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STANDARD PROTOCOL FOR ENCAPSULATION OF CANINE SEMEN

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Abstract: The study was conducted to standardize the protocol for encapsulation of canine semen. By digital manipulation of penis from six (06) healthy dogs semen was collected. Immediately after semen collection, the macroscopic and microscopic attribute was calculated. The sperm rich fraction of semen was utilized for preparation of encapsulated semen capsules. From the study we concluded that at a concentration of 1.5 per cent Sodium Alginate, 1 per cent Calcium Chloride and 0.1 per cent Poly-l - lysine concentration, the encapsulation of canine semen yield spherical shaped capsules.
Keyword: Dog, Encapsulation, Cryopreservation, Semen Preservation.

Introduction

Artificial insemination (AI) and semen freezing have become services available to dog owners worldwide and the demand for services to freeze semen is increasing. Clearly, artificial insemination facilitates the use of a male to sire several females by diluting the ejaculate, increases breeding hygiene and allows crossing between species with slightly different breeding seasons.

The remarkable success with bull semen cryopreservation has not been matched in other mammals. When cells are frozen they are subjected to stresses resulting from the water-solute interactions that arise through ice crystallization. The sperm plasma membrane lipids respond to temperature changes by alterations in their physical phase state. Although regions of fluid and gel phase lipids coexist at physiological temperatures, reductions of temperature favour fluid to gel transitions; the presence of sterols is thought to inhibit these phase changes.

Thus, cryopreservation induces a series of osmotic, chemical and mechanical stresses to sperm, causing death of some sperm and severe post-thaw damage in surviving cells, reducing fertilizing ability (Watson, 1995). Therefore, current pregnancy and live birth success rates of assisted reproduction techniques (ART) are not completely satisfactory with frozen-thawed dog semen (Kim *et al*., 2010).

To overcome current limitations, cryopreservation of microencapsulated canine sperm could be a viable alternative. Encapsulation is the process whereby living cells or tissues are completely encased by a semipermeable membrane, permitting the exchange of nutrients and

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metabolites (Lim, 1984) and encapsuled sperm used for AI maintains sperm viability and sperm concentrations in the uterus during oestrus, thereby allowing their release over an extended interval (Nebel *et al.*, 1993). Microencapsulation of sperm has been applied successfully for the enhancement of reproductive performance for various purposes, including semen controlled release (Torre, 2000), sperm preservation (Huang *et al.*, 2005), *in vitro* oocyte maturation (Torre *et al.*, 2006) and embryo culture to improve *in vitro* fertilization yield. So the present study is to standardise the protocol for encapsulation of canine semen.

Material and Methods

Collection of semen

The semen is collected from 6 healthy mature dogs at one week interval for six times. After collection of semen macroscopic and microscopic parameter judged at laboratory like colour, initial motility, progressive motility etc. If the parameter is satisfactory the sperm rich fraction is utilized for preparation of semen capsules.

Encapsulation of sperm: Encapsulation of canine semen was performed as per the procedure adopted by Shah *et al.* (2011) with little modifications. The sperm rich fraction was used for encapsulation. A neat semen sample (pre-sperm sperm rich and post-sperm fractions) was mixed (1:2) with 1.5% (w/v) sodium alginate solution dissolved in physiological saline to reach a final concentration of 1% sodium alginate. The sperm suspension was forced through a 24 gauge needle attached to 2 mL syringe into a 100 ml glass beaker containing 20 ml of 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 4.5 cm to ensure the shape of capsules. 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 120 seconds. The microgels were collected by filtration using a muslin cloth, rinsed three times with physiological saline. Then, the microgels were transferred into 0.1% (w/v) poly-L-lysine 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 muslin cloth and then rinsed three times with physiological saline. The membrane bound capsules were filtered and were used for cryopreservation. The volume of each microcapsule was obtained by measuring the number of capsules from the total volume of sperm suspension used for encapsulation. In the present study, in sodium alginate mixed semen

sample of 1.2 ml (average 0.8 ml semen + 0.4 ml sodium alginate) – average total of 160 number of encapsules were prepared each of 7.50 µl capacity containing 1.85 X 10^6 sperm / capsule. The encapsules were suspended in equal volume of a tris glucose based dilutor and virtual dilution was performed. To check the effect of encapsulation on sperm motility, viability, plasma membrane integrity, immediately after rinsing, some of the microgels were transferred into physiological saline, (pH 7.4) for 5 min. The alginate gel core of the microgels was liquefied by chelation of calcium ions.These encapsulated semen sample was filled manually in 0.25 ml capacity German Mini Straw, sealed with steel balls and then stored at 5^0C in a refrigerater and equilibrated for next 60 minutes duration. All the procedures described above for the encapsulation of canine semen were performed at room temperature in sterile environment.

Result and Discussion

Immediately after collection the sperm rich fraction of canine ejaculated semen was measured and half of the volume was thoroughly mixed with 1.5 per cent Sodium Alginate solution (Moderate Viscosity) at a ratio of 2:1 and placed at room temperature.

The composite mixture of sperm rich fraction of semen and 1.5 per cent Sodium Alginate solution was titrated in 100 ml capacity sterilized beaker containing 25 ml of 1 per cent Calcium Chloride solution from a distance of 5 cm.

Polymerization of alginate with calcium resulted in the formation of calcium alginate, which is observed in the form of a capsules containing 7.50 µl of sperm rich fraction of canine semen in its core having average semen concentration of 1.85 X 10^6 sperm / capsule.

These capsules were stabilized in 0.1 per cent Poly – L – Lysine solution which results in the formation of a polymer coat over the surface of capsule, thereby preventing its dilution in citrate containing semen dilutor used for semen cryopreservation.

After separation with the help of sterilized muslin cloth, the capsules are virtually diluted with Tris-Citrate-Glucose-Egg Yolk dilutor. Manually with the help of 16 G needle attached to 2 ml syringe, these capsules in virtually diluted form were deposited in 0.25 ml capacity German mini straws and sealed manually with steel balls. Capsule filled semen straws were then equilibrated at 5^0C for next 60 minutes in refrigerator and subsequently conventional LN_2 vapour freezing of 0.25 ml German Mini semen Straws filled with capsules is performed.

After 24 hours of cryopreservation, the capsule filled semen straws were thawed in at 37^0C for 60 seconds and the capsules are dissolved the in normal saline solution instead of sodium

citrate solution for 2 minutes, the post freeze semen sample showed arrested motility for first 10 – 15 minutes followed by resumption of progressive motility in the semen sample.

At 0.5 and 1 per cent Sodium Alginate concentration and 1 per cent calcium chloride solution in the present experiment, encapsules in the form of concave disc like structure are produced, thus interfering in the normal process of encapsulation of canine semen.

In the present study, at 1.5 per cent Sodium Alginate, 1 per cent Calcium Chloride and 0.1 per cent Poly-l - lysine concentration, the encapsulation of canine semen yield spherical shape capsules.

Huanga *et al.* (2005) used 0.75 to 1.5% alginate and 0.1% poly-L-lysine for encapsulation of sperm in cattle, pigs, and sheep and is in agreement with the present observations.

In the present experiment 1.5 per cent sodium alginate was used for the encapsulation of canine semen which is in complete agreement with the findings of Shah *et al.* (2010) who stated that to maintain the spherical shape of microcapsules, a higher concentration of alginate (above 1.0 per cent) is required.

Nebel and Saacke (1994) stated that to stabilize the capsule by an additional poly-l-lysine shell, alginate/$Ca^{2\pm}$-based encapsulation protocols required multiple steps so as to avoid capsule dissolution by $Ca^{2\pm}$ chelating buffer components like citric acid present in semen extenders. In the present study, to form a polymer coat over the surface of capsule, these were stabilized in 0.1 per cent Poly – L – Lysine solution, thereby prevent its dilution in citrate containing semen dilutor used for semen cryopreservation.

In the present experiment, the spermatozoa encapsulated with physiological seminal plasma in the sperm rich fraction of canine semen was suspended rather than diluted in the semen dilutor, thus ensuring molecular nutrient and metabolite exchanges but providing pH buffering to avoid ‘‘dilution shock’’ facilitating a virtual dilution after microencapsulation which is in complete agreement with the earlier findings of Torre *et al.* (2000) and Johnson *et al.* (2000). Our findings are in accordance with the findings of Conte *et al*., 1999 who stated that microencapsulation method has advantage as these capsules consist of a core of highly concentrated spermatozoa surrounded by a polymeric membrane which separate the cells from the extender.

Conclusion

The semen capsule prepared from canine semen with standard protocol at 1.5 per cent Sodium Alginate, 1 per cent Calcium Chloride and 0.1 per cent Poly-l - lysine concentration.

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