THE VIABILITY AND FERTILITY OF BOVINE SPERMATOZOA
ENCAPSULATED IN MICROCAPSULES AND MICROGELS

by

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submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Dairy Science

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August, 1989

Blacksburg, Virginia
Four experiments were conducted to evaluate the viability and fertility of bovine spermatozoa encapsulated in microcapsules and microgels. In Experiment 1, one of two morphologically distinct sperm types i.e. marker or unmarked bull spermatozoa (100 x 10^6 sperm/bull) were encapsulated in protamine sulfate microcapsules and simultaneously inseminated with the reciprocal sperm type unencapsulated. Insemination of both sperm types unencapsulated served as a control. Accessory sperm embedded in the zona pellucida were counted and morphologically classified 6 to 7 d post insemination. From microencapsulated inseminates, accessory sperm populations did not increase over the unencapsulated controls, but contributed 25.7% of the accessory sperm population. In Experiment 2, an in vitro study was performed to evaluate the maintenance of viability for bovine spermatozoa encapsulated in PIPES, HEPES, or saline microgels. Neat semen was pooled from five bulls (50 x 10^6 sperm/bull), encapsulated in alginate microgels, and incubated at 37 C for 8 h. The unencapsulated control displayed greater maintenance of viability for percent in-
tact acrosomes and motility when compared to all treatments. By 8 h incubation, PIPES and HEPES were not significantly different, but demonstrated greater maintenance of viability when compared to saline microgel treatments.

In Experiment 3, PIPES microgels were heterospermically inseminated with equal numbers (20 x 10^6 sperm/bull) of frozen-thawed marker bull and normal bull spermatozoa as explained in Experiment 1. Microencapsulated treatments contributed significantly lower numbers of accessory sperm when compared to unencapsulated controls.

In Experiment 4, one of the two morphologically distinct sperm types (20 x 10^6 frozen-thawed sperm/bull) were encapsulated in protamine sulfate microcapsules and the reciprocal sperm type was encapsulated in PIPES microgels. A total of 21 accessory sperm were recovered from 30 embryos which demonstrates the ability of microencapsulated spermatozoa to fertilize an oocyte.
ACKNOWLEDGEMENTS

The author would sincerely like to thank Dr. Raymond Nebel for his guidance, support, and encouragement throughout the completion of this research. I would also like to express my gratitude to the members of my graduate committee, Dr. R.G. Saacke and Dr. D.J. Sprecher. Their expertise and advice was invaluable throughout the completion of this research.

I would like to express thanks to
for their technical assistance.

I would like to thank Select Sires Inc., for their financial support of this research.

Finally, special gratitude is extended to my parents, family, and friends for their continuing support and encouragement.
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INTRODUCTION

Approximately 85% of the dairy farmers in the United States use AI to breed at least some percentage of the cows in their herd (Hoard's Dairymen Survey, 1988). Conception rates using AI range from 20 to 75% and this variation is traced to many complex causes such as: reproductive soundness of the cow, heat detection accuracy, semen quality, semen handling, insemination techniques, and timing of insemination. Hawk (1987) estimated 12 to 15% of the reproductive inefficiency in dairy cattle is attributed to the lack of spermatozoa available in the oviduct near the time of ovulation. Upon insemination, millions of sperm cells are deposited into the female reproductive tract. With natural mating, an average ejaculate from a mature bull contains 4 to 18 x 10^9 sperm cells (Sorenson, 1979). Sperm concentrations decrease 125 to 550 fold in artificially inseminated cattle with inseminate numbers ranging between 10 to 30 x 10^6 cells. Once inseminated, sperm cells are either transported to the oviduct or lost from the reproductive tract. Decreased efficiency of sperm transport or lack of sperm available for sperm transport are thought to be the major causes of fertilization failure in artificially inseminated dairy cattle.
Sperm Transport

Spermatozoa are transported to the site of fertilization, the ampullary-isthmus junction, in two phases. Most spermatozoa transported during the rapid or initial phase of sperm transport are nonviable (Hunter and Wilmut, 1984). Therefore, they play no direct role in fertilization. Distention of the vagina via coitus or tactile manipulation during AI causes contractions of the uterus and oviduct which propel sperm to the anterior regions of the female reproductive tract (Vandemark and Hayes, 1952). Within seconds to a few minutes, a spermatozoal population of low viability appears in the upper ampulla and peritoneal cavity (VanDemark and Moeller, 1951). Motile spermatozoa were recovered from the ampulla in 1 out of 7 does observed 1 to 15 min post-coitum. Of these only 8% were motile. Of the sperm recovered from the ampulla, 98% had disrupted acrosomal membranes, and 15% had dissociated heads (Overstreet and Cooper, 1978).

The second or sustained transport phase begins within a few minutes after insemination and lasts for approximately 8 to 16 h. Viable spermatozoa having the potential for fertilization are transported to the oviduct via spermatozoal motility, uterine contractions, and oviductal ciliary movement (Hawk, 1983). The number of spermatozoa transported to the oviduct gradually increases and then declines (Dobrowolski and Hafez, 1970). During migration through the female reproductive tract, spermatozoa undergo capacitation or gain the ability to fertilize an oocyte. Within 10 to 12 h, sufficient sperm numbers are available in the
caudal region of the isthmus which is thought to be a specialized storage site for bovine sperm (Hunter and Wilmut, 1984; Wilmut and Hunter, 1984). While stored in the caudal isthmus, sperm cells are continuously released to the anterior oviduct prior to fertilization. At the time of ovulation, sperm release occurs as a limited migration from the caudal isthmus (Overstreet and Cooper, 1978).

Sperm Recovery and Distribution within the Female Reproductive Tract

Spermatozoa are either transported to the site of fertilization or lost from the female genital tract within a short period of time after insemination. Spermatozoal disappearance from the female reproductive tract results from retrograde loss, phagocytosis, and passage of sperm into the body cavity.

I. Sperm Retention

Retention of sperm in the female reproductive tract is low with respect to the total sperm inseminated. Dobrowolski and Hafez (1970) vaginally inseminated 2.0 x 10⁹ neat spermatozoa into estrual cows and recovered 13.4, 3.8, and 0.9% of the inseminate from the genital tract at 1, 8, and 24 h, respectively. El-Banna and Hafez (1970) recovered 1.61 and 0.03% of the inseminate in vaginally inseminated cows at 16 and 40 h, respectively. Twelve h post insemination, Mitchell et al., (1985) recovered 6.3 and 6.5% of 1.0 x 10⁹ and 420 x 10⁶ neat
spermatozoa inseminated into the uterine body of estrual cows. Studies have shown that sperm cells subjected to freeze-thawing are unable to traverse the genital tract as effectively as neat semen. Lineweaver et al., (1970) inseminated 32 × 10^6 frozen-thawed and 34 × 10^6 fresh spermatozoa into heifers (6 per treatment) and recovered an average of 191 and 458 sperm cells, respectively, from the oviducts at 4 h after insemination.

II. Sperm Distribution

Of the 420 × 10^6 neat spermatozoa inseminated into the uterus of estrual cows, 6.5% were recovered from the female reproductive tract 12 h post insemination (Mitchell et al., 1985). Of the spermatozoa inseminated, 6.0% were lost to the vagina and 0.4% were lost to the cervix, 0.1% remained in the uterus, and 0.1% or 8 × 10^3 were transported to the uterotubal junction and isthmus. Overall, 98% of the inseminate recovered was lost to the posterior portion of the female reproductive tract. Dobrowolski and Hafez (1970) and El-Banna and Hafez (1970) reported similar distributions with neat semen throughout the female reproductive tract.

Larsson and Larsson (1985) artificially inseminated 160 × 10^6 frozen-thawed spermatozoa into the posterior uterus of estrual heifers, and recovered 14.6 and 0.6% from the female tract at 2 and 12 h, respectively. Of the sperm inseminated, 98.6 and 88.4% were recovered from the vagina and only 0.05 and
1.8% were recovered from the oviduct. Larsson and Larsson (1986) artificially inseminated three heifers with $20 \times 10^6$ frozen-thawed spermatozoa into the posterior uterus, and recovered spermatozoa from segments within the oviduct. A total of 46 sperm cells were found throughout the oviducts of three heifers. By 12 h post insemination, relatively few spermatozoa from an inseminate were transported to the caudal isthmus, and most spermatozoa were lost to the cervix and vagina.

III. Retrograde Loss

Mitchell et al. (1985), inseminated $420 \times 10^6$ neat spermatozoa into the uterine body of estrual cows. All mucus and urine discharged between the time of insemination and slaughter was collected. By 12 h post insemination, 73% of the inseminated sperm were accounted for within the mucus discharged and the female genital tract. Components were: inseminate lost from the genital tract in discharged mucus, 60.7%; lost in urine, 0.06%; aspirated from the vagina, 4.4%; adhered to equipment, 1.3%; retained in the genital tract, 6.5%. By 6 h post insemination, 51.8% of the inseminate recovered was voided from the genital tract. Retrograde loss accounted for over 90% of the total sperm recovered from the inseminate, and is considered the primary route of sperm loss.
IV. Phagocytic Loss

Phagocytosis of spermatozoa has been postulated as a second major route of sperm loss from the female reproductive tract. Roark and Herman (1950) reported a infiltration of leukocytes at the onset of estrus, and an increase in the uterine leukocytic response with in the presence of sperm (Howe and Black, 1963). Polymorphonuclear leukocytes containing ingested spermatozoa were found throughout all divisions of the bovine female genital tract (Mattner, 1968). Both live (motile) and dead bovine spermatozoa were ingested by polymorphonuclear leucocytes extracted from bovine blood. (Mattner, 1969). Mitchell et al., (1985) suggested spermatozoa unaccounted for from the inseminate (27%) were lost to phagocytosis.

V. Peritoneal Loss

Spermatozoa are lost to the peritoneal cavity in both the rapid and sustained phase of sperm transport (Overstreet and Cooper, 1978; Overstreet et al., 1978). However, sperm transported through the oviduct to the peritoneal cavity is thought to contribute little to sperm loss (Larsson, 1986). Ligatures were placed around the proximal uterus and inseminate doses of $85 \times 10^6$ were deposited into the posterior uterus of 4 estrual heifers. Histological examination 2 h post insemination recovered only one sperm cell from the oviduct containing ligatures.
This literature review will focus mainly on microencapsulation of living cells with special attention to the microencapsulation of bovine spermatozoa.

Microencapsulation may be thought of as a process that encases small discrete solid particles, liquid droplets, gases, or living cells within a protective polymeric coating (Chang, 1972). Impermeable or semipermeable membranes are formed by several techniques which yield microcapsules ranging in size from 0.2 μm to several millimeters in diameter. Release of microcapsule contents or by-products from encapsulated cells is dependent on membrane construction and may result from osmotic pressure, moisture, pH, temperature, physical force or combinations thereof. Leaching, erosion, or rupture are mechanisms for release of substrates or materials produced by or released from microcapsules (Luzzi, 1970).

Microencapsulation has a wide range of practical applications. The first commercial microencapsulated product was carbonless carbon paper where inert
materials were encapsulated in impermeable membranes and released by physical rupture (Green and Schneidcher, 1957). Extensive use of microencapsulation technology has been employed in the pharmaceutical industry for products such as microencapsulated aspirin for decreased stomach trauma, masking of bitter tasting drugs, protection from moisture, and protection for light sensitive drugs. Microencapsulation has been employed in animal agriculture. For example, administration of follicle stimulating hormone (FSH) for induction of superovulation requires numerous injections because of the short half-life of FSH. Demoustier et al., (1988) tested a biodegradable microcapsule that releases porcine pituitary extracts to induce superovulation in cattle. In an in vivo study, a lactic acid polymeric implant released FSH over a 90 h period with a mean plasma concentration of 0.3 ng/ml. Microcapsules placed in the uterus or inseminated into the vagina were examined for a possible long term delivery system in human contraception (Hassler, 1974). Retention of uterine inseminated microcapsules increased as capsule size increased, but very few microcapsules were transported to the uterus in vaginally inseminated rabbits. Release of progesterone from the microcapsules was controlled by varying the thickness of the microcapsule membrane with maximum release of progesterone being 3.6 μg/d.

Deposition of a polymer around an aqueous droplet containing biologically active materials produced the first “artificial cell” or microcapsule with a semipermeable membrane (Chang, 1964). Selective diffusion of small molecular weight substrates or by-products without loss of core contents was the major advantage
of the semipermeable membrane. Microcapsule size varied between 1 to 100 μm in diameter and membrane thickness was controlled by varying polymer concentrations. After encapsulation, red blood cells did not leak from nylon microcapsules and catalytic activity of carbonic anhydrase was "almost as efficient as for unencapsulated controls". The rate of molecules diffusing across the membrane was postulated as the rate limiting step to catalytic activity. Enzymatic activity was retained "after weeks of storage". Subcutaneous or intraperitoneal microcapsule injection was nontoxic to recipients and enzyme activity was detected. Enzymatic activity of trypsin and urease was "fairly efficient after the microencapsulation process". Living cells were unable to survive this microencapsulation process due to toxic organic solvents required for the condensation reaction.

**Microencapsulation of Living Cells**

Lim (1982) was the first to successfully develop an all aqueous microencapsulation process which enclosed bioactive compounds or viable cells within a semipermeable membrane. Cellular nutrients, metabolites, and waste products were free to diffuse into and out of the polyelectrolyte membrane comprised of polycations and polyanions. A polylysine-alginate membrane was the most successful membrane developed. Alginate or alginic acid (the polyanion), a polysaccharide extracted from seaweed, was easily converted from a viscous liquid to a solid gel. While in the presence of sodium (Na⁺), alginate remains in a
liquid state. Increasing the valency of the medium by addition of calcium
\((Ca^{++})\) causes gelation of alginate. Suspension of the alginate gel with multiva-
lent polyamines (polycation) such as polylysine, polyvinylamine, polyarginine, or
protamine sulfate initiates a crosslinking reaction between the alginate and
polyamine which forms a semipermeable hydrogel membrane.

Lim and Sun (1980) were the first to microencapsulate pancreatic islet cells
within a semipermeable polylysine alginate membrane. In an in vitro study,
microencapsulated and unencapsulated islet cells were assessed for their ability
to respond to a glucose challenge. Microencapsulated islet cells remained
morphologically and functionally intact for up to 4 months and secreted insulin
in concentrations comparable to the unencapsulated controls. In an in vivo
study, microencapsulated and unencapsulated islets were injected into the
peritoneal cavity of streptozotocin-induced diabetic rats. Microencapsulated
islets alleviated the diabetic condition for approximately 3 weeks while the control
islets survived for only 6 to 8 d.

Sun and O'Shea, (1985) microencapsulated rat islets of langerhans in polylysine
microcapsules and transplanted them into streptozotocin-induced diabetic mice.
The diabetic condition was alleviated within 3 d and for as long as 648 d with
no immune response observed. Encapsulated islets produced insulin at levels
sufficient to meet the physiological demands of the mice. Also,
microencapsulated hepatocytes were injected into rats with induced liver failure.
Survival rates of the rats increased after injection. Overall, viable cells
encapsulated in a semipermeable membrane can remain active in vivo and in vitro with little observation of an immune rejection. Water content of the semipermeable polylysine membrane was 93\% and scanning electron micrographs revealed both sides of the membrane to be smooth. The membrane was 4.00 ± 0.28 \mu m thick.

Bisected bovine embryos were placed into a recipient zona pellucida and microencapsulated in polylysine membranes (Hollingsworth and Page, 1988). Embryos were frozen and thawed, then evaluated for development and viability. Microencapsulated embryos matured at a faster rate and maintained higher morphological scores when compared to unencapsulated controls. Adaniya et al., (1986) microencapsulated murine embryos in saline microgels at the 2 cell stage and monitored their development for 72 h. No detrimental growth effect was observed when compared to unencapsulated controls.

Microencapsulation of Spermatozoa

Nebel et al., (1985) modified Lim's microencapsulation process and was the first to successfully microencapsulate bovine spermatozoa. Several experiments were conducted to evaluate the viability of bovine sperm cells suspended within semipermeable microcapsules. In an in vitro study, neat semen encapsulated in 0.75 and 1.5 mm polylysine microcapsules at concentrations of 45, 90, and 180 \times 10^6 cells were incubated at 37 C for 24 h. Sperm motility and acrosomal integrity
were estimated at 2, 12, and 24 h. Microencapsulated spermatozoa showed no significant difference with respect to motility and intact acrosomes when compared to an unencapsulated control. Unencapsulated spermatozoa (180 x 10⁶) exhibited lower motility (p < 0.05) at 12 h and lower (p < 0.05) motility and intact acrosomes at 24 h. Microcapsule integrity decreased as sperm concentration increased and 0.75 mm capsules appeared more fragile than 1.5 mm capsules.

Frozen-thawed semen with varying concentrations (0, 5, 10, 15%) of egg yolk was examined for viability after the microencapsulation process (Nebel et al., 1985). Spermatozoal motility and intact acrosomes evaluated at 2, 4, 8, 12, and 24 h was not affected by varying levels of egg yolk, but microcapsule uniformity (size and shape) decreased as egg yolk concentration increased to 15%. Minimal cellular injury for neat or frozen-thawed semen was observed from the microencapsulation process.

Varying polylysine concentrations of 0.05, 0.15, 0.25, and 0.35 % were used to determine the effects of polymer concentrations on microcapsule membrane integrity (Nebel, 1987). Bovine spermatozoa, encapsulated at 45 x 10⁶ sperm/ml, were evaluated for intact acrosomes and motility at 2, 4, and 8 h. Spermatozoal viability was not affected by polymer concentrations. At 0.05% polylysine, thinner-walled microcapsules were produced and appeared more fragile than microcapsules produced using higher polymer concentrations. Thickness and fragility of microcapsules did not significantly differ at polylysine concentrations.
of 0.15, 0.25, and 0.35%. Therefore, at concentrations of 0.15% and above, maximum crosslinking of polylysine and alginate resulted.

A heterospermic study designed to evaluate the potential retention of microencapsulated bovine spermatozoa in the female reproductive tract was conducted (Nebel et al., 1987). Morphologically normal spermatozoa (unmarked bull) and spermatozoa of a distinguishable tapered morphology (marker bull) were encapsulated with polylysine (biodegradable) or polyvinylamine (nonbiodegradable) microcapsules. Equal numbers of both cell types at concentrations of 200 x 10⁶ cells (each) were simultaneously artificially inseminated into the posterior uterus of estrual cows. One sperm type was microencapsulated while the other remained unencapsulated. Using a nonsurgical endometrial flushing procedure, uterine contents were recovered twelve hours after insemination. Isolated sperm cells were counted and morphologically classified. Spermatozoa contained within polyvinylamine microcapsules showed significant uterine retention over unencapsulated sperm, but polylysine microcapsules resulted in inferior sperm retention at 12 h post insemination. Intact polyvinylamine microcapsules were occasionally obtained from the uterine flushings. Nebel et al. (1987) suggested that polyvinyl microcapsules exhibited a stickiness property which allowed for resistance to retrograde flow or other physical forces encountered by unencapsulated spermatozoa. Because polyvinyl microcapsules resisted physical rupture, retained spermatozoa may not have reflected a sperm population capable of fertilization. Spermatozoa contained in polylysine microcapsules did not show favorable uterine retention and were...
thought to be affected by early rupture, retrograde movement, and/or phagocytosis.

Experiments similar to the microencapsulation of bovine sperm were conducted to evaluate the effects of microencapsulation on porcine spermatozoa (Esbenshade et al., 1987). In an in vitro study, minimal loss of cellular integrity and viability due to microencapsulation were reported when compared to unencapsulated controls. Polylysine microcapsules containing porcine spermatozoa were inseminated into the uterine body of estrual sows. Intact microcapsules containing motile sperm cells were recovered 3 and 6 h post insemination and were transported to the anterior uterus. Motility loss over time was similar for both in vitro and in vivo incubation conditions.

To summarize the microencapsulation studies of porcine and bovine spermatozoa the following general statements can be made: 1) sperm cells of both species survived the microencapsulation process and incubation with no decrease in sperm viability over unencapsulated controls 2) within the microcapsule, porcine spermatozoa remained motile in the female genital tract for up to 6 h and 3) increased uterine retention was observed for bovine spermatozoa encapsulated in polyvinylamine microcapsule.
Accessory Sperm Populations

There have been no reports regarding the effects of microencapsulation on sperm populations in the oviduct or fertility of microencapsulated sperm. The data presented in this thesis addresses the ability of microencapsulated bovine spermatozoa to gain access to the oviduct. Quantitative measurements were made of sperm cells attached and penetrated to the zona pellucida (accessory sperm) of the 6 to 7 d developing embryo. The number of accessory sperm has been suggested as an indicator of the efficiency of spermatozoa inseminated to be transported to the site of fertilization (Saacke et al., 1988; Hunter and Wilmut, 1983; Hawk and Tanabe, 1986). Some assumptions of accessory sperm as a fertility indicator must be made to use this method for the evaluation of sperm transported to the oviduct. Whether fertilization has or has not occurred, accessory sperm should be considered capable of fertilization because they have demonstrated the ability to be transported to the oviduct (site of fertilization), to be capacitated, to undergo a true acrosome reaction, and attach and penetrate the zona pellucida.

Fertilized embryos generally contain accessory sperm, and unfertilized embryos contain little or no accessory sperm (Hunter and Wilmut, 1983; Hill et al., 1971). Saacke et al., (1988) inseminated semen (100 or 200 x 10⁶) from bulls with morphologically normal and abnormal sperm cells. The average number of accessory sperm per 6 to 7 d embryo was 21.1 ± 30.5 and 1.1 ± 2.1 SD or single and superovulated cows, respectively. Accessory sperm cells of normal
morphology were more abundant than abnormally shaped sperm cells. As the severity of sperm cell head distortion increased, the greater exclusion of abnormal cells was observed. Degenerate to lower quality embryos had lower numbers of accessory sperm when compared to good to excellent quality embryos. In single ovulated eggs, 64 of the 68 (94%) embryos recovered 3 d post insemination cleaved with an average of 34 ± 6 SEM accessory sperm per egg (Hawk and Tanabe, 1986). In two of the three unfertilized ova, no accessory sperm were found but one contained 16 accessory sperm. Again, accessory sperm in superovulated cows were much lower than in single ovulated cows. Saacke et al., (1988) attributed lower accessory sperm numbers to increased abnormal sperm cells within inseminates when compared to the data of Hawk and Tanabe.

Conclusions

Most sperm contained within an inseminate never reach the site of fertilization. Many researchers have focused on improving sperm transport with expectations of increasing sperm numbers to the ampullary-isthmus junction by adding compounds to the semen such as: oxytocin (Hays and VanDemark, 1953), prostaglandins (Hawk, 1979; Lauderdale, 1975), and estrogens (Battalia, 1980). Gallagher and Senger 1989 reported high doses (20 mg/ml) of phenylepherine and ergonovine to be detrimental to sperm viability, but norepinephrine and oxytocin did not affect sperm viability. Little research has dealt with reduction of
retrograde flow and phagocytosis of spermatozoa. The objective of this research is to improve conception rates in artificially inseminated cattle through insemination of microencapsulated bovine spermatozoa. Microcapsules have been shown to be a protective barrier for capsule content to leukocytic invasion (Sun and O'Shea, 1985) and polyvinylamine microcapsules increased sperm retention in the bovine female reproductive tract (Nebel et al., 1987). By decreasing sperm loss to retrograde flow and phagocytosis, microencapsulation has the potential to increase sperm populations at the site of fertilization. Two specific microspheres i.e. microcapsules and microgels were evaluated in this thesis. A microcapsule is considered to be a hollow microsphere in which inert or bioactive materials are freely suspended within a polymeric membrane. A microgel is considered to be a solid microsphere which encases inert or bioactive materials within the inner gel matrix. Four experiments were conducted to test the potential use of microencapsulated bovine spermatozoa in the artificial insemination industry of cattle. In Experiment 1, a new membrane, protamine sulfate, was evaluated due to the high cost of polylysine, the carcinogenic potential and nonbiodegradability of polyvinylamine. Protamine sulfate is a naturally occurring polymer which is extracted from salmon semen. Rationale for use of the protamine sulfate membrane was: (1) the cost of protamine sulfate was over 200 dollars less than polylysine, (2) protamine sulfate exhibited the “stickiness” characteristic of polyvinylamine, and (3) protamine sulfate is biodegradable. Therefore, Experiment 1 was designed to compare the retention of spermatozoa encapsulated in protamine sulfate microcapsules and unencapsulated
spermatozoa. Both encapsulated and unencapsulated spermatozoa were simultaneously inseminated in estrual cows. After artificial insemination, spermatozoa encapsulated in protamine sulfate microcapsules were assessed for their ability to be released from the microcapsule, transported to the site of fertilization, attach and penetrate the zona pellucida, and ultimately fertilize an oocyte. These properties were evaluated through observations of accessory sperm. Experiment 2 was designed to determine if spermatozoa could maintain viability while encapsulated in the matrix of the microgel over time. The microgel is formed in the first step of the microencapsulation procedure. The rationale for testing sperm viability in the microgel was that microgels have a "stickiness" property which may enhance sperm retention in the female reproductive tract. Also, the time of the encapsulation procedure was reduced from approximately 30 to 5 min, and the cost of the polyamine membrane and chemicals was eliminated. Therefore, PIPES, HEPES, and saline buffered microgels were evaluated for percentage of intact acrosomes and motile sperm over 8 h at 37 C. At 8 h incubation, PIPES microgels supported greater maintenance of sperm viability when compared to HEPES and saline microgels, but no significant difference was observed between PIPES microgels and the control. Experiment 3 was designed to compare the retention of spermatozoa encapsulated in PIPES microgels over unencapsulated spermatozoa. Retention was evaluated utilizing accessory sperm populations as stated in Experiment 1. In Experiments 1 and 3, both PIPES microgels and protamine sulfate microcapsules contributed to accessory sperm populations. Experiment 4 was designed to evaluate the retention of sperm with equal compe-
tition of spermatozoa encapsulated in protamine sulfate microcapsules and PIPES microgels. Therefore, both protamine sulfate microcapsules and PIPES microgels were inseminated simultaneously and accessory sperm were evaluated as a measure of sperm retention in the female reproductive tract.
MATERIALS AND METHODS

Experiment 1. Accessory Sperm Populations after Heterospermic Insemination of Spermatozoa Encapsulated in Protamine Sulfate Microcapsules vs. Unencapsulated Spermatozoa.

With the high cost of polylysine, carcinogenic potential and nonbiodegradability of polyvinylamine, protamine sulfate was evaluated as a new possible polymer for the microencapsulation of bovine spermatozoa. Protamine sulfate has the "stickiness" qualities of polyvinylamine and costs approximately 200 dollars/g less than polylysine. Therefore, the objective of this study was to evaluate the ability of spermatozoa encapsulated in protamine sulfate microcapsules to be retained in the female reproductive tract. After insemination, there were three questions: (1) did microcapsules increase the retention of sperm in the uterus, (2) were spermatozoa released from the microcapsules, and (3) did the spermatozoa have the ability to be transported to the oviduct? Another important question of microencapsulated semen deal with their potential to undergo capacitation, the true acrosome reaction, attach and penetrate the zona pellucida, and ultimately fertilize an oocyte.
In a completely random design, a heterospermic study was conducted with two morphologically distinct sperm types which were artificially inseminated in accordance to three treatments: (1) Semen encapsulated in protamine sulfate microcapsules from a fertile bull providing sperm cells predominantly of a normal morphology (unmarked bull), and unencapsulated semen from a fertile bull providing sperm cells predominantly of a tapered morphology (marker bull), (2) Marker bull semen encapsulated in protamine sulfate microcapsules and unmarked bull semen unencapsulated, (3) Control, semen from both marker and unmarked bulls unencapsulated. In each treatment, ten cows were inseminated with equal numbers of 100 x 10^6 sperm cells from each bull.

To the authors knowledge, there have been no reports of an experiment of this nature. In this heterospermic study, two morphologically distinct sperm types i.e. marked or unmarked spermatozoa were simultaneously inseminated with one of the two sperm types microencapsulated. Simultaneous insemination of both unencapsulated sperm types served as a control. Accessory sperm were counted and morphologically classified. Therefore, this heterospermic study will examine the feasibility of applying a treatment such as microencapsulation to one of two morphologically distinct sperm populations and evaluate the effect of microencapsulation on accessory sperm populations. A study of this nature will hopefully allow the author to evaluate the effects of microencapsulation over a limited population of cows.

MATERIALS AND METHODS
Semen Preparation

An artificial vagina was used to collect semen from the unmarked bull on the day of insemination. A calibrated spectrophotometer was used for determination of spermatozoal concentration and a phase contrast microscope (100 and 400x) with heated stage (37°C) was used to estimate progressive motility. Frozen-thawed semen from the marker bull was thawed in a 35°C water bath for 45 s.

Microencapsulation

The microencapsulation procedure for Islets of Langerhans cells (Lim, 1982) as modified for bovine spermatozoa by Nebel et al., (1985) was used in this study. In this process, 100 x 10⁶ sperm cells to be encapsulated (respective to treatment) were placed in 1% sodium alginate and drawn into a 12 ml syringe. This aqueous sperm alginate suspension was extruded by a syringe pump technique (Ennis and James, 1950; Sparks et al., 1969) at 1.5 ml/min and forced through a 19 gauge hypodermic needle encased within an encapsulating jet. At the same time of extrusion, air controlled by an air flow meter was forced through the air inlet nozzle which caused a homogeneous aqueous 1.5 mm microdroplet to be expelled. Droplet size was controlled by varying the air velocity.

The bottom of the encapsulating jet was placed 3.54 cm above 80 ml of 1.5% CaCl₂-HEPES solution, (pH = 6.8). Upon submersion, an ionic exchange of
$Ca^{++}$ for $Na^+$ caused solidification of the microdroplet which formed the microgel. The CaCl₂-HEPES solution was aspirated, and microgels were rinsed three times with 0.9% physiological saline. Microdroplets were suspended in 1% protamine sulfate for 5 min. A crosslinking polymerization reaction between the protamine sulfate (polycation) and the sodium alginate (polyanion) formed the semipermeable membrane around the microgel. Excess protamine sulfate was aspirated. Microcapsules were rinsed in a CHES buffer (pH = 8.2) which terminates the crosslinking reaction by binding the unbound reactive sites on the semipermeable membrane (Lim, 1983). Microcapsules were rinsed three times with physiological saline and placed in 3% sodium citrate solution (pH = 7.4), which liquifies the microcapsules inner alginate gel core by chelation of the $Ca^{++}$.

Insemination

Microcapsules containing progressively motile (100 x 10⁶ cells) in the liquified core were transferred to a 12 ml syringe along with equivalent numbers of unencapsulated sperm. A breeding pipette was manipulated through the cervix and placed approximately 1.75 cm into the uterine body. The syringe was connected to the breeding pipette and sperm cells deposited. Insemination was per-
formed approximately 12 h after the onset of estrus. Cows were synchronized with Lutalyse\textsuperscript{1} and observed for estrus twice daily after injections.

**Uterine Flush**

Flushing procedures were based on techniques developed at The Colorado State University (Elsden and Seidel, 1982). Nonsurgical flushes of the uterine endometrium were performed for morula to blastocyst stage embryos 6 to 7 d after insemination. Modified Dulbecco's phosphate-buffered saline (PBS) supplemented with heat-treated fetal calf serum and antibiotic/antimycotic was used for flushing media. Prior to flushing, the corpus luteum was located by rectal palpation of the ovaries to determine which side of the reproductive tract ovulation occurred. The tailhead was clipped, scrubbed 3 times with iodine soap, and coated with 70\% isopropyl alcohol. Approximately 5 ml of Lidocaine\textsuperscript{2} was administered between the coccygeal vertebrae in order to decrease peristaltic waves of the rectum. A 20 gauge French 5cc balloon 2-way Foley catheter was manipulated through the cervix and placed into the uterine body. The 5cc balloon was inflated and 400 to 500 ml of PBS was deposited in 50 and 100 ml increments. Rectal manipulation of the uterus was necessary for PBS retrieval through the catheter.

\textsuperscript{1} Lutalyse, UpJohn Co., Kalamazoo, MI

\textsuperscript{2} Lidocaine Hydrochloride 2\%, Am-Vet Pharmaceuticals, Fort Collins, CO

**MATERIALS AND METHODS**
Embryo Recovery

Once the flush was completed, the recovered PBS was filtered with a 75 μm microfilter and filtrate was placed in petri dishes. Embryos within the filtrate were identified and isolated with a stereomicroscope (1 to 7x). Once recovered, embryos were rated for quality: (1) excellent, (2) good, (3) fair, (4) poor, (5) unfertilized ova (figure 1), and (6) degenerate. Embryo characteristics used in assessing quality were: Compactness of cells, symmetry of embryo shape, uniformity of blastomere size, uniformity of cytoplasmic density throughout all blastomeres, and presence of extraneous materials (Shea, 1981; Elsden and Seidel, 1982).

Accessory Sperm Evaluation

Accessory sperm (figure 2) were prepared for counting and morphological examination according to the following procedures (Saacke et al., 1986). A coverslip with petroleum jelly spread around the edges and a 3 μl drop of Pronase placed in the center of the coverslip was prepared. Pronase is a combination of commercially prepared enzymes capable of digesting the zona pellucida. Once recovered, the embryo was washed in a 5 μl droplet of phosphate-buffered saline and then transferred to the pronase droplet. The concave side of a hanging drop slide was placed over the 3 μl pronase droplet. A phase contrast microscope was used to monitor the digestion of the zona pellucida by Pronase. Therefore, a
Figure 1. Unfertilized ova with no accessory sperm embedded in the zona pellucida.
Figure 2. Embryo with multiple accessory sperm embedded in the zona pellucida.
Figure 3. Unmarked (arrow) and marker bull spermatozoa embedded in the zona pellucida at 400x magnification.
Figure 4. Unmarked (arrow) and marker bull spermatozoa embedded in the zona pellucida at 1000x magnification.
hanging drop preparation permitted evaluation of the digestion of the intact zona pellucida with phase contrast microscopy. When the zona pellucida became visibly wavy or softened, the coverslip with the intact embryo was transferred from the hanging drop slide to a siliconized slide. The embryo was gently compressed between the siliconized slide and the coverslip. Morphological evaluation of marked and unmarked accessory sperm was possible under a differential interference contrast microscope at 400 (figure 3) and 1000 times (figure 4) magnification.

Statistical Analysis

General linear regression procedures were performed for data analysis with the use of Statistical Analysis System, 1985. Percentage of unmarked to total (unmarked + marker) accessory sperm and total accessory sperm data were analyzed for the model displayed in table 10 of the appendix. Orthogonal contrasts and Fisher’s protected LSD tests were used to separate means. The residual error term was used in the denominator for testing all dependent variables.
Experiment 2. Effect of Extenders on the Viability of Bovine Spermatozoa Encapsulated in Alginate Microgels.

The objective of this research was to determine if the solid microgel could maintain spermatozoal viability equivalent to an unencapsulated control over time. The rationale for testing the microgel over the microcapsule was to decrease processing time, eliminate the expense of an outer membrane, and most importantly microgels possess a “stickiness” property which may enhance sperm retention. Motile spermatozoa encased within the microgel matrix are not free to move and therefore early rupture of the acrosomal membranes could result. Without the semipermeable membrane, diffusion of substrates and waste products across the microgel matrix was also in question. Microgels were prepared with HEPES, PIPES, and saline inside the gel matrix to test the importance of the local environment on maintenance of sperm viability. Microgels were suspended in HEPES, PIPES, and saline extenders to evaluate any exchange of substrates or waste product exchange across the microgel matrix.

A split plot design with five treatments was replicated six times. Treatments were: semen encapsulated in HEPES alginate microgels and suspended in HEPES extender (HEPES), semen encapsulated in PIPES alginate microgels and suspended in a PIPES extender (PIPES), semen encapsulated in sodium alginate microgels and suspended in a HEPES extender (Saline-HEPES), and semen...
encapsulated in sodium alginate microgels and suspended in 0.9% physiological saline (Saline). Unencapsulated semen extended in a TRIS buffer served as a control.

Semen Preparation

First ejaculates from five bulls were collected with an artificial vagina. Using a calibrated spectrophotometer, spermatozoal concentration was determined for each ejaculate. A phase contrast microscope (100 and 400x) with stage heated to 37 C was used for estimation to the nearest 10% of percentage progressive sperm cell motility. Pooled ejaculates, 50 x 10⁶ cells/bull, were placed in a TRIS extender for a final concentration of 250 x 10⁶ cells/ml.

Microencapsulation

Microgels were formed by utilizing the first steps of Experiment 1 for the microencapsulation of bovine spermatozoa (Nebel et al., 1985). After extrusion, microgels were rinsed 3 times in saline, transferred to their respective buffer, and incubated at 37 C for 8 h.
Sperm Evaluation

At 0, 2, 4, and 8 h, 15 to 20 microgels were placed in 0.5 ml of TRIS buffer. Microgels were dissolved by the chelation of Ca$^{++}$ with citric acid, a component of the TRIS buffer. Approximately 15 min later, spermatozoa free in suspension were estimated for percentage of motile sperm and intact acrosomes. Sperm motility was estimated as previously described. Percentage of intact acrosomes based on the integrity of the apical ridge was determined under a differential interference contrast microscope at 1000x magnification (Saacke and Marshall, 1968). Two separate wet smear counts of 100 sperm cells each were averaged for intact acrosome evaluation. In order to reduce bias, each treatment was coded then decoded after all counting was completed.

Statistical Analysis

General linear model procedures were performed for analysis of data with the use of Statistical Analysis System 1985. Orthogonal contrasts and Fisher's protected LSD test were used to separate means. Residual error term was used in the model (table 11, appendix) to test all dependent variables and interactions except for treatment where the error term for treatment by trial interaction was used.
Experiment 3. Accessory Sperm Populations after Heterospermic Insemination of Spermatozoa Encapsulated in PIPES Microgels vs. Unencapsulated Spermatozoa.

In Experiment 2, no significant difference was observed between PIPES microgels and the unencapsulated control at 8 h incubation. Therefore, the objective of this research was to evaluate the ability of spermatozoa encapsulated in PIPES microgels to: (1) be retained in the female reproductive tract, (2) be released from PIPES microgels, (3) be transported to the oviduct, (4) undergo capacitation and the true acrosome reaction, (5) attach and penetrate the zona pellucida, and (6) ultimately fertilize an oocyte.

In a completely random design, a heterospermic study was conducted in which two morphologically distinct sperm types were artificially inseminated in accordance to three treatments: (1) Semen encapsulated in PIPES microgels from a fertile bull providing sperm cells predominantly of a normal morphology (unmarked bull), and unencapsulated semen from a fertile bull providing sperm cells predominantly of a tapered morphology (marker bull), (2) Marker bull semen encapsulated in PIPES microgels and unmarked bull semen unencapsulated, (3) Control, semen from both marker and unmarked bull unencapsulated.
Insemination

Equal numbers (20 x 10⁶ cells/bull) of frozen-thawed marker and unmarked bull spermatozoa were inseminated approximately 12 h after the onset of estrus. Ten cows were inseminated in each treatment. After thawing in a 35 C water bath for 45 s, spermatozoa were encapsulated in PIPES microgels according to their respective treatments as previously discussed in Experiment 2. Once completed, microgels containing their respective morphological sperm cell type were placed into a 12 ml syringe. The reciprocal morphological sperm cell type was thawed in a 35 C water bath for 45 s then placed into the same 12 ml syringe. A breeding pipette was manipulated through the cervix and placed approximately 1.75 cm into the uterine body. Syringe contents were then deposited.

Uterine flushes, embryo recovery, accessory sperm evaluation, and statistical analysis were performed as discussed in Experiment 1.

Experiment 4. Accessory Sperm Populations after Heterospermic Insemination of Spermatozoa Encapsulated in Protamine Sulfate Microcapsules and PIPES Microgels.

The rationale behind this experiment was to examine the competitive retention after simultaneous insemination of spermatozoa encapsulated both in protamine
sulfate microcapsules and PIPES microgels. In Experiments 1 and 3, microencapsulated sperm were capable of transport, capacitation, the true acrosome reaction, and attachment and penetration of the zona pellucida. However, it is still unknown if sperm microencapsulated in microcapsules or microgels are capable of fertilizing an oocyte. In Experiment 4, all sperm in the inseminates were microencapsulated. Therefore, not only will this study allow for evaluation of accessory sperm, but also will demonstrate the ability of microencapsulated sperm to fertilize an oocyte.

In a completely random design, a heterospermic study was conducted in which two morphologically distinct sperm types were artificially inseminated in accordance with three treatments: (1) Unmarked bull spermatozoa encapsulated in protamine sulfate microcapsules, and marker bull spermatozoa encapsulated in PIPES microgels, (2) Unmarked bull semen encapsulated in PIPES microgels and marker bull semen encapsulated in protamine sulfate microcapsules, (3) Control, semen from both marker and unmarked bull unencapsulated.

**Microencapsulation**

Protamine sulfate microcapsules and PIPES microgels were produced as discussed in Experiments 1 and 3, respectively.
Insemination

Equal numbers of $20 \times 10^6$ sperm/bull were microencapsulated and placed in a 12 ml syringe. A breeding pipette was manipulated through the cervix and placed approximately 1.75 cm into the uterine body. The syringe was connected to the breeding rod and sperm cells were deposited. The combined spermatozoa from each bull were artificially inseminated approximately 12 h after the onset of estrus.

Uterine flushes, embryo recovery, accessory sperm evaluation, and statistical analysis were performed as previously discussed in Exp. 1.
RESULTS AND DISCUSSION

Experiment 1. Accessory Sperm Populations after Heterospermic Insemination of Spermatozoa Encapsulated in Protamine Sulfate Microcapsules vs. Unencapsulated Spermatozoa.

Sources of variation for accessory sperm populations after heterospermic insemination of bovine spermatozoa encapsulated protamine sulfate microcapsules are listed in tables 12 and 13 of the appendix. Treatment least squares means for percent unmarked to total accessory sperm recovered from the zona pellucida were significantly different (table 1). After heterospermic insemination of equal numbers (100 x 10^6 cells/bull) of unencapsulated neat unmarked bull spermatozoa and unencapsulated frozen-thawed marker bull spermatozoa, the unmarked bull spermatozoa “outcompeted” the marker bull spermatozoa 65.2 ± 0.10 percent of the time. This differential may be attributed to higher quality semen produced by the unmarked bull in comparison to the marker bull. However, higher numbers of unmarked bull accessory sperm may be explained by the fact that the marker bull semen had been subjected to freeze-thawing which has been shown to decrease the number of sperm able to be transported to the site.
TABLE 1. Accessory sperm recovered after heterospermic insemination with equal numbers (100 X10^6) of spermatozoa encapsulated in protamine sulfate microcapsules.

<table>
<thead>
<tr>
<th>Inseminates</th>
<th>Morphological sperm types</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Marker (n)</td>
<td>Unmarked (n)</td>
<td>Mean^1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both unmarked and marker sperm unencapsulated (control)</td>
<td>100</td>
<td>193</td>
<td>.65 ± 0.10^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unmarked sperm encapsulated/Marker sperm unencapsulated</td>
<td>142</td>
<td>78</td>
<td>.28 ± 0.10^2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marker sperm encapsulated/Unmarked sperm unencapsulated</td>
<td>65</td>
<td>271</td>
<td>.80 ± 0.09</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^1Least squares means ± SEM for percent unmarked to total sperm recovered.

^2Unmarked sperm encapsulated decreased the proportion (P < 0.01) of accessory sperm when compared to the control.
of fertilization (Lineweaver et al., 1970). In Experiment 3 of this thesis, a similar heterospermic study was performed with semen from the same bulls in Experiment 1. In Experiment 3, both unmarked and marker bull spermatozoa were frozen. Inseminate doses were decreased to \(20 \times 10^6\) sperm/bull. Least squares means for percent unmarked to total accessory sperm for the unencapsulated control decreased to 51.7 ± 0.05. Therefore, unequal competition between bulls in Experiment 1 was probably attributed to the fact that the marker bull was subjected to freeze-thawing, and spermatozoa from the unmarked bull was probably not higher in quality when compared to the marker bull spermatozoa.

When compared to the unencapsulated control, insemination of unmarked bull spermatozoa encapsulated in protamine sulfate microcapsules and marker bull spermatozoa unencapsulated showed a decrease (\(p < 0.01\)) for percent unmarked to total bull accessory sperm (table 1). Protamine sulfate microcapsules probably interfered with either retention or transport of the unmarked bull spermatozoa. Additionally, the timing of insemination of the encapsulated treatment may have favored the unencapsulated sperm populations.

Insemination of marker bull spermatozoa in protamine sulfate microcapsules and unencapsulated unmarked bull spermatozoa did not significantly alter the percent of unmarked to total accessory sperm when compared to the unencapsulated control (table 1). Although, a 35% decrease in marker accessory sperm was observed when compared to the unencapsulated control.
Although, encapsulation of the marker bull spermatozoa decreased the marker accessory sperm recovered by 35% when compared to the unencapsulated control.

Protamine sulfate microcapsules may have been prone to early rupture which exposed spermatozoa to retrograde loss, phagocytosis, loss to the peritoneum, or a combination thereof. Nebel et al., 1987 reported similar results for polylysine microcapsules which were heterospermically inseminated and recovered from the uterus 12 h post insemination. Also, protamine sulfate microcapsules may not have been able to adhere to the uterine endometrium which would result in intact microcapsules containing sperm to be lost by retrograde flow. Early rupture of microcapsules would expose the spermatozoa to retrograde flow. No gross observation of purulent debris was apparent upon observation of uterine flushes 6 to 7 d post insemination. No intact microcapsules or microcapsule remnants were observed in flushes performed 6 to 7 d post insemination.

Timing of insemination may have been a factor resulting in decreased accessory sperm with respect to microencapsulated sperm. Insemination took place 12 h after the onset of estrus which is most beneficial to unencapsulated spermatozoa (Trimberger, 1943). Optimum timing for insemination of microencapsulated spermatozoa may be closer to the onset of estrus because of potential increased retention prior to release for transport to the oviducts. In this case, microencapsulated spermatozoa may reach maximum sperm reserves in the oviducts approximately 24 h post insemination. In the pig, motile sperm within

RESULTS AND DISCUSSION
intact polylysine microcapsules were recovered from the uterus of sows 12 h post insemination (Esbenshade et al., 1987). Therefore, timing of insemination for encapsulated spermatozoa should be earlier than conventional timing for unencapsulated sperm. Timing of insemination for encapsulated sperm would probably be optimum as soon as the animal is detected in estrus. A experiment should be performed which would evaluate accessory sperm populations after insemination of microencapsulated spermatozoa at the onset of estrus and 12 h after the onset of estrus.

Encouraging aspects of this research were, not all microencapsulated spermatozoa were lost from the female reproductive tract. For treatments where microencapsulated sperm were inseminated, microencapsulated spermatozoa contributed 25.7% (143 of 556) of the accessory sperm population (table 1). These spermatozoa survived the microencapsulation process, were released from the microcapsules, and were transported to the site of fertilization. By attaching and penetrating the zona pellucida, spermatozoa released from protamine sulfate microcapsules demonstrated the ability to capacitate and undergo a true acrosome reaction. Therefore, spermatozoa released from protamine sulfate microcapsules show the competence to potentially fertilize an oocyte.

Simultaneous insemination of encapsulated and unencapsulated spermatozoa did not interfere with the retention and transport of the unencapsulated sperm populations accessed by accessory sperm. Therefore, heterospermic studies can be
considered an invaluable method for determining sperm transport when spermatozoa are subjected to treatments such as microencapsulation.

Embryo quality was significant (p < 0.01) in the model for total accessory sperm recovered (table 13 of appendix). For the thirty one embryos collected, 100% were fertilized, 93.5% contained accessory sperm, and only two were recovered without accessory sperm (table 2). The average number of accessory sperm per embryo was 27.39 ± 39.06. Wilmut and Hunter 1983 reported that embryos contain abundant accessory sperm and unfertilized embryos contained few accessory sperm. Saacke et al., 1988 reported similar data with an average of 21.1 ± 30.5 accessory sperm per 6 to 7 d old embryo/ova after inseminate doses of either 100 x 10^6 or 200 x 10^6 cells. Additionally, inseminates used in their study contained high proportions of abnormal sperm which were postulated to lower accessory sperm numbers. Hawk and Tanabe 1986 reported an average 34 ± 6 accessory sperm per 3 d old embryo/ova with inseminate doses of 20 x 10^6 cells or greater.

A trend was observed between the homogeneity of blastomeres and the number of accessory sperm attached to the zona pellucida. Least squares means for embryo quality were significant when analyzed for total accessory sperm (table 3). Comparisons between least squares means revealed excellent rated embryos to have greater numbers of accessory sperm when compared to degenerate (p < 0.05), fair (p < 0.01), and good (p < 0.01) rated embryos. High numbers of accessory sperm in poorly graded embryos cannot be accounted for

RESULTS AND DISCUSSION
TABLE 2. Embryos recovered following AI of spermatozoa encapsulated in protamine sulfate microcapsules with respect to fertility and accessory sperm.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total embryos recovered</td>
<td>31</td>
</tr>
<tr>
<td>Embryos containing accessory sperm (AS)</td>
<td>29</td>
</tr>
<tr>
<td>% Fertilized</td>
<td>100</td>
</tr>
<tr>
<td>Total AS recovered</td>
<td>849</td>
</tr>
<tr>
<td>Total AS contributed by microcapsulated sperm</td>
<td>143</td>
</tr>
<tr>
<td>Total AS recovered from inseminations containing microcapsules</td>
<td>556</td>
</tr>
<tr>
<td>Average number of accessory sperm per embryo (mean ± std. dev.)</td>
<td>27.39 ± 39.06</td>
</tr>
</tbody>
</table>
TABLE 3. Least squares means for total accessory sperm recovered after heterospermic insemination of equal numbers of neat (100 X10^6) and frozen thawed (100 X10^6) semen encapsulated in protamine sulfate microcapsules.

<table>
<thead>
<tr>
<th>Embryo quality</th>
<th>Number</th>
<th>Accessory sperm</th>
<th>Accessory sperm/embryo(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degenerate</td>
<td>3</td>
<td>5</td>
<td>1.19 ± 34.02</td>
</tr>
<tr>
<td>Poor</td>
<td>2</td>
<td>152</td>
<td>72.46 ± 26.86</td>
</tr>
<tr>
<td>Fair</td>
<td>5</td>
<td>52</td>
<td>9.89 ± 16.02</td>
</tr>
<tr>
<td>Good</td>
<td>17</td>
<td>303</td>
<td>11.23 ± 9.73</td>
</tr>
<tr>
<td>Excellent</td>
<td>4</td>
<td>337</td>
<td>77.35 ± 18.65</td>
</tr>
</tbody>
</table>

\(^1\)Least squares means ± SEM for total accessory sperm recovered.
except for the fact that only two embryos were examined with a poor rating and large variations in accessory sperm numbers were obtained. Also, at the time of fertilization when accessory sperm attach to the zona pellucida an embryo could have been of excellent quality, but environmental or individual cow factors (stress) may have caused degradation of the zygote between the time of fertilization and the endometrial flush performed 6 to 7 d post insemination. Therefore, an embryo which would have been rated excellent immediately after fertilization would be rated poor 6 to 7 d post insemination due to extraneous conditions encountered by the cow. Overall, as the degree of extruded blastomeres increased and the homogeneity decreased, the numbers of accessory sperm decreased. Embryos with homogeneous blastomeres (excellent rating) had significantly greater numbers of accessory sperm except when compared to embryos with a poor rating. Embryo quality vs. accessory sperm numbers are consistent with the data presented by Saacke et al., 1988.

The distribution of accessory sperm/embryo is not normal but skewed. Approximately 48% of the ova contain 10 or less accessory sperm and less than 10% of the ova contain more than 100 accessory sperm (figure 5). Saacke et al., 1988 observed similar distributions of accessory sperm.
Figure 5. Distribution of embryos by numbers of accessory sperm per embryo after insemination with protamine sulfate microcapsules.
RESULTS AND DISCUSSION

% of Embryos

Number of Accessory Sperm

0-10  11-20  21-30  31-50  51-100  >100

(n=29)
Experiment 2. Effect of Extenders on the Viability of Bovine Spermatozoa Encapsulated in Alginate Microgels.

Sources of variation for maintenance of sperm viability with respect to percentage of intact acrosomes and motility are displayed in tables 14 and 15 of the appendix. Treatment differences (p < 0.05) were observed between buffered microgels, nonbuffered microgels and the unencapsulated control. Treatment least squares means for percent intact acrosomes and motility are listed in tables 4 and 5. Orthogonal contrasts among means for percent intact acrosomes and motility were significant between the unencapsulated control vs. all other microgel treatments. No significance was detected by Fisher’s protected LSD test between all microgel treatments (PIPES, HEPES, saline-HEPES, and HEPES). Therefore, the unencapsulated control supported a greater maintenance of sperm viability when compared to the spermatozoa encapsulated in alginate microgels. The TRIS extender may have increased the maintenance of sperm viability when compared to PIPES, HEPES, and saline. This decreased maintenance of sperm viability for spermatozoa encapsulated in alginate microgels is most likely a result of limited diffusion of metabolites across the matrix of the microgel.

Ionically bound calcium alginate microgels structurally have spermatozoa entrapped within a solid gelled microsphere. Spermatozoa have restricted movement within the microgel. During incubation, exchange of spermatozoal metabolic by-products through the microgel appear to be the major factor limit-
### TABLE 4. Effect of buffers within microgels and suspension of microgels within an extender on maintenance of intact acrosomes during incubation at 37 C for 8 h.

<table>
<thead>
<tr>
<th>Buffer within microgels</th>
<th>Extender</th>
<th>Hours of Incubation (37 C)</th>
<th>Mean²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>PIPES</td>
<td>PIPES</td>
<td>79.3</td>
<td>70.3</td>
</tr>
<tr>
<td>HEPES</td>
<td>HEPES</td>
<td>76.0</td>
<td>74.8</td>
</tr>
<tr>
<td>Saline</td>
<td>HEPES</td>
<td>78.2</td>
<td>78.3</td>
</tr>
<tr>
<td>Saline</td>
<td>Saline</td>
<td>79.8</td>
<td>73.7</td>
</tr>
<tr>
<td>Unencapsulated Control</td>
<td></td>
<td>85.9</td>
<td>81.9</td>
</tr>
</tbody>
</table>

¹Least squares means for percent intact acrosomes for six replicates, each replicate being a pool of five ejaculates (standard error = 3.41).

²Treatment least squares means for percent intact acrosomes (standard error = 1.71).
TABLE 5. Effect of buffers within microgels and suspension of microgels within extenders on the maintenance of percent motility during incubation at 37 C for 8 h.

<table>
<thead>
<tr>
<th>Buffer within microgel</th>
<th>Extender</th>
<th>Hours of Incubation (37 C)</th>
<th>Mean²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>PIPES</td>
<td>PIPES</td>
<td>65.0</td>
<td>58.3</td>
</tr>
<tr>
<td>HEPES</td>
<td>HEPES</td>
<td>65.0</td>
<td>56.7</td>
</tr>
<tr>
<td>Saline</td>
<td>HEPES</td>
<td>65.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Saline</td>
<td>Saline</td>
<td>65.0</td>
<td>58.3</td>
</tr>
<tr>
<td>Unencapsulated Control (TRIS extender)</td>
<td>70.0</td>
<td>70.0</td>
<td>66.6</td>
</tr>
</tbody>
</table>

¹Least squares means for percentage of motile sperm cells for six replicates, each replicate being a pool of five ejaculates (standard error = 3.11).

²Treatment least squares means for percent motile sperm (standard error = 1.55).
ing sperm viability. Therefore, the decrease in maintenance of sperm viability appears to be a result of the matrix or the calcium bound alginate microgels.

Nebel et al., 1985, was the first to successfully suspend viable bovine spermatozoa within a semipermeable polylysine membrane. During incubation, no detrimental affects to microencapsulated cells were encountered in comparison to unencapsulated controls. Free nutrient and waste product exchange across the semipermeable membrane allowed for maintenance of sperm viability within the microcapsule. In contrast, microgels may have limited capacity for nutrient and by-product exchange when compared to the semipermeable microcapsules.

The treatment by time interaction was significant. Comparisons of least squares means for percent intact acrosomes and motility for pooled semen encapsulated in microgels and incubated in their respective extenders over 8 h at 37 C are displayed in tables 4 and 5. At 0 h incubation, no differences between all treatments with respect to maintenance of percent intact acrosomes and motility was observed. The only exception was HEPES which decreased (p < 0.05) in maintenance of percent intact acrosomes when compared to the unencapsulated control. Initially, microencapsulation procedures have no detrimental affects with respect to the maintenance of sperm viability. Forces encountered during extrusion of the sperm/alginate suspension through the encapsulating jet did not significantly decrease the viability of spermatozoa in all other treatments when compared to unencapsulated controls. This data is similar with Nebel et al., 1985 who re-

RESULTS AND DISCUSSION

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ported no adverse affects to maintenance of sperm cell viability immediately after spermatozoa were subjected to the microencapsulation procedure.

At 2 and 4 h incubation, sperm cell viability among treatments began to separate. Maintenance of percent intact acrosomes and motility were not different among all treatments in which microgels were incubated, but differences between the unencapsulated control vs. encapsulated spermatozoa were encountered. Maintenance of percent motility for the spermatozoa encapsulated in all microgel treatments decreased (p < 0.01) when compared to the control. Maintenance of percent intact acrosomes decreased (p < 0.05) for spermatozoa encapsulated in PIPES at 0 and 4 h, and saline at 4 h when compared to the control. Up to 4 h incubation, no significant decrease with respect to maintenance of sperm viability was observed among treatments where spermatozoa were encapsulated in alginate microgels.

By 8 h incubation, treatment means were considerably separated. Unencapsulated spermatozoa showed significantly greater (p < 0.01) maintenance of percent intact acrosomes and motility when compared to spermatozoa encapsulated in saline microgels suspended in a HEPES extender, and saline microgels extended in saline. Unencapsulated spermatozoa were not different with respect to percent intact acrosomes and motility when compared to spermatozoa encapsulated in PIPES microgels suspended in a PIPES extender. Unencapsulated spermatozoa showed a greater maintenance of percent motility when compared to spermatozoa encapsulated in HEPES microgels suspended in
Figure 6. Effect of encapsulation on percentage of intact acrosomes of spermatozoa extended in TRIS (control), PIPES, HEPES, or saline at 37C.
Figure 7. Effect of encapsulation on percentage of motile sperm extended in TRIS (control), PIPES, HEPES, or saline at 37°C.
HEPES extender (p < 0.05), but no significant difference was observed for the HEPES treatment with respect to percentage of intact acrosomes (figures 6 and 7).

PIPES and/or HEPES microgels suspended within their respective extenders supported greater maintenance of sperm viability when compared to saline alginate microgels which contained no buffer. In this case, a decrease in pH within the saline microgel would be encountered due to acids produced from sperm metabolism. With buffers such as HEPES or PIPES suspended within the microgel, an environment more compatible with sperm viability can be maintained within the microgel. This suggests the importance of a buffer within the microgel to support greater maintenance of sperm viability.

Significant decreases were observed between PIPES and/or HEPES microgels suspended within their respective extenders vs. saline microgels suspended within a HEPES extender. Limited waste product exchange between the saline microgel and the HEPES extender will not neutralize an acid challenge as readily as microgels with buffers suspended within the inner microgel matrix.

Spermatozoa encapsulated in saline microgels and extended in saline exhibited lower (p < 0.01) sperm viability than spermatozoa encapsulated in saline microgels and extended in HEPES. This decrease in viability for saline microgels suspended in saline suggests some neutralization of metabolites between the HEPES extender and spermatozoa encapsulated in saline microgels. Limited
diffusion of metabolites through the microgels explains the ineffectiveness of a buffered media to maintain a constant pH within the microgel. The combination of a microgel prepared with a buffer within the alginate and suspended within a buffered extender appear to be the most stable environment to support sperm viability.

No significant difference was observed between the PIPES or HEPES treatments. PIPES microgels appeared to support greater maintenance of sperm viability when compared to the unencapsulated control. Additionally, Parks et al., 1981 reported that a PIPES buffer supported greater maintenance of sperm viability when compared to a TRIS and HEPES buffer. Therefore, PIPES microgels were tested for their potential retention within the female reproductive tract.

Another possible explanation for the decrease in viability for saline microgel treatments when compared to PIPES, HEPES, and the control would be a latent injury affect. That is, suspension of spermatozoa in saline microgels did not significantly decrease sperm viability at 0 h when compared to all other treatments. At 8 h, the rate of decline for sperm viability was significantly greater than PIPES, HEPES, and the control. Therefore, initial exposure of spermatozoa to encapsulation in saline microgel may have caused damage to the sperm which was not expressed until the end of the 8 h incubation.
Experiment 3. Accessory Sperm Populations after Heterospermic Insemination of Spermatozoa Encapsulated in PIPES Microgels vs. Unencapsulated Spermatozoa.

Sources of variation for accessory sperm populations after heterospermic insemination of bovine spermatozoa encapsulated in PIPES microgels are listed in table 16 of the appendix. Criteria for evaluation of 39 ova recovered was at least one accessory sperm from either bull must have been present in the zona pellucida. A total of thirty ova were evaluated.

Treatment least squares means for percent unmarked bull to total (unmarked + marker) accessory sperm were significantly different (table 6). Competition between equal numbers of frozen-thawed unencapsulated spermatozoa (control) from both bulls was almost equivalent. The ratio of unmarked bull spermatozoa was slightly higher than the marker bull spermatozoa. Least squares means for percent unmarked to total accessory sperm were 51.7%. Preinseminate frozen-thawed sperm counts of the heterospermic mixture were made with the use of a differential interference microscope. Means for percent unmarked to total were 51.12 ± 3.91 for the preinseminate counts. Unmarked and marker accessory sperm recovered from the zona pellucida were almost equal to the preinseminate ratio. In comparison to Experiment 1, least squares means for percent unmarked to total accessory sperm decreased from 65.2 ± 0.01 to 51.7 ± 0.05. This decrease in the ratio of accessory sperm can be attributed to freeze-thawing of spermatozoa from both bulls prior to insemination. Overall, the ratio of marked
TABLE 6. Accessory sperm recovered after heterospermic insemination with equal numbers (20 X10^6) of frozen-thawed spermatozoa encapsulated in PIPES microgels.

<table>
<thead>
<tr>
<th>Inseminates</th>
<th>Morphological sperm types</th>
<th>Mean¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Marker (n)</td>
<td>Unmarked (n)</td>
</tr>
<tr>
<td>Both unmarked and marker sperm unencapsulated (control)</td>
<td>147</td>
<td>238</td>
</tr>
<tr>
<td>Unmarked sperm encapsulated/Marker sperm unencapsulated</td>
<td>126</td>
<td>4</td>
</tr>
<tr>
<td>Marker sperm encapsulated/Unmarked sperm unencapsulated</td>
<td>12</td>
<td>249</td>
</tr>
</tbody>
</table>

¹Least squares means ± SEM for percent unmarked to total sperm recovered.

²Unmarked and marked sperm encapsulated decreased proportion (P < 0.01) of accessory sperm when compared to control.
and unmarked unencapsulated spermatozoa contained within the inseminate was recovered in the same proportion as accessory sperm.

Encapsulation of either unmarked or marker bull spermatozoa in PIPES microgels decreased (p < 0.01) the proportion of unmarked or marker bull spermatozoa able to gain access to the zona pellucida of an embryo. When compared to the unencapsulated control, insemination of unmarked or marker bull spermatozoa encapsulated in PIPES microgels (both treatments) decreased (p < 0.01) the number of accessory sperm. PIPES microgels interfered with the retention and/or transport of unmarked and marker bull spermatozoa. Many different phenomenon can account for a decrease in accessory sperm numbers after AI of spermatozoa encapsulated in PIPES microgels. In endometrial flushes performed 6 to 7 d post insemination, intact PIPES microgels were recovered in 25% of the flushes. The ability of the uterine endometrium to release chelating agents in high enough concentrations to dissolve the microgels may be a limiting factor. Therefore, release of spermatozoa from the microcapsule would be the primary cause for decreased sperm population in the oviducts. Polyvinylamine microcapsules increased uterine retention of spermatozoa, but intact microcapsules were observed in flush media 12 h post insemination. Degradation or rupture of these microcapsule contents was in question (Nebel et al., 1985). In contrast, polylysine microcapsules were thought to be prone to early rupture, retrograde removal, phagocytosis, or a combination thereof. Retrograde removal of intact PIPES microgels could have been another route of sperm loss. No evidence of purulent debris in the female reproductive was observed within the

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uterine flushes. Timing of insemination may have played a critical role in the decreased accessory sperm numbers after microencapsulation (discussed in Experiment 1).

Although an increase in accessory sperm was not observed after microencapsulation of spermatozoa within PIPES microgels, 16 accessory sperm (released from microcapsules) were observed within the zona pellucida of ova recovered (table 7). These spermatozoa survived the microencapsulation process, were released from PIPES microgels within the female reproductive tract, and were transported to the site of fertilization. By attaching and penetrating the zona pellucida of an embryo in the oviduct, spermatozoa released from microcapsules demonstrated the ability to capacitate and undergo a true acrosome reaction. Therefore, microencapsulated spermatozoa show the competence necessary to fertilize an ova.

Embryo quality accounted for a significant portion of variation for percent unmarked to total accessory sperm (table 16 of the appendix). Accessory sperm recovered was consistent with the findings of Wilmut and Hunter 1983. They reported abundant accessory sperm in fertilized ova but few in unfertilized ova. Average number of accessory sperm per fertilized ova was 24.1 (p < 0.01) and the average number of accessory sperm per unfertilized ova was 0.5 ± 0.84.

Although not significant, a trend was observed between the homogeneity of blastomeres, morula to blastocyst stage, and the number of accessory sperm at-
TABLE 7. Ova recovered following AI of spermatozoa encapsulated in PIPES microgels with respect to fertility and accessory sperm.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total embryo/ova recovered</td>
<td>39</td>
</tr>
<tr>
<td>Embryo/ova containing accessory sperm (AS)</td>
<td>30</td>
</tr>
<tr>
<td>% Fertilized</td>
<td>82</td>
</tr>
<tr>
<td>Unfertilized ova with no AS</td>
<td>4</td>
</tr>
<tr>
<td>Unfertilized ova with AS</td>
<td>2</td>
</tr>
<tr>
<td>Embryos with no AS</td>
<td>5</td>
</tr>
<tr>
<td>Embryos with AS</td>
<td>28</td>
</tr>
<tr>
<td>Total AS recovered</td>
<td>776</td>
</tr>
<tr>
<td>Total AS contributed by microcapsulated sperm</td>
<td>16</td>
</tr>
<tr>
<td>Total AS recovered from inseminations containing microcapsules</td>
<td>391</td>
</tr>
<tr>
<td>Average number of accessory sperm embryo/ova (mean ± std. dev.)</td>
<td>19.90 ± 51.96</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION
attached to the zona pellucida. As the number of accessory sperm increased, embryo quality also increased (table 8). Embryos graded poor to fair averaged 9.6 ± 6.9 sperm per embryo and embryos graded good to excellent averaged 19.95 ± 39.3 sperm per embryo. Correlations were most likely not significant due to high variations of accessory sperm recovered. Trends between accessory sperm and embryo quality are consistent with Saacke et al., 1988.

A skewed distribution of accessory sperm per embryo (figure 8) was consistent with Experiment 2 and the observations of Saacke et al. 1988. Overall 66.7% of the embryos contained 10 or less accessory sperm and 7.7% contained over 50 accessory sperm.

**Experiment 4. Accessory Sperm Populations after Heterospermic Insemination of Spermatozoa Encapsulated in PIPES Microgels and Protamine Sulfate Microcapsules.**

Heterospermic insemination of marker and unmarked bull spermatozoa encapsulated in PIPES microgels and protamine sulfate microcapsules significantly reduced accessory sperm numbers when compared to the unencapsulated control. When unmarked bull spermatozoa were encapsulated in PIPES microgels and marker bull spermatozoa were encapsulated in protamine sulfate microcapsules, 10 unmarked and 2 marker bull spermatozoa were recovered as accessory sperm. When unmarked bull spermatozoa were encapsulated in protamine sulfate microcapsules and marker bull spermatozoa were encapsulated in PIPES microgels, 2 unmarked and 7 marker bull spermatozoa were recovered.
TABLE 8. Least squares means for total accessory sperm recovered after heterospermic insemination of equal numbers of frozen-thawed (20 X10⁶) semen encapsulated in PIPES microgels.

<table>
<thead>
<tr>
<th>Embryo quality</th>
<th>Number</th>
<th>Accessory sperm</th>
<th>Accessory sperm/embryo¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>UFO</td>
<td>6</td>
<td>3</td>
<td>0.5 ± 0.84</td>
</tr>
<tr>
<td>Degenerate</td>
<td>5</td>
<td>287</td>
<td>57.4 ± 123.91</td>
</tr>
<tr>
<td>Poor/fair</td>
<td>7</td>
<td>67</td>
<td>9.6 ± 6.90</td>
</tr>
<tr>
<td>Good/excellent</td>
<td>22</td>
<td>443</td>
<td>19.95 ± 39.28</td>
</tr>
</tbody>
</table>

¹ mean ± standard deviation
Figure 8. Distribution of embryos by numbers of accessory sperm per embryo after insemination with PIPES microgels.
as accessory sperm. A total of 21 accessory sperm were recovered. Unencapsulated inseminates from Experiment 3 were used as controls with 385 accessory sperm being recovered. Therefore, insemination of both microcapsules significantly reduced the total spermatozoa recovered. Retrograde loss, early rupture, phagocytosis, peritoneal loss, timing of insemination, limited sperm release were all possible reasons for decreased accessory sperm numbers which were discussed in Experiments 1 and 3.

Fertilization rate (70%) was reduced when compared to Experiments 1 and 3. Experiment 1 had 100% and Experiment 2 had a 82% fertilization rate. With low numbers of accessory sperm harvested, no trend was noticed with respect to accessory sperm numbers and embryo quality. The number of unfertilized ova increased to 6. Insemination of frozen-thawed microencapsulated spermatozoa increased the numbers of unfertilized ova and decreased accessory sperm numbers. This decrease in accessory sperm numbers was consistent with the data presented in Experiments 1 and 3.

By fertilizing 70% of the oocytes, microencapsulated spermatozoa demonstrated the ability to be transported to the site of fertilization, undergo capacitation, attach and penetrate the zona pellucida, and fertilize an oocyte. Knowing microencapsulated spermatozoa have the capacity to fertilize an oocyte, new polymers should be researched and tested for potential resistance to retrograde flow.
CONCLUSIONS

One application of microencapsulation is to suspend living cells within a semi-permeable membrane or within a calcium alginate matrix (microgel). For the AI industry, microencapsulation has been tested as a potential solution to retrograde loss and phagocytosis of spermatozoa in the female reproductive tract. Although in this thesis accessory sperm populations decreased after insemination of microencapsulated spermatozoa when compared to unencapsulated spermatozoa. Bovine spermatozoa encapsulated in protamine sulfate microcapsules and PIPES microgels demonstrated the ability to be transported to the site of fertilization, undergo capacitation, attach and penetrate the zona pellucida, and fertilize an oocyte. Many factors such as timing of insemination, early microcapsule rupture, retrograde loss, etc. may explain the decrease in accessory sperm populations reported in this thesis. A possible new method for testing microcapsule retention of spermatozoa in the female reproductive tract would be to inseminate microencapsulated spermatozoa and examine accessory sperm populations in superovulated cows. With ovulation taking place over an extended period of time, and microcapsules theoretically releasing spermatozoa over an extended
period of time, microcapsules may increase sperm reserves in the oviduct when compared to unencapsulated sperm. Another area of future research would be to examine new polyamine membranes for potential increased sperm retention. The timing of insemination appears to be a critical factor. Therefore, insemination of microencapsulated sperm at the onset of estrus would be another valid experiment to evaluate the influence on accessory sperm.

Currently in the embryo transfer industry, embryos are recovered with cracked or no zona pellucida. After micromanipulation of embryos, the zona pellucida can be damaged or in some cases destroyed. Therefore, development of a procedure that would encase an embryo in a artificial recipient zona pellucida would be another application of microencapsulation. Embryos encapsulated in a semi-permeable membrane may have the potential to increase the retention and protection of the embryo during transfer. Microencapsulation has potential for increased protection of embryos during cryopreservation. In the future, this technology, microencapsulation, could be an integral part of the AI and embryo transfer industries. Heterospermic insemination of two morphologically distinguishable sperm types has been shown in this thesis as an invaluable method for testing microcapsule retention. Significance was achieved in each heterospermic experiment where 30 eggs were examined. Insemination of only one sperm type would probably require a much larger amount of embryos for significance at the same level.
REFERENCES


Hoard’s Dairyman, Herd Health. p. 36 on 33rd Annual Hoard’s Dairyman


Table 9. Chemicals used in the microencapsulation procedure.

**CaCl₂-HEPES**
1.5% CaCl₂ Dihydrate
50 mM HEPES (N-2-Hydroxymethylpiperazine-N'-2-Ethanesulfonic Acid)
pH adjusted to 6.8

**CHES Buffer**
2% CHES (2[N-Cyclohexylamino]Ethane-Sulfonic Acid)
0.6% NaCl
pH Adjusted to 8.2/ Osmolarity adjusted to 290-300 mOsm
Dilute above solution 1:20 with sterile physiological saline (0.9%)

**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

**PIPES Buffer**
3.0% PIPES [Piperazine-N,N'-bis (2-ethanesulfonic acid)]
pH adjusted to 7.0
Osmolarity adjusted to 290-300 mOsm

**HEPES Buffer**
2.4% HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)
pH adjusted to 7.0
Osmolarity adjusted to 290-300 mOsm
TABLE 10. Linear regression model for accessory sperm populations after heterospermic insemination of spermatozoa encapsulated in protamine sulfate microcapsules and PIPES microgels.

Model

\[ Y_{ijkl} = \mu + \alpha_i + \beta_j + \Gamma_k + e_{ijkl} \]

- \( \mu \) = overall mean
- \( \alpha_i \) = fixed effect of the \( i \)th treatment
- \( \beta_j \) = fixed effect of the \( j \)th embryo quality
- \( \Gamma_k \) = fixed effect of the \( k \)th stage of embryo development
- \( e_{ijkl} \) = random residual
## APPENDIX

**TABLE 11.** Linear regression model for maintenance of percent intact acrosomes and motility after encapsulation of spermatozoa in alginate microgels.

<table>
<thead>
<tr>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Y_{ijkl} = \mu + \alpha_i + f_{ik} + \beta_j + \Gamma_k + \alpha\beta_{ij} + \beta\Gamma_{ik} + \alpha\Gamma_{ik} + e_{ijkl}$</td>
</tr>
</tbody>
</table>

- $\mu$ = overall mean
- $\alpha_i$ = fixed effect of the $i^{th}$ treatment
- $f_{ik}$ = error term ($\alpha\Gamma_{ik}$) used in the denominator for treatment
- $\beta_j$ = fixed effect of the $j^{th}$ time
- $\Gamma_k$ = fixed effect of the $k^{th}$ trial
- $\alpha\beta_{ij}$ = fixed effect of the $ij^{th}$ treatment by time
- $\beta\Gamma_{jk}$ = fixed effect of the $jk^{th}$ time by trial
- $\alpha\Gamma_{ik}$ = fixed effect of the $ik^{th}$ treatment by trial
- $e_{ijkl}$ = random residual
<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>0.4499&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Embryo quality</td>
<td>4</td>
<td>0.0315</td>
</tr>
<tr>
<td>Embryo development stage</td>
<td>1</td>
<td>0.0034</td>
</tr>
<tr>
<td>Residual</td>
<td>21</td>
<td>0.0442</td>
</tr>
</tbody>
</table>

<sup>1</sup>Significant p < 0.01
### TABLE 13. Linear regression analysis for total accessory sperm recovered after heterospermic insemination of spermatozoa encapsulated in protamine sulfate microcapsules.

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>372.6653</td>
</tr>
<tr>
<td>Embryo quality</td>
<td>4</td>
<td>4759.9384¹</td>
</tr>
<tr>
<td>Embryo development stage</td>
<td>1</td>
<td>3003.8410</td>
</tr>
<tr>
<td>Residual</td>
<td>21</td>
<td>1034.1489</td>
</tr>
</tbody>
</table>

¹Significant p < 0.01
**TABLE 14.** Linear regression analysis for maintenance of percent intact acrosomes of spermatozoa encapsulated in alginate microgels.

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>4</td>
<td>5590.9562$^2$</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>2031.4278$^1$</td>
</tr>
<tr>
<td>Replicate</td>
<td>5</td>
<td>763.1750$^1$</td>
</tr>
<tr>
<td>Treatment*Time</td>
<td>12</td>
<td>173.0909$^1$</td>
</tr>
<tr>
<td>Treatment*Replicate</td>
<td>20</td>
<td>140.3375$^2$</td>
</tr>
<tr>
<td>Time*Replicate</td>
<td>15</td>
<td>146.8494$^2$</td>
</tr>
</tbody>
</table>

$^1$Significant $p < 0.01$$^2$Significant $p < 0.05$
TABLE 15. Linear regression analysis for maintenance of percent motility of spermatozoa encapsulated in alginate microgels.

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>4</td>
<td>921.6666(^1)</td>
</tr>
<tr>
<td>Time</td>
<td>3</td>
<td>4100.8333(^1)</td>
</tr>
<tr>
<td>Replicate</td>
<td>5</td>
<td>238.8333(^1)</td>
</tr>
<tr>
<td>Treatment*Time</td>
<td>12</td>
<td>188.3333(^1)</td>
</tr>
<tr>
<td>Treatment*Replicate</td>
<td>20</td>
<td>134.6666(^1)</td>
</tr>
<tr>
<td>Time*Replicate</td>
<td>15</td>
<td>105.5000(^1)</td>
</tr>
</tbody>
</table>

\(^1\)Significant p < 0.01
**TABLE 16.** Linear regression analysis for percent normal to total accessory sperm populations after heterospermic insemination of spermatozoa encapsulated in PIPES microgels.

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2</td>
<td>1.6442&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Embryo quality</td>
<td>4</td>
<td>0.0226&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Embryo development stage</td>
<td>1</td>
<td>0.0161</td>
</tr>
<tr>
<td>Residual</td>
<td>20</td>
<td>0.0054</td>
</tr>
</tbody>
</table>

<sup>1</sup>Significant p < 0.01
<sup>2</sup>Significant p < 0.05
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The two page vita has been removed from the scanned document. Page 2 of 2