EFFECTS OF CULTURE CONDITIONS ON DEVELOPMENT OF EARLY MURINE AND BOVINE EMBRYOS

by

Amy Elizabeth Thuemmel

Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Dairy Science

APPROVED:

Francis C. Gwazdauskas, Chairman	Ronald E. Pearson
Ludeman A. Eng	William E. Vinson, Department Head

July 11, 1989

Blacksburg, Virginia

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(ABSTRACT)

Early mouse (n = 501) and bovine (d = 6; n = 172) embryos were obtained to evaluate the effect of a deproteinized hemodialysate (CLB1107) on embryo development in vitro. Bovine morulae also were cultured to examine the effect of agar embedding and the environment of immature mouse uteri on embryo development.

Mouse embryos were cultured for up to 96 h in M2 medium or M2 supplemented with CLB1107. One-and two-cell embryos did not develop beyond the two-cell stage in vitro. Degeneration of one-cell embryos occurred within 36 h. Two-cell embryos degenerated sooner when cultured in M2 plus 1% CLB1107 (27.4 ± 2.5 h) than in M2 alone (41.7 ± 3.0 h). Mean final development classification of embryos cultured from the morula stage (6) in M2 supplemented with CLB1107 was higher (9.0 ± 2) than that for morulae (8.2 ± 1) cultured in unsupplemented M2 medium. Development of embryos cultured from the blastocyst (8) or expanded blastocyst (9) stages was not affected by treatment.

Agar embedded bovine embryos were cultured in Ham's F-10 and 10% steer serum either 1) immediately after collection or 2) 24 h after storage in immature mouse uteri. Non-embedded embryos were cultured in Ham's F-10 containing 3) 10% steer serum, 4) 1% CLB1107 or 5) 1% CLB1107 and 10% steer serum. A greater percentage of the embryos reached the hatched blastocyst stage after culture in treatments 1, 3, 4, and 5 (38.1%, 34.6%, 28.6% and 21.1%) than embryos stored in immature mouse uteri for 24 h prior to in vitro culture (9.5%). Mean final development scores for non-embedded and agar embedded embryos cultured in Ham's F-10 and 10% steer serum were not different ($5.46 \pm .34$ and $4.89 \pm .44$), but were higher than embryos cultured in CLB1107 ($4.19 \pm .39$), CLB1107 and steer serum ($4.22 \pm .44$) or immature mouse uteri ($3.39 \pm .43$).

Medium supplemented with CLB1107 did not support mouse embryo development beyond the 2-cell stage nor did it enhance bovine embryo development. However, it appeared to enhance development of mouse morulae in vitro. Additionally, bovine morulae were not affected by agar embedding in vitro and they were able to develop following short term storage in the immature mouse uterus.

Acknowledgements

I would like to sincerely thank Dr. Frank Gwazdauskas for his guidance, encouragement and patience throughout the course of this project. Gratitude is also extended to Dr. Ronald Pearson, Dr. Ludeman Eng, and Dr. William Vinson for serving on my graduate committee.

Special thanks is expressed to my mentor and friend, Mr. Rodolfo Canseco, for his knowledge, assistance and patience.

Appreciation is also extended to Dr. Richard Saacke for the gift of semen for artificial insemination, Mrs. Judy Bame for technical assistance, Dr. Wolfgang Jochle for the gift of CLB1107, and Mr. Chuck Miller, Harold Nester and the rest of the employees at the Virginia Tech Dairy Center for their help and patience.

Lastly, I would like to thank my fiance, Mr. Walter Sparks, and my parents, Mr. Douglas Thuemmel and Mrs. Nancy Thuemmel, for their encouragement, love and support.

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Introduction

In vitro culture of embryos has provided researchers a unique method of examining early mammalian development. Several media have been used to successfully culture early murine and bovine embryos in vitro. Despite numerous advances regarding in vitro culture systems, development of bovine embryos through the 8- to 16-cell stage is inhibited in vitro. Embryos from certain strains of random bred mice experience a similar developmental block at the 2-cell stage in vitro. These developmental blocks are overcome by transfer of embryos to oviducts of foster mothers or coculture of bovine embryos with oviductal cells or trophoblastic vesicles in vitro. The factor(s) provided by these biological environments which promote murine and bovine embryo growth have yet to be identified.

Deproteinized hemodialysates from calf blood have been successfully used as media additives to promote healing and growth of deliberately damaged cell cultures. These blood products also have been shown to accelerate mouse embryo development in vitro, enhance mouse embryo survival after micromanipulation, and improve mouse and bovine embryo viability after freezing.

Embryo micromanipulation techniques have allowed researchers to investigate areas such as embryo development, cell differentiation, and gene expression. The ability of inbred and F1 cross mouse embryos to develop from the 1-cell to

blastocyst stage in vitro has facilitated a great deal of embryo micromanipulation research. Micromanipulation techniques such as gene microinjection and cloning involve 1- to 8-cell embryos. Successful development of micromanipulated embryos requires an environment which supports early embryo growth and promotes embryo recovery following microsurgery. Viablility of micromanipulated murine embryos is assessed by transfer of the embryos to pseudopregnant mice. Use of bovine recipients to determine micromanipulated bovine embryo viability is not economically feasible so viability is now evaluated by in vivo culture in ovine or rabbit oviducts. The accessibility of these animals is hindered by the cost of both species of animals and their maintenance. The use of immature mouse uteri for in vivo culture of early bovine embryos would significantly reduce in vivo culture expenses.

Microsurgical techniques such as microinjection and embryo bisection cause varying degrees of damage to the zona pellucida and the embryo itself. The zona pellucida prevents dispersal of blastomeres and protects the embryo from leukocytes and other foreign cells. The presence of a relatively intact zona pellucida is neccessary for early embryonic development, especially in situations such as in vivo culture in oviducts of foster mothers where the embryo is likely to encounter leukocytes. Researchers have shown that agar embedding is an effective method of sealing the zona pellucida and allowing for nutrient transport to the embryo.

Development of in vitro culture systems which support early mammalian embryonic growth and promote embryo recovery following micromanipulation would significantly hasten embryo research. Until such systems are established, the expense of in vivo culture systems must be reduced. The objectives of this study were to determine if the immature mouse uterus can be utilized as an in vivo culture system for bovine morulae, examine the effect of agar embedding on bovine morulae cultured in vitro, and evaluate mouse and bovine embryonic development in deproteinized hemodialysate supplemented media in vitro.

Specific objectives were: 1) to examine mouse 1-cell, 2-cell, morula, blastocyst and expanded blastocyst development in 1% v/v deproteinized hemodialysate and M2 medium; 2) to evaluate the effect of steer serum, deproteinized hemodialysate, and their combination on bovine morula development; 3) to evaluate the effect of agar embedding on bovine morula development in vitro; and 4) to test the effect of culturing agar embedded bovine morulae in immature mouse uteri for 24 h.

Review of Literature

In Vitro Culture Systems

The evolution of in vitro culture systems for embryos has allowed researchers to study and frequently monitor early mammalian development in controlled environments. Qualities such as high embryo production and low maintenance costs have made the mouse an excellent candidate for embryo research. The successful culture of 8-cell mouse embryos to the blastocyst stage in a medium based on Krebs-Ringer bicarbonate solution supplemented with bovine serum albumin (BSA), glucose, and antibiotics was one of the many early achievements which opened doors to a great deal of preimplantation embryo research (Whitten, 1956). McLaren and Biggers (1958) extended Whitten's (1956) experiment by demonstrating that blastocysts recovered from this culture system developed into normal mice after transfer to foster mothers. The medium described by Whitten (1956) has been modified several times and some of the more significant modifications will be discussed in this review.

Whitten (1957) reported that addition of lactate to the culture medium supported development of late 2-cell embryos to the blastocyst stage, but earlier stage embryos did not cleave in this medium. Brinster (1965) demonstrated that pyruvate, oxaloacetate and phosphoenolpyruvate could replace lactate in the

medium. Biggers et al. (1962) cultured mouse oviducts containing one-cell embryos on medium BGJb which contained lactate and glucose for energy sources. Seventy-four percent (159/215) of the embryos developed to the morula stage. These results suggested that components of the environment of the oviduct were necessary for the first three cleavage divisions to occur. Whitten and Biggers (1968) successfully cultured 1-cell mouse embryos to the blastocyst stage in medium similar to that described by Brinster (1965). The medium was modified by lowering the sodium chloride content from 119.32 to 68.49 mM and increasing the concentration of crystalline BSA from 1 to 4 g/l. By decreasing the sodium chloride content, osmolarity was reduced from 308 to 256 mOsmols. Development to the blastocyst stage was limited to embryos recovered from hybrid mice. None of the random bred 1-cell embryos reached the blastocyst stage. This in vitro cleavage block has been isolated to random bred strains of mice and is now believed to be of a cytoplasmic nature (Muggleton-Harris et al., 1982). Other mammalian embryos experience developmental blocks similar to the mouse and this phenomenon will be discussed later in this review.

Cross and Brinster (1973) determined that development of 1-cell random bred embryos to the blastocyst stage was sensitive to the type and concentration of energy source in the culture medium. Pyruvate best supported development from the 1- to 2-cell stage. Addition of lactate to pyruvate or lactate alone greatly reduced the proportion of embryos completing the first cleavage division. Lactate and pyruvate were, however, required for development beyond the first cleavage division. The best development of 1-cell embryos to the blastocyst stage was

achieved in media containing 0.25 mM pyruvate for the first cleavage division and 0.25 mM pyruvate and 30.0 mM lactate for subsequent cleavage. Neider and Corder (1983) examined ampullar and isthmic pyruvate and lactate content in cycling, pseudopregnant, and pregnant mice. In pregnant mice ampullar pyruvate increased to 0.67 mM approximately 12 h after ovulation while the isthmic pyruvate concentration remained at 0.40 mM throughout the preimplantation period. Ampullar lactate peaked to 6.6 mM 12 h after ovulation, but this level was considerably lower than levels tested by Cross and Brinster (1973;10-90 mM) and may be adequate to support 2-cell embryo development yet low enough to not adversely affect the first cleavage. With pregnancy isthmic lactate remained at approximately 5.5 mM throughout the preimplantation period while isthmic lactate levels in cycling and pseudopregnant mice declined within 48 h after ovulation. Pomp et al. (1988) modified Whitten's medium (1968; osmolarity = 280 mOsmols) by reducing the sodium lactate content from 21.6 to 11.6 mM (osmolarity = 250). The percentage of 1-cell embryos developing to blastocysts in vitro per female donor was significantly higher for outbred ICR and C57BL/6J F1 cross 1-cell embryos cultured in low sodium lactate medium than embryos cultured in Whitten's medium. The effects of sodium lactate and osmolarity could not be differentiated. However, the osmolarities of the both media fell into the osmolarity range required for optimal mouse embryo culture (Whitten, 1971).

Abramczuk et al. (1977) observed that development of random bred ICR 1-cell mouse embryos through the blastocyst stage in vitro required trace amounts of

EDTA (Ethlenadiamintetraacetic Acid) in the culture medium. One-cell embryos were cultured in modified Whitten's medium (1971) with 0.25 mM pyruvate and 4 g/l BSA. Medium supplemented with 10.8 uM of EDTA supported development of 74 % of the embryos to the blastocyst stage while 13 % of the embryos cultured in unsupplemented medium achieved the same stage of development.

Menino et al. (1985) examined the effects of replacing BSA with various amounts of bovine uterine fluid (BUF) or heat-treated bovine serum (HTBS) in Whitten's medium. Mouse embryo development in all media was positively correlated with the log total protein concentration. Embryo development in BSA and BUF supplemented media did not differ. Culture medium supplemented with HTBS supported significantly more embryo development than medium supplemented with BSA. Biery and Kraemer (1988) compared the development of 8-cell mouse embryos in Ham's F-10, modified Whitten's medium (with .1mM EDTA) or Dulbecco's Modified Eagle's Medium supplemented with either .2% BSA or 10% new born calf serum (NBCS). Whitten's medium supplemented with NBCS supported expansion and hatching rates greater or equal to all other treatments.

High maintenance costs and low embryo production have limited bovine embryo research and development of adequate culture systems for early bovine embryos. Several media and serum supplements have been evaluated for their ability to promote early bovine embryo development in vitro. With the exception of one rare incident (Wright et al., 1976a), bovine embryos cultured in vitro from 1-, 2- or 8-cell stages rarely develop past the 9- to 16-cell stage (Thibault, 1966; Wright

and Bondioli, 1981; Camous et al., 1984). In contrast, bovine morulae cultured in vitro frequently develop through the hatched blastocyst stage (Canfield et al., 1986; Canseco et al., 1988; Rajamehendran et al., 1986; Toole et et al., 1988).

Wright et al. (1976a) evaluated Modified Eagle's Medium (MEM), Tissue Culture Medium 199 (TCM-199), Ham's F-10, Synthetic Oviduct Fluid, Brinster's Mouse Ova Culture Medium (BMOC-3) and Whitten's medium for their ability to promote development of 2- to 8-cell bovine embryos. Media were supplemented with heat treated fetal calf serum (FCS) or bovine serum albumin (BSA). Ham's F-10 with 10 or 50% FCS was the only medium to support expanded blastocyst formation from 8-cell embryos. One of 9 expanded blastocysts hatched from the zona pellucida after 160 h in culture. This was the first reported in vitro hatching of a bovine embryo cultured from the 8-cell stage in a simple, chemically defined meduim.

Seidel et al. (1971) cultured two-to three-cell bovine embryos in TCM-199 plus 1.5% BSA or modified Ham's F-10 plus 1.5 BSA. Neither medium supported substantial embryo development, however, Ham's F-10 tended to promote more embryo cleavage (1.3 divisions/embryo) than TCM-199 (0.7 divisions/embryo). Onuma and Foote (1969) tested bovine serum, rabbit serum, Kerbs-Ringer bicarbonate solution and 10% sera, follicular fluid and Ham's F-10 for their ability to promote development of 1- to 16-cell bovine embryos. Approximately 45% of all embryos cleaved in culture, but development beyond the 16-cell stage

was not observed. No significant differences were found among the five media in their ability to promote early embryo cleavage.

Allen et al. (1982) compared embryo growth promotion of heat inactivated FCS, NBCS and normal steer serum (NSS) in Ham's F-10. Serum concentrations of 5 and 10% were tested. Ham's F-10 with 10% NSS supported significantly more hatching (67%, n = 15) than NBCS (33%, n = 15) and FCS (0%, n = 15). Forty percent of the embryos cultured in 5% NSS hatched while only 15% and 7% of the embryos cultured in 5% NBCS and 5% FCS reached the same stage of development. In contrast, Smith et al. (1986) reported hatching rates much lower than Allen et al. (1982) using Ham's F-10 with Hepe's Buffer (25%, n = 28) and Dulbecco's Phosphate Buffered Saline (D-PBS; 6.45%, n = 31) supplemented with 20% FCS. It must be recognized that Smith et al. (1986) added Hepes buffer to Ham's F-10 and to our knowledge, the Ham's F-10 used by Allen et al. (1982) did not contain Hepes, therefore, a direct comparison of the two studies is not possible. Additionally, Allen et al. (1982) cautioned that there is variation in commercial lots of FCS and similar variations may be observed in different collections of NSS. Work by Canfield et al. (1986) revealed no significant difference between the abilities of NSS and highly purified BSA to support bovine embryo development.

Rajamahendran et al. (1985) examined bovine morula development in Dulbecco's Phosphate Buffered Saline (D-PBS) and Ham's F-10 supplemented with 10% NSS. Nineteen of 27 embryos (70.4%) cultured in Ham's F-10 developed to

hatched blastocysts while only 11.5% (n = 26) of the embryos cultured in D-PBS initiated hatching. All of the embryos cultured in D-PBS and NSS did, however, reach the expanded blastocyst stage which suggests that D-PBS can be used for short term storage and culture of bovine embryos. Wright et al. (1976b) examined bovine blastocyst expansion and in vitro hatching as affected by two culture media supplemented with either 10% heat treated FCS or 1.5% BSA. Blastocyst expansion and hatching of 2- and 8-cell embryos was achieved in Ham's F-10 and Modified Ham's F-10 supplemented with 10% FCS. Neither media supplemented with 1.5% BSA promoted development of 1-, 2- or 8-cell embryos past the early blastocyst stage. The authors suggested that heat treated FCS may have factors essential for bovine blastocyst expansion and hatching in vitro.

Peters et al. (1978) compared viability of bovine morulae surgically transferred to synchronized recipients immediately after collection and embryos cultured for 24 h in Ham's F-10 supplemented with 10% heat treated FCS prior to transfer. No significant difference in viability was observed between embryos cultured in vitro (59.4%, n = 32) and controls (74.3%, n = 35) following transfer to recipients.

Developmental Block In Vitro

The most significant aspect of results reported by Wright et al. (1976a) was the fact that a 2-cell embryo developed to the hatched blastocyst stage in vitro. The development of 1- to 8-cell bovine embryos is typically arrested at the 8- to 12-cell stage when cultured in simple media in vitro. Thibault (1966) defined this un-

compromising stage of development as the "critical stage" or "block stage". This cleavage block may be similar to that observed in mouse embryos which are arrested at the 2-cell stage in vitro. While all bovine embryos experience a developmental arrest in vitro, only embryos from randomly bred strains of mice demonstrate the development block in vitro. Muggleton-Harris et al. (1982) demonstrated that the developmental block observed in random bred mouse embryos can be overcome by injecting small amounts of cytoplasm from inbred strain or F1 hybrid embryos which do not exhibit the block in vitro. These results led the authors to believe that the development block exhibited by certain mouse embryos in vitro to be of cytoplasmic nature.

Initiation of embryonic genome transcription begins at the mid 2-cell stage (Johnson, 1981). Muggleton-Harris et al. (1982) also reported that injection of two-cell embryonic cytoplasm into two-cell embryos promoted further development in vitro than injection of one-cell embryonic cytoplasm into one-cell embryos. The authors suggested that cytoplasm from two-cell embryos may provide stored maternal RNA or enzymes which assist the blocked embryo through the genetic transcription transition.

Researchers have shown that the block stage experienced by bovine, murine and hamster embryos can be overcome in vitro by coculture with trophoblasts or oviductal cells, or transfer to oviducts maintained in organ culture (Camous et al., 1984; Eyestone and First et al., 1988; Whittingham, 1968; Minami et al., 1988). Additionally, the cleavage block is not observed in embryos transferred

to oviducts of homogeneous or hetergeneous females (Minami et al., 1988; Eyestone et al., 1987; Sirard et al., 1985).

Based on conclusions from studies conducted by Muggleton-Harris et al. (1982), Camous et al. (1984) conducted experiments to determine whether fragments of bovine embryos recovered beyond the block stage were able to transmit compounds via culture medium to early bovine embryos and promote cleavage through the block stage in vitro. One- to eight-cell embryos were cultured for 3 - 4 d in medium B2 supplemented with 15% FCS or medium B2 supplemented with FCS and one trophoblastic vesicle. More embryos reached the morula stage (46%) in coculture with trophoblastic vesicles than when cultured alone (18%) regardless of initial stage of development.

Heyman et al. (1987) evaluated the roles of the trophoblastic vesicles and serum during coculture. Significantly more embryos developed to the morula stage (42%, n = 55) when cultured in serum supplemented medium with trophoblastic vesicles than embryos in coculture with trophoblastic vesicles without serum (12.7%, n = 47). One- to two-cell embryos also were cultured in media that had supported trophoblastic vesicles. Thirty-eight percent of the 1- to 2-cell embryos developed to the 16-cell stage after culture in trophoblastic vesicle conditioned medium while 41.8% of the embryos in coculture reached the same stage of development. Media preconditioned by trophoblastic vesicles was separated into a low molecular weight (MW:180-2500) and a high molecular weight (MW: > 10,000) fraction. None of the 2-cell embryos cultured in B2 medium

supplemented with high molecular weight fraction developed beyond the 8-cell stage. B2 medium supplemented with low molecular weight fraction supported 23.8% (n = 21) of the embryos to the morula stage.

Coculture of bovine embryos with oviductal tissue is another method of in vitro culture which has been shown to overcome the 8-cell block. Eyestone and First (1988) cultured 1-cell embryos generated from in vitro fertilization in TCM-199, TCM-199 and oviductal cells or TCM-199 and oviductal cell conditioned medium. Coculture and oviductal cell conditioned medium supported 25% (17/69) and 23% (19/82) of the embryos past the 16-cell stage. None of the embryos cultured in TCM-199 alone developed past the 16- cell stage.

Whittingham (1968) transferred fertilized 1-cell mouse ova into various regions of explanted oviducts and uterine horns maintained on a chemically defined medium. Zygotes developed to the blastocyst stage within the ampullar region of the oviduct. All other areas of the reproductive tract failed to support embryo growth. No differences were found in the ability of the ampullae to maintain development of native or transferred ova. Whittingham and Biggers (1967) cultured 1-cell mouse embryos in vitro through the first cleavage and subsequently transferred the 2-cell embryos into organ cultures of the ampullary regions of oviducts obtained from mature females in metoestrus I. Thirty-six percent of the embryos reached the blastocyst stage after 72 h of culture in explanted ampullae. These results demonstrate that the oviductal environment is only required to support development between the first and second cleavage.

Tarkowski (1962) transferred embryos between rats and mice. Mouse 2- and 8-cell and rat 2-, 4- and 8-cell embryos developed into blastocysts when reciprocally transferred to the oviduct of pseudopregnant females of the other species. Re-transferred rat blastocysts, which were recovered from mouse oviducts on d 8, to recipient rats failed to continue development. Mouse blastocysts were not re-transferred to recipient mice. This experiment was repeated by Beyer and Zeilmaker (1973) using prepubertal, cyclic and pregnant recipients. Eighty-three and 44% of the mouse and rat embryos recovered after 96 h of culture in foreign oviducts reached the morula stage. Zygote development was independent of recipient hormonal status. Morulae (n = 43) were transferred to the uterus of pseudopregnant hosts, 63% of which developed into living young. Similar results were reported by Papaioannou and Ebert (1986) who compared the development of mouse embryos in pseudopregnant and immature recipients. Embryos recovered from immature females exhibited a similar rate of implantation in normal females (77%) to those recovered from pseudopregnant females (66%).

Minami et al. (1988) demonstrated that isolated mouse ampullae maintained in organ culture supported the development of hamster 2-cell embryos which had previously failed to cleave in vitro. Ampullae isolated at 14-16 and 30-32 h after injection of human chorionic gonadotropin supported the greatest amount of development . Eyestone and First (1986) transferred 1- and 2-cell bovine embryos to sheep oviducts either immediately following collection or after in vitro culture for five days with Ham's F-10 and 10% FCS. Only 4% (n = 48) of the embryos

transferred after culture developed past the 8-cell stage, compared to 42% (n = 45) of the embryos transferred immediately. The authors concluded that the "cleavage block" in bovine embryos is a function specifically of the 8-cell stage, and that the "block" is irreversible.

Storage and development of early bovine embryos in oviducts of rabbits has been extensively researched (Maurer, 1976; Boland, 1984). Lawson et al. (1972a) examined the development of ovine and bovine (Lawson et al., 1972b) embryos in the rabbit oviduct and their viability after re-transfer to ewes and heifers, respectively. Two-, four-, eight- and sixteen-cell sheep embryos were transferred to the ligated oviducts of pseudopregnant or estrous rabbits. Eighty-seven percent of the embryos were recovered, 93% of which continued development in the rabbit. The best rate of survival after re-transfer was obtained with 2- and 4-cell embryos which had been cultured in pseudopregnant rabbits for 3 days. However, endocrine status of the surrogate did not significantly affect embryo development. In the second study 1-, 2-, 4- and 8-cell bovine embryos were transferred to oviducts of untreated, adult does. Eighty-five percent of the embryos were recovered, 83% of which appeared normal. Re-transfer of fifteen embryos to synchronized heifers yielded eleven calves.

Cow embryos fertilized in vitro were transferred to ligated oviducts of pseudopregnant rabbits (Sirard et al., 1985). A significantly higher recovery rate (84%) was achieved when 2- to 8-cell embryos were transferred to the oviduct rather than 1-cell embryos (39%). However, there was no significant difference

in the developmental potential of 1-cell embryos compared to cleaved embryos. Seventy-seven percent of the embryos were recovered after less than 99 h of incubation in the rabbit oviduct while 50% of the embryos incubated for more than 99 h were recovered. Zygotes ranging from the 8-cell stage to blastocyst were transferred to the uterus of synchronized virgin heifers. Non-surgical transfer yielded a 44% pregnancy rate while a 40% pregnancy rate resulted from surgical transfer. No pregnancies were obtained from transfer to the oviduct of synchronized cows.

Eyestone et al. (1987) cultured 1- and 2-cell bovine embryos in ligated oviducts of synchronized, ovariectomized and anestrous ewes. Recovery rates and development were not affected by hormonal status of the ewe. The viability of 13 morulae and blastocysts was tested by transferring them to eight recipient heifers. Five heifers (63%) became pregnant and four (50%) produced calves.

Immature mouse oviducts also have hosted rabbit and porcine embryos. Development of 1-cell rabbit and porcine embryos after 2 days in the oviduct had progressed to the morula and 4-cell stage, respectively (Ebert and Papaioannou, 1989). An additional day of culture in the oviduct led to a significant increase in the proportion of degenerating rabbit embryos. Porcine embryos did experience a cleavage block at the 4-cell stage that longer culture periods could not overcome. Late porcine 4-cell embryos and morulae cultured in immature mouse oviducts for up to 3 d contained significantly more cells than embryos cultured in vitro for the same duration. Krisher et al. (1989) reported successful devel-

opment of 1-cell porcine embryos to the blastocyst stage after 6 days of culture in pseudopregnant and pregnant mouse oviducts maintained in organ culture.

Agar Embedding

Recent advances in the area of embryonic micromanipulation have led to the production of monozygotic twins (Ozil et al., 1982), chimaeras (Fehilly et al., 1984), transgenic mice (Petters et al., 1987), and cloned bovine embryos (Barnes et al., 1987). The microsurgical techniques involved with these manipulations often cause varying degrees of damage to the zona pellucida. The zona pellucida prevents dispersal of blastomeres, direct contact between the embryo and alien cells, and facilitates the passage of the embryo through the oviduct (Willadsen, 1982). Willadsen (1979) described a method of protecting embryos that have damaged zona pellucidae or are void of the critical membrane. The technique involved coating embryos with agar after microsurgery in order to seal the zona pellucida. Monozygotic pairs of agar-coated single blastomere eggs were transferred to ligated oviducts of sheep. Twenty pairs (65%) of monozygotic blastomeres were recovered 3.5 to 4.5 days after transfer. Blastomeres were removed from the agar and transferred to recipient ewes. Ten ewes (63%) went to term and produced five sets of twins and five single lambs.

Fehilly et al. (1984) demonstrated that dissociated blastomeres embedded in agar cylinders followed by culture in vivo was an effective procedure for the pro-

duction of chimaeric sheep blastocysts. Thirty-eight (74.5%) recipient ewes went to full term, producing a total of 53 lambs which corresponded to a 71% pregnancy rate.

Agar embedding also has been shown to be beneficial for the frozen storage of whole and bisected embryos (Tsunoda et al., 1987). Fifty percent of the bisected embryos embedded in agar were undamaged after thawing while only 5% of the demi-embryos frozen without agar were undamaged. Additionally, a greater proportion of whole embryos frozen with agar (58%) were classified as morphologically undamaged after thawing than embryos frozen without agar (26%). These results suggested that agar embedding was beneficial to successful freezing of both bisected and whole embryos.

Eyestone et al. (1987) transferred 1- and 2-cell bovine embryos embedded in agar chips to ligated oviducts in the ewe. Most agar chips (81%, n = 43) were either fragmented or could not be recovered 5 days after transfer. Seventy-seven percent of the embryos recovered were free of agar. Break down of agar chips also was observed after 2-4 days in the rabbit oviduct (Boland, 1984). These data are in direct contrast to Willadsen (1982), who reported a 100% recovery rate of agar cylinders after 3.5-4.5 days in the ovine oviduct.

Hemodialysates

Solcoseryl (Solco-Basle Ltd., Birsfelden-Basel Switzerland) and CLB1107 (Candace Lane Corp., Parsippany NJ) are deproteinized hemodialysates from calf blood. Solcoseryl has been shown to accelerate embryo development in vitro and enhance embryo survival after micromanipulation and freezing. It has been proposed that hemodialysates increase glucose transport rate which leads to a high ATP/ADP ratio, thus enhances cell proliferation (W. Jochle, personal communication; Bradley and Culp, 1974). Hahn (1984) demonstrated that addition of 0.1% Solcoseryl (v/v) to Whitten's medium and 20% FCS (v/v) significantly increased the proportion of 2- to 4-cell mouse embryos developing to the blastocyst stage after 44 h of culture (86.2% embryos in supplemented medium; 66.2% in unsupplemented medium). Additionally, Hahn (1984) reported that significantly more split 16-cell embryos reached the blastocyst stage after 24 h (78.1%) than demi-embryos cultured in unsupplemented medium (44.1%). Transfer of blastocysts from both experiments revealed that Solcoseryl was associated with an increased proportion of implantations and absence of resorptions. Reed et al. (1987) cultured nonmanipulated and gene-microinjected one-cell mouse embryos in Whitten's medium with 0.1mM EDTA with or without 0.1% Solcoseryl. A greater proportion of both nonmanipulated and gene-microinjected embryos developed to the blastocyst stage after 120 h of culture in Solcoseryl supplemented medium (36.6%, 40.0%) than in unsupplemented medium (26.3%, 23.6%). In contrast, Reed et al. (1989) found that one-cell mouse embryo devel-

opment did not differ after culture in 0.1% Solcoseryl (v/v), 0.1% CLB 1107 (v/v) or unsupplemented, modified BMOC3 medium.

Viker et al. (1984) examined the effect of .01%, .05% and 0.1% Solcoseryl (v/v) on the viability of d 7 fresh or frozen bovine embryos in vitro. Embryos were cultured in Ham's F-10 supplemented with 10% FCS (v/v) or various concentrations of Solcoseryl. Forty-three percent of the embryos cultured in Solcoseryl were classified as viable after 72 h of culture, whereas, 0% of the embryos were viable after 72 h of culture in Ham's F-10 and 10% FCS (v/v). Addtionally, 18%, 8%, 33% and 50% of embryos which had previously been frozen were classified as viable after 72 h of culture in 10% FCS, .01%, .05% and 0.1% Solcoseryl (v/v). These results led the authors to suggest that .05% and 0.1% Solcoseryl (v/v) tended to enhance viability of d 7 frozen bovine blastocysts in Ham's F-10.

In Vitro Culture of Mouse Embryos in Medium Supplemented With a Deproteinized Hemodialysate

Abstract

Mouse embryos (n = 501) were cultured for up to 96 h in M2 medium or M2 supplemented with a deproteinized hemodialysate (1%) from calf blood (CLB1107) to assess in vitro development. One- and two-cell embryos did not develop beyond the 2-cell stage in vitro. Degeneration of one-cell embryos occurred within 36 h in culture. However, two-cell embryos degenerated sooner when cultured in M2 plus 1% CLB1107 (27.4 h) than in M2 alone (41.7 h). Mean final development classification of embryos cultured from the morula stage in M2 supplemented with CLB1107 was higher (9.0) than that for morulae (8.2) cultured in unsupplemented M2 medium. Development of embryos cultured from the blastocyst or expanded blastocyst stages was not affected by treatment. Media supplemented with 1% CLB1107 does not support early mouse embryo development beyond the 2-cell stage, however, it appears to enhance development of morulae in vitro.

Introduction

Mouse embryos are routinely cultured from the 2-cell to blastocyst stage in simple, chemically defined media (Gwatkin, 1966; Whitten and Biggers, 1968; Whittingham, 1971). Embryo development in vitro, however, has yet to be in synchrony with development in vivo. Development in vitro from the 1-cell to blastocyst stage is restricted to certain inbred strains and F1 crosses (Goddard and Pratt, 1983). Embryos from some inbred and most random bred strains fail to cleave beyond the 2-cell stage in vitro. This phenomenon is referred to as the "2-cell block" and is believed to be of cytoplasmic nature (Muggelton-Harris et al., 1982). Bowman and McLaren (1970) demonstrated that development of mouse embryos in vitro was delayed compared to that in vivo after the first 24 h of culture. Solcoseryl (Solco Basle, Ltd., Birsfelden-Basel Switzerland) and CLB1107 (Candace Lane Corp., Parsippany NJ) are identical deproteinized hemodialysates from calf blood (W. Jochle, personal communication). Solcoseryl has been used as a culture media supplement for enhanced growth of nonmanipulated and micromanipulated mouse embryos F1 cross mouse embryos (Hahn, 1984; Reed et al., 1987). Reed et al. (1989) reported that mouse embryo development did not differ when embryos were cultured in Solcoseryl or CLB1107 supplemented media. The objective of this study was to determine the effect of CLB1107 supplemented medium on random bred mouse embryo development from 1-cell through expanded blastocyst stages in vitro.

Materials and Methods

Immature female random bred ICR albino mice were superovulated with 5 IU pregnant mare serum gonadotropin (Ayerst Laboratories Inc., New York, NY), followed 46 h later by 5 IU human chorionic gonadotropin (hCG; Sigma, St. Louis, MO) and mated with mature ICR albino males. Embryos were collected approximately 20, 44 and 92 h after hCG injection to obtain 1-cell to expanded blastocyst embryos. Fertilization of ova recovered at 20 h was confirmed by the presence of both pronuclei under Hoffman modulation contrast optics. A total of 296 1-cell, 141 2-cell, 32 morulae, 14 blastocysts and 18 expanded blastocysts were randomly assigned to one of two treatments. Treatments consisted of M2 medium (Appendix A; Hogan et al., 1986) and M2 supplemented with 1% CLB1107. Zygotes were cultured individually in 100 ul medium under paraffin oil at 39 C in an atmosphere of 90%N_2, 5% O_2, 5% CO_2 for up to 96 h. Embryonic development was classified every 12 h as: 1: degenerate, 2: 1-cell, 3: 2-cell, 4: 3- to 4-cell, 5: 5- to 8-cell, 6: morula, 7: early blastocyst, 8: blastocyst, 9: expanded blastocyst, 10: hatching blastocyst, 11: hatched blastocyst. Treatment differences for development scores, time to developmental stages and time to degeneration were evaluated by analysis of variance. Independent variables were treatment, mouse, time (hours of incubation), initial stage of development and selected interactions. Initial stage of development was used as a covariate.

Results and Discussion

Time to develop to the 2-cell stage, time to degeneration and final development classification of 1-cell embryos were significantly affected by mouse ($P < 0.01$), but were not affected by treatment (Table 1; $P > 0.05$). Less than 4% ($n = 9$) of all 1-cell embryos developed to the 4-cell stage in M2 and M2 plus CLB1107.

Final development score of 2-cell embryos (3.1 ± .2) was significantly affected by mouse ($P < 0.01$), but was not affected by treatment ($P > 0.05$; Table 2). Of the 141 2-cell embryos cultured, 5.6% (8) went to the 4-cell stage after 60 h in culture. Initiation of embryonic genome transcription begins at the mid 2-cell stage (39 to 42 h post-hCG; Johnson, 1981). The 2-cell embryos collected at 44 h post-hCG may not have completed the transition from maternal to embryonic mRNA. Goddard and Pratt (1983) collected 2-cell CFLP and F1 cross embryos 42 to 46 h post-hCG and reported that greater than 90% of the CFLP embryos failed to cleave beyond the 2-cell stage whereas a majority of the F1 embryos developed to the blastocyst stage. Additionally, they reported that after 48 h in culture, the "blocked 2-cells" were still structurally and macroscopically intact. From these observations, they suggested that the embryos were in a state of developmental arrest rather than degeneration during culture.

In the current study, time to degeneration for 2-cell embryos was significantly shorter ($P < 0.01$) in M2 plus 1% CLB1107 (27.4 ± 2.48 h: Table 2) than in M2 alone (41.7 ± 3.06 h). Previous work has shown successful culture of 2-cell

Table 1. Time to develop to the 2-cell stage, time to degeneration and final classification of development of 1-cell mouse embryos cultured in M2 or M2 plus 1% CLB1107.

	Treatment	
	M2	M2 + 1% CLB1107
2-cell Stage (h)	25.3 ± 0.5[1]	26.2 ± 0.5*
Degeneration (h)	34.5 ± 1.7	36.0 ± 1.9*
Final Classification[2]	2.5 ± 0.1	2.5 ± 0.1*

[1] $\bar{X}$ ± S.E.

[2] Development scored as: 1 = degenerate, 2 = 1-cell, 3 = 2-cell

* $P > .05$

Table 2. Time to degeneration and final classification of development of 2-cell mouse embryos cultured in M2 or M2 plus 1% CLB1107.

	Treatment	
	M2	M2 + 1% CLB1107
Degeneration (h)	41.7 ± 3.1	27.4 ± 2.5**
Final Classification	3.1 ± .03[2]	3.0 ± .03**

[1] $\bar{X}$ ± S.E.

[2] Development scored as: 1 = degenerate, 2 = 1-cell, 3 = 2-cell, 4 = 3- to 4-cell

** $P > .01$

embryos (Hahn, 1984) as well as 1-cell embryos (Reed et al., 1989) from strains which do not display the 2-cell block in vitro. Hahn et al. (1984) cultured 2- to 4-cell CB6/F1 mouse embryos in Whitten's medium and 20% fetal calf serum (FCS) with and without 0.1% Solcoseryl. A greater percentage (86.2%) of embryos cultured in Solcoseryl reached the blastocyst stage than those cultured in unsupplemented media (66.2%). Reed et al. (1989), however, cultured 1-cell B6C3/F1 x B6D2/F1 mouse embryos in unsupplemented modified BMOC3 medium or supplemented BMOC3 with 0.1% Solcoseryl or 0.1% CLB1107 in embryo/volume ratios of 15, 5 and 1 zygote/ul of medium. The percentage of embryos that developed to the blastocyst stage was not different among culture media or embryo/volume ratios.

Stage of development of embryos collected at 92 h post-hCG was significantly affected ($P < 0.01$) by treatment, mouse, mouse by treatment interaction, initial stage of development, embryo nested within mouse by treatment interaction, time and treatment by time interaction (Table 3). Embryos cultured in 1% CLB1107 had a significantly higher mean stage of development ($9.8 \pm .1$) than those cultured in M2 alone ($9.1 \pm .1$). Embryos of the morula stage or greater development at 92 h post-hCG developed in a stepwise manner to 84 h in vitro (Table 4). Individual analyses of variance by initial stage of development at the time of culture showed that the mean development score of morulae cultured in 1% CLB1107 supplemented M2 media was significantly higher ($P < 0.05$) than for morulae cultured in M2 alone (Table 5).

Table 3. Analysis of variance for stage of development of embryos collected 92 h post-hCG and cultured in M2 or M2 plus 1% CLB1107.

Source	df	MS
Treatment (TRT)[1]	1	33.63**
Mouse[2]	4	11.53**
Mouse x TRT[3]	4	.69
Initial Stage of Development[4] (I)	1	25.33**
Embryo (Mouse x TRT)	53	2.02**
Time	7	16.08**
TRT x Time	7	1.92**
Residual	203	0.32

[1]TRT tested by Mouse x TRT
[2]Mouse tested by Embryo (Mouse x TRT)
[3]Mouse x TRT tested by Embryo (Mouse x TRT)
[4]I was used as a covariate and was tested by Embryo (Mouse x TRT)

**$P < 0.01$

Table 4. Developmental classification of embryos collected 92 h post-hCG and cultured for up to 96 h.

Time in Culture (h)	Classification $\overline{X} \pm$ S.E.	(N)
12	8.0 ± 2[1]	(64)
24	8.7 ± .1	(59)
36	9.4 ± .2	(54)
48	9.4 ± .1	(38)
60	9.4 ± .1	(30)
72	10.2 ± .2	(15)
84	10.2 ± .2	(15)
96	9.3 ± .2	(6)

[1]Development scored as: 8 = blastocyst, 9 = expanded blastocyst, 10 = hatched blastocyst, 11 = hatching blastocyst

Table 5. Mean development classification[1] of mouse embryos collected 92 h post-hCG after 96 h in culture in M2 medium or M2 plus 1% CLB1107.

Initial Stage of Development	Treatment: M2 $\bar{X} \pm$ S.E.	n	Treatment: M2 + CLB1107 $\bar{X} \pm$ S.E.	n
Morula	8.2 ± .1[2]	18	9.0 ± .2*	14
Blastocyst	9.1 ± .1	5	9.5 ± .2	9
Expanded Blastocyst	9.5 ± .1	8	9.7 ± .1	10

[1]Mean development score is the mean of development scores assigned after 12 h of culture and at 12 h intervals until degeneration, hatching of the blastocyst or 96 h of culture.

[2]Development scored as: 8 = blastocyst, 9 = expanded blastocyst, 10 = hatching blastocyst

*$P < 0.02$

Mean development classification of blastocysts and expanded blastocysts was not different ($P > 0.05$) between treatments (Table 5). Time to degeneration was not different ($P > 0.05$) between treatments and was 55 h for morulae, 60 h for blastocysts and 68 h for expanded blastocysts (Table 6). These results suggest that hemodialysate supplementation may only enhance development of embryos initiating culture prior to the blastocyst stage. Viker et al. (1984) cultured frozen bovine morulae, blastocysts and expanded blastocysts in Ham's F-10 supplemented with 10% FCS and 0.01%, 0.05%, 0.1% or 0.5% Solcoseryl. After 72 h in culture, embryo viability was significantly greater in 0.5% Solcoseryl than in all other treatments. The authors, however, did not report any differences in development nor did they examine the effect of Solcoseryl supplementation on development of embryos initiating culture at the various stages of development.

In conclusion, the results from culture of 1- and 2-cell random bred mouse embryos indicate that 1% CLB1107 may retard development from the 1-cell to the 2-cell stage and neither treatment supported significant development beyond the 2-cell block. Development of blastocysts and expanded blastocysts was not affected by 1% CLB1107. However, mean development score of morulae cultured in 1% CLB1107 was significantly greater than that of morulae cultured in unsupplemented media.

Table 6. Time to degeneration for embryos collected 92 h post-hCG and cultured for 96 h in M2 or M2 plus 1% CLB1107.

Initial Stage of Development	Treatment $\bar{X} \pm$ S.E.	(N)
Morula	55.2 ± 1.6[1]	(20)
Blastocyst	60.0 ± 2.7	(8)
Expanded Blastocyst	68.0 ± 1.2	(3)

[1]Means calculated from embryos which degenerated prior to the hatched blastocyst stage.

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Culture of Bovine Embryos in Deproteinized Hemodialysate Supplemented Media and Immature Mouse Uteri

Abstract

Bovine morulae (d 6; n = 172) were used to evaluate the effects of a deproteinized hemodialysate (CLB1107), agar embedding and the environment of immature mouse uteri on embryo development. Agar embedded embryos were cultured in Ham's F-10 and 10% steer serum either 1) immediately after collection or 2) 24 h after storage in immature mouse uteri. Non-embedded embryos were cultured in Ham's F-10 containing 3) 10% steer serum, 4) 1% CLB1107 or 5) 1% CLB1107 and 10% steer serum. A greater proportion embryos reached the hatched blastocyst stage after culture in treatments 1, 3, 4, and 5 (38.1%, 34.6%, 28.6%, and 21.1%) than embryos stored in immature mouse uteri for 24 h prior to in vitro culture (9.5%). Mean final development scores for non-embedded and agar embedded embryos cultured in Ham's F-10 and 10% steer serum were not different (5.5 ± .3 and 4.9 ± .4), and were higher than embryos cultured in CLB1107 (4.2 ± .4), CLB1107 and steer serum (4.2 ± .4) or immature mouse uteri (3.4 ± .4). These results suggest deproteinized hemodialysate supplementation at the concentration used in this study did not enhance embryo development in vitro. Moreover, bovine morulae were not affected by agar embedding and were able to develop following short term storage in the immature mouse uterus.

Introduction

Research involving early bovine embryos has been restricted due to lack of adequate culture systems. Embryos cultured from the 1-cell stage in chemically defined media fail to cleave beyond the 8- to 16-cell stage (Thibault, 1966; Wright and Bondioli, 1981). In contrast, bovine morulae cultured in similar conditions develop through the hatched blastocyst stage. Successful culture of bovine embryos past the 8- to 16-cell stage has been achieved in culture systems which provide biologically active environments either in vitro (Camous et al., 1984; Eyestone and First, 1988; Pool et al., 1988) or in vivo (Eyestone et al., 1987; Sirard et al, 1985; Ebert and Papaioannou, 1989). Ovine (Eyestone et al., 1987) and rabbit oviducts (Sirard et al., 1985) have been shown to support development of 1- and 2-cell bovine embryos through the blastocyst stage. Recently, Ebert and Papaioannou (1989) demonstrated that the immature mouse oviduct was capable of supporting development of late 4-cell porcine embryos and rabbit 1-cell embryos.

Recent advances in the area of embryonic micromanipulation have led to the production of chimaeras (Fehilly et al., 1984) clones (Barnes et al., 1987) and monozygotic twins (Tsunoda et al., 1987). Microsurgical techniques involved with micromanipulation often cause varying degrees of trauma and damage to the embryo (Willadsen, 1982). Research has shown that agar embedding can be used to seal or repair the zona pellucida of micromanipulated embryos (Fehilly

et al., 1984; Tsunoda et al., 1987; Willadsen, 1982) and culture nonmanipulated embryos in rabbit and ovine oviducts (Westhusin et al., 1989; Willadsen, 1979). Several embryos can be embedded in a single agar cylinder which can facilitate the handling of a large number of embryos, embryo recovery, and storage of embryos from different treatments within one oviduct.

Solcoseryl (Solco Basle Ltd., Birsfelden-Basel, Switzerland) and CLB1107 (Candace Lane Corp., Parsippany, NJ) are identical deproteinized hemodialysates from calf blood. Solcoseryl has been reported to enhance growth of nonmanipulated and micromanipulated mouse embryos (Hahn, 1984; Reed et al., 1987). Viker et al. (1984) reported enhanced viability of frozen bovine blastocysts when cultured in Solcoseryl supplemented media for up to 72 h. Reed et al. (1989) showed that mouse embryo development did not differ when embryos were cultured in Solcoseryl or CLB1107 supplemented media. The objectives of this study were to determine if the immature mouse uterus can support development of bovine morulae; to evaluate the effect of agar embedding on bovine morulae development; and to examine the effect of deproteinized hemodialysate supplemented media on bovine morulae development in vitro.

Materials and Methods

Embryo Collection

A total of 42 superovulations were conducted on 25 non-lactating Holstein cows (11 used 1X; 12 used 2X; 1 used 3X; 1 used 4X). Cows were superovulated with 32 mg of follicle stimulating hormone (FSH; Burns-Biotech, Omaha, NB) using a decreasing dose regime over 4 consecutive days, initiated between d 9 to d 13 of the estrous cycle. Prostaglandin F2 alpha (Lutalyse; Upjohn, Kalamazoo, MI) was administered i.m. (12.5 mg) at 60 and 72 h after the initial FSH injection to induce luteal regression. Cows were inseminated at 0, 12 and 24 h after the onset of estrus. Embryos were recovered nonsurgically with 500 ml of Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, NY) on d 6 after the first insemination. Embryos were scored as excellent, good, fair and poor according to morphological criteria described by Renard and Heyman (1979) and all embryos were randomly assigned to culture treatments (Appendix B).

Culture Treatments

Agar embedded embryos were cultured in Ham's F-10, 1.3% antibiotic-antimycotic solution (v/v; penicillin 10,000 U/ml, Fungizone 25 ug/ml, streptomycin 10,000 ug/ml; Gibco) and 10% steer serum (SS; v/v), either immediately after collection or following 24 h of culture in immature mouse uteri.

Embryos assigned to the in vivo culture treatment were transferred to uterine horns of immature mice (21 to 28 d) under pentobarbital anesthesia. One to three embryos were transferred to each uterine horn with a glass micropipette as described by Hogan et al. (1986). Embryos were flushed from the uterus with approximately 1 ml Ham's F-10 24 h after transfer and subsequently placed in Ham's F-10 and 10% SS (v/v) for further in vitro culture.

Non-embedded embryo culture treatments consisted of Ham's F-10 supplemented with an antibiotic-antimycotic solution and 10% SS (v/v); 1% CLB1107 (CLB; v/v); and 1% CLB and 10% SS (v/v). This concentration was selected based on beneficial effects of increased concentrations Solcoseryl on bovine embryo development in vitro (Viker et al., 1984). Serum was heat inactivated at 56 C for 30 min.

Agar Embedding

Embryos assigned to the agar embedding treatments were embedded in cylinders containing 1.5% agar (v/v; Sigma, St. Louis, MO) and 1.3% antibiotic-antimycotic solution (v/v) in saline (NaCl 9 g/l). Each cylinder contained one to two embryos and one colored density marker bead (Pharmacia, LKB, Uppsala, Sweden) which facilitated visualization of agar cylinders during transfer and recovery. Each cylinder measured approximately .12 x .3 mm. Embryos were re-

moved from the agar cylinders 24 h after the initiation of culture to allow expansion and hatching of the blastocyst.

Culture Procedures

Culture procedures were similar to those described by Wright et al. (1976). Following morphological evaluation, embryos were held in a petri dish containing Ham's F-10 and then placed in culture. For treatments with CLB, CLB and SS or control with SS, 1 ul of CLB and 10 ul of SS and a sufficient amount of Ham's F-10 to make a total volume of 90 ul were added to the culture dish (LUX 5250, 150 ul; Miles Laboratories, Inc., Naperville, IL). Next, 10 ul of Ham's F-10 containing a randomly selected embryo was added to the culture well. A drop of paraffin oil was placed above each culture well to buffer gas exchange and minimize evaporation. Embryos were cultured in an incubator at 37 C with an atmosphere of 5 O_2, 5% CO_2 and 90% N_2, bubbled through distilled water. Embryos were examined microscopically at 60X every 12 h for up to 96 h and development was recorded.

Statistical Analysis

Treatment differences were examined using the evaluation scheme of Wright et al. (1976). Each embryo was assigned a numerical value of 1 to 7, representing the embryo's most advanced stage of development. A score of 1 corresponded to

a degenerate embryo, 2 = morula, 3 = early blastocyst, 4 = blastocyst, 5 = expanded blastocyst, 6 = hatching blastocyst, and a score of 7 represented development to the hatched blastocyst stage. Final development scores, developmental times, and time to degeneration for each treatment were calculated and data were analyzed by analysis of variance. The independent variables in the models were treatment, initial embryo quality and cowflush, which represented an individual cow and her specific flush effect.

Results

Analysis of variance for final development score revealed significant treatment ($P < .01$) and cowflush ($P < .05$) effects. Least squares means for final development score for embryos cultured in each treatment are shown in Table 7. Final development scores for agar embedded and non-embedded embryos cultured in SS were not different ($P > .05$), however, final development scores of embryos cultured in mouse uteri or CLB supplemented media were significantly ($P < .05$) lower than embryos cultured in SS alone.

Nine (34.6%) of the embryos cultured in SS developed to the hatched blastocyst stage, whereas 6 (28.6%), 4 (21.1%) and 8 (38.1%) embryos cultured in CLB, CLB + SS, and embryos embedded in agar and cultured in SS, reached the same stage of development, respectively (Table 8). Twenty-one (27%) of the 78 embryos transferred were recovered from mouse uteri following 24 h of culture.

Table 7. Least squares means for final development score for bovine embryos.

Treatment[1]	n	Final Score	S.E.
SS	26	5.5^{a}	.3
CLB	21	$4.2^{b,c}$	.4
CLB + SS	19	$4.2^{b,c}$	.4
A + SS	21	$4.9^{a,c}$	.4
Mouse	21	3.4^{b}	.4

[1]Non-embedded embryos were cultured in Ham's F-10 medium containing: 10% steer serum (SS), 1% CLB1107 (CLB), 1% CLB1107 and 10% steer serum (CLB + SS); agar embedded embryos were cultured in Ham's F-10 and 10% steer serum (A + SS) or immature mouse uteri (Mouse).

^{a,b,c}means with different superscripts differ at $P < .02$

Table 8. Stage of embryo development after culture in vivo and/or in vitro.

Stage of Development	Treatment[1] SS (n)	SS (%)	CLB (n)	CLB (%)	CLB+SS (n)	CLB+SS (%)	A+SS (n)	A+SS (%)	Mouse (n)	Mouse (%)
Morula	26	100	21	100	19	100	21	100	21	100
Early Blastocyst	23	88.5[a]	18	85.7[a]	16	84.2[a]	19	90.5[a]	10	47.2[b]
Blastocyst	23	88.5[a]	16	76.2[a]	16	84.2[a]	18	85.7[a]	9	42.9[b]
Expanded Blastocyst	23	88.5[a]	14	66.7[a]	16	84.2[a]	17	81.0[a]	8	38.1[b]
Hatching Blastocyst	10	38.5	7	33.3	7	36.8	8	38.1	4	19.0
Hatched Blastocyst	9	34.6[a,b]	6	28.6[a,b]	4	21.1[a,b]	8	38.1[a]	2	9.5[b]

[1]Non-embedded embryos cultured in Ham's F-10 medium containing: 10% steer serum (SS), 1% CLB1107 (CLB), or 1% CLB1107 and 10% steer serum (CLB+SS); agar embedded embryos were cultured in Ham's F-10 and 10% steer serum (A+SS) or immature mouse uteri (Mouse).

[a,b] numbers in the same row with different superscripts differ $P < .05$

The percentage of degenerated embryos after 24 h in the mouse uterus 52.3% (11/21) was significantly higher ($P < .01$) than those cultured for 24 h in vitro in SS 14.3% (3/21). Four (19.1%) of the embryos recovered from the mouse uteri developed during culture within the uterus and 9.5% (2) of the recovered embryos developed to the hatched blastocyst stage after subsequent culture in Ham's F-10 and 10% SS .

Least squares means for developmental times to each stage of development were determined (Table 9). Development times to the early blastocyst and hatching blastocyst stages were significantly shorter ($P < .05$) for embryos cultured in 1% CLB supplemented medium than for those cultured in 1% CLB and 10% SS (Table 9). Time to the hatching blastocyst stage was significantly ($P < .05$) longer for embryos cultured in CLB and SS (71.1 h) than embryos cultured in CLB alone (38.6 h). Embryos recovered from the mouse uterus required significantly ($P < .05$) more time in culture than embryos cultured only in vitro to reach the early blastocyst, blastocyst and expanded blastocyst stages, however, time to reach the hatching and hatched blastocyst stages was not different ($P > .05$) from that required by embryos cultured in vitro for the entire culture period. Mean times to degeneration were 57.4 ± 6.4, 56.2 ± 6.2 , 57.7 ± 7.1, 48.3 ± 8.3, and 49.6 ± 7.2 h for embryos cultured in SS, CLB, SS and CLB, agar and SS, and in the mouse for 24 h, respectively, and were not different among treatments ($P > .05$).

Table 9. Least squares means for time (h) to each stage of development for embryos cultured in vivo and/or vitro

Stage of Development	Treatment[1] SS $\bar{X} \pm S.E.$	CLB $\bar{X} \pm S.E.$	CLB+SS $\bar{X} \pm S.E.$	A+SS $\bar{X} \pm S.E.$	Mouse $\bar{X} \pm S.E.$
Early Blastocyst	$20.1 \pm 2.2^{a,c}$	18.4 ± 1.9^{a}	$25.5 \pm 3.2^{b,c}$	14.2 ± 2.8^{a}	33.9 ± 5.1^{b}
Blastocyst	26.8 ± 3.6^{a}	$29.4 \pm 3.9^{a,b}$	$33.4 \pm 4.4^{a,b}$	27.1 ± 4.2^{a}	40.1 ± 5.6^{b}
Expanded Blastocyst	32.6 ± 2.4^{a}	39.8 ± 3.6^{a}	38.6 ± 3.4^{a}	38.1 ± 3.4^{a}	56.2 ± 5.0^{b}
Hatching Blastocyst	$47.6 \pm 13.2^{a,b}$	38.6 ± 12.5^{a}	71.1 ± 12.5^{b}	$46.5 \pm 15.2^{a,b}$	$38.1 \pm 17.1^{a,b}$
Hatched Blastocyst	51.3 ± 9.4	63.9 ± 11.2	82.5 ± 12.4	62.8 ± 9.8	69.9 ± 18.8

[1]Non-embedded embryos were cultured in Ham's F-10 containing: 10% steer serum (SS), 1% CLB1107 (CLB), or 1% CLB1107 and 10% steer serum (CLB+SS); agar embedded embryos were cultured in Ham's F-10 and 10% steer serum (A+SS) or immature mouse uteri (Mouse).

^{a,b}means with different superscripts in the same row differ at $P < .05$

Discussion

Greater than 65% of all embryos cultured in in vitro treatments reached the expanded blastocyst stage while less than 40% of all embryos developed to the hatched blastocyst stage. Previous experiments which cultured bovine embryos in Ham's F-10 and 10% steer serum reported that 49% to 92% of all bovine embryos reached the hatching blastocyst stage (Rajamahendran et al., 1986; Canseco et al., 1988; Toole et al., 1988). The lower percentage of embryos reaching the hatched blastocyst stage in the current study may be due to the use of all qualities of embryos while only excellent and good quality embryos were used in previous studies.

Our study revealed that bovine embryos were able to develop following short term storage in the immature mouse uterus. After 24 h of culture in the immature mouse uterus, fewer embryos had developed beyond the morula stage than embryos cultured in Ham's F-10 and 10% SS. However, the percentage of embryos recovered from the mouse uterus that reached the hatched blastocyst stage was only different from that of embryos embedded in agar and cultured in vitro and was not different from that of embryos cultured in the other in vitro treatments. Although only 27% of all embryos transferred to the mouse uterus were recovered, technique improvement led to greater recovery as the study progressed. To our knowledge, this is the first report in which bovine morulae were cultured in immature mouse uteri.

In the present study few embryos developed during culture within the mouse uterus. However, once removed from the uterus and placed in an in vitro culture system, the embryos retarded at the morula stage resumed cleavage and developed to the hatching and hatched blastocyst stages at the same rate as those cultured in vitro. Ebert and Papaioannou (1989) recently reported successful short-term storage of rabbit and porcine embryos in immature mouse oviducts. One-cell rabbit embryos reached the morula stage after 2 d in the immature mouse oviduct. A further day of culture in the oviduct led to a significant increase in the proportion of degenerating embryos. One-cell porcine embryos developed to the 4-cell stage after 2 d in culture, but failed to develop beyond the 4-cell stage after an additional day of culture. Late 4- to 6-cell porcine embryos as well as morulae cultured in immature mouse oviducts for up to 3 d contained significantly more cells than embryos cultured in vitro for the same duration. Krisher et al. (1989) reported successful development of 1-cell porcine embryos to the blastocyst stage after culture in pseudopregnant and pregnant mouse oviducts maintained in organ culture for 6 d.

Agar embedding did not appear to adversely affect embryo development. Addition of the colored density bead to the cylinder allowed us to visualize the cylinder within the uterine horn. We found that the agar cylinder did not leave the uterine horn, but rather, adhered to the lumen of the uterine horn and that flushing the horn with media did not provide enough pressure to dislodge the cylinder from the uterine wall. Embryos embedded in agar and cultured in SS in vitro achieved

a mean final development score not different from non-embedded embryos cultured in SS. Additionally, agar embedded embryos developed at approximately the same rate as non-embedded embryos. These results are consistent with previous studies that reported normal embryonic development following agar embedding (Tsunoda et al., 1987; Westhusin et al., 1989; Willadsen, 1982).

Final development of embryos cultured in CLB supplemented medium was not as advanced as embryos cultured in SS supplemented medium. Previous research has shown that Solcoseryl significantly enhanced the effects of fetal calf serum (FCS) in mouse embryo culture and tissue culture (Hahn, 1984; Miltenburger, 1985). Viker et al. (1984) suggested that Solcoseryl tended to enhance viability of d 7 frozen bovine blastocysts when cultured for up to 72 h. They also cultured fresh d 7 bovine embryos in Ham's F-10 supplemented with 10% FCS, .01%, .05% or .10% Solcoseryl (v/v) and reported that 42.7% of the embryos cultured in Solcoseryl and 0% of those cultured in FCS were viable after 72 h of culture. These results are in contrast with our findings which suggest that CLB supplementation does not enhance the effects of steer serum supplementation in bovine embryo culture medium. However, Viker et al. (1984) did not mention what effects Solcoseryl had on embryo development nor did they describe their method of determining viability. We observed embryo development and made no attempt to determine embryo viability.

Our results demonstrate that CLB1107 supplementation alone did not promote bovine embryo development in vitro. CLB1107 did not enhance the effects of

steer serum in embryo culture nor did it support embryonic development as well as steer serum supplemented medium. Additionally, bovine embryos were able to develop in vitro following short-term storage in the immature mouse uterus. However, the immature mouse uterus may not provide an optimum environment for bovine embryo development. While the immature mouse uterus did not support later stage bovine embryo development per se, the effect of the immature mouse oviduct on bovine embryo development has yet to be determined.

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General Summary

These studies demonstrated that addition of a deproteinized hemodialysate (CLB1107) to culture media generally did not enhance bovine or murine embryo development.

Addition of CLB1107 to M2 culture medium did not support devleopment of 1- and 2-cell random bred mouse embyros beyond the 2-cell stage in vitro. The ability to overcome the 2-cell block by injection of cytoplasm from non-blocking strain embryos (Muggleton-Harris et al., 1982) indicates that a cytoplasmic adaptation may be necessary for "2-cell blocked" embryos to develop in simple medium in vitro. Whether the cytoplasmic alteration is incorporation of enzymes and/or cofactors or replication of organelles for increased production of the factor(s) required for development in vitro has yet to be determined. Bovine embryos cease to develop beyond the 8-cell stage in vitro (Thibault, 1966). The bovine embryo block can be overcome in vitro to a limited extent (up to 40% of all embryos) by coculture with oviductal cells (Eyestone and First, 1988), trophoblastic vesicles (Camous et al., 1984) or culture in medium preconditioned by either cell type. Previous reports (Allen and Wright, 1984; Kuzan and Wright, 1982) had suggested that the coculture effect was dependant upon cell-embryo contact. The ability of preconditioned medium to overcome the block confirms that cell to embryo contact is not necessary for development of outbred embyros.

The inability of random bred mouse embryos to overcome the "2-cell block" in medium containing 1% deproteinized hemodialysate suggests that the supplement did not contain the embryotrophic factor(s) required for early embryo development or a higher concentration of the supplement is required.

Development of murine embyros cultured from the morula stage was significantly enhanced by CLB1107 supplementation. However, the deproteinized hemodialysate did not significantly affect blastocyst and expanded blastocyst development in vitro. In the rabbit the transition from late morula to expanded blastocyst involves an increase Na + uptake and an increase in Na +/K + pumps. Once the blastocyst is formed ATP consumption is shifted to other metabolic demands (Benos and Biggers, 1981). The stage specific effect of the deproteinized hemodialysate may be due to the high energy demand during this transition. Hemodialysates are believed to increase glucose transport rate (W. Jochle, personal communication). The resulting elevated cellular glucose content would lead to a high ATP/ADP ratio, thus facilitate the ATP dependent Na +/K + pump activity. This may lead to hastened blastocoele fluid accumulation therefore, reducing the time required to reach the hatched blastocyst stage. Embryos cultured at the blastocyst and expanded blastocyst stages are obviously past this energy demanding transition and continued development may not be enhanced by elevated glucose transport.

Bovine morula development was best supported by Ham's F-10 and steer serum in vitro. Supplementation of culture media with 1% CLB1107 did not affect

embryo development and it appeared to hinder the effects of steer serum in culture. The ineffectiveness of the hemodialysate may be due to the low concentration (1% v/v). A higher concentration may lead to a proportional increase in glucose transport rate, thus elevate cellular glucose to levels which would allow significant enhancement of embryo metabolism and development.

Agar embedding did not appear to impair bovine embryo development in vitro. Embryos embedded in agar and cultured in Ham's F-10 and steer serum developed at approximately the same rate as non-embedded embyros cultured in the same medium. The agar appeared to allow optimal transport of nutrients and macromolecules between the embryo and culture medium.

Bovine morulae were able to develop in vitro following 24 h of culture in the immature mouse uterus. However, little development occurred within the mouse uterus. The limited development may be due to transplantation shock which was first described by Tarkowski (1959) after transfer of mouse ova. Delayed or complete lack of development for up to 24 h are characteristic of transplantation shock. Time to reach the early blastocyst, blastocyst and expanded blastocyst stages for embryos stored in the mouse uterus was longer than that for embryos cultured in vitro. However, time to reach the hatching and hatched blastocyst stages was not different than the time required by embryos cultured in vitro. The ability of bovine embryos to resume development after short-term storage in the

immature mouse uterus suggests that the environment of the immature mouse reproductive tract is not lethal to the bovine morula. The resumption of development also supports the transplantation shock hypothesis. Longer culture periods must be tested before any solid conclusions are made in regard to the use of immature mouse uteri for bovine embryo culture.

Future Research

The ultimate goal of our research as well as previous experiments cited here is to create in vitro culture systems which provide environments representative of those found in vivo. Until such a system is available, early mammalian embryos such as 1- to 8-cell bovine embryos and 1- to 2-cell random bred mouse embryos must either be transferred to oviduct of foster mothers or placed in a biologically active in vitro culture system

Addition of deproteinized hemodialysates to mouse embryo culture medium enhanced mouse morula development, but did not significantly affect development of later stage mouse embryos. Additionally, 1- to 2-cell random bred mouse embryos failed to cleave beyond the 2-cell stage in deproteinized hemodialysate supplemented medium. Mouse embryos previously had been cultured in media containing 0.1% deproteinized hemodialysate while mouse embryos in the current study were cultured in medium containing 1.0% deproteinized hemodialysate. Future experiments should test the effects of several different concentrations of deproteinized hemodialysate supplements on mouse embryo growth and viability at various stages of development.

Bovine morula development was not affected by addition of 1.0% deproteinized hemodialysate to Ham's F-10 or Ham's F-10 and 10% steer serum. Only one other study tested the effects of this tissue culture supplement on bovine embryo

development. Three concentrations of a deproteinized hemodialysate in the culture medium were tested (.01%, 0.05% and 0.1%) and higher concentrations tended to enhance the viability of frozen bovine blastocyst. As with the mouse embryo research, several concentrations of deproteinized hemodialysate supplements should be evaluated for effects on embryonic growth and viability. Additionally, deproteinized hemodialysates should be tested for their ability to enhance bovine embryo survival after micromanipulation.

Agar embedding did not adversely affect bovine morula development in vitro. Previous work has demonstrated that agar can be utilized as a zona pellucida sealant or replacement. Agar, unlike the zona pellucida, must be removed from the embryo's surface to allow hatching of the expanded blastocyst from its protective membrane. Development of an artificial zona pellucida which either is broken by the pressure of the expanded blastocyst or dissolves in a time release fashion would eliminate the need to remove the embryos from the recipient, removal of the substitute membrane and re-transfer of the embryos into a recipient.

Our results demonstrate that bovine morulae do not develop when cultured in the immature mouse uterus for 24 h, but growth does resume during subsequent culture in vitro. Recently, porcine embryos were transferred to immature mouse oviducts in vivo and in organ culture in vitro. One-cell embryos failed to cleave beyond the 4-cell stage in vivo, but developed through the blastocyst stage when cultured in explanted oviducts maintained in vitro. The immature mouse oviduct may serve as an economical alternative to the rabbit and ovine oviducts currently

used to allow bovine embryo development through the critical block stage. Until a simple in vitro system is developed for early bovine embryos future experiments should assess the effects of oviductal hormonal status as well as storage in vivo and in organ explants on early bovine embryo development.

The need for simple in vitro culture systems for early mammalian embryos which experience a developmental block in vitro remains. Recent research has demonstrated that culture medium preconditioned by trophoblastic vesicles or oviductal cells can support bovine embryo development beyond the critical 8- to 16-cell stage. The embryotrophic factor(s) provided by the preconditioned media must be isolated and the mechanism of the factor(s) determined. Identification of the factor(s) should facilitate the development of culture systems for early bovine embryos and may hasten the development of culture systems for other early mammalian embryos.

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Appendix A. M2 Culture Medium

Component	mM	Molecular Weight	g/liter
NaCl	94.66	58.450	5.533
KCl	4.78	74.557	0.356
$CaCl_2$ $2H_2$ O	1.71	147.200	0.252
KH_2 PO_4	1.19	136.091	0.162
$MgSO_4$ $7H_2$ O	1.19	246.500	0.293
$NaHCO_3$	4.15	84.020	0.349
HEPES	20.85	238.300	4.969
Sodium lactate	23.28	112.100	2.610
Sodium pyruvate	0.33	110.000	0.036
Glucose	5.56	179.860	1.000
BSA			4.000
Penicillin G potassium salt			0.060
Streptomycin sulfate			0.050
Phenol Red			0.010
2X distilled H&sub.2O			up to 1 liter

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Appendix B. Initial quality of bovine morulae randomly assigned to culture treatments.

Culture treatments consisted of Ham's F-10 supplemented with 10% steer serum (SS), 1% CLB1107 (CLB), 1% CLB and 10% SS (CLB + SS), and agar embedded embryos cultured in immature mouse uteri (Mouse) and/or 10% SS and Ham's F-10 (A + SS).

	Initial Quality			
Treatment	Excellent	Good	Fair	Poor
SS	6	9	5	4
CLB	7	6	5	3
CLB + SS	3	12	3	1
A + SS	4	11	7	1
Mouse	5	12	4	0

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