Controlled in vivo culture of mammalian embryos and isolated blastomeres

by

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ABSTRACT

Hydrogel chambers made from polymerized 2-hydroxyethyl methacrylate were used for the in vivo culture of rabbit embryos or isolated blastomeres. In Experiment 1, culture of 119, 1-cell embryos for 72 hours in saline filled chambers implanted in the peritoneal cavity of male mice resulted in 72 morulae (61%) and 25 blastocysts (21%). Transfer of these 97 embryos to recipient does resulted in the birth of 3 live offspring (3%). Culture of 119, 1-cell embryos for 72 hours in saline filled chambers implanted in the peritoneal cavity of female mice resulted in 23 morulae (19%) and 68 blastocysts (57%). Transfer of these 91 embryos to recipient does resulted in the birth of 20 live offspring (22%). All of the 119 control, 1-cell embryos had degenerated after 72 hours of in vitro culture in saline filled chambers. In Experiment 2, culture of 80 blastomeres isolated from 4-cell embryos for 72 hours in compartmentalized, medium filled chambers implanted in the peritoneal cavity of female mice resulted in 21 morulae (26%), 43 blastocysts (54%), and 4 blastomeres (5%) that cleaved, but did not develop to the morula stage. For both experiments, the recovery rate for 238 embryos or 80 blastomeres cultured in vivo was 100%.

LITERATURE REVIEW

Embryo Culture

Development and successful application of manipulative technology to early stages of mammalian embryos require culture systems which, mimicking the maternal environment, allow for the subsequent cleavage and maintenance of totipotent viability of the cultured embryos. Despite numerous attempts to culture embryos in defined media (for review, see: Brinster, 1969, Bavister, 1981, and Wright and Bondioli, 1981), only embryos from certain strains of mice (Whitten and Biggers, 1968) and rabbits (Kane and Foote, 1970, Maurer et al., 1969, 1970, and Maurer, 1978) can be cultured in vitro in chemically defined media from the 1- or 2-cell to the blastocyst stage. Embryos from other strains of mice (Whittingham and Biggers, 1967) or from other mammalian species (Yanamigachi and Chang, 1964, Brinster, 1969, Whittingham and Bavister, 1974, Thibault, 1966, Lindner and Wright, 1978, Wright and Bondioli, 1981, Brackett et al., 1982, Heyman et al., 1987) display an in vitro block when cultured from the early stages of embryonic development. For some species, this in vitro block has been overcome by culture of embryos in synthetic oviductal fluid (Tervit et al., 1972, Tervit and Rowson, 1974) or by the co-culture of early embryos with oviducts (Biggers et al., 1962, Whittingham, 1968, Bavister and Minami, 1986), oviductal epithelium (Eyestone et al., 1987) or trophoblastic vesicles (Camous et al., 1984, Heyman et al., 1987).

Preimplantation mouse embryos cleave when placed in ectopic sites. The kidney (Fawcett, 1950, Kirby, 1962) and testicular capsules (Kirby,

1963a), spleen (Kirby, 1963b), anterior chamber of the eye (Fawcett et al., 1947, Runner, 1947) and peritoneal cavity of male or female mice (Onanoff, 1893, Fawcett et al., 1947) support cleavage, development of trophoblastic tissue, and grafting of these embryos to the ectopic sites. Rabbit morulae developed to blastocysts when implanted in the abdominal cavity of female mice (Briones and Beatty, 1954). Others have used the oviducts of intermediate, intra or interspecies, recipients for the in vivo culture of early embryos to bypass the in vitro block and obtain development to the blastocyst stage (Adams, 1973, De Mayo et al., 1980, Willadsen, 1982, Boland, 1984, Eyestone et al., 1985, Eyestone and First, 1986, Sirard et al., 1985, Sirard and Lambert, 1986, Robl et al., 1987). However, recovery of these embryos from the intermediate recipient is cumbersome and frequently results in loss of embryos and poor recovery rates.

After microsurgery, isolated blastomeres or blastomere clusters from preimplantation embryos require some form of protection, as the integrity of the zona pellucida is compromised (Moore et al., 1968, Willadsen, 1979). Manipulated embryos sealed in double agar chips maintained totipotency and produced live offspring after transfer (Willadsen, 1979). The agar chip system prevented direct exposure of nude blastomeres to the oviductal environment and, in addition, the chips temporarily maintained the blastomeres in identifiable groups. However, degradation of agar over time (Boland, 1984, Eyestone et al., 1985) decreases the effectiveness of this system.

Embryo Transfer

Mammalian preimplantation embryos were first successfully transferred by Walter Heape in 1890, utilizing genetically marked donor rabbit does and pregnant recipients (Heape, 1891). From this initial study, work has continued over the past century, providing information pertaining to synchronization of donor and recipient (Chang, 1950), superovulation of donor females (Pincus, 1940, Kennelley and Foote, 1965), and fertilization and culture in vitro (Onanoff, 1893, Pincus and Enzmann, 1934). Further investigations have examined the effect of in vitro culture on embryonic survival following transfer (Adams, 1970), the effect of the number of embryos transferred on the number of offspring developing to term (Adams, 1962), cold storage of mammalian embryos (Chang, 1948), and the effect of blastomere isolation on embryonic survival (Seidel, 1952). Throughout all of these foundational studies, the rabbit was selected as the species of choice because its embryos are easily obtained and withstand the rigors of manipulation. The rabbit doe also has the anatomical advantage of having independent uterine horns (Kozma et al., 1974), which allows for the segregation of treatment groups. In addition, the rabbit is an induced ovulator (Harper, 1961, Foote et al., 1963), which greatly simplifies the synchronization of donor and recipient for embryo transfer.

Totipotency of Blastomeres

The totipotency of single blastomeres isolated from mammalian embryos at early cleavage stages was first investigated by the disruption of a single blastomere within the zona-pellucida of two-cell

rabbit embryos (Seidel, 1952). The birth of normal offspring resulting from the transfer of these embryos stimulated further studies in the mouse (Tarkowski, 1959), pig (Moore et al., 1969), sheep (Willadsen, 1979 and 1981), cow (Willadsen and Polge, 1981), and horse (Allen and Pashen, 1984). From these studies, it appears that single blastomeres maintain their totipotency when isolated before the compaction occurring at the morula stage. After compaction, the development of isolated blastomeres is restricted to trophoblastic cell outgrowth. Recently, single blastomeres isolated from rabbit embryos at 2-, 4-, 8- and 16-cell stage were tested for their ability to develop to the blastocyst stage in vitro (Lioi et al., 1987). The percent of blastomeres that reached the blastocyst stage was 100, 58.9, 33.3 and 15.5% for the 2-, 4-, 8- and 16-cell stages, respectively.

Hydrogel

A polymeric structure is considered to be a hydrogel if it satisfies three basic criteria: 1) is comprised of polymeric chains; 2) is insoluble in water at physiological temperature, pH, and ionic strength; and 3) contains between 10 to 98% of its total weight in water (Ratner, 1981).

Hydrogels have been used in various clinical applications as they are among the most inert substances known. Many structural families of hydrogel have been used in surgical prosthetic (Voldrich et al., 1975) and implantation (Cerny et al., 1970) studies, but have been most widely used in the construction of soft contact lenses (Gasset and Kaufman, 1972). Because of their diffusible nature, hydrogels have also been

extensively used for the release of various chemical products when surgically implanted in animals (Graham, 1978).

The degree of diffusion through crosslinked hydrogels can be precisely controlled by altering the molar concentration of the crosslinker within the polymerizing solutions. For poly-2-hydroxyethyl methacrylate (pHema), the rate of diffusion declined linearly with an increasing concentration of the crosslinker tetraethylene glycol dimethacrylate (Lee et al., 1978).

The structure of pHema and other related hydrogels is comprised of water embedding polymeric strands. These strands combine to form a translucent acrylic matrix containing open pores. When casts made from polymeric hydrogels are emersed within a fluid environment of physiological temperature and pH, the pores expand to form channels through which fluid components can freely pass, by simple diffusion, across their respective concentration gradients. The change in diffusibility observed at varying levels of crosslinker concentration can thus be attributed to a differing number of open channels within the polymerized hydrogel.

pHema is an inert, translucent, biocompatible, diffusible and nonbiodegradable hydrogel with a long history of clinical application in human medicine. In addition, pHema can be easily polymerized and cast in any shape or size (Pinchuk and Eckstein, 1981, Ratner, 1981).

EXPERIMENTAL OBJECTIVES

In seeking an alternative to the agar chip system for the control of in vivo culture, I became aware of the properties of poly-2-hydroxyethyl methacrylate (pHema).

The objective of this study was to develop and test a sealable pHema hydrogel chamber into which embryos or embryonic cells can be secured over the course of in vivo culture.

EXPERIMENTAL HYPOTHESES

Experiment 1

Transfer of rabbit embryos cultured in pHema hydrogel chambers in the peritoneal cavity of intermediate mouse recipients.

Hypotheses

1) Embryonic development will not occur in hydrogel chambers filled with 0.9% saline, when cultured in vitro; 2) embryonic development will occur in hydrogel chambers filled with 0.9% saline, when cultured in vivo; 3) the sex of the intermediate recipient will not influence preimplantation embryonic development; and 4) the sex of the intermediate recipient does not influence development to term of the transferred embryos.

Experiment 2

Blastomere isolation and culture in compartmentalized pHema hydrogel chambers in the peritoneal cavity of female mouse recipients.

Hypothesis

pHema hydrogel chambers will allow cleavage of zona-free, isolated rabbit blastomeres when cultured in vivo.

MATERIALS AND METHODS

Construction of Hydrogel Chambers

The hydrogel chambers were made from the mixture of low acid 2-hydroxyethyl methacrylate as a monomer, and tetraethylene glycol dimethacrylate (TGD) and ethylene glycol (EG) as crosslinkers (Polysciences, Inc., Warrington, PA). The formulation of reactants was that described by Lee et al. (1978, Table 1) for a ratio of 10 ml Hema to 0.1 ml TGD (25.0 mM) and 3.0 ml EG (3.69 M) for a total crosslinker content in Hema of 3.72 M.

Before casting the chambers, 3 stock solutions were prepared and placed in glass test tubes: solution A - a mixture of 10 ml Hema, 0.1 ml TGD, 3.0 ml EG, and 2.0 ml distilled water; solution B - initiator, 1.0 ml of ammonium persulfate, 40 g per 100 ml distilled water or 1.75 M; solution C - co-initiator, 1.0 ml of sodium metabisulfite, 15 g per 100 ml distilled water or 0.79 M. Each stock solution was purged with nitrogen and the reactants were mixed and polymerized under pressure, as described by Pinchuk and Eckstein (1981) for the casting of ureteral anastomotic nipples.

The hydrogel chambers were cast at room temperature within the barrel of 71 mm long, 3.5 mm ID, 0.5 ml insulin syringes (No. 8471 single use, plastipak LO-dose U-100; Becton Dickinson and Co., Rutherford, NJ). The needle and its hub were removed to free the needle connector of the syringe. The plunger was withdrawn from the syringe and the rubber gasket at the tip of the plunger was removed and inverted so that the hollow cavity that previously attached the gasket to the

plunger was directed toward the barrel of the syringe. The inverted gasket was then reinserted in the barrel and aligned with the 50-unit mark of the syringe. The syringe with the gasket in position was hand-held vertically with the needle connector directed upwardly. The reactants, 1.51 ml of solution A, 0.1 ml of solution B, and 0.1 ml of solution C were then mixed within a 3 ml polyethylene syringe just before loading the casting insulin syringes. The syringe containing the polymerizing mixture was fitted with a 1 1/2-inch (38.1 mm), 18-gauge needle which was passed through the connector to fill the barrel of the insulin syringe with 0.5 ml of the polymerizing mixture. Immediately after filling the casting syringe, a 5.6-cm long, 0.9 mm OD stainless steel rod, inserted into a 5.6-cm long, 1.7 mm OD Teflon tubing (Cole-Parmer International, Chicago, IL) was passed through the needle connector and the mixture of reactants in the barrel of the insulin syringe to the rubber gasket in order to form a hollow chamber in the polymerizing gel. The Teflon tubing with the inserted steel rod was held in the center of the barrel by the needle connector and the hollow cavity of the inverted rubber gasket. A 6.0-cm long, 16 mm OD plexiglass rod, drilled through with a 7/32-inch bit (5.56 mm), was used to brace the barrel of the insulin syringe and prevent breakage during pressurized polymerization. A 4-cm long, 3.5 mm OD stainless steel rod was inserted into the flanged end of the barrel of the syringe and used to apply pressure to the gasket and polymerizing mixture. The braced syringe was then placed between size 9 neoprene stoppers attached to the jaws of a pipe clamp. Pressure was applied for 15 minutes by closing

the jaws of the clamp until all visible air bubbles were displaced from the polymerizing gel. No attempt was made to measure the pressure applied with the clamp. After this 15 minute period of pressurized polymerization, the cast hydrogel was removed from the syringe and the centrally located Teflon tubing with the rod was withdrawn from the cast hydrogel which now was a hydrogel tube of approximately 5 cm long, with a 1.7 mm lumen and 0.9 mm thick wall. The pHema hydrogel tubes, in batches of 10 tubes, were then placed for 96 hours in a 100 ml glass beaker containing 95% ethanol which was changed every 12 hours to remove nonpolymerized Hema. After ethanol washing, the pHema tubes were placed in a beaker containing 500 ml of distilled water and heated at a slow boil for 4 hours. The distilled water was changed and the procedure repeated 12 times. After boiling, the pHema tubes were cut into 1-cm long segments with a razor blade under observation with a stereoscopic microscope at 10 X. Segments containing visible flaws were discarded. Solid plugs, 2 mm long were made from 0.085 inch (2.16 mm) OD Silastic tubing (Dow Corning Corp., Medical Products, Midland, MI) filled with Silastic adhesive and used to close the end of the pHema tube to form a chamber (Fig. 1A). Each 1-cm segment of hydrogel tube and the 2 solid plugs to close the end of the chamber, were placed into a 2 ml glass ampule (Wheaton Scientific, Millville, NJ) containing distilled water. The ampules were then autoclaved for 40 minutes at 120°C, firesealed, and stored at room temperature until used (Fig. 1B).

Animals

Twenty-three uniparous, crossbred rabbit does ranging from 1.0 to

1.5 years of age were used as embryo donors or recipients for Experiments 1 and 2. Does were individually caged for at least 21 days before assignment to the experiments, fed commercial rabbit feed, and maintained during the experimental period in a room with controlled temperature (20 to 22°C) and light (12 hours' light/12 hours' dark). Two crossbred, mature rabbit bucks of known fertility were individually caged, maintained in the same room, and fed, as described for the does.

Fifteen mature, cycling female and 10 mature male Balb/c mice were used as intermediate recipients for the 2 experiments of this study. Mice were caged by sex in groups of 4 or 5 females or 2 males per cage, fed commercial mouse feed, and maintained in a room with controlled temperature (20 to 22°C) and light (14 hours' light/10 hours' dark). For the female mice intermediate recipients, the stage of the estrous cycle was determined by vaginal smears and only females which were late in the afternoon of the day of estrus (D1) were used.

Experiment 1. Transfer of Rabbit Embryos Cultured in pHema Hydrogel Chambers in the Peritoneal Cavity of Intermediate Mouse Recipients

Embryo Recovery and Culture Ten does were randomly selected from the 23 does to serve as embryo donors and 10 does were selected to serve as recipients. To induce superovulation, each donor doe was given a subcutaneous injection of 0.5 mg of follicle stimulating hormone (FSH-P, Burns Biotec, Omaha, NE) every 12 hours for 72 hours. Donor does were mated twice to each of the 2 bucks, 24 hours after the last FSH injection. Each of the 10 mated donors was then randomly paired with a recipient. To synchronize donors and recipients, each unmated

recipient was induced to ovulate by a single intramuscular injection of 50 IU of human chorionic gondotropin (hCG, Fort Dodge Laboratories, Fort Dodge, IA) given 14 hours after the fourth mating of the corresponding paired donor.

To recover 1-cell rabbit embryos, does were anesthetized with Halothane (Fort Dodge Laboratories, Fort Dodge, IA) 18 hours after the 4th mating and each oviduct was flushed from the uterotubal junction with 3 ml of 0.9% (or 0.154 M sodium chloride) sterile saline solution. After flushing and recovery, embryos were examined with an inverted miscroscope at 100 X, while still in the collecting dish and flushing fluid. Oocytes that had spermatozoa in the perivitelline space or embryos that had extruded the second polar body were considered to be 1-cell embryos. The 1-cell embryos from each doe were washed 3 times by transfer between 10 X 35 mm culture dishes (Lux, Miles Laboratories, Inc., Naperville, IL) containing sterile 0.9% saline solution before loading the pHema hydrogel chambers.

Three pHema hydrogel chambers were prepared for each doe before embryo collection. The sealed tip of each glass ampule was broken and the tube and plugs were withdrawn with sterile forceps and placed in 10 X 35 mm culture dishes containing sterile saline solution. With the aid of forceps, one of the solid plugs was inserted into one end of each of the 3 tubes and the tubes with the uninserted plugs were then incubated in a dish containing sterile saline solution for at least 110 minutes at 37°C, in an incubator with 5% CO_2 in humidified air before loading the chamber with embryos.

The 1-cell embryos recovered from each donor doe were subdivided into 3 groups of equal number of embryos. The embryos from each group were transferred into the lumen of a saline solution-filled pHema tube while the tube was immersed in saline. The open end of the tube was then sealed with the second Silastic plug and the sealed chambers were examined at 20 X with a stereoscopic microscope to verify the number of embryos and the integrity of the chamber. The 3 chambers containing the embryos from each donor were then randomly assigned to one of the following treatment groups: Group 1: in vitro controls cultured for 72 hours in a 10 X 35 mm culture dish containing 3 ml of 0.9% sterile saline solution in an incubator at 37°C with 5% CO_2 in humidified air; Group 2: cultured in vivo for 72 hours in the peritoneal cavity of an adult female Balb/c mouse on D1 of the cycle; Group 3: cultured in vivo for 72 hours in the peritoneal cavity of an adult male Balb/c mouse. The embryo loaded chambers assigned to Groups 2 and 3 were surgically implanted in anesthetized (Metofane, Pitman-Moore, Washington Crossing, NJ) female or male mice through a 1-cm long incision in the ventral abdominal wall. After 72 hours of either in vitro or in vivo culture, the chambers were recovered and examined at 25 to 100 X with an inverted microscope to determine the stage of embryonic development. Embryos were classified as follows: embryos that did not cleave or had fragmented blastomeres were considered degenerated; embryos that cleaved beyond the 1-cell stage, had recognizable, intact blastomeres, but did not reach the morula stage, were considered retarded; morulae were embryos that cleaved beyond the 16 cell stage, but had not yet developed

a blastocoele, while blastocysts were embryos with a clear, defined blastocoele. The number of degenerated or retarded embryos and the number of morulae and blastocysts obtained from each donor were recorded.

Embryo Transfer The morulae and blastocysts obtained after in vivo culture in the pHema chambers were transferred to the paired recipient. Recipients were anesthetized with Halothane, as described for the donor does. The ventral area of the abdominal wall was clipped free of hair, disinfected, draped, and the uterine horns were exposed through a 6-cm long midventral incision. For each paired recipient, the left or right uterine horn was alternated as to receive embryos cultured in vivo in either male or female mouse. Each horn was punctured with the eye of a No. 22 suture needle and the embryos from treatment groups 2 or 3 were transferred to the lumen of the assigned horn using a 5 µl Wiretrol pipet (Fisher Scientific, Springfield, NJ). The abdominal incision was sutured and each doe was fitted with an Elizabethian plastic collar until recovery from surgery.

Twenty-one days after transfer, recipient does were laparotomized. Before laparotomy, each doe was sedated by an intramuscular injection of 1 mg per kg of body weight of Acepromazine (Ceva Laboratories, Inc., Overland Park, KS). The midventral abdominal area was disinfected and then infiltrated with 10 mg/kg body weight of a 2% solution of Lidocaine (Astra Pharmaceutical Products, Inc., Worcester, MA). The uterine horns were exposed and the number of fetuses within each horn was recorded. Fetuses from embryos cultured in male mice were marked in utero by an

injection of 1 µl of Indian ink deposited, as a subcutaneous drop, in the rump area of each fetus. At parturition, which occurred 6 to 8 days after laparotomy, the number of offspring derived from each of the 2 treatment groups was recorded. Bunnies were observed daily for general health until weaning.

Experiment 2. Blastomere Isolation and Culture in Compartmentalized pHema Hydrogel Chambers in the Peritoneal Cavity of Female Mouse Recipients

Four-cell rabbit embryos were recovered from the 3 remaining does. Superovulation was induced and the embryos recovered, as described for Experiment 1, except that the oviducts were flushed 32 hours after the 4th mating with Whitten's medium (WM, Whitten and Biggers, 1968) supplemented with 1 mg bovine serum albumin (BSA, Fraction V, Sigma Chemical Co., St. Louis, MO) per ml of medium. The flushing medium was filtered through a 0.2 µm filter.

To isolate blastomeres, the 4-cell embryos recovered from each doe were placed in 10 X 35 mm culture dishes pretreated with Prosil 28 (PCR, Inc., Gainesville, FL) to decrease adhesion of the blastomeres to the culture dish. The embryos were washed as a group 3 times at room temperature in filtered WM-BSA medium and then incubated for 15 minutes at 37°C with 5% CO_2 in humidified air in Ca^{+}-and Mg^{++}-free, modified Dulbecco's phosphate buffered saline solution (DPBS, Dulbecco and Vogt, 1954), supplemented with 0.02% (or 0.68 mM) EDTA and 0.25 M glycerol. Next, the embryos were incubated for 15 minutes in modified DPBS, supplemented with 0.50 M glycerol and then transferred to Prosil 28

treated culture dishes containing modified DPBS supplemented with 1.0 M glycerol to remove the zona pellucida. The zona was removed under observation with inverted microscope at 100 X with a hand-held microknife made from a piece of razor blade. The zona-free blastomere clusters were then transferred and incubated 2 times for 15 minute periods, first in a culture dish containing modified DPBS supplemented with 0.50 M glycerol and then in modified DPBS supplemented with 0.25 M glycerol. To separate the blastomeres, each blastomere cluster was transferred to a culture dish containing modified DPBS only and then subjected, during observation with a stereoscopic microscope at 20 X, to repeated aspiration into and expulsion from a 50 μm ID silicone capillary tube (Polymicro Technologies, Phoenix, AZ) attached to a 5 μl Wiretrol pipet. The isolated blastomeres were then washed 5 times by transfer between dishes containing WM-BSA medium. Only those embryos from each of the 3 donor does that yielded 4 intact blastomeres were used in this experiment. The 4 blastomeres isolated from each embryo, hence a monozygotic group, were placed within individual compartments of a sterile pHema hydrogel chamber, under observation with a stereoscopic microscope at 20 X. These chambers were made from Hema, as described for Experiment 1, except that the length of each chamber was increased to 2 cm in length. One of the open ends was sealed with a plug and the lumen of the chamber was partitioned into 4 compartments (Fig. 2) by the insertion of 3, 1.7 mm OD x 2 mm thick pHema discs, while in a 10 X 35 mm culture dish containing WM-BSA medium. These discs were made from Hema hydrogel polymerized at room temperature within a 1.7 mm ID Teflon

tubing without applying pressure. The first blastomere was placed in the chamber and one of the pHema discs was inserted in the lumen of the chamber and positioned at approximately 2.5 mm from the sealed end of the chamber. This procedure was repeated until each of the 4 blastomeres was loaded into the compartmentalized chamber. The chamber was then sealed with the remaining plug (Fig. 2). The chambers containing 4 isolated blastomeres were surgically implanted in the peritoneal cavity of a female Balb/c mouse on D1 and incubated in vivo for 72 hours, as described in Experiment 1. Because of the number of monozygotic groups obtained, 4 chambers were implanted in the peritoneal cavity of each of 5 female mice. At the end of the 72 hour period of incubation, the compartmentalized chambers were recovered and the isolated blastomeres were examined for development with an inverted microscope at 25 to 100 X.

Statistical Analysis

End points for stage of development of embryos cultured in vivo and for the results of transfer of morulae and blastocysts to recipient does between groups 2 and 3 of Experiment 1 were compared by Chi-square analysis, with 1 degree of freedom and Yates correction (Steel and Torrie, 1960). Significance was established at $P \leq 0.05$. Data for embryos cultured in vitro (control group 1 of Experiment 1) were not statistically compared with those of groups 2 and 3 due to the 0 values obtained.

Due to the preliminary nature of this study, the confounding effect of intermediate recipient and the number of 0 values obtained in group 1 of Experiment 1, and the lack of controls for Experiment 2, no attempt was made to determine overall treatment and donor effects for either experiment.

RESULTS

Experiment 1

A total of 357, 1-cell embryos recovered from 10 donor does was used in this experiment (Table 1). All of the 238 embryos that were placed in the pHema chambers and cultured in vivo in the peritoneal cavity of male or female mice were recovered at the end of the 72 hours of in vivo culture (Table 1). All of the 119 embryos incubated in vitro in pHema chambers had degenerated during the 72 hours of culture. In comparison, only 10.1% of embryos cultured in male mice and 8.4% of the embryos cultured in female mice degenerated during the in vivo culture period. These differences were not significant ($P > 0.05$, Table 1). More ($P < 0.0005$, Table 1) of the embryos cultured in female mice developed to blastocysts (68/119) than those cultured in male mice (25/119). The transfer of 188 morulae and blastocysts (Table 1) recovered from pHema chambers implanted in the peritoneal cavities of male and female mice (Figs. 3A, B) resulted in 23 (12.2%) live offspring (Table 2). Fewer ($P < 0.005$) offsprings were born from the transfer of embryos cultured in male mice (3/97, Table 2) than those cultured in female mice (20/91). Survival to term was not influenced ($P > 0.05$) by the horn to which embryos were transferred. The 23 bunnies developed in an apparently normal fashion (Fig. 4) and were released for adoption after weaning.

Experiment 2

A total of 43, 4-cell embryos was collected from 3 donor does and eighty intact blastomeres were isolated from 20 of these 4-cell embryos. All of the blastomeres that were cultured in vivo were recovered from the compartmentalized pHema chambers after the 72 hours of culture. Of these 80 isolated blastomeres, 16 (20%) were retarded or degenerated, 21 (26%) developed to the morula and 43 (54%) to the blastocyst stages (Table 3). Eleven (44 blastomeres) of the 20 originally cultured monozygotic groups developed, apparently in synchrony, to become either morulae or blastocysts. Including the 4 retarded blastomeres that cleaved but did not develop to the morula or blastocyst stages, 85% (68/80, Table 3) of the isolated blastomeres survived the isolation procedure and cleaved, while cultured in vivo in the compartmentalized pHema chambers. However, many of these de novo formed morulae and blastocysts were fragile and fragmented during withdrawal from the chamber.

TABLES

Table 1. Development of one-cell rabbit embryos during 72 hours of in vitro or in vivo culture in saline-filled pHema hydrogel chambers

Treatment	Number of embryos				
	Cultured	Degenerated	Retarded[a]	Morula	Blastocyst
In Vitro Controls	119	119	0	0	0
Male Mouse	119	12	10	72*	25
Female Mouse	119	10	18	23	68*

[a]Embryos that cleaved beyond the 1-cell but did not advance to the morula stage.

*Significantly ($P < 0.0005$) different from the corresponding in vivo treatment group.

Table 2. Viability after transfer of rabbit embryos cultured in vivo in saline-filled pHema hydrogel chambers

Recipient number	Sex of intermediate mouse recipient	Number (stage)[a] transferred	Uterine horn	Fetuses day 25[b]	Offspring born alive
1	Male	7 (M), 3 (B)	Left	0	---
	Female	1 (M), 9 (B)	Right	0	---
2	Male	8 (M), 4 (B)	Right	0	---
	Female	11 (B)	Left	3	3
3	Male	6 (M), 2 (B)	Left	0	---
	Female	9 (B)	Right	3	3
4	Male	10 (M), 1 (B)	Right	0	---
	Female	13 (B)	Left	4	4
5	Male	7 (M), 1 (B)	Left	0	---
	Female	7 (M)	Right	0	---
6	Male	7 (M), 2 (B)	Right	0	---
	Female	1 (M), 7 (B)	Left	2	2
7	Male	11 (M), 2 (B)	Left	1	1
	Female	3 (M), 9 (B)	Right	4	4
8	Male	5 (M), 5 (B)	Right	2	2
	Female	9 (B)	Left	4	4
9	Male	7 (M), 1 (B)	Left	0	---
	Female	6 (M)	Right	0	---
10	Male	4 (M), 4 (B)	Right	0	---
	Female	5 (M), 1 (B)	Left	0	---
Totals	Male	97 (72M), (25B)	------	3	3
	Female	91 (23M), (68B)	------	20	20

[a]Stage: (M), morula; (B), blastocyst.

[b]Determined by laparotomy on day 25 of gestation.

Table 3. Development of single blastomeres isolated from 4-cell rabbit embryos during 72 hours of in vivo culture in media-filled pHema hydrogel chambers

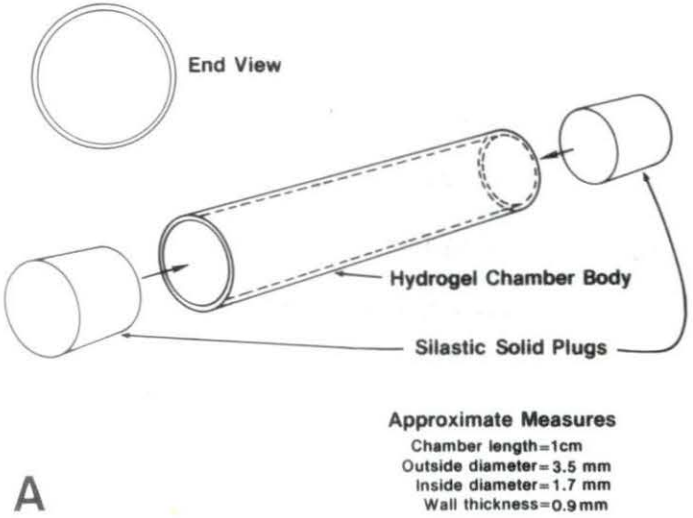
Blastomeres isolated and cultured	80
Of these:	
Degenerated	12
Retarded[a]	4
Morulae	21
Blastocysts	43
Total recovered after culture	80
Total developing to the morula or blastocysts stages	64 (11)[b]

[a]Blastomeres that cleaved but did not advance to the morula or blastocyst stages.

[b]Brackets indicate monozygotic groups.

FIGURES

Figure 1. pHema hydrogel chamber for the in vivo culture of embryos. Schematic representations of the chamber depicting general features and measures (A) and photograph of a pHema hydrogel chamber stored in a glass ampule (B)


End View
Hydrogel Chamber Body
Silastic Solid Plugs
Approximate Measures
Chamber length=1cm
Outside diameter=3.5 mm
Inside diameter=1.7 mm
Wall thickness=0.9mm
A

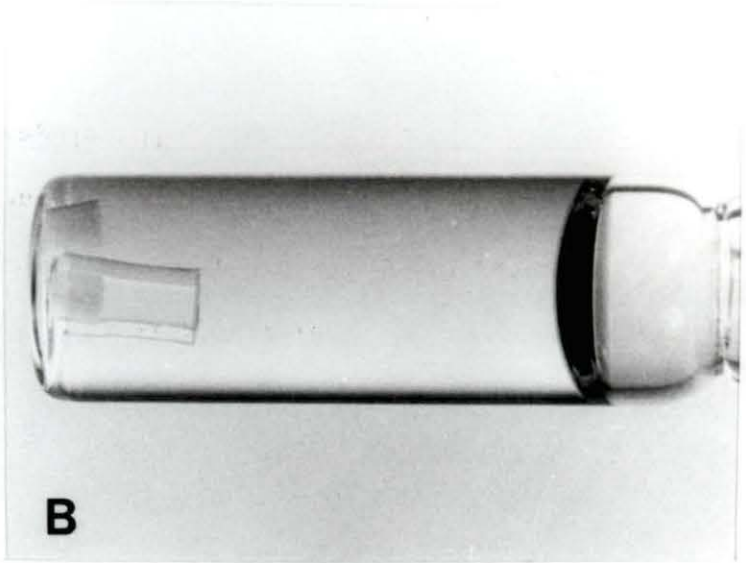

B

Figure 2. Schematic representation of a compartmentalized pHema chamber containing isolated blastomeres

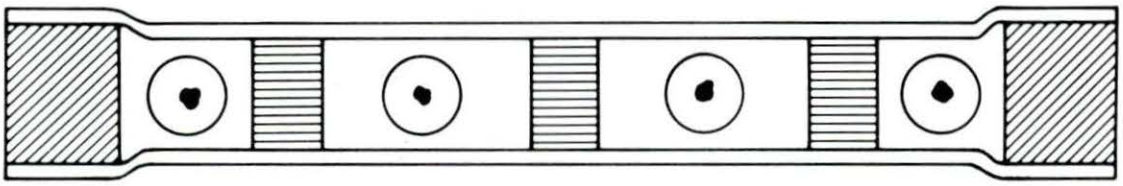


Figure 3. Rabbit embryos contained in pHema hydrogel chambers. These embryos were derived from 1-cell embryos after 72 hours of culture in the peritoneal cavity of a male mouse (A, compacted morula, 320 X) or female mouse (B, blastocyst, 380 X). Magnifications are approximate

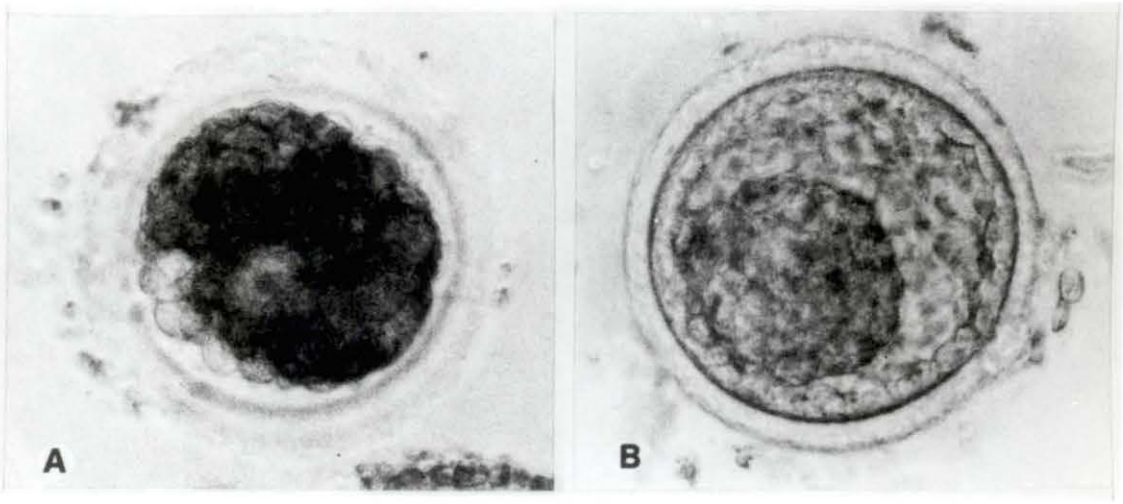

A
B

Figure 4. Bunnies obtained after transfer of embryos cultured in pHema chambers implanted in the peritoneal cavity of a male mouse (gray and white bunnies) and of a female mouse (black and white bunnies). The male and female mice shown were littermates of the intermediate recipients used

DISCUSSION

The loss of embryos and the impossibility to reliably maintain separation of treatment groups during in vivo culture seriously limits the evaluation of treatment effects on embryonic survival (Boland, 1984, Eyestone et al., 1985). In the ligated rabbit or sheep oviduct, relatively size-restricted environments, embryonic losses averaged 30% of the total number of embryos transferred (Boland, 1984), and may reach even higher percentages (Lawson et al., 1972, Willadsen, 1979, Eyestone et al., 1985, Sirard et al., 1985, Sirard and Lambert, 1986). The overall recovery rate for the interspecies, in vivo culture of embryos in the peritoneal cavity was 22% (Briones and Beatty, 1954).

In the present study, all of the embryos and blastomeres cultured within pHema chambers were recovered following the 72 hour period of culture. This high rate of recovery makes the pHema hydrogel chambers particularly well suited for paired studies designed to evaluate the totipotent viability of embryos at early stages of development or to culture individual blastomeres isolated from preimplantation embryos.

The development of 1-cell rabbit embryos to the morula and blastocyst stages within saline filled pHema chambers implanted in the peritoneal cavities of mice, as well as the birth of live offspring resulting from the transfer of these embryos, demonstrate that the pHema hydrogel chambers permit the passage of essential factors from the peritoneal cavity into the lumen of the chamber, such that embryonic development could occur. These results also indicate that the peritoneal cavity of mice can support the development of 1-cell rabbit

embryos to the blastocyst stage. The capability of the murine peritoneal environment to support development of rabbit embryos from the late morula to the blastocyst stages, was previously reported (Briones and Beatty, 1954). However, we believe this is the first report of interspecific peritoneal culture of rabbit embryos from the 1-cell to the blastocyst stages which resulted in the birth of live offspring after transfer to recipient does.

The percentage of 1-cell rabbit embryos that developed to the morula and blastocyst stages during culture for 72 hours in the peritoneal cavity of female mice is comparable to that reported for 2- and 4-cell embryos cultured within the ligated oviducts of estrous does (Adams, 1973). The development of embryos cultured in male mice was retarded when compared to the development of embryos cultured in female mice in our study or when compared to the rate of development in ligated oviducts (Adams, 1973). This suggests that the peritoneal cavity of the male mouse is a less favorable environment for embryonic development than that of the female mouse. Preimplantation rabbit embryos cultured in vitro display developmental retardation due to impaired cell proliferation, which can be corrected by supplementing the culture medium with uterine flushings (Fisher, 1987). It remains to be determined whether the differences in embryonic developmental capability of the peritoneal cavities of female and male mice are qualitative, quantitative, or both, since it is possible that the peritoneal cavity of female mice may contain growth factors absent in the male.

The percentage of offspring born from the transfer of morulae or blastocysts cultured from the 1-cell stage in the peritoneal cavity of female mice (20/91, 22%) appears to be greater than that resulting from embryos cultured in vitro for 72 hours (7.0%, Adams, 1970, 14%, Maurer, 1978) and compares with the percentages resulting from the transfer of 2- to 4-cell embryos cultured in the ligated oviduct of estrous does (17%, Adams, 1973) or obtained after transfer of noncultured embryos to asynchronous recipients (27%, Yang et al., 1986).

The fewer offspring born from the transfer of 1-cell rabbit embryos cultured in the peritoneal cavity of male mice (3/97, 3%) could be the result of the transfer of more morulae than blastocysts (72 morulae and 25 blastocysts, Table 2) than for the female mice (23 morulae and 68 blastocysts).

The number of isolated blastomeres that developed during in vivo culture to morulae and blastocysts, while contained in the compartmentalized pHema chambers, as well as the recovery at the end of the incubation period of all of the resulting product of each of the isolated blastomeres attest to the protective nature of the pHema chamber and emphasizes its potentials. In addition, a system that would allow for the identification of monozygotic groups at the end of an in vivo incubation period seems ideal for studies on the totipotency of blastomeres isolated from mammalian embryos. It remains to be determined, however, whether it is possible to retrieve intact de novo formed morulae or blastocysts from a hydrogel chamber and obtain live

offspring after transfer. In an earlier report (Moore et al., 1968), rabbit blastomeres isolated from 2-, 4-, or 8-cell embryos failed to survive after transfer to the rabbit oviduct.

The development of apparently normal blastocysts and the lack of trophoblastic vesicles in the blastocysts derived from isolated blastomeres suggests complete development while in the pHema chamber. Trophoblastic vesicles devoid of inner cell mass develop from isolated blastomeres when cultured in vitro (Tarkowski and Wroblewska, 1967) or in the oviduct of an intermediate recipient (Willadsen and Fehilly, 1983).

In summary, the results of this study demonstrate that pHema hydrogel can be cast into sealable and easily retrievable chambers for the in vivo culture of embryos. The mechanisms of solute movement through the pHema hydrogel, at the formulation of reactants used in this study, was reported (Lee et al., 1978) to follow a pore flow diffusion model. The diffusivity of hydrogel can be controlled by changes in the ratios of Hema to TGD and EG (Lee et al., 1978, Ratner, 1981) to allow for the passage of substances over a wide range of molecular weights. Thus, constituents of biological fluids, based on their size and concentration gradient, could be excluded from or collected within the chamber to identify growth factors or simply to collect naturally produced fluids for the culture of embryo or somatic cells. In addition, the pHema chamber also offers potentials for the in vivo culture of isolated blastomeres and for the selection of suitable intermediate recipients for the xenogenous in vivo culture of mammalian embryos.

CONCLUSIONS

1. Poly-2-hydroxyethyl methacrylate can be cast into chambers for the in vivo culture of embryos or isolated embryonic cells.

2. The use of pHema hydrogel chambers allows for the recovery of all of the embryos or embryonic cells placed within the chamber after a period of in vivo culture.

3. Physiological saline cannot support in vitro development of rabbit zygotes.

4. The pHema membrane allows for the diffusion of nutrients and for other factors such that rabbit zygotes, held initially in physiological saline, developed to the blastocyst stage during the period of in vivo culture.

5. The peritoneum and peritoneal fluid of both male and female mice of the Balb/c strain support development of rabbit embryos from the one-cell to the blastocyst stage.

6. The totipotency of embryos cultured in the peritoneal cavity of either male or female mice was maintained.

7. pHema hydrogel chambers protect zona-free embryonic cells during in vivo culture.

8. All of the blastomeres of the four-cell rabbit embryo are potentially capable of individually developing to the blastocyst stage.

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APPENDIX A:

USE OF AGAR CHIPS FOR THE IN VIVO CULTURE OF ISOLATED PORCINE BLASTOMERES WITHIN THE LIGATED OVIDUCTS OF INTERMEDIATE RECIPIENT GILTS

Ten pre-puberal F1 crossbred (Landrace X Yorkshire) gilts, weighing between 170 to 185 pounds, were used in this experiment. Gilts were randomly allocated to one of two groups: donors (n=5); recipients (n=5). Each donor was randomly assigned to a recipient with each pair being maintained over the course of the experiment in a single holding pen. Both the donor and the recipient of each pair were synchronized to cycle and ovulate by the intramuscular injection of 1500 IU pregnant mares serum gonadatropin (PMSG), followed in 72 hours with the administration of 500 IU human chorionic gonadatropin (hCG). Donor gilts were artificially inseminated 36 hours after the injection of hCG. Embryos were surgically recovered from donor gilts, under halothane anesthesia, 96 hours after the administration of hCG. Four-cell embryos were selected from amongst those recovered. The zona was removed and blastomeres were isolated from these embryos in a similar fashion as to the procedure used in experiment 2 of this thesis. Isolated blastomeres were then individually placed within empty surrogate zona's obtained from porcine oocytes. Monozygotic groups of manipulated embryos were embedded in a double layered agar chip, as described by Willadsen (1979), and the agar chips were surgically transferred to the ampullary portion of the paired recipient's oviducts. Each ampulla was ligated at both the isthmal-ampulla junction and fimbria. Agar chips and/or embryos were recovered after 72 hours of in vivo culture.

Of the 39 agar chips transferred to the ligated ampullas of the 5 recipient gilts, only 2 (5%) were recovered following 72 hours of in vivo culture. The disintegration of the agar chips within the oviductal lumen subsequently resulted in the recovery of only 58 (52%) embryos. Of the embryos recovered, 12 (21%) showed evidence of cleavage while 46 (79%) had degenerated.

Table A1. Use of agar chips for the in vivo culture of isolated porcine blastomeres within the ligated oviducts of intermediate recipient gilts

Recipient gilt number	Number of agar chips transferred	Number of isolated blastomeres transferred	Agar chips recovered	Embryos		
				Recovered	Degenerate	Cleaving
1	8	20	-	8	8	-
2	10	23	-	14	12	2
3	6	24	1	12	8	4
4	5	20	-	8	8	-
5	10	25	1	16	10	6

APPENDIX B:

IN VITRO AND IN VIVO CULTURE OF FOUR-CELL PORCINE EMBRYOS WITHIN pHEMA CHAMBERS

Five pre-puberal crossbred gilts were used to obtain four-cell porcine embryos within this study. Donor gilts were treated and embryos were recovered as described in Appendix A. The number of four-cell embryos recovered from each gilt was randomly allocated to two groups of equal number of embryos. Each embryo from each group was placed within a pHema hydrogel chamber filled with Whitten's medium supplemented with 15 mg/ml bovine serum albumin (BSA). Each chamber was then randomly assigned to either in vitro or in vivo culture. The chamber assigned to in vitro culture was placed within a 35 x 10 mm plastic tissue culture dish containing 3 ml Whitten's medium supplemented with 15 mg/ml BSA. Each culture dish, containing the medium and chamber, was then incubated at 37°C with 5% CO_2 in humidified air. The chamber assigned to in vivo culture was transferred into the peritoneal cavity of a female Babl/c mouse on day 1 of her estrous cycle. Both in vitro and in vivo groups were cultured for 72 hours.

The use of pHema hydrogel chambers for the in vitro and in vivo culture of 4-cell porcine embryos resulted in the recovery of all 86 treated embryos. The pHema chambers did not inhibit the cleavage of embryos during in vitro culture as 38 (88%) developed beyond the 8-cell stage. The peritoneal cavity of the cyclic female mouse, however, proved to be completely inhibitory to development as all of the 43 porcine embryos cultured in vivo blocked at the 4-cell stage.

Table B1. In vitro and in vivo culture of four-cell porcine embryos within pHema chambers

Donor gilt number	Culture site	Number of Embryos cultured	Embryos recovered	4-Cell	8-Cell	Morula	Blastocyst	% of embryos developing beyond 8-cell stage
1	In Vitro	10	10	---	---	5	5	100
	In Vivo	10	10	10	---	---	---	0
2	In Vitro	8	8	---	2	2	4	75
	In Vivo	8	8	8	---	---	---	0
3	In Vitro	5	5	1	---	2	2	80
	In Vivo	5	5	5	---	---	---	0
4	In Vitro	8	8	---	1	4	3	80
	In Vivo	8	8	8	---	---	---	0
5	In Vitro	12	12	1	---	7	4	92
	In Vivo	12	12	12	---	---	---	0

APPENDIX C:

IN VITRO AND IN VIVO CULTURE OF PREIMPLANTATION FELINE EMBRYOS WITHIN pHEMA CHAMBERS

Five anestrous mixed breed queens obtained from local shelters were induced to superovulate with a total of 3.0 mg FSH-P, utilizing a decreasing regime (1.0, 1.0, .5, .25, .25 mg, FSH-P). These doses were delivered over a 5 day period in equal bi-daily doses. Queens were allowed to breed freely to each of two toms on the 7th day following initiation of superovulation treatment. One-, 2- and 4-cell feline embryos were surgically recovered 48 hours following the last natural mating by flushing of each oviduct and uterine horn with Whitten's medium plus 1 mg/ml BSA. Embryos collected from each donor were classified, under stereomicroscopic examination, into sub-groups according to developmental stage. Each of the two equal numbered embryo groups were then sealed within individual pHema hydrogel chambers which had been previously equilibrated in Whitten's medium supplemented with 1 mg/ml BSA. One of the two pHema chambers was then selected at random for culture in vivo and was subsequently placed within the peritoneal cavity of a Balb/c female mouse on day 1 of her estrous cycle. The remaining chamber was placed within a 35 x 10 mm tissue culture dish containing 3 ml of Whitten's medium plus 1 mg/ml BSA. The culture dish was then transferred into an in vitro culture incubator maintaining a 37°C temperature with an atmosphere of 5% CO_2 in air. Culture continued in vitro and in vivo over a 72 hour period.

All of the 120 feline embryos cultured in vivo and in vitro within pHema chambers were recovered at the end of the culture period. Evaluation of these embryos bore evidence of the superior environment that the murine peritoneal cavity maintains over that of the in vitro incubator. Of the 60 feline embryos cultured in vivo, 33 (55%) developed to the blastocyst stage, while only 3 (5%) of the embryos cultured in vitro formed a blastocoele.

Table C1. In vitro and in vivo culture of preimplantation feline embryos within pHema chambers

Donor queen number	Treatment	Number of embryos cultured	Embryo number following 72 hours of culture			
			Degenerate	8-Cell	Morula	Blastocyst
1	In Vivo	10	2	---	2	6
	In Vitro	10	4	---	6	---
2	In Vivo	20	6	---	2	12
	In Vitro	20	8	---	9	3
3	In Vivo	12	4	---	3	5
	In Vitro	12	7	1	4	---
4	In Vivo	8	1	---	3	4
	In Vitro	8	3	2	3	---
5	In Vivo	10	3	---	1	6
	In Vitro	10	3	---	7	---

APPENDIX D:

IN VITRO AND IN VIVO CULTURE OF ONE-CELL RAT EMBRYOS WITHIN pHEMA CHAMBERS

Twenty cycling female outbred rats were used within this experiment. The estrous cycle was monitored by vaginal smear. Rats displaying proestrous smears were paired. Rats within each pair were then randomly assigned to serve as either embryo donor or recipient. Donor females were mated to an intact male of proven fertility, while recipients were mated to a vasectomized male. Approximately 10 hours after the expected time of ovulation, one-cell embryos were collected from the donors' oviducts with Whitten's medium. Embryos were then randomly allocated into one of two groups which were subsequently sealed within individual pHema chambers previously filled with Whitten's medium supplemented with 15 mg/ml BSA. The hydrogel chambers were then randomly assigned to either in vitro or in vivo culture. Chambers assigned to in vitro culture were placed within a 35 x 10 mm tissue culture dish containing 3 ml of Whitten's medium supplemented with 15 mg/ml BSA and cultured in an incubator maintaining a temperature of 37°C with an atmosphere of 5% CO_2 in humidified air. Chambers assigned for in vivo culture were transferred into the peritoneal cavity of the corresponding paired recipient rat. Both in vitro and in vivo treatment groups were cultured 72 hours.

The use of pHema chambers resulted in the recovery of all 104 rat embryos initially placed into culture. None of the rat zygotes cultured either in vitro or in vivo developed past the 2-cell stage. Embryos cultured in vivo, however, demonstrated a much greater tendency to degenerate over the culture period then those cultured in vitro.

Table D1. In vitro and in vivo culture of one-cell rat embryos within pHema chambers

Rat donor number	Culture site	Number of					
		Embryos cultured	Embros recovered	Degenerated	1-Cell	2-Cell	4-Cell
1	In Vivo	4	4	2	---	2	---
	In Vitro	4	4	---	---	4	---
2	In Vivo	5	5	3	1	1	---
	In Vitro	5	5	---	---	4	---
3	In Vivo	4	4	4	---	---	---
	In Vitro	4	4	---	---	4	---
4	In Vivo	6	6	2	2	2	---
	In Vitro	6	6	---	1	5	---
5	In Vivo	5	5	4	---	1	---
	In Vitro	5	5	---	---	5	---
6	In Vivo	5	5	3	---	2	---
	In Vitro	5	5	---	1	4	---
7	In Vivo	6	6	6	---	---	---
	In Vitro	6	6	---	---	6	---
8	In Vivo	7	7	3	1	3	---
	In Vitro	7	7	---	1	6	---
9	In Vivo	6	6	6	---	---	---
	In Vitro	6	6	---	---	6	---
10	In Vivo	4	4	2	---	2	---
	In Vitro	4	4	---	---	4	---

APPENDIX E:

IN VITRO CULTURE OF ONE- AND TWO-CELL RABBIT EMBRYOS IN LOW AND HIGH PROTEIN SUPPLEMENTED MEDIUM

One- and 2-cell rabbit embryos recovered from superovulated donors were used in this experiment. Embryos were subdivided into two groups containing equal numbers of 1- or 2-cell rabbit embryos. Each subgroup was then washed 3 times in protein free DPBS followed by random allocation to culture in either low (< 30 μg total protein) or high (15 mg/ml BSA) protein supplemented HL-1 (Ventrex Corp.) medium. Individual embryos from each subgroup were then placed into 1 ml of the appropriate medium which was held within a single compartment of a 24 well plastic tissue culture plate. Culture plates were then placed within an incubator maintaining a 37°C temperature with an atmosphere of 5% CO_2 in humidified air. Culture was continued for a 120 hour period. Embryonic development was monitored every 24 hours.

All embryos cultured in HL-1, without macromolecular supplementation, successfully cleaved to the morula stage, but only 6/20 embryos formed a blastocoele following 120 hours of in vitro culture. HL-1 fortified with 15 mg/ml BSA proved to be a superior medium as all 20 cultured embryos developed to the blastocyst stage, with 12/20 hatching from the zona pellucida.

Table E1. In vitro culture of one- and two-cell rabbit embryos in low and high protein supplemented medium

Medium	n	Time in culture	Number of			
			Morula	Blastocyst	Hatching	Hatched
HL-1[a]	20	72	20	---	---	---
		96	18	1	1	---
		120	14	4	1	1
HL-1 + 15 mg/ml	20	72	15	5	---	---
		96	1	10	9	---
		120	---	2	6	12

[a]Commercial medium containing less than 30 µg of total protein. (Donated by Ventrex Corp.)