LOW TEMPERATURE PRESERVATION OF MICROENCAPSULATED CANINE SPERM

カプセル化した犬精子の低温保存

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LOW TEMPERATURE PRESERVATION OF MICROENCAPSULATED CANINE SPERM

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SUMMARY

Semen preservation and artificial insemination (AI) in dogs has become a common practice in veterinary medicine. Preservation of dog semen has been used for assisted reproductive technology in this species. Semen preservation technology has become a valuable tool for the conservation of genetic materials of superior breeds of dogs. Its use is increasing among breeders to improve the breeding potential of their stud dogs. To maintain the fertilizability of sperm for more than several days, semen diluted with appropriate extenders should be preserved either by chilling or freezing. Chilled sperm has the short lifespan and the fertilizability of frozen-thawed semen after insemination remains variabie. Poor post-thaw quality and short sperm lifespan after thawing are major drawbacks for using cryopreserved canine sperm. To overcome current limitations, preservation of microencapsulated canine sperm could be a viable alternative.

The concept of microencapsulation of sperm for AI is to maintain sperm viability and high sperm concentrations in the uterus during estrus allowing their release over an extended interval, so that the viable population of sperm cells are available at the time of ovulation. Maintaining adequate numbers of viable sperm in the uterine tract

during the time of ovulation plays a vital role for maximizing conception rates. Microencapsulation is the process of encasing living cells by a semipermeable membrane, permitting the exchange of nutrients and metabolites. Sperm have been successfully microencapsulated in bovine and porcine. These microencapsulated cells remained viable for longer duration than the unencapsulated cells. And microencapsulation has received much interest with respect to enhancement of reproductive performance in animals for semen controlled release and sperm preservation, in vitro oocytes maturation and embryo culture to improve in vitro fertilization yield. As the bitch has a long estrus compared with that of the other domestic animals, microencapsulation of sperm could be an innovative and alternative technique for maintaining the sperm survivability and fertility and for successful AI in dogs. Therefore, insemination of microencapsulated sperm could improve conception rates in animals inseminated during ovulation.

Therefore, the microencapsulation technique for the preservation of canine sperm needs to be established. The objectives of the first study were to develop the method for chilling canine sperm using microencapsulation, to characterize the microencapsulation of canine sperm using 0.75 and 1.0% sodium alginate and 0.1% poly-L-lysine and to evaluate the influence of microencapsulation on sperm motility

and viability during storage at 4 °C. In the second study the objectives were to develop a method for cryopreserving microencapsulated canine sperm and to investigate whether: i) glycerol equilibrium time affected microencapsulated canine sperm survival; and ii) quality of frozen-thawed canine sperm was improved if the sperm was microencapsulated before cryopreservation.

The beagle dogs kept in the Laboratory Animal Research Center of Tottori University were used in this study. Semen was collected twice a week from each dog by digital manipulation and the ejaculates were pooled to obtain a sufficient volume. Pooled ejaculates were extended in egg yolk Tris extender and were encapsulated in gel (alginate only) or polycation (poly-L-lysine membrane bound) microcapsules at 0.75 and 1.0% alginate concentration at room temperature. Unencapsulated sperm suspension served as control. Characteristics of microcapsule and microencapsulated sperm were evaluated during chilling storage for 48 hrs. Gel microcapsules at 0.75% alginate concentration were fragile and had a tear drop-like structure, whereas those at 1.0% alginate concentration had a solid spherical structure. In all groups, diameter of the microcapsules increased with duration of storage ($P < 0.05$). Membrane thickness of polycation microcapsules decreased significantly with the prolongation of storage both at 0.75 and 1.0% alginate concentration. Total average recovery rate of

sperm from polycation microcapsules was significantly lower than that of gel microcapsules. Alginate concentration did not affect the sperm recovery rate from microcapsules. Progressive motility of polycation microencapsulated sperm and unencapsulated sperm was higher than that of the gel microencapsulated sperm, both at 0.75 and 1.0% alginate concentration ($P < 0.05$), although viability of sperm was similar among the three groups. To evaluate the sperm progressive motility and viability after chilling storage, sperm were microencapsulated in polycation microcapsules at 1.0% alginate concentration, stored at 4 ^{o}C for 0, 1, 4 and 7 days, and then cultured at 38.5 ^{o}C for 0, 6, and 24 hrs. Progressive motility and viability of microencapsulated sperm were higher than those of unencapsulated spermatozoa at 0 to 24 hrs of culture after 4 and 7 days of chilling storage ($P < 0.05$).

In the second study ejaculates from beagle dogs collected manually were pooled and extended in egg yolk Tris extender and encapsulated using alginate and poly-L-lysine at room temperature. The microcapsules were cooled at 4 ^{o}C, immersed in pre-cooled extender (equivalent in volume to the microcapsules) to reach final concentration of 7% glycerol and 0.75% Equex STM paste, and equilibrated for 5, 30 and 60 min at 4 ^{o}C. Thereafter, microcapsules were loaded into 0.5 mL plastic straws

and frozen in liquid nitrogen. Unencapsulated sperm suspension were also loaded into plastic straws, frozen in liquid nitrogen and served as control. Characteristics of microencapsulated canine sperm were evaluated after glycerol addition at 4 ^{o}C. Glycerol exposure for 5, 30 and 60 min did not significantly affect progressive motility, viability, or acrosomal integrity of microencapsulated sperm compared with pre-cooled unencapsulated sperm. Characteristics of frozen-thawed canine microencapsulated sperm were evaluated at 0, 3, 6, and 9 hrs of culture at 38.5 ^{o}C. Pre-freeze glycerol exposure for 5, 30, and 60 min at 4 ^{o}C did not influence post-thaw quality in unencapsulated sperm. Post-thaw motility and acrosomal integrity of microencapsulated sperm decreased more than those of unencapsulated sperm ($P < 0.05$) following glycerol exposure for 5 min. However, motility, viability and acrosomal integrity of microencapsulated sperm after 30 and 60 min glycerol exposure were higher than unencapsulated sperm cultured for 6 or 9 hrs ($P < 0.05$).

The effectiveness of microencapsulation on sperm motility and viability seems better with prolongation of chilling storage. Thus, the microencapsulation of canine sperm provided a better environment and protects the sperm allowing the exchange of nutrients, which enhanced the motility and viability with prolongation of storage. Also,

the microencapsulation prevented a rapid decrease in the motility and viability of encapsulated canine sperm during chilling storage and protected the sperm from the detrimental effect of freezing process enhancing post-thaw longevity, motility and viability during the culture period.

In conclusion, polycation microencapsulation at 1.0% alginate concentration can be successfully applied for chilling storage of canine sperm by maintaining the motility and viability for up to 7 days. And the microencapsulated canine sperm were also successfully cryopreserved, and this could be a viable alternative to the conventional sperm cryopreservation method in this species.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

The canine is the species in which artificial insemination was ever performed for the first time in 1780, by an Italian scientist abbey Lazzaro Spallanzani who not only was the first one to achieve a litter by artificial insemination (AI), but was also the first one to note that a reduction of temperature could lower metabolic activity of sperm in a reversible manner. Since then semen preservation has attained a stage of being an indispensable and integral part of assisted reproductive technology. Successful preservation of canine semen is therefore important for improving the results of the artificial reproductive techniques (ARTs), such as mainly artificial insemination (AI) and in vitro fertilization in dogs (Luvoni, 2006).

Semen for the use of AI can be prepared in different ways and can be used either fresh, or extended fresh or chilled, or frozen-thawed. Freshly ejaculated semen for AI is usually used when both the male and the females are present at the site of collection and the AI can be performed. The freshly ejaculated semen can be extended in order to maintain the sperm survivability. The fresh semen has the advantage of not being processed and therefore of not being damaged by chilling or freezing procedures which has to be used immediately after semen collection. For any further transport or for assisted reproductive technologies for short storage, either the semen has to be chilled or for long-term storage, semen has to be frozen.

The combinations of storage temperature, cooling rate, chemical composition of the extender, cryoprotectant concentration and the hygienic control are the key factors that affect the life span of sperm. In the last few decades, a slow progress have been made in improvement of storage technology, however, the recent advancement in reproduction technology and well understanding of the reproductive physiology have opened the door to a new era in preservation of sperm (Yoshida, 2000).

Development of assisted reproductive techniques in canids has been relatively slow; however, during the last 10 years some breakthroughs have been made. Semen preservation and artificial insemination in the canine has become a common practice in veterinary medicine. Chilled dog semen is easy to handle, and also several extenders can be used. The use of chilled extended semen for AI in the canine has become increasingly popular over the last 2 decades. Different types of semen extenders have been evaluated for their capability to keep chilled dog semen motile over time (Foote and Leonard, 1964; Province et al., 1984; Bouchard et al., 1990).

The domestic dog (*Canis familiaris*) is one of the first domesticated species (Morey, 1994). Dogs has been used for several purposes since its domestication for hunting, as guide dogs for the blind, and for investigation purposes as sniffing dogs. The large numbers of dogs are used as companion animals. The domestic dog has been treated as an useful models for research in reproduction of endangered wild canids (Goodrowe et al., 1998). The expanding use of dogs for different purposes and the demand for higher breeds among the people has compelled the researchers for preservation of sperm.

The success of semen storage depends on numerous factors which may be peculiar to each species and are optimized according to the type of semen to be preserved. Long-term storage of semen by cryopreservation, with high recovery rates on thawing, is essential for the establishment of genetic resource banks of canine species. Artificial insemination and semen preservation have been identified as powerful tools in breeding programs, which would allow the storage of semen from genetically valuable breed of dogs. The physiological and morphological properties of spermatozoa led to special procedures of preservation in liquid storage as well as cryopreservation of sperm cells. Assisted reproductive techniques developed for domestic dogs have been applied to captive wild animals (Goodrowe et al., 1998).

Preservation of dog semen for artificial insemination is becoming increasingly popular since it allows for the transporting of genetic material. The use of extended semen eliminates the animal shipping cost, reduces the chances of disease transmission and stress due to time consuming animal transportation allowing the breeders to use semen from genetically superior dogs both within and between countries.

1.1.1 Chilling

Semen may be preserved for short term storage either by chilling/cooling or for long term storage by freezing. The main disadvantage of short term storage of dog semen is the limited lifespan of sperm, as the extended chilled semen should be used within 4.9 days after its collection (England and Ponzio, 1996). As the length of

storage exceeds the quality of chilled semen deteriorates, it would be prudent to freeze the semen samples. AI with chilled semen has become an interesting tool in dog breeding. Many reasons explain this success. First, is relatively simple to chill dog semen; also the generalized use of in-clinic progesterone assays allows the achievement of good pregnancy rates and optimum litter size. As well, the development of pure dog breeding has initiated an increasing demand of import and export for semen. Chilled dog semen has allowed a relatively inexpensive, simple and successful method to achieve this demand. Dog semen is chilled to, and maintained at 4 ^{o}C for several days. Properly chilled semen can maintain motility and membrane integrity for at least 5 to 7 days, and after this period of time these decrease slowly. However, under practical circumstances canine chilled semen should be used as soon as possible after collection, ideally within the first 48 hrs. It is important to note that the fertilizing ability of sperm decreases 1 wk before a substantial drop in motility can be seen. The optimal cooling rate for chilling canine semen has been suggested to be from –0.3 to –1 ^{o}C/min (Bouchard et al., 1990).

1.1.2 Cryopreservation

Cryopreservation procedures induces a series of osmotic, physical-chemical alterations that lead to rupture of the sperm membrane, causing death of some sperm cells, removal of important membrane proteins, ionic rearrangement, alterations in the lipid-protein bi-layer and severe post-thaw damage in surviving cells reducing fertilizing ability (Watson, 1995; Holt, 2000). Frozen-thawed spermatozoa should be assessed to

determine the degree of cell integrity caused by cryopreservation (Pena-Martinez, 2004). Most studies on canine semen cryopreservation are focused mainly on post thaw sperm motility analysis (Heywood and Sortwell, 1971; Ivanova-Kicheva et al., 1997). The ultimate goal of frozen semen evaluation is to predict the fertility of semen after insemination into a bitch of normal fertility (Eilts, 2005). The combined evaluation of multiple morphological parameters with fertility of canine frozen-thawed semen is utmost important.

Frozen semen is of great interest in dog breeding mainly for two reasons. It allows international shipping of semen and also establishment of gene banks of sires of superior genetic value. Although chilled semen permits the first objective in many cases, while the second reason is only possible to achieve only through deep frozen semen. In addition, freezing technologies are important tools for the conservation of endangered wild canids (Marks et al. 1994; Goodrowe et al. 1998; Zindl et al. 2006). Although some different methods have been described (Alamo et al. 2005), dog semen is frozen and maintained in LN_2 at –196 ^{o}C. Recently, it has been found that canine spermatozoa can be frozen after 2 to 3 days of storage at 4 ^{o}C without a significant decrease in the sperm quality when compared with the semen frozen immediately after collection (Hermasson and Linde-Forsberg 2006). Frozen canine semen can be stored practically indefinitely, and the techniques and extenders for dog sperm cryopreservation developed in recent years have resulted in post-thaw motility of up to 70% (Pena and Linde-Forsberg, 2000a; Pena et al., 2003) and whelping rates of up to 85% (Rota et al., 1999; Linde-Forsberg et al., 1999; Thomassen et al., 2001). However, pregnancy rates and whelping rates are generally lower with frozen semen

than with chilled, extended semen, when the same optimal methods are used for AI (Farstad, 1984; Linde-Forsberg, 2001a). It is technically easier to chill than to freeze semen, and the shipment of chilled semen is less expensive and regulations for import and export are often less complicated than for frozen semen. Consequently, the use and international shipment of chilled semen in dog breeding is becoming increasingly more common. One disadvantage with chilled semen is the relatively short survival time, which is thought to necessitate use within a few days after collection (Gill et al., 1970; Morton and Bruce, 1989; Linde-Forsberg, 1991; Kumi-Diaka and Badtram, 1994). Therefore, the transportation of chilled semen to its destination requires careful planning so that it both remains fertile and is available on the most suitable insemination day for the bitch. To preserve viability and fertilizing capacity of sperm cells, semen to be stored or shipped needs to be extended and chilled. Many extenders for chilled dog semen have been evaluated, and egg-yolk–tris (EYT) extenders appear superior to the other extenders tested in vitro and in vivo (Harrop, 1954; Foote, 1964; Province et al., 1984; Bouchard et al., 1990; Kumi-Diaka and Badtram, 1994; Linde-Forsberg, 1995; Rota et al., 1995; Pinto et al., 1999; Strom et al., 2000).

1.2 Microencapsulation

Chang reported the development of microcapsules having semipermeable membranes and was the first to encapsulate biologically active materials and named these microcapsules “artificial cells” (Chang, 1964). He encapsulated living cells using

cross-linked protein membranes and proposed their use for hepatocytes and pancreatic islet cells (Chang et al., 1966). Lim and Sun (1980) applied this method and described a simple all aqueous system that permitted successful microencapsulation of pancreatic islet cells in an alginate poly-L-lysine membrane. Encapsulated islets implanted intraperitoneally temporarily restored normal glycemia in streptozotocin induced diabetic rats. A single implantation of the microencapsulated islets into rats with streptozotocin induced diabetes neutralized the diabetic state for 3 wk while unencapsulated control islets survived for only 6 to 8 days under the same conditions. Microencapsulated islets remained morphologically and functionally intact for 4 months during in vitro culture at 37 ^{o}C (Lim and Sun, 1980; Lim and Moss, 1981; Sun et al., 1984). Lim and Moss (1981) reported the microencapsulation of hepatoma cells grew and multiplied at the same rate as the unencapsulated controls.

The concept of using microencapsulated sperm for prolonged storage or sustained release of sperm within the reproductive tract of the female mammals has a biological precedent in several classes of animals. In all mammals sperm remains viable during storage in the cauda epididymis. A technique for microencapsulation of sperm has been developed with minimal sperm injury and thus, a potential use in artificial insemination. In concept, the potential role of microencapsulated semen in AI is to maintain sperm viability while reducing their susceptibility to retrograde action of the uterus and phagocytocis by leucocytes and allowing their release over an extended period.

Although millions of sperm are deposited in the female reproductive tract, during natural or artificial insemination, sperm disappear rapidly and relatively few

sperm reach the site of fertilization (Austin, 1949; Braden, 1953; El-Banna and Hafez, 1970). Many sperm fail to gain access to the oviducts as a result of anatomical barriers or physiological events that foster their disappearance (Hawk, 1983). Maintaining adequate numbers of viable sperm in the uterine tract during the time of ovulation plays a vital role maximizing conception rates. Therefore, insemination of microencapsulated sperm could improve conception rates in animals inseminated during ovulation. The concept of sperm microencapsulation is to be able to achieve sustained release of sperm in the female reproductive tract over a period of time so that viable population of sperm cells are available at the time of ovulation.

Sperm encapsulation is a strategy whereby a pool of live cells is entrapped within a semipermeable membrane. Microencapsulation is defined as a process of enclosing cells, tissues, or substances within a semipermeable membrane (Lim, 1984; Wheatley et al., 1985). Several types of cells and tissues have been successfully encapsulated, including hepatocytes, islet of Langerhans, tumor cells, adrenal cortical cells and sperm cells (Lim and Sun, 1980; Lim and Moss, 1981; Lim, 1984; Nebel et al., 1985; Esbenshade and Nebel, 1990). The encapsulated cells remain viable since the semipermeable membrane permits the exchange of nutrients and metabolites. Microencapsulation is an evolving branch of biotechnology with numerous applications including the enhancing of reproductive performance both in humans and other mammal species. Over the last twenty years male and female gametes and embryos have been encapsulated with or without somatic cells, for different purposes, such as semen controlled release, in vitro gametogenesis, embryo culture after in vitro fertilization and cell preservation.

1.2.1 Methodology

Microencapsulation procedure (Lim and Sun, 1980) involves three steps: (a) the production of a calcium alginate sperm cell-containing matrix; (b) the formation of a semipermeable membrane by interfacial polymerization with a multivalent polyamine; and (c) the liquefaction of the semisolid matrix by chelation of calcium with sodium citrate. The use of a biocompatible polymer as the alginate led to the maintenance of optimal cell viability levels both in vivo and in vitro. The capsule membrane is permeable to small molecules as glucose and oxygen but impermeable to large molecules such as immunoglobulins (Lim and Sun, 1980).

Alginates have been used to microencapsulate a variety of biological materials, including spermatozoa. Alginate or alginic acid is a linear polysaccharide copolymer of (1–4)-linked *b*-mannuronic acid (M) and *a*-guluronic acid (G) monomers, derived primarily from brown seaweed kelp (Smidsred and Skjak-Braek., 1990; Johnson et al., 1997). Within the alginate polymer, the M and G monomers are sequentially assembled in either repeating (MM or GG) or alternating (MG) blocks. The amount and distribution of each monomer depends on the species and age of seaweed from which the alginate is isolated (Smidsred and Skjak-Braek., 1990; Johnson et al., 1997). Alginate hydrogels are formed when divalent cations, such as Ca^{2+}, interact with blocks of G monomers to form ionic bridges between different polymer chains (Smidsred and Skjak-Braek., 1990).

Thus, in the presence of divalent cations, an aqueous solution of alginate will become a gel and this gel formation occurs due to the ionic interaction between guluronic acid residues from two or more alginate chains and cations. Thus the addition of calcium (Ca^{2+}) causes gelation of alginate and formation of microdroplets (hydrogels) upon which a semipermeable membrane can be formed. The outer semipermeable membrane is constructed by immersion of microgels in a solution of multivalent polyamines. Various polyamines with different concentrations of these polyamines have been used to construct the outer membrane such as 0.04 to 1.5% poly-L-lysine (Nebel et al., 1985), 0.01% polyvinylamine (Nebel et al., 1986), and 1% protamine sulfate (Munkittrick et al., 1992). A cross linking reaction between alginate and the polyamine occurs which displaces surface layer of Ca^{2+} ions and forms a semipermeable membrane. Liquefaction of semi solid gel core is accomplished be chelation of Ca^{2+} with sodium citrate resulting in a microcapsules surrounded by semipermeable membrane containing viable sperm cells.

The microencapsulation of bovine sperm with calcium alginate and poly-L-lysine has been successfully applied and achieved prolonged release (Nebel et al., 1985, 1993; Nebel and Saacke, 1994, 1996). However, this method has not been proven suitable for microencapsulation of boar sperm, by which viability has not been maintained for more than 16 hrs after encapsulation using bovine method (Nebel and Saacke, 1996). Therefore, a new microencapsulation method specifically for swine has been developed (Torre et al., 1998, 2000). Using barium alginate and protamine–barium alginate for microencapsulation, Torre et al. (2002) found that encapsulation enhances semen preservation, providing protection against membrane damage on

dilution. The in vitro release test of sperm revealed massive cell delivery from barium alginate microcapsules within 6 hrs, and slow release from protamine–barium alginate capsules.

One-step reverse technique for encapsulating swine sperm in barium alginate has been developed (Torre et al., 2002). Briefly, barium chloride solution is added to the semen and the resulting suspension is dropped into a sodium alginate solution. The suspension upon contact with sodium alginate, barium ions contained into the suspension diffuse out of the droplets and when they reach the interface, react with the alginate by ionic interaction, leading to the formation of a barium alginate gel membrane around the semen droplet. The thickness of the gel membrane increased until the diffusion of the barium ions through the semen droplet ends. After 1 hr the capsules are collected by filtration, rinsed twice with a specific swine sperm extender (aqueous solution containing glucose 60 g/L and $NaHCO_3$ 1.2 g/L, pH 7.2) and re-suspended in the same extender. The semipermeable membrane is constructed by suspension of capsules in 1% w/v aqueous solution of protamine sulphate and stirred for 20 min using a magnetic stirrer. Thus obtained microcapsules are collected by filtration, rinsed twice and then transferred to the swine sperm extender. This method has two advantages: firstly, these capsules consist of a core of highly concentrated sperm surrounded by a polymeric membrane which separate the cells from the extender. The sperm encapsulated with physiological seminal plasma were thus suspended rather than diluted in the storage medium, and a virtual dilution could be obtained after encapsulation (Torre et al., 2000, 1998). Secondly, barium ions were

employed as gelling agent in place of calcium, as Ba^{++} inhibit a precocious capacitation process that is promoted by Ca^{2+} (Munoz-Garay et al., 2001).

Microencapsulation Methodology

Microencapsulation procedures developed by Nebel et al., (1985) to encapsulate bovine sperm are illustrated as below:

1. Sperm (up to 150 x 10^6 sperm/mL) are suspended in 1.0 to 1.5% sodium alginate dissolved in physiological solution and loaded into a 10 to 50 mL syringe.

2. Microdroplets containing sperm are then produced by a syringe pump extrusion technique. Using a syringe pump, the sperm suspension in a syringe is forced through a 19 gauge hypodermic needle contained within an encapsulating jet at a rate of approximately 1.5 mL/min.

3. The bottom of the encapsulating jet is placed above a flask containing 80 mL of a 1.5% calcium chloride- HEPES buffer (50 mM), pH 6.8. The surface of the solution was 3.84 cm from the base of the encapsulating jet. Immediately upon contact with calcium chloride-HEPES solution, the droplets absorb Ca^{2+} ions causing solidification of the entire cell suspension resulting in a round shape retaining high viscosity microgel for the future microcapsules.

4. The calcium chloride-HEPES solution is aspirated and the microgels are rinsed three times with physiological saline.

5. Semipermeable membrane is constructed on the surface of the microgels by interfacial polymerization. Microgels are suspended in a physiological solution containing 0.04 to 1.5 % poly-L-lysine. Exposure time, polymer concentration and molecular weight influence the membrane thickness by limiting the cross linking of alginate and polyamine.

6. Membrane bound microgels are then rinsed three times with physiological solution and suspended in 3% sodium citrate (pH, 7.4) to liquefy the gel core by chelation of Ca^{2+} ions.

7. Microcapsules containing motile sperm are then transferred to desired environment or culture medium.

1.3 Microencapsulation of germ cells

1.3.1 Sperm

Sperm microencapsulation has been used since the development of bovine sperm microencapsulation for the first time by Nebel et al., (1985). A technique for microencapsulation of bovine sperm has been developed with minimal sperm injury and for potential use in artificial insemination. An intended function of microencapsulated semen for artificial insemination was to maintenance of sperm viability, to make the sperm less susceptible to retrograde action of the uterus and to phagocytosis by leucocytes following insemination, and to enable their slow release over an extended period of time, so that they are less dependent on timing of

insemination for high fertility. Microcapsules has shown the protective effect on sperm from uterine macrophage phagocytosis and promoted bioadhesion of the delivery system, preventing the semen reflux (Nebel et al., 1985; Munkittrick et al., 1992; Nebel et al., 1993; Nebel et al., 1996). An increase in fertility with microencapsulated semen at the optimal time of insemination (48 hrs after CIDR removal) has been obtained in CIDR synchronized heifers (Vishwanath et al., 1997). Recent studies has confirmed that the microencapsulation of boar sperm could prolong the viability of boar sperm and in vivo fertility results obtained inseminating boar sperm has shown that calcium alginate microencapsulation improves the fertility of sperm with better farrowing rates and litter size (Huang et al., 2005). Boar spermatozoa have been successfully microencapsulated maintaining the motility of encapsulated sperm similar to that of unencapsulated sperm when examined immediately after encapsulation and proposed that microencapsulation may enhance the potential uses of AI by reducing the number of sperm required for each breeding (Esbenshade and Nebel, 1990).

1.3.2 Oocyte

Several attempts have been made to design oocyte in vitro maturation (IVM) and/or in vitro fertilization (IVF) culture systems providing optimal yield. Recent studies showed that three-dimensional co-culture of follicular wall cells with immature oocytes is a good methodological approach for oocyte encapsulation (Pangas et al., 2003; Kreeger et al., 2005; Combelles et al., 2005; Heise et al., 2005). A three-dimensional culture of granulosa cells encapsulated in barium alginate protamine membranes has

been proposed because capsules allow self organization of round shaped bovine and porcine granulosa cells into clusters. A three-dimensional approach for COCs/oocyte co-culture in alginate matrix has also been applied using barium alginate capsules to mimic a basal membrane to enhance in vitro maturation yield of oocytes. An oocyte and a pool of granulosa cells, has been enclosed in an alginate fluid core and once in the core, the granulosa cells organized themselves into a pseudo follicular structure, morphologically resembling a mature, Graafian follicle, and thus creating an environment capable of inducing oocyte maturation without exogenous hormones (Vigo et al., 2005). A number of three-dimensional granulosa/oocyte co-culture systems have been evaluated and the encapsulation seems to be a valid technology for obtaining a three-dimensional environment suitable for co-culture of oocytes. Pangas et al., (2003) developed an alginate beads three-dimensional culture system designed for cumulus/oocyte complexes (COCs) yielded good results in terms of structural development and meiosis resumption for murine ova. The alginate based three-dimensional co-culture system can easily be employed in several murine oocyte development stages when a three-dimensional COCs co-culture system, is supplemented with follicle stimulating hormone, in a synthetic extracellular matrix with specific components (RGD peptide and laminin) (Kreeger et al., 2005). The positive influence of the three-dimensional co-culture system has been shown in the meiotic competence of mouse oocytes (Kreeger et al., 2006; Xu et al., 2006a), and also obtaining the viable and fertile offspring from this kind of co-culture technique (Xu et al., 2006b). This technique when applied to murine models could positively affect attempts of oocyte banking as well as the preservation of fertility in human reproduction.

A three-dimensional, follicle mimicking structure enhances in vitro maturation yields of human oocytes without hormonal supplementation in an in vitro maturation. A three-dimensional co-culture with granulosa cells enclosed in the core of barium alginate microcapsules applied to human oocytes obtained higher oocyte maturation yields when compared to a routine method i.e. microdrop oocyte culture (90.3% vs. 52%, after 48 hrs of culture). The co-cultured maturational yield of oocytes has been shown eightfold higher than that achieved with a microdrop maturation technique providing a higher number of gametes available for IVF programs (Torre et al., 2006).

1.3.3 Embryo

Several efforts have been made to find a valid method for the amelioration of in vitro conditions for the preservation and the early development of in vitro obtained embryos. Recently sodium alginate and poly-L-lysine has been utilized to microencapsulate embryos and has been proved to be a promising technique.

Alginate encapsulation has been used to protect micromanipulated bovine embryos (Hollingsworth and Page, 1988; Hsu et al., 1995). The artificial membrane of a microcapsule affords protection to intact or manipulated embryos (Willadsen, 1979). Krentz et al., (1993) reported that microencapsulated mouse morulae either in alginate or poly-L-lysine gave results similar to unencapsulated morulae, showing 87.5% of pregnancies for both encapsulation methods, and 71.4% for unencapsulated morulae. However, the smaller percentage of viable fetuses was noted, because mortality occurred in vivo due to an asynchronous condition between the uterine environment

and the embryos. Microencapsulation provided an adequate environment for in vitro development of mouse embryos to the expanded blastocyst stage which did not detrimentally affect embryonic size or cellular development in vitro (Krentz et al., 1993).

Yaniz et al., (2002) has evaluated the in vitro development of microencapsulated embryos and their ability to hatch from the pellucida and capsule. He proposed the bovine model, merging the calcium alginate semisolid three-dimensional matrix and the co-culture with oviduct cells in which the in vitro development rate to the blastocyst at day 7 was similar between encapsulated and unencapsulated embryos. Nevertheless, in practice, the microencapsulation method may offer several advantages over the use of common culture media. The benefits of encapsulation include, easier identification and manipulation of single embryo or groups of embryos, embryos maintain their three-dimensional structure much better than in culture medium (Elsheikh et al., 1997), and encapsulated embryos are more protected against freezing and thawing procedures (Niemann et al., 1986). It is also possible that the capsule around the embryo could be designed to act as a filter of known pore size to restrict the access of certain molecules and viruses (Klein et al., 1983). In vitro production of bovine embryos is rapidly growing due to high commercial prospects (Wrathall, 1995).

Microencapsulation significantly improved the development of embryos to the blastocyst stage regardless of the presence of the natural zona pellucida. The encapsulated embryos developed at a higher rate in the absence of protein as compared with unencapsulated embryos. And also the cell contacts at the 4-cell stage were significantly improved with microencapsulation. Microencapsulation has

improved the in vitro development of pronuclear stage embryos with intact or completely removed zona pellucida as well as micromanipulated embryos to the blastocyst stage (Elsheikh et al., 1997). In spite of this, embryo development rates are far from satisfactory (Bavister, 1995). Alginate encapsulation has been proven to be a viable alternative to culture media for in vitro development of embryo.

1.4 Objectives

The objectives of the study were as following;

1. To develop the preservation method for canine sperm using microencapsulation
2. To characterize the microencapsulation of canine sperm using 0.75% and 1.0% sodium alginate and 0.1% poly-L-lysine
3. To evaluate the influence of microencapsulation on sperm motility and viability during chilling storage
4. To develop a method for cryopreserving microencapsulated canine sperm
5. To investigate whether glycerol equilibrium time affects microencapsulated canine sperm survival
6. And to study whether the quality of frozen-thawed canine sperm improves by microencapsulation

CHAPTER 2

MICROENCAPSULATION OF CANINE SPERM AND ITS PRESERVATION AT 4 °C

2.1 Abstract

The objective of this study was to develop the preservation method for canine sperm using microencapsulation. Pooled ejaculates from three beagles (*Canis familiaris*) were extended in egg yolk Tris extender and were encapsulated in gel (alginate only) or polycation (poly-L-lysine membrane bound) microcapsules at 0.75 and 1% alginate concentration. In Experiment 1, characteristics of microcapsule and microencapsulated sperm were evaluated during chilling storage for 48 hrs. Gel microcapsules at 0.75% alginate concentration had a tear drop-like structure with fragility, whereas those at 1% alginate had a solid spherical structure. In all groups, diameter of the microcapsules increased with duration of storage ($P < 0.05$). Alginate concentration did not affect the sperm recovery rate from microcapsules. Total average recovery rate of sperm from polycation microcapsules was lower than that of gel microcapsules ($P < 0.05$). Progressive motility of polycation microencapsulated sperm and unencapsulated sperm (control) was higher than that of the gel microencapsulated sperm, both at 0.75 and 1.0% alginate concentration ($P < 0.05$), although viability of sperm was similar among the three groups. In Experiment 2, to evaluate the sperm longevity after chilling storage, sperm were microencapsulated in polycation microcapsules at 1.0% alginate concentration, stored at 4 °C for 0, 1, 4 and 7 days, and then cultured at 38.5 °C for 0, 6, and 24 hrs. Progressive motility and viability of microencapsulated sperm were higher than those of unencapsulated

spermatozoa at 0 to 24 hrs of culture after 4 and 7 days of chilling storage ($P < 0.05$). In conclusion, polycation microencapsulation at 1.0% alginate concentration can be successfully applied for chilling storage of canine sperm by maintaining the motility and viability for up to 7 days.

2.2 Introduction

Semen preservation and artificial insemination (AI) in dogs has become a common practice in veterinary medicine. The first successful AI in dogs using chilled semen was reported in 1954 by Harrop et al. Since then, the use of chilled semen for AI has been investigated (Tsutsui et al., 2003; Verstegen et al., 2005) and has tremendously increased in popularity among breeders to obtain genetically superior dogs. The longevity of fresh undiluted semen is very limited (Linde-Forsberg, 1995). To maintain the fertilizability of sperm for more than several days, semen diluted with appropriate extenders should be preserved either by chilling or by freezing. Although chilled sperm has a short life span (less than 4 days), chilling storage of extended semen may be preferable to freezing, due to high fertility rates (Linde-Forsberg, 1995; Linde-Forsberg, 1991; England and Ponzio, 1996). To overcome the limitation of chilling storage by prolonging the survivability and fertility of sperm, an alternative preservation technique using microencapsulation of the semen in alginate and poly-L-lysine has been developed in cattle, pigs, and sheep (Nebel et al., 1993; Huang et al., 2005; Maxwell et al., 1996).

Microencapsulation is the process whereby living cells or tissues are completely encased by a semipermeable membrane, permitting the exchange of nutrients and metabolites (Lim, 1984; Wheatley et al., 1985). Several types of cells and tissues such as hepatocytes, tumor cells, and adrenal cortical cells have been successfully encapsulated (Lim, 1984; Lim and Sun, 1980; Lim and Moss, 1981). These microencapsulated cells remained viable for longer duration than the unencapsulated cells. In the last few decades, the field of cell encapsulation has received much interest with respect to enhancement of reproductive performance both in humans and other mammals for different purposes, including semen controlled release (Nebel et al., 1993; Nebel et al., 1985; Munkittrick et al., 1992; Nebel et al., 1996; Vishwanath et al., 1997; Torre et al., 2000), sperm preservation (Huang et al., 2005; Maxwell et al., 1996; Herrler et al., 2006), in vitro oocytes maturation (Torre et al., 2006), and embryo culture to improve in vitro fertilization yield (Krentz et al., 1993; Elsheikh et al., 1997; Yaniz et al., 2002). The procedures of microencapsulation were first applied by Nebel et al. (Nebel et al., 1985) for bovine sperm. Nebel's concept of microencapsulation of sperm for AI was to maintain sperm viability and high sperm concentrations in the uterus during estrus allowing their release over an extended interval (Nebel et al., 1993; Nebel et al., 1985; Nebel and Saacke, 1995). Promising results of microencapsulation have been published for maintenance of membrane integrity of bovine (Nebel and Saacke, 1995) and porcine (Huang et al., 2005; Esbenshade and Nebel, 1990) sperm and for in vivo fertilization in cattle (Nebel et al., 1996; Nebel and Saacke, 1995), pigs (Huang et al., 2005; Esbenshade and Nebel, 1990), and sheep (Maxwell et al., 1996). That the bitch has a long estrus compared with that of the other

domestic animals, microencapsulation of sperm could be an innovative and alternative technique for maintaining the sperm survivability and fertility and for successful AI in dogs.

In cattle, pigs, and sheep, 0.75% to 1.5% alginate and 0.1% poly-L-lysine were used for microencapsulation of sperm (Huang et al., 2005; Maxwell et al., 1996; Nebel et al., 1985; Nebel et al., 1996). The microencapsulation was successfully applied for chilling storage of porcine and ovine sperm (Huang et al., 2005; Maxwell et al., 1996). However, no study has been reported on microencapsulation of canine sperm. Therefore, the microencapsulation technique for the preservation of canine sperm needs to be established. The objectives of this study were to characterize the microencapsulation of canine sperm using 0.75% and 1.0% sodium alginate and 0.1% poly-L-lysine and to evaluate the influence of microencapsulation on sperm motility and viability during chilling storage.

2.3 Materials and methods

2.3.1 Animals

Three healthy male beagles *Canis familiaris*, aged from 2 to 4 yr, were used. The dogs were kept in the Laboratory Animal Research Center of Tottori University, following the Guide for the Care and Use of Laboratory Animals established by Tottori University (Tottori, Japan).

2.3.2 Semen collection and preparation of sperm suspension

Semen was collected twice a week from each dog by digital manipulation, as described by Linde-Forsberg (2001), in a 15-mL prewarmed calibrated sterile plastic tube (Falcon; Becton Dickinson Labware, Lincoln Park, NJ, USA). The pre-sperm and sperm-rich fractions of the ejaculate were collected and immediately transported to the laboratory. Each ejaculate was analyzed to determine total volume, sperm concentration, progressive motility, and viability. Sperm concentration was determined using a Bürker-Türk hematocytometer. Only ejaculates with sperm concentration $>200\times10^6$ sperm/mL and progressive motility >70% were included in this study. For each replicate, ejaculates collected from three dogs were pooled to allow for the availability of sufficient volume of semen and to avoid individual differences between the ejaculates. Pooled ejaculates were centrifuged at 500 × g for 5 min. The supernatant was discarded, and the sperm pellets were suspended in egg yolk Tris extender (EYT) composed of 2.4 g Tris-hydroxymethyl aminomethane, 1.4 g citric acid, 0.8 g glucose, 100,000 IU Na-benzylpenicillin, 0.1 g streptomycin sulphate, 20 mL egg yolk, and 80 mL distilled water (Pena and Linde-Forsberg, 2000; Michael et al., 2009). The concentrated semen was diluted with EYT to achieve sperm concentration of 100×10^6 sperm/mL. Half of the diluted sperm suspension was used as control, and remainder was used for microencapsulation.

2.3.4 Evaluation of motility and viability of sperm

A 15-µL drop of sperm suspension was placed on a prewarmed glass slide and covered with a coverslip. The percentage of progressively motile sperm was assessed

by subjective microscopic examination using a light microscope at × 400 magnification. Sperm viability was assessed by eosin-nigrosin stain (England and Ponzio, 1996; Christiansen, 1984). A 10-µL drop of sperm suspension was smeared on a clean glass slide. Live and dead sperm were examined on eosin nigrosin stained smears by counting 200 sperm on each slide at × 1000 magnification.

2.3.5 Microencapsulation of canine sperm

Microencapsulation procedures developed for bovine and porcine sperm (Huang et al., 2005; Munkittrick et al., 1992) were used with minor modifications. Briefly, sperm suspension diluted with EYT was mixed with 1.5% (wt/vol) sodium alginate (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) solution dissolved in physiological saline to reach a final concentration of 0.75 and 1.0% sodium alginate. The sperm suspension was forced through a 21-gauge needle attached to 1-mL syringe into a 60 mm plastic dish (Falcon 1007; Becton Dickinson Labware) containing 1.5% (wt/vol) calcium chloride dissolved in physiologic saline. The distance between the tip of the needle and the surface of the calcium chloride solution was maintained at 8.5 cm to ensure the shape of microcapsule. The sperm suspension immediately upon contact with the calcium chloride solution resulted in a solidification of the entire droplet to form a high viscosity microgel. The microgels were swayed gently and allowed to react with calcium ions for 30 sec. The microgels were then collected by filtration using a stainless mesh (40 µm pore size), rinsed three times with physiologic saline, and termed *gel microcapsules*.

The microgels were transferred into 0.1% (w/v) poly-L-lysine (Sigma-Aldrich Chemical Co.) in physiologic saline for 5 min to make a semipermeable membrane on the surfaces of microgels. These poly-L-lysine membrane bound microgels were filtered with the stainless mesh and then rinsed three times with physiologic saline. Immediately after rinsing, the microgels were transferred into 55 mM sodium citrate dissolved in physiologic saline (pH 7.4) for 5 min (Castro et al., 2005). The gel core of the microgels was liquefied by chelation of calcium ions. The poly-L-lysine membrane bound microcapsules were filtered and termed *polycation microcapsules*. All procedures described above for the microencapsulation of canine sperm were performed at room temperature.

2.3.6 Experimental design

2.3.6.1 Experiment 1

This experiment was conducted to characterize the gel and polycation microcapsules and to study the influence of microencapsulation on chilling storage of canine sperm. Two concentrations (0.75% and 1.0%) of alginate were used for microencapsulation. Gel and polycation microcapsules were stored in 2 mL EYT in a 35 mm plastic dish (Falcon 1008; Becton Dickinson Labware) at 4 ^{o}C for 48 hrs. The 2 mL of unencapsulated sperm suspension diluted with EYT (control) was stored in a 15 mL sterile plastic tube at 4 ^{o}C for 48 hrs.

2.3.6.1.1 Evaluation of diameter and membrane thickness of gel and polycation microcapsules

The microcapsules were removed from the samples immediately after encapsulation (0hr) and after 24 and 48 hrs of storage at 4 ^{o}C. Diameters of the microcapsules were measured by an inverted microscope (TS-100; Nikon, Tokyo, Japan). Membrane thickness of microcapsules was measured from photomicrographs of each microcapsule taken by CCD camera (DP-12; Olympus, Tokyo, Japan) attached to the microscope.

2.3.6.1.2 Evaluation of recovery rate of sperm from the gel and polycation microcapsules

The recovery rate of sperm from the microcapsules was evaluated immediately after encapsulation. Initial concentration of sperm suspension in gel and polycation microencapsulation was counted (C_I). Then, the initial volume of microcapsule (V_I) was obtained by measuring the number of capsules from the total volume of sperm suspension used for microencapsulation. Five microcapsules were taken from the samples in a 1.5 mL of microcentrifuge tube containing 400 µL sodium citrate solution. The gel core of the gel microcapsules was dissolved, and the poly-L-lysine membrane of polycation microcapsules was ruptured by vortexing at room temperature. A total volume of sodium citrate solution including microcapsules was evaluated and expressed as V_T. The sperm suspension was used for counting the sperm

concentration in gel and polycation microcapsules (C_C). The recovery rate of sperm from the gel and polycation microcapsules was evaluated as below.

$$\text{Recovery rate (\%)} = (V_T \times C_C) / (V_I \times C_I) \times 100$$

2.3.6.1.3 Evaluation of motility and viability of microencapsulated sperm

Five microcapsules were taken in a 1.5 mL microcentrifuge tube containing 400 µL of sodium citrate solution after 0, 24, and 48 hrs of storage at 4 °C and were vortexed to release the sperm from the microcapsules. Then, 1 mL prewarmed modified Brackett and Oliphant (mBO) medium (Brackett and Oliphant, 1975) was added and centrifuged at 500 × g for 5 min. The supernatant was removed, the pellets were resuspended in mBO medium, and sperm motility and viability were evaluated.

2.3.6.2 Experiment 2

This experiment was conducted to evaluate the longevity of polycation microencapsulated sperm after chilling storage. The polycation microcapsules prepared at 1.0% alginate and 0.1% poly-L-lysine were stored at 4 °C in EYT for 0, 1, 4, and 7 days. Twenty microcapsules preserved at 4 °C were washed with prewarmed mBO medium, transferred into 1 mL mBO medium in the center well of an in vitro fertilization (IVF) culture dish (CLS 3260; Corning Costar Corp, Cambridge, MA, USA), and cultured at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 0, 6, and 24 hrs. In parallel, 200 µL of unencapsulated sperm suspension preserved at 4 °C was

taken in a 1.5 mL microcentrifuge tube, washed with prewarmed 1 mL mBO medium by centrifugation at 500 × g for 5 min, and resuspended in 1 mL mBO medium. The sperm suspension was transferred into an IVF culture dish and cultured for 0, 6, and 24 hrs as control. The motility and viability of encapsulated and unencapsulated sperm were evaluated.

2.3.6.3 Statistical analysis

Statistical analysis was performed using Statview 4.5 software (Abacus Concepts Inc., USA). In Experiment 1, three-way analysis of variance (ANOVA) was used to determine the effect of alginate concentration, microencapsulation method, and duration of chilling storage and their interaction on the diameter of microcapsules, sperm motility, and viability. The effect of alginate concentration and duration of chilling storage and their interaction on the membrane thickness of microcapsules, and the effect of alginate concentration and the microencapsulation method and their interaction on the sperm recovery rate were analyzed by the two-way ANOVA, followed by post hoc analysis using Fisher's protected least significant difference (PLSD). In Experiment 2, three-way ANOVA was used to analyze the interaction among treatment of sperm microencapsulation, storage, and culture period on both motility and viability, followed by post hoc analysis using PLSD. All percentage data were subjected to an arcsine transformation before analysis. Values were presented as means ± SD, and considered statistically significant at $P < 0.05$.

2.4 Results

2.4.1 Experiment 1

2.4.1.1 Diameter and membrane thickness of gel and polycation microcapsules

Gel microcapsules at 0.75% alginate concentration had a tear drop like structure and easily ruptured during the microencapsulation process, whereas those at 1.0% alginate had a spherical shape and were hard to rupture. Diameters of gel and polycation microcapsules at 0.75 and 1.0% alginate concentration are shown in Table 2.1. The three-way ANOVA revealed interaction between alginate concentration, microencapsulation method, and duration of chilling storage. Diameter of the microcapsules increased with the prolongation of storage period in all groups ($P < 0.05$). Membrane thicknesses of gel and polycation microcapsules at 0.75 and 1.0% alginate concentration are given in Table 2.2. Membrane thickness of the gel microcapsules was higher than that of polycation microcapsules when measured immediately after encapsulation (at 0 hr storage) both at 0.75 and 1.0% alginate concentration ($P < 0.05$). Membrane thickness was not detected in gel microcapsules at measurement after 24 and 48 hrs of storage, due to complete gelatinization of the gel core. For the two-way ANOVA, there was no interaction between alginate concentration, microencapsulation method, and duration of storage. Membrane thickness of polycation microcapsules decreased with the prolongation of storage ($P < 0.05$).

Table 2.1 Influence of gel and polycation encapsulation on the diameter of microcapsule

Concentration of alginate (%)	Microcapsule	Diameter of microcapsule at each storage period (mm)		
		0 hr	24 hr	48 hr
0.75	Gel	2.72 ± 0.02^{aA}	2.73 ± 0.02^{aA}	2.96 ± 0.03^{bA}
	Polycation	2.70 ± 0.05^{aA}	2.89 ± 0.08^{bB}	3.01 ± 0.01^{cB}
1	Gel	2.73 ± 0.02^{aA}	3.01 ± 0.01^{bC}	3.42 ± 0.04^{cD}
	Polycation	2.92 ± 0.12^{aB}	3.22 ± 0.03^{bD}	3.32 ± 0.04^{cC}

Values are expressed as Mean ± SD (n= 25).

$^{a-c}$ Within a row, values without a common superscript differed ($P < 0.05$).

$^{A-D}$ Within a column, values without a common superscript differed ($P < 0.05$).

Table 2.2 Influence of gel and polycation encapsulation on the thickness of microcapsule

Concentration of alginate (%)	Microcapsule	Thickness of microcapsule at each storage period (μm)		
		0 hr	24 hr	48 hr
0.75	Gel	325.00 ± 0.03^{A}	ND	ND
	Polycation	1.84 ± 0.20^{aB}	1.49 ± 0.30^{b}	1.29 ± 0.28^{c}
1	Gel	415.00 ± 0.02^{A}	ND	ND
	Polycation	1.80 ± 0.28^{aB}	1.30 ± 0.25^{b}	1.24 ± 0.22^{b}

Values are expressed as Mean ± SD (n=25).

$^{a-c}$ Within a row, values without a common superscript differed ($P < 0.05$).

^{A,B} Within a column, values without a common superscript differed ($P < 0.05$).

ND refers not detected.

2.4.1.2 Recovery rate of sperm from gel and polycation microcapsules

The recovery rates of sperm from gel and polycation microcapsules are given in Table 2.3. The two-way ANOVA revealed no interaction between alginate concentration and the microencapsulation method. The alginate concentration did not affect the sperm recovery rate from microcapsules. Total average recovery rate of sperm from polycation microcapsules was lower than that of gel microcapsules ($P < 0.05$).

2.4.1.3 Motility and viability of microencapsulated sperm

The three-way ANOVA revealed no interaction among alginate concentration, microencapsulation method, and the duration of storage on both progressive motility and viability. Progressive motility and viability of the sperm in all treatments decreased with the prolongation of storage at 4 ^{o}C (Tables 2.4 and 2.5; $P < 0.05$). Progressive motility of the polycation microencapsulated sperm was higher than that of the gel microencapsulated sperm both at 0.75 and 1.0% alginate concentration (Table 2.4; $P < 0.05$), and did not differ from that of unencapsulated sperm. Viability of gel and polycation microencapsulated sperm was similar to that of the unencapsulated sperm both at 0.75 and 1.0% alginate concentration (Table 2.5; $P < 0.05$).

Table 2.3 Influence of gel and polycation encapsulation on the recovery of canine sperm from the microcapsule

Concentration of alginate (%)	Sperm recovery rate from microcapsule (%)	
	Gel	Polycation
0.75	89.9 ± 3.3	76.6 ± 4.1
1	80.8 ± 15.5	71.0 ± 12.1
Total	85.3 ± 11.5[a]	73.8 ± 8.9[b]

Values are expressed as Mean ± SD for 4 replicates each using 5 microcapsules.
[a,b] Within a row, values without a common superscript differed ($P < 0.05$).

Table 2.4 Progressive motility of microencapsulated canine sperm stored at 4 °C

Concentration of alginate (%)	Microcapsule	Sperm motility at each storage period (%)			
		0 hr	24 hr	48 hr	Total
0	Control	82.4 ± 2.5^{a}	68.4 ± 5.2^{b}	60.4 ± 6.1^{c}	70.4 ± 10.5^{A}
0.75	Gel	69.4 ± 1.3	57.4 ± 5.5	53.4 ± 7.0	60.1 ± 8.6^{B}
	Polycation	80.6 ± 2.6	67.6 ± 7.5	62.0 ± 7.5	70.1 ± 10.0^{A}
	Total	75.0 ± 6.2^{a}	62.5 ± 8.2^{b}	57.7 ± 8.2^{c}	
1	Gel	69.4 ± 1.3	59.0 ± 7.4	49.0 ± 7.4	59.1 ± 10.3^{B}
	Polycation	83.0 ± 2.7	71.0 ± 5.4	63.0 ± 6.7	72.3 ± 9.8^{A}
	Total	76.2 ± 7.4^{a}	65.0 ± 8.8^{b}	56.0 ± 9.9^{c}	

Values are Mean ± SD for five replicates each being a pool of three ejaculates.

$^{a-c}$ Within a row, values without a common superscript differed ($P < 0.05$).

^{A,B} Within a column, values without a common superscript differed ($P < 0.05$).

Control refers unencapsulated sperm.

Table 2.5 Viability of microencapsulated canine sperm stored at 4 °C

Concentration of alginate (%)	Microcapsule	Sperm viability at each storage period (%)		
		0 hr	24 hr	48 hr
0	Control	89.0 ± 1.8[a]	76.2 ± 5.0[b]	71.0 ± 3.2[c]
0.75	Gel	83.9 ± 2.4	74.9 ± 2.9	70.5 ± 2.9
	Polycation	85.0 ± 3.4	75.5 ± 4.5	72.5 ± 3.7
	Total	84.4 ± 2.9[a]	75.2 ± 3.6[b]	71.5 ± 3.3[c]
1	Gel	84.8 ± 1.6	74.2 ± 5.0	71.2 ± 5.0
	Polycation	86.9 ± 1.0	76.3 ± 5.0	73.3 ± 3.8
	Total	85.8 ± 1.6[a]	75.2 ± 4.9[b]	72.2 ± 4.3[c]

Values are Mean ± SD for five replicates each being a pool of three ejaculates.
[a-c] Within a row, values without a common superscript differed ($P < 0.05$).
Control refers unencapsulated sperm.

2.4.2 Experiment 2

The results for progressive motility and viability of polycation microencapsulated sperm after culture are given in Tables 2.6 and 2.7. There was a significant interaction between treatment of sperm microencapsulation and culture period for both progressive motility and viability. The progressive motility and viability of microencapsulated sperm did not differ from that of the unencapsulated sperm (control) immediately after 1 day storage at 4 ^{o}C ($P < 0.05$). The motility and viability of both microencapsulated and unencapsulated sperm decreased with prolongation of the storage and culture periods. However, the motility and viability of microencapsulated sperm were higher than that of unencapsulated sperm at 24 hrs of culture without storage (after 0 day storage), at 6 and 24 hrs of culture after 1 day storage, and 0 to 24 hrs of culture after 4 and 7 day of chilling storage ($P < 0.05$). Furthermore, the viability of microencapsulated sperm was higher than that of unencapsulated sperm at 6 hrs of culture without storage ($P < 0.05$).

Table 2.6 Progressive motility of polycation microencapsulated canine sperm cultured at 38.5 °C after storage at 4 °C

Storage period (Day)	Treatment	Sperm motility at each culture period (%)		
		0 hr	6 hr	24 hr
0	Control	84.0 ± 3.5^{a}	66.6 ± 6.0^{b}	36.6 ± 4.9^{Ac}
	Encapsulated	82.5 ± 2.7^{a}	76.6 ± 4.0^{b}	50.8 ± 4.9^{Bc}
1	Control	68.6 ± 4.7^{a}	36.6 ± 6.8^{Ab}	8.3 ± 0.5^{Ac}
	Encapsulated	70.5 ± 5.6^{a}	62.0 ± 2.4^{Bb}	32.5 ± 6.8^{Bc}
4	Control	49.8 ± 4.0^{Aa}	30.8 ± 2.4^{Ab}	4.3 ± 1.2^{Ac}
	Encapsulated	62.0 ± 4.4^{Ba}	53.8 ± 5.8^{Bb}	34.1 ± 5.6^{Bc}
7	Control	36.1 ± 3.1^{Aa}	16.1 ± 3.7^{Ab}	3.0 ± 0.6^{Ac}
	Encapsulated	50.0 ± 3.1^{Ba}	38.1 ± 6.9^{Bb}	22.1 ± 5.8^{Bc}

Values are Mean ± SD for six replicates each being a pool of three ejaculates.

$^{a-c}$ Within a row, values without a common superscript differed ($P < 0.05$).

^{A,B} Values without a common superscript differed between treatments at the same culture period ($P < 0.05$).

Control refers unencapsulated sperm.

Table 2.7 Viability of polycation microencapsulated canine sperm cultured at 38.5 °C after storage at 4 °C

Storage period (Day)	Treatment	Sperm viability at each culture period (%)		
		0 hr	6 hr	24 hr
0	Control	89.8 ± 2.9^{a}	74.0 ± 3.2^{Ab}	52.1 ± 2.6^{Ac}
	Encapsulated	87.0 ± 1.0^{a}	80.5 ± 3.0^{Bb}	66.1 ± 5.6^{Bc}
1	Control	77.0 ± 4.9^{a}	53.0 ± 2.9^{Ab}	40.7 ± 3.0^{Ac}
	Encapsulated	77.4 ± 5.2^{a}	69.5 ± 5.3^{Bb}	57.3 ± 5.8^{Bc}
4	Control	60.8 ± 2.0^{Aa}	42.4 ± 2.5^{Ab}	27.5 ± 4.9^{Ac}
	Encapsulated	71.0 ± 4.1^{Ba}	64.1 ± 2.2^{Bb}	53.6 ± 2.7^{Bc}
7	Control	52.7 ± 2.4^{Aa}	37.5 ± 3.9^{Ab}	22.8 ± 6.6^{Ac}
	Encapsulated	66.3 ± 3.7^{Ba}	55.5 ± 3.5^{Bb}	44.3 ± 4.9^{Bc}

Values are Mean ± SD for six replicates each being a pool of three ejaculates.

$^{a-c}$ Within a row, values without a common superscript differed ($P < 0.05$).

^{A,B} Values without a common superscript differed between treatments at the same culture period ($P < 0.05$).

Control refers unencapsulated sperm.

2.5 Discussion

In the current study, the microencapsulation method and duration of chilling storage affected the shape of microcapsules. This is apparently the first report that describes successful encapsulation of canine sperm for chilling storage.

The gel and polycation microcapsules were swollen during the storage, which caused an increase in diameter. In polycation microcapsules, the membrane thickness decreased during storage. Morphological changes of microcapsules during the chilling storage have not been reported. The current results indicate absorption of extender used for preservation in the alginate gel and influx of the extender through semipermeable polycation membrane. The alginate gel at 0.75 to 1.5% concentration has been used for microencapsulation of sperm (Huang et al., 2005; Maxwell et al., 1996; Nebel et al., 1985; Nebel et al., 1996). In this study, gel microcapsules at 0.75% alginate concentration had a tear drop-like structure with fragility, whereas those at 1.0% alginate had a solid spherical structure. Therefore, in our microencapsulation technique, maintenance of the spherical shape of microcapsules requires a higher concentration of alginate (1.0%).

This study also evaluated the recovery rate of canine sperm from microcapsules immediately after encapsulation. In the previous report, sperm were recovered from the microcapsules by stirring (Huang et al., 2005). An in vitro release test revealed that sperm release from the microcapsules was enhanced at 110 rpm stirring but resulted in low release rate with short incubation (9% to 15%)(Huang et al., 2005). In the current study, sperm were easily recovered from the alginate gel microcapsules by vortexing without incubation, and the alginate concentration did not influence sperm

recovery from the microcapsules. Polycation microcapsule revealed significantly lower sperm recovery rate than that of gel microcapsules (73.8% vs. 85.3%). Sperm may be tightly trapped in the poly-L-lysine membrane and cannot be easily released, even by vortexing.

In this study, progressive motility of gel encapsulated canine sperm both at 0.75% and 1.0% alginate concentration was significantly lower than that of the polycation encapsulated and unencapsulated sperm, without significant reduction in sperm viability. Decrease in the motility and velocity of gel encapsulated sperm has also been reported in pig (Torre et al., 2000). In this study, complete gelatinization of the gel core was observed in the gel microcapsules stored for 24 and 48 hrs at 4 ^{o}C. Therefore, viscous alginate matrix in the gel microcapsule may physically interfere with kinetic activity of canine sperm. The motility of polycation encapsulated sperm did not differ from that of the unencapsulated sperm during 48 hrs of chilling storage. Liquefaction of the alginate gel core during preparation of the polycation microcapsules may prevent damage to the sperm kinetic system. The diameter and membrane thickness of the microcapsules showed no relation to the motility and viability of microencapsulated bovine sperm (Nebel et al., 1985; Nebel et al., 1996; Nebel and Saacke, 1994). The swelling of microcapsules during chilling storage observed in this study may indicate that permeability of alginate gel and polycation membrane facilitated maintenance of the viability of encapsulated canine sperm by exchange of nutrients from the extender. Various extenders have been used for microencapsulation of sperm (Huang et al., 2005; Maxwell et al., 1996; Nebel et al., 1996). A component of the extender might affect longevity of the microencapsulated sperm.

Considering the results of microcapsule formation and the motility and viability of recovered sperm from the microcapsules (Experiment 1), the polycation microcapsules at 1.0% alginate concentration is recommended for microencapsulation of canine sperm for chilling storage, despite a low sperm recovery rate.

In Experiment 2, it was clearly indicated that progressive motility and viability of the polycation microencapsulated canine sperm at 1.0% alginate concentration was enhanced compared with that of unencapsulated sperm after 4 and 7 days of storage at 4 ^{o}C. Furthermore, the microencapsulated sperm had high motility at 24 hr of culture at 38.5 ^{o}C without storage and at 6 and 24 hrs of culture after 1 day of chilling storage. The polycation microencapsulation was first demonstrated to prevent a rapid decrease in the motility and viability of encapsulated canine sperm during chilling storage. The polycation encapsulated porcine sperm had higher motility than that of unencapsulated sperm at 56, 24, and 8 hrs of culture at 37 ^{o}C after 2, 3, and 4 to 7 days of storage at 5 ^{o}C, respectively (Huang et al., 2005). Therefore, effectiveness of polycation microencapsulation on sperm motility seems to become significant with prolongation of chilling storage. The microencapsulation of canine sperm provided a better environment and protects the sperm allowing the exchange of nutrients, which enhanced the motility and viability with prolongation of storage.

In conclusion, polycation microencapsulation at 1.0% alginate concentration was successfully applied for chilling storage of canine sperm and maintained the motility and viability up to 7 days. Further studies are needed to examine the membrane integrity and fertility of microencapsulated canine sperm both in vivo and in

vitro. In addition, the application of microencapsulation for cryopreservation of canine sperm remains to be investigated.

CHAPTER 3

CRYOPRESERVATION OF MICROENCAPSULATED CANINE SPERM

3.1 Abstract

The objective was to develop a method for cryopreserving microencapsulated canine sperm. Pooled ejaculates from three beagle dogs were extended in egg yolk tris extender and encapsulated using alginate and poly-L-lysine at room temperature. The microcapsules were cooled at 4 °C, immersed in pre-cooled extender (equivalent in volume to the microcapsules) to reach final concentration of 7% (v/v) glycerol and 0.75% (v/v) Equex STM paste, and equilibrated for 5, 30 and 60 min at 4 °C. Thereafter, microcapsules were loaded into 0.5 mL plastic straws and frozen in liquid nitrogen. In Experiment 1, characteristics of microencapsulated canine sperm were evaluated after glycerol addition at 4 °C. Glycerol exposure for 5, 30 and 60 min did not significantly affect progressive motility, viability, or acrosomal integrity of microencapsulated sperm compared with pre-cooled unencapsulated sperm (control). In Experiment 2, characteristics of frozen-thawed canine microencapsulated sperm were evaluated at 0, 3, 6, and 9 hrs of culture at 38.5 °C. Pre-freeze glycerol exposure for 5, 30, and 60 min at 4 °C did not influence post-thaw quality in unencapsulated sperm. Post-thaw motility and acrosomal integrity of microencapsulated sperm decreased more than those of unencapsulated sperm ($P < 0.05$) following glycerol exposure for 5 min. However, motility, viability and acrosomal integrity of microencapsulated sperm after 30 and 60 min glycerol exposure were higher than

unencapsulated sperm cultured for 6 or 9 hrs ($P < 0.05$). In conclusion, since microencapsulated canine sperm were successfully cryopreserved, this could be a viable alternative to convention sperm cryopreservation in this species.

3.2 Introduction

Cryopreservation of dog semen has been used for assisted reproductive technology in this species (Ivanova-Kicheva et al., 1997). The first successful AI in dogs using frozen-thawed semen was well documented by Seager in (1969). Since then, freezing technology has become a valuable tool for the conservation of genetic materials of superior breeds of dogs, and for conservation of endangered canids. Furthermore, its use is increasing among breeders to improve the breeding potential of their stud dogs (Pena et al., 2006; Zindl et al., 2006). Several methods, using different types of packaging systems (including straws, pellets, ampoules and aluminum tubes) have been described for freezing canine sperm (Ivanova-Kicheva et al., 1997; Battista et al., 1988; Thomas et al., 1993; Olar et al., 1989; Govette et al., 1996). Regardless, fertilizability of frozen-thawed semen after insemination remains variable (Linde-Forsberg, 1991; Linde-Forsberg et al., 1999). Poor post-thaw quality and short sperm lifespan after thawing are major impediments for using cryopreserved canine sperm (Oettle, 1986; England, 1993). To overcome current limitations, cryopreservation of microencapsulated canine sperm could be a viable alternative.

Microencapsulation is the process whereby living cells or tissues are completely encased by a semipermeable membrane, permitting the exchange of nutrients and metabolites (Lim, 1984; Wheatly et al., 1985). Microencapsulation of sperm for AI

maintained sperm viability and sperm concentrations in the uterus during estrus, thereby allowing their release over an extended interval (Nebel et al., 1993; Nebel et al., 1985; Nebel and Saacke, 1995). Microencapsulation of sperm has been applied successfully for the enhancement of reproductive performance for various purposes, including semen controlled release (Nebel et al., 1993; Nebel et al., 1985; Munkittrick et al., 1992; Nebel et al., 1996; Vishwanath et al., 1997; Torre et al., 2000), sperm preservation (Huang et al., 2005; Maxwell et al., 1996; Shah et al., 2010), in vitro oocyte maturation (Torre et al., 2006), and embryo culture to improve in vitro fertilization yield (Krentz et al., 1993; Elsheikh et al., 1997; Yaniz et al., 2002). Microencapsulation of sperm has been reported, but not widely adopted in cattle, pigs and sheep, by prolonging the survivability and fertility of sperm at chilling storage (Nebel et al., 1993; Huang et al., 2005; Maxwell et al., 1996). Promising results of microencapsulation at 5 ^{o}C storage have been reported for maintenance of membrane integrity and for in vivo fertilization in pigs and sheep (Huang et al., 2005; Maxwell et al., 1996). Preservation of microencapsulated canine sperm has been applied by prolonging motility and viability relative to unencapsulated sperm after chilling storage for 4 to 7 days (Shah et al., 2010).

However, apparently there are no reports regarding cryopreservation of microencapsulated sperm in any species, including dogs. The objectives of the present study were to investigate whether: i) glycerol equilibrium time affected microencapsulated canine sperm survival; and ii) quality of frozen-thawed canine sperm was improved if the sperm was microencapsulated before cryopreservation.

3.3 Materials and methods

3.3.1 Animals

Three healthy male beagle dogs, aged 2 to 4 yrs, were used. The dogs were kept in the Laboratory Animal Research Center of Tottori University, in accordance with the Guide for the Care and Use of Laboratory Animals, approved by Tottori University, Japan.

3.3.2 Semen collection and preparation of sperm suspension

Semen was collected twice weekly from each dog by digital manipulation, as described (Linde-Forsberg, 2001), in a 15 mL pre-warmed calibrated sterile plastic tube (Falcon; Becton Dickinson Labware, Lincoln Park, NJ, USA). Fifteen ejaculates were collected from the three dogs (five from each dog). The pre-sperm and sperm-rich fractions of the ejaculate were collected and immediately transported to the laboratory. Each ejaculate was analyzed to determine its volume, sperm concentration, progressive motility, viability, acrosomal integrity, and morphological abnormalities. Sperm concentration was determined using a Bürker-Türk hematocytometer. Only ejaculates with $>200\times10^6$ sperm/mL, progressive motility >70%, and normal morphology >80% were included in this study. For each replicate, ejaculates collected from three dogs were pooled to allow for the availability of sufficient volume of semen and to avoid individual differences among ejaculates. Pooled ejaculates were centrifuged at 500 × g for 5 min. The supernatant was discarded and sperm pellets were suspended in egg yolk tris extender (EYT) composed of 2.4 g Tris-hydroxymethyl

aminomethane, 1.4 g citric acid, 0.8 g glucose, 100,000 IU Na-benzylpenicillin, 0.1 g streptomycin sulphate, 20 mL egg yolk and 80 mL distilled water (Pena and Linde-Forsberg, 2000; Michael et al., 2009). The concentrated semen was diluted with EYT to achieve sperm concentration of 100 × 10^6 sperm/mL. The diluted sperm suspension was used as a control and for microencapsulation.

3.3.3 Semen evaluation

A 10 µL drop of sperm suspension was placed on a pre-warmed glass slide and covered with a 18 mm × 18 mm size coverslip. The percentage of progressively motile sperm was assessed by subjective microscopic examination, using a light microscope at × 400 magnification. Motility was subjectively classified into the following five grades: +++, rapid forward progression; ++, moderate forward progression; +, motile with no progression; ±, motile without any vigour; and -, immotile. Sperm exhibiting a motility grade of rapid forward progression and moderate forward progression were used as sperm progressive motility. Sperm viability was assessed by eosin staining with a minor modification (Burgos and Paola, 1951). The sperm suspension was diluted 1:1 (v/v) with 0.125% eosin Y dissolved in 2.9% sodium citrate solution, placed on a slide, cover slipped, and evaluated. Dead sperm cells were stained red, whereas the viable sperm remained unstained, with or without tail movement. Live and dead sperm were examined by counting 200 sperm on each slide at × 400 magnification. For examination of acrosomal integrity, a drop of sperm was micro-pipetted on a slide to make a thin smear. Then, the air dried smear of semen was fixed for 15 min in 10%

buffered formal saline and stained for 90 min in a 7.5% (v/v) buffered solution of Giemsa stain (Kanto Chemical Co., Inc., Tokyo, Japan) (Watson, 1975; Didion et al., 1989). Stained slides were dried and mounted and then assessed by microscopy with oil immersion at ×1000 magnification. Acrosomal integrity was assessed as a percentage of sperm with dark acrosomal caps. For evaluation of sperm acrosomal status, at least 200 sperm were counted.

3.3.4 Microencapsulation of canine sperm

Microencapsulation procedures developed for canine sperm (Shah et al., 2010) were used, with minor modifications. Briefly, a sperm suspension diluted with EYT was mixed (1:2) with 1.5% (w/v) sodium alginate (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) solution dissolved in physiological saline to reach a final concentration of 1.0% sodium alginate. The sperm suspension was forced through a 21 gauge needle attached to 1 mL syringe into a 60 mm plastic dish (Falcon 1007; Becton Dickinson Labware) containing 1.5% (w/v) calcium chloride dissolved in physiological saline. The distance between the tip of needle and the surface of calcium chloride solution was maintained at 8.5 cm to ensure the shape of microcapsule. The sperm suspension immediately upon contact with the calcium chloride solution resulted in a solidification of the entire droplets to form a microgel. The microgels were swayed gently and allowed to react with calcium ions for 30 sec. The microgels were then collected by filtration using a stainless mesh (40 μm pore size), rinsed three times with physiological saline. Then, the microgels were transferred into 0.1% (w/v) poly-L-lysine

(Sigma-Aldrich Chemical Co.) in physiological saline for 5 min to make a semipermeable membrane on the surface of microgels. These poly-L-lysine membrane bound microgels were filtered with the stainless mesh and then rinsed three times with physiological saline. Immediately after rinsing, the microgels were transferred into 55 mM sodium citrate dissolved in physiological saline, (pH 7.4) for 5 min. The alginate gel core of the microgels was liquefied by chelation of calcium ions. The membrane bound microcapsules were filtered and used for cryopreservation. The concentration of sperm in microcapsules was 33×10^6 sperm/mL after dissolving the alginate. The volume of each microcapsule was 10 uL, which was obtained by measuring the number of capsules from the total volume of sperm suspension used for microencapsulation. All the procedures described above for the microencapsulation of canine sperm were performed at room temperature.

3.3.5 Experimental design

3.3.5.1 Experiment 1

This experiment was conducted to study the effect of glycerol equilibrium duration on characteristics of microencapsulated canine sperm after cooled storage. Microcapsules were cooled to 4 ^{o}C in a 35 mm plastic dish (Falcon 1008; Becton Dickinson Labware) in a refrigerator. After cooling and storage at 4 ^{o}C, the pre-cooled second extender, which was equivalent volume to the microcapsules and composed of EYT supplemented with final concentration of 7% (v/v) of glycerol and 0.75% (v/v) Equex STM paste (Nova Chemical Sales, Scituate, MA, USA) was added to the plastic

dish, and microcapsules were stored for equilibrium for 5, 30 and 60 min at 4 °C. The unencapsulated sperm suspension was also stored in 15 mL sterile plastic tube at 4 °C, as mentioned above.

3.3.5.1.1 Evaluation of microencapsulated sperm

After glycerol equilibration at 4 °C, 15 microcapsules were taken in the 1.5 mL microcentrifuge tube and were pipetted to release the sperm from the microcapsules. Then, 500 uL of pre-warmed modified Brackett and Oliphant (mBO) medium (Brackett and Oliphant, 1975) was added and sperm characteristics were evaluated as described above.

3.3.5.2 Experiment 2

This experiment was conducted to study the effect of pre-freeze glycerol exposure on the characteristics of frozen-thawed microencapsulated canine sperm. Freezing of microcapsules were performed as previously described for freezing canine sperm (Abe et al., 2008; Rota et al., 1997), with modifications. The microcapsules were cooled to 4 °C in a refrigerator, then the equivalent volume of the pre-cooled second extender was added to the microcapsules, and the extended semen was kept for 5, 30 and 60 min at 4 °C for equilibration (as in Experiment 1). Thereafter, 20 microcapsules were loaded into a 0.5 mL plastic straw (Fujihira, Tokyo, Japan) and sealed with polyvinyl alcohol powder (Sigma Chemical Co., St. Louis, MO, USA). Straws were placed horizontally in

liquid nitrogen (LN_2) vapor, 7 cm above the surface of LN_2, in a closed Stainless Dewar Flask (185 mm×270 mm, 6 Lt) for 5 min, and then plunged into the LN_2. The straws were stored in the LN_2 container for at least 1 day. After removal from storage, straws were thawed in a water bath at 37 °C for 30 sec, and their contents expelled into a culture dish.

Intact microcapsules were used for further experiment. Microcapsules were washed with pre-warmed mBO medium. Eighty microcapsules were transferred into 1 mL mBO medium in the center well of IVF culture dish (CLS 3260; Corning Costar Corp, Cambridge, MA, USA) and cultured under 5% CO_2, 95% humidity in air at 38.5 °C for 0, 3, 6, and 9 hrs. Characteristics of microencapsulated sperm were evaluated at each culture period. The unencapsulated sperm suspension (control) was also frozen in 0.5 mL plastic straw, thawed and centrifuged at 500 × g for 5 min, suspended in 1 mL mBO, and cultured as mentioned above.

3.3.6 Statistical analysis

Statistical analysis was performed using StatView for Windows 5.0 software (SAS Institute Inc., Cary, NC, USA). In Experiment 1, the effects of microencapsulation and glycerol equilibration time and their interaction on the sperm motility, viability and acrosomal integrity were analyzed by two-way ANOVA. In Experiment 2, the effect of microencapsulation, glycerol equilibration time and the duration of culture period and their interaction on the sperm motility, viability and acrosomal integrity were analyzed by three-way ANOVA, followed by post hoc analysis using Fisher's protected least

significant difference (PLSD). All percentage data were subjected to an arcsine transformation before analysis. Values are presented as means ± SD and considered statistically significant at $P < 0.05$.

3.4 Results

3.4.1 Experiment 1

The results of the effect of glycerol equilibrium duration on the characteristics of microencapsulated canine sperm during cooled storage are shown (Table 3.1). There was no significant interaction between microencapsulation and equilibrium time. Progressive motility, viability and acrosomal integrity after equilibrium with glycerol did not differ significantly from those of unencapsulated and microencapsulated sperm.

3.4.2 Experiment 2

The effects of pre-freeze glycerol exposure on the characteristics of frozen-thawed unencapsulated and microencapsulated canine sperm evaluated at 0, 3, 6, and 9 hrs of culture at 38.5 °C are shown (Tables 3.2, 3.3, and 3.4). There was an interaction among microencapsulation, glycerol equilibration time, and the duration of culture period on the sperm motility, viability, and acrosomal integrity. Sperm motility and acrosomal integrity immediately after thawing decreased in microencapsulated sperm exposed to glycerol for only 5 min ($P < 0.05$).

Table 3.1 Characteristics of microencapsulated canine sperm after glycerol exposure at 4 °C

Treatment		Glycerol exposure (min)	Sperm characteristics (%)*		
			Motility	Viability	Acrosomal integrity
Control	Initial		83.6±4.6	89.4±3.6	91.4±2.8
	Cooled	5	80.6±2.7	86.7±2.5	89.1±3.3
		30	81.4±2.5	86.8±2.9	89.4±2.8
		60	81.6±4.9	87.1±3.2	89.8±2.5
Encapsulated	Initial		82.8±4.1	88.8±3.0	91.1±3.0
	Cooled	5	81.1±3.2	86.1±1.7	89.6±1.8
		30	80.8±3.0	85.6±3.7	89.5±2.5
		60	81.4±3.3	86.5±3.2	89.7±2.3

Control = unencapsulated sperm.

Initial = ejaculated sperm.

*Values are mean ± SD for five replicates, each being a pool of three ejaculates.

Sperm motility, viability and acrosomal integrity decreased both in unencapsulated and encapsulated sperm with prolongation of the culture period ($P < 0.05$). Progressive motility and viability of microencapsulated sperm with glycerol exposure for 30 and 60 min was higher than the unencapsulated sperm after culture for 6 and 9 hrs, whereas with 5 min glycerol exposure, both progressive motility and viability were lower than all the glycerol exposed microencapsulated and unencapsulated sperm. Percentages of intact acrosomes were higher in microencapsulated sperm with glycerol exposure for 30 and 60 min after culture for 6 and 9 hrs than the unencapsulated sperm, whereas with 5 min glycerol exposure, intact acrosomes were lower than the all treatments during the culture period ($P < 0.05$).

Table 3.2 Progressive motility of cryopreserved microencapsulated canine sperm cultured at 38.5 °C

Treatment	Glycerol exposure (min)	Sperm motility at each culture period (%)*			
		0 hr	3 hr	6 hr	9 hr
Control	5	43.8 ± 5.2^{a}	29.0 ± 4.4^{b}	13.8 ± 1.7^{c}	6.0 ± 1.2^{d}
	30	42.6 ± 4.3^{a}	29.2 ± 2.3^{b}	13.0 ± 2.0^{c}	6.2 ± 1.3^{d}
	60	43.6 ± 5.6^{a}	30.6 ± 2.4^{b}	13.6 ± 2.0^{c}	6.6 ± 1.5^{d}
Encapsulated	5	$20.4\pm2.7^{Aa**}$	$17.2\pm1.7^{Aa**}$	$7.8\pm1.3^{Ab**}$	$2.4\pm0.5^{Ab**}$
	30	43.4 ± 5.1^{Ba}	31.2 ± 6.8^{Bb}	$21.6\pm3.1^{Bc**}$	$12.4\pm2.0^{Bd**}$
	60	43.2 ± 4.6^{Ba}	31.0 ± 4.9^{Bb}	$20.8\pm3.4^{Bc**}$	$12.6\pm2.6^{Bd**}$

Control = unencapsulated sperm.

$^{a-d}$Within a row, values without a common superscript differed ($P < 0.05$).

^{A,B}Within a column, values without a common superscript within a treatment differed ($P < 0.05$).

**Within a column, values of corresponding glycerol exposure differed from control ($P < 0.05$).

*Values are mean ± SD for five replicates, each being a pool of three ejaculates.

Table 3.3 Viability of cryopreserved microencapsulated canine sperm cultured at 38.5 °C

Treatment	Glycerol exposure (min)	Sperm viability at each culture period (%)*			
		0 hr	3 hr	6 hr	9 hr
Control	5	73.4±4.4^{a}	62.8±3.8^{b}	47.0±4.7^{c}	34.2±4.8^{d}
	30	74.4±3.9^{a}	61.4±3.2^{b}	47.2±4.7^{c}	35.4±3.7^{d}
	60	74.0±4.2^{a}	63.2±2.2^{b}	49.0±3.3^{c}	35.8±3.9^{d}
Encapsulated	5	69.8±4.4^{a}	48.6±4.2^{Ab**}	37.4±4.3^{Ac**}	22.2±3.8^{Ad**}
	30	73.8±3.8^{a}	63.8±4.4^{Bb}	59.0±4.8^{Bb**}	46.6±3.7^{Bc**}
	60	74.0±3.9^{a}	64.8±5.4^{Bb}	59.8±4.7^{Bb**}	47.2±4.3^{Bc**}

Control = unencapsulated sperm.

$^{a-d}$Within a row, values without a common superscript differed ($P < 0.05$).

^{A,B}Within a column, values without a common superscript within a treatment differed ($P < 0.05$).

**Within a column, values of corresponding glycerol exposure differed from control ($P < 0.05$).

*Values are mean ± SD for five replicates, each being a pool of three ejaculates.

Table 3.4 Acrosomal integrity of cryopreserved microencapsulated canine sperm cultured at 38.5 °C

Treatment	Glycerol exposure (min)	Acrosomal integrity at each culture period (%)*			
		0 hr	3 hr	6 hr	9 hr
Control	5	77.4 ± 1.8^{a}	66.8 ± 2.9^{b}	57.6 ± 3.0^{c}	43.8 ± 2.1^{d}
	30	78.1 ± 2.3^{a}	66.8 ± 3.0^{b}	57.6 ± 2.0^{c}	44.2 ± 3.1^{d}
	60	78.2 ± 2.5^{a}	67.4 ± 3.2^{b}	58.2 ± 2.0^{c}	44.4 ± 2.8^{d}
Encapsulated	5	$70.6\pm4.2^{Aa**}$	$58.4\pm2.5^{Ab**}$	$47.0\pm4.4^{Ac**}$	$34.8\pm3.2^{Ad**}$
	30	78.8 ± 3.3^{Ba}	68.8 ± 3.0^{Bb}	$65.0\pm3.0^{Bb**}$	$53.2\pm5.5^{Bc**}$
	60	79.0 ± 2.5^{Ba}	69.6 ± 3.2^{Bb}	$64.4\pm3.1^{Bb**}$	$54.0\pm5.2^{Bc**}$

Control = unencapsulated sperm.

$^{a-d}$Within a row, values without a common superscript differed ($P < 0.05$).

^{A,B}Within a column, values without a common superscript within a treatment differed ($P < 0.05$).

**Within a column, values of corresponding glycerol exposure differed from control ($P < 0.05$).

*Values are mean ± SD for five replicates, each being a pool of three ejaculates.

3.5 Discussion

In the present study, post-thaw progressive motility, viability and acrosomal integrity were evaluated after culture at 38.5 °C in unencapsulated and microencapsulated canine sperm with pre-freeze glycerol exposure for 5, 30 and 60 min. Although there are numerous reports on cryopreserving canine semen, this was apparently the first to describe the successful cryopreservation of microencapsulated canine sperm.

The preferred criteria for the evaluation of cryopreservation methods have been assessment of post-thaw motility and fertility. Sperm survival during post-thaw incubation at body temperature is also considered important to assess its potential fertilizing ability. To evaluate sperm post-thaw recovery and survival over time, samples were incubated at 38 °C, and motility and proportion of sperm cells with an intact plasma membrane were assessed (Rota et al., 1997).

In Experiment 1, the effects of cooling and glycerol addition at 4 °C on the microencapsulated canine sperm characteristics were determined. Progressive motility, viability and acrosomal integrity after cooling and glycerol addition compared with initial sperm were not significantly different in unencapsulated and microencapsulated sperm. Therefore, for both unencapsulated and microencapsulated sperm we concluded that cooling and glycerol exposure at 4 °C had no effect on pre-freeze canine sperm quality compared with ejaculated sperm.

In parallel with our results, cooling and glycerol equilibrium time at 5 °C had no effect on pre-freeze canine sperm motility compared with initial sperm motility; there

was no significant decline in motility due to cooling and glycerol exposure for 1 and 2 hrs, compared with initial motility (Olar et al., 1989). In another study, glycerol exposure for 30 min at 5 °C had no significant effect on motility and plasma membrane integrity when compared with fresh and equilibrated sperm (Rota et al., 1997). Motility, sperm membrane integrity and acrosome-reacted sperm did not significantly differ between the fresh and chilled semen samples stored at 5 °C for 24 hrs (Kumi-Diaka and Badtram, 1994). Therefore, canine sperm motility, viability and acrosomal integrity were not affected, and sperm withstand cooling and glycerol equilibrium in unencapsulated and microencapsulated sperm at 4 °C.

In Experiment 2, post-thaw motility, viability and acrosomal integrity decreased when sperm were incubated at 38.5 °C, with increasing culture duration in unencapsulated and microencapsulated sperm. Pre-freeze glycerol exposure for 5, 30, and 60 min did not cause any significant changes during the culture period in unencapsulated sperm. Although, pre-freeze exposure for 30 and 60 min did not cause any significant differences during the culture period for microencapsulated sperm, these end points were significantly decreased in microencapsulated sperm exposed to glycerol for 5 min. Progressive motility and viability at all glycerol exposure declined more quickly in unencapsulated sperm and were significantly lower than in microencapsulated sperm after 6 and 9 hrs of culture.

Effects of pre-freeze glycerol exposure on the post-thaw characteristics have been well described for canine (Pena et al., 1998; Okano et al., 2004) and boar sperm (Almlid and Johnson, 1988). In that regard, pre-freeze glycerol exposure for 0 to 4 hrs had no significant effect on the post-thaw progressive motility and acrosomal integrity

in canine sperm (Okano et al., 2004). Further, glycerol equilibration may take only a few seconds if sperm were sufficiently cooled (at 4 °C) before addition of glycerol. There were no significant differences in post-thaw progressive motility, viability and intact acrosome after incubation up to 4 hrs at 39 °C between 5 min and 1 hr pre-freeze glycerol exposure (Pena et al., 1998). In boar sperm, pre-freeze glycerol exposure for 0.5 to 75 min had no significant difference on post-thaw sperm motility, viability and plasma membrane integrity (Almlid and Johnson, 1988). The present findings were consistent with these findings and demonstrated that glycerol rapidly penetrated canine sperm. Furthermore, boar sperm penetrated within a few seconds and reached equilibrium within <30 sec (Pena et al., 1998; Okano et al., 2004; Almlid and Johnson, 1988). Therefore, we inferred that prolonged pre-freeze glycerol exposure had no advantage for unencapsulated canine sperm.

In microencapsulated canine sperm, pre-freeze glycerol exposure for 30 and 60 min resulted in higher post-thaw progressive motility, viability and acrosomal integrity than exposure for only 5 min. This might have been due to insufficient penetration of glycerol into the microencapsulated sperm when glycerol exposure was such a short interval. As in the previous study, microcapsules were swollen during the preservation at 4 °C, indicating influx of the extender through the microcapsules semipermeable membrane (Shah et al., 2010). Therefore, glycerol may require sufficient exposure (>5 min) to penetrate the microcapsules effectively, and thereby preserve the longevity of post-thaw sperm characteristics. It was noteworthy that the decline in motility, viability and acrosomal integrity in unencapsulated sperm disappeared quickly, which lead to significantly higher sperm characteristics after 6 and 9 hrs of culture at 38.5 °C for

microencapsulated canine sperm exposed to pre-freeze glycerol for 30 and 60 min. Furthermore, we inferred that microencapsulation provided a better environment and protected the sperm from the detrimental effect of freezing process enhancing post-thaw longevity, motility and viability during the culture period.

In a previous study, the proportion of sperm with an intact acrosome was significantly higher than the unencapsulated sperm after 24 hrs of culture than at 18, 24 and 38 °C, which was consistent with our findings (Torre et al., 2002). Thus, based on the present study, microencapsulation significantly reduced the rate of acrosomal damage, with better sperm preservation compared to unencapsulated sperm.

Glycerol is the cryoprotectant most commonly used for dog semen cryopreservation; although several concentrations have been used in dogs, optimal results were obtained with concentrations from 2 to 8 **%** (Olar et al., 1989; Pena et al., 1998; Cardoso et al., 2003). Cryopreservation in the absence of glycerol significantly reduced post-thaw motility and survivability of canine sperm (Olar et al., 1989). In general, there were no significant differences in progressive motility, viability and acrosomal integrity among any of the treatments when comparing cooled and after glycerol addition in unencapsulated and microencapsulated sperm. However, in frozen-thawed semen, a decrease in sperm characteristics was observed. This decrease was expected, as cryopreservation inevitably induced sperm damage.

In conclusion, microencapsulated canine sperm was successfully cryopreserved by prolonging the post-thaw motility, viability, and acrosomal integrity. Also, this technique could be applied as an alternative to conventional cryopreservation of

canine sperm. Further studies are needed to examine the fertility of cryopreserved microencapsulated canine sperm. In addition, fertility following the use of cryopreserved microencapsulated canine sperm for AI remains to be investigated.

CHAPTER 4

GENERAL DISCUSSION

Microencapsulation of semen has been described for prolonged storage or sustained release of sperm within the female reproductive tract (Nebel and Saacke, 1996) and has been successfully used for storage of porcine, ovine and bovine sperm. But, the application of microencapsulation on canine sperm has not been reported yet. This study evaluated the effect of microencapsulation on extending the longevity of canine spermatozoa. As the bitch has a long estrus compared with that of the other domestic animals, microencapsulation of sperm could be an innovative and alternative technique for maintaining the sperm survivability and fertility in dogs.

The author has described the successful microencapsulation of canine sperm for chilling/freezing storage. The characteristics of microcapsules showed that the microencapsulation method and duration of chilling storage affected the shape of microcapsules. The morphological changes of microcapsules during the chilling storage were observed which showed that gel and polycation microcapsules were swollen during the storage, which caused an increase in diameter. In polycation microcapsules, the membrane thickness decreased during storage. These changes in the morphological characteristics were observed due to the absorption of extender used for preservation in the alginate and influx of the extender through semipermeable polycation membrane. In other species, several concentration of alginate has been used for microencapsulation of sperm. Gel microcapsules at 0.75% alginate concentration showed a tear drop-like structure with fragility, whereas those at 1.0% alginate showed a solid spherical structure. These findings demonstrated that, for the

maintenance of the spherical shape of microcapsules requires a higher concentration of alginate (1.0%).

Recovery rate of canine sperm from microcapsules evaluated immediately after encapsulation showed that the alginate concentration had no influence on sperm recovery from the microcapsules and polycation microcapsules had significantly lower sperm recovery than that of gel microcapsules which was inferred as a trapping of sperm in the poly-L-lysine membrane.

Progressive motility of gel encapsulated canine sperm both at 0.75% and 1.0% alginate concentration was significantly lower than that of the polycation encapsulated and unencapsulated sperm, without significant reduction in sperm viability was observed due to the viscous alginate matrix in the gel microcapsule which may have physically interfered with the kinetic activity of canine sperm. Decrease in the motility and velocity of gel encapsulated sperm has also been reported in pig (Torre et al., 2000).

The swelling of microcapsules during chilling storage observed in this study may indicate that permeability of alginate gel and polycation membrane facilitated maintenance of the viability of encapsulated canine sperm by exchange of nutrients from the extender. The diameter and membrane thickness of the microcapsules showed no relation to the motility and viability of microencapsulated bovine sperm (Nebel et al., 1985; Nebel and Saacke, 1994; Nebel et al., 1996).

Progressive motility and viability of the polycation microencapsulated canine sperm at 1.0% alginate concentration was enhanced compared with that of unencapsulated sperm after 4 and 7 days of storage at 4 ^{o}C. The microencapsulated sperm had high

motility at 24 hr of culture at 38.5 °C without storage and at 6 and 24 hrs of culture after 1 day of chilling storage. This demonstrated that the polycation microencapsulation prevented a rapid decrease in the motility and viability of encapsulated canine sperm during chilling storage. Thus, effectiveness of polycation microencapsulation on sperm motility seems to become significant with prolongation of chilling storage. This inferred that microencapsulation of canine sperm provided a better environment and protects the sperm allowing the exchange of nutrients during chilling storage, which enhanced the motility and viability with prolongation of storage. After the successful preservation of microencapsulated canine sperm at chilling storage, microcapsules were used for cryopreserving the canine sperm. And this was apparently the first to describe the successful cryopreservation of microencapsulated canine sperm.

Post-thaw progressive motility, viability and acrosomal integrity were evaluated after culture at 38.5 °C in unencapsulated and microencapsulated canine sperm with pre-freeze glycerol exposure for 5, 30 and 60 min. Progressive motility, viability and acrosomal integrity after cooling and glycerol addition compared with initial sperm were not significantly different in unencapsulated and microencapsulated sperm. This concluded that for both unencapsulated and microencapsulated sperm cooling and glycerol exposure at 4 °C had no effect on pre-freeze canine sperm quality compared with ejaculated sperm. The present study showed that, canine sperm motility, viability and acrosomal integrity were not affected, and sperm withstand cooling and glycerol equilibrium in unencapsulated and microencapsulated sperm at 4 °C. Post-thaw motility, viability and acrosomal integrity decreased when sperm were incubated at

38.5 °C, with increasing culture duration in unencapsulated and microencapsulated sperm. Pre-freeze glycerol exposure for 5, 30, and 60 min did not cause any significant changes during the culture period in unencapsulated sperm.

Although, pre-freeze exposure for 30 and 60 min did not cause any significant differences during the culture period for microencapsulated sperm, but significantly decreased in microencapsulated sperm exposed to glycerol for 5 min. Progressive motility and viability at all glycerol exposure declined more quickly in unencapsulated sperm and were significantly lower than in microencapsulated sperm after 6 and 9 hrs of culture.

The effect of pre-freeze glycerol exposure for 0 to 4 hrs had no significant effect on the post-thaw progressive motility and acrosomal integrity in canine sperm (Okano et al., 2004). Glycerol equilibration may take only a few seconds if sperm were sufficiently cooled (at 4 °C) before addition of glycerol. There were no significant differences in post-thaw progressive motility, viability and intact acrosome after incubation up to 4 hrs at 39 °C between 5 min and 1 hr pre-freeze glycerol exposure (Pena et al., 1998). The present findings demonstrated that glycerol rapidly penetrated canine sperm. And the prolonged pre-freeze glycerol exposure had no advantage for unencapsulated canine sperm.

In microencapsulated canine sperm, pre-freeze glycerol exposure for 30 and 60 min resulted in higher post-thaw progressive motility, viability and acrosomal integrity than exposure for only 5 min. This might have been due to insufficient penetration of glycerol into the microencapsulated sperm when glycerol exposure was such a short

interval. Therefore, glycerol may require sufficient exposure (>5 min) to penetrate the microcapsules effectively, and thereby preserve the longevity of post-thaw sperm characteristics.

Significantly higher sperm characteristics after 6 and 9 hrs of culture at 38.5 °C for microencapsulated canine sperm exposed to pre-freeze glycerol for 30 and 60 min was observed due to quick decline in motility, viability and acrosomal integrity in unencapsulated sperm. These findings demonstrated that microencapsulation provided a better environment and protected the sperm from the detrimental effect of freezing process enhancing post-thaw longevity (motility and viability) and acrosomal integrity during the culture period.

The microencapsulation technology can be successfully applied for the low temperature preservation of canine sperm which in turn could lead to application of microencapsulation in AI. However the potential of the microencapsulation technique for AI needs further development in several areas including the ability to maintain the longevity of microencapsulated sperm in vivo, the ability of the microencapsulated sperm to be retained by the inseminated bitch, and the ability of the inseminated microcapsules to rupture over a sufficient period of time to make sperm available in sufficient numbers for fertilization. Further studies are needed to examine the fertility of low temperature preserved microencapsulated canine sperm.

In conclusion, polycation microencapsulation at 1.0% alginate concentration was successfully applied for chilling storage of canine sperm and maintained the motility and viability up to 7 days. Also, the microencapsulated canine sperm was successfully cryopreserved by prolonging the post-thaw motility, viability, and

acrosomal integrity. This technique could be applied as an alternative to conventional preservation of canine sperm.

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REFERENCES

Abe Y, Lee DS, Sano H, Akiyama K, Ueta YY, Asano T. Artificial insemination with canine spermatozoa frozen in a skim milk/glucose-based extender. J Reprod Dev 2008;54:290–294.

Alamo D, Batista M, Gonzalez F, Rodriguez N, Cruz G, Cabrera F, Gracia A. Cryopreservation of semen in the dog: use of ultra-freezers of –152 ^{o}C as viable alternative to liquid nitrogen. Theriogenology 2005;63:72–78.

Almlid T, Johnson LA. Effects of glycerol concentration, equilibration time and temperature of glycerol addition on post thaw viability of boar spermatozoa frozen in straws. J Anim Sci 1988;66:2899-2905.

Austin CR. Fertilization and the transport of gametes in the pseudo-pregnant rabbit. J Endocrinol 1949;6:63.

Battista M, Parks J, Concannon P. Canine sperm post-thaw survival following freezing in straws or pellets using PIPES, Lactose, Tris or Test extenders. Proc 11th Int Congr Anim Reprod (ICAR) 1988;3:229-231.

Bavister BD. Culture of preimplantation embryos: facts and artifacts. Human Reprod Update 1995;1:91-148.

Bouchard GF, Morris JK, Sikes JD, Youngquist RS. Effects of storage temperature, cooling rates and two different semen extenders on canine spermatozoal motility. Theriogenology 1990;34:147-157.

Brackett BG, Oliphant G. Capacitation of rabbit spermatozoa in vitro. Biol Reprod 1975;12:260-274.

Braden AWH. Distribution of sperm in the female tract of the rabbit after coitus. Australian J Biol Sci 1953;6:693.

Burgos MH, Di Paola G. Eosin test for the evaluation of sperm viability. Fertil Steril 1951;2:542-544.

Cardoso R, Silva A, Uchoa D, Silva L. Cryopreservation of canine semen using a coconut water extender with egg yolk and three different glycerol concentrations. Theriogenology 2003;59:743-751.

Castro MDE, Orive G, Hernandez RM, Gascon AR, Pedraz JL. Comparative study of microcapsules elaborated with three polycations (PLL, PDL, PLO) for cell immobilization. Microencapsulation 2005;22:303-315.

Chang TMS. Semipermeable microcapsules. Science 1964;146:524-525.

Chang TMS, Macintosh FC, Mason SG. Semipermeable aqueous microcapsules. Preparation and properties. Can J Physiol Pharmacol 1966;44:115-128.

Christiansen IBJ. Reproduction in the dog and cat. London: Bailliere Tindall, 1984;115-123.

Combelles CM, Fissore RA, Albertini DF, Racowsky C. In vitro maturation of human oocytes and cumulus cells using a co-culture three-dimensional collagen gel system. Hum Reprod 2005;20:1349-1358.

Didion BA, Dobrinsky JR, Giles JR, Graves CN. Staining procedure to detect viability and the true acrosome reaction in spermatozoa of various species. Gamete Res 1989;22:51-57.

Eilts BE. Theoretical aspects of canine cryopreserved semen evaluation. Theriogenology 2005;64:685-691.

El-Banna AA, Hafez ESE. Sperm transport and distribution in rabbit and cattle reproductive tract. Fertil Steril 1970;21:534.

Elsheikh AS, Takahashi Y, Hishinuma M, Mohammad Nour MS, Kanagawa H. Effect of encapsulation on development of mouse pronuclear stage embryos in vitro. Anim Reprod Sci 1997;48:317-324.

England GCW. Cryopreservation of dog semen: a review. J Reprod Fertil 1993;47 Suppl:243-255.

England GCW, Ponzio P. Comparison of the quality of frozen-thawed and cooled rewarmed dog semen. Theriogenology 1996;46:165-l7l.

Esbenshade KL, Nebel RL. Encapsulation of porcine spermatozoa in poly-L-lysine microsphere. Theriogenology 1990;33:499–508.

Farstad W. Bitch fertility after natural mating and after artificial insemination with fresh or frozen semen. J Small Anim Pract 1984;25:561–565.

Foote RH. The effects of electrolytes, sugars, glycerol, and catalase on survival of dog sperm stored in buffered-yolk mediums. Am J Vet Res 1964;25:32–36.

Foote RH, Leonard EP. Influence of pH, osmotic pressure, glycine and glycerol on survival of dog sperm in buffered yolk extenders. Cornell Vet 1964;54:78-89.

Gill HP, Kaufman CF, Foote RH, Dirk RW. Artificial insemination of beagle bitches with freshly collected, liquid-stored and frozen-stored semen. Am J Vet Res 1970;31:1807–1813.

Goodrowe KL, Hay MA, Platz CC, Behrns SK, Jones MH, Wadell WT. Characteristics of fresh and frozen-thawed red wolf *(Canis rufus)* spermatozoa. Anim Reprod Sci 1998;53:299–308.

Govette G, Linde-Forsberg C, Strom B. A successful concept for freezing of dog semen. 13th Int Congr Anim Reprod (ICAR), 1996;2:5-8 (Abstr).

Harrop AE. Artificial insemination of bitch with preserved semen. Vet Rec 1954;110:194–196.

Hawk HW. Sperm survival and transport in the female reproductive tract. J Dairy Sci 1983;66:2645.

Heise M, Koepsel R, Russell AJ, McGee EA. Calcium alginate microencapsulation of ovarian follicles impacts FSH delivery and follicle morphology. Reprod Biol Endocrinol 2005;3:47.

Hermasson U, Linde-Forsberg C. Freezing of stored, chilled dog spermatozoa. Theriogenology 2006;65:584–593.

Herrler A, Eisner S, Bach V, Weissenborn U, Beier HM. Cryopreservation of spermatozoa in alginic acid capsules. Fertil Steril 2006;85:208-213.

Heywood R, Sortwell RJ. Semen evaluation in the beagle dog. J Small Anim Pract 1971;12:343-346.

Holt WV. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 2000;53:47-58.

Hollingsworth TS, Page RD. The effects of microencapsulation on freezing bisected bovine embryos. Theriogenology 1988;29:262 (Abstract).

Hsu TT, Yang JG, Hsu H. Studies on microencapsulation and cryoconservation of the biopsed bovine embryos after PCR sexing. J Chin Soc Anim Sci 1995;24:139-151.

Huang SY, Tu CF, Liu SH, Kuo YH. Motility and fertility of alginate encapsulated boar spermatozoa. Anim Reprod Sci 2005;87:111-120.

Ivanova-Kicheva MG, Bobadov N, Somlev B. Cryopreservation of canine semen in pellets and in 5-ml aluminum tubes using three extenders. Theriogenology 1997;48:1343-1349.

Johnson FA, Craig DQM, Mercer AD. Characterization of the block structure and molecular weight of sodium alginates. J Pharm Pharmacol 1997;49:639–643.

Klein J, Stock J, Vorlop KD. Pore size and properties of spherical Ca alginate biocatalysts. Eur J Appl Microbiol Biotechnol 1983;18:86-91.

Kreeger PK, Deck JW, Woodruff TK, Shea LD. The in vitro regulation of ovarian follicle development using alginate extracellular matrix gels. Biomaterials 2006;27:714-723.

Kreeger PK, Fernandes NN, Woodruff TK, Shea LD. Regulation of mouse follicle development by follicle-stimulating hormone in a three-dimensional in vitro culture system is dependent on follicle stage and dose. Biol Reprod 2005;73:942-950.

Krentz KJ, Nebel RL, Canseco RS, McGilliard ML. In vitro and in vivo development of mouse morulae encapsulated in 2% sodium alginate or 0.1% poly-L-lysine. Theriogenology 1993;39:655-667.

Kumi-Diaka J, Badtram G. Effect of storage on sperm membrane integrity and other functional characteristics of canine spermatozoa: In vitro bioassay for canine semen. Theriogenology 1994;41:1355-1366.

Lim F. Microencapsulation of living cells and tissues—theory and practice. In: Lim F (Ed.), Biomedical Applications of Microencapsulation. CRC Press, Boca Raton, FL, 1984, p.137–154.

Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 1980;210:908-910.

Lim F, Moss RD. Microencapsulation of living cells and tissue. J Pharm Sci 1981;70:351–354.

Linde-Forsberg C. Achieving canine pregnancy by using frozen or chilled extended semen. Small Anim Prac 1991;21:467-485.

Linde-Forsberg C. Artificial insemination with fresh, chilled extended and frozen thawed semen in the dog. Semin Vet Med Surg (Small Anim) 1995;10:48–58.

Linde-Forsberg C. Regulations and recommendations for international shipment of chilled and frozen canine semen. In: Concannon PW, England GCW, Verstegen J (Eds.). Recent Advances in Small Animal Reproduction. A1209.0501. Ithaca: International Veterinary Service; 2001a. http://www.ivis.org/.

Linde-Forsberg C. Biology of reproduction and modern reproductive technology. In: Ruvinsky A, Sampson J, editors. The genetics of the dog. New York, CABI Publishing, 2001b.p.402–423.

Linde-Forsberg C, Strom Holst B, Govette G. Comparison of fertility data from vaginal vs. intrauterine insemination of frozen-thawed dog semen: a retrospective study. Theriogenology 1999;52:11–23.

Luvoni GC. Gamete cryopreservation in the domestic cat. Theriogenology 2006;66:101–111.

Marks SL, Dupuis J, Mickelsen WD, Memon MA, Platz CC. Conception by use of post-mortem epididymal semen extraction in a dog. J Am Vet Med Assoc 1994;204:1639–1640.

Maxwell WMC, Nebel RL, Lewis GS. Survival and fertility of micro-encapsulated ram spermatozoa stored at 5 °C. Reprod Dom Anim 1996;31:665-673.

Michael AJ, Alexopoulos C, Pontiki EA, Hadjipavlou-Litina DJ, Saratsis Ph, Ververidis HN, Boscos CM. Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. Anim Reprod Sci 2009;112:119-135.

Morey DF. The early evolution of the domestic dog. American Scientist 1994;82:336–347.

Morton DB, Bruce SG. Semen evaluation, cryopreservation and factors relevant to the use of frozen semen in dogs. J Reprod Fertil Suppl 1989;39:311–316.

Munkittrick TW, Nebel RL, Saacke RG. Accessory sperm numbers for cattle inseminated with protamine sulfate microcapsules. J Dairy Sci 1992;75:725-731.

Munoz-Garay C, De la Vega-Beltran JL, Delgado R. Inwardly rectify K^+ channels in spermatogenic cells: functional expression and implication in sperm capacitation. Dev Biol 2001;234:261-274.

Nebel RL, Bame JH, Saacke RG, Lim F. Microencapsulation of bovine spermatozoa. J Anim Sci 1985;60:1631-1639.

Nebel RL, Fultz SW, Saacke RG. A series of studies on the microencapsulation of bovine spermatozoa. Proc. 13th Int`l Symp. Contr Rel of Bio Mat 1986;214-215.

Nebel RL, Saacke RG. Technology and applications for encapsulated spermatozoa. Biotech Adv 1994;12:41-48.

Nebel RL, Saacke RG. Spermatozoal microencapsulation and capsule behavior in the female tract. Reprod Dom Anim 1996;31:75-85.

Nebel RL, Vishwanath R, McMillan WH, Pitt CJ. Microencapsulation of bovine spermatozoa: effect of capsule membrane thickness on spermatozoa viability and fertility. Anim Reprod Sci 1996;44:79-89.

Nebel RL, Vishwanath R, McMillan WH, Saacke RG. Microencapsulation of bovine spermatozoa for use in artificial insemination: a review. Reprod Fertil Dev 1993;5:701-712.

Niemann H, Brem G, Sacher D, Smidt, Krausslich H. An approach to successful freezing of demi-embryos derived from day 7 bovine embryos. Theriogenology 1986;25:519-524.

Oettle EE. Changes in acrosome morphology during cooling and freezing of dog semen. Anim Reprod Sci 1986;12:145-150.

Okano T, Murase T, Asano M, Tsubota T. Effects of final dilution rate, sperm concentration and times for cooling and glycerol equilibration on post thaw characteristics of canine spermatozoa. J Vet Med Sci 2004;66:1359-1364.

Olar TT, Bowen RA, Pickett BW. Influence of extender, cryopreservative and seminal processing procedures on post-thaw motility of canine spermatozoa frozen in straws. Theriogenology 1989;31:451-461.

Pangas SA, Saudye H, Shea LD, Woodruff TK. Novel approach for the three-dimensional culture of granulosa cell-oocyte complexes. Tissue Eng 2003;9:1013-1021.

Pena AI, Barrio F, Quintela LA, Herradon PG. Effects of different glycerol treatments on frozen thawed dog sperm longevity and acrosomal integrity. Theriogenology 1998;50:163-174.

Pena A, Linde-Forsberg C. Effects of Equex, one or two-step dilution, and two freezing and thawing rates on post-thaw survival of dog spermatozoa. Theriogenology 2000a;54:859-875.

Pena A, Linde-Forsberg C. Effects of spermatozoal concentration and post thaw dilution rate on survival after thawing of dog spermatozoa. Theriogenology 2000b;54:703-718.

Pena AI, Lugilde LL, Barrio M, Herradon PG, Quintela LA. Effects of Equex from different sources on post-thaw survival, longevity and intracellular Ca concentration of dog spermatozoa. Theriogenology 2003;59:1725-1739.

Pena FJ, Nunez M, Moran JM. Semen technologies in dog breeding: an update. Reprod Domest Anim 2006;41:21-29.

Pena-Martinez I. Canine fresh and cryopreserved semen evaluation. Anim Reprod Sci 2004;82-83:209-224.

Pinto CRF, Paccamonti DL, Eilts BE. Fertility in bitches artificially inseminated with extended, chilled semen. Theriogenology 1999;52:609–616.

Province CA, Amann RP, Pickett BW, Squires EL. Extenders for preservation of canine and equine spermatozoa at 5°C. Theriogenology 1984;22:409-415.

Rota A, Iguer-ouada M, Verstegen J, Linde-Forsberg C. Fertility after vaginal or uterine deposition of dog semen frozen in a tris extender with or without Equex STM paste. Theriogenology 1999;51:1045–1058.

Rota A, Strom B, Linde-Forsberg C. Effects of seminal plasma and three extenders on canine semen stored at 4 °C. Theriogenology 1995;44:885–900.

Rota A, Strom B, Linde-Forsberg C, Rodriguez-Martinez H. Effects of equex STM paste on viability of frozen-thawed dog spermatozoa during in vitro incubation at 38°C. Theriogenology 1997;47:1093–1101.

Seager SJW. Successful pregnancies utilizing frozen dog semen. A.I. Digest 1969;17:6-16.

Shah S, Nagano M, Yamashita Y, Hishinuma M. Microencapsulation of canine sperm and its preservation at 4 °C. Theriogenology 2010;73:560-567.

Smidsred O, Skjak-Braek G. Alginate as immobilization matrix for cells. Trends Biotechnol 1990;8:71–78.

Strom Holst B, Larsson B, Linde-Forsberg C, Rodriguez-Martinez H. Evaluation of chilled and frozen-thawed canine spermatozoa using a zona pellucida binding assay. J Reprod Fertil 2000;119:201–206.

Sun AM, O'Shea GM, Goosen MFA. Injectable microencapsulated islet cells as a bioartificial pancreas. Applied Biochem Biotech 1984;10:87-99.

Thomas PGA, Larsen RE, Hahn CN. A comparison of three packaging techniques using two extenders for the cryopreservation of canine semen. Theriogenology 1993;40:1199-1205.

Thomassen R, Farstad W, Krogenaes A, Fougner JA, Berg KA. Artificial insemination with frozen semen in dogs: a retrospective study. J Reprod Fertil Suppl 2001;57:341–346.

Torre ML, Faustini M, Norberti R, Stacchezzini S, Maggi L, Maffeo G, Conte U, Vigo D. Boar semen controlled delivery system storage and in vitro spermatozoa release. J Contr Rel 2002;86:83-89.

Torre ML, Maggi L, Giunchedi P, Conte U, Vigo D, Maffeo G. Calcium alginate beads containing an hydrophilic polymer for the encapsulation of swine spermatozoa. STP Pharma Sci 1998;8:233-236.

Torre ML, Maggi L, Vigo D, Galli A, Bornaghi V, Conte U. Controlled release of swine semen encapsulated in calcium alginate beads. Biomaterials 2000;21:1493-1498.

Torre ML, Munari EM, Albani E, Levi-Setti PE, Villani S, Faustini M, Conte U, Vigo D. In vitro maturation of human oocytes in a follicle-mimicking three-dimensional co-culture. Fertil Steril 2006;86:572-576.

Tsutsui T, Tezuka T, Mikasa Y, Sugisawa H, Hirofumi, Kirihara N, Hori T, Kawakami E. Artificial insemination with canine semen stored at low temperature. J Vet Med Sci 2003;65:307-312.

Verstegen JP, Onclin K, Iguer-Ouada M. Long-term motility and fertility conservation of chilled canine semen using egg yolk added Tris glucose extender: in vitro and in vivo studies. Theriogenology 2005;64:720–733.

Vigo D, Villani S, Faustini M. A follicle-like model by granulosa cell encapsulation in a barium alginate/protamine membrane. Tissue Eng 2005;11:709-714.

Vishwanath R, Nebel RL, McMillan WH, Pitt CJ, Macmillan KL. Selected times of insemination with microencapsulated bovine spermatozoa affect pregnancy rates of synchronized heifers. Theriogenology 1997;48:369-376.

Watson PF. Use of a Giemsa stain to detect changes in acrosomes of frozen ram spermatozoa. Vet Rec 1975;97:12-15.

Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995;7:781-891.

Wheatley MA, Eisen H, Langer R. Alginate/poly-L-lysine microcapsules as a vehicle for controlled release of macromolecules. (Proc. Int. Symp.) Contr Rel Bioreact Mater 1985;12:245–246.

Willadsen SM. A method for culture of micromanipulated sheep embryos and its use to produce monocygotic twins. Nature 1979;277:298-300.

Wrathall A. Embryo transfer and disease transmission in livestock a review of recent research. Theriogenology 1995;43:81-88.

Xu M, Erin W, Shea LD, Woodruff TK. Identification of a stage specific permissive in vitro culture environment for follicle growth and oocyte development. Biol Reprod 2006a;75:916-923.

Xu M, Kreeger PK, Shea LD, Woodruff TK. Tissue-engineered follicles produce live, fertile offspring. Tissue Eng 2006b;12:2739-2746.

Yaniz JL, Santolaria P, Lopez-Gatius F. In vitro development of bovine embryos encapsulated in sodium alginate J Vet Med A Physiol Pathol Clin Med 2002;49:393-395.

Yoshida M. Conservation of sperms: current status and new trends. Anim Reprod Sci 2000;60-61:349-355.

Zindl C, Asa CS, Gunzel Apel AR. Influence of cooling rates and addition of Equex paste on cooled and frozen thawed semen of Generic gray (*Canis lupus*) and Mexican gray wolves (*C.l. baileyi*). Theriogenology 2006;66:1797-1802.