EARLIEST STAGE AT WHICH MOUSE EMBRYOS LACKING ZONAE PELLUCIDAE CAN BE SUCCESSFULLY TRANSFERRED TO THE UTERUS

by

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A B S T R A C T

This experiment is designed to determine the earliest stage at which mouse embryos lacking zonae pellucidae, when transferred to recipient uteri, produce viable offspring.

Donor mice were superovulated using Pregant Mare Serum Gonadotropin (PMSG) and Human Chorionic Gonadotropin (HCG). On the fourth day post-coitum, each mouse was euthanized by cervical dislocation and uteri removed by blunt dissection. Embryos were flushed from each horn into embryonic watch glasses using Phosphate Buffered Saline (PBS). The stage of development was consistantly between late morula and early blastocyst. The embryos were divided into two equivalent groups. The zona pellucida was removed from one group using pronase. Recipients were superovulated to be synchronous with donors and were mated with vascectomized males to induce pseudopregnancy and prevent maternal embryos from developing. Each recipient was anesthetized with Inovar (10%) and a mid-ventral laparotomy performed to expose the uterus. Each recipient recieved either normal or zona-free embryos. Some recipients were allowed to go to term and others terminated after ten days to detect fetal development.

The technique was prooven adequate since a pregnancy did result in a control transfer with normal embryos, but, no pregnancies were detected in zona-free transfers.

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INTRODUCTION

This study is an important part of a larger project which deals with the production of identical twins in mice. More specifically, it is a pilot project for producing identical offspring as animal models for research and livestock production. One need only consider the improvement in experimental accuracy, made possible by the lack of genetic variation in subject models, to appreciate the scope of this study. Also, one can look at this from the standpoint of the farmer or rancher. The purebred cattle industry thrives on the production of superior animals. Producing two identical animals having some outstanding characteristic, permits that trait to be spread amoung more offspring. Of course, one runs the risk of narrowing the genetic base of a breed, but that problem has been met and delt with before.

It is important to note the limitation of twinning. Previous studies have had little success with methods leading to producing multiple identical offspring (Rossant, 1976; Tarkowski & Wroblewska, 1967). The design of this project is to produce only two offspring from a single embryo. In affect, this would be an effort to increase the incidence of the natural phenomenon of identical twinning, thus, overcoming some of the objections to other investigators efforts in the field, such as cloning.

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*The basic format of this paper will follow that of the Journal of Reproduction and Fertility. (J. Reprod. Fertil.)

With this limitation, chances for success are greatly enhanced.

In previous studies, one or more blastomeres were destroyed within the zona pellucida of two to eight cell embryos. Subsequent normal development occured in most cases (Nicholas & Hall, 1942; Daniel & Takahashi, 1965) and normal offspring resulted in separate studies (Tarkowski 1959; Seidel, 1952, 1960). When the zona pellucida is removed from early cleavage stage embryos, the individual blastomeres can be separated, in most cases, without damage. Normal cleavage may resume, but without the zona pellucida, there is less intimacy between cells, leading to seperation and ultimate death. This larger study will involve dividing later stage morulae into two blastomere masses (Trouson & Moore, 1974), culturing them in vitro to a stage at which successful implantation may occur, and transfer of the resulting embryos to recipient uteri. The earliest stage at which successful implantation and development from Zona-free embryos has occured, is the eight cell stage (Bronson & McLaren, 1969). But only one in twenty-nine embryos transferred to the oviduct produced a viable fetus. In comparison, fourteen of twenty-eight Zona-free blastocysts produced viable fetuses when transferred to the oviduct.

This study was to determine the earliest stage at which Zona-free embryos transferred to recipient uteri, survive and produce normal offspring. The choice of uterine transfer is more complicated than the fact that the uterus is easily accessable. Mechanical

division of these embryos creates two embryos, equivalent to some lesser stage of development. Donor embryos are likely to be uterine stage morulae. Following division they may appear to be from the oviduct. Uterine transfer would be more effective if the age of the cells are considered as opposed to embryo stage. Establishing uterine transfer would provide an option to transfer to the oviduct, depending on the age and condition of the embryo.

Why not merely culture all embryos to the blastocyst stage at which the zona is not necessary? This would be ideal, but the longer the embryos must be maintained in culture, the more embryo mortality can be expected. Culturing embryos takes considerable apparatus and special techniques (Brinster, 1963). An even more ideal situation would be to divide morulae and culture the halves only long enough that each will survive transfer. This method would reduce both expense and time spent in the laboratory,. leaving most of the work to the mouse recipient.

MATERIALS & METHODS

I. Animal Model & Timing of Events

This study involves transfer of Zona-free and normal embryos to the uterus, to detect survival of Zona-free embryos. It is necessary to use normal embryos as a control, so as to confirm the effectiveness of the technique utilized. To simplify the technique and also reduce problems associated with larger laboratory animals, mice were chosen as the experimental model. To show successful transfers with 100% certainty, a color marker was chosen to identify transferred offspring. In most cases, outbred albino mice were used as donors, with inbred C57 black mice as recipients. (In some trials the color scheme was reversed.)

The mouse (Mus musculi) is an excellent model for studies of this type. The major drawback to their use is their size. But, the vast amount of data available on them plus a little experience, negates the size aspect and adds a tremendous amount of experimental control. For the mouse, data on the timing of estrus and stages of gestation are very precise (Rugh, 1968). In this study, follicular development and ovulation were artificially induced, so reference to the estrous cycle may be negated. The most important aspect here, is the timing of the basic stages the embryo passes through from fertilization to implantation (See table 1, next page).

Table 1

AGE AND LOCATION OF PREIMPLANTATION EMBRYOS

Post Copulation	# of cells	Location
0-24 hrs	1	Ampulla
24-38	2	Upper oviduct
38-50	3-4	Mid-lower oviduct
50-64	5-8	Lower oviduct and uterus
60-80	morula	Uterus
74-82	blastocyst	Uterus

(Lewis & Wright, 1935; Rugh, 1968)

Fertilization generally occurs early in the morning. The morning at which evidence of copulation is found is designated as the start of day one. The morning of day two, cleavage has occured and two cell embryos can be observed in the oviduct. During day three several divisions occur and the exhisting morulae leave the oviduct and enters the uterus. The next morning late stage morulae and early blastocysts may be observed in the uterus. From previous studies it was determined that this stage of development may be critical to this study. On that assumption, all transfers to date have been performed on the morning of the fourth day of embryonic development. Normally, in vivo loss of the zona pellucida and reactivity with the uterus are synonomous with day five, with over 96% of the zonae shed within ten hours (Restall & Bindon, 1971). Late morulae and blastocysts were used initially because it has been shown that this stage may not require zonae pellucidae

for normal development and subsequent implantation (Modlinski, 1970; Bronson & McLaren 1970; McLaren, 1969).

II. Superovulation & Synchronization

Under normal circumstances a mouse ovulates between 10-15 ova. For the purpose of this study, large numbers of embryos are required, so one would like to obtain as many embryos as possible per mouse, since donors will be sacrificed. The induction of estrus and ovulation in rats and mice is well documented (Fowler & Edwards, 1957). Two gonadotropins in particular, Pregnant Mare Serum Gonadotropin (PMSG) and Human Chorionic Gonadotropin (HCG) are used extensively (Rugh, 1968). Pregnant Mare Serum Gonadotropin was first described by Cole and Hart in 1930 (Cole & Saunders, 1935). In the pregnant mare, it is produced in detectable quantities between the 40th and 170th days of pregnancy. PMSG mimmicks the action of Follicle Stimulating Hormone (FSH) which originates from the anterior pituitary gland. HCG is found in the blood and urine of pregnant women (Diczfalusy, 1960). It has an entirely different effect. In the normal estrous cycle of the mouse, FSH would cause maturation of follicles and another hormone, Lutienizing Hormone (LH), also from the anterior pituitary would cause ovulation. HCG mimmicks the effect of LH, thus inducing ovulation.

The technique used to induce estrus and ovulation in this study, is similar to that described by Gates in Methods in

Mammilian Embryology (Daniels, 1971). Each mouse is given an intraperitoneal injection of 10 I.U. (International Units) PMSG at approximately 4:00 PM. Fourty-four hours later, or about 12:00 AM, 10 I.U. of HCG is given in the same manner. Thus, HCG induces ovulation of mature follicles, and ova will be present in the oviduct.

At this time one must seperate donors from recipients. At the time of HCG injection, donors are placed with normal males of the same color. Recipients are placed with vascectomized males. Evidence of copulation is seen as presence of vaginal plugs. Although recipients will not have fertilized ova, the act of copulation will induce pseudopregnancy. That is, normal physiologic changes will occur as if viable embryos were present. The fact that viable embryos are not present in the recipients is important. Otherwize, large numbers of embryos may compete with and exclude transferred embryos.

Superovulation has another important aspect in that of the synchrony induced in all animals. When mice are superovulated, natural systems are overridden and all mice injected at that time should be synchronous. Thus, superovulation instills recipients with two important criteria: First, estrus is induced and following mating with vasectomized males, pseudopregnancy results. Secondly, if injected at the same time as the donors, they should be physiologically synchronized. For example: If the donors have 32 cell morulae in the uterus, the recipients system should

be at a stage equivalent to that containing 32 cell morulae.

The question arises whether or not recipients should be in exact phase with donors or possibly earlier or later. It is a complex question, but in most of the studies referred to previously, recipients are in phase with donors and pregnancies have resulted. As such, all transferred embryos were in phase to the recipients in this study.

III. Collection and Treatment of Embryos

It was decided from data in previous studies that early fourth day embryos would be studied initially. All viable embryos at this stage, have been found to be late morulae to early blastocysts. Any variation in stage may be accounted for by a few hours uncertainty in the time of ovulation. Because there stages were desired, only uterine recovery was necessary (See Table 1). Any earlier stage may require recovery from the oviduct.

The method used for recovery of the embryos is simple but for satisfactory collection it is necessary to sacrifice the donor. Females were killed by cervical dislocation. The uterus and oviduct were then exposed by blunt dissection. The ovaries were usually observed for presence of corpora lutea. In most instances gross examination was sufficient to determine if ovulation had actually occurred. Recovery of the uterus was accomplished by first severing it near the cervical end and grasping it with forceps. Raising the tract creates the proper tension for cutting away the mesentery

and fat as close to the serosal surface as possible along the entire length of the uterus and oviduct. (The oviduct was usually left attached.) Further tension on the tract allows the oviduct to be easily teased away from the ovary. Both horns were recovered and placed in small dishes containing flushing media.

The media used for embryo recovery was Phosphate Buffered Saline (PBS) with Penicillin (50u/ml) and Streptomycin (50mcg/ml). This is a commonly used media, but it is commonly supplemented with fetal calf serum. Due to the short period of time the embryos were maintained, supplementation was not considered necessary (McLaren, 1969).

Embryologic watch glasses were used as recovery dishes. Each dish was sterilized and prepared by adding 3mls of PBS and then covered. These, along with all necessary apparatus, were then placed in a dissecting scope cabinet which was maintained at approximately 35° C. A dish was prepared for each horn and labeled accordingly. The remaining procedures, except the actual transfer, were conducted under the dissecting microscope within a temperature controlled environment.

Two instruments were used to flush the uterus. First, a sterile 30 gauge needle and 1ml tuberculin syringe have been found sufficient for flushing both uterus and oviduct. The second instrument is a pair of fine watch makers forceps which hold the needle in place while flushing.

One horn is placed in a watchglass and completely covered

by media. This was placed on the microscope stage. Under medium power, the needle was inserted in the tubal end of the uterus and clamped with the forcep to stop backflow. The entire ml of media was flushed through the horn. In some cases embryos were actually seen exiting the end of the horn. The horn was agitated as it was removed to avoid any embryos adhering to it. With proper lighting, the embryos are easily seen at 20 - 30 power. The embryos were then collected together and identified to stage.

Mouth suction using finely drawn pastuer pipets has been found to be excellent for assembling all the embryos into the center of the dish. All stages present were recorded and unfertilized ova and degenerative forms were separated. In most cases, if a mouse responds to treatment with gonadotropins, one mouse may produce thirty or more viable embryos.

Each trial consisted of three donors and two recipients. Embryos were rarely collected from all three donors for varying circumstances. If sufficient numbers were collected, the embryos were collected into two equivalent groups. One group to each recipient.

All the embryos transferred to the control recipient were Zona-intact or untreated embryos. All those transferred to the other recipient were Zona-free. The zona is removed with Pronase (Mintz, 1962) .5 percent Pronase is placed in a separate dish and the embryos are transferred into it. About three minutes is

required to thin the zona, at which time the embryos were removed and rinsed twice with PBS. The removal of the zona was completed mechanically with a pipet (Mystkowska & Tarkowski, 1968). When the zona had been removed from all embryos in the second group, they were again assembled together in a fresh dish and preperations are then made for transfer.

IV. Uterine Transfer

There is an important difference between normal and Zona-free embryos: Adhesiveness. A great deal of care has been exersized when manipulating these. Following each transfer it was not uncommon to find a couple of Zona-free embryos adhered to the pipet, which required forceful pipeting to expel. Thus, only those embryos not rerecovered can be considered transferred. Conversely, after using glass pipets, 100% transfer of normal embryos was attained.

Recipients were prepared with .5ml of 10% Inovar, a general anesthetic, given intramuscularly. Within ten minutes marked analgesia is apparent with little or no loss of reflex. Though apparently conscious, there is complete loss of sensitivity to pain. The abdomen is clipped and cleaned before the animal is transferred to the operating surface, which was previously disinfected. The mouse is placed on her back and taped to the surface. This method of restraint is simple and effective. Both rear legs are secured and one strip is used to secure the upper torso.

The sterility of all instruments is maintained within a large beaker containing dilute Nolvasan disinfectant. Final preperation was made by applying Betadine solution liberally to the abdomen and operating surface.

A one centimeter incision was made through the skin and muscle layers along the ventral midline. An ovary and horn were then exposed and proper tension is achieved by clamping the ovarian fat pad. A puncture was made in the uterus with a 25 gauge hypodermic needle near the middle of the horn. The embryos (either zona intact or zona-free) were then drawn into a finely calibered pipet, in as tight a group as possible. As such, the embryos are then transferred in a minimum volume of media (McLaren, 1969). The pipet was inserted into the puncture wound a short distance toward the tubo-uterine junction. The embryos should be expelled without introducing air into the uterus. If necessary the same procedure was applied to the opposite horn.

Following transfer, each pipet is immediatly examined for untransferred embryos by flushing with PBS. The reproductive tract was finally returned to the peritoneal cavity and the muscle layers and skin were sutured separately. Recipients were returned to their cages to recover.

V. Collection of Data

Recording data was the most simple aspect of this study. It consisted primarily of keeping an adequate record of how many

embryos were transferred to which mouse who may or may not have concieved. There were three important criteria involved in maintainting an accurate record of data.

After each transfer, the pipet used was flushed, to dislodge any embryos which may have adhered to the inner surface. Any recovered in this manner were subtracted from the total and this figure was considered as the actual number transferred.

Each mouse was identified by cage number and the ears of mice recieving zona-free embryos were notched immediately following the procedure. This would infact negate any uncertainty as to the origin of a pregnancy.

Two methods were used to detect viable fetuses. The recipients were either allowed to carry pregnancies to term or sacraficed on day ten to detect vesicles before fetal mortality due to overcrowding is excpected to occur.

R E S U L T S

The first two attemts were performed with little proficiency. The technique was crude and the actual transfers were performed using a thirty gauge needle and small syringe. This proved highly ineffectual since no pregnancies occured and recipient mortality was high. Of the four mice that were

Table 2

RESULTS OF PRELIMINARY EMBRYO TRANSFER ATTEMPTS

Transfer Number	Recipient Z/ZF	Number of Embryos transferred	Recipient Mortality	Fetuses
1	ZF	7	X	-
	ZF	3		0
2	Z	6	X	-
	ZF	9		0

used in the first two transfers, the mortality rate ran precisly at 50%. (See Table 2) This was attributed to poor technique and post operative infection. It should be noted that fewer embryos were transfered initially because technique predisposed embryos to be retained by the needle, (both zona-free and normal) thus, following transfers, subsequent flushing of the needle inevitably revealed untransferred embryos.

Transfer #3 was a controled zona-intact transfer. Of the

Table 3

MOUSE EMBRYO TRANSFERS WITH (Z)
AND WITHOUT (ZF) ZONA PELLUCIDAE

Transfer Number	Z/ZF Recipient	Number of Embryos Transferred	Fetuses
3	Z	5	3
4	ZF	16	0
	Z	20	0
5	Z	20	0

five embryos transferred, three live offspring resulted. This technique was followed closely with only minor changes made in surgical preperation, but no further pregnancies could be demonstrated through transfer #5.*

*Similar attempts were made following these, but evidence of conception was not yet apparent, so as to be included in this report.

DISCUSSION

The alterations incorporated into the technique of the final three transfers, aided both the mechanical and psychological aspects of the procedure. One of the most important changes was that of the transfer apparatus. In the final three transfers, a finely drawn Pastuer pipet was used. The media/air interface could be observed easily in the thinnest portion of the clear pipet. Mouth suction easily controlled the flow within this area, with less control observed when fluid was allowed to collect in the body of the pipet. With this tremendous increase in control, a minimum volume of inoculum could be used and eliminate the possibility of injecting air into the uterus.

A second very important change, was in surgical preparation. Following the death of a recipient with peritonitis, complete surgical asepsis was maintained with the exception of gloves. Because of the ultimate importance which post operative infections may represent, asepsis was considered utmost in following transfers.

Ultimately, the most important factor is the man behind the transfer. Though confidence has little to do with implantation of an embryo in the uterus, it is none the less a very important tool to the researcher. Experimental variables have overcome confidence in this study as far as accomplishing the

goal it was originally intended for. Confidence in the procedure developed by this study dictates that pregnancies will be achieved with zona-free blastocysts, making it possible for earlier stages to be studied.

The greatest finds in science are never found by looking for them. And indeed the importance of this study was not finding the earliest stage at which transfer of mouse embryos lacking zona pellucidae can be successfully achieved. It may even be found that it is not relevant to producing identical twins, but it has set a base on which this research may continue at a slightly more advanced level than if this study had not been undertaken. And that it should continue is most important.

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