CRYOPRESERVATION OF MICROENCAPSULATED BOVINE SPERMATOZOA

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(**Key words**: microencapsulation, bovine spermatozoa, poly-L-lysine, poly-D-lysine)

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(ABSTRACT)

The ultimate design of a microencapsulated AI dose is to continuously release sperm over a period of time in the female reproductive tract, thus alleviating the need for estrus detection. The objective of Trial 1 was to determine in vitro sperm release times for three microcapsule membranes. Semen was collected from four bulls, pooled, extended in 20% egg yolk TEST to a concentration of 80×10^6 cells/ml, and encapsulated. Microcapsule membranes were constructed from isomers of polylysine: .1% poly-L-lysine (PLL), .1% poly-D-lysine (PDL), and a 50:50 mixture of the isomers (PLPD). Microcapsules were incubated at 37°C in a buffer containing .5% heparin or .5% trypsin and evaluated at 0.5, 1, 2, 4, 8, and 16 h post-encapsulation. For sperm encapsulated there were no significant differences in sperm motility. However, peak time of maximum sperm release differed between PLL and PDL membranes at 2 and 4 h of incubation. In Trial 2, sperm viability and microcapsule membrane stability were assessed post-thaw using PLL or PDL, two encapsulating temperatures (5°C or 23°C) and two times of glycerol addition (prior or post encapsulation at 5°C). Semen was extended to 80×10^6 cells/ml and encapsulated.

Capsules from all treatment combinations were incubated in .5% trypsin and evaluated as in Trial 1. In addition, motility was estimated at 1, 3, 6, and 9 h post-thaw. Motility from the unencapsulated control and capsules with glycerol addition prior to encapsulation, was superior ($P < .05$). Additionally, sperm release from capsules prepared at 5°C with glycerol addition post encapsulation was greater than all other treatments ($P < .05$). Time of peak sperm release for capsules was similar to the previous trial. There was a positive correlation between average capsule diameter and sperm release for both trials ($P < .05$). These data suggest that a combination of PLL and PDL capsules may complement each other in timing of sperm release and may be utilized in an inseminate mixture for extending the effective release in the female.

(Key words: microencapsulation, bovine spermatozoa, poly-L-lysine, poly-D-lysine)

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Introduction

Microencapsulation enables encased cells to continuously exchange nutrients and waste products or be released over a period of time. An ultimate practical goal of a microencapsulated semen dose system is to alleviate the need for estrus detection with estrous synchronization programs without compromising fertility. Use of microencapsulated semen may achieve this goal in various ways: through increased sperm retention in the female reproductive tract due to properties of the polymer, through ability of the capsules to rupture over a period of time and release sperm, and by preventing phagocytosis of sperm by leukocytes. Bovine, porcine, and ovine semen has been microencapsulated. In the bovine, fertility trials have measured capsule retention, while capsule retention has been studied in the porcine through capsule retrieval following insemination (Esbenshade et al., 1990; McMillan et al., 1994; Nebel et al., 1996; Maxwell et al., 1996). In vitro experiments conducted to estimate motility and sperm acrosomal intactness showed that there was no difference between encapsulated and unencapsulated samples (Nebel et al., 1985). Data from previous studies with encapsulated bovine spermatozoa indicates that the polymer currently being used as a microcapsule membrane, poly-L-lysine (PLL), breaks down too quickly in the female reproductive tract (Nebel et al., 1996; Vishwanath et al., 1996). Due to this early rupture, other polymer membranes or combinations need to be investigated in order to obtain the above stated goals.

Development of a polymer that allow for sperm release over a period of 8 to 24 hours are the next step in the development of a practical semen microencapsulation system. Polymers tested include PLL, protamine sulfate, and polyvinylamine (Nebel et al., 1986). A polymer that has not been tested is poly-D-lysine (PDL), an isomer of PLL. One possible disadvantage of this isomer is that naturally occurring enzymes do not recognize binding sites on D-isomers of amino acids, thus PDL membranes may not be broken down chemically, but may be able to release sperm only due to manual rupture such as uterine contractions. However, another alternative would be to have a mixture of the isomers which may allow for prolonged rupture and sperm release. The PDL may serve as a backbone or lattice-work for the easily ruptured PLL membrane. As the PLL breaks down, sperm may slowly be released from the PDL matrix.

Another area that needs further investigation is the determination of in vitro breakdown times of the various polymers that are used to microencapsulate sperm. A system for doing this may involve the incubation of various microcapsule types with the glycosaminoglycans normally present in the female reproductive tract. The most prevalent of these include heparin, heparan sulfate, chondroitin sulfate, and hyaluronic acid (Lee et al., 1986). Heparin has been shown to cause an acrosome reaction in bovine spermatozoa (Lenz et al., 1983; Lee et al., 1985; Varner et al., 1991) Additionally, heparin can be used to uncouple PLL bonds (Lim, 1995). In vivo studies examining the timing of insemination of estrous synchronized heifers have been conducted (McMillan, et al., 1994; Nebel et al., 1996), and indicate that sperm encapsulated in PLL membranes may be released quickly

Fertility trials to date utilized fresh semen extended in Caprogen (Nebel et al., 1995). Caprogen is an extender designed for storage of semen at 5°C. This extender is saturated with nitrogen gas which enables sperm to live longer due to decreased oxygen tension (Shannon, 1965). A procedure for cryopreservation of microencapsulated semen needs to be developed in order to enhance its use. Currently, the encapsulation procedure is carried out at 23°C (ambient temperature). In order to be compatible with current procedures for cryopreserving semen, the microencapsulation process needs to be adapted to 5°C. This can be accomplished by encapsulating after the extended semen equilibrates to 5°C. Glycerol can be added in increments after encapsulation and the fully extended, microencapsulated semen can be processed in straws and frozen conventionally.

The objectives of this research were: 1) to evaluate the breakdown characteristics of membranes made with PLL and PDL, and 2) to develop a procedure for the cryopreservation of microencapsulated sperm.

Literature review

Microencapsulation of sperm has the potential to increase sperm retention in the female and possibly prevent capacitation, thus increasing sperm life. Control of these factors can help to increase conception rates in synchronized animals. This literature review will include research from these areas: microencapsulation of bovine spermatozoa, cryopreservation of bovine semen, and role of glycosaminoglycans in the female reproductive tract.

Microencapsulation of Spermatozoa

One of the proposed advantages of microencapsulated sperm is increased sperm retention in the female reproductive tract. Indeed increased retention has been demonstrated in field trials using estrous synchronized heifers. The first person to microencapsulate sperm was Nebel in 1985. Through a modified jet pump extrusion technique, sperm were encapsulated at three different concentrations (45, 90, and 180 $\times$ 10^6 sperm/ml) in .75 or 1.5 mm microcapsules and incubated in vitro in Cornell University Extender at 37° for 24 h. Estimates of sperm motility percent and intact acrosomes were obtained at 2, 12, and 24 h of incubation. Percent motility and intact acrosomes did not differ between encapsulated treatments and unencapsulated controls, except the unencapsulated controls extended to a concentration of 180 $\times$ 10^6 sperm/ml exhibited

lower motility at 12 hours of incubation and lower motility and intact acrosomes after 24. This was possibly due to the accumulation of waste products and limited nutrient availability at higher sperm concentrations in these incubation conditions. In addition, it was found that as sperm concentration increased, capsule fragility increased. Increased sperm concentrations could interfere with the cross linking of Na alginate and PLL due to sperm cells becoming trapped in the membrane during formation. This initial sperm microencapsulation study showed that sperm cells could be encapsulated with minimal loss in sperm viability.

Nebel et al. (1985) also measured the effect of egg yolk and glycerol content on the microencapsulation procedure. Capsule integrity and spermatozoal viability during a 24 h incubation at 37°C was evaluated using frozen-thawed semen extended in 2.9% sodium citrate buffer with glycerol (7% v/v) and either 0, 5, 10, or 15% egg yolk (v/v). The control containing no egg yolk and the treatment containing 5% egg yolk did not differ significantly in the ability to produce uniform capsules. However, as egg yolk concentration increased, capsule uniformity decreased and there was a tendency to produce tear drop shaped capsules. This irregular shape was believed to be caused by interference of high egg yolk concentrations with ion exchange during the gelation of the sperm-alginate droplets. Neither glycerol or the presence of non-motile sperm affected capsule formation. Extended semen without egg yolk was lower in viability as compared to the other treatments. This was probably due to the lack of protection during the freezing procedures. Viability of encapsulated sperm was highest for the 10 and 15% yolk

levels. It was concluded that neat or frozen semen can be encapsulated with minimal cellular injury.

Nebel et al. (1986) evaluated four PLL concentrations (.05, .15, .25, and .35%) and the effect on capsule membrane thickness, integrity of the capsule following stress, and spermatozoal survival. Sperm were encapsulated at 45 $\times 10^6$ cells/ml and incubated for 8 h at 37°C. Motility estimates and counts for intact acrosomes were performed at 2, 4, and 8 h. There was no difference in sperm viability between the four treatments. Capsules formed by the .05% PLL were thinner walled than all other concentrations. There was no difference in capsule thickness between all other treatments. This suggests that maximum cross-linking of the sodium alginate and PLL occurs at concentrations between .05 and .15%.

An in vivo study was conducted to look at competitive retention of sperm in the uterus after heterospermic insemination with marked and unmarked bull sperm (Nebel et al., 1986). Two capsule types were evaluated, PLL (biodegradable) and polyvinylamine (non-biodegradable). Polyvinylamine has a characteristic "stickiness" which may assist in the retention of capsules in the female reproductive tract. Polyvinyl capsules showed a significant increase in sperm retention in the uterus 12 h post insemination compared to PLL capsules, 25.8 vs 18.5%. This disadvantage of PLL capsules could be due to early rupture of capsules or retrograde removal of capsules from the female reproductive tract.

Porcine sperm were encapsulated by Ebenshade and Nebel in 1990. In their first experiment, they compared the relationship between sperm concentration and incubation temperature on motility. Semen was extended to produce five concentrations (7.5, 15, 30,

60, and 120 × 10^6 sperm/ml) and incubated at four temperatures (4, 15, 20, and 37°C). Motility was evaluated at 1, 2, 4, 8, and 16 h of incubation. Semen incubated at 4°C had significantly lower motility as compared to other treatments. Additionally, as sperm concentration increased, motility was maintained for longer periods of time.

In a second experiment, Ebenshade and Nebel (1990) encapsulated porcine sperm and evaluated motility and intact acrosomes at three different concentrations (30, 60, and 120 × 10^6 sperm/ml) and three different in vitro temperatures (4, 15, and 20°C). After 8 h of incubation, only 7% of the encapsulated sperm were motile, and there were no motile sperm at 24 h of incubation. Acrosomal integrity of encapsulated sperm did not differ from unencapsulated controls. Additionally, they determined if microencapsulated sperm could be inseminated into sows and evaluated the viability of encapsulated sperm in vivo. Sperm were encapsulated at a concentration of 120 × 10^6 cells/ml and inseminated into sows on the second day of estrus. Sows were divided into three groups, and the uterus of each was flushed at either 3, 6, or 24 h post insemination. After collection, capsules were ruptured and motility was estimated.

No capsules were obtained from sows flushed 24 h post insemination. Those flushed at 3 and 6 h post insemination did have uterine fluid containing capsules. Motility for capsules recovered from these sows was similar to that found in the previous experiments, with encapsulated semen showing a lower motility over time. They concluded that rate of motility loss was not dependent on incubation conditions. Capsule breakdown could be estimated to occur between 6 and 24 h with viable encapsulated sperm present at 6 h post insemination.

Munkittrick et al., (1991) attempted to combine the desirable characteristics of biodegradability and "stickiness" with a series of experiments designed to evaluate protamine sulfate microcapsules. Semen was frozen at a concentration of 100×10^6 cells/ml and stored in liquid nitrogen, prior to encapsulation as in previous experiments for fresh semen. Cows were inseminated in the uterine body 12 h after estrus was first detected with either of two treatments: inseminates containing encapsulated marked sperm and unencapsulated unmarked sperm or encapsulated unmarked sperm and unencapsulated marked sperm. Embryos were recovered 6 d post insemination and numbers of accessory sperm were determined. Proportion of embryos with accessory sperm and accessory sperm numbers were unaffected by encapsulation, though encapsulation did reduce numbers of accessory sperm for the specific sperm type encapsulated and also reduced the range of accessory sperm obtained. They concluded that timing of insemination of microencapsulated semen needs to be considered due to the need for release of semen from capsules.

Until recently, the effects of polymer concentration on sperm motility had not been considered. A series of experiments evaluated the relationship between polymer concentration (PLL), capsule membrane thickness, sperm motility, and pregnancy rates in estrous synchronized heifers (Nebel et al., 1996). Four concentrations of the polymer PLL (.025, .05, .075, and .1% w/v) were used. The thickness of the corresponding membranes were measured from micrographs generated by transmission electron microscopy. Membrane thickness were $3.22 \pm .54$, $5.30 \pm .31$, $7.12 \pm .41$, $7.44 \pm .85$ μm, respectively. All treatments were incubated in Caprogen, a nitrogen gassed ambient temperature

extender, for 120 h at 23°C. Sperm motility estimates were taken at 24 h intervals and results indicated that polymer concentration had no influence on sperm motility.

In the next experiment, 342 heifers were synchronized for fertility evaluation. Synchronization was accomplished using the progesterone-impregnated CIDR-B® device containing a 10 mg estradiol benzoate capsule inserted for 10 d with administration of 12.5 mg of prostaglandin $F_{2\alpha}$ on day 6 of CIDR insertion. One of the advantages to using this system was the precise synchrony of estrous, with 88.4% of the heifers detected in estrus within 48 h of CIDR removal. Heifers were inseminated in the uterus 24 h post CIDR removal, which coincided with pro estrus stage of the cycle, with either unencapsulated or encapsulated semen with one of the four polymer concentrations. Inseminate dose was 5×10^6 spermatozoa in .25 ml straws. Pregnancy rates for the heifers receiving the unencapsulated control (48.6%) did not differ from those that received encapsulated semen (49.4%). The capsules made with .025% PLL had the highest pregnancy rate of 53.7%. It was concluded that as polymer concentration increased from .075 to .1%, there was no difference in membrane thickness. Additionally, there was no effect on fertility due to membrane thickness.

McMillan et al., (1994) conducted a study to investigate the relationship of timing of insemination with unencapsulated and encapsulated semen (.075% PLL). They inseminated a total of 220 heifers at 12, 24, 36, 48, and 60 h post CIDR removal and reported that there was no difference between treatments (59 versus 52%), although pregnancy rates increased in a linear manner with time of AI. Highest pregnancy rates were at 48 h post CIDR-B® removal. This study combined with Vishwanath et al., 1996,

suggests that sperm in PLL capsules are released within 18 to 24 h post insemination. The possible increased retention of encapsulated semen in the reproductive tract and decreased phagocytosis by leukocytes of sperm that are encapsulated could explain why pregnancy rates are similar between the two treatments.

In a subsequent study, Vishwanath et al., 1996 evaluated in vitro motility of microencapsulated semen and in vivo fertility of 417 estrous synchronized heifers inseminated with .075% PLL microcapsules. Sperm motility was estimated daily for 96 h and incubated in Caprogen. There was no difference in motility between encapsulated and unencapsulated sperm except at the 96 h sampling. At this time, unencapsulated sperm had an advantage over encapsulated treatments (26.7% vs 11.7%). All heifers were synchronized with CIDR devices and inseminated at either 24 or 48 h post CIDR removal. Those animals assigned to the group to be inseminated 48 h post CIDR removal were observed for estrus for four days.

There was no significant difference in pregnancy rates between the 24 and 48 h insemination times for the control group (61 vs 60.6%). There was a significant difference ($P < .01$) in pregnancy rates between insemination times for the encapsulated treatment (45.1 vs 68.6% respectively). At 24 h post CIDR removal, pregnancy rates between unencapsulated and encapsulated treatments differed significantly (61 vs 45.1%). There was no difference in pregnancy rates between these two treatments at 48 h . For those animals detected in estrus visually, encapsulated sperm had a 12% advantage in pregnancy rate at 48 h. This experiment agreed with results obtained from McMillan et al., in 1994 in which there was a linear trend in fertilizing potential with encapsulated semen. This

advantage of microencapsulated sperm at 48 h post CIDR removal could be due to decreased retrograde flow of capsules from the female reproductive tract when compared to unencapsulated sperm. If only one capsule is retained in the uterus, 25,000 sperm cells are available at the time of ovulation.

Recently, Maxwell et al., (1996) conducted a series of experiments to evaluate the viability of encapsulated ram sperm stored at 5°C. Two capsule types were evaluated, PLL and PDL. After encapsulation, sperm were incubated for 8 days at 5°C. Unencapsulated controls had a higher percentage of sperm that were motile and had intact acrosomes when compared to encapsulated treatments. Additionally, there was no difference between capsule types.

Following the in vitro trial, an experiment was conducted to evaluate the fertility rates of ewes inseminated with encapsulated and unencapsulated sperm (Maxwell et al., 1996).Unencapsulated and PLL and PDL microcapsules were inseminated. The fertility trials did not suggest lowered viability of sperm encapsulated in PLL or PDL microcapsules. There was no difference in the proportion of embryos recovered between the unencapsulated control and each polymer type. Nor was there a difference in the proportion of ewes with fertilized ova. There was a significantly lower mean number of accessory sperm in those embryos that were fertilized by encapsulated sperm and a reduced range of accessory sperm obtained, which agrees with results exhibited by Munkittrick et al., (1991). Maxwell concluded that the decreased motility in the in vitro experiment was possibly due to the dilution effect of mixing sperm with Na alginate.

These experiments demonstrate that PLL capsule breakdown occurs between 6 and 24 h after deposition in the female reproductive tract. To increase pregnancy rates at insemination times early in the reproductive cycle (pro estrous) a capsule membrane needs to be developed that breaks down at a later time. Additionally, microcapsules may have an advantage in uterine retention in the female reproductive tract as compared to unencapsulated semen.

Cryopreservation of bovine spermatozoa

Cryopreservation of animal cells allows for long term preservation in a viable state and a cessation or slowing of biological processes (Mazur, 1984). The ultimate goal in developing storage methods for bull semen has been to maintain the quality and fertility of ejaculated sperm through processing, storage, and insemination (Coulter, 1992).

Robbins et al. (1976) conducted a study to determine the influence of freeze rate, thaw rate, and glycerol level on acrosome retention and bovine spermatozoal survival for semen packaged and frozen in French straws (.5 ml). They examined glycerol levels of 1, 4, 7, 10, and 13% and freeze rates between -10 and -80°C of 6.5, 13.1, 26.3, 52.5, and 105°C/hour. Additionally, thaw rates were studied by plunging straws into water at 5°C for 2 m, 20°C for 1 m, 35°C for 30 s, 50°C for 15 s, and 65°C for 7.5 s. Percent motility and percent intact acrosomes were recorded to the nearest 10% at 0, 3, 6, and 9 h of incubation at 37°C.

Twelve ejaculates from 12 different bulls were evaluated. Egg yolk-citrate-glycerol extender was split into fraction A and fraction B (with glycerol). Immediately

after collection, semen was extended with fraction A. After cooling to 5°C, fraction B was added in increments of 10, 20, 30, and 40% by volume at 10 min intervals. Freezing was accomplished using 2-methyl-butane or liquid nitrogen vapor, and freeze rate was measured using a copper-constant thermocouple.

Results indicated the glycerol level required for optimum sperm motility and acrosome retention was dependent on thaw bath temperature and rate of thaw. As glycerol level was increased to maintain sperm viability, thaw bath temperature and rate of thaw also increased. In addition, freeze rate had no significant effect on sperm viability. They determined that the optimum level of glycerol was 8.5% with a thaw bath temperature of 65°C.

Rodriguez et al. (1982) conducted an experiment similar to that of Robbins et al., (1976). Comparing glycerol levels of 5, 7, 9, or 11%, they evaluated fast (3.5 s), moderate (20 m), and slow (40 m) freeze rates and their effect on bovine spermatozoal post-thaw motility. Additionally, they compared thaw temperatures ranging from 5°C to 90°C and thaw times ranging from 5 s to 3 min. Finally, post-thaw motility was compared for straws frozen either horizontally or vertically, at the above rates.

Unlike the results presented by Robbins et al., (1976), it was determined that there was a significant difference in post-thaw motility between fast and slow rates of freezing. There was no difference between the fast and moderate freezing rates which suggests that bovine spermatozoa can tolerate a wide range of freezing rates. The highest post-thaw motility was obtained from those treatments extended with the addition of 7% glycerol and frozen by the fast method (from 5°C to -130°C in 3.5 m). Increasing thaw bath

temperature resulted in higher post-thaw motility, though a thaw bath temperature of 90°C did not significantly improve motility. Straws frozen horizontally were superior in motility when compared to those frozen vertically. When frozen at a slow rate, the higher level of glycerol (11%) was optimum.

Glycosaminoglycans and the female reproductive tract

Secretions of the female reproductive tract play an important role in preparing spermatozoa for fertilization by initiating capacitation and the acrosome reaction. Glycosaminoglycans (GAG) generally exist as side-chains covalently attached to serine residues on a protein core (Ax et al., 1984). The entire GAG-protein complex is referred to as a proteoglycan. They are an important part of the intercellular matrix and of biological fluids and make up the secretions of the female reproductive system including mucus, which is necessary for sperm and ova transport (Lee and Ax, 1984). In the bovine reproductive system the GAGs that are most prevalent and biologically active are chondroitin sulfate A, B, and C, hyaluronic acid, heparin and heparan sulfate (Lee et al., 1986). This portion of the literature review will focus on experiments conducted in three different species, measuring the levels of GAGs in the female reproductive tract.

In 1984, Lee and Ax tried to quantify the concentration and composition of GAGs in the female bovine reproductive tract. Female bovine reproductive tracts (n=104) were obtained immediately after slaughter. Stage of estrous cycle was determined by luteal morphology. Each oviduct, uterine horn and cervix was flushed with 5 ml of .1 M PBS,

and flushings were frozen and stored until analysis could be performed. Additionally, cervical mucous from estrual cows was obtained by placing a gauze pad in the cervix for 20 min. Glycosaminoglycans were isolated by alcoholic precipitation and concentrations were quantified by high performance liquid chromatography. Glycosaminoglycan concentrations significantly decreased anterior to the cervix. Although cervical mucus contained significantly higher levels of GAG then the rest of the reproductive tract, it did not elicit an acrosome reaction. This could serve to ensure that acrosome reactions do not occur prematurely as sperm travel through the reproductive tract. As ovulation approached (proestrus stage), the ratio of protein bound GAG in the uterine horns ipsilateral to the corpus luteum decreased. There was no significant difference in GAG concentration between ipsilateral and contralateral horns. In estrual mucus from live cows, amounts of heparin and chondroitin sulfate were higher when compared to nonestrual mucus.

In the bovine, GAGs seem to increase the incidence of acrosome reactions of sperm by either direct activation of Ca^{2+} influx, activation of phospholipase A_2, or activation of a cAMP-dependent protein kinase (Varner et al., 1991). Additionally, increased production of GAGs cause the expansion of cumulus cells surrounding oocytes which can lead to the formation of two pronuclei in bovine ova fertilized in vitro (Lee and Ax, 1984). Handrow et al.,1982, found that heparin is more effective in causing an acrosome reaction than any other GAG due to its high degree of sulfation.

In 1986, Lee et al. conducted a similar study measuring the GAG content in the ewe reproductive tract. Ewes were observed for estrus and slaughtered at either 0, 12,

24, 36, 72, or 144 h after first observed in estrus. Reproductive tracts were removed and the cervix, uterine horns, and oviducts flushed with a modified Tyrodes balanced salt solution. Glycosaminoglycans were isolated as per Lee and Ax, 1984.

Results obtained were similar to those reported for the bovine as GAG concentration decreased anterior to the cervix and concentrations were similar between the left and right uterine horns and left and right oviducts. There was a twofold increase in GAGs in the cervix at 144 h post estrus (luteal phase). This increase in GAGs could be due to the influence of progesterone which contributes to the high viscosity of cervical mucus during the luteal phase of the cycle and the formation of the cervical plug during pregnancy.

There was a greater enhancement of the acrosome reaction with estrual mucus as compared to mucus from the luteal phase when sperm were incubated in vitro. Furthermore, there was more heparin-like GAG present in the follicular phase of the cycle.

In a study conducted by Varner et al., (1991), the concentrations of various GAGs were measured in the reproductive tracts of mares. Ovariohysterectomies were performed on mares at either d 3 of behavioral estrus, d 8 of diestrus or within 6 h of ovulation. Tracts were flushed according to Lee et al., 1986, and stored at -20°C until analyzed for GAG content.

Results from this study concur with the previous two experiments. Mean GAG concentrations were similar for both ipsilateral and contralateral uterine horns (2.0 ± 0.3 and 2.0 ± 0.4 μg/ml, respectively). However, there was a greater concentration of GAGs in the uterine horns then in the oviducts. Additionally, GAG concentration was elevated in

the follicular phase of the estrous cycle (143.5 ± 9.6 μg/ml). Follicular fluid contained a high quantity of GAGs though it did not effect the concentration in the oviducts, possibly because the fluid does not enter the lumen after ovulation. It was determined that GAG concentrations vary with the stage of the cycle, being highest in the follicular phase. This supports the theory that capacitation in vivo is due to the GAGs present

Research with microencapsulated bovine semen is needed to determine the best capsule type that will allow for release of sperm over the period of time that encompasses maximum GAG concentrations, time of ovulation, and the fertile life span of the released ovum. Specifically, depending on synchronization methods and insemination times, microcapsules need to break down between 8 and 24 h post insemination so as to allow for adequate time for sperm transport to the site of fertilization and for capacitation to occur.

Glycosaminoglycans, specifically heparin, are thought to breakdown PLL microcapsules. A capsule type needs to be developed that can release sperm after exposure over a period of time to the secretions of the reproductive tract. This may be accomplished by in vitro evaluation of microcapsules incubated in heparin or other GAGs and determine breakdown times and sperm release.

Microencapsulation of bovine spermatozoa has been performed at room temperature. Cold room microencapsulation needs to be evaluated to determine optimal conditions prior to cryopreservation.

Materials and Methods

Experiment 1: Determination of breakdown time of various microcapsule membranes.

To the knowledge of this author, there has been no in vitro determination of breakdown times of microcapsules. Nebel et al. (1995) exhibited, through a breeding trial with 335 estrous synchronized Friesian heifers, similar pregnancy rates for inseminates of unencapsulated semen and inseminates containing capsules of various membrane thickness'. They concluded that a possible reason for no difference in pregnancy rates was early rupture of microcapsules in the female reproductive tract. Two additional field trials using 637 synchronized heifers reveal that PLL capsules rupture within 12 h after insemination. Additionally, when inseminated at 48 h post CIDR removal, PLL capsules had an advantage in conception rates over unencapsulated controls, possibly due to decreased retrograde flow of microcapsules (McMillan, 1995; Vishwanath, 1996). The objective of this experiment was to determine membrane breakdown times in vitro of three microcapsule polymers: PLL, PDL, and a 50:50 mixture of the two polymers. The enzyme trypsin, which cleaves down lysine-arginine peptide bonds (Lehninger et al., 1993), and heparin were used in the incubation medium to evaluate membrane breakdown times and sperm release. Heparin has been shown to initiate the acrosome reaction in sperm (Handrow et al., 1982; Lee et al., 1986; Parrish et al., 1988) and a 1% solution will

completely uncouple poly-lysine bonds through competitive binding to the side chains of the polyamino acid (Lim, pers. comm.).

Semen collection and extension.

Semen from five bulls, ranging from 12 to 18 months of age, was collected using an artificial vagina. First ejaculates of four of the five bulls were pooled based on minimums of 1×10^9 cells/ml and 60% motility. Immediately after collection, estimates of sperm motility were obtained using a phase contrast microscope (250×) equipped with heated stage (37°C). Sperm concentration was determined using a previously calibrated spectrophotometer. Semen was extended in either a 20% or 40% egg yolk TEST extender (Garcia et al., 1989) to a final concentration of 20×10^6 cells/ml. Samples that were to be encapsulated were extended in 40% egg yolk to allow for dilution after addition of Na alginate.

Microencapsulation procedures.

Immediately following transport to the laboratory, semen was encapsulated using the procedures for bovine spermatozoa as developed by Nebel et al., (1985), and later modified (Nebel et al., 1993). Extended semen was mixed 50:50 with 1.5% Na Alginate (Pronova Biopolymer a.s., Drammen, Norway) in a 35 ml syringe and extruded at a rate of 1.5 ml/m through a 20 gauge needle enclosed in an encapsulating jet. As the mixture was forced through the syringe, nitrogen flowed through the intake nozzle which caused the

formation of microdroplets. Capsule size was controlled by varying the nitrogen gas velocity.

As microdroplets were formed, they dropped into a solution of 1.5% $CaCl_2$-Hepes (pH = 6.8), which was 4.2 cm from the base of the jet. On contact with $CaCl_2$, Na^+ ions exchanged for Ca^{++} ions and form a solid microgel. After microgels were formed, they were rinsed three times with .9% physiological saline. Microgels were separated into equal aliquots and suspended for six Hours in either .1% PLL (MW 29,500), .1% PDL (MW 25,900), or a 50:50 mixture of the two polymers (PLPD) (Sigma Chemical Co, St. Louis, MO). Microcapsules were rinsed an additional 3 times in .9% physiological saline and suspended in 2.9% Na Citrate (Sigma Chemical Co., St. Louis, MO) (pH = 7.2) for a minimum of 1 h to liquefy the microgel core. After 1 h, capsules were rinsed 3 times in .9% physiological saline. Solutions used in the microencapsulation procedure are described in Table 13 of the appendix.

Microcapsules were incubated in TEST (control), and TEST containing either .5% heparin, or .5 % trypsin (Sigma Chemical Co., St. Louis, MO) for 16 h at 37°C. Capsules were evaluated at 0, .5, 1, 2, 4, 8, and 16 h for diameter (mm), sperm concentration in the incubation media, and percent motility. Prior to sampling for sperm, well plates were vortexed for 5 s to resuspend sperm. Capsule size was measured using a micrometer in the eyepiece diaphragm of a stereomicroscope (10 ×). Six capsules from each treatment were measured for mean diameter. Sperm numbers were measured using a hemocytometer (American Scientific Products, McGaw Park, IL) and calculated using the standard formula for white blood cells. Individual well plates were vortexed thoroughly to

ensure that there was an even distribution of sperm cells to include those no longer viable and those precipitated out of solution. Sperm motility's were obtained by gently crushing two capsules on a slide with a cover slip. Motility was estimated to the nearest 10%. After evaluating results from previous in vitro experiments in which there were no differences between sperm motility in unencapsulated and encapsulated treatments and looking at the time constraints in the present experiment, it was decided not to include an unencapsulated control.

Experiment 2: Cryopreservation of microencapsulated semen.

Previous field trials with microencapsulated semen have used liquid semen (McMillan et al., 1994; Maxwell et al., 1996; Nebel et al., 1996; Vishwanath et al, 1996, Methods to cryopreserve microencapsulated semen need to be developed to be marketable in countries where it is not feasible to use liquid semen. In experiment 2, two encapsulation temperatures were evaluated, 23°C and 5°C. Room temperature encapsulation has been the standard for sperm microencapsulation. It is desirable to evaluate a microencapsulation procedure at 5°C because it would be compatible with current freezing techniques used by US AI organizations. Nebel et al., (1985) evaluated encapsulating semen post-thaw that contained glycerol, and found no significant effect of glycerol on the encapsulation process. Studies evaluating sperm and capsule integrity with glycerol addition prior to or post encapsulation are necessary to determine the effect of glycerol passage across the capsule membrane. The objectives of this experiment were to

develop encapsulation and cryopreservation procedures for microencapsulated sperm that maintain capsule and sperm integrity.

Semen collection and Extension.

Semen collection and extending procedures were as previously described in experiment 1. Semen was extended to a final concentration of 20×10^6/ml in either 20 or 40% egg yolk TEST. Samples that were to be encapsulated were extended in 40% egg yolk to account for the 1:1 dilution effect with Na alginate. Four treatments were evaluated: 23°C encapsulation temperature with glycerol addition post-encapsulation, 5°C encapsulation temperature with glycerol addition pre-encapsulation, 5°C encapsulation temperature with glycerol post-encapsulation, and standard extended semen processing with glycerol addition at 5°C as a control. In addition, two capsule types were evaluated: .1% PLL, and .1% PDL for a total of 7 treatments. On return to the lab, a sample of semen was encapsulated (23°C) as in experiment 1 and then all four treatments poured in Erlenmeyer flasks and placed in beakers containing 20ml of 23°C water. All were allowed to equilibrate to 5°C for 3 h in the cold room (5°C).

After equilibration, glycerol was added to the 23°C and 5°C (glycerol prior to encapsulation) treatments, and the unencapsulated control in 10, 20, 30, and 40% increments by volume at 10 hour intervals to obtain a 1:1 ratio of capsules and sperm to extender (Robbins et al., 1976). At this time, the first of the two 5°C treatments was encapsulated, suspended in Na Citrate for 1 h, and cryoprotectant added at 10 min intervals (5°C encapsulation, glycerol addition post encapsulation). Following this, the

last 5°C treatment (glycerol prior to encapsulation) was encapsulated and suspended in Na Citrate for 1 h. Encapsulated semen was then put in .5 ml French straws (I.M.V. International Corporation, Minneapolis, MN). Freezing of straws was according to Robbins et al., 1976, where straws were placed in nitrogen vapors for 10 min, placed in goblets and canes, and submerged in liquid nitrogen and allowed to equilibrate for a minimum of 2 wks. Each unit of semen contained 200 capsules/straw.

Samples were evaluated post-thaw for motility at 0, 3, 6, and 9 h. In addition, capsules were incubated in TEST buffer (control) and .5% trypsin for 16 h. As described in experiment 1, six capsules were measured for mean diameter using a micrometer and sperm numbers in the incubation media were estimated after vortexing, using a hemocytometer at 0, 0.5, 1, 2, 4, 8, and 16 h post-thaw.

Statistical Analysis

General linear model procedures were used for data analysis (Statistical Analysis Systems, 1985). Collection days were used as replicates as new capsules were made daily. Capsule mean diameter, sperm motility, and sperm concentration outside of capsules were analyzed using the models in Tables 12 and 13 of the appendices. Analysis was performed using Hours as repeated measures. Non-orthogonal contrasts test was used to compare microcapsule membrane types and procedure of microcapsule preparation. Tukey's multiple range test was used to compare motility between treatments. For all analysis, the interactions by day were used as error terms. Anova tables for all models are found in tables 14, 15, 16, 17, 18, and 19 in the appendix.

Results and Discussion

Experiment 1: Effect of incubation in TEST buffer, .5 % heparin or .5 % trypsin for 16 h at 37°C on average capsule diameter, sperm release, and sperm motility of three polymer membranes

Three microcapsule membranes were evaluated in this experiment; PLL, PDL, and PLPD. Shown in Table 1 is the decline in sperm motility over a 16 h period for the three microcapsule membranes studied. This decline in motility normally occurs with incubation. There was no difference between motility for the various polymers, except at four hours of incubation where PLPD was at a slight disadvantage ($P < .05$). This was probably not biologically significant, as the difference was less than 10 % and evaluations were made to the nearest 10 %. This trend in motility agrees with previous in vitro work where there was no difference in sperm motility between unencapsulated and encapsulated sperm (Nebel et al., 1985; Nebel et al., 1995; Vishwanath et al., 1996).

The effect of incubation at 37°C with .5 % trypsin on sperm release from PLL, PDL, and PLPD microcapsules is shown in Table 2. Poly-L-lysine capsules released the highest numbers of sperm after 2 h of incubation, and the greatest numbers of sperm overall. Poly-D-lysine capsules consistently released the lowest numbers of sperm, whereas the mixture of the two polymer types, PLPD, was intermediate to both polymer types. During the first 2 h of incubation, sperm release for PLL microcapsules was significantly greater than other capsule types. Poly-D-lysine and PLPD microcapsules did

Table 1. Least square means for percent motile sperm encapsulated with Poly-L-lysine (PLL), Poly-D-lysine (PDL), or a PLL/PDL mixture (PLPD) at a final sperm concentration of 20×10^6 sperm/ml incubated at 37°C in TEST buffer

	Hours of Incubation at 37°C						
Polymer	0	.5	1	2	4	8	16
PLL	70	70	60	46	40[b]	12	0
PDL	70	70	60	50	40[b]	10	0
PLPD	70	70	58	48	34[a]	10	0
SEM	0.0	0.0	2.2	2.0	1.4	1.2	0.0

[ab]Means with different superscripts differ from others in column ($P < .05$)

Table 2. Least squares means for sperm release ($\times 10^5$) from Poly-L-lysine (PLL), Poly-D-lysine (PDL), and a PLL/PDL mixture (PLPD) microcapsules containing a final sperm concentration of 20×10^6 sperm/ml incubated at 37°C in .5 % trypsin

	Hours of Incubation at 37°C						
Polymer	0	.5	1	2	4	8	16	Total
PLL	0	8.0[a]	13.0[a]	22.1[a]	10.0	0	0[a]	53.1
PDL	0	.1[b]	.8[b]	0[b]	2.2	.3	0[a]	3.4
PLPD	0	.8[b]	5.9[b]	4.4[b]	7.5	5.0	6.7[b]	30.3
SEM	0	2.2	4.1	3.1	6.2	3.8	1.8	

[ab]Means with different superscripts within columns differ ($P < .05$)

not differ in sperm release until 16 h of incubation at which time, PDL microcapsules had degraded and PLPD microcapsules were still releasing sperm cells.

The effect of incubation at 37°C with .5% heparin on sperm release for the three microcapsule membranes is shown in Table 3. At 1 h of incubation, PLL microcapsules had greater numbers of sperm released than PDL microcapsules. Sperm release for PLL capsules peaked first, at 2 h of incubation. Poly-D-lysine and PLPD microcapsules peaked soon after at 4 h of incubation. Combining PLL and PDL microcapsules in an inseminate will therefore may allow for a greater range in sperm release.

Average microcapsule diameters after incubation at 37°C in .5 % trypsin are presented in Table 4. There was no change in capsule diameter until 1 h of incubation. At this time, PLL microcapsules expanded and were significantly larger than either PDL or PLPD microcapsules. This expansion continued until 4 h at which time they started to degrade. Though there was no significant difference between PDL and PLPD microcapsules, PLPD microcapsules tended to be intermediate in size to the other types. Poly-L-lysine microcapsules have been shown to expand after the Na alginate is liquefied due to the flexible nature of the polymer (Goosen, et al., 1985). Though capsules were not measured immediately after polymerization, this expansion was exhibited in PLL microcapsules after 1 h of incubation. Neither of the other microcapsules exhibited similar expansion. Poly-L-lysine microcapsules degraded completely by 8 h of incubation. This agrees with work conducted by Esbenshade et al., (1990) where uterine fluid flushed from sows inseminated with PLL microcapsules contained microcapsules at 3 and 6 h post insemination but no microcapsules 24 h post insemination. Poly-D-lysine or PLPD

Table 3. Least squares means for sperm release ($\times 10^5$) from Poly-L-lysine (PLL), Poly-D-lysine (PDL), and a PLL/PDL mixture (PLPD) microcapsules containing a final sperm concentration of 20×10^6 sperm/ml incubated at 37°C with .5 % heparin

	Hours of Incubation at 37°C						
Polymer	0	.5	1	2	4	8	16	Total
PLL	0	7.4	19.9[a]	24.8	24.1	6.4	3.0[ab]	85.6
PDL	0	9.9	7.3[b]	21.3	23.4	10.0	0[b]	71.9
PLPD	0	12.7	16.2[ab]	19.0	29.3	9.7	6.8[a]	93.7
SEM	0	2.2	4.1	3.1	6.2	3.8	1.8	

[ab]Means with different superscripts within columns differ ($P < .05$)

Table 4. Least squares means for average capsule diameter (mm) of Poly-L-lysine (PLL), Poly-D-lysine (PDL), and a PLL/PDL mixture (PLPD) microcapsules containing a final sperm concentration of 20×10^6 sperm/ml after 37°C incubation with .5 % trypsin

	Hours of Incubation at 37°C						
Polymer	0	0.5	1	2	4	8	16
PLL	1.0	.9	1.3^a	1.7^a	$.6^a$	0^a	0^a
PDL	1.0	.9	1.0^b	1.0^b	1.2^b	1.0^b	$.8^b$
PLPD	1.0	1.0	1.1^b	1.1^b	1.1^b	1.0^b	1.1^b
SEM	0	.05	.04	.06	.18	.13	.16

[ab]Means with different superscripts within columns differ ($P<.05$)

microcapsules showed no sign of deterioration at either 8 or 16 h. This could be easily explained as enzymes do not recognize binding sites on D-isomers of amino acids, so trypsin should not affect capsule size or sperm release.

The effect of .5% heparin on average capsule diameter after incubation at 37°C for all capsule types is shown in Table 5. In contrast to incubation with .5% trypsin, capsule size did not increase when incubated with .5% heparin; however, they did become very small in diameter. Heparin never totally degraded PLL or PLPD microcapsules, though PDL microcapsules decreased in size at 8 h of incubation and were totally degraded by 16 h. Heparin splits polylysine-polylysine bonds by attaching to the R-groups and splitting the bonds between them, thus uncoupling the inter-chain bonds.

Concentrations of trypsin and heparin in this experiment were not physiological, all comparisons made to in vivo events would be relative only. These in vitro studies on sperm release suggest that PLL microcapsules release sperm fairly early, the majority within 4 h in vitro. This supports theories that have been set forth by others, with in vivo experiments which suggest no advantage of sperm encapsulated in PLL microcapsules when compared to unencapsulated sperm due to early breakdown and release (McMillan et al., 1994; Nebel et al., 1996; Vishwanath et al., 1996). Actual breakdown times in vivo in the bovine have not been determined.

In the ovine, PLL and PDL microcapsules did not differ from unencapsulated controls in the number of embryos recovered (Maxwell, et al., 1996). There was a lower number of accessory sperm found in embryos from ewes that were inseminated with PDL capsules versus PLL microcapsules (Mean: 1.3 vs. 5.8). Though this was not significantly

Table 5. Least squares means for average capsule diameter of Poly-L-lysine (PLL), Poly-D-lysine (PDL), and a PLL/PDL mixture (PLPD) microcapsules containing a final sperm concentration of 20×10^6 sperm/ml after 37°C incubation with .5 % heparin

	Hours of Incubation at 37°C						
Polymer	0	.5	1	2	4	8	16
PLL	1.0	1.2	1.3[a]	1.4	1.1	.9[a]	.4[a]
PDL	1.0	1.0	1.1[b]	1.3	1.2	.4[b]	0[a]
PLPD	1.0	1.1	1.2[b]	1.3	1.3	1.1[a]	.9[b]
SEM	0	.1	.1	.1	.2	.1	.2

[ab]Means with different superscripts within columns differ (P<.05)

different, it supports the results of the present experiment showing that sperm release from PDL capsules is significantly lower when compared to other polymers.

As was expected, PLPD microcapsules were intermediate in sperm release and average size compared to the other two polymers. Poly-L-lysine and PDL microcapsules were chosen to be used for the cryopreservation experiment.

Experiment 2: Effect of 37°C incubation in TEST or .5 % trypsin on capsule diameter, sperm release, and motility of cryopreserved PLL and PDL capsules prepared at two encapsulation temperatures with two methods of glycerol addition.

Sperm motility for cryopreserved PLL and PDL microcapsules after 9 h of incubation are presented in Table 6. There was a significant difference between the motility of unencapsulated sperm and encapsulated sperm, regardless of encapsulating polymer ($P < .05$). There was no difference in sperm motility between polymer types across treatments. Within polymer types there was a significant difference between capsule preparation at 5°C with glycerol addition prior and post encapsulation, favoring post encapsulation addition of glycerol. At cold room temperatures, Na alginate is more viscous than at ambient temperature. This viscous nature of sodium alginate could lead to uneven distribution of glycerol during mixing. If the extender with glycerol was not thoroughly mixed with Na alginate before encapsulation, sperm cells could be more susceptible to freeze injury. Microcapsule preparation at 23°C was intermediate to the other treatments for maintenance of sperm motility during incubation. This treatment was

Table 6. Least squares means for sperm motility of cryopreserved unencapsulated semen and Poly-L-lysine (PLL) and Poly-D-lysine (PDL) capsules prepared at 23°C and 5°C with glycerol addition either prior or post encapsulation with a final sperm concentration of 20×10^6 sperm/ml incubated at 37°C in TEST buffer

			Hours of Incubation at 37°C			
Polymer	Temperature[a]	Glycerol[b]	0	3	6	9
Unencapsulated	-	-	50[c]	43[c]	21	3
PLL	23	post	38[ce]	33[ce]	19	6
PLL	5	prior	26[de]	21[de]	15	1
PLL	5	post	46[c]	39[ce]	24	5
PDL	23	post	35[ce]	30[ce]	15	0
PDL	5	prior	26[de]	24[ce]	9	3
PDL	5	post	43[ce]	39[ce]	24	6
SEM	-	-	4.7	4.7	3.6	1.6

[a]Temperature (°C) at which encapsulation was performed
[b]Glycerol addition either prior or post encapsulation
[cde]Means with different superscripts differ within column ($P < .05$)

at ambient temperature for 30 min longer than the others, which could explain the lower motility when compared to the 5°C treatments. Immediate cooling of extended semen lowers sperm metabolism and extends viability. Poly-L-lysine and PDL microcapsules prepared at 5°C with glycerol addition post encapsulation were not different from the unencapsulated control (46 and 43 % vs. 50 % motility, respectively). From 3 h of incubation until 9 h, there was no significant difference in sperm motility between unencapsulated controls and encapsulated treatments. This agrees with previous in vitro work (Nebel et al., 1985; Nebel et al., 1995; Vishwanath et al., 1996). The difference in initial motility may be due to the viscosity of Na alginate. On initial evaluation, sperm cells appeared sluggish. If sodium alginate within microcapsules was not completely liquefied after processing, sperm motility may have been impaired initially until the alginate was removed. These results indicate that microcapsules can be cryopreserved with minimal cell injury. Additionally, microencapsulating at 5°C with glycerol addition post encapsulation has an advantage in sperm motility as compared to other methods of processing. The molecular weight of the polymers used in this experiment allowed for the passage of glycerol through the membrane.

Sperm release from PLL capsules incubated in .5 % trypsin is presented in Table 7. Sperm release occurred earlier for microcapsules prepared at 5°C with glycerol addition post encapsulation and by 16 h of incubation all sperm were released. Microcapsules prepared at 5°C with glycerol addition prior to encapsulation released the lowest sperm numbers of the three preparations. The data suggest peak sperm release was between one to four hours as compared to the other 5°C treatment which appeared to peak within the

Table 7. Least squares means for sperm released ($\times 10^5$) from cryopreserved Poly-L-lysine (PLL) microcapsules prepared at 23°C and 5°C with glycerol addition either prior or post encapsulation with a final concentration of 20×10^6 sperm/ml incubated at 37°C with .5 % trypsin

			Hours of Incubation at 37°C						
Polymer	Temperature[a]	Glycerol[b]	0	.5	1	2	4	8	16
PLL	23	post	6.7[c]	9.4[c]	25.9[cd]	23.1[cd]	23.5[c]	18.9[c]	2.5
PLL	5	prior	7.2[c]	13.6[c]	18.2[d]	14.2[d]	4.0[d]	0[d]	0
PLL	5	post	15.6[d]	26.2[d]	27.8[c]	30.0[c]	11.6[cd]	3.3[d]	0
SEM	-	-	1.9	2.0	2.0	3.5	4.0	3.2	2.0

[a]Temperature (°C) at which encapsulation was performed
[b]Glycerol addition either prior or post encapsulation
[cd]Means with different superscripts differ from others in column ($P < .05$)

first two hours of incubation. Poly-L-lysine capsules prepared at 23°C released significantly fewer numbers of sperm in the first 30 min of incubation. Nebel et al. (1985) encapsulated sperm after thawing with varying glycerol levels and results indicated that there was no effect of glycerol on capsule formation. Their experiment was conducted at ambient temperature, in comparison to the present treatment for this experiment. It is possible that lower temperatures and the combination of glycerol and Na alginate could affect PLL polymerization.

The effect of incubation in .5 % trypsin on sperm release from PDL capsules is presented in Table 8. Overall, sperm release in PDL microcapsules for the first two hours of incubation was significantly lower than release from PLL microcapsules. Within PDL microcapsules, sperm released from microcapsules prepared at 23°C was significantly lower than all other treatments in the first hour. Following the first two hour of incubation, there was no difference in sperm numbers released. The sperm release pattern from PDL microcapsules tends to be more persistent than that of PLL microcapsules, which peaked in sperm release between two and four hours of incubation. Additionally, there did not seem to be an effect of glycerol on PDL polymerization as seen in PLL capsules prepared at 5°C with glycerol added prior to encapsulation. A combination of microcapsules constructed from these polymers in an inseminate could provide for an effective release of sperm to encompass the time of ovulation.

Average capsule diameters for PLL microcapsules incubated in trypsin for 16 h are presented in Table 9. Microcapsules tended to expand after 30 min of incubation similar to the previous experiment. Microcapsules prepared at 5°C with glycerol addition prior to

Table 8. Least squares means for sperm released ($\times 10^5$) from cryopreserved Poly-D-lysine (PDL) microcapsules prepared at 23°C and 5° with glycerol addition either prior or post encapsulation with a final concentration of 20×10^6 sperm/ml incubated at 37°C with .5 % trypsin

			Hours of Incubation at 37°C						
Polymer	Temperature[a]	Glycerol[b]	0	.5	1	2	4	8	16
PDL	23	post	2.8	3.8[c]	3.5[c]	4.6	3.6	3.1	3.9
PDL	5	prior	5.6	8.9[cd]	10.1[cd]	9.9	13.8	8.5	6.7
PDL	5	post	8.4	10.0[d]	13.8[d]	6.6	16.8	10.4	6.2
SEM	-	-	1.9	2.0	2.0	3.5	4.0	3.2	2.0

[a]Temperature (°C) at which encapsulation was performed
[b]Glycerol addition either prior or post encapsulation
[cd]Means with different superscripts differ from others in column ($P < .05$)

Table 9. Least squares means for average capsule diameter, after incubation in a solution containing trypsin at 37°C, of cryopreserved Poly-L-lysine (PLL) microcapsules prepared at 23°C and 5°C with glycerol addition either prior or post encapsulation with a final sperm concentration of 20×10^6 sperm/ml

			Hours of Incubation at 37°C						
Polymer	Temperature[a]	Glycerol[b]	0	.5	1	2	4	8	16
PLL	23	post	1.2	1.3[c]	1.2[cd]	1.2[c]	1.1[c]	.3	.2
PLL	5	prior	1.2	1.5[d]	.9[c]	.4[d]	0[d]	0	0
PLL	5	post	1.2	1.3[c]	1.4[d]	.5[d]	.2[d]	0	0
SEM	-	-	.02	.03	.1	.1	.1	.1	.1

[a]Temperature (°C) at which encapsulation was performed
[b]Glycerol addition either prior or post encapsulation
[cd]Means with different superscripts differ from others in column ($P < .05$)

encapsulation degraded earlier than the other treatments (4 vs. 8 h), further suggesting an effect of glycerol and cooler temperatures on polymerization. Poly-L-lysine microcapsules were significantly larger from 2 to 4 h and still intact at 16 h. Average capsule diameters for PDL microcapsules after incubation in .5 % trypsin for 16 h are illustrated in Table 10. Average capsule diameters did not change over time as in PLL microcapsules. This lack of change in diameter or breakdown is due to the lack of substrate binding sites for enzymes on D-isomers of amino acids. There is a significant difference between average capsule diameters of PLL and PDL microcapsules.

The next three figures compare PLL and PDL microcapsules prepared at the various temperatures. Figure 1 illustrates the effect of incubation in trypsin on the average capsule size and sperm release of PLL and PDL microcapsules prepared at 23°C with glycerol addition post encapsulation. Sperm release was different between polymer types from 1 to 8 h of incubation. Poly-L-lysine capsules peaked in sperm release, whereas sperm release from PDL capsules was constant over time. Average capsule diameters did not differ until 8 h of incubation, at which time PLL capsule were significantly smaller in size.

Figure 2 illustrates the effect of incubation in .5 % trypsin on the average capsule size and sperm release of PLL and PDL microcapsules prepared at 5°C with glycerol added prior to encapsulation. There was no difference in sperm release between PLL or PDL microcapsules. Average capsule size differed between polymers over time except at 1 h.

Table 10. Least squares means for average capsule diameter, after incubation in a solution containing .5% trypsin at 37°C, of cryopreserved Poly-D-lysine (PDL) microcapsules prepared at 23°C and 5°C with glycerol addition prior or post encapsulation with a final sperm concentration of 20×10^6 sperm/ml

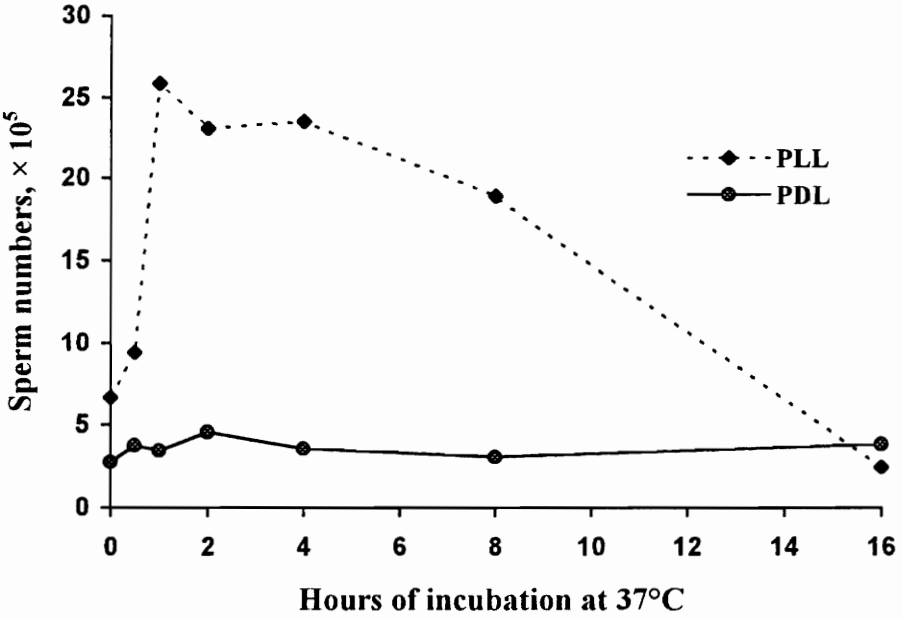
			Hours of Incubation at 37°C						
Polymer	Temperature[a]	Glycerol[b]	0	.5	1	2	4	8	16
PDL	23	post	1.1	1.1	1.0	1.1	1.1	1.1	1.1
PDL	5	prior	1.1	1.1	1.3	1.1	1.1	1.2	1.1
PDL	5	post	1.2	1.2	1.2	1.2	1.0	1.0	1.1
SEM	-	-	.02	.03	.1	.1	.1	.1	.1

[a]Temperature (°C) at which encapsulation was performed
[b]Glycerol addition either prior or post encapsulation

Figure 3 illustrates the effect of incubation in .5% trypsin on the average capsule size and sperm release of PLL and PDL microcapsules prepared at 5°C with glycerol added post encapsulation. There was a difference in sperm release between polymer times in the first two hours of incubation. After 4 h though, sperm release did not differ. There was no initial difference between average capsule diameter. Following the first hour of incubation, PLL microcapsules were significantly smaller in diameter. These figures suggest that peak sperm release for PLL capsules is between 0 and 4 h.

Within the first hour of incubation, there was a significant difference in sperm release between PLL microcapsules prepared at 5°C with glycerol addition post encapsulation and all other processing types. Sperm release for PDL microcapsules was significantly different in the first hour of incubation between microcapsules prepared at 23°C and 5°C with glycerol added post encapsulation. Sperm release for the 23°C processing was persistent over the entire incubation period, whereas it peaked slightly in the 5°C processing.

Figure 1. Effect of 37°C incubation in trypsin on sperm release and average capsule diameters of cryopreserved Poly-L-lysine and Poly-D-lysine microcapsules processed to a final sperm concentration of 20×10^6 sperm cells/ml at 23°C with glycerol addition post encapsulation


Sperm numbers, × 10^5
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
Hours of incubation at 37°C
PLL
PDL

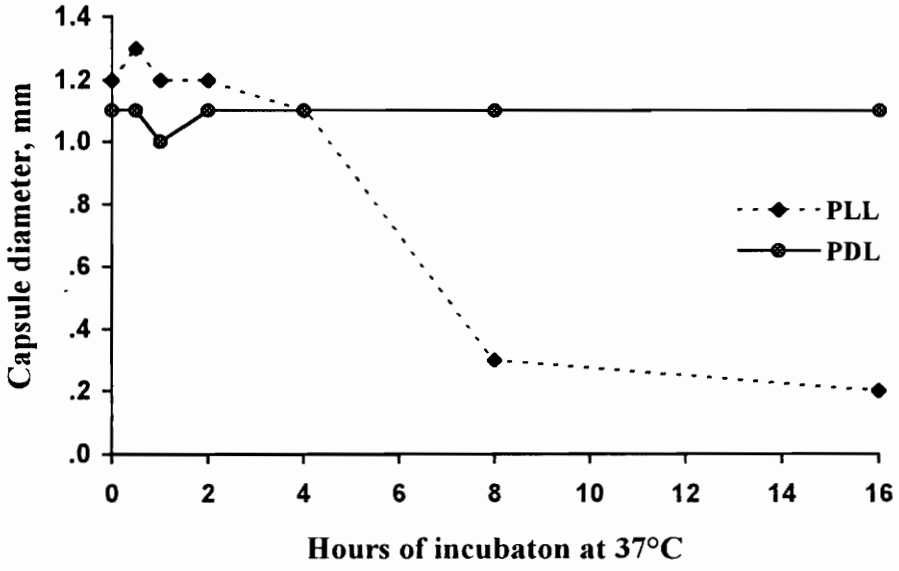
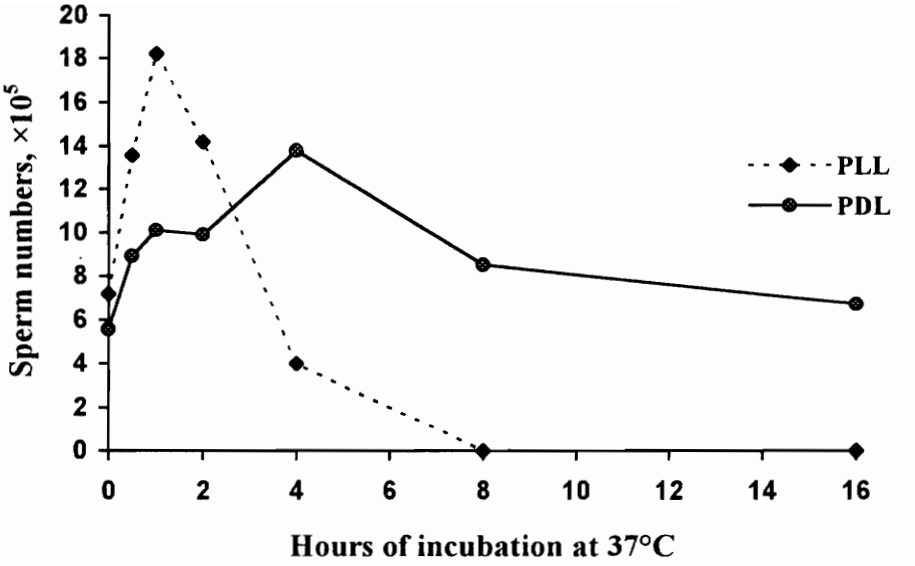

Capsule diameter, mm
1.4
1.2
1.0
.8
.6
.4
.2
.0
0
2
4
6
8
10
12
14
16
Hours of incubaton at 37°C
PLL
PDL

Figure 2. Effect of 37°C incubation in trypsin on sperm release and average capsule diameters of cryopreserved Poly-L-lysine and Poly-D-lysine microcapsules processed to a final sperm concentration of 20×10^6 sperm cells/ml at 5°C with glycerol addition prior to encapsulation


Sperm numbers, ×10^5
20
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
PLL
PDL
Hours of incubation at 37°C

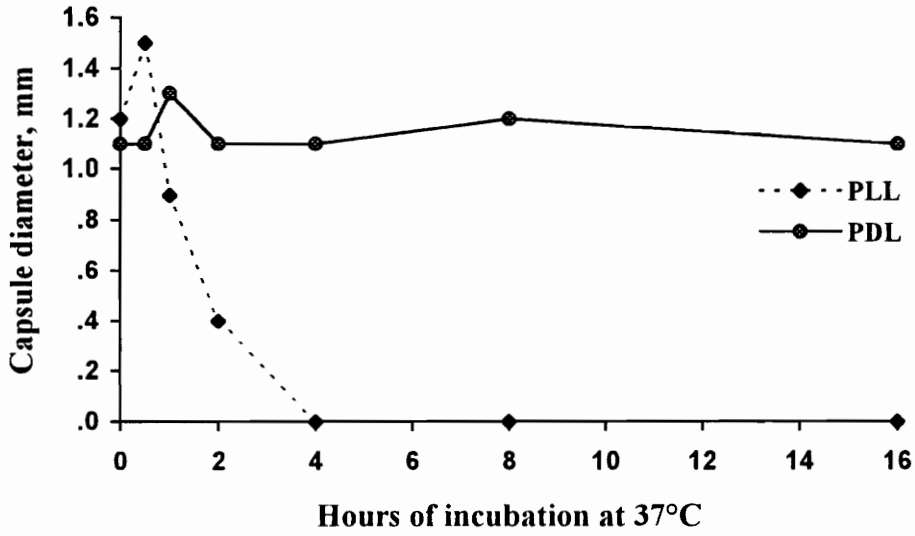
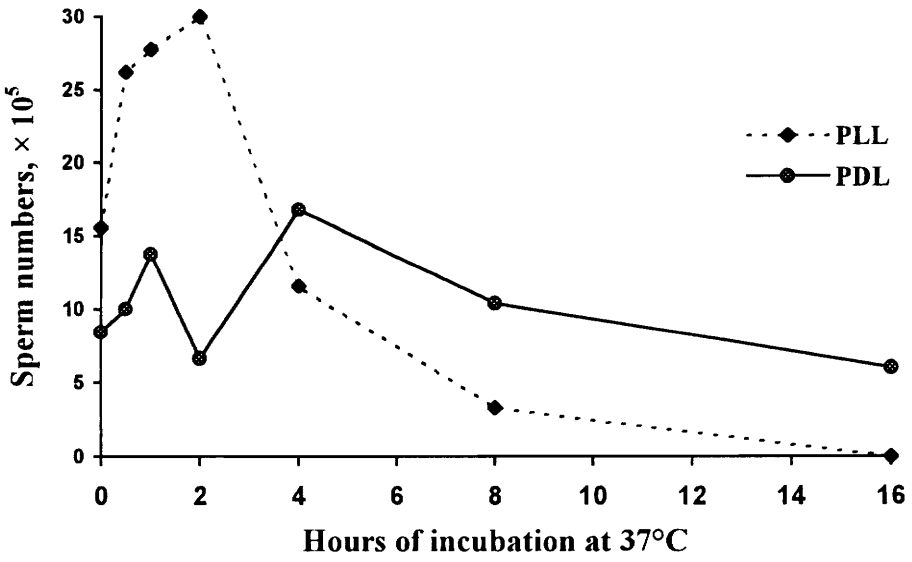
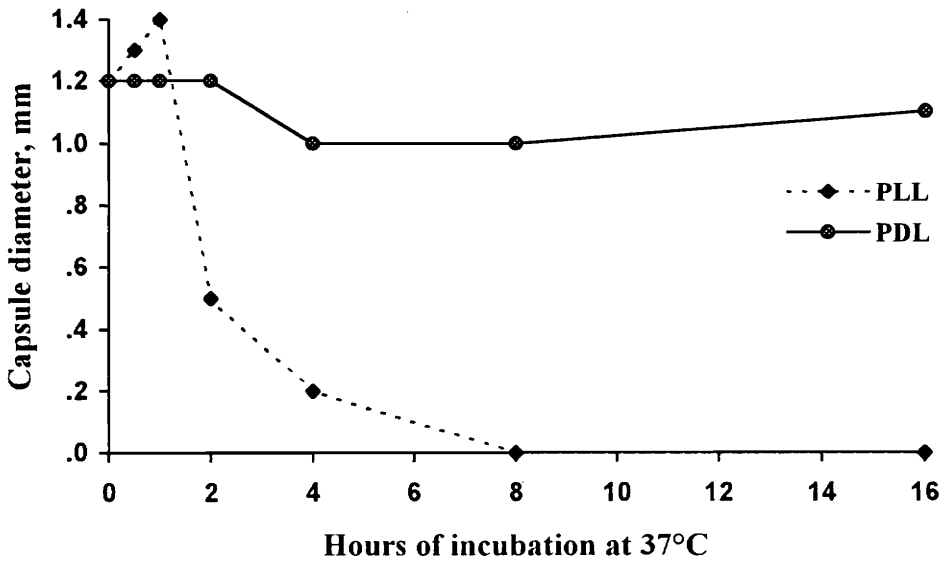

Capsule diameter, mm
1.6
1.4
1.2
1.0
.8
.6
.4
.2
.0
0
2
4
6
8
10
12
14
16
PLL
PDL
Hours of incubation at 37°C

Figure 3. Effect of 37°C incubation in trypsin on sperm release and average capsule diameters of cryopreserved Poly-L-lysine and Poly-D-lysine microcapsules processed to a final sperm concentration of 20×10^6 sperm cells/ml at 5°C with glycerol addition post encapsulation


Sperm numbers, × 10⁵
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
Hours of incubation at 37°C
PLL
PDL


Capsule diameter, mm
1.4
1.2
1.0
.8
.6
.4
.2
.0
0
2
4
6
8
10
12
14
16
Hours of incubation at 37°C
PLL
PDL

Conclusions

It has been determined through experimentation, that microencapsulation of sperm can be accomplished with different polymer types with minimal detrimental effects to sperm viability The technique developed here for in-vitro evaluation of sperm release and average capsule diameter appear to be accurate and useful to determine effects on capsule integrity. Some problems were encountered with obtaining accurate determination of sperm numbers in incubation media surrounding the capsules (released sperm). There are a number of alternative methods that may be substituted for this procedure in future experiments. Sperm viability may be evaluated to determine physiological effects by collecting uterine mucous and incubating microcapsules in vitro over a 24 to 48 h period. Capsule degradation and sperm viability in vivo could be evaluated in an experiment similar to that conducted in the porcine utilizing insemination of microcapsules. Also, evaluation of the interaction of microcapsules with uterine endometrial cells in an oviductal co-culture system would be feasible. Sperm numbers could possibly be measured more accurately with in vivo techniques, as it has been done numerous times in the past (Lee and Ax, 1984; Lee et al., 1986; Varner et al., 1991).

There was an effect of encapsulation temperature and timing of glycerol addition on viability of microencapsulated sperm cells. The treatment that obtained comparable sperm viability to unencapsulated controls was 5°C microcapsule preparation with glycerol addition post encapsulation, regardless of polymer type. This procedure for microencapsulation is suitable for incorporation into current cryopreservation methods.

There have been conflicting results in the bovine and ovine on the issue of intact acrosomes. In the bovine, there was no difference between unencapsulated controls and encapsulated sperm in the percentage of intact acrosomes (Nebel et al., 1985). On the other hand, in the ovine, sperm encapsulated in either PDL or PLL microcapsules had significantly lower percentage of intact acrosomes when compared to unencapsulated controls (Maxwell et al., 1996). In further experimentation it would be reasonable to estimate percent intact acrosomes to determine if viability is indeed affected. Additionally further research could be done to determine if there is a species difference, especially since ovine and porcine sperm are more susceptible to dilution effects (Maxwell et al., 1996).

Vishwanath et al., (1996) and Maxwell et al., (1996) hypothesized that PLL was causing capacitation of sperm cells which could account for the decreased viability in vitro in the ovine experiment. If this were happening, fertility rates in vivo between encapsulated and unencapsulated sperm would be different in both the bovine and ovine trials. Additionally, at the time of polymer addition, there is little chance for sperm cells to come into contact with PLL as they are suspended in Na alginate microgels. Those cells that do come into contact with the polymer are incorporated into membrane formation and not available to fertilize. The number of sperm cells incorporated into the membrane has been found to be less than 10 % (Nebel, pers. comm.).

One other factor that may affect sperm viability in cryopreserved microcapsules is distribution of capsules in individual straws and its effect on freeze rate. Capsules tended to congregate towards the cotton plug due to the vacuum drawing the solution into the straws. There was some settling that occurred between the time straws were filled and

frozen, but it may not have been sufficient enough for the capsules to become evenly distributed throughout the straw. This may have affected the rate at which individual capsules and sperm cells were frozen. When sperm cells freeze too fast, intracellular ice forms, thus affecting viability. When frozen slowly, "solution effects" or increasing solute concentration, cause damage (Saacke, 1982).

Field trials have not been conducted to evaluate fertility rates in cattle inseminated with cryopreserved microencapsulated semen. Utilizing a prostaglandin synchronization program, heifers could be inseminated with one of three treatments: inseminated containing both PLL and PDL microcapsules; PDL microcapsules alone; and unencapsulated semen. The combination of PLL and PDL capsules in an inseminate may be more advantageous due to their differing sperm release. Additionally, if only one microcapsule is retained in the female reproductive tract, there are 50,000 sperm cells available to fertilize the ova. It would be feasible to conduct trials to evaluate fertility utilizing an inseminate with reduced encapsulated sperm numbers.

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Appendix

Table 11. Chemicals used in the microencapsulation procedure

$CaCl_2$-HEPES

1.5% $CaCl_2$ Dihydrate
50 mM HEPES (N-2-Hydoroxyethylpiperazine-N'-2-Ethanesulfonic Acid)
pH adjusted to 6.8

Citrate-Saline

3% sodium citrate dihydrate
Dilute above 1:3 with sterile physiological saline
pH adjusted to 7.0
Osmolarity adjusted to 290-300 mOsm

TEST buffer

.2M TEST (N-tris[Hydroxymethyl] methyl-2-aminoethanesulfonic acid)
.09M TRIS
.01M Fructose
pH adjusted to 7.0
Osmolarity adjusted to 290-325 mOsm

Table 12. Statistical model for average capsule diameter (avgdim) and sperm release (sp) for experiments 1 and 2 using hours as repeated measure

Model

$$Y_{ijkl} = \mu + A_i + \beta_j + \gamma_k + A\beta_{ij} + A\gamma_{ik} + \beta\gamma_{jk} + A\beta\gamma_{ijkl} + \delta_l + A\delta_{il} + \beta\delta_{jl} + A\beta\delta_{ijl} + \gamma\delta_{kl} + A\gamma\delta_{ikl} + \beta\gamma\delta_{jkl} + E_{ijkl}$$

μ = overall mean
A_i = random effect of the i^{th} day
β_j = fixed effect of the j^{th} capsule
γ_k = fixed effect of the k^{th} enzyme
$A\beta_{ij}$ = random effect of the ij^{th} day by capsule
$A\gamma_{ik}$ = random effect of the ik^{th} day by enzyme
$\beta\gamma_{jk}$ = fixed effect of the jk^{th} enzyme by capsule
$A\beta\gamma_{ijk}$ = random error for the above terms
δ_l = fixed effect of the l^{th} hour
$A\delta_{il}$ = random effect of the il^{th} day by hour
$\beta\delta_{jl}$ = fixed effect of the jl^{th} capsule by hour
$A\beta\delta_{ijl}$ = random effect of the ijl^{th} day by capsule by hour
$\gamma\delta_{kl}$ = fixed effect of the kl^{th} enzyme by hour
$A\gamma\delta_{ikl}$ = random effect of the ikl^{th} day by enzyme by hour
$\beta\gamma\delta_{jkl}$ = fixed effect of the jkl^{th} capsule by enzyme by hour
E_{ijkl} = random residual

Table 13. Statistical model for sperm motility (mot) for experiments 1 and 2 using Hours as repeated measures

Model
$Y_{ij} = \mu + A_i + \beta_j + A\beta_{ij} + \gamma_k + A\gamma_{ik} + \beta\gamma_{jk} + E_{ijk}$

μ = overall mean
A_i = random effect of the i^{th} day
β_j = fixed effect of the j^{th} capsule
$A\beta_{ij}$ = random error for the above variables
γ_k = fixed effect of the k^{th} hours
$A\gamma_{ik}$ = random effect of the ik^{th} day by hour
$\beta\gamma_{jk}$ = fixed effect of the jk^{th} capsule by hour
E_{ijk} = random residual

Table 14. Repeated measures analysis of variance for sperm motility of sperm, incubated at 37°C, encapsulated in Poly-L-lysine (PLL), Poly-D-lysine (PDL) and a PLL/PDL mixture

Sources of variation	Degrees of freedom	Mean square
Day	4	5.71
Capsule	2	20.00
Residual 1	8	5.71
Hours	6	11664.13**
Hours by day	24	7.38
Hours by cap	12	12.22
Residual 2	48	9.05

**Significant $P < .05$

Table 15. Repeated measures analysis of variance for sperm motility of sperm , after incubation at 37°C, encapsulated in Poly-L-lysine, (PLL) and Poly-D-lysine (PDL) prepared at 23°C and 5°C, with glycerol addition either prior or post encapsulation

Source of variation	Degrees of freedom	Mean square
Day	7	125.9
Treatment	6	1109.1**
Residual 1	42	361.9
Hours	3	13342.1**
Hours by day	21	43.1
Hours by treatment	18	128.2**
Residual 2	126	39.9

**Significant $P < .05$

Table 16. Repeated measures analysis of variance for sperm release from Poly-L-lysine (PLL), Poly-D-lysine (PDL), and a PLL/PDL mixture (PLPD), microcapsules incubated at 37°C in .5 % heparin and .5 % trypsin

Sources of variation	Degrees of freedom	Mean square[a]
Day	4	1.7**
Capsule	2	2.6**
Day by capsule	8	.46
Enzyme	2	.39
Day by enzyme	8	.8
Enzyme by capsule	4	1.3**
Residual 1	16	.4
Hours	6	7.5**
Hours by day	24	1.3**
Hours by capsule	12	.9
Hours by day by capsule	48	.7
Hours by enzyme	12	3.5**
Hour by day by enzyme	48	.7
Hours by enzyme by capsule	24	.4
Residual 2	96	.6

[a] Mean square $\times 10^{12}$

**Significant $P < .05$

Table 17. Repeated measures analysis of variance for average capsule diameter for Poly-L-lysine (PLL), Poly-D-lysine (PDL), and a PLL/PDL mixture (PLPD) microcapsules incubated at 37°C in .5 % heparin and .5 % trypsin

Sources of variation	Degrees of freedom	Mean square
Day	4	2.3**
Capsule	2	.4**
Day by capsule	8	.04
Enzyme	2	.03
Day by enzyme	8	.3**
Enzyme by capsule	4	.3**
Residual 1	16	.04
Hours	6	1.4**
Hours by day	24	.1
Hours by capsule	12	.4**
Hours by day by capsule	48	.1
Hours by enzyme	12	.5**
Hours by day by enzyme	48	.1
Hours by enzyme by capsule	24	.2**
Residual 2	96	.1

**Significant $P < .05$

Table 18. Repeated measures analysis of variance for sperm release, after 37°C incubation in .5% trypsin, from Poly-L-lysine (PLL) and Poly-D-lysine (PDL) capsules prepared at 2 different temperatures and with glycerol addition either prior or post encapsulation

Sources of variation	Degrees of freedom	Mean square[a]
Day	7	8.9**
Capsule	5	16.6**
Day by capsule	35	1.9
Enzyme	1	.8
Day by enzyme	7	.6
Capsule by enzyme	5	4.6
Residual 1	42	1.4
Hours	6	5.4**
Hours by day	42	.6
Hours by capsule	30	1.2**
Hours by day by capsule	210	.4
Hours by enzyme	6	8.9**
Hours by capsule by enzyme	30	1.9**
Hours by day by enzyme	42	.6
Residual 2	210	.5

[a]Mean square error $\times 10^{12}$
**Significant $P < .05$

Table 19. Repeated measures analysis of variance for average capsule diameters, after 37°C incubation in .5% trypsin, of Poly-L-lysine (PLL) and Poly-D-lysine (PDL) capsules prepared at 23°C and 5°C and with glycerol addition either prior or post encapsulation

Sources of variation	Degrees of freedom	Mean square
Day	7	.2
Capsule	5	1.2**
Day by capsule	35	.1
Enzyme	1	9.1**
Day by enzyme	7	.1
Capsule by enzyme	5	2.3**
Residual 1	35	.1
Hours	6	1.6**
Hours by day	42	.03
Hours by capsule	30	.4**
Hours by day by capsule	210	.0
Hours by enzyme	6	2.0**
Hours by capsule by enzyme	30	.4**
Hours by day by enzyme	42	.03
Residual 2	210	.04

**Significant $P < .05$

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