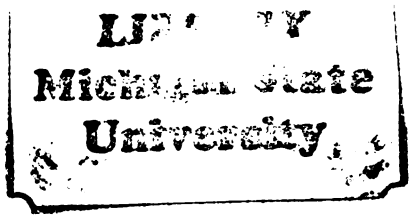

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Blastomere separation of preimplantation embryos in the golden hamster and squirrel monkey as a method for production of monozygotic twins.

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BLASTOMERE SEPARATION OF PREIMPLANTATION EMBRYOS IN THE GOLDEN HAMSTER AND SQUIRREL MONKEY AS A METHOD FOR PRODUCTION OF MONOZYGOTIC TWINS

By

Richard Graham Rawlins

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ABSTRACT

BLASTOMERE SEPARATION OF PREIMPLANTATION EMBRYOS IN THE GOLDEN HAMSTER AND SQUIRREL MONKEY AS A METHOD FOR PRODUCTION OF MONOZYGOTIC TWINS

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Development of techniques for blastomere separation, production of viable multiplet embryos, and the in vitro culture of isogenic and control embryos to development stages suitable for embryo transfer were investigated using hamster (Mescocricetus auratus) and squirrel monkey (Saimiri sciureus) embryos. Experimental protocols were first applied to hamster embryos of 2-, 4- and 8-cells, fertilized in vivo; they were then applied to monkey embryos fertilized and cultured in vitro.

Viable multiplet hamster embryos (assayed by morphology and the exclusion of the vital dye trypan blue) were produced by mechanical separation and by chemical and mechanical separation of blastomeres. Pretreatment of parent embryos with a 0.25% solution of pronase lysed the zona pellucida and a solution of calcium and magnesium-free phosphate buffered saline with 0.02% EDTA reduced

intercellular adhesion prior to microsurgery and increased the post-surgical survival of multiplet embryos. Mechanical separation was used to produce a viable half-embryo from an in vitro fertilized 4-cell squirrel monkey embryo. Chemical pretreatment had no detrimental effect on control embryos. None of the multiplet embryos produced underwent further development after transfer to in vitro culture at 37°C in 5% CO2 in air. Control embryos of the same developmental stages were successfully cultured to morula and blastocyst stage in TALP. A total of 193 hamster embryos were subjected to blastomere separation and 1285 were cultured in vitro to test the efficacy of four different culture media. These included TC-199 with pyruvate, TC-199 with 20-30% pregnant hamster serum, Tyrodes solution with 20-30% pregnant hamster serum, and TALP.

With love and gratitude for their patience and support:

To my parents,

Herbert Lee and Eleanor Graham

Rawlins

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INTRODUCTION

Microsurgical production of viable monozygotic multiplet embryos by blastomere separation of single parent embryos has been routinely performed in rodents and has resulted in the live birth of normal, identical offspring. In the last five years, artifically produced sets of normal twins, triplets and quadruplets have been derived from domestic livestock. The technique has not been applied to nonhuman primate embryos, but the recent success of embryo transfer in two species of nonhuman primates now offers the possibility that monzygotic embryos generated by microsurgery can now be transferred and maintained in utero to term. The present study was directed at the development of techniques for blastomere separation, production of multiplet embryos, and in vitro culture of the isogenic embryos to cell stages suitable for embryo transfer in the hamster (Mesocricetus auratus) and squirrel monkey (Saimiri sciureus). Experimental protocols for blastomere separation and embryo culture were first attempted using hamster embryos fertilized in vivo prior to use on the less readily available in vitro fertilized monkey embryos.

The goals of the study were to develop a successful micro-surgical procedure for blastomere separation in the hamster and squirrel monkey, to determine the viability of the surgically produced multiplet embryos, and to evaluate in vitro culture methods that would support continued growth and differentiation of the monozygotic embryos.

The first objective of using blastomere separation to produce multiplet primate embryos is maximization of output of in vitro fertilization systems utilizing primates by increasing the numbers that can be generated. Blastomere separation and subsequent embryo culture in vitro also permits sex determination and assay of chromosomal and biochemical normality of preimplantation embryos while identical multiplets are maintained undisturbed and continue to develop without risk in culture. Monozygotic multiplet embryos provide a valuable resource as isogenic animals for experimental use in physiology and behavior. Finally, the successful use of blastomere separation to produce multiplets in primates has direct application to the growing field of human in vitro fertilization and embryo transfer, since the methods developed to assay the normality of half embryos in nonhuman primates can also be used to determine viability of human embryos as well. Thus removal of blastomeres and subsequent culture in vitro can provide a valuable tool for the early detection of genetic or metabolic abnormalities in in vitro fertilized human embryos prior to the costly and often unsuccessful transfer procedure.

LITERATURE REVIEW

BLASTOMERE SEPARATION

An Historical Perspective

Modern techniques for the micromanipulation of mammalian preimplantation embryos are derived from experimental work on embryonic development and differentiation which began 100 years ago. The experimental analysis of development was inaugurated largely by Wilhelm Roux, whose work on the physiology of amphibian embryological development in the 1800's provided the first proof that early embryonic growth is self-differentiated (Roux, 1888). Roux demonstrated that once division had begun, the component blastomeres were totipotent and capable of further development. By introducing a hot needle into one of two blastomeres of a frog (Rana esculenta) 2-cell embryo, half of the embryo was killed and the capacity of the other blastomere to develop was tested (Roux, 1888). The intact blastomere survived and developed while the destroyed blastomere decayed. The experiment produced the first known viable half-embryo and was subsequently replicated many times (Morgan, 1895).

Roux's work was criticized because the killed blastomere had not been removed from contact with the viable half embryo, and it was argued that total isolation of the living

blastomere had not been obtained. Embryologists were not concerned about the dead blastomere interacting metabolically, but they worried that there may have been mechanical constraints placed on the development of the intact blastomere by the presence of the decaying tissue (Spemann, 1938).

Driesch (1891, 1892) was the first to completely separate and maintain two viable blastomeres. Sea urchin embryos (Parechinus sp.) at the two cell stage were placed in sea water and violently shaken for several minutes until the blastomeres dissociated. The isolated blastomeres continued to develop at first as if still attached, producing a half blastula with an opened blastocoele, but the open edges then curved together and produced two normally proportioned embryos of half the normal size. Similar results were later shown for amphibians (Morgan, 1895; Spemann, 1938).

Hertwig (1893) made the first attempt at production of twin embryos in amphibians by bisection of the newt (Triton taeniatus) two-cell embryo. Although he did not succeed at isolating the blastomeres, he introduced a new method for embryo microsurgery which improved upon the crude dissociation of cells by agitation. He constricted the two cell embryo along the first cleavage plane with a hair loop to cut the blastomeres apart. The same technique was later used by Endres (1895) to produce the first set of viable Triton twins by blastomere separation, and by Herlitzka (1897) and Spemann (1938) who confirmed Endres' work. Again, the twins produced were morphologically normal, but

of half the normal size.

It is important to note that these early experiments were directed at understanding the regulation of early embryonic development rather than the production of monozygotic half embryos. At that time, little was known about the capacity of the early embryo for self-regulation during differentiation and it was the work of both Roux and Driesch which gave rise to the concept of totipotency for early embryonic development. Driesch (1891) thought the isolated blastomeres were of equal developmental potential and could regulate development, once disturbed, towards a whole embryo, providing its initial structure was not complex. It was Roux (1888) who provided the evidence that self differentiation in early embryonic development subsequently gave way to what he called dependent differentiation which resulted in the loss of totipotency of the blastomeres and subjected them to the limiting influences of the other cells such that independent development was lost and mosaic development occurred (Spemann, 1938; Nicholas and Hall, 1942).

Studies of half embryo development in echinoderms and amphibians continued into the 1900's, but the emphasis shifted from examination of the developmental potential of the half embryo toward consideration of the interactions between blastomeres as assessed by isolation of individual cells. The goal was resolution of the conflict between Driesch's concept of totipotency and Roux's theory of mosaic

development. In time, it was shown that neither concept was correct. Species-specific differences in the capacity of embryonic tissue to reconstitute itself following manipulation were identified, and attention turned away from earlier work, which had shown only the potency of an isolated part of the embryo to develop, toward more detailed analyses of the mechanics of total organization of the embryo during ontogeny (Spemann, 1938; Nicholas and Hall, 1942).

In the first three decades of this century, blastomere isolation was confined experimentally to echinoderms and amphibians. Huber (1915) suggested the use of mammalian embryos in isolation studies, but there are no reports of any such work available. The prevailing view was that higher life forms could not be used for blastomere separation studies since, from the beginning of embryogenesis, potentiality was limited as a result of the increased specialization of mammalian species relative to lower life forms (Nicholas and Hall, 1942).

Use of Mammalian Embryos for Blastomere Separation and New Microsurgical Methods

The first known attempt to use mammalian embryos for blastomere separation studies was undertaken by J. S. Nicholas and B. V. Hall at the Osborn Zoological Laboratory of Yale University in 1933. They removed 2-cell stage rat embryos from the oviducts, rinsed them in acidified calcium-free Ringer's solution to remove the zona pellucida, and

separated the blasomeres mechanically with a fine hair or glass needle. Once bisected, the cells were transferred to the left uterine horn of a host female nat for culture. Most of the blastomeres were lost, but a few did implant and produce embryos which were dissected from the uterus at 10 days (Nicholas and Hall, 1933).

Nicholas and Hall (1933, 1942) introduced important new techniques for embryo manipulation. A glass pipette was developed for holding and stabilizing the embryo during surgery. The pipette was drawn to a distal aperture of about 25% the diameter of the embryo and placed in a universal joint clamp so that the point would lie in the field of view of the microscope. The zone pellucida of the embryo was then aspirated into the pipette and the suction tube closed with a clamp to anchor the embryo in place. Then a needle pipette was introduced into one blastomere through the zona pellucida and the blastomere removed to produce a half embryo (Nicholas and Hall, 1942). The incidence of damage to the surviving blastomeres due to the effects of mechanical separation and aspiration was apparently high since, of half embryos produced, none ever developed following transfer (Pincus, 1936).

Hall's (1935) finding that the zona pellucida dissolved in the presence of an acid solution at a rate proportional to the degree of acidity led to development of an acid bath (0.0002 M HCl in Calcium-free Ringer's solution) which would

remove the zona without damage to the blastomeres over a period of about three hours. Once denuded, the blastomeres were washed in Calcium free Ringer's solution which had been buffered to pH 7, and blastomere separation induced by cutting with a glass needle or eyelash, or by forcing them apart with a jet of fluid from a capillary pipette (Nicholas and Hall, 1942). Acid removal of the zona pellucida of the embryo was employed because the resiliency of the zona prevented application of the microsurgical instruments (either a fine knife, glass needle, or eyelash) and reduced the chance of successful blastomere isolation. The chemical method was preferred because it produced two intact half embryos from the same parent embryo and eliminated the risk of mechanical damage to one or both blastomeres. The methods developed by Nicholas and Hall for embryo microsurgery are virtually identical to those employed today.

Despite improved microsurgical techniques and the fact that removal of the zona pellucida did not inhibit further embryonic development, the separated rat blastomeres did not develop beyond the egg cylinder stage after transplantation, and viable offspring were never produced.

During this same period, Pincus (1936) claimed to have transplanted single blastomeres of 2-cell stage rabbit embryos into a host oviduct and produced normally differentiated, but small blastomdermic vesicles from the pseudo pregnant uteri of the recipient does. However, there are no

published data to support the work.

Live Births from Embryos Produced from Isolated Blastomeres

From 1942 to 1952, no additional studies of half embryo production and development were published, but the results of Nicholas and Hall were noticed by Frederick Seidel at the Max Planck Institute, who began working on the isolation and transfer of blastomeres from rabbit 2-cell and 4-cell embryos. Seidel obtained two live rabbits from 2-cell half embryos produced by destruction of one blastomere with a needle (Seidel, 1952). Later work focused on the development of isolated blastomeres from 4-cell stage rabbit (*Oryctolagus cuniculus*) embryos, but only midterm fetuses were obtained (Seidel, 1956, 1960). Identical twins were not produced since Seidel was destroying blastomeres rather than separating them. The work was significant because it was the first report of live births resulting from transplantation of half embryos in mammals and because it represented the first known attempt to culture these embryos in vitro to study embryogenesis after blastomere destruction (Seidel, 1960).

Laser Destruction of Blastomeres

Blastomere isolation by selective destruction and in vitro culture of the resulting embryos was carried a step further by Daniel and Takahashi (1965), who used a ruby laser as a microsurgical tool to destroy individual blastomeres of

2-, 4-, and 8-cell rabbit embryos. The remaining blastomeres did survive and were cultured in vitro, but did not progress beyond midmorula stage (32-64 cells). Typically, the 1/2 blastomeres cleaved four times to produce 16 cells, the 1/4 blastomeres cleaved 3 times and reached 8 cells, and the 1/8 blastomeres divided to 4 cells (Daniel and Takahashi, 1965). The laser produced embryos were not transferred to recipient does to test their viability in an in vivo system, but the results of in vitro culture of these isolated blastomeres were not particularly good in comparison to Seidel's success with in vitro culture of isolated rabbit blastomeres.

The Role of the Zona Pellucida in the Development of Manipulated Embryos

Moore et al. (1968) extended Seidel's work by examining the developmental potential of single blastomeres from rabbit embryos at the 2-, 4-, and 8-cell stage to determine if blastomeres isolated from the later developmental stages remained totipotent. Blastomeres were by mechanical removal of the zona pellucida and mechanical separation of the cells with a glass or silvered steel needle attached to a micromanipulator. Blastomeres were also left within the zona, and these were produced by needle destruction of the surrounding blastomeres after puncture of the zona pellucida. Rabbit serum was injected into the embryo after destruction of the blastomeres to disperse the cytoplasm and the damaged blastomeres were removed. The dissections were carried out

in homologous serum with 300-500 I.U. of pencillin per ml. of fluid in a welled glass slide. The isolated blastomeres of 2-, 4-, and 8-cell embryos, zona-free, with zona, or injected into a surrogate zona pellucida, were then transferred to the oviducts of synchronized recipient does. None of the zona-free single blastomeres survived, but 30%, 19%, and 11% of the 2-, 4-, and 8-cell blastomeres left in the zona developed into normal live offspring. About 34% of the 4-cell blastomeres placed in surrogate zonas underwent one or two additional cleavages, but none survived to blastocyst stage. No 8-cell blastomeres were placed in host zonae in these experiments. The results of mechanical separation of rabbit blastomeres showed a progressive decrease in the survival rate of single blastomeres with increasing cell stage of the parent embryo. It is also worth noting that mechanical separation of the 4- and 8-cell stage embryos resulted in greater damage to the blastomeres because of increased intercellular adhesion and the diminished size of the blastomeres, so that the results may not have actually related to a loss in potential for independent development with advancing cell stage, but rather to an artifact of the experimental procedure. Failure of the zona-free blastomeres to develop after transfer was attributed to leucocytic invasion and digestion of the cells (Moore et al., 1968).

These studies did little to extend Seidel's findings, since the only live births were also produced from

blastomeres isolated by destruction of surrounding blastomeres. However, they are significant because they represent the first attempt to produce identical offspring by mechanical separation of individual blastomeres from rabbit embryos. They also represent the first use of the technique of reintroduction of isolated blastomeres into surrogate zonae pellucidae to protect the cytoplasm from attack by the maternal immune system.

Seven years after Seidel (1952) published the first results of blastomere isolation experiments with rabbits, Andrzej Tarkowski of the University of Warsaw Zoological Institute developed isolated blastomeres of mouse embryos (Tarkowski, 1959a, 1959b). The work was intended to investigate the regulatory capacity of half embryos and to determine the fate of cells arising from blastomeres isolated at simple and complex cell stages.

Experimental manipulation of the embryos was unchanged from Seidel's earlier report. Blastomeres were destroyed under a dissecting microscope by piercing them with a glass needle held in a micromanipulator. The 2-cell and 4-cell embryos were anchored by suction through a patent glass pipette. The microsurgery was done in mouse serum diluted 1:1 with normal saline under a dissecting microscope, and the procedure lasted from 16 to 28 minutes (Tarkowski, 1959b).

After the blastomeres had been destroyed, the embryos were transfered to the oviducts of host females mated the previous night by vasectomized males. The embryos were introduced by making a slit in the ovarian capsule and injecting the material directly into the lumen of the oviduct through the infundibulum. The females received embryos from donor females of a different coat color for genetic confirmation of the transfer should young be produced (Tarkowski, 1959a).

Of 175 half embryos produced from 2-cell embryos, 30.8% implanted and 17.1% developed normally but were not allowed to go to term. Of 21 3/4 embryos transferred, 47.6% implanted and 33.3% developed normally. Three females gave birth to young derived from 1/2 embryos and one female delivered a litter from 3/4 embryos. All of the offspring were normal and reproduced (Tarkowski, 1959b).

In addition to the transfer of blastomeres isolated by destruction of adjacent cells and left within the zona, a series of transfers of denuded half embryos derived from 2-cell parent embryos was attempted. The zona pellucida was removed mechanically by aspirating the embryo into a micro-pipette (70u diameter) and expelling it to rupture the zona. The blastomeres were then separated by a glass needle or a jet of medium from the pipette. The denuded blastomeres did not develop in vivo (Tarkowski, 1959b); Tarkowski and Wroblewska, 1967), but would culture to the blastocyst stage in vitro (Tarkowski, 1971).

Both Seidel (1959, 1960) and Tarkowski (1959) found that isolated blastomeres of the 2-cell and 4-cell embryo would develop to normal blastocysts that were reduced in size, but culture of later cell stage blastomeres resulted in a high incidence of formation of trophoblastic vesicles (blastocysts without an inner cell mass).

Chemical Removal of the Zona Pellucida and Chemical Dissociation of the Blastomeres

Tarkowski and Wroblewska (1967) hypothesized that the cause of trophoblast formation was an epigenetic effect and that the fate of an embryo cultured from an isolated blastomere was linked to its spatial position within the embryo at the morulae stage. Therefore, both the number and arrangement of the blastomeres were significant variables.

Because blastomere isolation induced by destruction of adjacent cells did not permit a test of the epigenetic hypothesis, Tarkowski and Wroblewska (1967) returned to the chemical isolation of blastomeres, as devised by Nicholas and Hall (1942), to collect data on developmental capacities of all blastomeres from a single parent embryo. The study was particularly important methodologically, since 1) chemical removal of the zona pellucida by lysing with a 0.5% solution of pronase (Mintz, 1962) was employed, 2) the blastomeres of the denuded embryos were dissociated by soaking the embryos in a 0.02% concentration of Versene (EDTA) in calcium- and magnesium-free Dulbecco's solution, and 3) the denuded and

isolated blastomeres were cultured in vitro using Brinster's medium (Brinster, 1963). Within 48 hours of incubation, blastocysts formed from the denuded blastomeres, but only 15% of the 8-cell derived embryos were true blastocysts. Transfer of the denuded embryos was not tried due to the previous failure of zona-free embryos to develop in the mouse. Despite the occurrence of trophoblastic vesicle formation instead of normal development to blastocyst stage in the 1/8 embryos, the experiment showed that at least up to the 8-cell stage, the blastomeres remained totipotent. This finding was later substantiated by Kelly (1975, 1977). Tarkowski did not attempt to replace the zona pellucida of denuded embryos derived from in vitro culture of single blastomeres, but he did suggest, probably as a result of the successful in vivo development of single blastomeres which were left within the zona, that such transfers might implant and undergo further development in utero (Tarkowski, 1971).

Tarkowski was apparently unaware that Moore, et al. (1969) had already attempted embryo transfer of pig embryos produced by mechanical separation of 4- and 6-cell parent embryos with subsequent replacement of the zonae pellucidae. They suggested that the failure of the microsurgically produced embryos to develop might relate to a critical interaction of the blastomeres with the parent zona pellucida such that introduction of a blastomere into a foreign zona would

result in cell death, that damage to the zona itself could be responsible, or that leucocytic invasion and digestion of the blastomeres via the rent made in the zona killed the embroys.

The first two ideas were discounted because isolated and denuded blastomeres developed in vitro without any interaction with the parent zona (later substantiated by Menino and Wright, 1979a, 1979b) and because damage to the zona, in the absence of manipulation of the blastomeres, did not significantly decrease embryo survival in vivo following transfer. They concluded that a significant change must occur in either the embryo or the uterine environment once the blastocyst hatches from the zona pellucida in order for the embryo to survive (Moore et al., 1969).

Production of Monozygotic Embryos by Blastomere Separation

Shortly after publication of Moore's (1969) paper, Mullen et al. (1970) produced the first set of identical twins in the mouse produced by microsurgically splitting a 2-cell embryo and culturing each half embryo in separate containers until compaction occurred. The embryos were then transferred to the reproductive tract of a surrogate mother and live offspring were born (Mullen et al., 1970). Similar results were obtained by Fiser and Macpherson (1975) who produced 54 pairs of identical twin blastocyst embryos by chemical dissociation (Calcium- and Magnesium-free Brinster's medium plus 0.02% EDTA) of 2-cell mouse embryos.

They argued that the reproductive potential of selected females of a mammalian species could be multiplied by a process of superovulation, embryological cloning, and embryo transfer (Fiser and Macpherson, 1975).

Trounson and Moore (1974) continued attempts to produce identical offspring in domestic animals by embryo splitting. The earlier work with rabbits and pigs clearly showed that survival and development of single blastomeres in recipient females required the enclosure of the half embryo in a relatively intact zona pellucida to protect the denuded blastomeres from desruction in utero.

Two approaches were considered. The blastomeres could be protected from direct exposure to the uterine environment, or denuded blastomeres could be cultured in vitro to blastocyst stage, when they would normally shed the zona, and then be transferred to a host female. The second method produced live offspring. Fertilized ova from superovulated Merino ewes were reocovered on days 4-7 by lavage with Dulbecco's phosphate buffered saline (PBS) and the morula were treated with a 5% solution of pronase (Sigma) for 1.5 to 8 minutes to remove the zona pellucida or reduce it to a thin shapeless membrane. The embryos were then washed in PBS with 20% serum to remove residual pronase and transferred to a glass container in the same medium for micromanipulation at 35°C. Sterile glass needles were used to remove any remnants of the zona and to cut the inner cell

mass of each morula in two. In later trials, the enzymatic removal of the zona was discontinued because it was found mechanical removal did not damage the blastomeres (Trounson and Moore, 1974).

Once divided, the half embryos were rinsed in PBS with 20% serum, then incubated for 48 hours at 37.5°C in polystyrene tubes. Over 80% of the embryos continued development to expanded blastocysts. The embryos were then transferred to recipient ewes. Fourteen half embryos, prepared from seven parent embryos, reached blastocyst stage and were transferred individually to surrogate mothers. Only two of these females lambed (14.3%), but the experiment produced the first live births of domestic livestock from half embryos. They were not identical twins (Trounson and Moore, 1974).

Tarkowski and Rossant (1976) extended microsurgery on mouse embryos to include production of haploid mouse blastocysts by mechanical separation of the pronucleate embryo into two havles, each containing one of the pronuclei. Haploid embryos remained viable and developed to blastocysts with a distinct inner cell mass. The haploid cell lines provided a new source of material for the study of gene action. Tarkowski (1977) also reported that preimplantation development in these embryos required the presence of the X chromosome since none of the Y bearing, androgenetic embryos developed beyond the 4-cell stage.

Epstein et al. (1978) published an additional and previously unreported use for blastomere separation in genetic analysis the following year. Because the most reliable method for embryonic sex determination is chromosome analysis, and because preparation of the cells for karyotyping is destructive, embryos could not assayed if other cytological or metabolic work was to be done. To circumvent this problem, half embryos were prepared by dissociation of 2-cell mouse embryos and culturing them in vitro, reaching twin blastocyst stage after 2.5 days. One half embryo was frozen for later use in enzyme assays and the other prepared for chromosome analysis. The twin blastomeres developed to blastocysts 71% of the time and readable karyotypes were obtained from 47% of the half-blastocysts. Frozen embryos were pooled in groups of five each of the same sex and tested for HGPRT and APRT activity (hypoxanthine-guanine-phosphoribosyl transferase and adenine phosphoribosyl transferase) to assay X chromosome function in vitro. No embryo transfers were done (Epstein et al., 1978).

Moustafa and Hahn (1978) improved on Mullen's (1970) success with mouse blastomere separation and embryo development by splitting morulae. Sixteen cell embryos were collected from superovulated females, and divided with micromanipulators following mechanical removal of the zona pellucida or chemical digestion with pronase (Moustafa and Brinster, 1972). Half embryos were cultured in Brinster's

medium at 37.5°C in 5% CO_2 and air. Over 80% of the embryos developed to the blastocyst stage after 72 hours of culture. Twenty pairs of half embryos, incubated for 30 hours following surgery, were transferred to host females and 8 sets of identical twins were delivered (40%). Fourteen of these reached puberty and showed normal reproductive performance. The results showed the production of monozygotic twins in mice could be routinely accomplished in at least one mammalian species, but only under conditions where the bisected embryo could be cultured in vitro prior to transplantation and a relatively low success rate tolerated. Later work confirmed these results (Dyban and Sekirina, 1981; Tsunoda and McLaren, 1983).

Tsunoda and McLaren (1983) improved the survival rate of mouse half embryos produced by bisection of 2-cell parent embryo by avoiding in vitro culture following surgery and using the ligated oviduct of a host female to culture the half embryos for two to four days before transfer to surrogate mothers (8-12% in vitro vs. 65% in vivo culture). The methods used were developed by Willadsen (1979, 1982).

Production of Monozygotic Multiplets in Domestic Species by Blastomere Separation

Willadsen (1979) introduced new techniques for blastomere separation which permitted production of monozygotic twins (sheep) by intermediate culture of the half embryos in a host oviduct, rather than in vitro, prior to embryo transfer.

The methods were devised to provide protection for the embryo in vivo following surgery (Trounson and Moore 1969).

Preliminary experiments showed that 2-cell sheep embryos with damaged zonae pellucidae could be embedded in an agar chip, transferred to a ligated rabbit oviduct for in vivo culture, and would continue normal development as long as the agar plug remained intact (Willadsen, 1979). Two cell embryos were then collected and mechanically separated in PBS at room temperature. The half embryos were produced by slicing the zona pellucida along the interblastomeric groove with a glass needle mounted on a micromanipulator, aspirating the blastomeres out of the zona with a micropipette, separating the blastomeres by suction rather than cutting, and injecting the isolated cells back into surrogate zona prepared from previously evacuated embryos. The half embryos were then embedded in a 1.2% solution of agar (DIFCO) in 0.9% NaCl in distilled water cooled to 36-38°C. Pairs of monozygotic embryos were embedded and expelled into PBS as a solid cylinder. Several sets of twin embryos were then embedded into a larger cylinder of agar and transferred to the ligated oviducts of recipient ewes on the first or second day of the estrous cycle. The embryos were removed by lavage after 3.5 to 4.5 days and were dissected out of the plug. Embryos which reached late morula or early blastocyst stage were transferred to host females in days 5-7 of the estrous cycle. Sixteen sets of monozygotic twins

were transplanted, one pair to a single ewe. Eleven of 16 host females became pregnant (68%) and 10 went to term, producing 5 sets of identical twins and 5 single lambs (Willadsen, 1979). The success paved the way for further experiments in species that could not be cultured in vitro to developmental stages adequate for embryo transfer.

Willadsen next investigated the use of a surrogate zona pellucida, the agar embedding technique and cryopreservation for supporting development of multiplet embryos derived from single blastomeres obtained from 2-cell, and pairs of blastomeres from 4-cell, and 8-cell sheep (Ovis sp.) embryos. In this series, the ligated oviduct of a non-synchronized host ewe was used to incubate the embedded half embryos until they reached an age of 5.5 to 6.5 days. The embryos were recovered and if they had developed to late morulae or early blastocysts, one of each monozygotic pair was transferred immediately to a ewe in day 6 of the estrous cycle (all but a few of the host females entered estrous naturally, but some were induced with prostaglandin). The other half was frozen in 1.5 M DMSO in PBS to test the ability of the manipulated embryos to withstand cryopreservation.

Embryos were frozen by cooling at 0.3 C/min to -36°C, then at 0.1° C/min to -60°C before placement at 196°C in liquid nitrogen. Frozen embryos were thawed by rapid warming from -196° to -50°C, then a rate of 4°C/min to

-10°C prior to rapid thawing to room temperature (Willadsen, 1977). Frozen embryos were transplanted when the recipient of one of twin halves did not return to estrous (Willadsen, 1980).

Use of the nonsynchronized ewe as the primary recipient of the half embryos for the brief period of culture in the ligated oviduct had no detrimental effect on development. All sets of monozygotic embryos recovered developed at a normal rate, but were reduced in size relative to ordinary day 7 sheep embryos and contained only half the number of cells. The size reduction did not affect embryo survival. The pregnancy rate (78.6%) and survival rate (80.0%) were comparable to those observed for transfer of intact sheep embryos. There was no difference in survival rates for half embryos derived from 2-, 4- or 8-cell embryos, suggesting that at least up to the 8-cell stage the blastomeres remain totipotent. Out of 30 half embryos transferred, 25 lambs were born (83.3%). Of these, three sets of monozygotic twins (2 sets from 8-cell parent embryos) were produced. Nine of the 12 frozen half embryos were transferred and three lambs, each a monozygotic twin of a lamb produced in the first transfer series, were born (Willadsen, 1980). The success of this experiment proved that multiplet monozygotic embryos could be produced from domestic species from preimplantation embryos at a late stage of development and marked the first attempt to freeze and thaw half embryos for

subsequent embryo transfer. Although the survival rate of the cryopreserved twins was below that of both intact and half embryos transferred immediately to host females, the delivery of 25% of the frozen embryos as normal term neonates demonstrated that isogenic animals of different ages could be produced by delay of embryo transfer through freezing.

Having shown the utility of the embedding and in vivo culture techniques for production of monozygotic twins in sheep, Willadsen and Polge (1981) used the same methods to attempt preparation of monozygotic quadruplets in cattle (Bos taurus) since cattle twins had been shown to be very valuable as experimental animals for physiological research. Quarter cow embryos were derived by separating 8-cell embryos into four groups of two, injecting the blastomeres into surrogate zonae pellucidae, and embedding them in agar plugs for culture in the ligated oviducts of sheep for 90 hours. Of 44 quarter embryos transferred to the sheep, 91% continued to develop and 77% became blastocysts. Twenty-six blastocysts, 13 pairs of monozygotic embryos, were transferred to the tip of the uterine horns of each surrogate mother (cows) on day 7 of the estrous cycle. Nine of the 13 females became pregnant (69%) with six carrying twins (46%). Eight went to full term and delivered one set of monozygotic triplets, two sets of monozygotic twins and one singleton (31%). Normal development was obtained even though the

multiplet embryos had been cultured to blastocyst stage in a foreign species prior to transfer. Also, the first artificially produced quadruplet embryos developed to term.

Willadsen et al. (1981) next integrated the microsurgical production of multiplet embryos in cattle to routine embryo transfer in livestock following artificial insemination. This required evaluation of half and quarter embryos generated by splitting intact parent embryos at the 32 to 60 cell stage. Previous blastomere separation had been done on 2- to 8-cell embryos recovered by laparotomy, but the objective here was to flush day 5 and day 6 embryos at morula stage for manipulation without resort to standing surgery for embryo recovery. The embryos would be obtained just as they are for commercial embryo transfer.

Hereford x Friesian heifers were superovulated with 2000 i.u. of PMSG on day 9 to 14 of the estrous cycle and injected 48 hours later with 750 g Cloprostenol. Ten hours after the onset of estros the females were inseminated with fresh or frozen Hereford semen. Embroys were flushed from the oviducts with PBS on day 5 or day 6 and manipulated as before. Noticeable differences in the microsurgery were noted. Early cell stages could be separated more easily than the morulae and considerable cell damage was induced by the microsurgery. However, the morulae tolerated the splitting and the survival rate of half and quarter embryos did not differ significantly from that of intact transferred

cow embryos (75%). Fourteen pairs of monozygotic twins were transferred and 10 twin pregnancies were reported (71%). Four groups of quadruplet embryos gave rise to three twin pregnancies, but no more than two offspring were derived from the original set of four isogenic embryos.

The results argued against the utility of producing quadruplets, but did demonstrate that late stage embryos would retain the capacity to develop into whole embryos after separation into half and quarter embryos by microsurgery. Live births of monozygotic twin horses were also obtained from quarter embryos produced by splitting 4-cell parent embryos into single blastomeres (Willadsen, 1982). It was subsequently shown that multiplet embryos from cows would survive cryopreservation at a rate (72%) equivalent to that obtained with intact cow embryos (Lehn-Jensen and Willadsen, 1983). For all practical purposes, artificial sets of twins from domestic species could now be consistently produced from nonsurgically recovered embryos and stored indefinitely by freezing.

Willadsen's methods transformed blastomere separation of mammalian embryos from a laboratory exercise directed at understanding the regulation of development in the preimplantation embryo to a manipulative technique of great utility and commercial value in animal husbandry.

Monozygotic twinning was formerly treated as a biological curiosity of the metatherian mammal, the nine-banded

armadillo (Patterson, 1913; Enders, 1966), an event of exceptionally low natural incidence in eutherian mammals (Hamilton and Mossman, 1972; Markert and Seidel, 1981), or the pathological result of induction by introduction of exogenous teratogens (Tuchmann-Duplessis and Mercier-Parot, 1961; Hay, 1964; Ferm and Hanover, 1969; Ferm 1978; Kaufman and O'Shea, 1978; Hsu and Gonda, 1980; Naruse et al., 1983). Willadsen demonstrated that monozygotic multiplet embryos could be artificially generated and transplanted at rates comparable to those obtained from transfer of intact livestock embryos and that normal and reproductively viable offspring would result from the procedure. The discovery was of major significance since the only repeatable methods for induction of twinning in cattle had previously involved the transfer of multiple embryos and polyzygotic embryos did not result in isogenic offspring (Rowson et al., 1971; Heyman and Renard, 1978).

Markert and Seidel (1981) conjectured that production of identical twins would benefit livestock producers by generating twice the number of offspring per embryo. The prospect of delayed transfer of a twin embryo offered the additional opportunity to evaluate phenotypic information from the transfer and maturation of one of the half embryos so that an estimate could be made concerning the value of transfer and maintenance of the frozen twin against the clone which had matured. They also suggested that the cost

of research could be substantially reduced by using artificial multiplet embryos since by eliminating genetic variation in experimental trials, the number of animals needed in a particular research series could be reduced without loss of experimental power.

Further simplification of bovine embryo microsurgery occurred with the discovery (Williams et al., 1982) that morulae could be bisected, introduced into host zonae pellucidae, and cultured in vitro for 24 hours prior to transfer to recipient females without the intermediate steps of embedding in agar and culture in a host oviduct. Even in vitro culture was abandoned when Ozil et al. (1982) showed that bisected morulae and early blastocysts from cattle could be directly transferred to the surrogate and still result in viable monozygotic twins. Although the survival mate of the half embryos, when produced by direct transfer, was lower (51%) than that obtained by Willadsen, the simplification of the microsurgical procedure and embryo culture phase offset this difference in terms of the labor required to generate multiplet embryos (Ozil, 1983; Williams, et al., 1983). Elimination of half embryo embedding and in vitro culture prior to transfer has not met with success in other mammalian species to date.

Today, bovine blastomere separation is routinely used in commercial embryo transfer facilities in the United States, France and England for increasing the numbers of

purebred livestock embryos available for transfer and for the generation of multiple embryos of preselected parentage (Williams et al., 1983; Baker et al., 1984).

Along with commercial applications, experimental work with blastomere separation and production of half embryos continues. Half embryo, conspecific chimeras have been produced by splitting 4- and 8-cell sheep embryos and joining the blastomeres of the two developmentally different embryos. The result is the differentiation of cells derived from the 8-cell blastomeres into the inner cell mass of the chimera and the generation of the trophectoderm from the younger 4-cell blastomeres (Willadsen and Fehilly, 1983). The use of blastomere separation and fusion to produce interspecific hybrids in domestic animals has also been reported, but the results are not yet published (Seidel, 1983a). Sheep-goat chimeras have been generated by joining the isolated blastomeres of 8-cell embryos from one species with blastomeres from 4-cell embryos of the other, so that the inner cell mass derives from the 8-cell line of blastomeres and develops into the fetus and the trophectoderm from the 4-cell line differentiates into the chorionic membranes, allowing the foreign species to be hosted in the surrogate mother since the placenta which supports the fetus matches the host (Seidel, 1983a; Willadsen and Fehilly, 1983). Finally, serial production of monozygotic embryos from the same parent embryo has been attempted in mice by culture of

daughter embryos cloned from the parent, but the method shows only limited potential. Of 174 2-cell embryos resplit after initial derivation from parent 2-cell embryos, only 19% continued development to the blastocyst stage (O'Brien et al., 1983). Continued reduction in cell mass to levels below a metabolic threshold may account for the failure of serially cloned embryos to develop further.

Applications of Blastomere Separation and Monozygotic Multiplet Embryos to Research on Nonhuman Primates and Humans.

To date, there have been no reports of the use of blastomere separation to produce monozygotic multiplet embryos in nonhuman primates. Although the South American marmosets (Callithricidae) routinely twin (Epple, 1967), the natural incidence of twinning in most primate species is very low (Wilson, 1978; Rawlins and Kessler, 1983) and usually produces fraternal rather than identical offspring. Artificial production of isogenic primates would allow genetic control in physiological and behavior research, and increase the reproductive potential of genetically valuable females. Microsurgical production of monozygotic primates could also be used to increase the numbers of embryos availabe for transfer to recipient females as a means of multiplying the numbers of endangered primate species. However, the utility of producing artificial twins through bastomere separation in primates is currently limited by the very low survival rates of the embryo transfers which have

been attempted in nonhuman species. Only five nonhuman primates have been produced by embryo transfer of in vivo and in vitro fertilized embryos to surrogate mothers. Before monozygotic primate embryos can be used in husbandry and conservation, a considerable amount of work must be done to enhance the success of intact nonhuman primate embryo transfers.

Blastomere separation and production of monozygotic nonhuman primate embryos has immediate use in the study of development of the preimplantation embryo. Long term studies of the biochemical and genetic normality of in vitro fertilized squirrel monkey (Saimiri sciureus) embryos have established the utility of nonhuman primates as an animal model for investigation of fertilization and differentiation of the human embryo (Dukelow et al., 1983). In vitro fertilized embryos from the squirrel monkey have been subjected to biochemical (Ghosh et al., 1982; Hutz et al., 1983) and chromosomal assays (Mizoguchi and Dukelow, 1981; Asakawa et al., 1982) to determine the normality of embryonic development. Given the rapid growth of human in vitro fertilization and embryo transfer programs world wide, these studies are of critical importance to the identification of abnormal embryonic development since current ethics and legislation prevent experimental investigation of human embryogenesis.

Despite high levels of fertilization, only a small percentage of the embryos fertilized in vitro develop beyond the 8-cell stage (Chan, 1983). This limits embryo production and research opportunities. Blastomere separation and the generation of multiplet embryos could double the number of embryos that can be obtained from the in vitro fertilization of a limited number of oocytes.

Blastomere separation may also be useful in assessing the genetic normality of primate embryos or chromosomal activation. A half embryo can be held in culture white its twin is subjected to gradual fixation and chromosomal analysis or placed in culture for investigation of biochemical and metabolic normality. Minimally, removal of blastomeres and their subsequent in vitro culture to morulae or blastocysts would provide sufficient material to determine the sex of the embryo by destructive karyotyping while retaining a viable identical twin for transfer and gestation.

MATERIALS AND METHODS

Production and Collection of Embryos

Mature female hamsters (8-10 weeks old) were used as embryo donors. They were maintained on a 14L:10D (lights on from 0600-2000 h) cycle. Females were examined for the presence of a postovulatory vaginal discharge and if a plug was present, they were placed on a superovulatory regimen. Each animal was injected with 30 I.U. of pregnant mare's serum (PMS) (Serotropin, Teizo, Tokyo, Japan) i.p. at 1000 h on the morning of Day 1 (day of ovulatory plug) and injected 76 hours later at 1400 h on Day 4 with 30 I.U. of human chorionic gonadotropin (HCG) (A.P.L., Ayerst Laboratories, Inc. New York, NY) i.p. (Mizoguchi and Dukelow, 1980). The females were mated with males on proven fertility on the evening of Day 4 following the injection of HCG.

At varying intervals after mating, the females were sacrificed by cervical dislocation to obtain embryos at different stages of development. Two-cell embryos were recovered from the oviducts 40 hours post coitum and 4-cell embryos at 60 hours. Eight-cell embryos were obtained from the uterine horns 72 hours after mating and morulae at 78 hours (Ghosh et al., 1982).

Embryos were collected by oviductal and uterine lavage. The oviducts were dissected away from the uterine horns. Once isolated, 0.5 ml of culture medium was injected into the fimbria of the oviduct with a blunted 25 gauge needle on a 1.0 ml sterile plastic tuberculin syringe (Becton-Dickinson) to flush the embryos into a welled glass slide. A retrograde flush with 0.5 ml of culture medium was also used to wash embryos from the uterine horns. Embryo recovery was carried out at room temperature, but the medium injected into the oviduct or uterine horn was maintained at 37°C by warming in a temperature controlled water bath. Embryos were sorted according to developmental stage and counted, under stereoscopic magnification (105x), by aspirating them into the a 5 ul micropipettor (SMI, Emeryville, CA). They were then transferred to an eight chamger LAB-TEK culture slide (Miles Laboratories, Inc., Naperville, IL) filled with 0.5 ml of medium per chamber at 37°C and incubated at 37°C in 5% CO_2 in humidified air, or subjected to experimental manipulation.

Squirrel monkey embryos were obtained by fertilization in vitro. Adult (500 to 600 grams body weight) Bolivian and Guyanan squirrel monkeys (Charles River Research Primates, Port Washington, New York) were housed indoors from November through May on a 12L:12D light cycle (lights on 0600 h to 1800 h) at 21$\pm$ 2°C, and outdoors in gange cages from June to October (Jarosz and Dukelow, 1976). The animals were fed

commercial high protein New World monkey diet (Ralston-Purina, St. Louis, MO) daily along with fresh fruit. Water was avilable ad libitum.

Females were induced to ovulate by exogenous hormone administration. Because the squirrel monkey does not menstruate, stages of the estrous cycle cannot be determined for indivudal animals without the use of vaginal smears or steroid assays. Therefore, induction of follicular growth and oocyte maturation was arbitrarily started five to six days before the scheduled time of laparoscopic oocyte recovery (Dukelow, 1970).

Four daily injections of 1.0 mg of follicle stimulating horomone (FSH-P, Burns-Biotec Labs., Inc., Omaha, NE) were given i.m., followed by a single injection of 250 I.U. of HCG i.m. on the final day of FSH treatment, 16 hours prior to laparoscopy for follicular aspiration (Dukelow, 1979). During the nonbreeding season (July through September) five days of FSH injections were required to induce follicular development (Kuehl and Dukelow, 1975).

Oocytes were recovered by laparoscopy (Dukelow et al., 1971). The monkeys were anesthetized with sodium pentobarbital (15 mg/600 gm animal) 16 hours after the HCG injection. A small midline incision was made in the abdominal wall with a scalpel and the trocar-cannula of the laparoscope (Karl Storz, Tuttlingen, W. Germany) was pushed into the peritoneal cavity through the incision. The

trochar was then removed and the 4 mm O.D. laparoscope inserted. The peritoneal cavity was then insufflated with CO_2 through the cannula.

Once the ovaries were visualized, a 25 gauge needle on a 1 ml sterile plastic tuberculin syringe was pushed through the abdominal wall and used to move the fimbria of the oviduct aside to expose the ovary. The ovarian follicles were then penetrated by the needle and the oocytes aspirated into the syringe, which contained 0.05 ml of tissue culture medium (TC-199, GIBCO, Grand Island, NY) modified to contain 1 mm pyruvate, 100 ug/ml gentamicin (Schering Corp., Kenilworth, NJ), 1 U/ml heparin and 20% heat inactivated (30 min at 56°C) fetal calf serum (FCS, GIBCO) (Asakawa et al., 1982; Chan et al., 1982). The medium was sterilized prior to use by filtration through a 0.45 um Millex@ filter (Millipore Corp., Bedford, MA) and stored in 10 ml Vacutainer (Becton-Dickinson Co.) tubes at 4°C, then warmed to room temperature before laparoscopy.

Aspirated oocytes were transferred directly into 0.25 ml of TC-199 per chamber in sterile 8-chamber culture slides and incubated at 37°C in 5% CO_2 in humidified air. Oocytes were matured for 21 hours in culture prior to insemination.

Semen was collected from adult male squirrel monkeys by electroejaculation (Kuehl and Dukelow, 1974). The animals were placed under physical restraint, and a rectal probe was inserted to deliver short pulses of current (120 pulses/sec

at 5 msec duration). Voltage was varied at 3 sec intervals by a cyclic increase and decrease. The ejaculated plug of coagulum was diluted in modified TC-199 (as above) and held at 37°C for 5-10 minutes to permit release of sperm. Sperm counts were made with a hemocytometer and the oocytes in each chamber of the culture slide were inseminated with 0.05 ml of a sperm cell suspension of 10^5 to 10^6 sperm/ml, 21 hours after collection of the ova (Chan, 1983).

Twenty four hours after insemination and incubation, the oocytes were reexamined on an inverted compound microscope at 450x to determine if fertilization had occurred. The following were used as criteria for fertilization (Chan, 1983).

1.) Presence of two or more polar bodies in the perivitelline space.
2.) Presence of two or more polar bodies and two pronuclei or two or more equal sized blastomeres by 24 hours post insemination.
3.) Observation of the sperm tail or midpiece within the cytoplasm.

Pyknotic and morphologicaly abnormal (shrunken or asymmetrical) oocytes were classed as degenerated. One cell monkey embryos were obtained 24 hours after insemination, 2-cell at 48 hours, and 4-cell embryos at 60 hours (Hutz, 1983).

Embryo Microsurgery

Mechanical removal of the zona pellucida and blastomere separation was based on a modification of techniques developed by Nicholas and Hall (1942) and Willadsen (1979). The zona pellucida was opened and the blastomeres removed by aspiration for division and subsequent replacement within the zona pellucida or culture as denuded embryos.

First, a 5 ul glass micropipette was drawn to a patent needle on an electric microelectrode puller (Stoelting, Chicago, IL.). The tip diameter was approximately 90 u. Second, another 5 ul glass micropipette was pulled to a non-patent needle of about 30 u diameter. Two right angles, opposed and in the same plane, were bent into the pipettes over the open flame of a gas microburner constructed from a 10 gauge stainless steel syringe needle. The bends were required to correct for the difference in height between the micromanipulators and the stage of the microscope (Figure 1). The needles were placed in the instrument holders (patent) of two Emerson Model B micromanipulators (J.H. Emerson Co., Cambridge, MA) which were mounted on either side of the stage of a 7x zoom stereomicroscope. Three dimensional movement of each instrument could be independently controlled, in the vertical Z axis by helical gear adjustment and vertically and horizontally in the X and Y axes of the microscope stage by movement of a single joystick control.

Figure 1: The micromanipulators used for blastomere separation.

For microsurgery, the needles were lowered to the surface of a 150 mm glass petri plate containing a 1 ml drop of culture medium in the center. Intact embryos were then transferred from a welled glass slide with a micropipette to the medium in the petri plate. No more than five embryos were removed from culture at any time. The remaining embryos were maintained at 37°C on a slide warmer while microsurgery was carried out at room temperature on the others.

After transfer, the embryos were located under magnification in an erect image field and each needle was then brought to either side of a single embryo to begin surgery. The patent (left side) pipette was connected to a ruber hose with a spring clamp and suction applied by mouth. When the tip of the 90 u patent needle was brought near the embryo, the suction aspirated the zona pellucida a slight distance into the pipette and the suction line was clamped, anchoring the embryo to the end of the moveable probe. Next the 30 u nonpatent needle was pushed through the zona pellucida on the right side and moved laterally to tear a linear cut across half the circumference of the zona (Figure 2). Care was taken not to damage any of the blastomeres.

Once the zona pellucida had been opened, the nonpatent probe was replaced with a second patent needle 45 u in diameter which was also attached to a suction line. The tip of this probe was blunted by snapping off the end with a

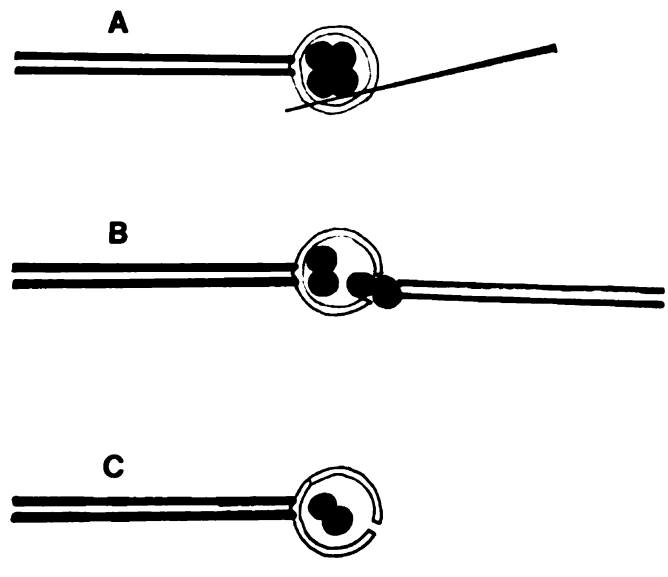

Figure 2. Procedure for mechanical separation of blastomeres. A) Needle is used to open the zona pellucida. B) Patent pipette is used to separate blastomeres. C) Isolated blastomeres are replaced into a host zona pellucida prior to culture or embryo transfer.

pair of microsurgical forceps. The 45 u probe was pushed through the rent in the zona pellucida and brought up against the blastomeres. Under gentle suction, the blastomeres were aspirated from the zona pellucida. While the objective was separation and removal of only half of the blastomeric contents of the embryo, the entire embryo was usually withdrawn due to strong intercellular adhesion. When this occurred, the denuded intact embryo was released from the micropipette and allowed to settle to the surface of the petri plate. The 90 u pipette was then used to prevent movement of the embryo by placing the tip lengthwise behind the blastomeres, and the 45 u probe was replaced with the 30 u needle which was used to divide the blastomeres by pressing down with the tip of the probe along the first cleavage plane of the embryo against the surface of the glass. The divided blastomeres were then picked up with the 45 u patent probe and replaced into a zona pellucida or left denuded.

A micropipette was used to collect the half embryos and transfer them directly to 0.5 ml of culture medium (21-22°C) per chamber in an eight chamber tissue culture slide for incubation, or to Dulbecco's phosphate buffered saline (PBS) (GIBCO) for viability assay by vital dye staining with trypan blue (Kodak, Rochester, NY), followed by culture. A 0.2% solution of trypan blue was used to assess mechanical damage to individual blastomeres incurred during surgery. Exclusion of the dye indicated the plasma membrane of the

embryo was intact and the blastomeres normal. Failure to incorporate the dye also provided an indirect measure of metabolic normality since exclusion of trypan blue correlates strongly with normal metabolic activity as measured by uptake of flourescein diacetate (Hutz, 1983; Ridha, 1983). After treatment with trypan blue, the embryos were rinsed twice in PBS for 30 seconds and placed in culture. All vital embryos were incubated for 24 hours at 37°C in 5% CO_2 and humidified air, then checked for further development. Intact embryos of the same developmental stage were cultured in parallel with the half embryos as controls.

Due to the high incidence of structural damage to individual blastomeres from mechanical removal of the zona pellucida and blastomere separation, chemical removal of the zona pellucida from the embryo and chemical reduction of intercellular adhesion of the blastomeres prior to mechanical separation (Tarkowski and Wroblewska, 1967; Fiser and Macpherson, 1975) was also used.

Initially, treatment consisted only of removal of the zona pellucida from the intact embryo by lysing in a filtered (45 um Millex@ filter) 0.25% solution of pronase (Grade B - Lot 71879, Calbiochem, Los Angeles) in PBS for 30 seconds at room temperature. The zonae pellucidae disappeared within 15 to 20 seconds. Denuded embryos were very sticky and adhered to the surface of the glass petri plate (even when the glass had been treated with organosilane

(Prosil-28, PCR, Inc., Gainesville, FL) as well as to the glass micropipette. This made it very difficult to separate and transfer the embryos, so intracellular adhesion was chemically reduced in later experiments. Denuded embryos were rinsed twice in PBS and transferred to a solution of calcium and magnesium - free PBS (GIBCO) containing 0.02% EDTA as a calcium chelating agent. After 5 - 15 minutes, the embryos were placed in culture medium for microsurgery.

Blastomere separation was accomplished without anchoring the embryo by suction to avoid damage to the cells. The shaft of the 90 u pipette was used to minimize embryo movement by placing it beside the blastomeres lengthwise. The 30 u needle was used to make a vertical cut down and across the cleavage plane of the embryo and the blastomeres usually dissociated without visible structural damage using this method. If an embryo rolled away from the needle, suction was applied to several blastomeres with the 90 u blunt pipette to stabilize the embryo. The half embryos were then assayed for structural damage with trypan blue and if viable, transfered to the incubator for 24 hours. Chemical pretreatment of the embryos prior to microsurgery reduced blastomere injury during manipulation and increased the speed with which the embryo could be bisected. This reduced the total amount of time the half embryo spent out of culture and the method improved experimental results. None of the half embryos was embedded in agar for culture

since it had been previously demonstrated (Seidel, 1960; Moustafa and Hahn, 1978; Menino and Wright, 1983) that zona-free mammalian embryos, especially those of rodents, could be successfully cultured in vitro.

Embryo Culture

Hamster embryos were cultured at 37°C in 5% CO_2 and humidified air for a minimum of 24 hours of incubation. Embryo development was assessed for the 2-, 4-, and 8-cell stage and for microsurgically produced multiplet embryos, with and without zonae pellucidae. Four culture media were tested. These were TC-199 (GIBCO) with 20% fetal calf serum plus pyruvate, TC-199 with 20% and 30% pregnant hamster serum (PHS), Tyrodes solution (GIBCO) with 20% and 30% PHS, and TALP (Tyrode's solution, albumin, lactate and pyruvate) (Bavister et al., 1983).

Modified TC-199 was used because it had previously been shown to support development of in vitro fertilized squirrel monkey embryos (Kuehl and Dukelow, 1979; Dukelow et al., 1983). It was prepared by adding 25 ml of heat inactivated (30 min at 56°C) fetal calf serum (GIBCO) to 100 ml of stock TC-199. After mixing, 1.05 mM of pyruvate (14.4 mg pyruvic acid) were added along with 1.25 ml of gentamicin (Schering Corp., Kenilworth, NJ). The medium was sterilized by filtration through a 0.45 um Millex@ filter and stored in 10 ml Vacutainer tubes at 4°C for no longer than 30 days. Before use the medium was rewarmed to 37°C in a water bath.

The pH ranged between 7.2 and 7.5.

TC-199 with 20 and 30% PHS was used under the assumption that the addition of homologous serum might enhance development of hamster embryos. It was prepared from a stock solution of TC-199 to which pregnant hamster serum was added just before use. The stock solution comprised 100 ml of TC-199, 14.4 mg of pyruvate (1.05 mM), 0.87 ml of sodium lactate syrup (50 mM) and 1.25 ml of gentamicin. The stock solution was filtered (see above) and stored in 10 ml Vacutainer tubes at 4°C for no more than 30 days. Pregnant hamster serum was obtained from embryo donors killed by cervical dislocation. About 2 ml of blood were drawn from each animal by cardiac puncture with a 20 gauge needle mounted on a 3 ml sterile plastic syringe. The blood was transferred to a 3 ml Vacutainer and held in an ice bath for 15 minutes to allow clot formation. Serum was separated by centrifugation for 30 minutes at 5000 rpm in a refrigerated (2°C) Sorvall RC2-B centrifuge with head type HS-4 and pipetted into 3 ml Vacutainers for indefinite storage at -20°C. For each experiment, 2 or 3 ml of frozen PHS were thawed and heat inactivated. Then 8 or 7 ml of TC-199 stock solution were added to the 2 or 3 ml of PHS and mixed for 30 seconds on a tube shaker. The medium was then sterilized by filtration and maintained at 37°C. The pH after addition of PHS was 7.2. Unused TC-199 with PHS was discarded after each trial.

The test of modified Tyrode's solution (GIBCO, Grand Island Biological Co., Grand Island, NJ) was based on the successful use of this medium by Whittingham and Bavister (1974) for culturing fertilized hamster ova to the 2-cell stage. It contained 9.5 g of Tyrode's powder in 1 l of distilled water, with 4.0 g of bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO), 1.0 g of sodium bicarbonate, 0.10 mM of sodium pyruvate, 800 I.U. of gentamicin (12.5 mg in 1.25 ml) and 20% or 39% PHS, heat inactivated and added immediately before use (Fan, personal communication). The pH of the medium was 7.6 after addition of PHS and equilibration with 5% CO_2 in air at 37°C.

TALP (Tyrode's solution, albumin, lactate and pyruvate) was the last culture medium tested. Bavister et al. (1983a, 1983b) had shown it to effectively support development of both hamster and rhesus monkey (Macaca mulatta) embryos in vitro and given the requirement of in vitro culture of rodent and primate embryo in the present study, its use was warranted. TALP was prepared according to Bavister et al. (1983a). It contained 114.0 mM NaCl, 3.2 mM KCl, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25.0 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5.0 mM glucose, 10.0 mM sodium penicillin-G, 1 mg per 100 ml of phenol red and 3 mg per ml of BSA. In addition, four basic amino acids required for the maturation of hamster oocytes in vitro (Gwatkin and Haidri, 1973) were added from a 100% concentrate stock solution which combined the four. Final

concentrations were glutamine (146 mg/l), phenylalanine (16 mg/l), methionine (8 mg/l), and isoleucine (26 mg/l) (Bavister et al., 1983A). The sodium pyruvate and BSA were added immediately before use and the solution was sterilized by filtration (45 um Millex@). The pH ranged between 7.6 and 7.8.

In all experiments, the same culture medium was used for embryo recovery, microsurgery, and embryo culture.

Statistical Analyses

All data were analysed using non-parametric statistics. Media efficacy and experimental protocols were evaluated by Chi-square (contingency table or differences among proportions) tests calculated according to Siegel (1956) and Freund (1971).

RESULTS

Production and Viability of Hamster Half-Embryos

A total of 193 hamster embryos were subjected to blastomere separation (Table 1). Two, 4-, and 8-cell embryos were used to produce multiplet embryos by splitting each embryo in half with the micromanipulators with or without chemical pretreatment. Of a possible 386 demi-embryos, 194 survived surgery, as assayed by either morphological integrity of the blastomeres (Tarkowski, 1971) or by exclusion of the vital dye, trypan blue (Hutz, 1983; Ridha, 1983). The survival rate before culture in vitro was 50.3%. Of all the multiplets produced, 27% were successfully replaced within homologous zonae pellucidae. There was a significant difference in the surgical survival rate of the embryos which varied according to the developmental stage of the parent embryo. The 2- and 8-cell parent embryo survival rates of 63.2% and 55.9% respectively did not differ significantly ($X^2 = 1.18$, $p >$ than .05), but both survival rates were significantly different from the 33.6% rate of 4-cell embryos ($X^2 = 20.94$, $p < .001$). There was no significant difference by developmental stage in the proportion of viable multiplets returned to zonae pellucidae ($X^2 = 1.18$, $p > .05$).

TABLE 1

BLASTOMERE SEPARATION OF HAMSTER EMBRYOS
(Both Methods Pooled)

Stage	Embryos	Multiplets Surviving	%	Multiplets Placed in Zona P.	%	Development in Culture
2-Cell	38	48/76	63.2	4	8.3	0.0
4-Cell	61	41/122	33.6	20	48.7	0.0
8-Cell	94	105/188	55.9	29	27.6	0.0
Total	193	194/386	50.3	53	27.3	0.0

Multiplet Survival by Developmental Stage

$X^2 = 20.94$

$df = 2$

$p < .001$

Note: Viability assayed by morphological appearance and exclusion of the vital dye, Trypan Blue (Kodak, Rochester, NY).

The differential survival of 2-cell and 8-cell half-embryos was thought to be an experimental artifact since the procedure for separation of blastomeres was changed during the study. Intercellular adhesion increases from the 2- to 8-cell stage and the expected result would be to see fewer numbers of the more developed embryos giving rise to viable multiplets than was observed, since separation of blastomeres should be progressively more difficult as development of the parent embryo proceeds. The survival rates for multiplet embryos produced by mechanical and chemical procedures were compared to determine if the change in protocol influenced the pooled results on survivorship.

The Effect of Chemical Pretreatment on Half-Embryo Viability

Table 2 shows the numbers of multiplet embryos derived from 4- and 8-cell parent embryos by mechanical and chemical separation. Survivorship of multiplets from chemically pretreated 4-cell embryos was 87.5% versus 25.4% for those divided mechanically. The difference was significant (X^2 = 23.97, $p < .001$). Similar results were found for the 8-cell embryos, of which 46.3% of the mechanically separated embryos survived versus 82.0% for the pretreated half-embryos. This difference was also significant (X^2 = 18.89, $p < .001$). Six times the number of 4-cell parent embryos were divided mechanically relative to chemically pretreated embryos, but only 3 times more 8-cell parents were separated. The pooled results for 4-cell embryonic

mortality following surgery reflect these proportions, but the factor responsible for the difference in survival for mechanically separated 4- and 8-cell embryos is not apparent. The survivorship of mechanically bisected 8-cell embryos was significiantly greater than that of the 4-cell derivatives (X^2 = 11.2, $p < .001$). There was no significant difference in multiplet survival between 4- and 8-cell embryos when chemically pretreated (X^2 = 0.26, $p > .05$). The data in Table 2 clearly show the efficacy of chemical removal of the zona pellucidae and reduction of intercellular adhesion in facilitating blastomere separation. Multiplet embryos produced by chemical pretreatment consistently had less injury following surgery and showed less structural deformation thatn did embryos produced by mechanical separation. Figure 3 compares the morphology of half embryos produced by both methods.

Despite development of a successful procedure for separating blastomeres from parent embryos without apparent cell damage, none of the multiplet embryos underwent further development in vitro. Retention of the zona pellucida had no effect. Morphologically normal half-embryos, assayed by trypan blue, placed immediately into four different types of culture media, and with or without the protection of a zona pellucida did not reorganize to whole embryos and died in vitro.

TABLE 2

A COMPARISON OF MULTIPLET EMBRYO PRODUCTION IN THE HAMSTER
BY
MECHANICAL AND CHEMICAL SEPARATION OF BLASTOMERES

Developmental Stage of Embryo

	4-Cell	8-Cell
Mechanical		
Embryos	53	69
Survivors	27/106	64/138
Percentage	25.4	46.3
Chemical		
Embryos	8	25
Survivors	14/16	41/50
Percentage	87.5	82.0
	$X^2 = 23.97$ df = 1 $p < .001$	$X^2 = 18.89$ df = 1 $p < .001$

Note: Survivorship assayed by morphological normality and by exclusion of the vital dye Trypan Blue (Kodak, Rochester, NY).

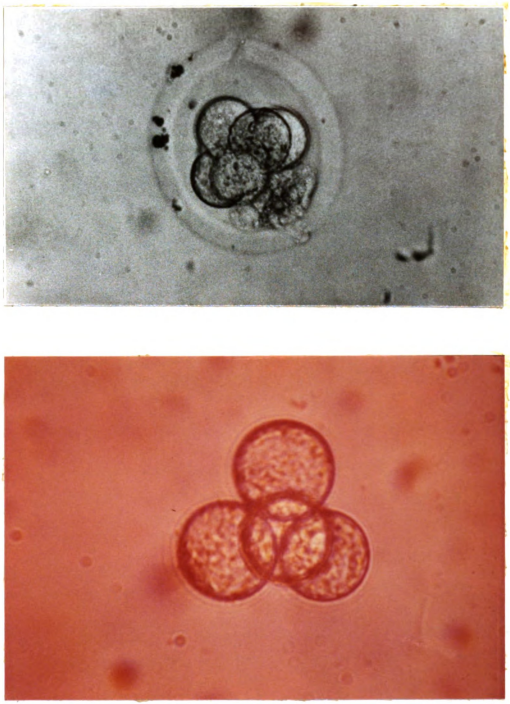

Figure 3. A comparison of the morphology of hamster half-embryos derived from blastomere separation of 8-cell embryos by mechanical (A) and chemical/mechanical (B) methods.

The Effect of Culture In Vitro on Half-Embryo Development

Five viable half embryos, derived from 8-cell parents, were replaced in zonae pellucidae and transferred to the tip of the left uterine horn (ligated) of a synchronized female (Rihda, 1983) which had been mated with a vasectomized male. Five days later the female was sacrificed and the uterus was flushed with culture medium. No embryos were recovered. The uterine horn was then opened and examined for implanation sites, but none were found. Half-embryos within the zonae pellucidae did not develop in vivo.

Differential Development of Control Embryos In Vitro

Given the differential survivorship of multiplet embryos by developmental stage, a comparison of in vitro culture of intact control embryos of the same cell numbers was made. Table 3 shows the proportion of 2-, 4-, 8-cell and morula stage embryos which underwent further development after 24 hours of in vitro culture (results from all culture media were pooled). A total of 1285 embryos were tested. The results show a significant increase ($X^2 = 160.4$, $p < .001$) in development with advancing embryonic age. Note that none of the 353 2-cell embryos placed in culture developed, regardless of the culture medium used. When the 2-cell embryos were excluded from analysis, the increased development of morulae relative to 8-cell embryos in culture was still significant ($X^2 = 19.89$, $p < .001$). The data suggested the 8-cell embryo would be the most suitable

TABLE 3

A COMPARISON OF CULTURE IN VITRO OF HAMSTER EMBRYOS BY DEVELOPMENTAL STAGE (POOLED FOR ALL MEDIA)

Stage	Embryos	Development 24h +	Percentage
2-Cell	353	0/353	0.0
4-Cell	297	72/297	24.2
8-Cell	593	379/593	63.9
Morula	42	41/42	97.6

All Stages	8-Cell vs. Morula
$X^2 = 160.4$	$X^2 = 19.89$
df = 2	df = 1
$p < .001$	$p < .001$

parent for blastomere separation given the rate of development of 8-cell controls. Morulae were not used in this study, despite their routine bisection for monozygotic twin production in domestic cattle, because this developmental stage exceeds the maximum development obtained with in vitro fertilized squirrel monkey embryos.

Efficacy of the Culture Media for Control Embryo Development

Next, the ability of the four culture media to support continued development of intact preimplantation hamster embryos was examined as an indirect test of ability to support half-embryo growth. Table 4 compares the development of 2-, 4-, 8- cell and morula stage control embryos in TC-199 with pyruvate, TC-199 with PHS, Tyrodes solution with PHS, and TALP.

TC-199 with pyruvate supported no further embryonic development regardless of developmental stage and was abandoned as a culture medium. It is excluded from statistical analysis here. The addition of 20-30% PHS to TC-199 markedly improved in vitro culture of 8-cell embryos, but had no effect on the 4-cell stage. Only 23.4% of the 8-cell controls underwent additional cleavage in TC-199 with PHS. Tyrodes solution with 20-30% PHS more than doubled devleopment relative to TC-199. In this medium 35.3% of 4-cell and 58-1% of 8-cell embryos developed to the 8-cell and morula stages respectively. None became blastocysts. In TALP, 2-cell embryos would not continue growth, but 30.1% of the

TABLE 4

A COMPARISON OF HAMSTER EMBRYO CULTURE BY DEVELOPMENTAL STAGE AND CULTURE MEDIUM

Medium	Stage	Embryos	Development 24h	Percentage
TC-199 + Pyruvate*	2-Cell	3	0	0.0
	4-Cell	33	0	0.0
	8-Cell	29	0	0.0
TC-199 +PHS*	4-Cell	28	0	0.0
	8-Cell	124	29 (a)	23.4
Tyrodes +PHS*	4-Cell	17	6 (a)	35.3
	8-Cell	31	18 (a)	58.1
TALP*	2-Cell	350	0	0.0
	4-Cell	219	66	30.1
	8-Cell	409	332 (b)	81.2
	Morula	42	41	97.6
TOTAL		1285	492	38.3

(a) None developed beyond morulae.
(b) Developed to late morulae and early blastocysts.

* See text for the description of the culture media.

4-cell and 81.2% of the 8-cell embryos developed to 8-cell and early morulae stages. In addition, 39.6% of the 8-cell embryos became blastocysts (Table 5). This stage was not reached by 8-cell embryos in the other media. Finally, 97.6% of the morulae cultured in TALP developed to blastocyst stage. The differences in the percentage of embryos developing in the three culture media (excluding TC-199 with pyruvate) were significant for both 4-cell ($X^2 = 11.95$, $p < .001$) and 8-cell embryos ($X^2 + 145.39$, $p < .001$). There was no significant difference in 4-cell development between Tyrodes solution with PHS and TALP ($X^2 = 0.198$, $p > .05$), but TALP was superior for the culture of 8-cell embryos ($X^2 = 9.45$, $p < .001$). Based on this finding, TALP was used as the culture medium for control and manipulated 8-cell embryos exclusively. However, none of the half-embryos developed in TALP, despite the fact that the control embryos routinely cultured into blastocysts (see Figure 4).

The Effects of Chemical Pretreatment on the Development of Intact Embryos

Detrimental effects on embryonic development from chemical pretreatment were evaluated next. Table 5 compares the development of 8-cell embryos left intact within the zona pellucida, denuded by removal of the zona pellucida with 0.25% pronase, and of 8-cell embryos denuded and washed from 5 to 15 minutes in a solution of calcium and magnesium-free PBS with .02% EDTA at room temperature (22° C) prior to incubation in TALP at 37° C in 5.0% CO in air. Of the

TABLE 5

A COMPARISON OF THE EFFECTS OF TREATMENT WITH PRONASE AND WITH CALCIUM AND AND MAGNESIUM-FREE PBS PLUS EDTA* ON THE DEVELOPMENT IN VITRO OF 8-CELL HAMSTER EMBRYOS IN TALP

	Embryos	Development 24h +	%	Blastocysts	%
Controls	409	332/409	81.2	162	39.6
Pronase Only	264	209/264	79.2	131	49.6
Pronase, Calcium Magnesium-free PBS with EDTA	111	62/111	55.9	35	31.5

All Development	Development to Blastocysts
$X^2 = 32.65$	$X^2 = 12.25$
df = 2	df = 1
$p < .001$	$p < .01$

*see text for concentrations

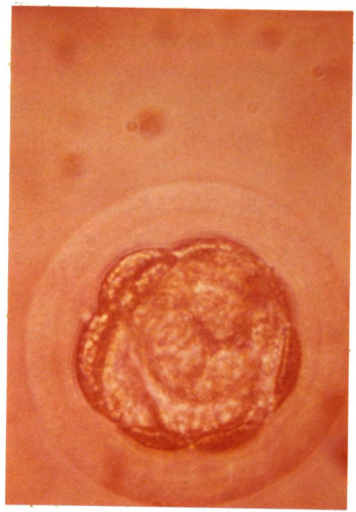

Figure 4: An example of a blastocyst, within the zona pellucida, cultured in vitro from an 8-cell hamster embryo in TALP.

controls, 81.2% underwent further growth and 39.6% developed into blastocysts. The embryos without zonae pellucidae were inclubated in TALP immediately following a 30 second treatment with pronase followed by two rinses in PBS at room temperature. Of these, 79.2% continued development and 49.6% became blastocysts. There was not significant difference in the general development of these two groups (X^2 = 0.41, df = 1, $p > .05$), but a significantly larger number of zona-free embryos developed into blastocysts (49.6%, X2 = 6.54, df = 1, $p < .02$).

Embryos denuded and treated in calcium and magnesium-free PBS with EDTA did not achieve the percent development seen in the other two groups. Only 55.9% of the 8-cell embryos continued cleavage, and showed a significant reduction relative to control (X^2 = 30.48, $p < .001$). However, the proportion of treated embryos which developed into blastocysts did not differ significantly (X^2 = 2.42, $p > .05$) from controls. Both of these groups differed significantly from the higher percentage of blastocysts developed from the zona-free embryos (X^2 = 12.25, $p < .01$).

The improved development of the zona-free 8-cell embryos in vitro is unexplained, but similar results have been observed in mice (Tsunoda and McLaren, 1983). This may be due to improved uptake of required nutrients in the absence of a barrier from the zona-pellucida. Decreased development of the denuded 8-cells with reduced intercellular adhesion could result from disintegration of the

cell-cell gap junctions which support compaction and spreading of 8-cell blastomeres into early morulae (Hillman, et al., 1972; Deuchar, 1975; Balakier and Pedersen, 1982; Trosko et al., 1982). However, the equivalent percentage of development in this group to blastocyst stage, relative to controls, argues against this as a detrimental effect.

Temperature effects were rules out since all embryos were maintained at 22°C prior to culture in TALP. Duration of the experimental treatments was not considered a factor since enzymatic removal of the zona pellucida requires less than 30 seconds, and the 8-cell embryos, denuded and chemically dissociated over a period of 15 minutes, developed just as well as the control embryos which were immediately transferred to the incubator.

Given the substantial improvement in multiplet survival following chemical pretreatment and the minimal difference in development of intact and denuded 8-cell parent embryos, chemical pretreatment was retained for microsurgical production of half-embryos. Interestingly, in two experiments where 8-cell embryos were chemically denuded and subjected to reduction of intercellular adhesion, three of the embryos spontaneously dissociated while in culture into isolated blastomeres or groups of two and four blastomeres (Figure 5). None of these developed further. This suggests that the physical contact of the microsurgical instruments on the chemically pretreated embryos was not adversely affecting the development of the multiplets, but that the loss of

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The Effect of Temperature Change on Embryo Development

Another factor which could cause developmental failure of the multiplets was the delay of entry into culture caused by the microsurgical procedure itself. While the 8-cell embryos were routinely split within 5 to 10 minutes after chemical pretreatment, the half-embryos were placed into 0.5 ml of TALP in a culture slide and held on a slide warmer at 37° C until all half-embryos in a series had been completed.

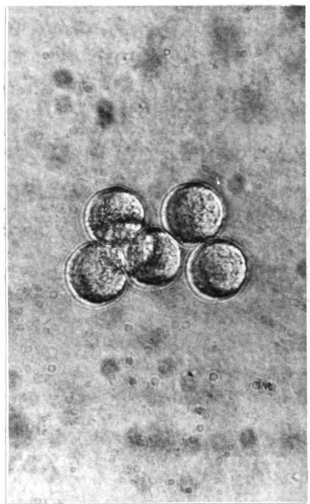

Figure 5. An example of spontaneously dissociated blastomeres from an 8-cell hamster embryo denuded with pronase and washed in calcium- and magnesium-free PBS (with 0.02% EDTA).

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This conflicted with earlier experiments in the study which had shown that dhilling hamster embryos to 4°C for 2 hours prior to microsurgery did not kill them. A total of

28 2-cell hamster embryos were tested for viability with fluorescein diacetate (Hutz, 1983) after being cooled to 4°C and maintained for four hours. All remained metabolically normal. The damage incurred by delay of incubation was probably linked to prolonged exposure of the culture medium to the atmosphere instead of temperature changes. Phenol red, used as an indicator of pH change in TALP and TC-199, shows a definite color shift from pink to purple as the medium is exposed to air and becomes more basic. Similar changes were observed in the medium during the delayed culture experiments and during microsurgery. A gradual change of the culture medium to a more basic pH may be responsible for the lack of embryonic development, but microsurgery under oil-covered microdrops of medium was not tried in this study, nor was in vitro culture under oil used since intact embryos were developing well in culture without it.

Micromanipulation of a Squirrel Monkey 4-Cell Embryo

Because of the lack of success with in vitro and in vivo development of the multiplet hamster embryos, the more difficult to obtain squirrel monkey embryos were diverted to other research. However, the methodology for blastomere separation developed on the hamster embryo was applied to the monkey in one experiment. Blastomere separation (mechanical only) was performed on an in vitro fertilized and in vitro cultured 4-cell squirrel monekey (Saimiri sciureus. The parent embryo was obtained by laparoscopic

collection of the oocyte following the ovulatory induction regimen given. The oocyte was incubated 21 hours prior to fertilization. Fresh sperm were colelcted by electroejaculation and suspended in TC-199 with pyruvate, and added to the oocyte in culture. At 60 hours after fertilization, a 4-cell embryo was obtained. The embryo was transferred to a 1.0 ml drop of TC-199 with pyruvate in the petri dish of the micromanipulator unit and one of the four blastomeres (degenerate - see Figure 6) was removed by aspiration (Figure 6). The manipulated embryo excluded the vital dye trypan blue and was cultured in TC-199 with pyruvate for 24 hours at 37°C in 5% CO^2 in air. The embryo did not develop further. However, the experiment resulted in the production of the first viable microsurgically produced nonhuman primate embryo (Rawlins et al., 1983). Subsequent experiments completed on oocytes recovered from squirrel monkeys indicate that a 0.25% solution of pronase will remove the zona pellucida from the ova within 30 seconds at room temperature.

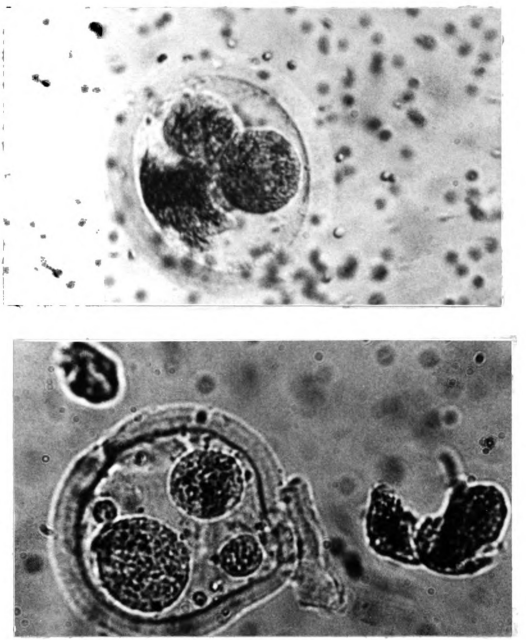

Figure 6. Microsurgical removal of a single blastomere from a 4-cell squirrel embryo fertilized in vitro. A) all blastomeres inact. B) degenerate blastomere removed.

DISCUSSION

The objective of this study was to develop a method for the production of monozygotic multiplet embryos in the hamster and squirrel monkey by blastomere separation. It has been shown that viable half embryos can be produced from 4- and 8-cell hamster embryos by either mechanical, or mechanical and chemical, separation of blastomeres; but the combination of the two methods results in the greatest percentage of post-surgical survival of the half-embryos. The methods developed on the hanster embryos for blastomere separation are also effective on in vitro fertilized squirrel monkey embryos. The use of the vital dye trypan blue proved effective as an assay of embryonic viability for both intact and manipulated embryos of both species. The results are consistent with other studies on the isolation of individual blastomeres in preimplantation embryos of eutherian mammals (Nicholas and Hall, 1942; Seidel, 1960; Tarkowski and Wroblewska, 1967; Moustafa and Hahn, 1978; Willadsen, 1979; Ozil, 1982; Williams, 1982).

Failure of the hamster multiplet embryos to develop in vitro is not surprising given the known sensitivity of hamster embryos to any form of manipulation in culture and especially to a basic pH (Whittingham and Bavister, 1974),

but there is no explanation for why this is the case. It is difficult to attack experimentally since the multiplet hamster embryos produced in this study were viable prior to entry into culture and control embryos, as well as embryos subjected to all phases of the separation protocol except physical dissociation, developed to blastocysts in the in vitro culture system. The problem rests with development of a suitable system for culture of the half embryos, not with the methods used to separate the blastomeres.

The lack of development in multiplet embryos returned to homologous zonae pellucidae and transferred to synchronized hamster females is also interesting. It has been known for many years that murine embryos would develop in vitro without a zona pellucida, but not in vivo (Tarkowski, 1959, 1971). Similar results were reported with sheep (Trounson and Moore, 1974). However, half-embryos have developed to full term and resulted in live births when transferred immediately following microsurgery (Ozil, 1982; Williams, 1982, 1983). In these experiments, the manipulated embryos were developed to morulae before being sectioned, and because they were advanced developmentally, they may have been more capable of survival. From a maturational standpoint, they were near the point in embryonic development when protection of the zona pellucida is no longer required. Transfer of half-embryos derived from hamster morulae and replaced in zonae pellucidae would likely have a better chance for further development in vivo than those

derived from 8-cell embryos since transfer of 8-cell embryos with intact zonae pellucidae has been successful in the golden hamster (Bavister el al., 1983a; Hoppe and Bavister, 1983; Ridha, 1983), but lowered survival and development occurs when the zonae pellucidae are removed (Hoppe and Bavister, 1983). From these studies, the transfer and implantation of hamster multiplet embryos should be possible.

The results of the tests on culture media for supporting further development of hamster embryos are clear. The more advanced embryonic stages show better rates of development in vitro than do early 2- and 4-cell embryos, regardless of the culture medium used. TALP is significantly better for maintaining growth in both 8-cell and morula embryos. Althoguh there are no published reports of successful culture of 2- and 4- cell embryos (Bavister el al., 1983), 30.1% of the 4-cell embryos cultured in TALP in this study underwent additional development. The significantly greater development of zona-free 8-cell embryos in TALP compared to controls in this study differs from the results of Hoppe and Bavister (1983) which, using the hamster and TALP, showed no difference between the groups. The percentage of embryos devleoping into blastocysts in their study ws 46% for controls and 44% for the denuded 8-cells (pronase - 0.25%), compared to 39.6% and 49.6% for the same groups in this study. TALP is a desireable culture medium for experimental work because, unlike the media which

contain serum, it is chemically defined and can be modified according to experimental design. Further, it is useful because the medium has been shown to support both hamster and monkey (Macaca mulatta) embryo growth in vitro, thereby providing a single nutrient system for both rodent and primate species.

Given the low numbers of 4- and 8-cell embryos obtained from in vitro fertilization of squirrel monkey oocytes (Chan, 1983), chemical pretreatment of these embryos prior to microsurgery should be used to minimize the risk of damage to the blastomeres and to maximize post-surgical survival of the multiplets. Although only one primate embryo was manipulated, the results indicate that production of monzygotic embryos from nonhuman primates is possible with existing technology. The recent success of embryo transfer in two primate species (Papio sp. and Macaca mulatta) makes transfer of half-embryos in nonhuman primates feasible. Based on the consistent sucdess of twin production by blastomere separation of morula and blastocyst embryos in domestic cattle, and if in vitro fertilized primate embryos can be developed in culture to morulae or early blastocysts, blastomere separation should be attempted on these later developmental stages instead of 4-cell and 8-cell embryos, as the probability of successful embryo transfer is greater.

While generation of artifical sets of monzygotic twins in exotic primate species has great utility for breeding programs, and the use of isogenic primates of a single

species for experimental research holds great promise for both physiological and behavioral work, and it is in genetics that blastomere separation provides the most novel application.

As mentioned previously, separation of blastomeres into twin embryos allows for both delayed implantation of one of the embryo and or the use of genetic assays on half of the daughter embryos of the parent (Epstein et. al., 1978; Epstein, 1981). Sex determination prior to embryo transfer by karyotyping one of the multiplets is a simple application. Chromosome activation can also be examined (Hoppe and Whitten, 1972; Epstein, 1978). Another interesting application is the assessment of in utero differences on subsequent embryonic development and animal behavior. This has been examined in mice by examining resulting differences in the body weight and stature of monozygotic multiplets carried to term in one or several different surrogate mothers. Clear differences in morphology were identified despite isogenecity of the embryos which correlated with in utero differences among the surrogate mothers. Behavioral differences were not considered (Gartner and Baunack, 1981). Similar studies of the influence of the genome relative to nurture, in utero and post-natal, could easily be carried out on nonhuman primates in conjunction with production of monozygotic multiplets by blastomere separation and embryo transfer.

Another important use of blastomere separation in primates is the detection of chromosomal abnormalities in in vitro fertilized human embryos. This problem is now receiving considerable attention (Schlesselman, 1979; Angell et al., 1983). Identification of monosomy, trisomy, mosaicism and polyploidy in preimplantation human embryos has not been accomplished despite the possibility that their occurrence after fertilization in vivo and in vitro may be very frequent. Production of half-embryos would permit chromosomal examination of one multiplet while the other remained in culture and useable for transfer.

As Seidel (1983a) has pointed out, one of the most important constraints on experimentation with mammalian embryos is the limited numbers which can be produced. Blastomere separation and the production of monozygotic multiplet embryos from a single parent embryo provides an elegant method to alleviate the shortage. It also allows for twinning by choice instead of chance.

SUMMARY AND CONCLUSIONS

This study was directed at the development of techniques for blastomere separation, production of viable multiplet embryos, and the in vitro culture of isogenic and control embyros to developmental stages suitable for embryo transfer in the hamster (Mesocricetus auratus) and squirrel monkey (Saimiri sciureus). Experimental protocols were first tried on in vivo fertilized 2-, 4-, and 8-cell hamster embryos prior to use on the less easily obtained, in vitro fertilized and in vitro cultured monkey embryo. The following conclusions can be made from the data:

1.) Blastomere separation can be used to produce viable monozygotic half-embryos in both the hamster and squirrel monkey.

2.) Mechanical removal of the zona pellucida and mechanical separation of the blastomeres of hamster embryos results in significantly greater post-surgical losses of multiplet embryos than that seen when embryos are chemically treated prior to surgery.

3.) The zona pellucida of both hamster and squirrel monkey embryos can be removed by lysing in a 0.25% solution of pronase in phosphate buffered saline

without damage to the embryo.

4.) A solution of calcium and magnesium-free phosphate buffered saline with 0.02% EDTA as a calcium chelating agent can be used to reduce intercellular adhesion between blastomeres prior to microsurgery without damage to hamster embryos.

5.) Mechanical separation of the blastomeres of a 4-cell squirrel monkey embryo resulted in production of a live 3/4 embryo.

6.) The vital dye, Trypan Blue, provides a rapid and effective means of assaying control and half-embryo viability prior to and after incubation.

7.) Despite the successful micromanipulation of hamster embryos into viable multiplets, none developed after 24 hours of incubation at 37°C in 5% CO_2 in air.

8.) Temperature changes that occurred during microsurgery were not responsible for the failure of multiplet hamsters to develop in culture.

9.) Transfer of hamster multiplet embryos to surrogate mothers did not result in implantation or development of the embryos.

10.) The success of culture in vitro of control hamster embryos was a function of their developmental stage at the time of entry into culture. Early 2-cell embryos did not develop and 4-cell embryos showed little growth, while 8-cell embryos and

morulae regularly developed further, regardless of the medium used.

11.) In vitro culture of control hamster embryos was best in TALP, compared to TC-199 with pyruvate, TC-199 with pregnant hamster serum, and Tyrodes solution with pregnant hamster serum. TALP regularly supported development of 8-cell embryos to morula and blastocyst stages.

12.) Chemical pretreatment of intact 8-cell hamster embryos had no effect on development to blastocyst stage compared to controls, but denuded 8-cell embryos showed greater overall development than did controls.

13.) Additional work is needed to isolate the factors responsible for failure of multiplet embryos to develop in culture. There is no obvious reason why blastomere separation and culture should not work in this rodent. The extreme sensitivity of hamster embryos to any form of manipulation may be responsible.

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APPENDIX A

PUBLICATIONS BY THE AUTHOR

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Forty years of rhesus research. R.G. Rawlins. New Scientist 82: 108-110, 1979.

Parturient and post-partum behavior of a free-ranging rhesus monkey (Macaca mulatta). R.G. Rawlins. J. Mammalogy 60: 432-433, 1979.

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APPENDIX B

CURRICULUM VITAE

Name: Richard Graham Rawlins

Born: January 15, 1950

Birthplace: Joliet, Illinois

Formal Education: Northwestern University, 1968-1973
Michigan State University, 1982 to present.

Degrees Received: Bachelor of Arts with Distinction and Departmental Honors, Northwestern University, 1972

Master of Arts, Northwestern University, 1973

Honors and Scholarships:

John Merrill Scholarship - University of Illinois, 1968
Illinois State Scholarship 1968 - 1972
National Merit Finalist 1968
Northwestern University Academic Scholarships 1968 - 1972
Phi Beta Kappa - Northwestern University 1972
National Science Foundation Fellow
National Science Foundation - Dissertation Improvement Grant
Sigma Xi Meritorious Research Award Michigan State University-1984

Professional Societies:

American Association for the Advancement of Science
American Society of Primatologists
International Primatological Society
Animal Behavior Society