for producing monozygotic twin offspring in farm animals. The developmental stage of the embryo to be micromanipulated dictates which technique is appropriate for use. Pre-compaction stage embryos are best suited for blastomere separation techniques, whereas postcompaction-stage embryos are easily bisected using a microsurgical blade or a fine glass needle.

The first successful attempt to artificially produce monozygotic twin offspring in farm animals was through blastomere separation of 2-cell sheep embryos (Willadsen, 1979). Previous attempts to micromanipulate early-stage embryos of farm animals had failed due to the inability of these early-stage embryos to survive in vivo without an intact or near intact zona pellucida (Moore et al., 1969; Trounson and Moore, 1974).

Willadsen (1979) eliminated this problem by agar embedding pre-compaction-stage embryos following micromanipulation, to produce an "intact" zona pellucida. The agar-embedded embryos were then transferred to the ligated oviduct of an anestrous or diestrous ewe for in vivo culture. After the embryos had developed to the morula or blastocyst stage in vivo, the agar-embedded embryos were recovered from the
oviduct of the ewe and removed from the agar. Those embryos which had normally developed were transferred to respective recipients. This approach was used to produce the first twin lambs (Willadsen, 1979), twin and triplet calves (Willadsen and Polge, 1981) and twin foals (Willadsen, 1982).

Although the technique developed by Willadsen has been proven to be very effective for the micromanipulation and culture of early-stage embryos, it was not considered to be practical for routine use by embryo transplant units. This method was time consuming, labor intensive and often resulted in lost embryos following the in vivo culture.

A number of more practical embryo bisection procedures have been developed for postcompaction-stage embryos (Ozil et al., 1982; Williams et al., 1982; Lambeth et al., 1982; Willadsen and Godke, 1984; Rorie et al., 1985). These procedures have been widely adapted by researchers and the embryo transplant industry because they can be performed on morulae and blastocysts routinely collected on day 7 to 8 post-estrus. With this approach to bisecting later-stage embryos, the embryos can then be directly transferred to recipients without the use of agar embedding or an intermediate recipient for in vivo culture.

Although the embryo bisection techniques developed for later-stage embryos are more practical and less time consuming than the present blastomere separation technique, the blastomere separation technique has an advantage. The bisection technique used for post-compaction-stage embryos destroys 15% or more of the embryonic cells during the bisection process (Picard et al., 1986; Skrzyszowska and Smorag, 1987; Chesne et al., 1987). In contrast, the blastomere separation technique results in little or no injury to the embryonic
63

cells, since the blastomeres are mechanically separated rather than dissected.

Embryo micromanipulation, using the blastomere separation technique, would become a more practical method for producing monozygotic twins if the requirement for agar embedding and in vivo culture could be eliminated. If an adequate in vitro culture system were available, late precompaction-stage embryos could be collected and blastomere separation performed to produce demi-embryos ("half" embryos). These demi-embryos could then be cultured for 1 or 2 days in vitro to the morula stage and transferred directly to recipient animals.

Embryos are not routinely cultured for more than 24 hours in vitro before transfer to recipients, since the viability of embryos cultured in medium alone declines after 24 hours of in vitro culture (Tervit et al., 1972; Willadsen et al., 1978). A number of embryo co-culture systems, however, have been reported in the literature which maintain embryo viability longer in vitro than medium alone. Kuzan and Wright (1982b) first reported on the successful use of a monolayer of bovine uterine or testicular fibroblasts for the culture of bovine morulae. The uterine fibroblast monolayer co-culture system has also been evaluated for the culture of day-7 bovine demi-embryos and has been shown to be superior to medium alone for the short-term as well as long-term culture of bisected embryos (Voelkel et al., 1984a, 1985a).

Recently, bovine embryos have been cultured from the 5- to 8-cell stage to the morulae or blastocyst stages using a co-culture system of oviductal epithelium (Eyestone et al., 1987). In addition, trophoblastic vesicles, produced from trophoderm sections of day 13 or 14
bovine embryos, have been used to culture bovine embryos from the 1- to 8-cell stages to morulae (Camous et al., 1984a). The trophoblastic vesicle co-culture system is the only co-culture system to date that has been shown to be effective for culturing bovine embryos from the 1-cell stage through the 8- to 12-cell developmental block stage to morulae. Trophoblastic vesicles secrete factors which promote the growth and development of bovine embryos (Heyman et al., 1987a). Conditioned medium, collected from cultures of trophoblastic vesicles, has been shown to be as effective for culturing precompaction-stage bovine embryos as the co-culture of embryos with trophoblastic vesicles (Heyman et al., 1987a). Since trophoblastic vesicles secrete factors which promote the development of intact early-stage bovine embryos, trophoblastic vesicle co-culture might also be of value in the culture of micromanipulated early-stage bovine embryos.

The objective of this study was to investigate the feasibility of culturing bovine demi-embryos, produced by blastomere separation of precompaction-stage embryos, with bovine trophoblastic vesicles.

**Materials and Methods**

**Embryo Donors**

Twelve mature Angus, Hereford and Brahman crossbred beef cows varying in age from 3 to 11 years served as embryo donors for this study. The cows were maintained on grass hay and bermudagrass pasture and were in excellent to good body condition. These donor cows ranged in weight from 565 to 660 kg. Starting on days 10 to 15 of the estrous cycle (day 0 = onset of estrus), the potential donor females were superovulated using a 4-day descending dose treatment of twice daily
injections (im) of follicle stimulation hormone (FSH:FSH-P, Schering Corp., Omaha, Nebraska). Each donor received a total FSH dose of 28 mg during the 4-day superovulation period. At 48 hours after the initial FSH injection, luteal regression was induced with a single 500 µg dose (im) of cloprostenol (Estrumate: Miles Laboratories, Shawnee, Kansas). The donor cows were mated naturally at estrus with an Angus bull of known fertility followed by artificial insemination with two units of frozen semen 24 hours after the onset of estrus.

**Embryo Recovery**

Embryos were non-surgically recovered from nine donors on day 4.5 for precompaction-stage embryos and on day 13.5 (from three donors) for bovine trophoblastic vesicle (bTV) production. Uterine horns were flushed using an 18 to 20 French Foley catheter and Dulbecco's phosphate-buffered saline (PBS) with 2% (v/v) fetal calf serum (FCS) and an antibiotic-antimycotic (Ab-Am) solution. The Ab-Am solution was used in the flushing medium at a concentration of 100 units of penicillin, 100 µg of streptomycin and .25 µg of amphotericin-B per ml of medium. The embryos were maintained at room temperature (20°C) in a modified PBS holding medium (HM) after recovery and prior to assignment to treatment. The HM was composed of PBS with 10% (v/v) FCS, 4.0 gm/l of bovine serum albumin, 1.0 gm/l of glucose, .036 gm/l of pyruvic acid and Ab-Am.

**Experimental Treatments**

The embryos recovered from each donor were evaluated for morphological quality and developmental stage using a stereomicroscope (100X) and following the guidelines as described by Lindner and Wright (1983).
Embryos (n=33/treatment) were assigned at random within donor and developmental stage across the treatments (figure 3). Within each group of three embryos assigned across treatments, one embryo was randomly selected for bisection. The embryo was bisected using blastomere separation and an equal number of blastomeres were placed into the original and a foreign zona pellucida (ZP). One of the resulting demi-embryos (DE) was assigned to Treatment A (cultured in medium alone) and the remaining DE of each DE pair was allotted to Treatment B (co-cultured with a bTV). Intact embryos were allotted to Treatment C (culture in medium alone) and Treatment D (co-cultured with a bTV). A rent was cut in the zonae pellucidae of the embryos in Treatments C and D, using a fine glass needle controlled by a micromanipulator, in order to simulate the opening in the zona pellucida of the bisected embryos allotted to Treatments A and B.

**Embryo Bisection**

The DE allotted to Treatments A and B were produced by microsurgically separating the blastomeres of each embryo into two equal portions similar to the procedure described by Willadsen (1982). The micromanipulation procedure was accomplished in an open 75 mm plastic petri dish (Lux) containing 30 ml of HM. A 150 µm fire polished suction pipette and a 70 µm beveled transfer pipette, each controlled by a separate Lietz micromanipulator, were used during the micromanipulation procedure. The embryo was positioned with the suction pipette, while the beveled transfer pipette was inserted through the ZP of the embryo. Medium was then introduced into the ZP from the transfer pipette to flush the blastomeres out of the ZP and into the sur-
FIGURE 3. ILLUSTRATION OF THE TREATMENTS IN EXPERIMENT III

Treatment A
Demi-embryos cultured in medium alone (n=33)

Treatment B
Demi-embryos co-cultured with a bTV (n=33)

Treatment C
Intact embryos cultured in medium alone (n=33)

Treatment D
Intact embryos co-cultured with a bTV (n=33)
rounding medium. The blastomeres were then separated by pipetting the blastomeres several times with a hand-held glass pipette. The separated blastomeres were counted and one-half of the blastomeres were drawn up into the transfer pipette for introduction into a ZP. The remaining blastomeres were placed into a previously prepared evacuated ZP obtained from an unfertilized or degenerate bovine ova.

**Trophoblastic Vesicle Production**

Bovine trophoblastic vesicles were prepared from day 13.5 to 14 elongated embryos 24 to 36 hours prior to the collection of precompaction-stage embryos. The elongated embryos were cut into embryonic sections using the procedure previously described by Rorie et al. (1987a). The embryos were then placed into a plastic petri dish with .1 to .2 ml of HM and cut into embryonic sections 1 to 2 mm² in size with a razor blade held by a pair of hemostats. The embryonic section containing the embryonic disc was discarded. Bovine trophoblastic vesicles of similar size and appearance, which had expanded to a diameter of 1 to 2 mm following the 24- to 36-hour culture period, were selected for co-culture with DE and intact embryos in Treatments B and D, respectively.

**In Vitro Culture**

The embryonic sections for bTV production were cultured in Nunc four-well culture plates containing 1 ml/well of CMRL 1066 medium (Sanyal and Naftolin, 1983) with 10% (v/v) FCS and Ab-Am. The embryos in Treatments A, B, C and D were individually cultured in 96-well culture plates with .3 ml/well of CMRL 1066 medium with 10% (v/v) FCS and Ab-Am. All cultures were maintained at 37°C in a humidified atmos-
phere of 5% CO₂ in air. Half of the medium in each well was replaced after 3 days of culture. The embryos were evaluated every 24 hours for morphological quality (1= excellent; 2= good; 3= fair; 4= degenerate) and developmental stage during the 96-hour culture period, using the criteria as established by Lindner and Wright (1983). Embryos which advanced in developmental stage and appeared normal, based on the evaluation criteria, were considered viable embryos in this study.

Statistical Analysis

The embryo viability scores assigned to embryos in this study were analyzed by assigning a score of "1" to viable embryos and a score of "0" to non-viable embryos. Also, the quality grade 1 or 2 embryos in each treatment were assigned a score of "1" and the grade 3 and degenerate embryos were assigned a score of "0". Embryo viability and quality scores for the treatments and treatment interactions were compared using one-way analysis of variance (Steel and Torie, 1980). Treatment means were compared, using Tukey's significant difference test (Steel and Torie, 1980).

Results

Embryo Recovery and Micromanipulation

A total of 99 viable appearing embryos were recovered from nine donor cows on day 4.5 of the cycle. These embryos were randomly assigned by donor and developmental stage across the treatments. Twenty-seven of the embryos were at the 8-cell stage of development at recovery and the remaining embryos contained 16 cells each.

A total of 21 viable elongated embryos were recovered from three donors on day 13.5 post-breeding. These elongated embryos were
successfully dissected into embryonic sections for bTV production. Greater than 90% of the embryonic tissue sections developed into expanded bTV after 24 to 36 hours of in vitro culture.

All 33 of the 8- to 16-cell embryos assigned to Treatments A and B were successfully bisected using the blastomere separation technique. The blastomere separation technique averaged 5 minutes to perform on each embryo. No apparent injury to the blastomeres was noted during the bisection procedure.

**In Vitro Culture**

The viability and morphological quality of the embryos following 48 and 96 hours of in vitro culture are presented in table 6. After 48 hours of culture, 8 of the DE (24.2%) in Treatment A were evaluated to be viable, with 5 DE (15.2%) being classified as excellent or good quality (embryo quality grade 1 or 2). At the end of the 96 hour culture period, 5 DE (15.2%) remained viable, but only 1 DE (3%) was of excellent to good quality.

In Treatment B, 17 DE (51.5%) and 10 DE (30.3%) were judged to be viable and of excellent or good quality after 48 hours in culture, respectively. At 96 hours in vitro, 13 of the DE (39.4%) in Treatment B were classified as viable, with 10 DE (30.3%) of good or excellent quality. There were a similar number of viable embryos (grades 1, 2 and 3) in Treatments A and B throughout the culture period (P> .05). The quality of the DE in Treatments A and B were similar after 48 hours of culture. However, there were more excellent or good quality DE (P< .05) in Treatment B at the end of the culture period.
<table>
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<td>Number(%)</td>
<td>Number(%)</td>
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<tr>
<td></td>
<td>of viable embryos</td>
<td>of grade 1&amp;2 embryos</td>
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<tr>
<td>A</td>
<td>33</td>
<td>8(24.2%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>33</td>
<td>17(51.5%)&lt;sup&gt;ad&lt;/sup&gt;</td>
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<tr>
<td>C</td>
<td>33</td>
<td>21(63.6%)&lt;sup&gt;bd&lt;/sup&gt;</td>
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<td>D</td>
<td>33</td>
<td>31(94.0%)&lt;sup&gt;c&lt;/sup&gt;</td>
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</table>

* Treatment A and B were DE cultured in the control medium alone and co-cultured with bTV, respectively. Treatment C embryos were cultured intact in the control medium alone and Treatment D embryos were intact embryos co-cultured with bTV.

<sup>a,b,c,d</sup> Numbers in the same columns with different superscripts differ (P<.05).
Twenty-one of the intact embryos (63.6%) cultured in medium alone in Treatment C were judged to be viable after 48 hours of in vitro culture. Of these, 13 (39.4%) were considered to be of excellent or good morphological quality. When the embryos in Treatment C were removed from culture at 96 hours, 16 (48.5%) of these were considered viable and 7 (21.2%) were judged as good or excellent quality.

Of the 33 embryos co-cultured with bTV in Treatment D, 31 (94%) were classified viable after 48 hours of culture. Twenty (60.6%) of these were excellent or good quality. At the termination of culture (96 hours), 28 of the embryos (84.8%) in Treatment D were judged to be viable and 21 (63.6%) were considered to be excellent or good quality. After 48 hours of culture, there were a similar number of quality grade 1 or 2 embryos in Treatments C and D, but there were more viable embryos (P<.05) in Treatment D than in Treatment C. At the end of the 96-hour culture period, there were more embryos considered viable and quality grade 1 or 2 in Treatment D than in the other treatments (P<.01).

A comparison of the number of viable embryos in Treatments A and C indicate that the overall viability was lower (P<.01) for bisected embryos than for embryos cultured intact (Treatment C). However, the co-culture of a bTV with a bisected embryo (Treatment B) resulted in the bisected embryos having viability and quality scores similar to that of intact embryos (Treatment C).

The number of viable and quality grade 1 or 2 bisected embryos (Treatments A and B) were lower (P<.01) throughout the 96-hour culture period than the number of viable and quality grade 1 or 2 intact embryos (Treatments C and D). Also, the culture of bisected and intact
embryos with bTV (Treatments B and D) resulted in more (P<.01) viable and excellent to good quality embryos at the end of the culture period than did the culture of embryos in the control medium alone (Treatments A and C). At the end of the 96-hour culture period 85%, 61%, 52% and 12% of the embryos allotted to Treatments A, B, C and D, respectively, were judged to be degenerate.

The final developmental stages of the embryos allotted to the treatments in this study are presented in table 7. In Treatment A, four of the five viable embryos (80%) were blastocysts after 96 hours of culture. The viable appearing embryos in Treatment B ranged from late morulae to expanded blastocysts at the end of the 96-hour culture period. Eleven of 13 embryos (84.4%) considered viable had developed to or beyond the blastocyst stage of development by the end of the culture period.

At the end of culture, the developmental stages of the 16 viable embryos in Treatment C ranged from late morulae to hatched blastocysts. Ten of 16 embryos (62.5%) had developed to the blastocyst or hatched blastocyst stage of development. Twenty-four (85.7%) late morula and hatched blastocyst stage embryos considered viable in Treatment D had achieved at least the blastocyst stage of development after 96 hours of in vitro culture.

Discussion

Previous studies with farm animal embryos have shown that the presence of an intact or near intact ZP is necessary for pre-compaction stage embryos to survive in vivo (Moore et al., 1969; Trounson and Moore, 1974; Massey et al., 1982). The only solution to this problem
<table>
<thead>
<tr>
<th>Embryo developmental stage</th>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
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<td>Late morula</td>
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<td>Total embryos/treatment</td>
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<sup>a</sup> Treatment A and B were DE cultured in the control medium alone and co-cultured with bTV, respectively. Treatment C embryos were cultured intact in the control medium alone and Treatment D embryos were intact embryos co-cultured with bTV.
to date has been the agar embedding of micromanipulated precompaction-stage embryos to produce an "intact" ZP and \textit{in vivo} culture to the morula or blastocyst stages, which have shown to be able to survive \textit{in vivo} without an intact ZP (Willadsen, 1979).

Seidel (1982) has reported that an intact ZP is not required for precompaction-stage bovine embryos to survive \textit{in vitro}. If an \textit{in vitro} culture system were available which would adequately maintain embryo viability for 2 or more days, then micromanipulation studies could be conducted with precompaction-stage embryos without the need for agar embedding or \textit{in vivo} culture. This would make micromanipulation studies with precompaction-stage embryos more practical than at present.

The present study was undertaken to evaluate whether the co-culture of micromanipulated precompaction-stage embryos with bTV would maintain the viability of these embryos for a 4-day culture period. The \textit{in vitro} co-culture systems reported in the literature have been shown to be superior to the culture of embryos in medium alone (Kuzan and Wright, 1982a,b; Voelkel et al., 1984a,1985a; Wiemer et al., 1987; Eyestone et al., 1987; Camous et al., 1984a,b,c; Heyman et al., 1987a). The bTV co-culture system has an advantage over other co-culture systems in that bTV have been shown to secrete growth promoting factors (Heyman et al., 1987b).

In the present study, the viability and quality of "half" and intact precompaction-stage embryos cultured with or without bTV were compared. The number of embryos assumed viable in each treatment were those embryos which were considered to be of excellent to fair quality at the time of evaluation. The quality grade 1 and 2 embryos are those
which were considered to be of a morphological quality that could be transferred to recipients with optimal potential for pregnancies.

A comparison of Treatments A and B tend to indicate that the co-culture of DE with bTV did not maintain embryo viability over that achieved with medium alone. However, there was a trend for more embryos to survive the *in vitro* culture in Treatment B. In this study, 30% of the DE co-cultured with bTV were of excellent or good quality at the end of the 96-hour culture period. In comparison, the agar embedding and *in vivo* culture technique for micromanipulated precompaction-stage embryos has resulted in 57 to 77% of the micromanipulated embryos being classified as normal at the time of recovery from the intermediate host (Willadsen, 1979; Willadsen and Polge, 1981).

The results of this study indicate that the culture of intact embryos with bTV is superior to the culture of intact or bisected embryos in medium alone. The number of viable embryos and embryo quality in Treatment D was consistently higher than for any other treatment in this study. The co-culture of intact day 4.5 embryos (Treatment D) compares favorably with the results achieved by Wiemer et al. (1987) who cultured day 5.5 to 6.5 bovine embryos on a fetal uterine fibroblast monolayer. In this study, 85% of the day 5.5 to 6.5 bovine embryos cultured on a fetal fibroblast monolayer were considered viable after 60 hours of *in vitro* culture.

In the present study, 85% of the intact embryos cultured with bTV were also judged to be viable at 96 hours *in vitro*. The same percent viability was maintained in the present study for 36 hours longer and with bovine embryos initially at the 8- to 16-cell stage of development, rather than with the initial developmental stage of morulae.
At the end of the culture period, more of the intact embryos (Treatment C) were considered viable and of quality grade 1 or 2 than were the number of bisected embryos (Treatment A). However, the results of this study do indicate that the number of viable and quality grade 1 or 2 DE co-cultured with bTV (Treatment B) were equal to that of intact embryos cultured in medium alone (Treatment C).

Treatments A and B (demi-embryos) were compared with Treatments C and D (intact embryos) to determine the overall effect of embryo bisection on embryo viability and morphological quality. The results of this study show that bisection of the 8- to 16-cell embryos used in this study reduced both the viability and the morphological quality of the embryos. This finding suggests it is not feasible to produce and culture early-stage bisected embryos under the conditions of this experiment. The embryos co-cultured with bTV (Treatments B and D) were also compared with Treatments A and C (culture medium alone) to determine the overall effect of the bTV co-culture system on embryo viability and quality. This comparison clearly shows that the bTV co-culture system was superior to medium alone for maintaining both embryo viability and morphological quality in vitro.

The embryos in this study were recovered on day 4.5 post-estrus and cultured to day 8.5 in vitro. Bovine embryos recovered on day 8.5 post-estrus are usually expanded, hatching or hatched blastocysts (Lindner and Wright, 1983). A comparison of the final developmental stages of the embryos in this study with the expected developmental stages of "fresh" day 8.5 embryos, indicates that the embryos in all treatments of the present study lagged behind in morphological development. This developmental lag, however, was less severe for the intact
embryos co-cultured with bTV (Treatment D). Previous studies have shown that the long-term in vitro culture of bovine embryos results in a developmental lag of 1 or more days (Camous et al., 1984b,c).

The results of this study do offer some encouragement for the in vitro culture of precompaction-stage bovine embryos. The embryos used in this experiment were recovered from the donor cows 4.5 days after the onset of estrus. Precompaction-stage bovine embryos as late as day 5 or day 6 can be bisected, using the blastomere separation technique (Willadsen et al., 1981). If day 5 or 6 embryos were used, the blastomeres could be separated and the resulting micromanipulated embryos would require only 24 to 36 hours of culture before they could be transferred to recipients. This shorter culture period should result in higher embryo quality in vitro and survival in vivo.

Since bTV secrete growth promoting substances which are present in bTV conditioned medium, the advantages of the bTV co-culture system are available without having to rely on the availability of "fresh" bTV for embryo culture. The bTV co-culture system or bTV conditioned medium might have application for the short-term culture of bisected bovine embryos prior to transfer to recipients or before and(or) after cryopreservation.

The embryos in the present study were not transferred to recipients, to evaluate in vivo survival. Further studies are necessary to evaluate the survival of micromanipulated and cultured precompaction stage bovine embryos following transfer to recipients.
CHAPTER V

EXPERIMENT IV

THE VIABILITY OF BOVINE 'HALF' EMBRYOS PRODUCED BEFORE OR AFTER LIQUID NITROGEN FREEZING

Introduction

Micromanipulation of bovine embryos to produce monozygotic twins is now being offered by some of the more progressive commercial cattle embryo transplant units around the world. One of the primary advantages of embryo splitting is the capability of increasing the number of progeny from genetically superior females. Pregnancy rates can be obtained in cattle from fresh "half" embryos (demi-embryos) which are comparable with those of intact embryos (Voelkel et al., 1984b; Takeda et al., 1986). When sufficient surrogate females are available, good quality embryos can be bisected and transferred, thus potentially doubling the number of offspring from embryos obtained from valuable donor animals.

The use of micromanipulation for the production of genetically identical twins may increase efficiency of comparative animal research. In one report, Hancock (1954) indicated that a pair of monozygotic twins can be worth as many as 72 pairs of unrelated individuals for research purposes, depending upon the parameter under study.

Before the full research and economic potential of embryo bissection can be realized, an efficient procedure must be developed which would result in a high percentage of the demi-embryos developing into
twin offspring. The versatility offered by induced monozygotic twins will likely be dependent upon demi-embryos surviving cryopreservation and long-term storage in liquid nitrogen (LN\textsubscript{2}) prior to their transfer to suitable recipients.

Lehn-Jensen and Willadsen (1983) were first to report results on the survival of frozen-thawed "half" and "quarter" bovine embryos. In this study, day 5 to 6 bovine embryos were collected and the blastomeres separated by micromanipulation. These dissected embryos were then embedded in 1.2% agar and transferred to the ligated oviducts of anestrous ewes for 1 or 2 days of in vivo culture. These embryos were then recovered from the oviducts and those which had developed were frozen and stored in LN\textsubscript{2}.

Twenty-six of the frozen "half" embryos (70.3%) survived the freeze-thaw procedure and 18 were selected for transfer. Eleven "half" embryos (61.1%) transferred developed in utero, including four pairs of monozygotic twins. Twenty-one of 28 "quarter" embryos frozen (75%) survived the freeze-thaw process. Four "quarter" embryos were selected for transfer; however, no pregnancies were reported. These preliminary findings suggested low in vivo survival would result from frozen-thawed bovine "half" embryos, and offered little encouragement for producing offspring from frozen-thawed "quarter" embryos.

In a subsequent study with cattle, Heyman and Chesne (1984) also investigated the feasibility of producing offspring from frozen-thawed "half" embryos. In their study, blastocyst-stage bovine embryos were bisected using micromanipulation, the embryo portions frozen and stored in LN\textsubscript{2}. Twenty demi-embryos were transferred individually to recipients resulting in six bovine recipients (30%) pregnant at 45 days of
gestation. However, by 90 days of gestation only four recipients (20%) remained pregnant.

Previous studies have suggested that an intact zona pellucida may be necessary for optimal survival of the cryopreservation process (Kanagawa et al., 1979). If this were true, embryos produced prior to cryopreservation would need to be enclosed in an "intact" zona pellucida. This would require agar embedding or some other means of closing the zona pellucida after micromanipulation.

In the present study, two trials were conducted to further investigate the feasibility of cryopreserving bovine demi-embryos. The primary objectives of Trial I and II were to evaluate the in vitro survival of bovine demi-embryos produced either prior to or following a freeze-thawing procedure. In Trial I, the embryos bisected prior to cryopreservation were cultured for 4 hours before cryopreservation in an attempt to allow for recovery from the bisection injury. Also, an effort was made in Trial II to evaluate the effect of an "intact" zona pellucida (simulated by agar embedding) on demi-embryo survival. This was to determine if protection from the zona pellucida would be necessary for optimal freeze-thaw survival.

Materials and Methods

Donor Preparation

Seventeen mature crossbred beef cows of the English, European and Brahman breeds were used as embryo donors in Trial I. In Trial II, 15 mature crossbred beef cows of the same breeding and selected from the same herd were used as embryo donors. Starting on days 9 to 15 of the estrous cycle, the donor cows were administered follicle stimulating
hormone (FSH-P: Schering Corp., Omaha, Nebraska) twice daily (im) using a 4-day descending dose treatment schedule (total dose = 36 mg FSH-P per donor female). At 48 and 60 hours after the initial 6 mg FSH injection, each female received injections (im) of 25 and 15 mg, respectively, of PGF$_2$α (Lutalyse: Upjohn Co., Kalamazoo, Michigan) to induce luteal regression. The donor cows were mated naturally to a fertile Angus bull during the early stages of standing estrus followed by artificial insemination with two units of high quality frozen-thawed beef semen at 24 hours after the onset of standing estrus. All donor females were non-lactating and in good to excellent body condition at the time of treatment. The donor cows ranged in weight from 522 to 610 kg at the onset of the study.

**Embryo Collection**

On day 7.5 to 8.0 (Trial I) or day 7.0 to 7.5 (Trial II) of the subsequent estrous cycle (onset of estrus = day 0), embryos were non-surgically collected from the donors by the procedure previously reported by Looney et al. (1981). The embryos were recovered using Dulbecco's phosphate-buffered saline (PBS: Gibco, Grand Island, New York) flushing medium with 2% (v/v) fetal calf serum (FCS) and an antibiotic-antimycotic (Ab-Am) solution containing 100 units of penicillin, 100 μg of streptomycin and .25 μg of amphotericin-B per ml of flushing medium. Embryos were maintained during the time prior to cryopreservation at room temperature in a modified PBS holding medium (HM) containing 10% FCS, 4.0 gm/1 of bovine serum albumin, 1.0 gm/1 of glucose, .036 gm/1 of pyruvic acid and Ab-Am.
Experimental Treatments - Trial I

A total of 132 excellent or good quality late morula to expanded blastocyst-stage embryos were allotted to four treatments in Trial I (figure 4). The embryos were randomly assigned by donor, morphological stage and quality grade across treatment groups. The treatments were: (A) embryos bisected into demi-embryos (DE) prior to cryopreservation (n=44), (B) embryos bisected into DE after freezing and thawing (n=44), (C) intact embryos (control) that were agar embedded prior to cryopreservation (n=22) and (D) intact (control) embryos frozen without agar embedding (n=22). After the embryos in Treatment A were bisected, the resulting DE were individually placed into a zona pellucida (ZP) and the embryos in all four of the treatments were cultured for 4 hours in CMRL-1066 medium (Sanyal and Nafolin, 1983) supplemented with 10% (v/v) FCS and Ab-Am. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. Following the 4 hour in vitro culture, the embryos in Treatments A, B and C were embedded in an agar solution and prepared for cryopreservation.

Experimental Treatments - Trial II

A group of 120 late morula to expanded blastocyst-stage embryos of quality grade 1 (excellent) or 2 (good) were assigned by donor, morphological stage and embryo quality grade across treatment groups. Forty embryos were equally assigned to each of three primary treatment groups (figure 5). The treatments were: (A) embryos bisected into DE prior to freezing, (B) embryos bisected into DE after freezing-thawing and (C) embryos frozen-thawed but not bisected (intact controls). One DE of each DE pair in Treatment A was replaced into the original ZP and
FIGURE 4. ILLUSTRATION OF THE TREATMENTS IN TRIAL 1 OF EXPERIMENT IV

Treatment A

Embryo (n=44) → Bisection → Agar Cylinder → Demi-embryos FROZEN → Thawed → Demi-embryos cultured post-thaw

Treatment B

Embryo (n=44) → Thawed → Intact embryo FROZEN → Embryo bisection post-thaw → Demi-embryos cultured post-thaw

Treatment C

Embryo (n=44) → Thawed → Intact embryo FROZEN → Intact embryo cultured post-thaw

Treatment D

Embryo (n=44) → Thawed → Intact embryo (not agar embedded) FROZEN → Intact embryo cultured post-thaw
FIGURE 5. ILLUSTRATION OF THE TREATMENTS IN TRIAL II OF EXPERIMENT IV

Treatment A

Embryo (n=40) → Bisection → Agar Cylinder → Demi-embryo

Treatment B

Frozen Embryo (n=40) → Thawed → Bisection → Demi-embryo

Treatment C

Frozen Embryo → Thawed (no bisection) → Intact Embryo
embedded in a .5 mm cylinder of 1.2% agar to simulate an 'intact' ZP (designated Treatment A-I). The remaining DE of each of the DE pairs in Treatment A was frozen ZP free (designated Treatment A-II). The embryos were frozen as soon as possible after micromanipulation of embryos in Treatment A.

**Micromanipulation Procedure**

A Leitz micromanipulator unit and a Wild M-8 stereomicroscope were used in the embryo micromanipulation procedures in Trial I and II. The embryos were bisected in an open, 75 mm, sterile petri dish containing 30 ml of HM. Late morula-stage embryos in Treatments A and B (Trial I and II) were bisected into DE using the microsurgical procedure previously described by Lambeth et al. (1983). A glass holding pipette was used to position the embryo while a fine glass surgical needle was used to make a rent in the ZP approximately 60% of the circumference of the embryo. The glass needle was then used to rotate the embryo until the rent was perpendicular to the base of the petri dish. The glass needle was then used to open the ZP and remove the embryonic cells. The ZP-free embryo was partially bisected by positioning the glass needle above the embryo resting on the floor of the petri dish and lowering the glass needle on a vertical plane. The bisection process was completed by repositioning the embryo between the holding pipette and the needle and using a gentle sawing motion with the glass needle.

After bisection of Treatment A embryos in Trial I, the glass needle was used to open the rent and simultaneously replace one DE back into the original ZP. The remaining DE was placed into a surrogate ZP obtained from a degenerate or unfertilized bovine ova. In Trial II,
only one DE of each DE pair was placed back into the original ZP (Treatment A-I). The remaining DE of each DE pair was frozen as ZP-free (Treatment A-II). The morulae-stage embryos in Treatment B (Trial I and II) were bisected after freezing-thawing using the same bisection procedure.

Blastocyst-stage embryos were bisected using a procedure similar to that described previously for ovine embryos by Willadsen and Godke (1984). Each embryo was positioned with the holding pipette so that the inner cell mass (ICM) was on the ventral side of the embryo as viewed through the stereomicroscope (100X). The fine glass needle was passed through the center of the embryo (directly above the center of the inner cell mass) to pierce the embryo from pole to pole. The embryo was released from the holding pipette and the glass needle was slowly lowered on a vertical plane to bisect the inner cell mass. The trophoblastic cavity contracts after the initial bisection procedure. With the needle still piercing the embryonic cell mass, the embryo was rotated to position the uncut trophoblast portion between the holding pipette and glass needle. The trophoblastic layer was then bisected with a sawing motion of the glass needle.

The contracted embryo was removed from the ZP and the bisection was completed by positioning the embryo between the holding pipette and the glass needle. A gentle sawing motion was again used to complete the bisection procedure. After the bisection, both DE (Trial I) or one DE of each DE pair (Trial II) was replaced back into the original or a surrogate ZP. The same bisection procedure was used to bisect blastocyst-stage embryos in Treatment B (Trial I and II) post-thaw.
**Agar Embedding**

In Trial I, the DE pairs in Treatment A and the intact embryos in Treatments B and C were agar embedded using a procedure similar to that described by Willadsen (1982). A 1.2% agar stock solution was prepared by adding 1.2 gm of fresh agar powder per 100 ml of distilled water and autoclaving for 15 minutes. This 1.2% agar solution was then allowed to cool to 38°C prior to embryo exposure. A .5 mm (I.D.) glass capillary tube was attached to a 1 ml precision Hamilton syringe by silastic tubing and was used to handle the embryos during the agar embedding procedures.

The DE or embryo to be embedded in agar was drawn up into the glass capillary tube and deposited into a petri dish containing FCS layered under PBS. The DE or embryo with a small volume of FCS was drawn up into the capillary tube and deposited into a petri dish containing 2 ml of 1.2% agar solution at 38°C. Finally, the DE or embryo in the agar solution was slowly drawn up into the capillary tube and deposited into a petri dish containing PBS at room temperature. The resulting agar cylinder was trimmed on both ends close to the DE or embryo with a razor blade held by a pair of small hemostats. The embedded DE or embryo was then placed into HM until the time of cryopreservation. In Trial II, this same agar embedding procedure was used to encase the DE replaced into a ZP in Treatment A-I following bisection. The remaining embryos in Trial I (Treatment D) and Trial II (Treatments A-II, B and C) were frozen without agar embedding.
Embryo Cryopreservation

The same cryopreservation procedures were used for all of the intact or DE in the treatments in Trials I and II. The DE or intact embryos were placed into HM containing 1.5 M glycerol solution (Leibo, 1984) and individually loaded into .25 ml plastic bovine semen straws. Both DE of each DE pair in Treatment A were placed into the same straw.

All embryos were frozen using the method described by Pool et al. (1986), which had been modified slightly from the procedure previously reported by Leibo (1984). After 10 minutes equilibration in the glycerol solution, the straws containing the DE and the intact embryos were placed into a Model R-201 Planner programmable LN$_2$ freezing unit and were cooled from room temperature (20-22°C) to -7°C at a rate of -1.0°C per minute. The straws were "seeded" with a pair of metal forceps cooled in LN$_2$ and were held at -7°C for 15 minutes prior to cooling to -33°C at a rate of -.3°C per minute. After 15 to 20 minutes equilibration at -33°C, the straws were then transferred directly to LN$_2$ (-196°C) for storage.

After 1 to 4 months of storage (Trial I) or 1 to 3 weeks of storage (Trial II) in LN$_2$, embryos were thawed by placing the straws in a warm water bath at 35°C for 20 seconds. The embryos were removed from the straws and then transferred to HM containing 1.08 M sucrose to remove the glycerol cryoprotectant. After 10 minutes, the embryos were removed from the sucrose solution and then transferred to fresh HM prior to culture.
In Vitro Culture

The DE in Treatment A of Trial I and II and the intact embryos in Treatment B and C in Trial I were removed from the agar cylinders using the method previously outlined by Willadsen (1982). The agar cylinder was positioned by a holding pipette while a 25 gauge hypodermic needle attached to a micromanipulator unit was used to trim the agar cylinder close to the embryo, thus releasing the embryo from the agar.

After the DE and the intact embryos were released from the agar cylinders and the embryos in Treatment B were bisected following thawing, all DE and intact embryos were transferred from HM to 24-well culture plates. Each well of the plate contained 1.5 ml of CMRL-1066 medium with 10% (v/v) FCS and Ab-Am. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air. The embryos were evaluated under a Wild M-8 stereomicroscope (100X) for morphological development and assigned viability scores at 12 and 24 hours of in vitro culture. The embryos were evaluated as grade 1 (excellent), grade 2 (good), grade 3 (fair) or grade 4 (degenerate) as previously defined by Lindner and Wright (1983).

Statistical Analysis - Trials I and II

In this study, embryo viability was analyzed by assigning a score of "1" to viable embryos and "0" to non-viable embryos. Similarly the number of transferable (quality grade 1 or 2) embryos were compared in each treatment by assigning a value of "1" to transferable embryos and "0" to embryos which were not of transferable quality. These data for Trial I and II were analyzed using one-way analysis of variance pro-
cedures (Steel and Torie, 1980). Treatment means were compared using Tukey’s significant difference test (Steel and Torie, 1980).

Results

Micromanipulation and Embryo Recovery - Trial I

A total of 132 excellent to good quality late morula to expanded blastocyst-stage embryos were used in this trial (table 8). The 17 donor cows produced an average of 7.8 excellent or good quality embryos (grades 1 or 2) per collection. The embryos were randomly assigned within donor, quality grade and developmental stage across treatments. All 44 embryos in Treatment A were successfully bisected to produce 44 DE pairs prior to cryopreservation. After thawing, the 88 DE were recovered from the agar cylinders and placed into culture.

The 44 embryos in Treatment B were successfully recovered from the agar embedding and bisected to produce 44 DE pairs. All embryos were bisected in Treatment B, regardless of the post-thaw quality. In Treatments C and D, the 22 intact embryos in each treatment were recovered post-thaw. All 22 embryos in Treatment C were successfully removed from the agar embedding.

Micromanipulation and Embryo Recovery - Trial II

The 120 bovine embryos used for this trial were distributed by embryo quality grade and developmental stage for each treatment (table 9). Each of the 15 donor cows used in this trial contributed an average of 8 per collection embryos which were assigned across Treatments A, B and C. In Treatment A, 40 embryos (100%) were successfully bisected into DE pairs at the time of micromanipulation. In Treatment A-I one DE was lost during the agar embedding process and two DE were
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<th>Treatment</th>
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### TABLE 9. DISTRIBUTION OF EMBRYOS BY INITIAL QUALITY GRADE AND DEVELOPMENTAL STAGE IN TRIAL II.

<table>
<thead>
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<th>Quality grade and morphological stage</th>
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</table>
damaged while attempting to remove them from the agar cylinder. Two of 40 DE in Treatment A-II were lost during thawing and removal from the plastic straws. The remaining 37 of the 40 DE (93%) in Treatment A-I and 38 of the 40 DE (95%) in Treatment A-II were placed into culture and evaluated for development in vitro.

In Treatment B, 40 embryos (100%) were recovered from the plastic straws following thawing. Only embryos designated as quality grades 1 or 2 following thawing were subjected to micromanipulation. Thirty-five of 40 embryos (88%) were bisected to produce 35 DE pairs. The DE pairs and the five embryos not bisected were placed into culture to evaluate in vitro development. Correspondingly, all 40 of the embryos in Treatment C were recovered from the straws following thawing. These embryos were placed into the culture system without bisection to serve as intact controls.

In Vitro Survival - Trial I

All embryos were evaluated at 12 hours of culture and again 24 hours later. Each embryo was assigned a quality grade (1 = excellent, 2 = good, 3 = fair, 4 = degenerate) and evaluated for morphological development at 12-hour intervals. Only embryos of quality grades 1 or 2 were considered to be of transferable quality in this experiment. All embryos were cultured for 24 hours and observed to substantiate the observations made at 12 hours post-thaw.

The embryo quality grades after 12 hours of in vitro culture are presented in table 10. In Treatment A, 10 DE (11.4%) were viable (quality grade 1, 2 or 3) after 12 hours of culture. Four DE (4.6%) were evaluated to be of transferable quality. Fifteen DE (17.1%) in
<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2/88(2.3%)</td>
<td>2/88(2.3%)</td>
<td>6/88(6.8%)</td>
<td>78/88(88.6%)</td>
</tr>
<tr>
<td>B</td>
<td>1/88(1.1%)</td>
<td>2/88(2.3%)</td>
<td>12/88(13.6%)</td>
<td>73/88(83.0%)</td>
</tr>
<tr>
<td>C</td>
<td>3/22(13.6%)</td>
<td>7/22(31.8%)</td>
<td>4/22(18.2%)</td>
<td>9/22(36.4%)</td>
</tr>
<tr>
<td>D</td>
<td>3/22(13.6%)</td>
<td>6/22(27.3%)</td>
<td>2/22(9.1%)</td>
<td>11/22(50.0%)</td>
</tr>
</tbody>
</table>

Embryo quality grade 1 = excellent, 2 = good, 3 = fair, 4 = degenerate
Treatment B were viable at 12 hours of culture with 3 (3.4%) considered to be of transferable quality. There were no differences between the number of viable or transferable embryos in Treatments A and B (P>.05).

In Treatment C, 14 intact embryos (63.6%) were viable after 12 hours in culture with 10 (45.5%) being of transferable quality. Of the 22 embryos in Treatment D, 11 (50%) were viable and 9 (41%) were of transferable quality after 12 hours of in vitro culture.

Although embryo viability and quality were slightly higher for the intact embryos frozen in an agar cylinder in Treatment C when compared with the intact embryos frozen without agar embedding in Treatment D, these differences were not significant (P>.05). Since there was no difference between Treatments C and D, these control treatments were combined for comparison with Treatments A and B (table 11). Overall, 25 of the intact control embryos (56.8%) (Treatments C and D combined) were viable, with 19 (43.2%) being of transferable quality. This resulted in more (P<.05) viable and transferable embryos in both the control treatments than either Treatments A or B.

**In Vitro Survival - Trial II**

All embryos were evaluated at 12 hours of culture and again 24 hours later. Each embryo was evaluated using the same criteria as was used in Trial I. Also, only embryos of quality grades 1 or 2 were considered to be of transferable quality in Trial II of this experiment. All embryos were cultured for 24 or more hours and observed to substantiate the observations made at 12 hours post-thaw.

The results at 12 hours of in vitro culture are presented in table 12. In Treatment A-I, 14 DE (35.0%) survived the freeze-thaw process.
TABLE 11. EMBRYO/DE VIABILITY AND QUALITY IN TRIAL I
AT 12 HOURS POST-THAW

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of embryos/DE</th>
<th>Number(%) of viable embryos</th>
<th>Number(%) of transferable embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>88</td>
<td>10 (11.4%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 (4.6%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>88</td>
<td>13 (14.8%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 (3.4%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C+D</td>
<td>44</td>
<td>25 (56.8%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19 (43.2%)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* There were no differences between treatments C and D, so these treatments were combined for comparison with treatments A and B.

<sup>a, b</sup> Numbers within columns with different superscripts are significantly different (P<.05).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-I</td>
<td>5(12.5%)</td>
<td>4(10.0%)</td>
<td>5(12.5%)</td>
<td>24(65.0%)</td>
</tr>
<tr>
<td>A-II</td>
<td>3(7.5%)</td>
<td>4(10.0%)</td>
<td>5(12.5%)</td>
<td>25(70.0%)</td>
</tr>
<tr>
<td>B</td>
<td>5(6.3%)</td>
<td>12(15.0%)</td>
<td>25(31.3%)</td>
<td>24(47.5%)</td>
</tr>
<tr>
<td>C</td>
<td>17(42.5%)</td>
<td>10(25.0%)</td>
<td>4(10.0%)</td>
<td>10(22.5%)</td>
</tr>
</tbody>
</table>

*aEmbryo quality grade 1 = excellent, 2 = good, 3 = fair, 4 = degenerate*
Nine DE (22.5%) were of transferable quality. Twelve DE (30.0%) in Treatment A-II survived the freezing-thawing process and 7 DE (17.5%) were considered to be of transferable quality. No differences (P>.05) were detected between Treatments A-I and A-II, so these treatments were combined for comparison with Treatments B and C (table 13). Overall, 26 DE (32.5%) in Treatments A-I and A-II produced prior to freezing survived the freezing-thawing process and a total of 16 DE (20.0%) were of transferable quality at 12 hours of in vitro culture.

Forty-two DE (52.5%) in Treatment B were classified as being viable at 12 hours in vitro, with 17 DE (21.3%) considered to be of transferable quality. There was no difference (P>.05) between the number of viable or transferable embryos in Treatments A and B.

In Treatment C, 31 of the intact control embryos (77.5%) were assessed as viable at 12 hours in vitro and 27 embryos (67.5%) were considered to be of transferable quality. There were more (P<.05) viable and transferable embryos in Treatment C than in Treatment A or B.

Discussion

Micromanipulation

The number of successful bisections produced in Treatments A and B of both Trials I and II confirmed that the micromanipulation procedures used in this study were acceptable for producing day-7 to day-8 bovine DE. Although some DE (≈20% in each trial) were unequal in proportion prior to the onset of culture, no discernable differences were noted in the viability among the DE after 12 hours of in vitro culture.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of embryos/DE</th>
<th>Number(%) of viable embryos</th>
<th>Number(%) of transferable embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(I+II)</td>
<td>80</td>
<td>26 (32.5%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16 (20.0%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>80</td>
<td>42 (52.5%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17 (21.3%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>40</td>
<td>31 (77.5%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27 (67.5%)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Since there were no differences between treatments A-I and A-II these treatments were combined for comparison with treatments B and C.

<sup>a</sup>,<sup>b</sup> Numbers within columns with different superscripts are significantly different (P<.05).
Furthermore, this slight size difference of some of the pre-culture DE was not associated with post-thaw in vitro viability scores.

The micromanipulation procedures used in these trials required only a glass holding pipette and a fine glass needle for the bisection or manipulation of the embryos, as compared with other methods which require elaborate micromanipulator units and(or) multiple microinstruments to complete the microsurgery (Ozil, 1982, 1983).

The agar embedding procedure proved to be efficient for encasing ZP intact embryos and for closing the ZP of DE after micromanipulation. However, this technique did require much practice prior to the onset of this study before the method could be consistently performed without losing embryos in the agar or destroying the embryo during removal from the agar cylinder.

In Vitro Survival

The intact and the demi-embryos in Trial I and II were evaluated at 12 hours post-thaw because this was considered to be the earliest time that a fair comparison between the DE produced before and after freezing could be made. Also from a practical consideration, 12 hours of culture following thawing would likely be the longest period that commercial transplant units would culture bovine embryos prior to transfer to recipient females. As previously mentioned, the embryos were evaluated using accepted criteria previously established by Lindner and Wright (1983).

A comparison of the post-thaw embryo viability and quality in Treatments C and D in Trial I would indicate that the cryopreservation of intact bovine embryos in agar has no detrimental or advantageous
effect on embryo survival post-thaw. Numerous types and solutions of agar have been evaluated for the embedding of bovine embryos prior to cryopreservation and none have resulted in positive or detrimental effects on post-thaw embryo viability (W.F. Rall, personal communication).

In Trial I, low in vitro embryo survival was evident in all treatments in this study. The cause of this low survival in vitro was not evident. The basic cryopreservation methodology used in this study has been shown to result in post-thaw viability for bovine embryos of greater than 60% for bovine embryos (Lehn-Jensen, 1979; Leibo, 1984). Therefore, it is unlikely that the cryopreservation procedure used for this study was the primary fault. The low embryo survival following culture post-thaw was observed across all donors in the study. Even though embryo donor variation exists in the survival of embryos following cryopreservation, it is unlikely that the overall low embryo survival could be attributed to the embryo donors.

A possible explanation of why embryo viability was low in all treatments of Trial I might be the additional time the embryos were in culture after collection and prior to cryopreservation. Other studies have shown that post-thaw embryo viability is inversely related to time in culture prior to cryopreservation (Wright, 1985; Chesne et al., 1987). The period from embryo collection to cryopreservation in this study ranged from 8 to 12 hours. This time period may have been excessive for optimal embryo survival post-thaw. The embryos in this study were cultured for 4 hours prior to cryopreservation in an attempt to allow the DE in Treatment A to recover from bisection injury prior
to cryopreservation. This culture period may have had the opposite effect of reducing rather than enhancing post-thaw viability.

Even though the viability and quality of the intact control embryos (Treatments C and D) in Trial I were lower than expected, viability and quality tended to be better in these treatments than in Treatment A or B. These results would indicate that the embryos in Treatments A and B could not overcome the bisection and freezing injury following thawing.

In Trial II, an effort was made to freeze the embryos as soon as possible after embryo recovery. This approach should reduce losses in embryo viability due to culture time. Therefore, embryos were frozen immediately after micromanipulation rather than culturing for a short period. No more than two to three donor females were collected on any one day in this trial, thus insuring that the embryos could be processed and frozen in an expeditious manner. The time from embryo collection and handling to freezing varied from 3 to 6 hours. This time is similar to that in large commercial transplant units.

The results from Trial II suggest that the lack of a ZP does not alter the survival rate of day 7 to 7.5 DE during freezing. There were no differences in either the viability or the number of transferable DE between Treatment A-I and Treatment A-II. These results are in agreement with recent findings of Chesne et al. (1987) and Takeda et al. (1987) who have recently reported that the ZP is not necessary for the optimal survival of frozen-thawed DE. In contrast, another study has indicated that an "intact" ZP is essential for the cryopreservation of bovine DE (Niemann et al., 1986). Others have also indicated there are no differences in the survival of intact bovine embryos frozen either
with or without an intact ZP (Blakewood et al., 1986; Niemann et al., 1987).

There were no differences in the number of transferable quality DE when the DE were produced before or after freezing-thawing. However, a higher, though not significant, percentage of DE produced after freezing-thawing were considered viable at 12 hours of in vitro culture (52.5% vs. 32.5%). Before one could suggest it would be preferable to produce bovine DE after freezing, further evaluation is necessary. In either case, however, only 20% of the DE in Treatments A combined (A-I and II) were transferable compared with 68% for the intact controls in Treatment C.

Prior to the present study, others have reported on the cryopreservation of bovine DE. Lehn-Jensen and Willadsen (1983) reported that 70% of the "half" embryos produced by blastomere separation of precompaction bovine embryos survived freezing and were viable post-thaw. Embryo survival following freezing-thawing in their study was much greater than that observed for frozen-thawed DE in the present study. This difference in post-thaw viability might be explained in the different bisection techniques used. The blastomere separation method allows for the separation of the embryo into equal portions without cell (blastomere) loss or injury. In contrast, the bisection techniques used now for later-stage compacted morulae and blastocysts results in cell loss and injury during the bisection process (Picard et al., 1986; Skrzyszowska and Smorag, 1987; Chesne et al., 1987). This mechanical injury, in addition to freezing injury and viability loss in vitro could be too great for the bisected embryos to overcome following thawing.
Heyman and Chesne (1984) reported pregnancy rates of 20% for DE produced from frozen-thawed blastocysts. This pregnancy rate is very similar to the percentage of transferable quality DE following in vitro culture in Trial II of the present study.

Previous studies have indicated that day 7.5 to 8 bovine embryos (blastocysts and expanded blastocysts) may survive cryopreservation better than slightly earlier stage embryos (Menezo, personal communication). However, in the present study no differences were noted in the viability or quality of embryos that were cryopreserved at the late morula through expanded blastocyst-stages.

As was previously mentioned, the results of both Trial I and II show that there is no difference in the in vitro survival of DE produced before or after cryopreservation. There tended to be more transferable quality embryos in Treatments A and B of Trial II (19 to 21%) than in Treatments A and B of Trial I (3 to 4%). The apparent difference between Trials I and II was the culture time from embryo recovery to cryopreservation. The lower embryo viability and quality evident across all treatments in Trial I could possibly be due to the extended time the embryos were in culture prior to cryopreservation.

The embryos in Trials I and II were not transferred to recipients for evaluation of in vivo survival. This would logically be the next step. However, developing a procedure that will stand up to in vitro evaluation is needed before expensive field trials are conducted. Correspondingly, results from this study further indicate the need to develop an efficacious procedure for the production of viable frozen-thawed bovine demi-embryos.
Vitrification has recently been evaluated for the cryopreservation of bovine DE (Massip et al., 1987). Vitrification differs from conventional cryopreservation methods in that the cryoprotectants used are at a higher concentration and form a solid when frozen. This avoids injury to the embryo from ice crystal formation, since ice crystals do not form. In this study, DE were produced from day 6.5 to 8 bovine embryos and frozen in either 10% glycerol (conventional freezing) or in a vitrification solution of propanediol-glycerol. The transfer of 10 DE pairs frozen by the conventional method resulted in two pregnancies (20%) compared with six pregnancies (28%) for DE pairs cryopreserved by the vitrification method.

More studies are necessary to evaluate the use of vitrification for cryopreserving DE. Refinement of the vitrification technique could result in a viable cryopreservation method for demi-embryos in the future. Also, the use of a fibroblast monolayer (Kuzan and Wright, 1982b) or a trophoblastic vesicle co-culture system (Camous et al., 1984a) with cryopreserved DE might also improve DE survival rates following thawing.
LITERATURE CITED


APPENDIX TABLE 1. ANALYSIS OF VARIANCE FOR THE NUMBER OF DEMI-EMBRYOS FORMED IN EACH TREATMENT IN EXPERIMENT I.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tech</td>
<td>1</td>
<td>.4464</td>
<td>9.60**</td>
</tr>
<tr>
<td>Zona</td>
<td>1</td>
<td>.4464</td>
<td>9.60**</td>
</tr>
<tr>
<td>Tech x Zona</td>
<td>1</td>
<td>.2857</td>
<td>6.14**</td>
</tr>
<tr>
<td>Error</td>
<td>220</td>
<td>.0465</td>
<td></td>
</tr>
</tbody>
</table>

^a Tech = Bisection Technique (slide micromanipulator or razor blade method).
Zona = Zona pellucida treatment (ZI or ZF).

APPENDIX TABLE 2. ANALYSIS OF VARIANCE FOR THE NUMBER OF BLASTOCYSTS FORMING FROM DEMI-EMBRYOS IN EXPERIMENT I.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tech</td>
<td>1</td>
<td>.5402</td>
<td>2.57</td>
</tr>
<tr>
<td>Zona</td>
<td>1</td>
<td>1.0045</td>
<td>4.78*</td>
</tr>
<tr>
<td>Tech x Zona</td>
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<td>.19</td>
</tr>
<tr>
<td>Error</td>
<td>220</td>
<td>.2098</td>
<td></td>
</tr>
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</table>

^a Tech = Bisection Technique (slide micromanipulator or razor blade method).
Zona = Zona pellucida treatment (ZI or ZF).
APPENDIX TABLE 3. ANALYSIS OF VARIANCE FOR THE NUMBER OF VIALBLE EMBRYOS AT 60 HOURS IN VITRO IN EXPERIMENT II.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
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<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td>.2871</td>
<td>1.37</td>
</tr>
<tr>
<td>Error</td>
<td>105</td>
<td>.2090</td>
<td></td>
</tr>
</tbody>
</table>

APPENDIX TABLE 4. ANALYSIS OF VARIANCE FOR THE NUMBER OF QUALITY GRADE 1 OR 2 EMBRYOS AT 60 HOURS OF CULTURE IN EXPERIMENT II.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
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<td>10.04**</td>
</tr>
<tr>
<td>Error</td>
<td>105</td>
<td>.2111</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX TABLE 5. ANALYSIS OF VARIANCE FOR THE NUMBER OF VIAL EMBRYOS AT 96 HOURS IN VITRO IN EXPERIMENT II.

<table>
<thead>
<tr>
<th>Source</th>
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<th>Mean square</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td>.6945</td>
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</tr>
<tr>
<td>Error</td>
<td>105</td>
<td>.2368</td>
<td></td>
</tr>
</tbody>
</table>

APPENDIX TABLE 6. ANALYSIS OF VARIANCE FOR THE NUMBER OF QUALITY GRADE 1 OR 2 EMBRYOS AFTER 96 HOURS OF CULTURE IN EXPERIMENT II.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
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<th>F ratio</th>
</tr>
</thead>
<tbody>
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<td>Treatment</td>
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<td>1.0093</td>
<td>4.75*</td>
</tr>
<tr>
<td>Error</td>
<td>105</td>
<td>.2124</td>
<td></td>
</tr>
</tbody>
</table>
### APPENDIX TABLE 7. ANALYSIS OF VARIANCE FOR THE NUMBER OF VIABLE EMBRYOS AT 48 HOURS OF IN VITRO CULTURE IN EXPERIMENT III.

<table>
<thead>
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<th>Mean square</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>5.9394</td>
<td>33.18 **</td>
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<td>1</td>
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<td>16.93 **</td>
</tr>
<tr>
<td>A x B</td>
<td>1</td>
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<tr>
<td>Error</td>
<td>128</td>
<td>.1790</td>
<td></td>
</tr>
</tbody>
</table>

*Treatment A = culture of intact or bisected embryos.  
Treatment B = culture of embryos with or without bTV.*

### APPENDIX TABLE 8. ANALYSIS OF VARIANCE FOR THE NUMBER OF QUALITY GRADE 1 OR 2 EMBRYOS AT 48 HOURS OF IN VITRO CULTURE IN EXPERIMENT III.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>2.4545</td>
<td>11.63 **</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>1.0910</td>
<td>5.17 *</td>
</tr>
<tr>
<td>A x B</td>
<td>1</td>
<td>.0305</td>
<td>.14</td>
</tr>
<tr>
<td>Error</td>
<td>128</td>
<td>.2110</td>
<td></td>
</tr>
</tbody>
</table>

*Treatment A = culture of intact or bisected embryos.  
Treatment B = culture of embryos with or without bTV.*
**APPENDIX TABLE 9. ANALYSIS OF VARIANCE FOR THE NUMBER OF VIABLE EMBRYOS IN EXPERIMENT III AT 96 HOURS OF IN VITRO CULTURE.**

<table>
<thead>
<tr>
<th>Source&lt;sup&gt;a&lt;/sup&gt;</th>
<th>df</th>
<th>Mean square</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>5.5227</td>
<td>29.53**</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>3.3409</td>
<td>17.86**</td>
</tr>
<tr>
<td>A x B</td>
<td>1</td>
<td>.1894</td>
<td>1.03</td>
</tr>
<tr>
<td>Error</td>
<td>128</td>
<td>.1870</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Treatment A = culture of intact or bisected embryos. Treatment B = culture of embryos with or without bTV.

**APPENDIX TABLE 10. ANALYSIS OF VARIANCE FOR THE NUMBER OF QUALITY GRADE 1 OR 2 EMBRYOS AT 96 HOURS OF IN VITRO CULTURE IN EXPERIMENT III.**

<table>
<thead>
<tr>
<th>Source&lt;sup&gt;a&lt;/sup&gt;</th>
<th>df</th>
<th>Mean square</th>
<th>F ratio</th>
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<tr>
<td>Treatment</td>
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<td></td>
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</tr>
<tr>
<td>A</td>
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<td>2.1894</td>
<td>13.28**</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>4.0076</td>
<td>23.32**</td>
</tr>
<tr>
<td>A x B</td>
<td>1</td>
<td>.1890</td>
<td>1.15</td>
</tr>
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<td>.1648</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Treatment A = culture of intact or bisected embryos. Treatment B = culture of embryos with or without bTV.
### APPENDIX TABLE 11. ANALYSIS OF VARIANCE FOR THE NUMBER OF Viable Frozen-Thawed Embryos/Df in Trial I of Experiment IV.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>3.39</td>
<td>23.85**</td>
</tr>
<tr>
<td>Error</td>
<td>217</td>
<td>.1423</td>
<td></td>
</tr>
</tbody>
</table>

### APPENDIX TABLE 12. ANALYSIS OF VARIANCE FOR THE NUMBER OF Transferable Quality Embryos/Df in Trial I of Experiment IV.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>2.7080</td>
<td>33.39**</td>
</tr>
<tr>
<td>Error</td>
<td>217</td>
<td>.0811</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX TABLE 13. ANALYSIS OF VARIANCE FOR THE NUMBER OF Viable embryos/DE POST-THAW IN TRIAL II OF EXPERIMENT IV.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>2.26</td>
<td>9.76**</td>
</tr>
<tr>
<td>Error</td>
<td>197</td>
<td>.2320</td>
<td></td>
</tr>
</tbody>
</table>

APPENDIX TABLE 14. ANALYSIS OF VARIANCE FOR THE NUMBER OF TRANSFERRABLE EMBRYOS/DE IN TRIAL II OF EXPERIMENT IV.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean square</th>
<th>F ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>3.6225</td>
<td>20.66**</td>
</tr>
<tr>
<td>Error</td>
<td>197</td>
<td>.1753</td>
<td></td>
</tr>
</tbody>
</table>
VITA

Rickey W. Rorie was born to A. F. and Helen Rorie in Harrison, Arkansas on January 5, 1954. He received his elementary and secondary education from Yellville-Summit Elementary and High School (1960-1972). He received his Bachelor of Science degree in Animal Science from the University of Arkansas at Fayetteville in May of 1977 and his Masters of Science degree in Animal Science from the University of Arkansas at Fayetteville in May of 1979.

From November of 1979 to December of 1983, he worked as a County Extension Agent - Agriculture for the University of Arkansas Cooperative Extension Service in Woodruff and Faulkner Counties. In January 1984, he entered graduate school at Louisiana State University and is currently working toward the Doctor of Philosophy degree in Reproductive Physiology under Dr. Robert A. Godke.
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Major Field: Animal Science (Reproductive Physiology)

Title of Dissertation: The Production, Culture and Cryopreservation of Bovine Demi-Embryos

Approved:

[Signatures]

EXAMINING COMMITTEE:

[Signatures]

Date of Examination: June 11, 1987