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IN VITRO DEVELOPMENT OF PREIMPLANTATION MOUSE EMBRYOS MICROENCAPSULATED IN SODIUM ALGINATE

By

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ABSTRACT

IN VITRO DEVELOPMENT OF PREIMPLANTATION MOUSE EMBRYOS MICROENCAPSULATED IN SODIUM ALGINATE

By

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The purpose of this study was to develop a technique for microencapsulating preimplantation mouse embryos and to test the procedure in vitro by culturing encapsulated embryos and assessing developmental potential. Embryos were collected from immature superovulated and mated SWW or B6D2-F1 female mice. Microencapsulation was accomplished by embryo aspiration in sodium alginate (1.1%) using a 5 μl micropipette, and extrusion into $CaCl_2$ (1.5%) with subsequent cross-linked matrix formation. The technique was developed, tested, and found to have no detrimental effect upon two cell and four cell stage mouse embryo development in vitro. Embryos encapsulated singly, doubly or triply developed at rates similar to non-encapsulated control embryos.

In addition, B6D2-F1 mice (a hybrid of cross C57BL/6J and DBA/2J) responded better than Swiss Webster white (SWW) mice to a superovulation regimen of 5/5 International Units PMSG/HCG. The mean number of embryos per female following superovulation was significantly higher for B6D2-F1 mice. Collection and culture of mouse preimplantation embryos was carried out in TALP-Hepes and TALP, respectively. This medium proved superior to modified TC199.

"Perhaps I ought to explain," added the badger, lowering his papers nervously and looking at the Wart over the top of them, "**that all embryos look very much the same.** They are what you are before you are born - and, whether you are going to be a tadpole or a peacock or a cameleopard or a man, when you are an embryo you just look like a peculiarly repulsive and helpless human being."

T. H. White,

The Once and Future King

To my parents,

Warren and Doris,

I dedicate this thesis for their continued support not only in my education, but in the further enrichment of my life.

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INTRODUCTION

The birth of Louise Brown on July 25, 1978 forever changed the course of human *in vitro* fertilization (IVF). This singular event established, with living proof, the efficacy of a notion once believed to be impossible, and thrust medical science into a brave new world.

Nevertheless, no procedure in biology is perfect, and IVF remains in its infancy as it enters a second decade. Term pregnancy rates following IVF and embryo transfer (ET) remain low compared to such rates following natural conception. A myriad of hypotheses exist as to why IVF conceptions may fail to develop to term. A few of these include abnormal karyotypes (Short, 1979; Biggers, 1987); altered levels of steroids at the time of ET (Gidley-Baird et al., 1986; Radwanska, 1988), and occurrence of ectopic pregnancy (Martinez and Trounson, 1986; Correy et al., 1988).

Although normal conceptions are subject to adversity in the guise of chromosome imbalance, unreceptive uterine endometrium, and leukocytic attack, to name a few, IVF conceptions must endure these and more. Minimal as opposed to optimal culture conditions, and forceful aspiration in lieu of gentle peristalsis, are burdens to be borne by the

IVF conception. A final blow to the transferred embryo appears to be an increased rate of ectopic implantation.

Recent analyses (Lopata, 1983; Speirs et al., 1983; Yovich et al., 1988) of ET following IVF reveal that pregnancy rates can be increased by the transfer of multiple embryos. The best figure to date is four embryos versus embryos numbering from one to three and five for maximal pregnancy. The goal of IVF and ET is a viable offspring, but many clinics report pregnancy rates in which the word "pregnancy" is ill-defined.

The primary consideration of this study was to determine how methods could be modified or improved to alleviate the detrimental effects associated with IVF and ET. As the fertilization rate *in vitro* appears to be similar to that with *in vivo* conception, an obvious procedural step that could benefit from improvement would be the transfer.

We studied the *in vitro* development of preimplantation mouse embryos encapsulated in sodium alginate. The potential advantages of encapsulating embryos in biodegradable materials are: 1) protection from hostile external factors, 2) ease in manipulation by increased size to macroscopic proportions, 3) prevention of ectopic implantation due to the weight and/or size, and 4) a means of grouping any number of embryos together for manipulation as a single entity.

A scenario for encapsulation of preimplantation embryos

is as follows: standard IVF embryos are encapsulated in any desired multiplet and placed in the uterus (confirmed visually by means of a laparoscope). The capsule degrades and the embryo(s) implant in the uterine endometrium.

The requirements other than biodegradability of the capsules include: control of degradation rates either through volume or amount of covalent cross-linking, permeability to incoming nutrients and outgoing wastes, and uninhibited cell cleavage rates.

Encapsulation could be a much needed improvement in ET procedures presently in use in human IVF.

LITERATURE REVIEW

In Vitro Fertilization

As with most research directed toward human medical application, in vitro fertilization (IVF) research was initiated in animals, predominantely the rabbit and mouse.

As far back as 1797, Cruickshank gave a detailed report on the stages and timing of the reproductive cycle in the rabbit drawing conclusions as to the site of fertilization, the time of ovulation, and the time course of the embryo's travel down the reproductive tract. These studies were performed for comparison to human ovulation.

Almost 100 years later (1890), Heape performed the first transfer of in vivo fertilized rabbit ova from an Angora donor to a Belgian recipient. The embryos were placed to the distal end of the oviduct, and live young resulted from the transfer.

With the establishment of embryo transfer (ET), IVF could then be addressed.

Pincus and Enzman (1934) were the first to report successful IVF in the rabbit which was demonstrated by subsequent transfer and birth of live young. Unfortunately, scientists were unable to repeat this work for over twenty years. In 1935, these same researchers showed that ova could

be recovered directly from the follicle and fertilized *in vitro*.

Not until 1951 was the phenomenon of capacitation discovered in the rabbit by Chang (1951). Capacitation is a term applied to mammalian sperm wherein after a given amount of time, the sperm achieve the capacity to fertilize an oocyte. Once the understanding for sperm capacitation was known, its application to IVF was obvious. In 1955, Chang successfully, and repeatedly, demonstrated IVF in the rabbit.

Mouse IVF-ET was first demonstrated by McLaren and Biggers (1958), who also attained live young after transfer to recipient females. Additionally, these workers established a unique culture medium for mouse embryos indicating species-specific energy requirements.

Human IVF had its beginnings with the first published description (Letheby, 1852) of ova in the oviducts of virgin females. Opinions differed at that time as to whether ovaries released ova spontaneously and independently of a male factor (Letheby, 1852). Almost 100 years later, Rock and Hertig (1944) conducted similar studies in which they estimated the time of ovulation by assessing the age of unfertilized and fertilized ova as well as the stage of uterine endometrial development. At this time, Rock and Hertig announced the first reliable report of *in vitro* fertilization of human ova. No transfer was attempted.

Interestingly, human IVF was first suggested in an anonymous letter published by the New England Journal of Medicine (1937), entitled "Conception in a Watch Glass."

Hayashi (1963) also fertilized human ova *in vitro*, and got twenty of 160 oviductal ova to cleave, two of which reached the morula stage. Additionally, Hayashi reviewed the present culture media in use for IVF in attempts to prolong the culture period and improve developmental rates in culture.

Later that year, Edwards and Steptoe first cultured human embryos with the goal of placing the fertilized ova back into the donor as therapy for infertility (Edwards and Steptoe, 1963). In 1966, Edwards' failed IVF attempt was accredited to a lack of capacitation (Edwards et al., 1966).

Kennedy (1969) achieved the first maturation of human oocytes in a chemically defined medium (CDM) thereby refuting the need for any maternal factor during the early cleavage stages. Steptoe and Edwards (1970) advanced the procedure of IVF by introducing the laparoscope as a means of nonsurgical oocyte recovery directly from the follicle, and in concert, used gonadotropins to superovulate the ovaries thereby increasing the number of mature oocytes per cycle.

Steptoe and Edwards (1976) reported the first human IVF pregnancy; nevertheless, the transferred embryo implanted in the oviduct resulting in a tubal (ectopic) pregnancy that

soon was aborted. Lopata et al. (1978) reported that human preovulatory oocytes undergo fertilization within three hours of insemination *in vitro*, as do pronuclear ova six hours after insemination. They were inconclusive as to the minimum time required for capacitation.

Louise Brown was born on July 25, 1978 following IVF and subsequent uterine implantation (Steptoe and Edwards, 1978) thereby marking the procedure as a success for tubal infertility. Since the success of Steptoe and Edwards, the number of IVFs and IVF clinics has risen around the world (Lopata, 1983).

Human IVF presently involves (1) placing the female patient on a superovulation regimen to induce follicular growth and oocyte maturation, (2) collecting mature oocytes directly from the ovarian follicle guided by laparoscopy or ultrasound, (3) fertilizing the oocytes *in vitro* with sperm, (4) culturing the fertilized oocytes in a nutritive medium to the four to sixteen cell stage, and (5) transferring the embryo(s) back to the donor's uterus (Hodgen, 1981; Radwanska, 1988). If successful, the embryo implants and subsequent normal development ensues (Lopata, 1983; Radwanska et al., 1986). IVF involves a deviation from normal *in situ* events beginning just prior to ovulation and ending about day 23 of the cycle (Hodgen, 1981).

The major event immediately following IVF and ET is implantation. Implantation commences with the settling of

the embryo (blastocyst) onto, and subsequent invasion of, the uterine epithelium by apoptotic cell death (Parr, Tung, and Parr, 1987). Apoptosis is a form of cell death, distinct from necrosis, characterized by surface blebbing, shrinkage and fragmentation of the cells, condensation of the chromatin, indentation and fragmentation of the nuclei, and cytoplasmic organelles remain morphologically intact.

A general summary of a human embryo's fate from ovulation to implantation is as follows: Upon ovulation, the ovary releases an oocyte which becomes trapped by the fimbria of the oviduct, and begins a descent down the oviductal lumen. Normally, the ovum is fertilized in the upper third or ampullar region of the oviduct (Biggers, 1981; McLaren, 1982), and will take about three days to traverse the oviduct before entering the uterus as a twelve to sixteen cell stage early morula (Biggers, 1979). Barring abnormality, the embryo will continue dividing, form a blastocoele, and undergo determination and differentiation into inner cell mass (ICM) and trophectoderm (McLaren, 1982; Short, 1979).

A week after ovulation (Steptoe and Edwards, 1976), or six days after fertilization (Biggers, 1981), the preimplantation period ends as the embryo settles onto and begins to invade the receptive endometrial tissue of the uterus. Uterine epithelial cells surrounding the embyro undergo apoptotic cell death, and are then phagocytized by

the trophoblast cells (Parr et al., 1987).

A better understanding of the influences acting upon successful IVF-ET begins with an understanding of the influences acting upon normal conception.

The conception rate for a normal couple per menstrual cycle having unprotected intercourse is nearly 50%, with a viable pregnancy rate approaching 25% (Biggers, 1981; Jones et al., 1983; Soules, 1985). Although not readily calculable for all treatments attempted, some reviewers have estimated a couple's chances for pregnancy following IVF-ET.

A major misrepresentation, though, in the field of IVF-ET is the ill-defined term "pregnancy." Pregnancy, to the IVF clinician, can have three distinct meanings (Jones et al., 1983; Soules, 1985) depending upon the stage. In the IVF clinic, an initial pregnancy is recognized by high levels of beta-human chorionic gonadotropin (b-hCG), and is classified as a biochemical or chemical pregnancy. If the fetus were to abort prior to the next expected menses, a preclinical abortion would apply, which would be indistinguishable from the normal menstrual flow. Many IVF clinics cannot or do not detect such pregnancies as large doses of b-hCG are given to mature the follicles prior to laparoscopy (Jones et al., 1983). The distinction between a chemical pregnancy and later stage pregnancy is not defined clearly in the literature leading to a misrepresentation of the chance for conception through IVF-ET.

The Norfolk IVF program (Jones et al., 1983) classifies pregnancy by outcome. There are preclinical abortions, clinical abortions, or viable deliveries. It has been argued that normal conceptions are subject to these distinctions, but that occurrences of the first category for obvious reasons go unnoticed in the general population. Miscarriages would fall into the second category occurring after a missed cycle.

Soules (1985) has voiced caution about inflated IVF pregnancy rates when a program includes all three types in their pregnancy rate, report only selected patients, or use a denominator to give the highest figure. Soules also argues that an IVF pregnancy can and should be broken down into the three categories. These three occur at incidences approximating normal conceptions where about 45% achieve implantation, of which one-third abort, and one-ninth spontaneously abort leaving a 25% chance of a viable pregnancy. This figure concurs with sources in predicting a natural conception (Biggers, 1981: Jones et al., 1983; Soules, 1985).

The following rates represent data from different parts of the world and include many different IVF clinics and procedures. After the first IVF baby was born, the pregnancy rate remained less than 5% (Soules, 1985) until such developmental breakthroughs as improved ovulation induction, multiple oocyte recovery, and optimal laboratory techniques.

A collective study of IVF for 1982 (Lopata, 1983) showed a 10 to 12% chance for term pregnancy. In 1984, 58 IVF teams pooled their results of 9641 IVF treatment cycles to reveal an overall viable pregnancy rate of 13% (Soules, 1985). Another collective study (Radwanska et al., 1986) gave a rate of 15%.

A seven year study (Yovich, 1988) of the IVF program at The PIVET Centre (Australia) showed their pregnancy rate rose from 12.1 to 19.1%. Yovich (1988) reports that the largest IVF clinics have rates of approximately 20%, whereas Radwanska (1988) reports a 20 to 30% rate per completed cycle. Given a normal viable pregnancy rate of 25%, IVF appears to be approaching this value steadily.

One feature of IVF-ET learned over the past decade is the advantage of transferring more than one embryo per treatment cycle.

Table 1. Number of embryos transferred and percent pregnant.

Source	Number of embryos			
	1	2	3	4(≥)
Edwards et al. (1984)	15.7	27.8	36.2	
Feichtinger and Kemeter (1984)	11.7	30.0	36.4	
Jones et al. (1984)	20	23	29	35
Kerin et al. (1984)	6	24	33	50
Lopata (1983)	11.2	18.6	22.6	35.3
Martinez and Trounson (1986)	9.4	27.6	58.2	
Quigley and Wolf (1984)	11.1	28.0	12.5	33.2
Seppala (1985)	9.5	14.6	19.3	24.1
Soules (1985)	10	15	19	
Speirs et al. (1983)	10	22	19	35
Testart et al. (1986)	18.3	26.0	30.0	
Trounson (1984)	7	25	23	0
Yovich et al. (1988)	0	2.1	12	19.4

Re-evaluating the pooled results of the 58 IVF teams (Soules, 1985) by the number of embryos transferred, the advantage becomes evident: 10% for a single embryo transferred, 15% for two, and 19% for three. Other studies (Table 1) have yielded similar results. The group at PIVET (Yovich et al., 1988) reported after IVF, that four embryos transferred yielded maximal results: 0.0% per single embryo transferred, 7.1% per two, 12% per three, 19.4% per four, and 14.8% per five. Table 1 shows that most IVF teams have reported maximal results with the transfer of four or more embryos.

By the time any in vitro fertilized embryo attains the stage of implantation, a substantial chance exists that embryo mortality will have occurred. Reasons for this include the following: abnormalities of genetic origin during gametogenesis (Short, 1979; Biggers, 1987); altered steroid levels at or shortly after the time of transfer wherein elevated estradiol (E2) levels and a lower ratio of progesterone (P) to E2 exhibited implantation failure (Gidley-Baird et al., 1986; Radwanska, 1988); subtle changes in the hormonal milieu induced by ovarian hyperstimulation; synthetic hormones that may exhibit antiestrogenic and antireceptor effects at the level of the endometrium (Birkenfeld, Beier, and Schenker, 1986; Aksel et al., 1986; Thatcher et al., 1988); premature luteolysis frequent in gonadotropin-stimulated cycles leading to implantation or

pregnancy failure (Radwanska, 1988). In approximately 15% of IVF treatments, the embryos remained in the transfer catheter, or were expelled into the cervix or fallopian tube (Radwanska, 1988); also, another possible factor of embryo mortality could be bacterial contamination and trauma to the endometrium (Radwanska et al., 1986). Of IVF abortions analyzed chromosomally, 77% were normal (Andrews et al., 1986) indicating a uterine or hormonal, not embryonic, explanation for post-IVF pregnancy loss.

Estimates show embryonic loss to increase from about 30% at ovulation to a plateau of about 60% by fourteen weeks after ovulation (Biggers, 1981). According to Biggers, the probability of conception with IVF is similar to that observed in vivo yet after IVF, a larger proportion of the embryos is lost. On this point, he ascribes the loss to problems of implantation.

Lopata (1983) suggested an improved chance of establishing a pregnancy by the transfer of multiple embryos; yet, this might increase the incidence of multiple pregnancy, and possibly ectopic pregnancy. Nevertheless, recent studies (Lopata, 1983; Radwanska, 1988; Yovich et al., 1988) indicate that the transfer of four embryos following IVF resulted in higher percentages of implantation versus the transfer of one, two, three, and five embryos. Also, twinning resulted at a rate of 30% (Lopata, 1983), which is more than thirty times the dizygotic twinning rate in the general population.

Lopata (1983) reported that it was unknown whether multiple embryo transfer resulted in a predisposition to ectopic pregnancy. Ectopic pregnancy was the cause for 33.3% wastage in IVF-ET (Yovich et al., 1988), compared to 32.9% in gamete intrafallopian transfer (GIFT), 19.4% in pronuclear stage transfer (PROST), and 33.3% in tubal embryo stage transfer (TEST) thus revealing a serious problem associated with IVF as well as other forms of embryo manipulation in infertility therapy. Values for the normal occurrence of ectopic pregnancy are 4 to 6% (Martinez and Trounson, 1986; Medical Research International, 1988).

ET is considered to be the easiest technical step of the IVF process, yet it seems to be the least efficient (Radwanska, 1988) as implantation failure occurs in approximately 70% of the treatment cycles.

The first recorded human IVF-ET pregnancy (Steptoe and Edwards, 1976) was an ectopic pregnancy. Ectopic pregnancy (EP) is the implantation of a fertilized ovum outside of the endometrial cavity of the uterus (Barnes, Wennberg, and Barnes, 1983). Although not causative of EP, a high incidence is associated with IVF-ET (Martinez and Trounson, 1986). Liu et al. (1988) reported that there exists a high incidence of pregnancy wastage in both IVF-ET and natural conception, but the incidence is greater following IVF. Their estimate of early pregnancy wastage following natural conception alone is 78%.

Recent analyses of the incidence of EP reveal this form of early pregnancy wastage to be severe. A study by Rubin et al., (1983) showed that EP rose from 4.5 to 9.4 per 1000 reported pregnancies between 1970 and 1978. Reported pregnancies did not include spontaneous abortions, illegal abortions, or stillbirths. A second study by Barnes et al. (1983) estimated the incidence of EP at 4 per 1000 pregnancies averaged from 1974 to 1976. Martinez and Trounson (1986) estimated between 2.7 to 12.9 EPs per 1000 diagnosed conceptions, pregnancies, or live births.

The severity of this idiopathy is evidenced by the death of 437 women (of 262,000 diagnosed) in the nine years of the Rubin et al. study (1983). In 1977 alone, EP was the single largest cause of maternal death in non-white women.

Nevertheless, directly trying to associate the incidence of EP to IVF-ET manipulations is not simple. The incidence of EP has been rising since 1970 (Rubin et al., 1983), and IVF-ET data is just beginning to be analyzed thoroughly. The collaborative data from various IVF clinics show EP to be just as prevalent if not more so. Correy et al., (1988) showed that of eighty-one clinical pregnancies, eight were ectopic, and nineteen aborted spontaneously. The 3rd World Congress on IVF-ET (1983) reported an incidence of EP of 1.8%, whereas one year later the 4th World Congress reported an incidence of 3.8%. All EPs occured in women with clinically diseased tubes (Correy et al., 1988).

Although researchers have not directly tied any of the procedures of IVF-ET to EP (Martinez and Trounson, 1986; Liu et al., 1988; Radwanska, 1988), there has been indication of altered tubal function causing EP in infertile women receiving IVF-ET causing EP (Correy et al., 1988). Correy et al. (1988) have suggested migration of the embryo after correct placement (in the uterus) as the major cause for EP. A tubal pregnancy results, which encompasses approximately 95% of EPs.

An obvious improvement to existing IVF procedures would be improved ET and implantation rates. IVF and ET have reached the status of clinical procedure; nevertheless, research is needed to improve today's standard procedures in IVF and ET. The improvement could come in the form of embryos encapsulated for protection in transfer and better implantation rates.

Embryo Embedding

Willadsen (1979) first embedded two cell sheep embryos in agar for protection; these agar blocks proved to be insoluble in the female genital tract as demonstrated in ligated rabbit oviducts. The zona pellucida of a two cell embryo was removed, the blastomeres were extracted, placed into evacuated zonae pellucidae, and the perivitelline space was filled with sheep serum. A cylinder was used to envelop the torn zona pellucida, which was then embedded in a larger

agar cylinder. The plug was then placed into the ligated oviduct of a receptive ewe, and remained until the embryo had developed to the blastocyst stage. The agar chip was then recovered, and the embryos released and transferred to definitive hosts.

Although embedding in agar proved not to be harmful and did support the manipulated embryo at a normal rate, this method required a second surgical procedure to remove the agar cylinder. An obvious improvement at this stage would be the selection of degradable embedding material. Also noted by Willadsen is the fact that single blastomeres may be embedded without a zona pellucida, yet he advised against it as releasing the embryo at a later stage would be difficult. Again, the desired encapsulating material should be biodegradable.

More recently Warfield, Seidel, and Elsden (1987) have encased zona pellucida-free bovine embryos in 7% gelatin for bisected embryo and monozygotic twin experiments. These researchers bisected an embryo into two roughly equal demi-embryos; they then transferred the demi-embryo to synchronized recipients as either zona pellucida intact demi-embryo, zona pellucida-free demi-embryo, or gelatin-embedded zona pellucida-free demi-embryo. No advantage or disadvantage was seen for either treatment group. Therefore, 7% gelatin embedding of bovine demi-embryos was no more detrimental to the embryo than a

simple transfer.

A novel method of encapsulating embryos is through microencapsulation, in which biodegradable materials are used. The field of microencapsulation is a relatively new one; nevertheless, the range of applications has become almost infinite.

Microcapsules, as defined by Luzzi (1970), are "discrete packages of material in the size range of 0.5-200 μ, ... produced by a number of techniques." Microcapsules have been used in industry for the encapsulation of dyes for "carbonless carbon paper," (Luzzi, 1970; Green and Schleider, 1956); in medicine for the encapsulation of enzymes for enzyme-replacement therapy (Chang, 1964; Luzzi, 1970); in aerospace studies for the encapsulation of bacteria as cosmic dosimeters (Luzzi, 1970); finally, and perhaps with greatest application, in the pharmaceutical field for the encapsulation of certain drugs (Luzzi, 1970; Phares and Sperandio, 1964), most commonly aspirin (Holliday, 1960).

Many methods exist to form microcapsules, yet until 1978 these all involved organic solvents or heating which prevented their application to living tissue. At this time, Lim (Lim and Moss, 1979) developed a novel method wherein biological materials, notably cells, could be encapsulated successfully without compromising viability. Preliminary studies included the microencapsulation of red blood cells,

hepatocytes, pancreatic endocrine tissue, and islet cells (Lim and Moss, 1979; Lim and Sun, 1980; Lim, 1984).

Simply, the Lim and Moss method involved *in situ* polysalt formation around the surface of a microdroplet. Cells were suspended in microdroplets of alginic acid, and dropped into a calcium chloride solution which gelled immediately.

Alginic acid is a polysaccharide derived from brown seaweed and is composed of three (1-4)-linked polyuronic acid blocks consisting of poly-beta-D-mannuronic acid segments, poly-alpha-L-guluronic acid segments, and segments alternating in the above two moieties. The saccharide chains, in the sodium form of free acid groups (sodium alginate), cross link upon the addition of calcium chloride. The divalent calcium ion displaces two monovalent sodium ions to cross link the polymer (Adinaya, 1987).

Recently, bioengineers in cooperation with reproductive physiologists, have looked at forming microcapsules around the preimplantation mouse embryo to assess the developmental potential *in vitro* (Adinaya, et al., 1987). Adinaya (1987) slightly modified the encapsulation technique of Lim and Moss by omitting the addition of polylysine which then created a faster degrading capsule. As these capsules allow nutrients in and wastes out, serve as a physical barrier, are nontoxic, and are biodegradable, this method was chosen to develop a possible aid for IVF programs.

Superovulation

Superovulation, the stimulated production of mature oocytes through hormone injection, is a standard method to obtain a great number of oocytes and embryos in many domestic and common laboratory animal species.

The possibility of inducing superovulation was first recognized (Smith and Engle, 1927) from experimental evidence involving anterior pituitary extracts injected intramuscularly. These researchers noted rapid sexual maturity of the female genital system in immature rats and mice. Significantly, the oviductal ova of superovulated rodents possessed polar bodies and spindles as did those of the untreated animals.

Friedman (1929) found that she could generate similar results in the rabbit by injecting pregnant human urine intraperitoneally or intravenously, which proved to be more feasible and economical. Non-pregnant human urine was ineffective. Rowlands (1942) achieved superovulation in intact as well as hypophysectomized immature rats through a single injection of mare serum gonadotropin followed by a single injection of chorionic (urinary) gonadotropin.

Runner and Palm (1953) superovulated mice to establish the time of ovulation, finding it occured twelve to sixteen hours after chorionic gonadotropin injection. They also found that ova obtained by superovulation would develop to term if transferred, to a foster mother, within four hours

after ovulation.

Hafez (1964) found a decreased rate of implantation and litter size in rabbits that had been superovulated compared to controls, which he attributed to overcrowding *in utero*. Fujimoto, Pahlavan, and Dukelow (1974) analyzed karyotypes of rabbit blastocysts obtained from superovulated does and found 9.7% chromosomal abnormalities compared to 0% in controls. Kaufman (1978) warns that researchers must weigh the benefits of a large number of oocytes against the possible detrimental effects of hormone induced ovulation, as a poorer pregnancy outcome exists in superovulated females. For the studies herein, two and four cell stage embryos were isolated from the oviduct into embryo culture medium.

Recent evidence shows a difference exists in the cell surface characteristics of spontaneous versus induced ovulated mouse embryos. Champlin, et al. (1987) found a significant difference in the amount of microvilli and percentage of smooth areas on the mouse embryo cell surface, whereas superovulated mice exhibited less microvilli and greater smooth areas.

Fowler and Edwards (1957) analyzed superovulation in mature mice using gonadotropin injections. They found two factors caused differences in the number of oocytes ovulated: the concentration of PMS and the strain of mouse. Variations in response to gonadotropins exist across strains, and

different hormone regimens are necessary for untested stocks. Wilson and Zarrow (1962) analyzed superovulation in immature mice and found the concentration of PMS, the concentration of HCG, and the time interval between the two to influence the response. Optimal concentrations for PMS were 5 to 10 International Units (IU), for HCG were 5 to 10 IU, and the optimal time interval between injections was thirty to fifty hours.

Presently, superovulation entails the injection of hormones to induce follicular growth and oocyte maturation (by follicle stimulating hormone), and to induce ovulation (by chorionic gonadotropin) in mammals. Standard protocols use 5 to 10 IU PMSG, followed forty-eight hours later with 5 to 10 IU human chorionic gonadotropin (HCG), dependent upon the strain of mouse, given intraperitoneally (i.p.) or subcutaneously (s.c.) (Rafferty, 1970; Hogan et al., 1986).

Culture Media

Media used for the culture of mammalian ova has a long and varied history. Basically, medium used for any cell culture is a plasma-based or physiological salt-based solution buffered and osmoregulated to mimic an *in vivo* environment.

One of the first successful media used for rabbit embryo culture *in vitro* was coagulated blood plasma (Brachet, 1912). Although this nutritive source was hardly ideal, it

did allow for development up to 40 hours. Pincus (1930) made up several mixtures of media which included rabbit plasma, chick plasma, rabbit embryo extract, and chick embryo extract. Using these media, Pincus got two and four cell embryos to develop to morulae. Plasma solutions remained the only viable medium until Mather (1950) reported primitive streak stage embryos underwent differentiation if cultivated on clots of Tyrode's solution, egg albumin and agar.

Pincus (1941) demonstrated that the energy for expansion (of the rabbit blastocyst) is derived from the glycolytic pathway through poisoning experiments using potassium cyanide. Nevertheless, he realized the elucidation of a definitive nutritive requirement for the preimplantation embryo would have to wait for the development of a chemically defined medium (CDM) (Biggers, 1987). By 1947, practical application of preimplantation embryo culture had been initiated (Biggers, 1987).

Culture of the rodent preimplantation embryo was successful first with Hammond (1949) in which he chose a medium based upon physiological saline supplemented with hen egg white and yolk. Hammond also identified the importance of cell stage to development at the time of explantation.

The next medium adopted was Krebs-Ringer bicarbonate as the physiological saline to control the pH level. Whitten (1956) supplemented these salts with glucose, penicillin, streptomycin, and 1% egg white, and gassed the solution with

5% carbon dioxide to give a pH of 7.4. Additionally, Whitten observed no development in the Krebs-Ringer alone, and found the essential factors in the egg white were non-dialysable and could be replaced with crystalline bovine serum albumin (BSA), 0.03 to 6%.

The next milestone occurred with the McLaren and Biggers (1958) IVF-ET experiment in mice demonstrating that blastocysts aquired by Whitten's culture method could develop into normal adults.

With few exceptions to this point, only eight cell embryos had developed in vitro until Whitten (1957) found that calcium lactate supplementation would support the development of two cell embryos to blastocysts. From this point on, a multitude of similar media have been developed incorporating minor changes. Improvements since have included optimized salt concentrations (Brinster, 1965a), and the use of metabolic intermediates (Whitten, 1957) as substitutes for lactate which included pyruvate, phosphoenolpyruvate, and oxaloacetate. Brinster (1965b) analyzed the joint effect of lactate and pyruvate, which led to an optimized ratio of the two energy sources and was termed BMOC (Brinster's medium for ovum culture). It was then found to be advantageous to include glucose along with pyruvate and lactate (Brinster, 1971) to get expanded blastocysts from two cell embryos.

Nevertheless, Mintz (1964) showed that mouse

preimplantation embryos could develop in body sera as she used a medium consisting of 50% fetal calf serum (FCS) and 50% Earle's balanced salt solution supplemented with lactate. Purshottam and Pincus (1961) concluded from their work that rabbit embryos cultured to the blastocyst stage have relatively simple nutritive requirements, but later developmental stages require other serum components.

Whitten and Biggers (1968) cultured mouse embryos in CDM for the first time thereby negating any necessity for a female factor. These researchers also found F1 hybrids to be most suitable especially if the female were a C57BL mouse.

Wales (1969) identified the importance of calcium and potassium ions in mouse culture medium. Although ions are essential for in vitro development, Wales found the embryos tolerated wide ranges in ion concentrations.

Whittingham (1971) reviewed optimal pH and osmolarity values for mouse embryo culture, and found them to be 7.2 to 7.4 and approximately 0.285 osmoles, respectively.

Present day medium for embryo culture, with slight variation, consists of a physiological salt base, the energy substrates lactate, pyruvate, and glucose, as well as antibiotics, BSA, and phenol red as an pH indicator. Extra bicarbonate or an additional buffer is added to the collecting medium. The media used in these experiments was based on a modified Tyrode's salt solution (Bavister et al., 1983), for mouse embryo culture.

MATERIALS AND METHODS

Animals

The animals selected for embryo production were two strains of Mus musculus: Swiss Webster white (SWW), and B6D2-F1. A cross of C57BL/6J by DBA/2J produced B6D2-F1 mice. Initially, all strains except B6D2-F1 were purchased from The Jackson Laboratory (Bar Harbor, Me.); subsequently, our own breeding programs maintained stocks of SWW, C57BL/6J, and DBA/2J. A specific breeding program was employed for the production of B6D2-F1 mice. B6D2-F1 mice were sexed at twenty one days of age. Mice were provided Breeder Blox (Wayne) and water ad libitum, and maintained on a 12:12 L:D cycle.

Superovulation

Female SWW or B6D2-F1 mice, twenty-one to twenty-five days old, were superovulated by subcutaneous (s.c.) injection with 8 International Units (IU) pregnant mare's serum gonadotropin (PMSG) (Serotropin, Teizo, Tokyo, Japan), followed by 8 IU human chorionic gonadotropin (HCG) (Sigma Chemical Co., St. Louis, Mo.) forty-eight to fifty-one hours later. (Identical regimens were followed for experiments using 5/5 IU PMSG/HCG, respectively.) Immediately following HCG injection, primed females were housed with males of proven fertility overnight at a ratio of one to one.

Embryo Collection

Forty-eight and seventy-two hours after injection of HCG the mice were sacrificed by cervical dislocation, the abdominal cavity exposed, and both oviducts removed. Embryos were recovered by gentle teasing of the oviducts using two fine forceps in a three-welled embryological watchglass with 0.5 ml TALP+Hepes. Embryos were picked up with a 5 μl SMI glass micropipet (Emeryville, Ca.) attached to a thumb-screw aspirator. A similar aspirator was used with a 100 μl micropipet for encapsulated embryo transfers. The embryos were washed once in 0.5 ml fresh TALP+Hepes, pooled in 1.0 ml TALP+Hepes in a Falcon organ tissue culture dish (Becton Dickinson, and Co., #3037, Cockeysville, Md.) and placed in the incubator at 37°C in 5% CO_2/air.

Upon collection all embryos were allocated randomly to control or treatment groups. Embryo collection (and subsequent encapsulations) were carried out under a dissecting microscope (15X-105X).

Media

The media used for collection and culture of mouse embryos were modified TC199, TALP and TALP+Hepes. Modified TC199, the first culture medium used, was attained commercially as TC199 (Gibco, Grand Island, N. Y.) and modified with the addition of sodium pyruvate, gentamicin, and fetal calf serum (FCS) (Bates, Kontio, and Dukelow,

1985). TALP (Tyrode's-albumin-lactate-pyruvate) was adapted from a medium used for hamster embyro culture (Bavister, Leibfried, and Lieberman, 1983).

TALP is based upon Tyrode's salt solution (Tyrode, 1910), first developed as a physiologically purgative salt solution. We modified the TALP only by retaining the concentration of bicarbonate at 25 mM when Hepes was added (collection), adding penicillin/streptomycin, and omitting the amino acids used by Bavister et al.(1983), as preimplantation mouse embryos require only the energy substrates in the salt solution (glucose, lactate, and pyruvate).

This medium was prepared fresh with maximum storage of three weeks for the salt solution. The balance of ingredients were added Day -1 of the experiment, except BSA and sodium pyruvate were added Day 0 to deter salt precipitation. TALP was prepared from a stock solution of 114.0 mM NaCl, 3.2 mM KCl, 2.0 mM $CaCl_2$-$2H_2O$, 0.5 mM $MgCl_2$-$6H_2O$, 0.4 mM NaH_2PO_4-H_2O, 5.0 mM glucose, 10,000 IU/100 ml penicillin-G, and 1 mg/100 ml phenol red. A working solution of 100 ml was prepared Day -1 of the experiment by adding 10.0 mM sodium lactate, 25 mM $NaHCO_3$ and 10,000 IU/100ml streptomycin/ penicillin (Sigma). The solution was filtered through a 0.22 µm Corning sterile filter system (Corning Glass Works, #25932, Corning, N.Y.). The sterile collecting vessel was secured under a laminar flow hood, and with the top loosened,

the medium was placed in the incubator for equilibration overnight. On the day of the experiment, 0.5 mM sodium pyruvate and 3 mg/ml bovine serum albumin (BSA) were added to the 100 ml solution. Two 50 ml aliquots were separated, and 240 mg/100 ml N-2-Hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (Hepes) was added to one aliquot. For collection, TALP+Hepes was filter-sterilized through a 0.22 μm Millex-GS filter unit (Millipore Corp., #SLGS0250S, Bedford, Ma.) into a 10 ml Vacutainer (Becton Dickinson, #6430 Rutherford, N.J.) and held in a 37°C water bath. Likewise, TALP was prepared for subsequent culture. Salts were dissolved in sterile water for injection.

Enzymatic Digestion

Zonae pellucidae were digested enzymatically using trypsin or pronase. Trypsin proved to be variable in effect, so pronase was substituted for further treatments. Embryos were aspirated from the collecting medium, and extruded into the 0.5% solution of pronase in phosphate buffered saline (PBS) for a maximum of 40 seconds until the zonae pellucidae disappeared. Denuded embryos were then washed in fresh medium once before further treatment.

Encapsulation

Sodium alginate (Sigma, type IV) was prepared on the day of experimentation by suspending 0.22 gms. in 20.0 ml 0.15 M saline to yield a 1.1% solution (wgt./vol.). Similarly, 0.3 gms. of $CaCl_2$ was dissolved in 20.0 ml 0.15 M saline to yield a 1.5% solution (wgt./vol.). These solutions were kept at 37°C on a slide warmer. The saline for both solutions was filter-sterilized through a 0.22 μm filter disk.

Encapsulation was performed by aspirating embryos into an acid-washed, sterilized 5 μl glass micropipet along with 1.0 μl of media, extruded into a 1.1% sodium alginate (SA) solution, mixed lightly to ensure even alginate envelopment, and re-aspirated in the order SA-embryo-SA for a total volume approximating 1.0 μl. Submerging the micropipet under a solution of 1.5% $CaCl_2$, the contents were expelled quickly resulting in immediate gelling of the matrix into a plug. The 1.5% $CaCl_2$ solution was agitated slightly for 30 seconds to allow adequate cross-linking of divalent calcium ions in the alginate matrix. A larger (inner diameter) micropipet was then used to aspirate the encapsulated embryos from the $CaCl_2$ solution into 1.0 ml of fresh TALP+Hepes to wash the excess calcium ions from the plug. A three-welled embryological watchglass facilitates the sequential transfers involved. After washing, the plug was transferred and expelled into a culture dish containing 1.0 ml TALP, covered with light mineral oil, and placed in the incubator. The

mineral oil was equilibrated (in the incubator) the evening prior to the experiment, and kept at 37°C on a slide warmer until used.

Cleavage stages of both control and encapsulated embryos were assessed at twenty-four hours on a Nikon Diaphot inverted microscope.

Media Analysis

Analysis of the TALP and TALP+Hepes media was performed as follows. A 50 μl sample of each medium was extracted from the vacutainer in a 37°C water bath immediately following culture of treatment and control embryos, and was quantitated in a blood/gas analyzer (Instrumentation Laboratory, #1304, Lexington, Ma.). The I.L. #1304 is a diagnostic blood/gas analyzer which quantitatively measures nine parameters of serum: pH, pCO_2, pO_2, bicarbonate, total CO_2, base excess, standard bicarbonate, base excess in extra cellular fluid, and $\%O_2$ saturation.

Statistical Analyses

Statistical methods performed on the results were taken from Gill (1987). Where statistical significance was found, confidence levels (probability) were indicated.

Results of superovulation trials were analyzed by (1) comparison of means between 5 IU and 10 IU gonadotropins for SWW mice, (2) comparison of means between 5 IU and 8 IU

gonadotropins for B6D2-F1 mice, and (3) comparison of means between SWW and B6D2-F1 mice for 5 IU gonadotropins. For cases (1) and (2), preliminary f ratio (variance) test indicated homogeneity and means were compared by the two-sample t test. For case (3), variances were found to be unequal, therefore Behren's t-like test (t') was used (Gill, 1987, p.71).

Encapsulation versus control trials were analyzed using two-way contingency tables for both two cell and four cell stage embryos. This test statistic closely follows the chi-square (q) distribution.

Encapsulation of embryos by number per capsule trials were analyzed using multidimensional contingency tables to test for independency and/or interaction of cell stage and embryo number per capsule. As the q value for the test of independence was low, an approximate q value for interaction was used. The q value for interaction was also low, therefore cell stage data were lumped and comparisons for number of embryos per capsule were made.

RESULTS

Superovulation

Trials were initiated using Swiss Webster white (SWW) mice, but these were replaced with the strain B6D2-F1, a cross between C57BL/6J and DBA/2J mice. The hybrid mice, standardly designated B6D2-F1 (Staats, 1976) showed greater response to superovulation by producing over a two-fold increase in the number of embryos compared to the SWW mice. Table 2 compares the mean embryo number collected per superovulated mouse from the two strains with the indicated concentrations of gonadotropins. Embryos initially collected from SWW mice and used in encapsulation trials developed in two of twenty-one cases compared to the control embryos where ten of sixteen developed.

Media

Modified TC199 was the first culture medium used, but problems with pH stability and poor developmental rates in culture led to the search for an alternate medium. A modified Tyrode's solution, Tyrode's-albumin-lactate-pyruvate (TALP), was chosen based upon its success with hamster ovum culture (Bavister et al., 1983), and was used exclusively after the initial trials.

Blood/gas analysis was performed on the medium for collecting and culturing embryos. Initially, we found that TALP+Hepes (25 mM bicarbonate) exposed to the atmosphere increased in pH approximately 0.15 units in 18 minutes. Bicarbonate (HCO3-) also rose (2.0 mmol/L) in the time indicated.

Table 2. Superovulation in SWW and B6D2-F1 mice.

	MOUSE STRAIN			
	SWW		B6D2-F1	
PMSG/HCG (units injected)	5/5	10/10	5/5	8/8
mice injected	10	25	50	50
mice responding	7	21	41	40
embryos collected	84	253	1146	1476
mean embryos/mouse responding	12.0	12.7	28.0	36.9
% responding	70	84	82	80
mean embryos/mouse injected	8.4(a,c)	10.1(a)	22.9(b,c)	29.5(b)

(a) Equal variance estimated by f ratio test; no difference between means.
(b) Equal variance; no difference between means.
(c) Unequal variance; 99.97% ($P<0.00025$) confidence of difference between means.

When TALP+Hepes (2 mM bicarbonate was used for culture and analyzed, pH levels dropped dramatically from 7.35 to 6.94 (average of two samples). TALP+Hepes (10 mM bicarbonate) pH levels dropped from 7.47 to 6.99 (average of two samples).

When TALP (25 mM bicarbonate) was used for culture and analyzed, pH levels dropped only from 7.59 to 7.37 (average of six samples). Bicarbonate levels dropped slightly from 18.2 to 16.7 mmol/L (average of six samples). Partila carbon dioxide (pCO_2) levels rose from 18.52 to 30.09 (%) (average of six samples), in the presence of 5% CO_2/air.

Encapsulation

Various methods of encapsulating embryos were attempted in an effort to develop a feasible, consistent technique. Preliminary work involved the use of an air flow regulator adaptd as a sleeve around a blunted 22 gauge needle through which the alginate was passed (Rehg, Dorger, and Chau, 1986). This apparaus offerred precise control of capsule diameter and rate of formation which was dependent solely upon liquid flow rate and gas flow rate. This method soon became infeasible as the technique involved placing the the embryos into the alginate solution randomly, drwaing a portion of the alginate into the a syringe and locking the syringe in the 22 gauge needle for expulsion. The technique demanded blind manipulation necessitating locating the embryo once extruded into the $CaCl_2$.

Other methods involved using glass (Pasteur) pipettes for capsule formation, but these capsules had too large diameters to be of use in *in vivo* studies or in the assessment stage of *in vitro* work. The cross linking formed the interaction of the alginate in $Cacl_2$ creates a light defracting structure which renders viewing through the capsule more difficult with increasing diameter. The method developed allows for precise control of capsule diameter and less precise control of capsule lenght. The inner diameter of the glass micropipette determines the cross sectional area whereas the amount of alginate aspirated into the micropipette determines the capsule lenght. Micropipettes may be scored a specified distance from the tip to mark the alginate level. Simple math will determine the approximate volume of alginate given the inner diameter and length of the cylinder.

Also crucial is that visual contact be maintained with the embryo(s) throughout all steps. The microcapsule is somewhat opaque (embryos are obscured viewed through a dissecting microscope); nevertheless, embryo stage assessment is accomplished easily on an inverted phase contrast microscope. Once this method was developed, no embryos were lost in the encapsulating process indicating its relative ease and repetitive nature. Two hunred, ninety seven embryos were encapsulated with none lost compared to preliminary encapsulating methods in which of the 49 attempted, 24 were

lost (49%). The method of encapsulating embryos that was used is detailed in the Materials and Methods section. Initial encapsulating trials varied in success and feasability, and embryos frequently were lost in the encapsulating process. Modifications were implemented and subsequently no embryos were lost in the encapsulating process.

The results of encapsulation of B6D2-F1 embryos cultured in modified TALP for 24 hours is shown in table 3. This lists the number of embryos that developed over the number cultured, with percentages in parentheses.

Table 3. Development of encapsulated and control B6D2-F1 embryos by cell stage. (a)

Cell Stage	Encapsulated (%)	Control (%)	Total
2(b)	40/97 (41.2)	65/123 (52.8)	220
4(c)	24/39 (61.5)	18/31 (58.1)	70
Total	64/136 (47.1)	83/154 (53.9)	290

(a) Criteria for development at 24 hours: 3 cell embryos are precocious 2 cell embryos, and minimum of one complete division event.
(b) Development was independent of treatment for 2 cell embryos.
(c) Development was independent of treatment for 4 cell embryos.

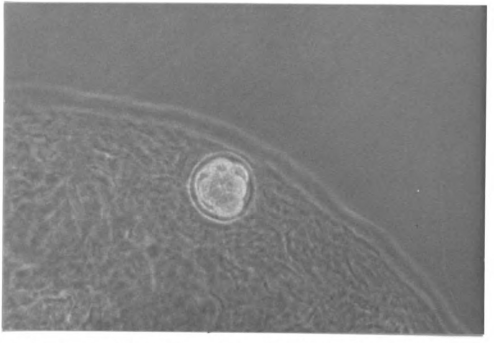

Figure 1. Morula (8 cell, beginning compaction) developed in vitro from a 2 cell B6D2-F1 embryo encapsulated in alginate (200X).

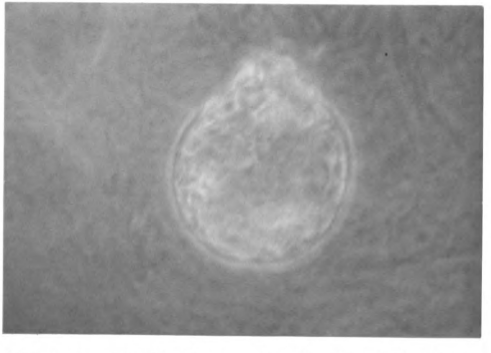

Figure 2. Hatching blastocyst developed in vitro from a 2 cell B6D2-F1 embryo encapsulated in alginate (400X).

Enzymatic Digestion

Preliminary trials also were carried out with enzymatic treatment of the embryos to digest the zona pellucida. Pronase or trypsin (0.5%) was used to remove the zona pellucida. Since there was no difference in the type of enzymatic digestion by either enzyme, the data were pooled and are shown in table 4.

A series of trials were carried out to compare the development by number of embryos encased per capsule. Embryos were encapsulated singly, doubly, or triply in a given amount of alginate and cultured under oil. Table 5 shows the results of these trials.

Table 4. Development of embryos after enzymatic removal of the zona pellucida with subsequent encapsulation and culture, or culture only. (a)

	Cell Stage				
Trial	1	2	3	4	single blastomere
encapsulation	0/2	6/40	0/3	2/6	1/2(b)
enzyme/encapsulation	0/2	4/14	---	0/2	2/2(c)
enzyme	---	4/6	1/1	---	---
culture only	---	6/13	---	0/2	---

(a) These trials included initial encapsulation trials in which some embryos are lost.
(b) One 2 cell embryo dissociated during the encapsulation procedure. One blastomere divided.
(c) One 2 cell embryo dissociated after enzymatic digestion of the zona pellucida. Both blastomeres divided.

Table 5. Development of B6D2-F1 embryos by cell stage per capsule for (A) treatment and (B) control.

(A) Treatment

Embryo Stage	Number of embryos per capsule (a)			
	1 (%)(b,c)	2 (%)(b,d)	3 (%)(c,d)	Total
2	7/9 (77.8)	16/22 (72.7)	12/24 (50.0)	35/55 (63.6)
4	3/4 (75.0)	10/10 (100)	11/15 (73.3)	24/29 (82.8)
Total	10/13 (76.9)	26/32 (81.3)	23/39 (59.0)	59/84 (70.2)

(a) Chi-square test for independence and interaction of cell stage and embryo number per capsule showed no significance. Therefore, cell stage data were lumped for within class comparisons of embryo number per capsule and analyzed by Bonferroni Chi-square.
(b) No significant difference (P>0.05).
(c) No significant difference (P>0.05).
(d) No significant difference (P>0.05).

Table 5, (continued)

(B) Control

Embryo stage (a)	Developed/Cultured (%)
2	52/57 (91.2)
4	18/24 (75.0)
Total	70/81 (86.4)

(a) Development was independent of cell stage (P>0.05).

DISCUSSION

Superovulation

B6D2-F1 mice were superior superovulators to the SWW mice. B6D2-F1 mice gave a higher response to standard doses of gonadotropins. Although in preliminary trials using embryos from SWW mice for encapsulation only 2 of 21 (9.5%) embryos developed, control embryos develpoed at a rate of 62.5% (10/16). It is believed inadequate ability of the culture medium to support development in some of the early experiments was at fault. Additionally, other workers have reported lower developmental rates with SWW mice compared to hybrids such as B6D2-F1 (Adinaya et al., 1987).

The percent of mice that responded to the various concentrations of gonadotropins, which ranged from 70.0% to 84.0% in SWW mice and 80.0% to 82.0% in B6D2-F1 mice, showed that in all cases mice responded equally well even though the numbers of embryos were varied. The doses of gonadotropins used for induced ovulation traditionally have ranged from 1 to 10 IU with the majority of researchers using between 5 and 10 IU (Spindle and Goldstein, 1975; Hogan et al., 1986). The present results with B6D2-F1 mice indicate that when 8 IU PMSG/8 IU HCG are given s.c., and the females placed with males overnight immediately following HCG injection, greater

than 15 embryos can be collected per mouse.

A recent reference (Hogan et al., 1986) has placed the limit of low superovulation at 15 or fewer embryos. A comparison of the means attained from superovulation of B6D2-F1 mice reveal that both 5/5 and 8/8 IU gonadotropins are equally effective in producing a high number (greater than 15) of preimplantation embryos. The comparison of means between 5 IU and 10 IU gonadotropins for SWW mice showed no difference. Not suprisingly, comparison of means between SWW and B6D2-F1 mice at 5 IU gonadotropins showed a highly significant difference ($P<0.0005$).

The number of SWW mice tested was low, but previous work at the Endocrine Research Center, as well as other reports, substantiate SWW mice to be low superovulators (Adinaya et al., 1987; Hogan et al., 1986). Given the results of the between means comparison of SWW and B6D2-F1 mice, the latter are more efficient in response to superovulation.

Media

Modified TALP was used for embryo collection (TALP+Hepes) and culture for all trials following preliminary encapsulation development.

TC199, used in preliminary trials, proved to be difficult to maintain an optimal pH, and culture of SWW mouse embryos in TC199 showed poor and varied developmental rates. Upon this, TALP was chosen as a recent report indicated it to

be better for culture of preimplantation mouse embryos (Bates et al., 1985). In their report, Bates et al. found overall rates of development for 2 cell and 4 cell embryos in TALP of 83.4% and 72.7%, respectively, compared to like stages in TC199 of 51.5% and 68.0%. Although the overall development of mouse embryos in TALP was about 50% in this study, later trials showed developmental rates approaching near 100% whereas earlier trials of embryos cultured in TC199 showed developmental rates of about 62% at best (10/16).

The results of the analysis of the media used during embryo manipulation confirm the necessity of an additional buffer (Hepes) during collection procedures. TALP is sufficient for culture procedures. The difference in embryo environment for collection (atmospheric) versus culture (5% CO_2 /air) suggests the need for differential media. When embryo culture was performed in TALP+Hepes (2 and 10 mM bicarbonate), pH levels sank to below 7.0. Hepes buffer under the influence of incubating conditions drives the pH level too low for optimal embryo development. Therefore, it can be concluded that bicarbonate is a sufficient buffer for <u>in vitro</u> culture which correlates with the <u>in vivo</u> environment being mimiced.

Encapsulation

The major question of this research problem is: Does encapsulation affect preimplantation embryonic development

in vitro? Table 3 lists the results of B6D2-F1 embryos encapsulated versus control embryos of equal cell stage. The evidence supports independence of encapsulation on development in that beginning either at the 2 or 4 cell stage the mouse preimplantation embryo developed just as well in the treatment groups as in the control groups. For encapsulation versus control, trials of 2 cell embryos developed 41.2% and 52.8%, respectively, and trials of 4 cell embryos developed 61.5% and 58.1%, respectively. Although disparity in developmental rates across cell stage appears unusual, 2 cell stage embryos may be more susceptible to lethal influence, whereas 4 cell stage embryos may be more fit to survive as the poor 2 cell embryos have been weeded out already.

The overall developmental rate for encapsulated embryos was 47.1% compared to the controls, which was 53.9%. Again, these numbers reflect inadequate culture conditions early in the experiment, and increased culture rates later in the experiment as a developmental rate near 100% was seen across treatments in later trials. The fact that the rate of embryo development for treatment versus control was not significantly different, even though both were low, substantiates a negative influence inherent in both treatments.

The results of encapsulation on mouse embryo development support a recent study which analyzed the

bioengineering parameters of alginate capsules. Adinaya et al., (1987), found no significant ($P>0.05$) difference in rate of development for mouse embryos cultured to blastocyst stage in 72 hours for encapsulated versus control trials. This was true for experiments using SWW mice and CB6F1 mice.

Adinaya et al., (1987), did report however that a significant ($P<0.05$) number of encapsulated SWW mouse embryos reached intermediate stages of development, but not in CB6F1 mice. Therefore, they concluded this to be coincidental. These researchers also changed from SWW mice to CB6F1 mice (hybrid) to improve the overall rate of development across treatments. Although the number of embryos developing to blastocysts was much greater for CB6F1 mice over SWW mice, there again was no diference within strain for encapsulated versus control embryos.

Pilot trials were conducted in which the zona pellucida of the embryo was removed chemically using pronase or trypsin at a 0.5% concentration. Trials with trypsin showed long and varied times for enzyme digestion of the zona pellucida so it was discontinued in lieu of pronase at the same concentration (Rawlins, 1984). This experiment was undertaken to see whether a denuded embryo would develop without its zona pellucida while encased in alginate versus a denuded embryo *in vitro*. The encapsulation-only treatment showed little potential with only 6 of 40 (15.0%) embryos developing. These trials also were performed when the culture

medium was often inadequate, allowing for extreme variability in developmental rates. In addition, these trials involved initial encapsulation methods in which some embryos were lost. Nevertheless, as seen in table 4, 28.6% of 2 cell embryos denuded of their zonae pellucidae and encapsulated, developed versus 66.7% denuded only and 15.0% encapsulated only. As these experimental numbers are only preliminary, no concrete conclusions may be drawn, but there is good indication for development of zonae pellucidae-free embryos in alginate capsules.

Willadsen (1982) has indicated the need for protection of the preimplantation embryo as it traverses the oviduct naturally in the form of a zona pellucida or artificially by means of an embedding material. He has experimented with agar embedding of sheep preimplantation embryos (Willadsen, 1979), and found this to substitute as protection for the embryo when the zona pellucida has been damaged or removed in monozygotic splitting procedures. Beginning with 33 zonae pellucidae-free, blastomere-separated embryos, Willadsen recovered 20 of them from recipient ewes. He also reported for recovered single blastomeres, that 35 developed to late morulae/early blastulae, one was retarded and four were degenerate.

The results in this study indicate that preimplantation embryos can be sripped (enzymatically) of their protective zonae pellucidae, encapsulated in alginate, and cultured in

vitro until a later transfer is desirable. An advantage of alginate embedding over the agar used by Willadsen is the biodegradability of the alginate. Agar embedded embryos and/or blastomeres must be removed surgically prior to implantation. This was a major consideration in choosing alginate as the artificial protecting material.

The only other known report of embedded micromanipulated mammalian embryos is that of Warfield et al., (1987). In this study, bisected bovine embryos (post-compaction) were transferred to recipient females after the following manipulations.

In one trial, demi-embryos with the zonae pellucidae intact developed in 29 of 88 cases (33%), for zona pellucida-free developed in 8 of 25 cases (32%), and for zona pellucida-free/gelatin embedded developed in 8 of 22 cases (36%). Again, this evidence supports the potential of embryos to manipulation and subsequent embedding with no detrimental effect on further development. Warfield et al. were trying to establish the viability of zona pellucida-free embryos for routine embryo transfer, and concluded that placing bisected embryos into a zona pellucida or embedding in gelatin was of no advantage. The alternative is just as important with regards to the findings of this study. Gelatin embedding was not detrimental.

It should be reiterated that the Warfield et al. study tested postcompaction embryos which do not have further need

of the zona pellucida. In addition, they believed the gelatin to have dissipateed soon after uterine deposition, however this was not tested.

A most interesting finding from the enzymatic digestion of the zona pellucida reported here was the two separate incidences of (1) a 2 cell embryo that hatched from its zona pellucida within the alginate capsule, one of which divided; and (2) a 2 cell embryo enzymatically denuded of its zona pellucida which also separated in the capsule and subsequently both blastomeres divided. Although somewhat like the Warfield et al. report, these again were precompacted embryos and were isolated cases. Nevertheless, these two incidences demonstrate that alginate encapsulation could be applied to existing embryo splitting technology.

A third trial, performed to study the effect of varying number of embryos per capsule consisted of embryos encapsulated singly, doubly, and triply. The volume of alginate was kept constant across treatment groups. These results in general agree with the previous encapsulation trials as all developmental rates were greater than 50% by the number of embryos per capsule. The greater rate seen in embryos encapsulated doubly over either capsules of single or three embryos is suprising. A tentative explanation could be that proximity of two embryos is optimal such that one helps the other in development, whereas a single embryo rests unaided, and three embryos compete for local

nutrients.

These figures hold true to the pattern seen in the earlier encapsulation trials (Table 3) although the rates are higher across cell stage. Therefore, it is concluded that the explanation for these results is the same.

Overall, the results presented in this study show microencapsulation of mouse preimplantation embryos is possible, and that such treated embryos develop *in vitro* at rates similar to control embryos at the 2 cell and 4 cell stage. As *in vitro* culture of mammalian preimplantation embryos remains a vital, yet ever changing, field in the study of reproductive physiology, improvements upon existing procedures become necessary to ensure the efficiency and validity of the results.

SUMMARY AND CONCLUSIONS

Mouse preimplantation embryos microencapsulated in sodium alginate can be cultured *in vitro* to further stages of development. A technique to microencapsulate the embryos was developed which can be accomplished easily with standard collecting apparatus. Any number of embryos may be encapsulated together with this technique. The following conclusions were drawn from the data:

1) Superovulation of hybrid mouse strain C57BL/6J x DBA/2J (B6D2-F1) produced a significantly greater number of preimplantation embryos over inbred mouse strain Swiss Webster white (SWW).

2) TALP and TALP+Hepes are adequate nutritive media for the culture and collection of, respectively, mouse preimplantation embryos with the modifications outlined in the Materials and Methods section.

3) Microencapsulation of 2 cell and 4 cell mouse embryos in sodium alginate does not inhibit their *in vitro* development.

4) The placement of one, two, or three embryos per microcapsule was of no advantage or disadvantage to *in vitro* development.

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APPENDIX

APPENDIX

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