

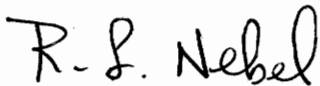
**DEVELOPMENT OF MOUSE MORULAE AFTER ENCAPSULATION IN
ALGINATE MICROGELS OR POLY-L-LYSINE MICROCAPSULES**

by

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(ABSTRACT)

Three experiments were conducted to evaluate *in vitro* and *in vivo* development of zona pellucida-intact (ZPI) and zona pellucida-free (ZPF) mouse embryos after encapsulation in either 2% sodium alginate or 0.1% poly-L-lysine (PLL). In Experiment 1, rate of development of ZPI embryos (n=150) from morulae to hatched blastocysts was measured after encapsulation in alginate or PLL and as unencapsulated controls. Following encapsulation, developmental stages were recorded every 24 h for 120 h. Percentage of encapsulated embryos completely hatched from the zona pellucida were not different from each other but were lower than unencapsulated controls at 48, 72, 96 and 120 h.

Development of ZPI and ZPF mouse embryos after encapsulation in either alginate or PLL was examined in Experiment 2. Developmental stages and diameters were recorded every 24 h for 72 h. At 72 h, embryos were stained and fixed on slides to examine nuclei. Percentage of ZPI embryos developing to expanded blastocysts, their diameters and nuclear counts were not different from

each other or from ZPF embryos. Percentage of ZPI embryos initiating hatching or completely hatched from the zona pellucida, their diameters and nuclear cell numbers were also similar.

In the final experiment, ZPI mouse morulae were unencapsulated or encapsulated in either alginate or PLL and transferred into recipients to examine in vivo development. Recipients were allowed to develop fetuses to term. Recipients receiving encapsulated embryos failed to deliver pups. However, five of six recipients of unencapsulated embryos (n=71) delivered a total of 16 live pups. Additional transfers were performed to examine viable fetuses and resorption sites on day 10 of gestation. Pregnancy rates, diagnosed by the presence of viable fetuses or resorption sites, were similar for all treatments: unencapsulated (71.4%), alginate (87.5%) and PLL (87.5%). However, the total number of viable fetuses present was higher for unencapsulated embryos (42.1%) when compared to embryos in alginate microgels (17%) and embryos in PLL microcapsules (14.6%). Additionally, recipients of alginate and PLL encapsulated embryos had more resorption sites (4% and 13.4%) when compared to recipients of unencapsulated embryos (0%).

These investigations demonstrated that development of encapsulated ZPI mouse morulae is impaired at the hatched blastocyst stage; however, encapsulated ZPI and ZPF mouse morulae develop similarly in size and nuclear counts. In vivo development of ZPI morulae was also impaired due to an asynchronous condition between the uterine environment and the developing embryos.

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Table of Contents

INTRODUCTION	1
LITERATURE REVIEW	4
Micromanipulation of Embryos	4
Bisected Embryos	6
Freezing of Embryos	9
Agar Embedding	12
Microencapsulation	14
Microencapsulation of Living Cells	16
Microencapsulation of Spermatozoa	18
Microencapsulation of Embryos	21
EFFECTS OF ENCAPSULATION ON IN VITRO DEVELOPMENT OF ZONA PELLUCIDA-INTACT AND ZONA PELLUCIDA-FREE MOUSE MORULAE	25
ABSTRACT	25
INTRODUCTION	27
MATERIALS AND METHODS	29
Embryo Collection	29
Removal of Zona Pellucida	30

Microencapsulation Procedures	30
Culture Procedures	31
Staining and Examination of Embryos	34
Statistical Analysis	34
RESULTS AND DISCUSSION	37
Experiment 1	37
Experiment 2	45
PREGNANCY MAINTENANCE IN MICE FOLLOWING TRANSFER OF	
 ENCAPSULATED EMBRYOS	62
ABSTRACT	62
INTRODUCTION	64
MATERIALS AND METHODS	66
Embryo Collection	66
Microencapsulation	66
Embryo Transfer	66
Fetal Development	67
Statistical Analysis	68
RESULTS AND DISCUSSION	69
CONCLUSIONS	75
REFERENCES	78
APPENDIX	88
VITA	93

List of Illustrations

Figure 1.	Microencapsulated zona pellucida-intact and zona pellucida-free mouse embryos.	32
Figure 2.	Percentage of embryos initiating hatching after encapsulation in alginate or poly-L-lysine compared to unencapsulated embryos. . .	39
Figure 3.	Percentage of embryos developing to hatched blastocysts after encapsulation in alginate or poly-L-lysine compared to unencapsulated embryos.	41
Figure 4.	Percentage of embryos developing to expanded blastocysts after encapsulation in alginate or poly-L-lysine compared to unencapsulated embryos.	52
Figure 5.	Percentage of encapsulated zona pellucida-intact embryos initiating hatching in alginate or poly-L-lysine compared to unencapsulated embryos.	53
Figure 6.	Percentage of encapsulated zona pellucida-intact embryos completely hatched in alginate or poly-L-lysine compared to unencapsulated embryos.	54

List of Tables

Table 1.	Percentage of embryos developing after encapsulation in either alginate microgels or poly-L-lysine microcapsules compared to unencapsulated embryos.	38
Table 2.	Mean developmental classification ¹ of unencapsulated embryos and embryos encapsulated in alginate microgels or poly-L-lysine microcapsules.	43
Table 3.	Repeatability of development scores between hours of incubation within mouse and treatment.	44
Table 4.	Percentage of embryos developing after encapsulation in alginate microgels or poly-L-lysine microcapsules compared to unencapsulated embryos.	46
Table 5.	Developmental classification ¹ of unencapsulated and encapsulated zona pellucida-intact (ZPI) and zona pellucida-free (ZPF) embryos.	47
Table 6.	Diameter (mm) means of unencapsulated and encapsulated zona pellucida-intact (ZPI) and zona pellucida-free (ZPF) embryos.	49
Table 7.	Nuclear counts ¹ of unencapsulated and encapsulated embryos at 72 h incubation.	50
Table 8.	Diameters (mm) ¹ of unencapsulated embryos and embryos encapsulated in alginate or poly-L-lysine developing to expanded blastocysts.	55
Table 9.	Diameters (mm) ¹ of unencapsulated embryos and embryos encapsulated in alginate or poly-L-lysine developing to hatching or hatched blastocysts.	57
Table 10.	Nuclear counts ¹ of embryos after encapsulation in alginate microgels or poly-L-lysine microcapsules compared to unencapsulated embryos.	58

Table 11. Percentage of embryos with uncountable nuclei ¹ after staining with trypan blue and Hoechst 33342.	60
Table 12. Production of live young following transfer of unencapsulated and encapsulated morulae in alginate microgels or poly-L-lysine microcapsules.	70
Table 13. In vivo survival at mid-gestation of unencapsulated and encapsulated morulae in alginate microgels or poly-L-lysine microcapsules.	72
Table 14. M2 medium used for embryo collection and transfer.	89
Table 15. Solutions used for microencapsulation procedures.	90
Table 16. CZB medium for in vitro culture.	91
Table 17. Staining procedures for examining nuclei.	92

INTRODUCTION

Recent advancements in embryo micromanipulation have led to questions concerning the necessity of a zona pellucida, the acellular glycoprotein layer surrounding an embryo, not only during freezing but also for successful embryo transfer. Varying degrees of damage are inflicted upon the zona pellucida during microsurgical techniques such as microinjection and bisection. The necessity of the zona pellucida, which normally prevents dispersal of blastomeres and protects the embryo from foreign cells (Willadsen, 1982), is controversial in many species. Investigators have reported increased viability of micromanipulated embryos without a zona pellucida (Warfield et al., 1987; Hoppe and Bavister, 1983) and frozen embryos with a damaged zona pellucida (Niemann, 1985; Niemann et al., 1986). However, evidence exists that micromanipulated and/or frozen embryos with a damaged or absent zona pellucida have decreased in vitro survival rates (Warfield et al, 1987; Wagner and Graves, 1984) and lack the viability to maintain pregnancy (Heyman, 1985; Rottmann and Lampeter, 1981; Kanagawa et al., 1979). For these reasons, micromanipulated and/or frozen embryos have been

embedded in agar in an attempt to increase viability. Research has shown that agar embedding can increase viability of micromanipulated embryos (Yong and Jianchen, 1990; Tsunoda et al., 1987; Lehn-Jensen and Willadsen, 1983; Willadsen, 1982) and nonmanipulated embryos (Westhusin et al., 1989; Willadsen, 1979).

Previously reported studies indicate that agar embedding is beneficial to successful freezing of both intact and bisected embryos of various species. However, agar embedment appears to have the following disadvantages: 1) the critical temperature of agar necessary for the phase change from a liquid to a solid is very close to the lethal temperature of embryos, 2) agar gel is relatively fragile, and 3) agar gel is transparent making it difficult to see and manipulate under the microscope (Kojima et al., 1990). Therefore, another method of protecting embryos has been investigated.

Lim (1982) developed the first successful encapsulation procedure for living cells. His methods have since been adapted to encapsulate preimplantation embryos and may serve as a successful alternative to embedment in agar. Zona pellucida-intact (ZPI) and zona pellucida-free (ZPF) mouse embryos have been encapsulated in sodium alginate and develop similarly to unencapsulated embryos (Adaniya et al., 1987; Cosby and Dukelow, 1990). Microencapsulation of bovine (Hollingsworth and Page, 1988) and rabbit embryos (Kojima et al., 1990) prior to freezing has shown to enhance survival by reducing freeze/thaw injury. Pregnancy has been maintained after transfer of alginate encapsulated ovine

embryos (Meredith et al., 1990) and frozen/thawed rabbit embryos following the removal of the microgels (Kojima et al., 1990).

Two encapsulation methods, alginate microgels and poly-L-lysine (PLL) microcapsules, were examined in this study. Specific objectives were 1) to evaluate in vitro development of mouse ZPI embryos encapsulated in either alginate or PLL from morulae to hatched blastocysts; 2) to examine in vitro development of mouse ZPI and ZPF morulae after encapsulation in either alginate or PLL; and 3) to examine in vivo survival of encapsulated morulae. These studies may lead to encapsulation of micromanipulated and/or frozen embryos to enhance in vitro survival and for potential use in embryo transfer.

LITERATURE REVIEW

Micromanipulation of Embryos

Microsurgical techniques such as microinjection and bisection often cause varying degrees of damage to the zona pellucida, which normally prevents dispersal of blastomeres and protects the embryo from foreign cells (Willadsen, 1982). The necessity of a zona pellucida after micromanipulation is controversial. Investigators have reported increased viability of micromanipulated embryos without a zona pellucida (Warfield et al., 1987; Hoppe and Bavister, 1983) and frozen embryos with a damaged or absent zona pellucida (Niemann, 1985; Neimann et al., 1986). However, evidence exists that micromanipulated and/or frozen embryos with a damaged or absent zona pellucida have decreased in vitro survival rates (Warfield et al., 1987; Wagner and Graves, 1984) and lack the viability to maintain pregnancy (Heyman, 1985; Rottmann and Lampeter, 1981; Kanagawa et al., 1979). This literature review will examine problems that occur

following micromanipulation of embryos and will present possible solutions to these existing problems.

Reduced in vitro development has been shown for ZPF embryos when compared to ZPI embryos in mice (Rottmann and Lampeter, 1981) and cattle (McFarland et al., 1985). However, Hoppe and Bavister (1983) reported that bovine morulae and blastocysts, either enzymatically (93%, n = 14) or mechanically (93%, n = 29) denuded of their zona pellucida, develop in vitro to blastocysts and expanded blastocysts at rates higher but not significantly different than ZPI embryos (88%, n = 38). It was also reported that 10 to 15% of all attempts to remove the zona pellucida resulted in severe damage to the embryos and those embryos were discarded.

Reduced development of ZPF embryos has also been shown following transfer into recipients. Rottmann and Lampeter (1981) reported that 50% of ZPI rabbit morulae transferred into recipients implanted while none of the ZPF morulae implanted. Massey et al. (1982) also reported no pregnancies following transfer of bovine morulae and earlier stage embryos after removing the zona pellucida with 0.5% pronase. However, transfer of ZPF early blastocysts and blastocysts produced 4 of 11 (36.4%) pregnancies and 2 of 7 (28.6%) pregnancies. Calving rates after ZPI embryo transfer normally range from 40 to 70%, depending on method of transfer, recipient management and embryo quality (Heyman, 1985). Percentages reported were lower than normally obtained for transfer of ZPI embryos and implicates the necessity of an outer protectant layer for in vivo de-

velopment, especially for morula and earlier stage embryos. These studies are in direct contrast to Hoppe and Bavister (1983), who reported that transferred bovine morulae, mechanically denuded of their zona pellucidas, develop similarly to ZPI embryos after transfer (33 vs. 39%). However, all ZPF embryos developed in vitro to the blastocyst stage prior to transfer. These studies indicate that ZPF early blastocyst and blastocyst stage embryos may produce pregnancies; however, earlier stage embryos may need additional development until they are transferrable. Procedures for denudation, either enzymatic or mechanical, may also influence development (Hoppe and Bavister, 1983).

Bisected Embryos

Embryo micromanipulation is a general term that refers to alteration of an embryo's original form. Such manipulations include embryo cloning, microinjection and bisection. These techniques require penetration of the zona pellucida and will yield embryos with a damaged or absent zona pellucida. The viability of micromanipulated embryos may be influenced by the characteristics of the zona pellucida.

Ponzilius et al. (1987) bisected mouse embryos after digesting the zona pellucidas with pronase and compared their survival in vitro and in vivo. Exposure of 8- and 16-cell embryos to pronase followed by decompaction allowed a significantly

higher proportion of demi-embryos to develop into blastocysts (47.7%) after overnight culture than did exposure of demi-embryos to pronase alone (21.4%). Bisected 8-cell stage embryos (n = 58) cultured overnight yielded no live young after transfer into recipients. However, transfer of 196 bisected 16-cell stage embryos after decompaction and overnight culture yielded 10.2% live young. Bisected mouse embryos cultured within a surrogate zona pellucida have displayed slightly different results (Lawitts and Graves, 1988). Bisected 8-cell stage embryos developed into blastocysts in vitro at higher rates than bisected 16-cell stage embryos (93 vs. 75%). These results are higher than previously reported primarily due to the fact that embryos were placed into separate zona pellucidas. Overall development in vivo yielded 8% of 8-cell stage and 1% of 16-cell stage embryos developing into live fetuses. These two studies concluded that bisected embryos placed into surrogate zona pellucidas yield higher in vitro survival rates than bisected ZPF embryos; however, low in vivo survival rates are obtained regardless of developmental stage or presence of a zona pellucida.

Research on bisected mouse embryos yielded high in vitro survival and low in vivo survival rates. Bisected bovine morulae and blastocysts without a zona pellucida also exhibited high in vitro survival rates (88 vs. 81%; McEnvoy and Sreenan, 1990). However, bisected morulae without a zona pellucida produced less pregnancies (20%) and fewer young (30%) after transfer than bisected blastocysts (73% pregnancy rate, 91% live young). Similar results were reported by Williams et al. (1984). Bisected bovine morulae within a surrogate zona pellucida expressed a low pregnancy rate when compared to early blastocysts (16

vs. 60%); however, no data was presented for live births. These studies indicate that bisected morulae develop at lower rates than bisected blastocysts in vivo and may need additional protection to increase survival rates.

Warfield et al. (1987) conducted a series of studies in which bisected bovine morulae, blastocysts and expanded blastocysts, with and without surrogate zona pellucidas, were randomly assigned and transferred into recipients. In trials one and two, ZPF demi-embryos developed into fetuses at lower rates than embryos within surrogate zona pellucidas. In the last three trials, all demi-embryos developed into fetuses at similar rates, regardless of placement into surrogate zona pellucida. Transferring two demi-embryos per recipient did not influence survival rates when compared with transferring demi-embryos individually. These authors concluded that demi-embryos without a zona pellucida could be transferred without decreasing survival rates. Baker and Shea (1985) compared pregnancy rates of bisected bovine embryos within a zona pellucida. Recipients receiving two demi-embryos in separate uterine horns had the highest pregnancy rate (55%) while recipients receiving only one demi-embryo yielded a lower but not different pregnancy rate (30%). Calving results were not reported.

Freezing of Embryos

During cryopreservation, the role of the zona pellucida is expanded to act as a gradient for water and/or cryoprotectant diffusion and as a physical barrier for prevention of extracellular ice formation (Lehn-Jensen and Rall, 1983). Therefore, damage to or absence of a zona pellucida prior to freezing would permit dehydration and rehydration at a faster rate than in an intact embryo. For these reasons, cooling and warming rates may need to be adjusted according to the condition of the zona pellucida. Kanagawa et al. (1979) froze and thawed bovine embryos ($n = 23$) with a punctured zona pellucida and transferred them into nine recipients. Although the embryos transferred were morphologically indistinguishable after freezing, only one calf was born. Niemann (1985) reported a high pregnancy rate (8 of 12; 66.7%) after transferring frozen/thawed bovine embryos with apparently damaged zona pellucidas. Damages occurred during the freezing process and were not intentionally performed as was the case in the previous study.

Bovine embryos also have been studied to determine the optimum stage of development for freezing (Trouson et al., 1978). In vitro survival of day 8 blastocysts, following freezing and thawing was higher than survival of hatched blastocysts (56 vs. 25%). Following transfer into recipients, 5 of 11 (45%) recipients receiving blastocysts were pregnant 60 days after transfer but none of the ten recipients of hatched blastocysts were pregnant. These authors concluded that

embryos must be enclosed within a zona pellucida to achieve pregnancy following freeze/thaw procedures.

Wagner and Graves (1984) compared in vitro survival rates of ZPI and ZPF 2- and 8-cell mouse embryos. Forty percent of the 2-cell and 53% of the 8-cell ZPI embryos developed into blastocysts after freezing. None of the ZPF embryos survived freezing. Takeda et al. (1989) reported similar results after freezing bisected mouse morulae and blastocysts with and without zona pellucidas. Bisected ZPF morulae and blastocysts survived freezing but at rates lower than bisected embryos within a surrogate zona pellucida, regardless of stage of development and cryoprotectant used. These two studies concluded that the zona pellucida plays an important role during cryopreservation of mouse embryos.

Freezing of demi-embryos may play a critical role in the embryo transfer industry. Bisection of bovine embryos is employed to produce identical twins and/or more offspring per embryo. However, freezing of these embryos may be necessary until suitable recipients are acquired. Bielanski and Hare (1988) split bovine morulae and blastocysts and placed them into empty zona pellucidas prior to freezing. Immediately after thawing, demi-embryos frozen by a conventional slow cooling method had similar survival rates as embryos frozen by vitrification (77 vs. 75%). Reduced survival rates occurred in both treatments over 24 h in vitro culture (33 vs. 20%), indicating that demi-embryos may require additional protection against freeze/thaw injury. Heyman (1985) reported that frozen demi-embryos without a zona pellucida and transferred into recipients individ-

ually develop into fetuses at lower rates than frozen/thawed whole blastocysts that were transferred in pairs (20.0 vs. 63.6%). The author contributed the low pregnancy rate of one frozen demi-embryo to the weak embryonic signal within the uterus of the recipients, leading to premature luteolysis. Niemann et al. (1986) reported that recipients receiving bovine demi-embryos classified as excellent or good and enclosed within a surrogate zona pellucida prior to freezing showed no difference in pregnancy rates when compared to recipients receiving freshly bisected embryos (46.2 vs. 47.5%) . However, no pregnancies were obtained after transfer of frozen/thawed demi-embryos classified as poor, although two halves were transferred per recipient. They concluded that placing demi-embryos in a surrogate zona pellucida may improve their developmental capacity and that only excellent and good quality embryos should be frozen and transferred into recipients.

Many different freezing techniques and cryoprotectants have been implicated in the previously cited studies. However, many of these studies concluded that embryos without a zona pellucida, either whole or split, do not survive freezing at high rates. For these reasons, other methods need to be adapted to increase the viability of embryos after freezing.

Agar Embedding

Placing micromanipulated embryos within a surrogate zona pellucida prior to freezing may improve their developmental capacity. Another method of protecting micromanipulated embryos includes embedment in agar. Willadsen (1979) was the first to describe the method of coating ovine embryos with agar after microsurgery. Monozygotic pairs of agar-coated single blastomere embryos were transferred into ligated sheep oviducts. Sixty-five percent of the monozygotic blastomeres were recovered 3.5 to 4.5 days after transfer, removed from the agar and transferred into recipient ewes. Five sets of twins and five single lambs were born from ten ewes (69% pregnancy rate). A similar study was reported by Eyestone et al. (1987). One- and two-cell bovine embryos were embedded in agar and transferred into ligated oviducts of anestrous and ovariectomized ewes. Embryos were recovered 5 days after transfer and agar chips removed. Thirty-seven percent of the embryos recovered from anestrous ewes developed to late morula or blastocyst stages as compared to 45% in ovariectomized ewes. Two of six (33%) recipients receiving embryos cultured in anestrous ewes were pregnant and one of two (50%) recipients were pregnant after receiving embryos cultured in ovariectomized ewes. However, additional studies with more recipients need to be conducted to verify these results. Westhusin et al. (1989) also embedded one- and two-cell bovine embryos in agar, surgically transferred them into ligated sheep oviducts and recovered them 6 days after transfer. One hundred percent of the embryos and agar chips were recovered with 52% of the embryos

developing to compact morulae or blastocysts. These studies indicate that ovine and bovine embryos can develop after embedment in agar; however, agar chips must be removed prior to transfer into recipients.

An intact zona pellucida may increase viability of frozen bovine morulae and blastocysts (Lehn-Jensen and Rall, 1983; Trouson et al., 1978; Kanagawa et al., 1979). Furthermore, recent studies suggest that bisected bovine embryos may require a zona pellucida prior to freezing (Niemann et al., 1986). Embedding embryos in agar has been proven to protect embryos during the freezing process (Yong and Jianchen, 1990). Mouse demi-embryos without a zona pellucida were embedded in agar prior to freezing. The ZPF demi-embryos developed into blastocysts after embedment in agar at similar rates as demi-embryos within a surrogate zona pellucida and embedded in agar (69.2 vs. 67.5%). However, demi-embryos with a zona pellucida, not embedded in agar, developed into blastocysts at a lower rate (60%) than embryos embedded in agar, regardless of the presence of a zona pellucida. This study indicates an increase in developmental capacity of mouse demi-embryos after agar embedment. Tsunoda et al. (1987) demonstrated that higher percentages of bisected goat embryos were undamaged after embedment in agar and freezing (50%) as compared to bisected embryos without agar (5%). Additionally, a greater percentage of intact goat embryos were undamaged after embedment in agar and freezing (58%) than intact embryos frozen without agar (26%). Lehn-Jensen and Willadsen (1983) froze and thawed 'half' and 'quarter' bovine embryos after placement in a surrogate zona pellucida and embedment in agar. Seventy percent of 'half' embryos

survived freezing and 75% of 'quarter' embryos survived. Following removal of the agar chip and transfer into recipients, three of six 'half' embryos developed into fetuses after being transferred individually. Eight of twelve 'half' embryos developed following twin transfers. None of the four frozen/thawed 'quarter' embryos developed into fetuses following transfer. These studies conclude that agar embedding may decrease freeze/thaw injury and increase viability of micro-manipulated embryos.

Microencapsulation

Microencapsulation can be defined as the process of enclosing small discrete solid particles, liquid droplets, gases or living cells within an intact membrane (Chang, 1972). The functions of the capsular membrane includes protecting the material within the capsule and controlling the flow of materials across the membrane. Microcapsules may range in size from 0.2 μ m to several millimeters in diameter and may possess impermeable or semipermeable membranes, depending on the composition of the enclosed material. Contents within the microcapsule may be released depending on the construction of the capsular membrane and on moisture, pH, temperature and physical pressure within the area surrounding the capsule. The mechanism for release may rely on leaching, erosion, rupture or other actions, depending on the composition of the membrane wall (Luzzi, 1970).

Microencapsulation techniques originated by enclosing inert materials within impermeable membranes. The encapsulated material therefore was released only if the capsular membrane ruptured. The first commercial product utilizing this technique involved microencapsulating carbonless carbon paper that required physical force for the rupture of the membrane and disclosure of the contents (Green and Schneidcher, 1957). Biomedical industries have utilized microencapsulation procedures to encapsulate ion-exchange resins, enzymes, cofactors, hormones, proteins, antigens and antiserums (Chang, 1972; Chang, 1977; Lim and Sharpe, 1978; Lim and Moss, 1979). Gardner et al. (1980) examined the potential for a long term delivery system in human contraceptives by encapsulating ^{125}I -human serum albumin and ^{85}Sr -microspheres and monitoring their movement after insertion into vaginal canals of monkeys and baboons. They concluded that microcapsules placed in the vagina do migrate across the cervix. Although the migration was low, the potential exists for developing a controlled-release contraceptive system for the female reproductive tract.

Microencapsulation techniques have also been utilized within the agricultural industry by encapsulating enzymes and cell-free extracts to produce different flavors in cheese (Olson, 1986), encapsulating porcine pituitary extracts in an attempt to superovulate cattle (DeMoustier et al., 1988) and encapsulating soil pesticides to demonstrate long term release underground (Williams, 1977).

Research and development of semipermeable microcapsules produced the first "artificial cell" containing biologically active materials (Chang, 1964). These

microcapsules provided selective diffusion of small molecular weight substrates yet did not depend on physical rupture for the release of their contents. Capsular membranes vary in thickness due to the composition of encapsulating materials. Such materials used included: cellulose nitrate (Chang, 1957), protein (Chang, 1964; Chang et al., 1966; Chang, 1969), polysaccharide (Chang, 1967) and lipids (Mueller and Rudin, 1968; Pagano and Thompson, 1968).

Microencapsulation of Living Cells

Lim (1982) was the first to develop an all-aqueous microencapsulation procedure in which tissue and individual cells were encapsulated and remained viable over an extended period of time. These encapsulated cells, comprised of polycations and polyanions, were permeable to nutrients, ions, oxygen and other materials needed to maintain the cells yet impermeable to bacteria, lymphocytes and large proteins. Alginate is a water soluble polyanion that is often used as an encapsulation material. This biocompatible polysaccharide is reversible from a viscous liquid to a solid gel. Alginate remains in a liquid state in the presence of sodium (Na^+) but will solidify into a hydrogel upon suspension in a multivalent cation solution containing calcium (Ca^{++}). Multivalent polycations such as PLL and polyethylenimine initiate a cross linking reaction in the presence of alginate gels to form semipermeable membranes around the gels. The interior gel is then liquified by removing the calcium ions by simple ion exchange.

Lim and Sun (1980) utilized an all-aqueous system to microencapsulate pancreatic islet cells within semipermeable PLL microcapsules. Microencapsulated and unencapsulated islet cells were assessed by viability and integrity both in vitro and in vivo. The microencapsulated cells remained morphologically and functionally intact throughout 15 weeks in culture and secreted insulin comparable to unencapsulated islet cells. To test the response in vivo, islet cells, both encapsulated and unencapsulated, were injected into rats with chemically induced diabetes. Unencapsulated cells survived for six to eight days as expected but amazingly, microencapsulated cells neutralized the diabetic condition for almost three weeks.

This all-aqueous phase system was utilized again to microencapsulate hepatoma cells and pancreatic islet cells in PLL microcapsules (Lim and Moss, 1981). Microencapsulated hepatoma cells grew and multiplied in suspension at the same rate as unencapsulated control cells. Unencapsulated pancreatic islet cells showed signs of degeneration at three weeks but microencapsulated islet cells flourished over eight weeks, with more cell proliferation and insulin production than control cells. These investigators concluded that microencapsulated hepatoma cells and pancreatic islet cells can multiply and flourish for longer periods of time in vitro than unencapsulated cells and therefore, these cells will assist them to develop a better and simpler implantable artificial pancreatic endocrine organ for the treatment of diabetes.

The use of microencapsulation for a long term delivery system of hormones and other compounds has been investigated. Sun and O'Shea (1985) transplanted PLL encapsulated rat pancreatic islet cells into streptozotocin-induced diabetic rats. This transplantation reversed the diabetic condition for approximately 650 days, indicating a prolonged insulin release from the encapsulated islet cells. Intact capsules containing islet cells were recovered from animals approximately two years after implantation. These experiments demonstrate that PLL microcapsules can survive in the animal's body for nearly two years and have the potential as a long term delivery system.

Microencapsulation of Spermatozoa

Nebel et al. (1985) conducted a series of in vitro and in vivo studies to evaluate the efficiency of fertilization of encapsulated spermatozoa using microencapsulation procedures developed by Lim for pancreatic islet cells. They were the first to successfully encapsulate bovine sperm for the potential use in artificial insemination. Using three concentrations (45, 90 and 180 x 10⁶ sperm/ml), semen was encapsulated in 0.75 and 1.5 mm diameter microcapsules made from 0.04% PLL, incubated at 37 C and evaluated for viability, motility and acrosomal integrity at 2, 12 and 24 h. Neither sperm concentration nor capsule size affected spermatozoal viability. However, integrity of the microcapsules decreased as sperm concentration increased.

Nebel et al. (1987) determined the effects of polymer concentrations on microcapsule membrane integrity and spermatozoa survival. Bovine spermatozoa were encapsulated at 45×10^6 sperm/ml in varying PLL concentrations (0.05%, 0.15%, 0.25%, 0.35%) and evaluated for motility and intact acrosomes at 2, 4 and 8 h of incubation. Viability of encapsulated sperm was not affected by polymer concentrations over the 8 h period. Thinner-walled microcapsules were formed at 0.05% PLL than at higher concentrations, indicating maximal cross-linking between PLL and alginate at concentrations greater than 0.05%.

An *in vivo* study was conducted to evaluate the uterine retention of microencapsulated bovine spermatozoa following artificial insemination (Nebel et al., 1987). Two morphologically distinct sperm types were unencapsulated or encapsulated into either PLL (biodegradable) or polyvinylamine (nonbiodegradable) microcapsules and inseminated into 15 cows. Uterine contents were recovered at 12 h post insemination and sperm were isolated and counted differentially. Polyvinylamine microcapsules containing spermatozoa increased uterine retention 12 h post insemination while PLL microcapsules did not, indicating that polyvinylamine capsules may resist retrograde removal or may require additional physical pressure to rupture. The PLL microcapsules probably ruptured soon after insemination and were exposed to retrograde movement and/or phagocytosis similar to unencapsulated sperm.

The viability and fertility of encapsulated bovine spermatozoa has also been investigated using protamine sulfate microcapsules and sodium alginate microgels

(Munkittrick, 1989). In the first two experiments, morphologically distinct sperm from two bulls was encapsulated in microcapsules, microgels or unencapsulated and inseminated into cows. Alginate and protamine encapsulated semen reduced accessory sperm numbers within the zona pellucida of embryos recovered 6 to 7 days post insemination as compared to unencapsulated sperm. In the final experiment, two morphologically distinct sperm types were encapsulated in microcapsules and microgels and inseminated simultaneously. By achieving a fertilization rate of 70%, these encapsulated sperm demonstrated the ability to migrate to the site of fertilization, undergo capacitation, penetrate the zona pellucida and fertilize oocytes. However, only 21 total accessory sperm were recovered from 30 embryos. These experiments demonstrate that protamine sulfate and sodium alginate are able to sustain sperm viability and fertilization can be achieved; however, accessory sperm numbers are lower than normally achieved from unencapsulated sperm.

Experiments have been conducted to evaluate porcine spermatozoa after encapsulation in PLL microcapsules (Esbenshade and Nebel, 1990). Sperm were encapsulated at concentrations of 30, 60 or 120 x 10⁶ sperm/ml, incubated at 4, 15 or 20 C and evaluated for motility and intact acrosomes at 2, 4, 8 and 16 h. Sperm motility and acrosomal morphology were not affected; however, an accelerated loss of motility was visible in encapsulated sperm. In an in vivo study, encapsulated sperm (120 x 10⁶ sperm/ml) were inseminated into estrous sows. Three and six hours after insemination, intact microcapsules containing motile sperm were recovered by flushing the uterus; however, no microcapsules were re-

covered at 24 h. They concluded that encapsulated sperm can be inseminated into estrous sows, but the sperm lose motility at a higher rate than unencapsulated sperm.

Microencapsulation of Embryos

Adaniya et al. (1987) were the first to successfully encapsulate mouse embryos in sodium alginate. In an in vitro study, microencapsulated 2-cell mouse embryos from SWW strain mice developed to the blastocyst stage after 72 h in culture at a higher but not different rate than unencapsulated embryos (37.4 vs. 33.3%). Due to the low percentages obtained, the strain of mice was changed to CB6F1. In this new strain, encapsulated and unencapsulated embryos developed to blastocysts at similar rates (91.7 vs. 95.0%). This experiment demonstrated a simple and effective method to encapsulate embryos without detrimentally affecting development.

Embryonic growth was not affected after prolonged exposure of alginate coated rat embryos to calcium chloride for 3, 6, or 9 min (Meredith et al., 1990). Post-incubation areas of encapsulated blastocysts that were cultured for 24 h were not different from unencapsulated embryos. In an in vivo study, ovine embryos were encapsulated in alginate, exposed to calcium chloride for 3 min and transferred into recipients ewes. Five of seven (71%) ewes receiving embryos in alginate

microgels were pregnant by laparotomy between 23 to 28 days after transfer and were not different from ewes receiving unencapsulated embryos (four of seven; 57%). However, lambs developing to term were not reported. These experiments demonstrated that prolonged exposure of alginate to calcium chloride did not delay embryonic development in vitro and that encapsulated embryos exhibited no detrimental effect upon pregnancy maintenance in ewes.

Previously reported studies encapsulated embryos individually within a capsule. Cosby and Dukelow (1990) examined developmental rates of 2- and 4-cell mouse embryos microencapsulated in sodium alginate individually, in multiples of two or three and denuded of their zona pellucida. Two- and four-cell embryos encapsulated individually or in multiples developed similarly to unencapsulated embryos in vitro. The ZPF encapsulated embryos developed at a lower rate (55%) than encapsulated ZPI embryos (100%) but there was no difference between encapsulated and unencapsulated ZPF embryos (55 vs. 70%).

Split bovine embryos may be placed into surrogate zona pelluciditas prior to freezing to enhance survival. Microencapsulation of split embryos may also increase survival rates. Hollingsworth and Page (1988) microencapsulated and froze bisected bovine morulae in PLL microcapsules after placement into separate zona pelluciditas (n = 58). Microencapsulated demi-embryos exhibited more post-thaw embryonic growth, classified by reorganizing cell mass, stage advancement or cell number increase, than unencapsulated demi-embryos at 12 h of incubation. These authors concluded that microencapsulation of demi-embryos may

reduce mechanical damage normally occurring during the freezing and thawing procedures.

Development of frozen/thawed rabbit embryos following encapsulation in sodium alginate has also been investigated (Kojima et al., 1990). After rapid thawing and mechanical removal of the alginate gel, *in vitro* and *in vivo* embryonic development was examined. The percentage of embryos with an intact zona pellucida was higher in the encapsulated group than in the unencapsulated group (95.3 vs. 76.5%). The encapsulation process also reduced damage to the mucin coat from 43.1 to 8.5%. Encapsulated and unencapsulated frozen/thawed embryos developed similarly into viable fetuses 17 days after transfer as diagnosed by laparotomy (54.2 vs. 66.7%). Recipients developed further and delivered live offspring. These results suggest that encapsulation prior to freezing can improve the percentage of transferrable rabbit embryos after thawing and may enhance *in vivo* survival of embryos if the capsule is removed prior to transfer.

To summarize the microencapsulation experiments of embryos, the following general statements can be made: 1) development of 2-cell mouse embryos into blastocysts after encapsulation in sodium alginate was comparable to unencapsulated embryos, 2) encapsulation with sodium alginate improved the percentage of transferrable frozen and thawed rabbit embryos, 3) frozen and thawed encapsulated rabbit embryos developed into viable fetuses 17 days after mechanical removal of the alginate gel and transfer into recipients, 4) ovine

embryos encapsulated in alginate and transferred into recipients exhibited similar pregnancy rates to ewes receiving unencapsulated embryos.

**EFFECTS OF ENCAPSULATION ON IN VITRO DEVELOPMENT OF
ZONA PELLUCIDA-INTACT AND ZONA PELLUCIDA-FREE MOUSE
MORULAE**

ABSTRACT

Two experiments were conducted to evaluate in vitro development of zona pellucida-intact (ZPI) and zona pellucida-free (ZPF) embryos after encapsulation in either 2% sodium alginate or 0.1% poly-L-lysine (PLL). In Experiment 1, rate of development of ZPI embryos from morulae to hatched blastocysts was measured after encapsulation in either alginate or PLL and for unencapsulated controls. Following encapsulation, embryos were cultured for 120 h and developmental stages recorded every 24 h. Percentage of encapsulated embryos completely hatched from the zona pellucida were not different from each other but were lower than the unencapsulated controls at 48, 72, 96 and 120 h. In vitro development of ZPI and ZPF unencapsulated and encapsulated morulae were examined in Experiment 2. Developmental stages and diameters were measured every 24 h for 72 h. Following 72 h in vitro culture, embryos were stained with

Hoechst 33342 DNA stain and nuclei counted. At 48 and 72 h, percentage of ZPI embryos developing to expanded blastocysts and their diameters were not different from each other or from ZPF groups. Comparison of nuclear counts from embryos developing to expanded blastocysts showed no difference between ZPI and ZPF groups. Therefore, we conclude that in vitro development of encapsulated ZPI morulae to hatched blastocysts is not enhanced. However, development of encapsulated ZPI and ZPF morulae does not appear to detrimentally affect embryonic size or nuclear counts.

INTRODUCTION

Recent advancements in embryo micromanipulation (microinjection, nuclear transfer, sexing and splitting) have led to questions about the necessity of the zona pellucida during cryopreservation. It has been shown that an intact zona pellucida is necessary for bovine morulae and blastocysts to survive freezing (Kanagawa et al., 1979; Lehn-Jensen and Rall, 1983; Trounson et al., 1978). Studies on splitting of bovine morulae and blastocysts have reported that demi-embryos are also highly sensitive to freeze/thaw procedures, regardless of placement into surrogate zona pelluciditas (Bielanski and Hare, 1988; Heyman, 1985; Picard et al., 1985). Demi-embryos have been embedded in agar prior to freezing, in an attempt to increase viability. In vitro development of frozen/thawed mouse demi-embryos with and without surrogate zona pelluciditas and embedded in agar was similar but significantly higher than nonembedded demi-embryos (Yong and Jianchen, 1990). Embedding in agar also improved post-thaw survival of intact and bisected goat embryos (Tsunoda et al., 1987). However, agar appears to have disadvantages including a critical temperature for the phase change that is very close to the lethal temperature of embryos (Kojima et al., 1990).

Lim (1982) developed the first successful encapsulation procedure for living cells. His methods have been adapted to encapsulate preimplantation embryos and may serve as a successful alternative to embedment in agar. Adaniya et al. (1987) reported that 2-cell zona pellucida-intact (ZPI) mouse embryos

encapsulated in alginate exhibited no difference in the percentage of embryos developing to the blastocyst stage as compared to unencapsulated controls. Unencapsulated zona pellucida-free (ZPF) embryos and two- and four-cell ZPF encapsulated mouse embryos displayed similar developmental rates (Cosby and Dukelow, 1990). Questions concerning the viability of encapsulated ZPI and ZPF morulae need to be addressed.

The objective of Experiment 1 was to determine if encapsulation in either alginate microgels or poly-L-lysine (PLL) microcapsules affected in vitro development of ZPI mouse morulae to hatched blastocysts. In Experiment 2, effects of encapsulation in either alginate or PLL on in vitro development of ZPI and ZPF mouse morulae were determined.

MATERIALS AND METHODS

Embryo Collection

Immature female CD1 mice were superovulated with 5 IU of pregnant mare serum gonadotrophin (PMSG; Diosynth, Chicago, IL) and 5 IU of human chorionic gonadotropin (hCG; Lyphomed, Rosemont, IL) 48 h after PMSG, then placed with mature males. Embryos were recovered 60 h post coitum (vaginal plug) by flushing the uterus with M2 medium (Appendix; Table 14). A total of 150 morulae were recovered for Experiment 1 and randomly assigned to one of three treatments: unencapsulated control, alginate microgel and PLL microcapsule. Experiment 2 utilized 312 embryos that were assigned to the following treatments: 1) unencapsulated ZPI, 2) unencapsulated ZPF, 3) ZPI in alginate, 4) ZPF in alginate, 5) ZPI in PLL, and 6) ZPF in PLL. Four treatments were assigned to each mouse in a randomized incomplete block design and rotated as follows: 1) mouse one-unencapsulated ZPI and ZPF plus ZPI and ZPF in alginate, 2) mouse two-unencapsulated ZPI and ZPF plus ZPI and ZPF in PLL, and 3) mouse three-ZPI and ZPF in alginate plus ZPI and ZPF in PLL. This rotation continued for all mice in Experiment 2.

Removal of Zona Pellucida

Zona pellucidas were removed by placing embryos in phosphate buffered saline with 0.5% pronase (Protease; Behring Diagnostics, La Jolle, CA). Embryos were observed under stereomicroscope until zona pellucidas thinned, approximately 10 to 15 min. They were then washed three times in M2 medium (Appendix; Table 14).

Microencapsulation Procedures

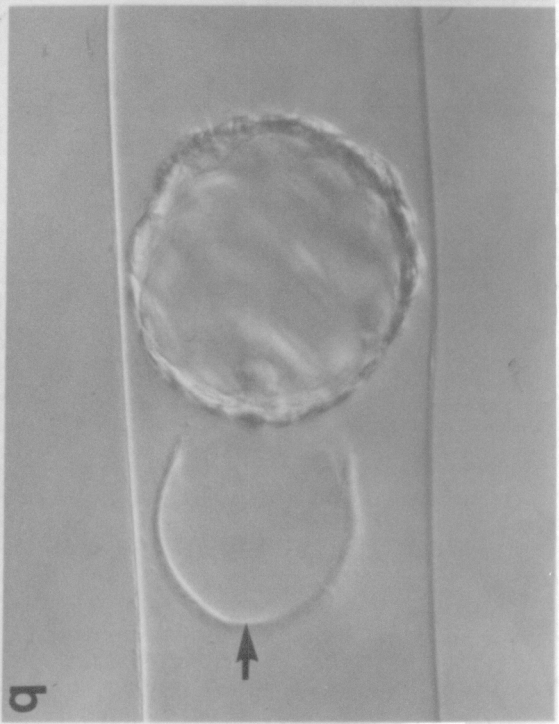
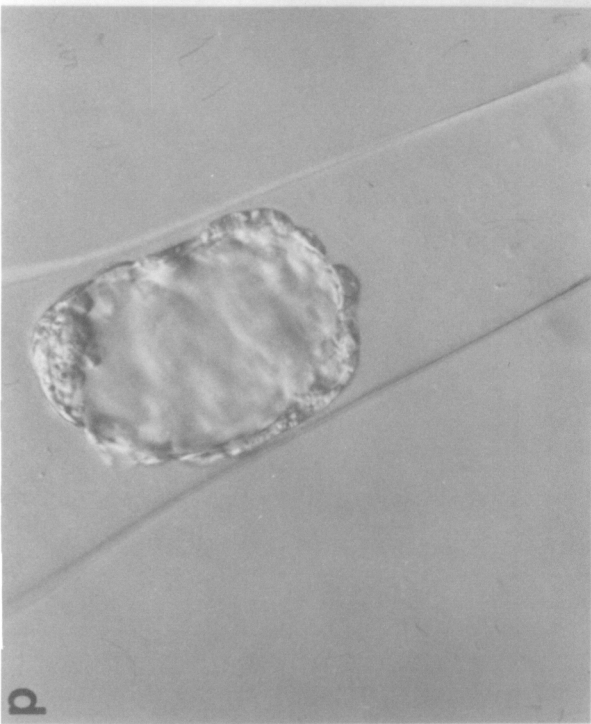
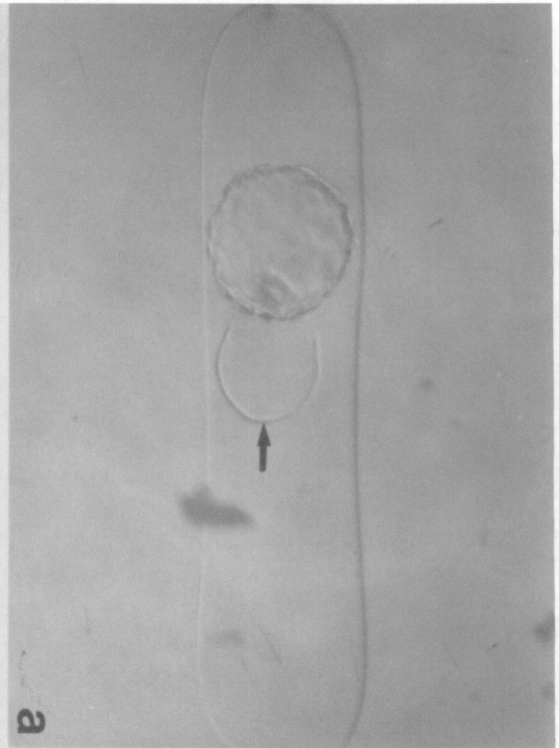
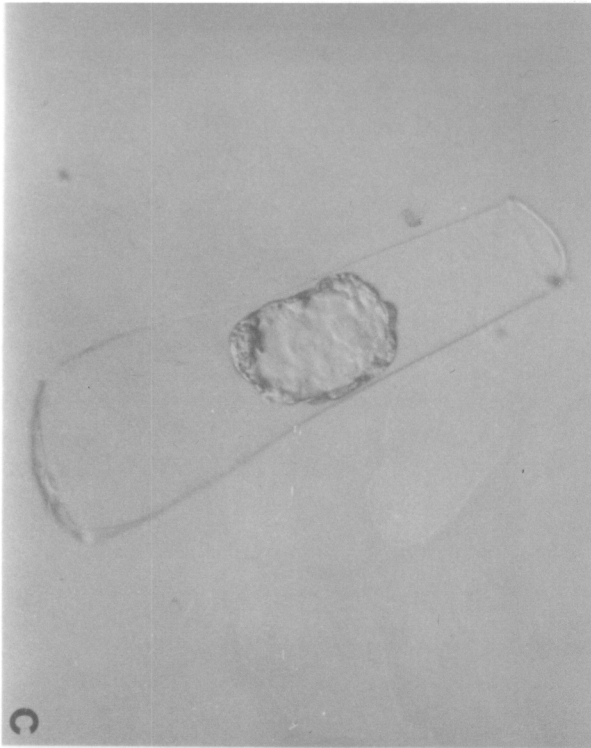
Microencapsulation procedures utilized were those developed for pancreatic islet cells (Lim, 1982) as modified for bovine spermatozoa (Nebel et al., 1985) and murine morulae (Adaniya et al., 1987). Embryos were immersed in 2% sodium alginate (Appendix; Table 15) and aspirated into Pasteur pipettes. Pipette tips were transferred to petri dishes containing 1.5% calcium chloride (Appendix; Table 15). Their contents were then expelled as a semi-soft alginate microgel that contained embryos. Semi-solid microgels were cut to obtain one embryo per gel and allowed to further solidify for approximately 30 min. They were then rinsed in 0.9% physiological saline and served as alginate microgels. To obtain PLL microcapsules, alginate microgels were suspended for 1 min in 0.1% PLL (Appendix; Table 15). This suspension initiated a crosslinking polymerization reaction forming a semipermeable membrane around the microgel. Microgels were

rinsed in CHES buffer (Appendix; Table 15), which terminated the crosslinking reaction and then rinsed with physiological saline to remove excess CHES. Microgels were placed in 3% sodium citrate (Appendix; Table 15) that dissolved the alginate core resulting in PLL microcapsules containing viable embryos.

Culture Procedures

Embryos were cultured individually in 25 μ l CZB medium (Appendix; Table 16), covered with silicone oil and incubated at 37 C in an atmosphere of 90% N₂, 5% O₂ and 5% CO₂ for 120 h. In Experiment 1, each petri dish contained three microdrops of CZB medium, with all treatments represented in each dish. Embryonic development was classified microscopically every 24 h using the following numeric values: 0=degenerate, 1=morula, 2=early blastocyst, 3=blastocyst, 4=expanded blastocyst, 5=hatching blastocyst (zona pellucida cracked), 6=hatched blastocyst (embryo completely hatched from zona pellucida). Experiment 2 utilized four microdrops per petri dish, with all treatments per mouse represented in each dish. The ZPI embryos were classified through the hatched blastocyst stage (Figures 1a and 1b) and ZPF embryos were only classified through the expanded blastocyst stage since a zona pellucida is necessary to classify hatching and hatched stages (Figures 1c and 1d). Measurements of the longest length were taken with a stage micrometer that was placed into the eyepiece of a stereomicroscope. These diameters were rounded to two

Figure 1. Microencapsulated zona pellucida-intact and zona pellucida-free mouse embryos: (a) Hatched blastocyst outside the zona pellucida (arrow) 72 h after encapsulation in poly-L-lysine (200x magnification). (b) Higher magnification of same hatched blastocyst. Note empty zona pellucida at arrow (500x). (c) Zona pellucida-free expanded blastocyst 48 h after encapsulation in an alginate microgel (200x). (d) Higher magnification of expanded blastocyst without a zona pellucida (500x).



decimal places and recorded every 24 h to determine if encapsulation affected embryonic size.

Staining and Examination of Embryos

Embryos were placed on glass slides, counterstained with trypan blue, stained with Hoechst 33342 and embedded between the slide and coverslip (Appendix; Table 17). Stained nuclei were examined under a Zeiss inverted scope with epillumination using a Hoffman 20x objective. Nuclei were counted three times per embryo and the average recorded.

Statistical Analysis

In Experiment 1, chi-square was used to determine the independence of treatments from developmental stages. Stages of development were analyzed by a linear model (Statistical Analysis System, 1985), of the independent variables treatment, mouse and their interaction. A separate analysis was performed for each hour of incubation. Orthogonal contrasts were used to test mean differences of the percentage of unencapsulated embryos developing to hatching and hatched blastocysts as compared to the two encapsulation treatments. Orthogonal contrasts comparing the percentage of hatching and hatched blastocysts within alginate and PLL were also used to test mean differences. Correlations of devel-

opmental stages among incubation times were examined, while holding constant the effects of mouse and treatment.

Experiment 2 was analyzed by chi-square to determine the independence of treatments from the developmental stages. Treatment differences of stages of development and diameters were analyzed by a linear model (Statistical Analysis System, 1985), using treatment, presence of zona pellucida, hour of incubation, mouse and their interactions as independent variables. Stained nuclei were analyzed similarly except hour of incubation was removed from the model. Correlations of stages of development, diameters and nuclei were examined, while holding constant the effects of mouse, treatment and presence of a zona pellucida.

Seven nonorthogonal contrasts compared treatment differences between stages of development, diameters and nuclear counts. The first set of three contrasts compared unencapsulated ZPI embryos to unencapsulated ZPF embryos, alginate ZPI embryos to alginate ZPF embryos and ZPI embryos in PLL to ZPF embryos in PLL. The second set of two contrasts compared unencapsulated ZPI embryos to ZPI embryos in both encapsulation treatments and compared ZPI embryos in alginate microgels to ZPI embryos in PLL microcapsules. The final set of two contrasts compared unencapsulated ZPF embryos to ZPF embryos in both encapsulation treatments and compared ZPF embryos in alginate microgels to ZPF embryos in PLL microcapsules.

Percentage of embryos developing to expanded blastocysts and their nuclear counts were analyzed using seven nonorthogonal contrasts as previously described with treatment, presence of zona pellucida, mouse and their interactions as independent variables. Percentage of embryos with uncountable nuclei were also analyzed using seven nonorthogonal contrasts with the same independent variables. Diameters of embryos developing to expanded blastocysts were analyzed similarly except hour of incubation was added to the model. Percentage of embryos initiating hatching or completely hatched and their mean nuclei number were compared using two orthogonal contrasts described in Experiment 1 with treatment, mouse and their interaction as independent variables. Diameters of hatching and hatched blastocysts were analyzed similarly except hour of incubation was added to the model. Estimable least square means were obtained by deleting the mouse interactions from the model. Contrasts and standard errors for those means were obtained from the appropriate interaction mean square in the full model.

RESULTS AND DISCUSSION

Experiment 1

Developmental stages of unencapsulated and encapsulated embryos were compared over a 120 h incubation period (Table 1). Stage of development was dependent on treatment at 48, 72, 96 and 120 h (X^2 ; $p < 0.001$). Twenty-four hours after encapsulation, embryos in alginate and PLL appeared to be developing at the same rate as the unencapsulated embryos. However, developmental changes began to occur at 48 h and continued throughout the 120 h incubation period, establishing delayed embryonic development within microgels and microcapsules.

The percentage of embryos initiating hatching after encapsulation in PLL were greater than the percentage hatching in alginate at 48, 72, 96 and 120 h (Figure 2; $p < 0.05$). It was apparent that alginate microgels did not allow embryos to penetrate the zona pellucida and therefore hatching was limited. During incubation, at approximately 48 to 72 h, blastocysts encapsulated in alginate microgels were observed expanding to a large diameter without hatching while PLL and unencapsulated embryos initiated hatching. The lack of hatching for embryos encapsulated within alginate probably was due to the extra resistance of the microgels that was not present within the single membrane layer of PLL microcapsules. Alginate microgels provide a solid mass surrounding the embryo and therefore, embryo expansion was restricted. Embryos in PLL microcapsules

Table 1. Percentage of embryos developing after encapsulation in either alginate microgels or poly-L-lysine microcapsules compared to unencapsulated embryos.

Hr of incubation	Stage of development	Treatment		
		Unencapsulated control	Alginate microgel	Poly-L-lysine microcapsule
24	Degenerate	4	10	12
	Morula	18	20	12
	Early Blastocyst	8	8	12
	Blastocyst	20	20	26
	Expanded Blastocyst	48	42	34
	Hatching Blastocyst	2	0	4
48 ¹	Degenerate	10	14	22
	Blastocyst	2	4	2
	Expanded Blastocyst	42	68	42
	Hatching Blastocyst	22	10	34
	Hatched Blastocyst	24	4	0
72 ¹	Degenerate	18	26	24
	Expanded Blastocyst	18	46	22
	Hatching Blastocyst	18	16	40
	Hatched Blastocyst	46	12	14
96 ¹	Degenerate	24	26	30
	Expanded Blastocyst	10	44	12
	Hatching Blastocyst	16	10	40
	Hatched Blastocyst	50	20	18
120 ¹	Degenerate	26	34	36
	Expanded Blastocyst	8	34	6
	Hatching Blastocyst	16	10	34
	Hatched Blastocyst	50	22	24

¹Stage of development is dependent on treatment (X^2 ; $p < 0.001$)

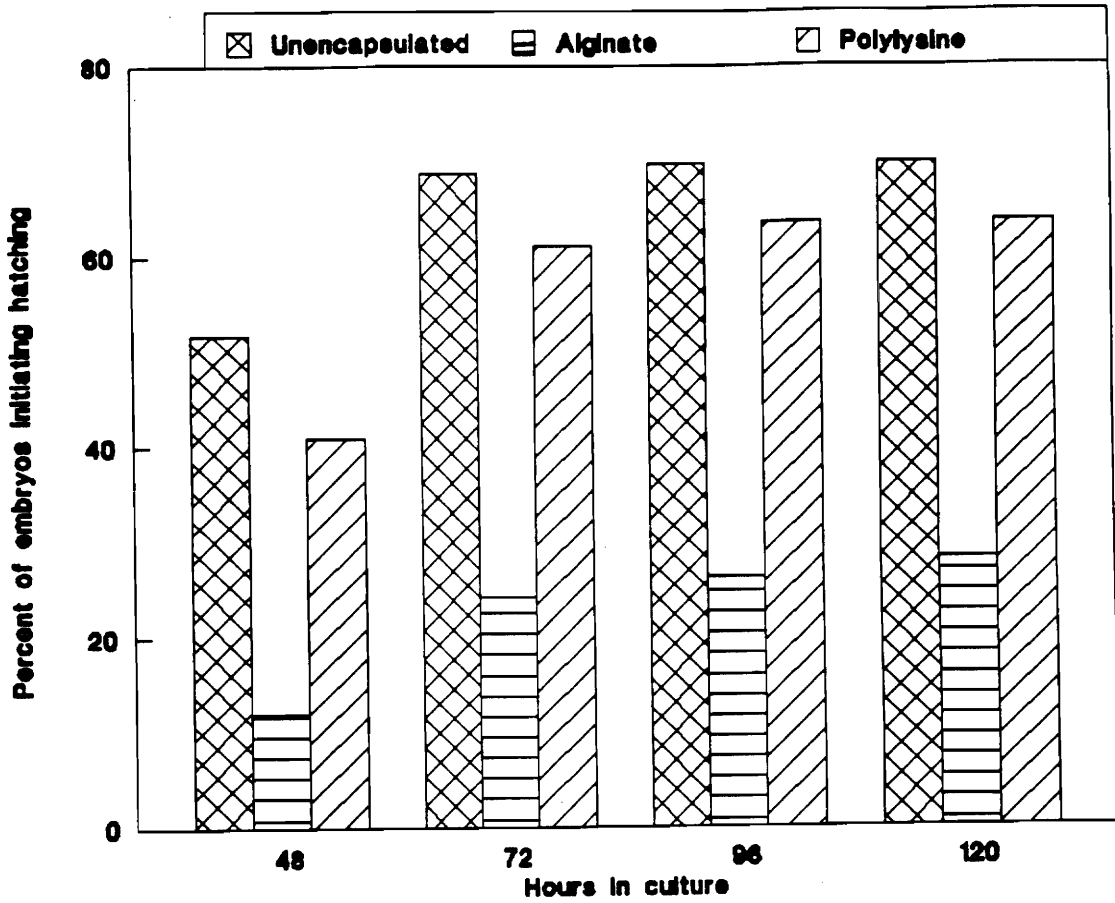


Figure 2. Percentage of embryos initiating hatching after encapsulation in alginate or poly-L-lysine compared to unencapsulated embryos.

were able to move and expand within the open space of the capsule. Unencapsulated embryos initiated hatching at percentages greater than both encapsulation treatments at 48, 72, 96 and 120 h ($p < 0.05$). These percentage differences were due primarily to the low hatching rates of embryos within alginate microgels.

The percentage of unencapsulated embryos that completely hatched from the zona pellucida peaked at 96 h (50.1%) with PLL and alginate still progressing at 120 h (25.6 and 20%; Figure 3). Comparison of least square means found no significant difference between the percentage of hatched embryos in PLL and alginate over the 120 h period. However, the percentage of unencapsulated hatched embryos was greater than the average percentage of encapsulated hatched embryos at 48, 72, 96 and 120 h ($p < 0.05$). These results indicate that encapsulation inhibits complete hatching both in PLL microcapsules and alginate microgels. Adaniya et al. (1987) reported that the percentage of 2-cell unencapsulated mouse embryos developing to the blastocyst stage was higher (95.0%) but not significantly different than alginate encapsulated embryos (91.7%). Cosby and Dukelow (1990) also found 2- and 4-cell stage embryos encapsulated in alginate developing to blastocysts at similar rates (47.1%) as unencapsulated controls (53.9%). These results support our conclusions that unencapsulated and encapsulated mouse embryos develop to the expanded blastocyst stage similarly and then the encapsulation material delays or inhibits

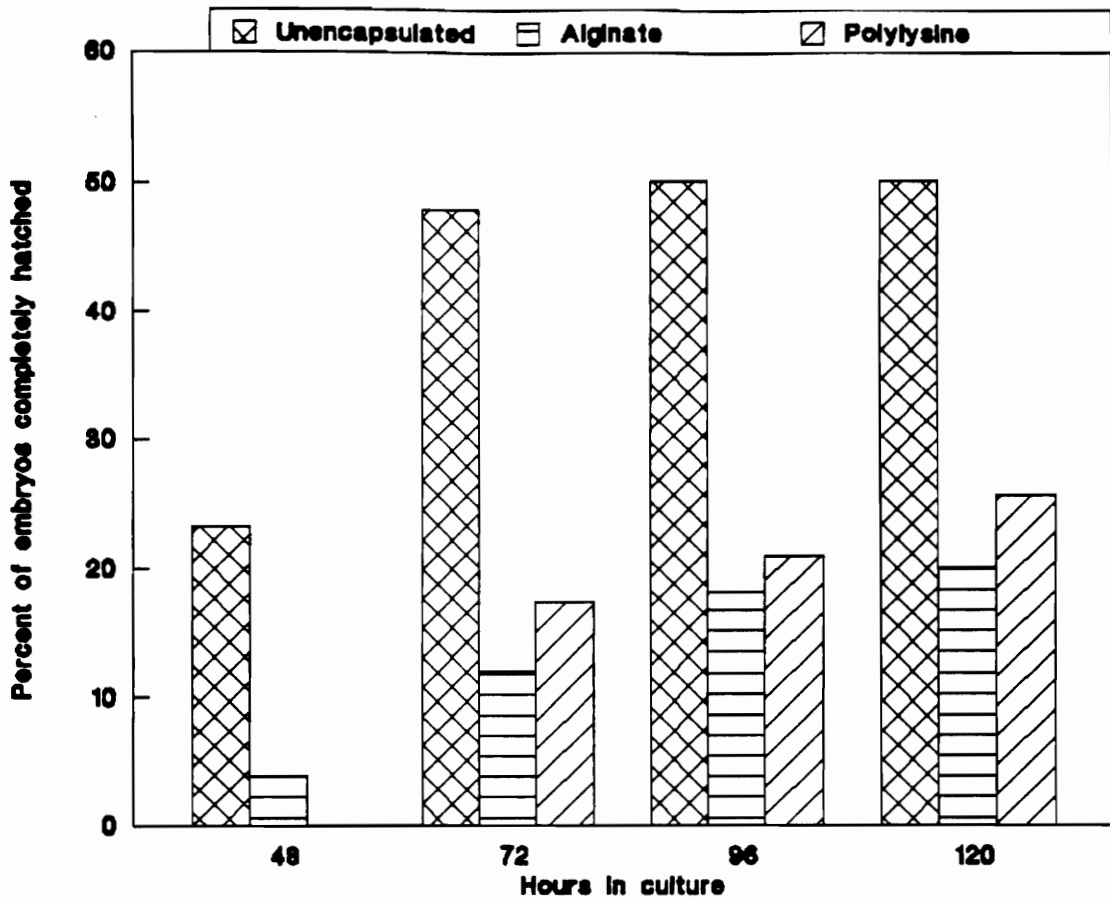


Figure 3. Percentage of embryos developing to hatched blastocysts after encapsulation in alginate or poly-L-lysine compared to unencapsulated embryos.

hatching. Therefore, it may be necessary to remove the encapsulation material to allow the embryo to completely hatch from the zona pellucida.

Least square means and orthogonal contrasts for developmental stages of embryos encapsulated in each treatment are shown in Table 2. Separate analysis of variance for each treatment by incubation time showed no difference between the mean developmental stages for embryos encapsulated in alginate and PLL throughout the 120 h incubation period. Mean developmental stage of unencapsulated embryos was greater than the average mean of embryos in alginate microgels and PLL microcapsules at 48 h ($p < 0.05$) but not significantly different at 24, 72, 96 and 120 h. A trend appeared with unencapsulated embryos maintaining a higher mean developmental stage over the incubation period.

Correlations of developmental stages were similar at 72, 96 and 120 h (Table 3). These high correlations indicated similar developmental changes occurring during the later incubation times and that data collection could have ceased at 72 h with no difference in conclusions.

These results demonstrate that ZPI mouse morulae encapsulated in alginate or PLL do not hatch completely from the zona pellucida at rates similar to unencapsulated embryos. However, embryos in PLL microcapsules do initiate hatching more frequently than embryos in alginate microgels. Because few embryos hatched out of the encapsulation material, we speculate that removal of

Table 2. Mean developmental classification¹ of unencapsulated embryos and embryos encapsulated in alginate microgels or poly-L-lysine microcapsules.

Treatment	Incubation time (h) ²					Overall
	24	48	72	96	120	
Unencapsulated	2.94 ± .2	4.33 ± .3 ³	4.50 ± .4	4.37 ± .4	4.31 ± .4	3.97 ± .3
Alginate	2.78 ± .2	3.69 ± .3	3.58 ± .4	3.66 ± .4	3.44 ± .4	3.43 ± .3
Poly-L-lysine	2.74 ± .2	3.54 ± .3	3.87 ± .4	3.75 ± .4	3.61 ± .4	3.44 ± .3

¹Development scored as: 0 = degenerate, 1 = morula, 2 = early blastocyst, 3 = blastocyst, 4 = expanded blastocyst, 5 = hatching blastocyst, 6 = hatched blastocyst

²Least square mean ± SE

³Unencapsulated control greater than the average of the two encapsulation treatments (p<0.05)

Table 3. Repeatability of development scores between hours of incubation within mouse and treatment.

	24 h	48 h	72 h	96 h	120 h
24 h	1.00	.78	.71	.62	.57
48 h		1.00	.84	.76	.73
72 h			1.00	.92	.86
96 h				1.00	.93
120 h					1.00

the encapsulation material may be necessary to allow a higher percentage of embryos to hatch completely.

Experiment 2

Developmental stages of unencapsulated and encapsulated ZPI and ZPF embryos were compared over a 72 h incubation period (Table 4). Stage of development was dependent on treatment at 24, 48, and 72 h, indicating a treatment effect throughout the incubation period (X^2 ; $p < 0.05$). The ZPF embryos developed similarly across the three treatments. A trend toward higher degeneration of encapsulated ZPF embryos was revealed when compared to unencapsulated ZPF embryos; however, ZPI embryos in PLL microcapsules exhibited the highest percentage of degeneration. A lower percentage of hatching and hatched embryos across all treatments was obtained in Experiment 2.

Least square means for developmental classifications of unencapsulated and encapsulated embryos are shown in Table 5. Analysis of the overall developmental stages by nonorthogonal contrasts showed no difference between ZPI and ZPF treatments although the unencapsulated controls maintained a higher classification throughout the 72 h period for all ZPI and ZPF treatments.

Table 4. Percentage of embryos developing after encapsulation in alginate microgels or poly-L-lysine microcapsules compared to unencapsulated embryos.

Hr of incubation	Stage of development	Treatment					
		ZPI ¹			ZPF ²		
		Control	Alginate	Poly-L-lysine	Control	Alginate	Poly-L-lysine
24 ³	Degenerate	8	8	24	4	16	16
	Morula	8	22	10	14	13	18
	Early Blastocyst	25	9	24	21	28	20
	Blastocyst	32	37	20	44	28	22
	Expanded Blastocyst	25	20	22	17	15	24
	Hatching Blastocyst ⁴	2	4	0			
48 ³	Degenerate	8	8	38	6	20	24
	Morula	0	4	2	2	0	2
	Early Blastocyst	2	4	0	6	4	6
	Blastocyst	12	18	8	23	15	12
	Expanded Blastocyst	46	54	36	63	61	56
	Hatching Blastocyst ⁴	15	6	10			
72 ³	Degenerate	8	13	44	12	24	28
	Early Blastocyst	0	4	0	0	0	2
	Blastocyst	4	4	4	12	2	8
	Expanded Blastocyst	40	48	22	76	74	62
	Hatching Blastocyst ⁴	19	13	16			
	Hatched Blastocyst ⁴	29	18	14			

¹ZPI = zona pellucida-intact

²ZPF = zona pellucida-free

³Stage of development through expanded blastocyst is dependent on treatment (X^2 ; $p < 0.05$)

⁴Combined with expanded blastocysts for chi-square

Table 5. Developmental classification¹ of unencapsulated and encapsulated zona pellucida-intact (ZPI) and zona pellucida-free (ZPF) embryos.

Treatment	Incubation time (h) ^{2,3}			n ⁴
	24	48	72	
Unencapsulated				
ZPI	2.5	3.8	4.2	52
ZPF	2.4	3.2	3.2	52
Alginate				
ZPI	2.6	3.5	4.0	54
ZPF	2.2	3.1	3.1	54
Poly-L-lysine				
ZPI	2.1	2.6	2.8	50
ZPF	2.2	2.7	2.8	50

¹Development scored as: 0 = degenerate, 1 = morula, 2 = early blastocyst, 3 = blastocyst, 4 = expanded blastocyst, 5 = hatching blastocyst, 6 = hatched blastocyst

²Least square mean

³SE = 0.2

⁴n = sample size

Diameters were measured every 24 h and analyzed by analysis of variance (Table 6). Tests of mean diameters indicated that ZPI embryos were not different from each other or from ZPF embryos. These results indicate that neither encapsulation method impaired embryonic size and therefore embryos grew similarly to unencapsulated embryos. In a similar study ZPI and ZPF mouse morulae were compared and embryonic diameter recorded after a 48 h incubation period (Rottmann and Lampeter, 1981). They reported no difference between ZPI and ZPF volumes, which is comparable to diameter measurements obtained in this experiment.

Analysis of nuclear counts by nonorthogonal contrasts indicated that unencapsulated ZPI and ZPF embryos were not different from each other or from encapsulated ZPI and ZPF embryos (Table 7). However, unencapsulated ZPI and ZPF embryos tended to have higher nuclear counts than encapsulated ZPI and ZPF embryos over the 72 h incubation period. The ZPI and ZPF embryos encapsulated in PLL exhibited the lowest mean nuclear counts, which may be contributed by the chemical composition of the capsule. These nuclear counts are higher than previously reported by Rottmann and Lampeter (1981), which is expected because of the differences in culture length of 48 versus 72 h. In contrast to our results, they found a significant difference between nuclear counts from unencapsulated ZPI (47.7) and ZPF (35.1) mouse morulae.

Table 6. Diameter (mm) means of unencapsulated and encapsulated zona pellucida-intact (ZPI) and zona pellucida-free (ZPF) embryos.

Treatment	Incubation time (h) ^{1,2}			n ³
	24	48	72	
Unencapsulated				
ZPI	.080	.104	.101	52
ZPF	.079	.097	.099	52
Alginate				
ZPI	.081	.098	.105	54
ZPF	.077	.095	.098	54
Poly-L-lysine				
ZPI	.071	.085	.089	50
ZPF	.073	.087	.092	50

¹Least square mean

²SE = .004

³n = sample size

Table 7. Nuclear counts¹ of unencapsulated and encapsulated embryos at 72 h incubation.

Treatment	ZPI ²	ZPF ³
Unencapsulated	51.9 ± 1.9	48.2 ± 2.0
Alginate	56.3 ± 1.8	47.1 ± 2.0
Poly-L-lysine	45.4 ± 1.9	41.8 ± 2.0

¹Least square mean ± SE

²ZPI = zona pellucida-intact

³ZPF = zona pellucida-free

Correlations within mouse and treatment were similar between developmental stage and nuclear count (.56) and diameter and nuclear count (.51). A higher correlation existed between stage of development and diameter (.77).

Percentage of ZPI embryos developing to expanded blastocysts were not different from each other or from ZPF embryos at 24, 48, and 72 h, indicating that ZPF embryos developed similarly to ZPI embryos regardless of encapsulation treatment (Figure 4). However, ZPI and ZPF embryos in PLL microcapsules exhibited the lowest percentages. These conclusions are supported by Cosby and Dukelow (1990), who reported no difference between alginate encapsulated and unencapsulated ZPF 2-cell mouse embryos. However, they reported that development of ZPF embryos was significantly lower than ZPI embryos. We also found no difference between the percentage of ZPI embryos initiating hatching or completely hatched at 48 and 72 h (Figures 5 and 6). Overall, there was a higher tendency for embryos in PLL microcapsules to initiate hatching and completely hatch as compared to embryos in alginate microgels. This is in direct contrast to results obtained in Experiment 1. In Experiment 1, embryos encapsulated in alginate microgels allowed minimal hatching to occur and therefore, complete hatching was limited. In Experiment 2, embryos in alginate both initiated and completed hatching at higher rates than embryos encapsulated in PLL microcapsules.

Comparison of diameters from embryos developing to expanded blastocysts showed no difference between ZPI and ZPF embryos (Table 8). All ZPF

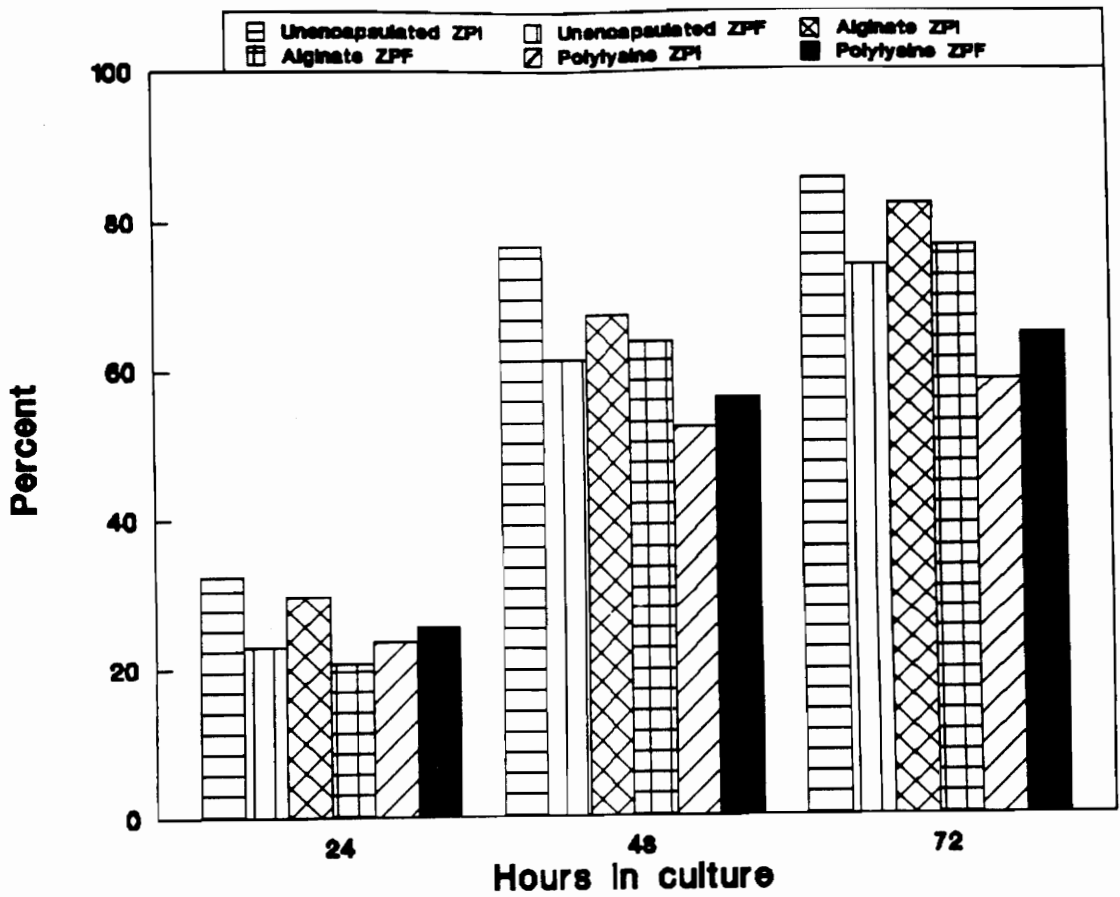


Figure 4. Percentage of embryos developing to expanded blastocysts after encapsulation in alginate or poly-L-lysine compared to unencapsulated embryos.

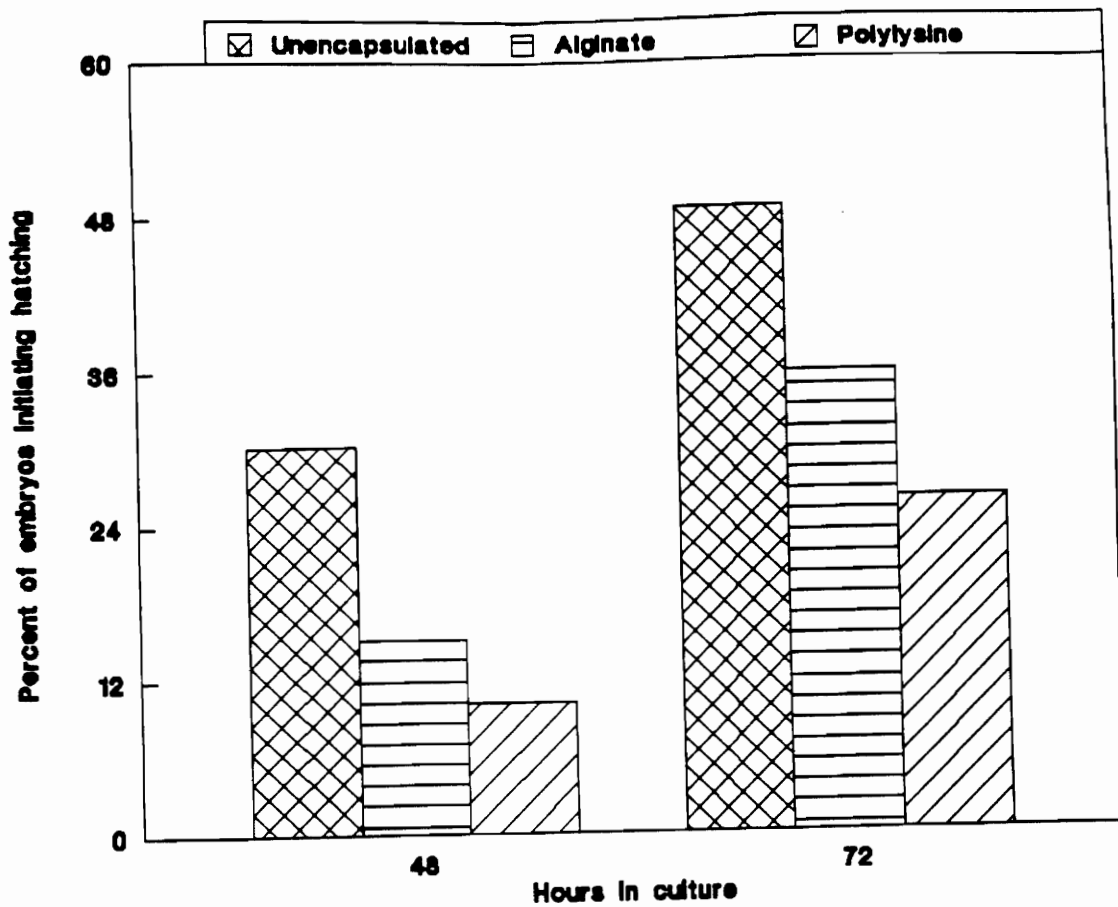


Figure 5. Percentage of encapsulated zona pellucida-intact embryos initiating hatching in alginate or poly-L-lysine compared to unencapsulated embryos.

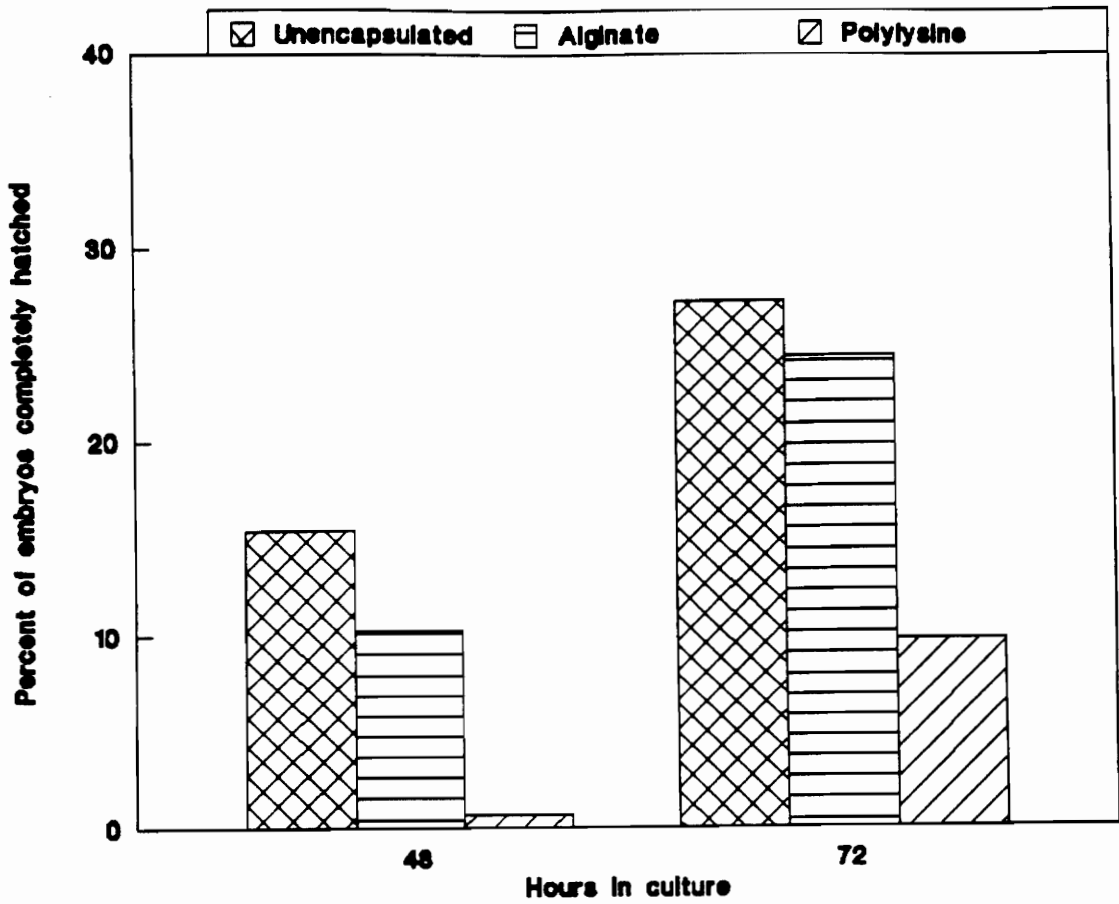


Figure 6. Percentage of encapsulated zona pellucida-intact embryos completely hatched in alginate or poly-L-lysine compared to unencapsulated embryos.

Table 8. Diameters (mm)¹ of unencapsulated embryos and embryos encapsulated in alginate or poly-L-lysine developing to expanded blastocysts.

Hr of incubation	Treatment					
	ZPI ²			ZPF ³		
	Control	Alginate	Poly-L-lysine	Control	Alginate	Poly-L-lysine
24	.099 ± .005	.096 ± .006	.096 ± .006	.100 ± .007	.097 ± .007	.096 ± .006
48	.116 ± .003	.110 ± .004	.114 ± .004	.108 ± .004	.114 ± .004	.107 ± .004
72	.109 ± .003	.115 ± .003	.121 ± .004	.109 ± .003	.114 ± .003	.114 ± .004

¹Least square mean ± SE

²ZPI = zona pellucida-intact

³ZPF = zona pellucida-free

embryos, regardless of encapsulation treatment, developed to a similar embryonic size. At 48 h, there was a tendency for ZPF embryos in alginate to have larger diameters than other ZPF embryos. This indicates that alginate microgels cause blastocysts to expand to larger diameters than unencapsulated embryos and embryos in PLL. At 72 h, there was a tendency for embryos from both encapsulation treatments to obtain larger diameters than unencapsulated embryos, indicating that the encapsulation material allows the embryo to increase in size but not advance in developmental stage.

Using orthogonal contrasts, no differences in diameters were found between encapsulated and unencapsulated ZPI embryos that either initiated hatching or completely hatched at 48 and 72 h (Table 9). All embryos at 48 h exhibited larger diameters than embryos at 72 h, which may be misleading. Through the hatching process, blastocysts elongate through the cracked zona pellucida yielding a larger measurement. After hatching, blastocysts retract into their original spherical shape which may explain the numeric differences occurring at 48 and 72 h. These findings support Meredith et al. (1990) who reported no difference between post-incubation areas of blastocysts in alginate and unencapsulated blastocysts after exposure to calcium chloride for 3, 6 or 9 min.

Stained nuclear counts after 72 h incubation indicated that unencapsulated ZPI and ZPF embryos developing to expanded blastocysts were not different from each other or from encapsulated ZPI and ZPF embryos (Table 10). A tendency toward lower stained nuclear counts was revealed for ZPF embryos developing

Table 9. Diameters (mm)¹ of unencapsulated embryos and embryos encapsulated in alginate or poly-L-lysine developing to hatching or hatched blastocysts.

Hr of incubation	Stage of development	Encapsulation Method		
		Unencapsulated	Alginate	Poly-L-lysine
48	Hatching Blastocyst	.126 ± .006	.124 ± .009	.132 ± .008
	Hatched Blastocyst	.123 ± .008	.127 ± .012	.131 ± .012
72	Hatching Blastocyst	.114 ± .005	.116 ± .005	.127 ± .006
	Hatched Blastocyst	.116 ± .007	.118 ± .007	.126 ± .009

¹Least square mean ± SE

Table 10. Nuclear counts¹ of embryos after encapsulation in alginate microgels or poly-L-lysine microcapsules compared to unencapsulated embryos.

Treatment	Expanded Blastocyst		Hatching Blastocyst	Hatched Blastocyst
	ZPI ²	ZPF ³	ZPI	ZPI
Unencapsulated	57.2 ± 2.8	55.8 ± 3.0	67.4 ± 3.5	74.8 ± 6.6
Alginate	64.0 ± 2.7	53.2 ± 3.0	60.3 ± 3.5	59.3 ± 6.6
Poly-L-lysine	66.0 ± 3.3	54.0 ± 3.3	73.1 ± 3.8	76.1 ± 9.0

¹Least square mean ± SE

²ZPI = zona pellucida-intact

³ZPF = zona pellucida-free

to expanded blastocysts and was probably due to the process of zona removal which could cause damage to the remaining cell mass. Nuclear counts from unencapsulated embryos initiating hatching and completely hatched were not different from encapsulated embryos; however, PLL microcapsules provided an adequate environment for the highest nuclear counts at both stages. There was a tendency for PLL microcapsules to allow hatching and hatched blastocysts to expand to larger diameters and obtain higher cell numbers than unencapsulated embryos and embryos in alginate microgels, even though a lower percentage of embryos in PLL achieved these stages of development. Nuclear counts of alginate encapsulated ZPI embryos were misleading because expanded blastocysts had a higher average cell mass than hatched blastocysts as evident by their high standard errors. Microgels may be reducing the cell mass without having a detrimental effect on stage of development.

The ZPF embryos in alginate and PLL had the highest percentage of embryos with uncountable nuclei but were not different from other treatments (Table 11). Embryos with uncountable nuclei were either lost during the staining procedure or undistinguishable when examined with fluorescence microscope. Therefore, an accurate count was impossible and these measurements were entered into the data set as missing values.

Alginate and PLL encapsulation solutions provide an adequate environment for the development of ZPI and ZPF mouse embryos. We found no difference between unencapsulated treatments (both ZPI and ZPF) and encapsulated treat-

Table 11. Percentage of embryos with uncountable nuclei¹ after staining with trypan blue and Hoechst 33342.

Treatment	ZPI ²	ZPF ³
Unencapsulated	5.3 ± 3.8	9.1 ± 3.8
Alginate	3.5 ± 3.6	14.6 ± 3.6
Poly-L-lysine	5.2 ± 3.8	13.2 ± 3.8

¹Least square mean ± SE

²ZPI = zona pellucida-intact

³ZPF = zona pellucida-free

ments (both ZPI and ZPF) for stage of development, diameter and nuclear count. These results indicate that encapsulation causes no detrimental effects on embryos regardless of the presence of the zona pellucida.

PREGNANCY MAINTENANCE IN MICE FOLLOWING TRANSFER OF ENCAPSULATED EMBRYOS

ABSTRACT

Zona pellucida-intact (ZPI) mouse morulae were encapsulated in either 2% sodium alginate or 0.1% poly-L-lysine (PLL) and transferred into pseudopregnant recipients to examine in vivo development. Transfer of unencapsulated ZPI mouse morulae served as controls. In the first study, a total of 220 embryos were transferred with an average of 12.2 embryos transferred per recipient. Five of six recipients receiving unencapsulated embryos (n = 71) delivered a total of 16 live pups. However, none of the recipients receiving embryos encapsulated with alginate or PLL delivered pups. For these reasons, a second study was performed to examine fetal developmental on day 10 of gestation. Pregnancy maintenance by recipients on day 10 of gestation was similar for all treatments: unencapsulated (71.4%), alginate (87.5%) and PLL (87.5%). However, the total number of viable fetuses present was higher for unencapsulated embryos (42.1%) when compared to either encapsulation treat-

ment (alginate, 17% and PLL, 14.6%). Recipients receiving PLL encapsulated embryos had a high number of resorption sites (13.4%) as compared to recipients of embryos in alginate microgels (4.0%) and recipients of unencapsulated embryos (0%). Embryos encapsulated in alginate or PLL do not develop into live young after transfer; however, small viable fetuses were present on day 10 of gestation. We conclude that transfer of encapsulated mouse morulae into uteri of recipients increases embryonic mortality due to an asynchronous condition between the uterine environment and developing embryos.

INTRODUCTION

Rabbit and cattle morula and earlier stage embryos of these species produced no pregnancies following enzymatic removal of the zona pellucida (Rottmann and Lampeter, 1981; Massey et al., 1982). Bisected murine and bovine morulae have high in vitro survival rates; however, in vivo development is low, regardless of placement into surrogate zona pellucidas (Lawitts and Graves, 1988; McEnvoy and Sreenan, 1990; Williams et al., 1984; Baker and Shea, 1985). Low pregnancy rates also have resulted after cryopreservation of nonmanipulated mouse blastocysts (Lopes et al., 1989; Massip et al., 1984), bovine embryos with a punctured zona pellucida (Kanagawa et al., 1979) and bovine demi-embryos without a zona pellucida (Heyman, 1985). For these reasons, micromanipulated and/or frozen embryos have been embedded in agar in an attempt to increase embryonic development and decrease cellular damage. Research has shown that agar embedding can increase viability of micromanipulated embryos (Yong and Jianchen, 1990; Tsunoda et al., 1987; Lehn-Jensen and Willadsen, 1983; Willadsen, 1982) and nonmanipulated embryos (Westhusin et al., 1989; Willadsen, 1979).

Recently, sodium alginate has been utilized to encapsulate embryos and provides a more stable medium than does agar gel. Viable offspring were born following freezing of alginate encapsulated rabbit embryos; however, the microgels were removed prior to transfer (Kojima et al., 1990). Meredith et al. (1990) reported

no detrimental effect of pregnancy maintenance in ewes 23 to 28 days after transfer of embryos within alginate microgels; however, lambs developing to term were not reported.

The necessity of the zona pellucida for normal embryonic development is controversial in many species. Microsurgical procedures such as bisection and manipulation of individual blastomeres often require removal of the zona pellucida, resulting in a decrease in embryo viability. Freezing of embryos with a damaged or absent zona pellucida often permits dehydration and rehydration at faster rates than in nonmanipulated embryos. For these reasons, it is necessary to know if frozen and/or manipulated embryos require an outer protectant layer to achieve normal embryonic development in vivo. The present study examined fetal development of alginate and poly-L-lysine (PLL) encapsulated zona pellucida-intact (ZPI) mouse morulae after transfer into pseudopregnant recipients. These procedures have the potential to be adapted for micromanipulated and/or frozen embryos and if successful, can be utilized in embryo transfer.

MATERIALS AND METHODS

Embryo Collection

Immature female CD1 mice were superovulated as previously described (page 29). Morulae were recovered, pooled and randomly assigned to one of three treatments: unencapsulated controls, alginate microgels and PLL microcapsules. CD1 virgin females designated to serve as pseudopregnant recipients were placed with vasectomized males at a ratio of 2:1 three days prior to transfer.

Microencapsulation

Microencapsulation procedures were performed as previously described (page 30). All embryos were placed in M2 medium (Appendix; Table 14) and maintained at 37 C prior to transfer.

Embryo Transfer

Recipients were anesthetized by injecting 0.2 ml sodium pentobarbital intraperitoneally. After wiping the lower back with alcohol, a longitudinal incision was made in the midline of the back at the last rib. The skin was gently moved to the left side until the incision was over the fat pad. Once the fat pad

was exposed, a second incision was made through the peritoneum to remove the fat pad, ovary, oviduct and cranial tip of the uterine horn. A seratine clip was applied to the fat pad and laid across the middle of the back. The transfer pipette was loaded with the desired number of embryos ($\bar{x}=12.2$), all from the same treatment. The mouse was then gently moved to the stereomicroscope stage. A small opening was made in the uterine wall with a 25 gauge needle. The transfer pipette was inserted into the small opening and the embryos transferred. Recipients receiving encapsulated embryos often required two transfers, one transfer per uterine horn, to reduce the amount of fluid placed in the uterus. The seratine clip was removed after transfers were completed and the mouse removed from the stereomicroscope stage. Fat pad, ovary, oviduct and uterus were placed within the body cavity and skin closed with a wound clip.

Fetal Development

In the first study, recipients were allowed to carry the transferred embryos to term (17 days after transfer). Once the pups were born, pregnancy rates and number of live births per treatment were calculated. Since recipients receiving encapsulated embryos did not deliver pups, a second study was performed to examine fetal development at mid-gestation. Recipients were sacrificed seven days after transfer and diagnosed pregnant if viable fetuses or resorption sites were present. Pregnancy and developing rates were calculated for all treatments.

Statistical Analysis

In the first study, no statistical analysis was performed because no live births were reported for recipients of encapsulated embryos. In the second study, viable fetuses, resorption sites and pregnancy rates were analyzed using chi-square.

RESULTS AND DISCUSSION

In the first study, recipients of unencapsulated embryos had a high pregnancy rate (83.3%) with 16 of 71 (22.5%) embryos developing into live pups (Table 12). Hoppe and Coman (1983) also transferred ZPI morulae into foster mothers. They achieved a 84.8% pregnancy rate with 34.5% of the total embryos transferred developing into live young, which is very similar to this study. Comparable results were also reported by Mullen and Carter (1973). Eighty-three percent of inbred recipients were diagnosed pregnant after developing viable fetuses on day 16 to 17 of gestation or after delivering live pups. From the total number of embryos transferred ($n = 112$), 33% developed into fetuses or live young.

Despite the live births recorded from recipients of unencapsulated embryos, no recipients receiving encapsulated embryos delivered live pups (Table 12). Recipients that had not given birth by day 22 of gestation were sacrificed and uteri examined. Many recipients of encapsulated embryos had discolored or swollen uteri. No resorption sites were evident in any recipients receiving encapsulated embryos. These findings lead us to believe that transferred encapsulated embryos are unable to implant due to circumstances created within the uterine environment or the restriction of the embryo by the encapsulation material.

In the second study, recipients were sacrificed on day 10 of gestation. No differences were found in pregnancy rates between recipients receiving unencapsulated embryos ($n = 7$; 71.4%), recipients of embryos in alginate ($n = 8$; 87.5%) and re-

Table 12. Production of live young following transfer of unencapsulated and encapsulated morulae in alginate microgels or poly-L-lysine microcapsules.

Treatment	No. of recipients	No. of pregnant recipients (%)	Total embryos transferred	No. of live young (%)
Unencapsulated	6	5 (83.3)	71	16 (22.5) ¹
Alginate Microgels	6	0 (0)	77	0 (0)
Poly-L-lysine Microcapsules	6	0 (0)	72	0 (0)

¹26.7% in pregnant females

recipients of embryos in PLL (n=8; 87.5%; Table 13). Forty two percent of the unencapsulated embryos developed into viable fetuses and were visible on day 10 of gestation. No resorption sites were evident in any recipients of unencapsulated embryos. Recipients of embryos in alginate microgels exhibited 17 viable fetuses and 4 resorption sites. Recipients receiving embryos in PLL microcapsules exhibited 12 viable fetuses and 11 resorption sites. Chi-square analysis revealed that the number of viable fetuses and resorption sites found were related to the treatment applied to the embryos prior to transfer ($p < 0.05$).

Two experiments involving the transfer of alginate encapsulated embryos also have been reported. Four of eight recipients of encapsulated frozen/thawed rabbit embryos were diagnosed pregnant by laparotomy on day 17 of gestation (Kojima et al., 1990). However, encapsulation materials were removed prior to transfer. Three of six recipients of frozen/thawed unencapsulated rabbit embryos were also pregnant. From the pregnant recipients, 13 of 24 encapsulated embryos and 8 of 12 unencapsulated embryos developed into viable fetuses. Out of the 21 total fetuses detected on day 17, 17 accomplished full term development. These authors concluded that no differences existed between pregnancy rates and developing rates of encapsulated and unencapsulated embryos. However, different results may have been obtained if the microgels were intact prior to transfer and if more transfers were performed to increase the number of embryos per pregnant recipient. Meredith et al. (1990) transferred ovine embryos in alginate microgels into ewes and found no difference between their pregnancy rates (5 of

Table 13. In vivo survival at mid-gestation of unencapsulated and encapsulated morulae in alginate microgels or poly-L-lysine microcapsules.

Treatment	No. of recipients	No. of pregnant recipients (%)	Total embryos transferred	No. of viable fetuses on day 10 (%)	No. of resorption sites (%)
Unencapsulated	7	5 (71.4)	95	40 (42.1) ¹	0 (0)
Alginate Microgels	8	7 (87.5)	100	17 (17) ²	4 (4)
Poly-L-lysine Microcapsules	8	7 (87.5)	82	12 (14.6) ³	11 (13.4)

¹53.3% in pregnant females

²19.5% in pregnant females

³16.7% in pregnant females

7) and recipients of unencapsulated embryos (4 of 7) 23 to 28 days after transfer. Even though no data was presented on live births, they concluded that alginate microgels have no detrimental effect upon pregnancy maintenance in ewes. To the knowledge of the author of this thesis, no embryo transfers have been reported using PLL microcapsules.

Alginate and PLL encapsulated embryos failed to develop into live pups but are present at mid-gestation gives rise to speculations that: 1) encapsulation materials prevented the embryo from hatching from the zona pellucida in the normal time period of 5 days, thus delaying implantation and causing an asynchronous condition between the uterine environment and developing embryo, and/or 2) the embryo implanted but the encapsulation material initiated a toxic reaction that caused embryonic death. These explanations, particularly the first, coincide with results reported in Experiments 1 and 2. In Experiment 1, the percentage of encapsulated ZPI embryos completely hatched from the zona pellucida in vitro was lower than unencapsulated embryos at 48 h after encapsulation and later. Twenty to twenty-five percent of embryos within alginate microgels hatched in Experiments 1 and 2, which corresponds to the percentage hatched and developed in vivo (21%). A wider range of PLL encapsulated embryos hatched in vitro (10 to 25%), yet a slightly higher percentage (28%) hatched in vivo. The uterine environment was unable to degrade the encapsulation material in the time required for normal hatching and implantation. Since the viability was decreased due to the presence of encapsulation materials, we theorize that removal or al-

teration of the encapsulation material prior to transfer is a requirement for embryos encapsulated within sodium alginate or PLL.

CONCLUSIONS

Our results indicate that alginate microgels and PLL microcapsules provide an adequate environment for nutrient transport to ZPI and ZPF embryos; however, embryonic development of morulae to hatched blastocysts is not enhanced. Conflicting results were reported for two studies that examined in vitro development. In Experiment 1, the percentage of ZPI mouse morulae initiating hatching in PLL was greater than the percentage for alginate microgels; however, the percentage of unencapsulated embryos hatching was greater than both encapsulation treatments. The percentage of unencapsulated embryos that hatched completely from the zona pellucida was greater than both encapsulation treatments. In Experiment 2, the percentage of encapsulated ZPI embryos initiating hatching or hatched completely from the zona pellucida were different from each other or from unencapsulated ZPI embryos. No differences were detected between the percentage ZPI and ZPF embryos developing to expanded blastocysts. Additionally, no differences were found upon comparison of diameters and nuclear counts from ZPI and ZPF embryos that developed to expanded blastocysts, in-

dicating that microgels and microcapsules do not impair the growth and development of embryos.

Research has shown that 2- and 4-cell mouse embryos in alginate microgels develop into blastocysts similar to unencapsulated embryos. Encapsulation also has been shown to decrease freeze/thaw injury and may increase viability of micro-manipulated embryos. Therefore, encapsulation of 2- and 4-cell embryos, frozen embryos or manipulated embryos may enhance survival; however, we speculate that the encapsulation material must be removed to allow hatching at normal or enhanced rates. Further experiments would need to be performed to substantiate these conclusions.

Because few encapsulated embryos hatched from the zona pellucida and hatched from the encapsulation material, we anticipated that microgels and microcapsules may degrade within the uterine environment and normal implantation could occur if embryos were transferred into recipients. However, the final experiment demonstrated that live births were impossible following the transfer of embryos encapsulated in alginate or PLL. Yet viable fetuses were present at mid-gestation. Therefore, an asynchronous condition may exist between the uterine environment and the transferred embryos, thus delaying or inhibiting implantation. This condition is due to the inability of the embryo to hatch properly from the zona pellucida and escape from the encapsulation material. Another possible explanation for results obtained in Experiment 3 is that embryos implanted but development was impaired due to a toxic interaction of the encapsulation mate-

rial and the uterine environment. This impaired development may be dependent on the amount of encapsulation material transferred into the uterus and the size of the recipient's uterus. The same impaired developmental effects may not be seen with sheep or cattle which have a larger uterine capacity. These conclusions can be tested by examining in vivo development following: 1) removal of encapsulation material prior to transfer, 2) transferring empty microgels and microcapsules along with unencapsulated embryos, or 3) transferring encapsulated morulae into asynchronous recipients. These future experiments would determine if our speculations are correct and may solve questions concerning in vivo development of encapsulated embryos in other species.

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APPENDIX

Table 14. M2 medium used for embryo collection and transfer.

Stock A	Component	g/100ml
(10x concentration)	NaCl	5.534
	KCl	0.356
	KH ₂ PO ₄	0.162
	MgSO ₄ * 7H ₂ O	0.293
	sodium lactate	2.610
	glucose	1.000
	penicillin	0.060
	streptomycin	0.050
	or 4.349 g of 60% syrup	
Stock B	Component	g/100 ml
(10x concentration)	NaHCO ₃	2.101
	phenol red	0.010
Stock C	Component	g/10 ml
(100x concentration)	sodium pyruvate	0.036
Stock D	Component	g/10 ml
(100x concentration)	CaCl ₂ * 2H ₂ O	0.252
Stock E	Component	g/100 ml
(10x concentration)	HEPES	5.958
	Phenol red	0.010
Adjust pH to 7.4 with 0.2 N NaOH		

If stored in refrigerator at 4 C, stocks A, D, and E can be kept up to 3 months, but it is important that stocks B and C are change every other week.

Stock	200 ml
A	20.0 ml
B	3.2 ml
C	2.0 ml
D	2.0 ml
E	16.8 ml
H ₂ O	156.0 ml
BSA	800 mg

After measuring osmolarity, add BSA to medium, allow to dissolve slowly, and mix gently. Adjust pH with 0.2 N NaOH to pH 7.2-7.4.

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Table 15. Solutions used for microencapsulation procedures.

Alginate

2% Alginate (Protanal LF-60, Protan, Drammen, Norway)
Diluted with 0.9% physiological saline

CaCl₂-HEPES

1.5% CaCl₂ Dihydrate (Sigma, St. Louis, MO)
50mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma, St. Louis, MO)
Diluted with distilled water
pH adjusted to 6.8
Osmolarity adjusted to 290-300 mOsmol

Polylysine

0.1% Poly-L-lysine Hydrobromide, Mol. Wt. 70,000-150,000 (Sigma, St. Louis, MO)
Diluted with 0.9% physiological saline
Osmolarity adjusted to 290-300 mOsmol

CHES Buffer

2% CHES (2[N-Cyclohexylamino] ethanesulfonic acid) (Sigma, St. Louis, MO)
0.6% Sodium Chloride (Sigma, St. Louis, MO)
pH adjusted to 8.2
Osmolarity adjusted to 290-300 mOsmol
Dilute above solution 1:20 with 0.9% physiological saline

Sodium Citrate

3% Sodium Citrate Dihydrate (Fisher, Fair Lawn, NJ)
Diluted with distilled water
Osmolarity adjusted to 290-300 mOsmol

Table 16. CZB medium for in vitro culture.

Component	g/Liter
NaCl	4.770
KCl	0.360
KH ₂ PO ₄	0.160
MgSO ₄ * 7H ₂ O	0.290
NaHCO ₃	2.110
CaCl ₂ * 2H ₂ O	0.250
sodium lactate (60% syrup)	5.847
sodium pyruvate	0.029
EDTA (disodium salt)	0.036
glutamine	0.146
BSA	5.000
sodium penicillin	0.060
streptomycin	0.070

Add glutamine from a fresh stock of 100 mM (1.46 g/100 ml) immediately before use. CZB (without glutamine) is good for 2 weeks when stored at 4 C. Osmolarity adjusted to 274-295 mOsmol.

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Table 17. Staining procedures for examining nuclei.

Solutions

Stock Hoechst 33342 (HO; bisbenzimidazole, Sigma, St. Louis, MO) was prepared by dissolving 1 mg HO/ml water. HO stock was stored indefinitely in the dark at room temperature. HO working solution was prepared daily by combining 0.75 ml of 2.3% sodium citrate dihydrate (Fisher Scientific, Fair Lawn, NJ), 0.25 ml ethanol (Pharmco Products, Dayton, NJ) and 10 μ l HO stock. Trypan blue (TB; Allied Chemical, Morristown, NJ) consisted of 0.01% TB dissolved in 2.3% sodium citrate.

Staining Procedures

- 1) 10 μ l drop of TB placed on siliconized slide (Sigmacote; Sigma, St. Louis, MO).
- 2) Embryo transferred to the TB drop with a pipette and incubated at room temperature for 1 min.
- 3) TB removed from embryo.
- 4) 10 μ l drop of HO placed on embryo immediately and incubated for 3 to 5 min at 37 C.
- 5) Excess HO removed.
- 6) Small drop of Flo-Texx (Lerner Laboratories, Stamford, CT) placed on embryo and 22 x 22 mm coverslip installed on the Flo-Texx.

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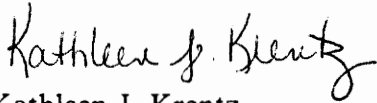
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