THE EFFECT OF POLY-L-LYSINE CONCENTRATION, MOLECULAR WEIGHT, AND ENCAPSULATION TEMPERATURE ON MICROENCAPSULATED BOVINE SPERMATOZOA/

by

Stanley Wakefield Fultz

Thesis 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

APPROVED:

R.G. Saacke, Chairman

R.E. Pearson

R.M. Akers

T.W. Keenan

M. White, Department Head

December, 1984
Blacksburg, Virginia

THE EFFECT OF POLY-L-LYSINE CONCENTRATION, MOLECULAR WEIGHT, AND ENCAPSULATION TEMPERATURE ON MICROENCAPSULATED BOVINE SPERMATOZOA

by

Stanley W. Fultz

(ABSTRACT)

A series of in vitro studies were conducted to evaluate the effect of poly-l-lysine concentration, molecular weight, and encapsulation temperature on the post encapsulation survivability of spermatozoa. Viability of spermatozoa encapsulated at 20±2 C using four poly-l-lysine concentrations (.05%, .15%, .25%, and .35%) did not differ over the 8 h incubation period. However, the viability of each of the four treatments was lower than that of the unencapsulated control ($p<.05$ and $p<.01$; percentage motility and percentage intact acrosomes, respectively), indicating spermatozoal damage occurred during the encapsulation process. Capsule wall thickness and integrity for the .15%, .25%, and .35% concentrations were greater ($p<.01$) than that of the .05% capsules.

Encapsulation at two temperatures (5 C vs 20 C) using two polylysine concentrations (.05% vs .25%) was performed to study the effect of thermally decreased metabolism during the encapsulation procedure on the post encapsulation spermatozoal survivability. Unencapsulated controls for each temperature demonstrated superiority ($p<.01$) of the 20 C temperature for maintenance of acrosomal integrity, indicating spermatozoal damage had occurred during neat semen cool-down. Despite this damage, the 5 C encapsulated sperm demonstrated superiority ($p<.01$) to that of the 20 C encapsulated sperm for percentage intact acrosomes.

Encapsulation at 5 C using two polylysine concentrations (.05% vs .25%) and three polylysine molecular weights (14,000; 55,000; and 90,000 daltons) was conducted to study the effect of any interaction between concentration and molecular weight. For motility, encapsulated treatments did not differ ($p>.05$). Also, percentage intact acrosomes were unaffected except for the 14,000/.25% combination maintaining viability better ($p<.01$) than the 55,000/.05% combination after 6 h incubation and equal to the 5 C unencapsulated control after incubation for 4 hours. Also, the 14,000/.25% combination was the only treatment to maintain intact capsules following agitation.

ACKNOWLEDGEMENTS

Foremost, I would like to thank my advisor, Dr. Richard G. Saacke. I am grateful for his assistance in my research, for the experience of learning from him, for the knowledge he has imparted to me in the field of reproductive physiology, and for the way he and his wife gave me continual support and friendship.

A special thanks is offered to Dr. Ray Nebel for introducing this project to me and for his advice during my research.

My thanks are also extended to Drs. R.E. Pearson, J.M. White, R.M. Akers, and T.W. Keenan, distinguished members of my graduate committee, for their time, effort, and guidance.

Thanks are also extended to Select Sires Inc., Plain City, Ohio, for their financial support of this project.

A special thanks is offered to

for their technical assistance and friendship.

Finally, I especially wish to thank my wife, , for her love, understanding, prayers, and continual encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter I

INTRODUCTION

The female bovine displays behavioral estrus for 13 to 17 h every 17 to 24 days. Ten to 12 h following the end of behavioral estrus, she releases one or two ova from the ovary into the oviduct, the site of fertilization. The fertile lives of the released ova and that of spermatozoa within the female reproductive tract have been estimated at 20 to 24 h and 30 to 48 h, respectively. This relatively brief fertile life of both the ova and the sperm renders the time of mating and breeding a matter of utmost importance. This is especially true in artificial insemination where man must recognize the signs of behavioral estrus (Hawk and Bellows, 1980; McLaren, 1980).

At the time of breeding, either naturally or artificially, millions of spermatozoa are deposited into the female's reproductive tract. However, relatively few sperm actually reach the site of fertilization (Austin, 1949; Braden, 1953; El-Banna and Hafez, 1970). Using nulliparous heifers, Dobrowolski and Hafez (1970) were able to recover only 24 X 10^3, 200 X 10^3, and 15 X 10^3 sperm from the oviduct 1, 8, and 24 h respectively, post insemination of 2 X 10^9 sperm at the external cervical os. Likewise, El-Banna

and Hafez (1970) reported the recovery of only 17,025 and 4,230 sperm per oviduct after 16 and 40 h respectively, post insemination of semen artificially deposited at the external cervical os.

Following insemination, the two major causes of sperm loss are: voiding by passage from the oviducts into the peritoneal cavity (Mattner, 1963) and phagocytosis due to an intensive leucocytic invasion (Howe and Black, 1963; Bedford, 1965; Mattner, 1968; Dobrowolski and Hafez, 1970; El-Banna and Hafez, 1970).

Salisbury et al., (1978) have reviewed evidence that leucocytic removal of sperm is the major factor responsible for sperm removal, especially in uterine-inseminated cattle. Therefore, decreased phagocytosis would mean an increased number of sperm remaining in the female reproductive tract to contribute to the fertilization process. Nebel (1983) proposed microencapsulation as a method for decreasing phagocytosis, and thereby prolonging sperm retention in the female reproductive tract, thus leading to improved conception rate in artificially bred cattle.

Using the microencapsulation procedure developed by Lim and Sun (1980), with slight modifications to accomodate spermatozoa, Nebel (1983) successfully encapsulated viable bovine spermatozoa. However, he found that when encapsulat-

ed (polylysine membrane) sperm were inseminated into the uterine body of the cow, they were preferentially removed compared to unencapsulated sperm. He postulated that this might have been due to an early rupture and/or retrograde removal of the capsule. A subsequent study using polyvinyl membrane encapsulated sperm resulted in a significant increase in uterine retention of encapsulated sperm following intrauterine insemination. These polyvinyl microcapsules exhibited a more rigid capsule membrane as well as a sticky surface that may have contributed to the capsule retention within the female reproductive tract. However, the use of polyvinyl as a membrane material is not practical since this compound is nonbiodegradable. It would appear that a successful microcapsule for sperm would protect sperm from phagocytosis and/or retrograde removal, and also be biodegradable to permit safe usage in food-producing animals. Microcapsule membranes must ultimately vary in thickness and/or rigidity, from those tough enough to insure the maintainance of intact capsules within the female for prolonged time periods, to those thin enough to break within close proximity to the time of insemination. Finally, the capsule wall must be permeable enough to permit sperm survival. For these reasons, the following objectives were undertaken.

1. To determine the effect of poly-l-lysine concentration used in capsular membrane formation on membrane thickness and subsequent ability of encapsulated sperm to survive in vitro.

2. To compare the effect of microencapsulation at 5 C and 20 C to determine if thermally decreased metabolic activity of spermatozoa during the encapsulation process is beneficial to spermatozoal survival following microencapsulation.

3. To determine the effect of poly-l-lysine molecular weight used in capsular membrane formation on capsule integrity and subsequent ability of encapsulated sperm to survive in vitro.

Chapter II

LITERATURE REVIEW

Microencapsulation has been defined as a process whereby small, discrete, solid particles, liquid droplets, gases, or living cells are completely enveloped by an intact membrane. Microcapsules range in size from .2μm to several millimeters and may have impermeable or semipermeable membranes of variable thickness depending upon the requirements and planned function of the enclosed material (Kondo, 1979).

The capsule membrane may be constructed to allow release of the encapsulated material under prescribed conditions that control or prolong the action of the capsular contents. The release of the capsule contents may be made dependent upon moisture, pH, temperature, physical pressure, or combinations of the above. The mechanism(s) of release of the enclosed material may be associated with leaching, erosion, rupture, or other similar actions, depending upon the capsule membrane composition (Nebel, 1983).

The industrial microencapsulation procedure first disclosed in the patent literature (Green and Shneidcher, 1957) utilized microcapsules possessing impermeable membranes in the development of a printing system now identified as "carbonless copying paper". Due to the impermeable membrane,

the encapsulated material was only effective when the wall of the microcapsule was ruptured, thus releasing the enclosed material. Due to the lack of membrane permeability in these capsules, they were not useful for the confinement of biologically active materials that require a continual transfer of nutrients and/or products across the membrane to provide the desired action of the microcapsule. To achieve such a transfer of materials both into and out of the microcapsule, the membrane composition had to be altered.

In a series of publications, Chang and co-workers (Chang, 1964; Chang and MacIntosh, 1964; Chang et al., 1966; Chang and Poznansky, 1968a) reported the development of semipermeable membrane enclosed microcapsules. Chang called these semipermeable microcapsules "artificial cells" due to the similar physical properties between naturally occurring cells and the semipermeable microcapsules developed in their laboratory (Chang, 1964). The semipermeable membrane enclosed microcapsules developed by Chang and co-workers differed from the impermeable membrane-bound microcapsules of earlier industrial use in three important areas. First, the materials encapsulated were aqueous solutions or suspensions of biologically active compounds, enzymes, or intact cells. Secondly, the encapsulated materials were not dependent upon membrane rupture for the desired action. Lastly, the selec-

tive permeability of the semipermeable membrane allowed for passage of small molecular weight solutes, but restricted the passage of macromolecules or cellular organelles.

Using the above semipermeable membrane, Chang (1964) encapsulated red blood cell contents. The enclosed hemoglobin and carbonic anhydrase would thus be prevented from entering the extracellular environment and becoming involved in an immunological reaction, or being excreted or metabolized. Since that introduction, much research has been done in the biomedical field using semipermeable membrane bound microcapsules. More recently, biologically important materials successfully encapsulated within semipermeable membranes have included: activated carbon granules (Chang, 1966), ion-exchange resins (Chang, 1966; Sparks et al., 1969), enzymes (Chang and Poznansky, 1968b; Chang, 1977; Lim and Moss, 1979), proteins (Chang, 1977), and antibodies (Lim and Sharp, 1978).

The microencapsulation technology developed by Chang and co-workers (Chang, 1964; Chang and MacIntosh, 1964; Chang et al., 1966; Chang and Poznansky, 1968a) involved one or two conditions too harsh for successful encapsulation of living cells: (a) contact of the aqueous phase, where cells would be located, with at least one organic solvent phase; and (b) exposure of cells to relatively high temperatures

during the encapsulation process. Obviously, both conditions must be avoided for cells to remain viable during and after the microencapsulation process.

Lim and Sun (1980) described a simple all-aqueous phase system for successful microencapsulation of viable pancreatic islet cells. The microcapsular membrane developed was composed of cross-linked alginate, a nontoxic polysaccharide, which was permeable to molecules such as glucose and insulin but impermeable to larger molecules such as immunoglobulins or albumin. The encapsulation technique avoided the use of both organic solvents and heat. They reported that a single intraperitoneal transplantation of microencapsulated pancreatic islets into streptozotocin-induced diabetic rats neutralized the diabetic state for 3 weeks. Microencapsulated islets remained morphologically and functionally intact in vitro at 37 C for as long as 4 months.

Using this system, developed by Lim and Sun (1980), Lim and Moss (1981) reported the successful encapsulation of hepatoma cells. They found that, in vitro, microencapsulated hepatoma cells grew and multiplied at approximately the same rate as nonencapsulated controls (doubling time of approximately 24 h). The overcrowding hepatoma cells stretched the capsule membrane prior to finally bursting the capsule mem-

brane at day 10 of culture. In vitro perfusion studies with 10 microencapsulated pancreatic islets and 10 nonencapsulated islets demonstrated that the amount of insulin released per islet per minute following stimulation by a combination of high glucose (5 mg/ml) and theophylline (10mM) were comparable between the two groups. Pancreatic islets used were 3 to 6 days old at the time of encapsulation (Lim and Moss, 1981).

Using the microencapsulation technology described by Lim and Sun (1980), Jarvis and co-workers (Jarvis and Spriggs, 1981; Jarvis et al., 1982) demonstrated that the process does not result in loss of cell viability for Friend erythroleukemic cells (Jarvis and Spriggs, 1981) or a hybridoma cell line (Jarvis et al., 1982) and was applicable for encapsulation of both suspension and monolayer cell types (Jarvis and Spriggs, 1981; Jarvis et al., 1982). In addition, Jarvis and co-workers (Jarvis and Spriggs, 1981; Jarvis et al., 1982) reported that the microcapsule wall could be dissolved without damage to the metabolic competence of the encapsulated cells.

With slight modifications, Nebel et al. (1984) reported the successful microencapsulation of viable bovine spermatozoa using the procedure of Lim and Sun (1980) and Lim and Moss (1981). Modifications to the encapsulation procedure

involved the following: (1) an increase of the sodium alginate level from .8% to 1.4%; (2) an increase in the $CaCl_2$ concentration from 1.2% to 1.5% in the "hardening bath"; (3) an increase in poly-l-lysine concentration from .02% to .04% and exposure time from 3 min to 5 min; and (4) removal of anhydrous $CaCl_2$ from the CHES-buffer by substituting .9% physiological saline.

After inclusion of the above changes in the encapsulation procedure, Nebel et al. (1984) conducted in vitro studies using two capsule sizes (.75mm and 1.5 mm) and three sperm concentrations (45×10^6, 90×10^6, and 180×10^6 sperm/ml). They demonstrated that encapsulated treatments did not differ from each other or from unencapsulated controls ($p>.05$) except at the 180×10^6 sperm/ml concentration in which the unencapsulated control exhibited significantly lower motility after 12 h and both lower motility and lower intact acrosomal integrity after 24 h of incubation ($p<.05$). The decreased viability of the unencapsulated control was undoubtedly due to the accumulation of lactic acid or availability of nutrients at this concentration. Permeability of the capsule membrane at 180×10^6 sperm/ml was apparently adequate to permit exchange of important materials between the capsule and culture medium.

Nebel (1983) also conducted a study of the in vivo retention of encapsulated sperm in the uterus following artificial insemination. In this study, two types of microcapsules were studied, biodegradable (polylysine membrane) and non-biodegradable (polyvinyl membrane). The two capsule types were used in conjunction with a heterospermic insemination using semen of two bulls, where the spermatozoa of one bull was morphologicaly distinguishable from that of another bull. The encapsulation of sperm in non-biodegradable (polyvinyl) microcapsules resulted in a significant increase in encapsulated sperm obtained from uterine flushings 12 h post insemination over that of unencapsulated sperm. In contrast, biodegradable (polylysine) microcapsules gave erratic results when evaluated by competitive retention with the overall result being more rapid disappearance of encapsulated sperm. This implied to Nebel (1983) that the polylysine microcapsules were affected by retrograde movement, early rupture, or other factors not affecting polyvinyl microcapsules.

Nebel (1983) suggested two major differences between the capsule types explaining the results of the study: (1) polyvinyl microcapsules had a "stickiness" property, and therefore, may have resisted retrograde removal resulting in physical retention of the capsules within the uterus and (2)

polyvinyl microcapsules had a much thicker capsule wall as compared to the polylysine microcapsules (R.L. Nebel, personal communication), thereby inhibiting early rupture.

Although sperm encapsulated in polyvinyl microcapsules were preferentially retained in the uterus 12 h post insemination over unencapsulated sperm, the capsules tend not to rupture, and therefore, would not provide a population of sperm capable of fertilization. Also, future research to modify the polyvinyl capsule membrane is not practical since this compound is nonbiodegradable, and therefore, not safe for use in food producing animals. Therefore, to obtain successful capsule retention in the uterus, it would appear most fruitful to modify the polylysine membrane to achieve the capsule membrane characteristics desired for the utilization of microencapsulated spermatozoa in artificial insemination.

Chapter III

MATERIALS AND METHODS

EXPERIMENT 1: EFFECT OF POLY-L-LYSINE CONCENTRATION ON VIABILITY OF MICROENCAPSULATED SPERMATOZOA

This experiment was designed to determine the effect of poly-l-lysine concentration used in capsule formation on the thickness of the capsule membrane, integrity of the capsule, and survival of encapsulated spermatozoa.

A randomized complete block design consisting of four treatments (.05%, .15%, .25%, and .35% poly-l-lysine hydrobromide) was employed utilizing a split ejaculate technique. Experiment 1 was replicated six times. The four levels of poly-l-lysine chosen for study were based upon pilot experiments which indicated that concentrations greater than that used by Nebel et al. (1984) could decrease capsule fragility. However, the effect of the increased polylysine concentration on the spermatozoal viability required study.

Semen was collected with an artificial vagina and for each replicate, first ejaculates from each of three bulls were pooled. Semen was allowed to gradually cool to room temperature (20±2 C). Spermatozoal concentration was determined using a previously calibrated spectrophotometer (Baush and Lomb Spectronic 20, Baush and Lomb, Inc., Rochester,

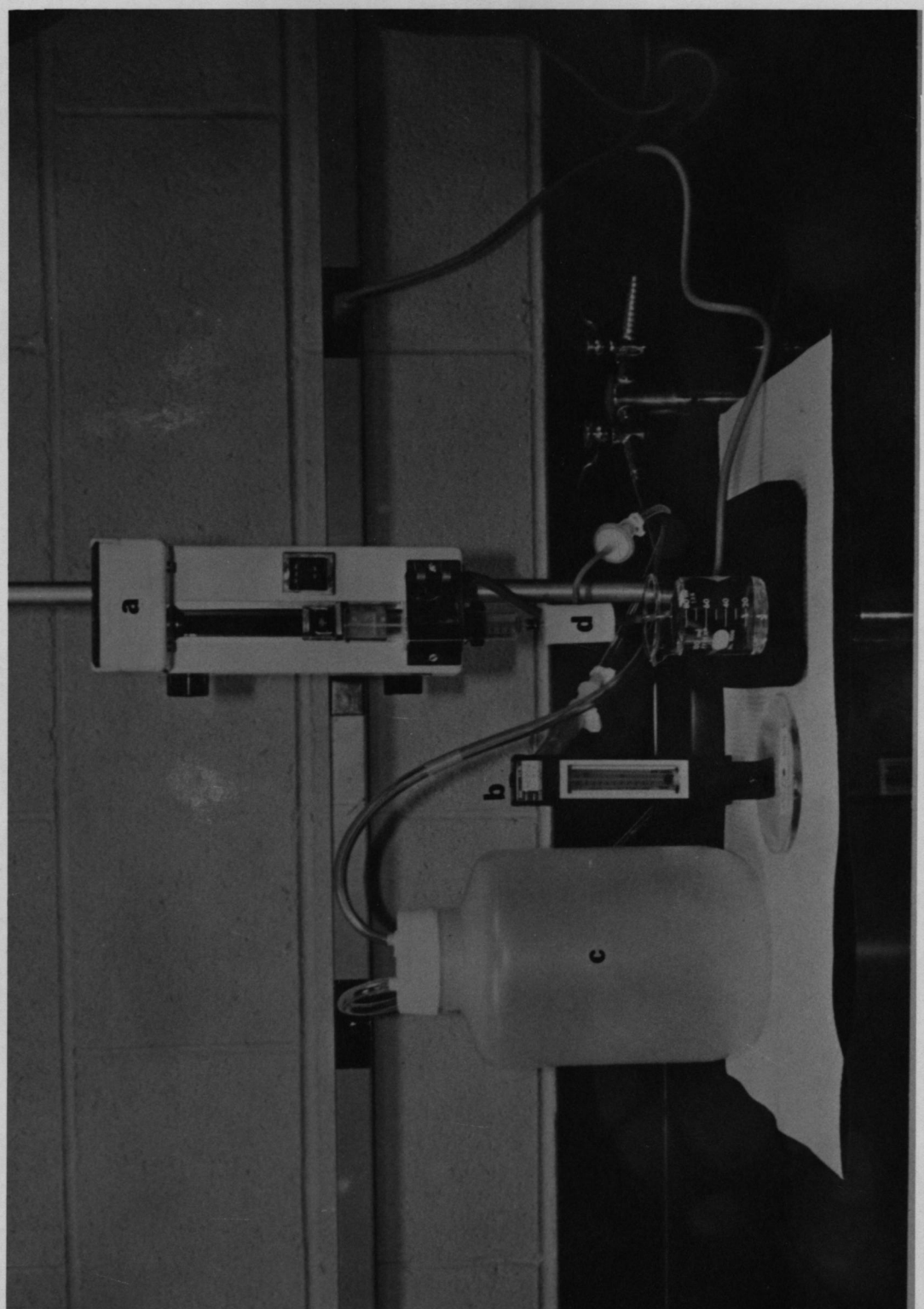
NY). An aliquot of the semen was extended to 45 X 10^6 sperm/ml in 2.9% sodium citrate dihydrate containing 20% egg yolk (v/v), as the unencapsulated control. Prior to use, all egg yolk-citrate (EYC) was clarified by centrifugation at 8000 X g for 30 minutes. Two wet-mount smears prepared from the control aliquot were used to determine initial percentage progressive motility and percentage intact acrosomes. Percentage progressive motility was subjectively estimated, to the nearest 10%, using a phase contrast microscope (100X) equipped with a warm stage (37 C) and percentage of intact acrosomes was objectively determined by differential counts of 200 cells using differential interference contrast optics (1250X) (Saacke and White 1972). Semen used had a minimum of 1 X 10^9 sperm/ml, 60% progressive motility, and 80% intact acrosomes.

Semen for encapsulation was extended to 45 X 10^6 sperm/ml in physiological saline containing 1.4% sodium alginate (w/v), pH 6.8 (75% Kelco Gel LV, Kelco, Division of Merck and Co. Inc., Rahway, NJ; 25% Protanol LF 60, Mult-Kem Corp., Ridgefield, NJ).

Microcapsules were produced according to the procedure of Lim and Sun (1980) as modified by Nebel (1983) with the exception of the syringe and hypodermic needle sizes used (see figure 1 for instrumentation). A 30-ml syringe was

used instead of the 10-ml syringe (Nebel, 1983) permitting production of sufficient numbers of capsules for all treatments, thereby eliminating the necessity of changing syringes and cleaning the jet head within a single experiment. A new improved jet head (figure 2), permitting more precise centering of the needle (F. Lim, personal communication) was substituted for the older type utilized by Nebel (1983). In addition, a 22-gauge needle was used instead of a 19-gauge (Nebel, 1983), because it was found to produce capsules more uniform in size using a lower air flow rate. Droplets were produced from the semen/alginate suspension using the syringe pump extrusion techniques adapted from Ennis and James (1950), as modified by Sparks et al., (1969). Using a Model A syringe pump (Razel Scientific Instruments, Inc., Stamford, CT), the solution to be encapsulated was forced through the 22-gauge hypodermic needle contained within a jet head attached to the 30-ml syringe, as shown in figure 1, at a rate of approximately 1.3 ml per minute. Simultaneously, air at constant pressure, was passed through the air inlet of the jet (figure 2) to achieve a capsule size of approximately 1.1 mm in diameter. The air pressure was controlled by a flowmeter (Airco, E.I. DuPont deNemours and Co., Inc., Wilmington, DE), to maintain constant droplet size.

Figure 1. Instrumentation used in microencapsulation procedure: (a) Razel® model A syringe pump, (b) Airco® flowmeter (c) Air reservoir tank, connected to (d) Encapsulation jet which is attached to 30-ml disposable syringe containing cell suspension to be encapsulated.


a
b
c
d

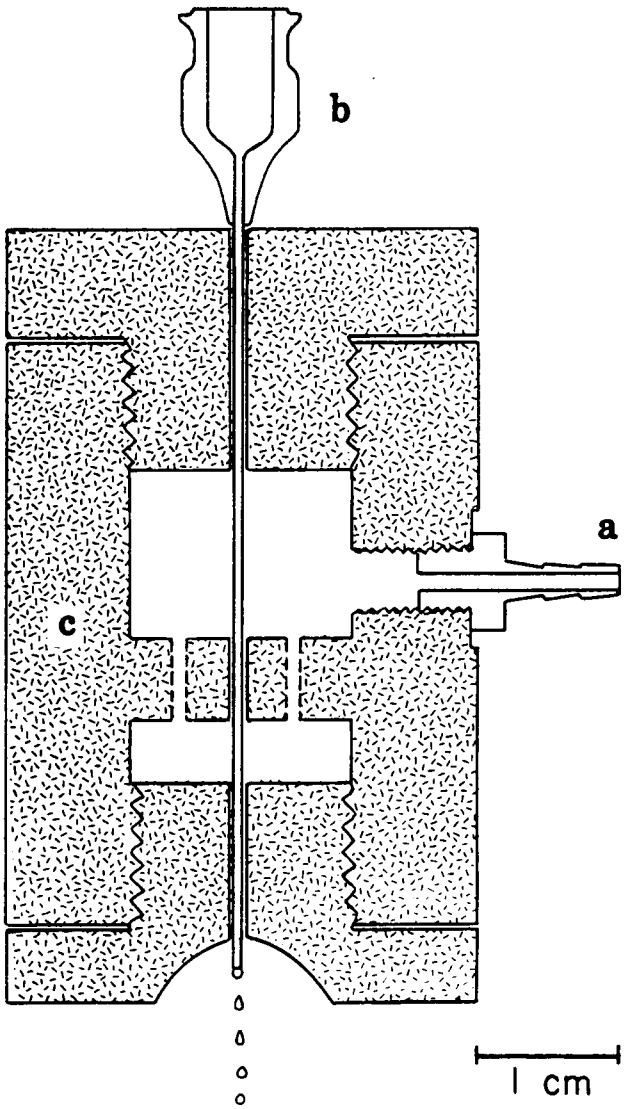


Figure 2. Two-dimensional drawing of self-centering encapsulating jet; (a) Air inlet nozzle, (b) 22 gauge stainless steel needle, (c) Nylon polymer casing.

Extruded droplets were collected in a beaker containing an aqueous solution (80 ml) of 1.5% $CaCl_2$-Hepes buffer, pH 6.8 (table 8, appendix). The surface of this solution was 4.3 cm from the end of the jet head producing the droplets. After the droplets settled in the beaker, the $CaCl_2$ solution was aspirated leaving the high viscosity molds of the future microcapsules. These molds were washed three times with physiological saline. A semipermeable membrane was deposited on the surface of the molds by suspending them in physiological saline containing either .05%, .15%, .25%, or .35% (w/v) poly-l-lysine hydrobromide (Sigma Chemical Co., St. Louis, MO) for 5 minutes. The polylysine used in Exp. 1 had an approximate molecular weight of 55,000 daltons (based on viscosity determination by Sigma Chemical Co.), range 30,000 to 70,000 daltons. The excess polylysine was aspirated and the capsules rinsed with CHES-buffer, pH 8.2 (table 8, appendix) to terminate "cross-linking" of the polylysine and alginate. The capsules were then rinsed three times with physiological saline and suspended in an isotonic sodium citrate-saline solution, pH 7 (table 8, appendix) for 5 min to liquify the alginate gel inside the microcapsules. Excess sodium citrate-saline solution was aspirated and EYC, prepared as previously described, was added as the incubation medium. Sixty-five-ml aliquots of each treatment were

placed into 65-ml capacity disposable tissue culture flasks (Falcon flasks, Div. Becton, Dickinson and Co., Oxnard, CA). The volume of the flask displaced by microcapsules was approximately 10 ml. All four treatments and control were incubated for 8 h in EYC at 37 C. Prior to removing capsules for evaluation, the culture flasks were gently inverted several times to thoroughly mix capsules. At 2-h intervals, approximately 30 capsules were removed from each treatment with the use of a 2-ml disposable pipet (eye dropper type). The capsules were placed into a concave cavity of a serological PYREX plate maintained at 37 C. Excess EYC was aspirated off and the capsules were gently crushed by direct vertical pressure with a glass rod. After gentle mixing, three wet-mount smears were prepared from the effluent and two were evaluated for percentage motility and percentage intact acrosomes as previously described. The third smear was used if a discrepancy of more than 10% occurred for either motility or intact acrosome evaluations.

Microcapsule fragility was determined for each of the four capsule treatments at 24 h post encapsulation. Prior to removing capsules for this test, the culture flasks were inverted several times to thoroughly mix the capsules. From each flask (treatment), 2 ml of capsules/EYC solution were drawn into a 2 ml pipet. From this sample, three replicates

were produced, each replicate represented by a .4 ml-aliquot placed into a separate 15 X 85 mm test tube. This allowed approximately 80 to 100 capsules per replicate. To each replicate, .6 ml of EYC was added as an extender. This decreased capsule concentration within the test tube to allow greater ease in counting intact capsules. To determine the number of intact capsules present in each test tube, the tube was covered with Parafilm "M" laboratory film (American Can Company, Dixie/Marathon, Greenwich, CT) and placed horizontally on a Darkfield Quebec Colony Counter (American Optical, Scientific Instrument Division, Buffalo, NY). The number of intact capsules present in each tube was determined by direct counts. Following this initial count, individual tubes were agitated for 1 min on a Vortex Genie Mixer (Distributed by Scientific Products, Division of American Hospital Supply Corporation, McGaw Park, IL) at speed setting 5. Speed setting 5 and agitation time of 1 min were used since pilot experiments showed that this speed and time was sufficient to rupture a few thicker-membraned capsules (.35% poly-l-lysine), and nearly all thinner-membraned capsules (.05% poly-l-lysine). Therefore, if variation in the degree of capsule wall thickness or strength existed among intermediate treatments, variation could be expected in capsules remaining intact after agitation. After vortexing,

the tube was placed on the colony counter and the number of intact capsules remaining was determined by direct counts. The percentage intact capsules remaining after agitation was determined using an average of the three subsamples of each treatment and expressed as a percentage of those intact before agitation.

Membrane thickness was measured from photomicrographs of sectioned capsules. From each treatment approximately 1 ml of capsules was fixed and washed according to Karnovsky (1965). Capsules were dehydrated in a series of alcohol washes followed by embedding in 50% propylene oxide:50% Epon-Araldite. The embedded samples were sectioned (.5µm) using an ultramicrotome and stained for one min with Azure II (Jeon, 1965). The stained sections were allowed to dry on a hot plate maintained at 60 C. These sections were examined and photographed using bright-field microscopy. The photographs were enlarged 736X and membrane thickness measured at the thinnest point on each photograph to eliminate the possibility of over estimating thickness due to oblique sections. Ten measurements were taken for each treatment.

To avoid biased evaluations, treatments were coded prior to encapsulation and decoded after evaluation.

EXPERIMENT 2: EFFECT OF ENCAPSULATION TEMPERATURE ON THE VIABILITY OF MICROENCAPSULATED SPERMATOZOA

To date, all microencapsulation of bovine spermatozoa has been carried out at room temperature (approximately 20 C). During the encapsulation procedure, the spermatozoa are suspended in a gelled state for approximately 45 minutes. During this time, the metabolic activity of the cells would be expected to continue, and metabolic byproducts such as lactic acid would increase, potentially resulting in a lowered pH. This increased acidity may be a cause of spermatozoal injury resulting from the microencapsulation procedure. To obtain optimum spermatozoal survival throughout the encapsulation procedure, it is important to identify where damage to spermatozoa is occurring. For the above reasons, a probable cause may be the time the spermatozoa are suspended in the gelled state. Therefore, if the metabolic activity of the cells was decreased during the encapsulation procedure, one might expect an increase in cell survival. This experiment was designed to test the effect of lowering sperm metabolism thermally during encapsulation on their survivability post encapsulation at 37 C.

A randomized complete block design with treatments in a 2 X 2 factorial arrangement (two encapsulation temperatures, 5 C and 20 C, and two poly-l-lysine concentrations, .05% and .25%) utilizing a split ejaculate technique, was employed to

study the interaction of encapsulation temperature and poly-lysine concentration on viability of encapsulated spermtozoa. The two concentrations utilized in this experiment were selected for their representation of thin and thick capsule membrane formation based on results from Exp. 1. Room temperature in this experiment was 20±2 C and cold room temperature was 5±1 C. Hereafter, room temperature and cold room temperature will be reported as 20 C and 5 C, respectively. Experiment 2 was replicated six times. Unencapsulated samples extended to 45 X 10^6 sperm/ml in EYC were prepared from semen at both temperatures to serve as controls.

Semen for this experiment was collected and initial evaluation performed as previously described (Exp. 1). Following the mixing of the neat semen with 10 ml of EYC as the 20 C control, neat semen was added to 10 ml of sodium alginate for encapsulation at 20 C. The remaining aliquot of semen (approximately 5 ml) was placed into a 250 ml beaker containing 200 ml of 20 C water, and cooled to 5 C in a walk-in cold room (5 C). Cooling rate was approximately .3 C per min for the first 45 min and entire cooling to 5 C required 65 min.

While the cold-room semen was cooling, the 20 C semen/alginate solution was encapsulated at room temperature. Two 5-ml aliquots of the semen/alginate mixture were extruded

with the high viscosity molds of the microcapsules being collected in the beaker of $CaCl_2$-Hepes buffer as described in Exp. 1. Following the saline rinses, one aliquot was used to form the .05% polylysine treatment while the other aliquot was used to form the .25% polylysine treatment. Remaining steps of encapsulation were as described in Exp. 1. Immediately following encapsulation, the samples were evaluated for percentage progressive motility and percentage intact acrosomes as described in Exp. 1. The two treatments and the 20 C control were then incubated at 37 C for 8 h post encapsulation with samples evaluated at 2-h intervals as previously described in Exp. 1.

After the room temperature semen had been encapsulated and the first post encapsulation evaluation completed (approximately 1.5 h from the time encapsulation was initiated at room temperature), the semen placed in the cold room was encapsulated. Again, an unencapsulated control was prepared by extending the cooled neat semen to 45 X 10^6 sperm/ml in 10 ml of precooled EYC. For encapsulation, cooled neat semen was added to 10 ml of precooled sodium alginate for encapsulation at 5 C. The microcapsules were formed, encapsulated, and liquified as previously described (Exp. 1). However, these steps all occurred at 5 C. Again, two 5-ml aliquots were extruded from the semen/alginate mixture. One

aliquot was encapsulated with .05% polylysine and the other with .25% polylysine. Following the liquification by sodium citrate, the capsules were removed from the cold room and EYC (20 C) was added to rapidly bring the temperature of the microcapsules to 20 C. A sample was then removed and evaluated for percentage progressive motility and percentage intact acrosomes as previously described. Again the treatments were incubated at 37 C for 8 h post encapsulation with samples evaluated at 2-h intervals. The first 5 C evaluation was approximately 2 h after the first 20 C evaluation.

To avoid biased evaluations, treatments were coded prior to encapsulation and decoded after evaluation. However, due to timing, 20 C and 5 C treatments were known.

EXPERIMENT 3: EFFECT OF POLY-L-LYSINE MOLECULAR WEIGHT ON THE VIABILITY OF MICROENCAPSULATED SPERMATOZOA

To date, all microencapsulation of bovine spermatozoa using poly-l-lysine as the membrane material has been carried out with approximate molecular weights of 40,000 and 55,000 daltons poly-l-lysine (range 30,000 to 70,000 daltons). The effect higher and/or lower molecular weight poly-l-lysine may have on the capsule membrane characteristics or the process of encapsulating spermatozoa is not known. Therefore, this experiment was designed to test the effect of different poly-l-lysine molecular weights employed

during the membrane formation phase of encapsulation on post encapsulation spermatozoal survivability when incubated at 37 C.

A randomized complete block design with treatments in a 2 X 3 factorial arrangement (two poly-l-lysine concentrations and three poly-l-lysine molecular weights) utilizing a split ejaculate technique, was employed to study the interaction of poly-l-lysine concentrations and molecular weights during encapsulation at 5 C. Encapsulation was conducted at 5 C to maximize post encapsulation survival as found in Exp. 2. Two poly-l-lysine concentrations (.05% and .25%) were employed to allow representation of the two membrane thicknesses capable of being formed as found in Exp. 1. Three poly-l-lysine molecular weights were represented in this experiment. Fifty-five thousand daltons (range 30,000 to 70,000 daltons) served as a control since this has been the conventional molecular weight employed. Fourteen thousand daltons (range 4,000 to 15,000 daltons) represented a lower molecular weight, and 90,000 daltons (range 70,000 to 150,000 daltons) (based on viscosity determination by Sigma Chemical Co.) represented a higher molecular weight. Experiment 3 was replicated six times. Unencapsulated aliquots of semen extended prior to cooling and after cooling served as 20 C and 5 C controls, respectively.

Semen for this experiment was collected and initial evaluation performed as described in Exp. 1. Immediately following concentration determination of the pooled ejaculate, a 20 C control was prepared by extending neat semen to 45 X 10^6 sperm/ml in 10 ml EYC. The remaining neat semen (approximately 5 ml) was then placed into a 600 ml beaker containing 400 ml of 20 C water and placed into a walk-in cold room to allow gradual cooling to 5 C (cooling rate of approximately .17 C per min). Cooling rate was decreased relative to that used in Exp. 2 in an attempt to decrease sperm damage caused by too rapid a cool-down. After the semen reached 5 C (approximately 90 min following placement into the cold room) a control was prepared by extending neat semen to 45 X 10^6 sperm/ml in 10 ml precooled EYC. For encapsulation, neat semen was extended to 45 X 10^6 sperm/ml in 40 ml precooled sodium alginate. Six 5-ml aliquots were extruded from the semen/alginate mixture and each aliquot of capsules was randomly used in one of the six polylysine concentration/molecular weight combinations. Following liquification, excess sodium citrate was aspirated and EYC was added as the incubation medium. Each treatment was placed into a disposable tissue culture flask as described in Exp. 1 and then removed from the cold room and placed into an incubator maintained at 37 C. This allowed gradual warming of

the treatment to 37 C as opposed to the rapid warming performed in Exp. 2 in an effort to decrease spermatozoal damage caused by rapid temperature changes. All six treatments and the two controls were incubated for 8 h post encapsulation in EYC at 37 C and post encapsulation evaluations were performed at 2-h intervals as described in Exp. 1.

Encapsulated treatments were evaluated for capsule fragility as described in Exp. 1.

To avoid biased evaluations, treatments were coded prior to encapsulation and decoded after evaluation.

STATISTICAL METHODS

Data in all experiments were analyzed by analysis of variance (Barr et al., 1979) and least square means were tested using Tukey's studentized range test for pairwise comparisons (Gill, 1978). Pooled ejaculates were considered replicates and were treated as a random variable. Based on the expected mean squares, the appropriate mean squares for testing significance of main effects were the pooled ejaculates x main effect interactions. Residual mean squares were used for testing significance of all three-way interactions. Experiment 2 main effects and main effect two-way interactions for intact acrosomes were tested using Satterthwaite approximations (Gill, 1978) as the expected mean squares.

Chapter IV

RESULTS AND DISCUSSION

EXPERIMENT 1: EFFECT OF POLY-L-LYSINE CONCENTRATION ON THE VIABILITY OF MICROENCAPSULATED SPERMATOZOA

Percentage motility and percentage intact acrosomes for sperm encapsulated in four poly-l-lysine concentrations are presented in tables 1 and 2, respectively (see tables 9 and 10, appendix, for analysis of variance for motility and intact acrosomes, respectively). For all four concentrations, viability of encapsulated sperm did not differ over the 8 h incubation period. However, the viability of each of the four encapsulated treatments was lower than that for the unencapsulated control ($p<.05$ and $p<.01$; percentage motility and percentage intact acrosomes, respectively). This suggests that the microencapsulation procedure had a detrimental effect on the maintenance of sperm viability. This is contradictory to the findings by Nebel et al. (1984). They found that the viability of incubated sperm encapsulated at 45 and 90 x 10^6 sperm/ml in .75 and 1.5 mm capsule sizes did not differ from unencapsulated sperm of the same concentration. An apparent difference in sampling technique could account for a major portion of this discrepancy. The technique used by this author harvested sperm to be evaluated by

TABLE 1. EFFECT OF POLY-L-LYSINE CONCENTRATION ON MAINTENANCE OF MOTILITY DURING INCUBATION AT 37 C

Poly-l-lysine concentration (%, w/v)	Hours of incubation (37 C)				
	0	2	4	6	8
	Percent motility[a]				
Unencapsulated control	63.3[c]	61.7[c]	58.3[c]	50.0[c]	45.0[c]
.05	25.0[b]	18.3[b]	15.0[b]	3.0[b]	[illegible].0[b]
.15	26.7[b]	18.3[b]	11.7[b]	.0[b]	.0[b]
.25	25.0[b]	16.7[b]	11.7[b]	.0[b]	.0[b]
.35	25.0[b]	18.3[b]	11.7[b]	.0[b]	.0[b]

[a]Mean percent motility for six replicates, each replicate being a pool of three ejaculates (SE=±1.5)

[bc]Means in same column with unlike superscripts are different ($p<.05$)

TABLE 2. EFFECT OF POLY-L-LYSINE CONCENTRATION ON MAINTENANCE OF INTACT ACROSOMES DURING INCUBATION AT 37 C

Poly-l-lysine concentration (%, w/v)	Hours of incubation (37 C)				
	0	2	4	6	8
	Percent intact acrosomes[a]				
Unencapsulated control	90.2[c]	86.4[c]	88.3[c]	86.7[c]	82.2[c]
.05	43.7[b]	40.1[b]	40.2[b]	35.5[b]	41.7[b]
.15	50.4[b]	39.1[b]	42.5[b]	41.7[b]	37.6[b]
.25	42.3[b]	38.2[b]	40.3[b]	39.3[b]	34.3[b]
.35	43.3[b]	40.5[b]	39.7[b]	33.9[b]	29.8[b]

[a]Mean percent intact acrosomes for six replicates, each replicate being a pool of three ejaculates (SE=±2.3)

[bc]Means in same column with unlike superscripts are different ($p<.01$)

forceful rupturing of capsules, and thorough mixing of the sample prior to preparation of the wet-mount used for microscopic evaluation. In contrast, the technique used by Nebel involved gentle rupture of capsules, and evaluation of the effluent without mixing. In the capsule membrane, many sperm are entrapped. These sperm are most likely dead or injured and, therefore, upon forceful rupture and thorough mixing, as done in this experiment, many of these dead or injured sperm were included in the sperm population sampled. The method of Nebel et al. (1984) would not have included these injured or dead cells. It is the opinion of this author that the method of evaluation employed in this experiment provided a more accurate account of the spermatozoal loss associated with the encapsulation process. Also, since future work in microencapsulation of spermatozoa should be directed toward improving viability of all spermatozoa post encapsulation, it is important to establish a method of evaluation which permits an accurate representation of spermatozoal damage associated with the entire encapsulation process. On the other hand, it is also important to distinguish between the spermatozoal injury and loss associated with capsule membrane formation vs the total sperm injury and spermatozoal loss due to the encapsulation procedure per se. From the work of Nebel et al. (1984) it was very possi-

ble that sperm in the central region of the capsule escaped injury, and therefore, represented a normal population and that a certain portion of the spermatozoa will have to be sacrificed in the encapsulation process.

Results for the effect of polylysine concentration on capsule diameter, capsule wall integrity, and capsule wall thickness are presented in table 3 (see table 11, appendix, for analysis of variance). Polylysine concentration of .05% yielded thinner-walled capsules ($p<.01$) than did the higher concentrations tested. This suggests that maximum "cross-linking" of the polylysine and alginate takes place with polylysine concentrations greater than .05%, but less than or equal to .15%. Representative micrographs illustrating the capsule wall thickness obtained for each treatment are shown in figure 3. The thinner walls of the .05% membranes subsequently resulted in greater ($p<.01$) capsule wall fragility after vortexing (table 3). Also, the .05% membrane was associated with larger ($p<.01$) diameter capsules (table 3).

The fact that viability of encapsulated sperm for the four treatments tested did not differ ($p>.05$) along with the fact that increased concentration of polylysine of .15% or greater results in thicker, more rigid capsules, demonstrates that capsule integrity can be enhanced by use of higher concentrations of polylysine without detrimental

TABLE 3. EFFECT OF POLY-L-LYSINE CONCENTRATION ON CAPSULE DIAMETER, CAPSULE WALL INTEGRITY, AND CAPSULE WALL THICKNESS

Poly-l-lysine concentration (%, w/v)	Capsule diameter[a] (mm)	Capsule wall integrity[b] (%)	Capsule wall thickness[c] (μm)
.05	1.16±.02[e]	.1± .1[d]	1.92±.21[d]
.15	1.03±.02[d]	75.0±10.6[e]	5.32±.54[e]
.25	1.04±.02[d]	79.0± 4.3[e]	5.55±.41[e]
.35	1.03±.02[d]	81.3± 7.7[e]	5.09±.38[e]

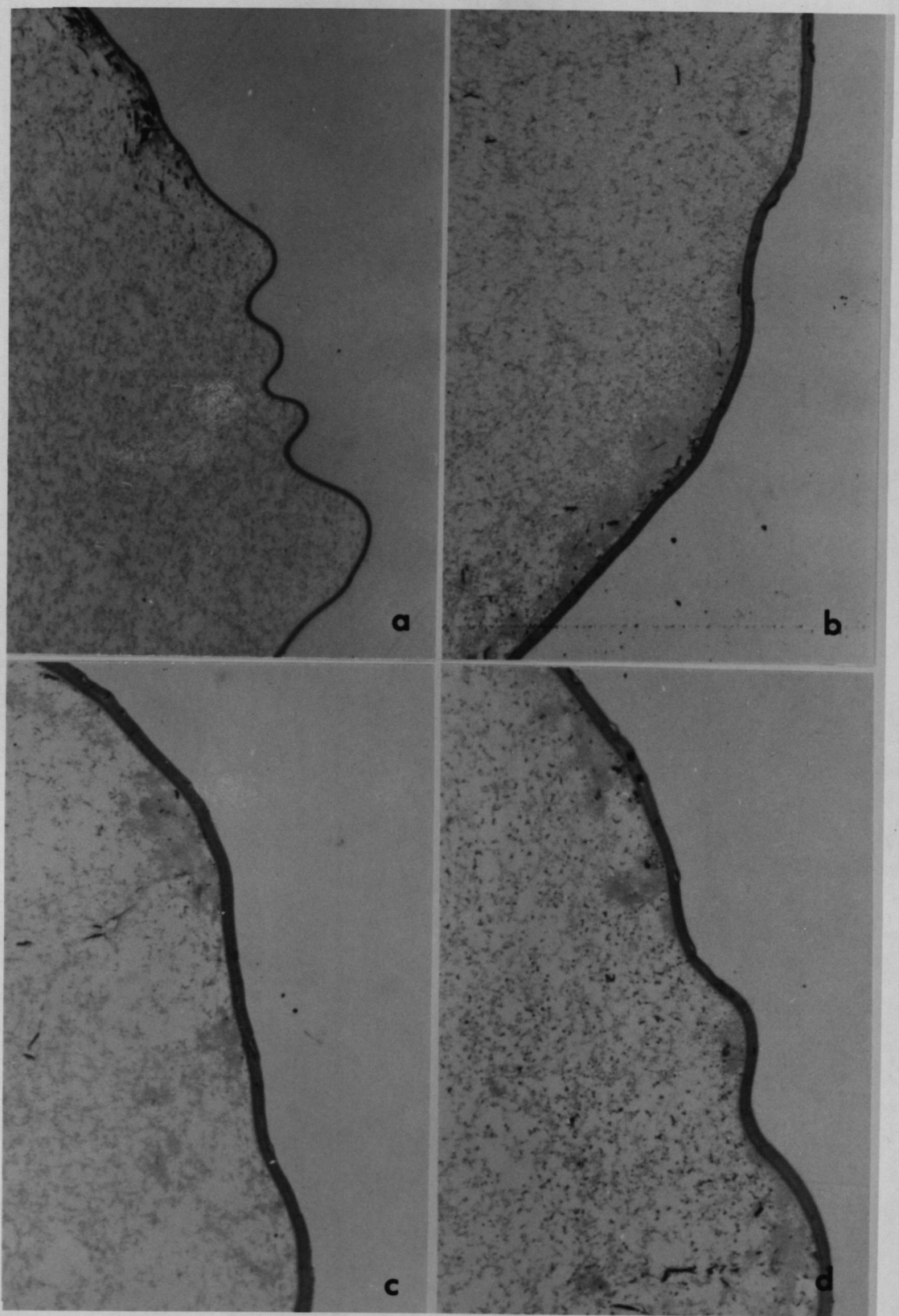
[a]Mean capsule diameter ±SD (mm) for six replicates. For each replicate, ten capsules were selected and measured using a stereoscope (7X)

[b]Mean percent ±SD capsules that survive vortexing treatment (six replicates). Each value represents a percent intact capsules calculated from a differential count of sampled capsules prior to and following vortexing treatment

[c]Mean capsule wall thickness±SD(μm) for six replicates as determined by measurement from micrographs (736X) taken of sectioned microcapsules

[de]Means in same column with unlike superscripts are different ($p<.01$)

Figure 3. Representative bright-field micrographs illustrating capsule wall thickness obtained for each poly-l-lysine concentration in Experiment 1; (a) .05%, (b) .15%, (c) .25%, and (d) .35%. (400X)


a
b
c
d

effects on the viability of the encapsulated sperm. The increase in capsule integrity appears to be related to capsule wall thickness. Capsule wall thickness, however, was concentration dependent, but in this experiment only two capsule wall thicknesses were achieved. Therefore, more work is necessary using polylysine concentrations between .05% and .15% to establish the relationship between capsule thickness and integrity.

EXPERIMENT 2: EFFECT OF ENCAPSULATION TEMPERATURE ON THE VIABILITY OF MICROENCAPSULATED SPERMATOZOA

From Exp. 1, we learned that microcapsule wall thickness could be increased to an apparent maximum (approximately 5.5 μm) without detrimental effects on sperm viability. However, sperm loss due to encapsulation per se was greater than anticipated. Therefore, in Exp. 2, we studied the effect of encapsulation temperature (5 C vs 20 C) for two polylysine concentrations (.05% vs .25%) to allow representation of both the thin and thick capsule walls.

Percentage motility and percentage intact acrosomes for unencapsulated controls and sperm encapsulated using two polylysine concentrations at two temperatures are presented in tables 4 and 5, respectively. It is clear from the controls that cooling neat semen to 5 C was injurious. Although no significance was detected for motility between the two con-

trols, when observed over time, there was a trend toward superiority of the 20 C over that of the 5 C. However, the mean percentage motility for all observations demonstrates superiority ($p<.01$) of the 20 C encapsulated control over that of the 5 C unencapsulated control (table 4). Percentage intact acrosomes for the 5 C and 20 C controls were not different at the initial observation ($p>.05$). However, by 2 h of incubation, the 5 C control began to lose viability rapidly as compared to the 20 C control (table 5). This indicated damage had occurred to the sperm cells during the cool-down period. This damage could be the result of a too rapid rate of cooling (.5 h to reach 5 C) or due to lactic acid build-up in the neat semen during prolonged cooling, thus resulting in increased acidity. Recall from the experimental procedures that the 5 C semen required the sperm cells to remain at high concentrations (approximately 1.2×10^9 sperm/ml) in the seminal plasma of the neat semen for 1.5 h while the 20 C semen was diluted to 45×10^6 sperm/ml in EYC.

The effect due to temperature on the maintenance of motility of encapsulated spermatozoa was opposite of that of the control. From table 4, we see that the 5 C encapsulation temperature was superior ($p<.01$) to that of the 20 C unencapsulated control for the mean percentage motility of

TABLE 4. EFFECT OF POLY-L-LYSINE CONCENTRATION AND ENCAPSULATION TEMPERATURE ON MAINTENANCE OF MOTILITY DURING INCUBATION AT 37 C

Poly-l-lysine concentration (%, w/v)	Temperature[a] (C)	Hours of incubation (37 C)					
		0	2	4	6	8	$\bar{X}$
		Percent motility[b]					
Unencapsulated control	5	48.3	41.7	35.0	28.3	11.7	33.0[e]
	20	63.3	63.3	58.3	51.7	46.7	56.7[f]
.05	5	26.7	21.7	18.3	10.0	.0	15.3[cd]
	20	25.0	21.7	10.0	1.7	.0	11.7[c]
.25	5	36.7	26.7	16.7	10.0	.0	18.0[d]
	20	26.7	18.3	11.7	1.7	.0	11.7[c]

[a]Temperature of semen, solutions, apparatus, and environment during the encapsulation process

[b]Mean percent motility for six replicates, each replicate being a pool of three ejaculates (SE=±2.4)

[cdef]Means in same column with unlike superscripts are different ($p<.01$)

TABLE 5. EFFECT OF POLY-L-LYSINE CONCENTRATION AND ENCAPSULATION TEMPERATURE ON MAINTENANCE OF INTACT ACROSOMES DURING INCUBATION AT 37 C

Poly-l-lysine concentration (%, w/v)	Temperature[a] (C)	Hours of incubation (37 C)				
		0	2	4	6	8
		Percent intact acrosomes[b]				
Unencapsulated control	5	82.8^{e}	72.6^{e}	62.7^{d}	60.9^{e}	54.7^{de}
	20	87.6^{e}	86.3^{f}	86.8^{e}	83.9^{f}	83.1^{f}
.05	5	66.5^{d}	55.3^{d}	52.4^{cd}	46.1^{cd}	46.2^{cd}
	20	42.4^{c}	42.0^{c}	44.7^{c}	41.6^{cd}	35.7^{c}
.25	5	67.3^{d}	62.0^{de}	53.6^{cd}	50.6^{de}	58.4^{e}
	20	53.1^{c}	37.7^{c}	42.5^{c}	38.3^{c}	34.9^{c}

[a]Temperature of semen, solutions, apparatus, and environment during the encapsulation process

[b]Mean percent intact acrosomes for six replicates, each replicate being a pool of three ejaculates (SE=±1.8)

[cdef]Means in same column with unlike superscripts are different ($p<.01$)

the .25% polylysine concentration. The .05% polylysine concentration demonstrated a similar trend although it was not significant. Likewise, the effect due to temperature on the maintenance of intact acrosomes of encapsulated spermatozoa was the opposite of that observed for the controls. From table 5, we see that the 5 C encapsulation temperature was superior ($p<.01$) to that of the 20 C. Since the effect of temperature acted differently on the unencapsulated controls and the encapsulated treatments, an analysis of variance was conducted without the inclusion of the controls to determine the effect of encapsulation temperature on the survivability of microencapsulated spermatozoa.

Analysis of variance (excluding unencapsulated controls) for motility (table 14, appendix) revealed a significant temperature effect ($p<.05$), with 5 C being superior to 20 C. However, when capsules were incubated over time, there was no detection of this significance as indicated in table 4. The analysis of variance (table 14, appendix) also revealed no significant effect on motility due to polylysine concentration. This is in agreement with results of Experiment 1.

Results for percentage intact acrosomes for sperm encapsulated using the two polylysine concentrations at the two temperatures are presented in table 5 (see table 15, ap-

pendix, for analysis of variance). For the encapsulated treatments, percentage intact acrosomes differed ($p<.01$) prior to 4 h of incubation, with the 5 C temperature being superior. By 8 h of incubation, the .25% polylysine concentration treatment encapsulated at 5 C was superior to the other three encapsulated treatments, and equal to the 5 C control. This demonstrates that increased concentrations of polylysine, with assumed increases in capsule wall thickness, was beneficial to sperm survival in vitro when encapsulated at 5 C. In contrast, the unencapsulated controls at 20 C were again superior to those at 5 C ($p<.01$) for intact acrosomes after 2 h of incubation (table 5).

Despite the damage incurred during the cooling period, the 5 C encapsulated sperm demonstrated superiority to the 20 C encapsulated sperm for intact acrosomes over the 8 h incubation period (table 5). Therefore, if the damage caused by cooling neat semen could be reduced, a more distinct benefit of encapsulation at 5 C would be expected.

EXPERIMENT 3: EFFECT OF POLY-L-LYSINE MOLECULAR WEIGHT ON THE VIABILITY OF MICROENCAPSULATED SPERMATOZOA

Percentage motility and percentage intact acrosomes for sperm encapsulated at 5 C using two polylysine concentrations and three polylysine molecular weights are presented in tables 6 and 7, respectively. For motility, encapsulated

treatments did not differ from each other over the 8-h incubation period. Also, percentage intact acrosomes were unaffected by polylysine concentration or molecular weight except for the 55,000 molecular weight/.05% concentration treatment which differed ($p<.01$) from the 14,000 molecular weight/.25% concentration after 6 h incubation at 37 C with the 14,000/.25% combination being superior (table 7).

The 14,000/.25% combination was also the only treatment not to differ for percentage intact acrosomes after 4 h incubation from the unencapsulated control prepared from semen cooled to 5 C prior to extending to 45 X 10^6 sperm/ml in EYC. Prior to 4 h post encapsulation, all encapsulated treatments had lower ($p<.01$) viability than the unencapsulated controls (table 7). This indicated damage had occurred to the encapsulated sperm cells, as in Experiments 1 and 2. However, since the 5 C control did not differ from the 14,000/.25% treatment after 4 h post encapsulation, it can be assumed the capsule wall may have protected the remaining viable sperm cells within the membrane from factors causing decreased viability. These results suggest that damage to spermatozoal cells by the encapsulation procedure occurs after the membrane has been placed on the viscous mold. Therefore, capsules with thin membranes (.05% polylysine) would allow more damage to occur than thick membranes

TABLE 6. EFFECT OF POLY-L-LYSINE MOLECULAR WEIGHT AND CONCENTRATION ON MAINTENANCE OF MOTILITY DURING INCUBATION AT 37 C

Poly-l-lysine molecular weight[a]	Poly-l-lysine concentration (%,w/v)	Hours of incubation				
		0	2	4	6	8
		Percent motility[b]				
Unencapsulated control (5 C)		48.3^{d}	41.7^{de}	38.3^{d}	35.0^{d}	25.0^{d}
Unencapsulated control (20 C)		60.0^{d}	53.3^{e}	51.7^{d}	51.7^{e}	40.0^{e}
14,000	.05	31.6^{c}	28.3^{cd}	18.3^{c}	6.7^{c}	1.7^{c}
	.25	30.0^{c}	23.3^{c}	13.3^{c}	3.3^{c}	$.0^{c}$
55,000	.05	28.3^{c}	20.0^{c}	18.3^{c}	5.0^{c}	$.0^{c}$
	.25	33.3^{c}	30.0^{cd}	18.3^{c}	6.7^{c}	$.0^{c}$
90,000	.05	26.7^{c}	23.3^{c}	13.3^{c}	3.3^{c}	$.0^{c}$
	.25	28.3^{c}	23.3^{c}	13.3^{c}	5.0^{c}	$.0^{c}$

[a]Approximate molecular weight based on viscosity determination by Sigma Chemical Co., St. Louis, MO

[b]Mean percent motility for six replicates, each replicate being a pool of three ejaculates (SE=±2.6)

[cde]Means in same column with unlike superscripts are different ($p<.05$)

TABLE 7. EFFECT OF POLY-L-LYSINE MOLECULAR WEIGHT AND CONCENTRATION ON MAINTENANCE OF INTACT ACROSOMES DURING INCUBATION AT 37 C

Poly-l-lysine molecular weight[a]	Poly-l-lysine concentration (%, w/v)	Hours of incubation				
		0	2	4	6	8
		Percent intact acrosomes[b]				
Unencapsulated control (5 C)		78.4^{d}	70.5^{d}	65.2^{d}	68.1^{e}	63.2^{e}
Unencapsulated control (20 C)		83.2^{d}	81.3^{d}	80.4^{e}	80.3^{f}	82.9^{f}
14,000	.05	60.3^{c}	54.9^{c}	52.1^{c}	48.0^{cd}	47.7^{cd}
	.25	65.7^{c}	55.4^{c}	55.0^{cd}	57.9^{de}	52.8^{de}
55,000	.05	60.8^{c}	51.2^{c}	44.5^{c}	45.1^{c}	40.0^{c}
	.25	63.6^{c}	56.2^{c}	52.3^{c}	48.6^{cd}	45.3^{cd}
90,000	.05	59.7^{c}	59.2^{c}	46.2^{c}	48.8^{cd}	45.2^{cd}
	.25	63.3^{c}	51.2^{c}	51.7^{c}	50.7^{cd}	47.1^{cd}

[a]Approximate molecular weight based on viscosity determination by Sigma Chemical Co., St. Louis, MO

[b]Mean percent intact acrosomes for six replicates, each replicate being a pool of three ejaculates (SE=±1.8)

[cdef]Means in same column with unlike superscripts are different ($p<.01$)

(14,000/.25%) would allow. This would also suggest that the sperm cells in close association to the membrane would be more subject to injury than those toward the center of the capsule. A possible cause of this injury is the solutions used during the encapsulation process. The solution of most concern should be the CHES-buffer and its possible toxicity to spermatozoa needs investigated. These finding also support the discrepancy observed between this author's results and those of Nebel et al. (1984). Their procedure of evaluation resulted in sampling the effluent from the center of the evaluated capsule, thus allowing the more damaged cells to remain within the capsule. This author evaluated a mixed sample of capsule contents, thus releasing damaged and/or injured cells to the sample population.

As in Exp. 2, the unencapsulated control prepared from gradually-cooled semen (5 C) had lower viability than the unencapsulated control prepared at 20 C without cooling. However, the difference was smaller and only evident after 6 h incubation for motility and 4 h for acrosomal integrity. In Exp. 2, the difference was apparent after 2 h incubation. This illustrates that change in rate of cooling between Exp. 2 and Exp. 3 did decrease the damage to the sperm cells caused by cooling. The larger volume of water surrounding the 5 ml of neat semen provided a larger temperature buffer-

ing action to allow a more gradual cool-down with subsequently lower sperm damage. The addition of egg yolk to the neat semen would provide another possible means of maintaining spermatozoal viability during the prolonged cooling time. Nebel et al. (1984) found that the inclusion of egg yolk up to 2.9% of the encapsulating medium provided adequate control of capsule size and shape. Yolk concentrations above 2.9% resulted in the loss of uniform capsule size and shape due to interaction between yolk and membrane formation.

Results from the integrity test performed on capsules produced from the three polylysine molecular weights and two polylysine concentrations yielded surprising results. Only the 14,000 daltons molecular weight/.25% concentration treatment maintained intact capsules after vortexing. Following vortexing, 89±9% of the capsules vortexed remained intact. All capsules from all other treatments were 100% ruptured during vortexing. It was expected that the 55,000/.25% treatment combination would also maintain intact capsules as was reported in Exp. 1. Since the 55,000/.25% did not maintain integrity after vortexing, we can assume the lower temperature of encapsulation reduced the degree of cross-linking between the sodium alginate and the polylysine, thus resulting in a reduced capsule wall thickness

and/or strength from that obtained during encapsulation at 20 C. These results suggest an interaction between polylysine concentration, molecular weight, and encapsulation temperature.

Future work exploring the extent of this interaction needs to be pursued. Using the two polylysine concentrations and the three polylysine molecular weights, in conjunction with the two encapsulation temperatures (5 C vs 20 C) would allow observation of this interaction. However, due to the difficulty in processing more than six treatment per experiment, it would be advantageous to evaluate each molecular weight seperately. Observations should include viability evaluations (i.e., motility and acrosome integrity), capsule wall integrity test, and measurement of capsule wall thickness.

In conclusion, these experiments demonstrated that capsule wall thickness can be increased to an apparent maximum (approximately 5.5 μm) by increasing the polylysine concentration without detrimental effect on post encapsulation spermatozoal survivability. However, capsule wall thickness differences need to be studied for polylysine concentrations between .05% and .15% when encapsulation is performed at 20 C. It was also shown that encapsulation at 5 C resulted in an increased maintenance of spermatozoal viability over that

of encapsulation at 20 C regardless of polylysine concentration. Microcapsules produced from a polylysine concentration of .25% and molecular weight of 14,000 daltons yielded capsules less likely to rupture than those produced from 55,000 or 90,000 daltons molecular weight polylysine regardless of concentration. An interaction between polylysine concentration, polylysine molecular weight, and encapsulation temperature was suspected from the increased viability of the 14,000/.25% treatment combination. Therefore, more work directed toward the evaluation of capsule membranes from the concentration, molecular weight, temperature interaction is required.

Lastly, researchers performing viability tests on encapsulated spermatozoa must decide which sampling techniques are best suited to provide the answers to their research question. That is, should they evaluate only the "normal" cells that are a part of the center of the capsule and evaluated as unmixed effluent (Nebel et al., 1984), or should they be concerned with the total spermatozoal loss by evaluating thoroughly mixed capsule contents, as done in these experiments.

LITERATURE CITED

Austin, C.R. 1949. Fertilization and the transport of gametes in the pseudo-pregnant rabbit. J. Endocrinol. 6:63.

Barr, A.J., J.H. Goodnight, J.P. Sall, W.H. Blair and D.M. Chilko. 1979. SAS User's Guide. SAS Institute. Cary, NC.

Bedford, J.M. 1965. Effect of environment on phagocytosis of rabbit spermatozoa. J. Reprod. Fertil. 9:249.

Braden, A.W.H. 1953. Distribution of sperm in the female tract of the rabbit after coitus. Aust. J. Biol. Sci. 6:693.

Chang, T.M.S. 1964. Semipermeable microcapsules. Science 146:524.

Chang, T.M.S. and F.C. MacIntosh. 1964. Semipermeable aqueous microcapsules. Pharmacologist 6:198 (Abstr.).

Chang, T.M.S., F.C. MacIntosh, and S.G. Mason. 1966. Semipermeable agueous microcapsules: Preparation and properties. Canad. J. Physiol. Pharmacol. 44:115.

Chang, T.M.S. 1966. Semipermeable aqueous microcapsules ("artificial cells"): with emphasis on experiments in a extracorpeal shunt system. Trans. Amer. Soc. Artif. Intern. Organs 12:13.

Chang, T.M.S. and M.J. Poznansky. 1968a. Semipermeable aqueous microcapsules (artifical cells): Permeability characteristics. J. Biomed. Mat. Res. 2:187.

Chang, T.M.S. and M.J. Poznansky. 1968b. Microencapsulated catalase for acatalasemia. Proc. Canad. Fed. Biol. Sci. 11:74.

Chang, T.M.S. 1977. Biomedical Applications of Immobilized Enzymes and Proteins, Vol. 1 pp 69-90 and 147-153. Plenum, New York, NY.

Dobrowolski, W. and E.S.E. Hafez. 1970. Transport and distribution of spermatozoa in the reproductive tract of the cow. J. Anim. Sci. 31:940.

El-Banna, A.A. and E.S.E. Hafez. 1970. Sperm transport and distribution in rabbit and cattle reproductive tract. Fertil. Steril. 21:534.

Ennis, W.B. and D.T. James. 1950. A simple apparatus for producing droplets of uniform size from small volumes of liquids. Science 112:434.

Gill, J.L. 1978. Design and Analysis of Experiments in the Animal Medical Sciences. Iowa State Univ. Press, Ames.

Green, B.K. and L. Schneidcher. 1957. Microcapsules with hydrophobic contents. U.S. Pat. 2,800,457; U.S. Pat. 2,800,258.

Hawk, H.W. and R.A. Bellows. 1980. Beef and dairy cattle. In: E.S.E. Hafez (Ed.) Reproduction in Farm Animals. pp 339. Lea and Febiger, Philadelphia, PA.

Howe, G.R. and D.L. Black. 1963. Spermatozoan transport and leucocytic responses in the reproductive cycle of calves. J. Reprod. Fertil. 6:305.

Jarvis, A.P. and T.A. Spriggs. 1981. Microencapsulation of tissue culture cells; characterization of growth and specific macromolecular synthesis. J. of Cell Biology 91:202a (Abstr.).

Jarvis, A.P., T.A. Spriggs, W.R. Chipura, M. Sullivan, and G.A. Koch. 1982. Microencapsulation of hybridoma cells; characterization of growth and monoclonal antibody production. In Vitro 18:276. (Abstr.).

Jeon, K.W: 1965. Simple method for staining and preserving epoxy resin-embedded animal tissue sections for light microcopy. Life Sci. 4:1839.

Karnovsky, M.J. 1965. A formaldehyde glutamaldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 27:137 (Abstr).

Kondo, A. 1979. Microcapsule Processing and Technology, Ed. J.W. Van Valkenburg. Marcel Dekker, Inc., New York, NY.

Lim, F. and A.R. Sharpe. 1978. A liquid/solid phase RIA system for thyroxine (T4). Clin. Chem. 24:1040 (Abstr.).

Lim, F. and R.D. Moss. 1979. Microencapsulated enzymes as stable reusable reagents. Clin. Chem. 25:1071 (Abstr.).

Lim, F. and A.M. Sun. 1980. Microencapsulated islets as bioartifical endocrine pancreas. Science 210:908.

Lim, F. and R. D. Moss. 1981. Microencapsulation of living cells and tissues. J. Pharm. Sci. 70:351.

McLaren, A. 1980. Fertilization, cleavage and implantation. In: E.S.E. Hafez (Ed.) Reproduction in Farm Animals. pp 227-228. Lea and Febiger, Philadelphia, PA.

Mattner, P.E. 1963. Spermatozoa in the genital tract of the ewe. II. Distribution after coitus. Aust. J. Biol. Sci. 16:688.

Mattner, P.E. 1968. The distribution of spermatozoa and leucocytes in the female genital tract in goats and cattle. J. Reprod. Fertil. 17:253.

Nebel, R.L. 1983. Microencapsulation of bovine spermatozoa. Ph.D., Dissertation, Virginia Polytechnic Institute and State University, Blacksburg.

Nebel, R.L., J.H. Bame, R.G. Saacke and F. Lim. 1984. Microencapsulation of bovine spermatozoa. J. Anim. Sci. (In press).

Saacke, R.G. and J.M. White. 1972. Semen quality tests and their relationship to fertility. Proc. 4th Tech. Conf. Artif. Insem. and Reprod. NAAB. 22.

Salisbury, G.W., N.L. VanDemark and J.R. Lodge. 1978. Physiology of Reproduction and Artificial Insemination of Cattle. W.H. Freeman and Company, San Francisco and London.

Sparks, R.E., R.M. Salemme, P.M. Meier, M.H. Litt, and O. Lindan. 1969. Removal of waste metabolites in uremia by microencapsulated reactants. Trans. Am. Soc. Artif. Intern. Organs. 15:353.

APPENDIX

TABLE 8. COMPOSITION OF SOLUTIONS USED IN MICROENCAPSULATION PROCEDURE

$CaCl_2$-HEPES Solutions

1.5% $CaCl_2$-Dihydrate

50 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid)

.001% Tween 20 (polysorbate 20 NF)

Adjust solution to pH 6.8 with NaOH

CHES Buffer

2% CHES (2[N-Cyclohexylamino]ethane-sulfonic acid)

.6% NaCl

Adjust solution to pH 8.2 with Citric Acid

Above solution diluted 1:20 with sterile .9% physiological saline

Citrate-Saline Solution

100 mM Sodium citrate dihydrate

Diluted 1:3 with .9% physiological saline

Adjust solution to pH 7.0

TABLE 9. ANALYSIS OF VARIANCE FOR MOTILITY IN EXPERIMENT 1

Sources of variation	Degrees of freedom	Mean square
Ejaculate[a]	5	55.600**
Polylysine concentration[b]	4	11804.333**
Hour[c]	4	3181.000**
Ejaculate X polylysine concentration	20	59.933**
Ejaculate X hour	20	30.600**
Polylysine concentration X hour	16	25.167*
Residual	80	13.267

[a]Pooled ejaculates

[b]Includes unencapsulated control

[c]Hours of incubation at 37 C

*$p<.05$

**$p<.01$

TABLE 10. ANALYSIS OF VARIANCE FOR INTACT ACROSOMES IN EXPERIMENT 1

Sources of variation	Degrees of freedom	Mean square
Ejaculate[a]	5	1150.598**
Polylysine concentration[b]	4	26806.893**
Hour[c]	4	656.492**
Ejaculate X polylysine concentration	20	632.638**
Ejaculate X hour	20	128.835**
Polylysine concentration X hour	16	80.235
Ejaculate X polylysine concentration X hour	80	66.065**
Residual	154	17.663

[a]Pooled ejaculates

[b]Includes unencapsulated control

[c]Hours of incubation at 37 C

*$p<.05$

**$p<.01$

TABLE 11. ANALYSIS OF VARIANCE FOR CAPSULE DIAMETER, CAPSULE WALL INTEGRITY, AND CAPSULE WALL THICKNESS IN EXPERIMENT 1

Sources of variation	Degrees of freedom	Mean square		
		Diameter	Integrity	Thickness
Ejaculate[a]	5	.006314**	664.710*	2.153*
Polylysine concentration	3	.022715**	9300.028**	17.502**
Residual	15	.000279	156.355	.574

[a]Pooled ejaculate

*p<.05

**p<.01

TABLE 12. ANALYSIS OF VARIANCE FOR MOTILITY IN EXPERIMENT 2

Sources of variation	Degrees of freedom	Mean square
Ejaculate[a]	5	667.222**
Polylysine concentration[b]	2	18835.556**
Temperature[c]	1	933.889*
Hour[d]	4	4567.222**
Ejaculate X polylysine concentration	10	101.556**
Ejaculate X temperature	5	109.889*
Ejaculate X hour	20	38.889
Polylysine concentration X temperature	2	4135.556**
Polylysine concentration X hour	8	50.139
Temperature X hour	4	156.111**
Polylysine concentration X temperature X hour	8	51.528
Residual	110	36.101

[a]Pooled ejaculates

[b]Includes unencapsulated controls

[c]Temperature of encapsulation

[d]Hours of incubation at 37 C

*p<.05

**p<.01

TABLE 13. ANALYSIS OF VARIANCE FOR INTACT ACROSOMES IN EXPERIMENT 2

Sources of variation	Degrees of freedom	Mean square
Ejaculate[a]	5	1492.71**
Polylysine concentration[b]	2	31465.19**
Temperature[c]	1	1095.65
Hour[d]	4	2385.44**
Ejaculate X polylysine concentration	10	411.10**
Ejaculate X temperature	5	389.41**
Ejaculate X hour	20	54.11**
Polylysine concentration X temperature	2	11599.29**
Polylysine concentration X hour	8	85.03
Temperature X hour	4	645.76**
Ejaculate X polylysine concentration X temperature	10	392.55**
Ejaculate X polylysine concentration X hour	40	46.30**
Ejaculate X temperature X hour	20	41.58**
Polylysine concentration X temperature X hour	8	249.65**
Ejaculate X polylysine concentration X temperature X hour	40	40.79**
Residual	196	23.03

[a]Pooled ejaculates

[b]Includes unencapsulated controls

[c]Temperature of encapsulation

[d]Hours of incubation at 37 C

*p<.05

**p<.01

TABLE 14. ANALYSIS OF VARIANCE FOR MOTILITY IN EXPERIMENT 2 EXCLUDING UNENCAPSULATED CONTROLS

Sources of variation	Degrees of freedom	Mean square
Ejaculate[a]	5	279.333**
Polylysine concentration	1	53.333
Temperature[b]	1	750.000*
Hour[c]	4	3272.916**
Ejaculate X polylysine concentration	5	97.333*
Ejaculate X temperature	5	110.000*
Ejaculate X hour	20	51.417
Polylysine concentration X temperature	1	53.333
Polylysine concentration X hour	4	38.750
Temperature X hour	4	60.417
Polylysine concentration X temperature X hour	4	42.917
Residual	65	35.974

[a]Pooled ejaculates

[b]Temperature of encapsulation

[c]Hours of incubation at 37 C

*$p<.05$

**$p<.01$

TABLE 15. ANALYSIS OF VARIANCE FOR INTACT ACROSOMES IN EXPERIMENT 2 EXCLUDING UNENCAPSULATED CONTROLS

Sources of variation	Degrees of freedom	Mean square
Ejaculate[a]	5	1194.798**
Polylysine concentration	1	405.364
Temperature[b]	1	13130.123*
Hour[c]	4	1462.771**
Ejaculate X polylysine concentration	5	678.317**
Ejaculate X temperature	5	334.587**
Ejaculate X hour	20	57.932**
Polylysine concentration X temperature	1	400.094
Polylysine concentration X hour	4	107.086
Temperature X hour	4	338.817*
Ejaculate X polylysine concentration X temperature	5	675.323**
Ejaculate X polylysine concentration X hour	20	42.499*
Ejaculate X temperature X hour	20	49.163*
Polylysine concentration X temperature X hour	4	254.840*
Ejaculate X polylysine concentration X temperature X hour	20	58.670**
Residual	132	25.657

[a]Pooled ejaculates

[b]Temperature of encapsulation

[c]Hours of incubation at 37 C

*$p<.05$

**$p<.01$

TABLE 16. ANALYSIS OF VARIANCE FOR MOTILITY IN EXPERIMENT 3

Sources of variation	Degrees of freedom	Mean square
Ejaculate[a]	5	698.590**
Subclass[b]	6	6566.319**
Hour	4	6277.372**
Ejaculate X subclass	30	35.486
Ejaculate X hour	20	50.917
Subclass X hour	24	73.958*
Residual	150	45.167

[a]Pooled ejaculates

[b]Poly-l-lysine concentration by poly-l-lysine molecular weight subclass

[c]Hours of incubation at 37 C

*p<.05

**p<.01

TABLE 17. ANALYSIS OF VARIANCE FOR INTACT ACROSOMES IN EXPERIMENT 3

Sources of variation	Degrees of freedom	Mean square
Ejaculate[a]	5	915.569**
Subclass	6	8133.727**
Hour	4	2924.354**
Ejaculate X subclass	30	47.968
Ejaculate X hour	20	50.439
Subclass X hour	24	92.512**
Residual	390	46.496

[a]Pooled ejaculates

[b]Poly-l-lysine concentration by poly-l-lysine molecular weight subclass

[c]Hours of incubation at 37 C

*p<.05

*p<.01

The vita has been removed from the scanned document