

A STUDY ON CRYOPRESERVATION OF ENCAPSULATED DOG SEMEN

Chetan K. Lakde¹, M.S. Patil², S.K. Sahatpure³ and A.P. Gawande⁴

¹Ph.D. Scholar, ²Assistant Professor, ³Associate Professor and Incharge, ⁴Associate Professor; Department of Animal Reproduction, Gynaecology and Obstetrics; Nagpur Veterinary College; Nagpur – 440 006.

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The study was conducted on cryopreservation of encapsulated dog semen. Pre and post freeze seminal characteristics and conception rate were estimated. Average volume for fraction – I, II and III of dog semen ejaculates and sperm concentration were 0.69 ± 0.06 , 1.60 ± 0.11 and 2.33 ± 0.13 ml and $369.94 \pm 13.45 \times 10^6$ sperm / ml, respectively. Initial motility and initial viability of neat semen were reduced significantly ($P < 0.01$) in Group I (conventional) and II (encapsulation). Plasma membrane intact spermatozoa was observed to be 81.64 ± 1.25 % in the fresh sperm rich fraction of dog semen which showed significant reduction in Group I and II. The average post thaw motility, viability and plasma membrane integrity were noted 13.06 ± 0.64 , 54.36 ± 1.06 , 24.13 ± 1.41 % in Group I and 22.36 ± 1.28 , 59.04 ± 1.53 , 31.64 ± 2.31 % in Group II, respectively. The average conception and whelping rate recorded in Group I and II was of 50.00, 83.33 and 16.67, 66.66 %, respectively.

Keywords: Encapsulation, Intrauterine insemination, Motility, Plasma membrane integrity, Viability, Sperm.

Freezing and cooling of spermatozoa during cryopreservation for artificial insemination causes ultrastructural changes in the acrosome and plasma membrane which reduces longevity and fertility. Cryopreservation-induced capacitation-like changes and reduced ability of spermatozoa to bind with the cells of the reproductive tract of the bitch may contribute to the reduced fertility of cryopreserved spermatozoa. Before fertilisation, there is requirement for spermatozoa to be stored within the reproductive tract to prolong their viability and motility which inhibits capacitation until the released oocyte matures. 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). Poor post-thaw quality and short sperm lifespan after thawing are major impediments of cryopreserved canine sperm (England, 1993).

Preservation of microencapsulated dog sperm has been applied by prolonging motility and viability related to unencapsulated sperm after chilling storage for 4-7 days (Shah *et al.*, 2011).

Materials and Methods

Part of M.V.Sc. Thesis

Collection of Semen

Total 36 ejaculates collected from 6 male dogs (6 from each) were used for semen evaluation and cryopreservation. The pre-sperm, sperm rich and post-sperm fractions of semen were collected into 15 ml graduated sterile glass tube attached to the artificial vagina end coned by digital manipulation.

Evaluation of Semen

After collection, the semen evaluation was carried out which include sperm concentration, mass activity, percentage of progressively motile sperm and percentage of spermatozoa with intact plasma membrane was estimated.

Cryopreservation of Semen

The sperm rich fraction of collected semen was divided into two equal parts. Part one was utilized for the preparation of encapsulated semen capsules followed by its further cryopreservation. The other half part of semen was utilized for conventional method of semen cryopreservation. Encapsulation of dog semen was processed on 0.5 ml of sperm rich fraction. After collection the sperm rich semen sample was diluted with equal volume of the tris glucose dilutor immediately. The semen was then filled manually into 0.25ml plastic straws and

sealed with steel balls. The semen straws were then transferred to a refrigerator at 5°C and were kept for a further 60 min, for equilibration, after that vapour freezing by LN₂ and directly immerse into LN₂.

Thawing of semen

Straws of cryopreserved semen (from both groups) were removed from the LN₂ and placed into water bath at 37°C for 30 seconds. The semen samples were used for evaluation of post-thaw parameters of semen.

Vaginal Artificial Insemination

Vaginal artificial insemination was performed using a simple bovine artificial insemination sheath which was cut to an appropriate length which attach, to which a simple plastic disposable syringe containing the semen.

Results and Discussion

Macroscopic Characteristics

The sperm rich second semen ejaculate fraction has milky opalescent and thick consistency, which was in accordance with Filho *et al.*, (2011). The mass activity observed in the sperm rich fraction of dog semen was 2.99 ± 0.10 which was similar to observed by Raut (2009). Dog semen ejaculates were collected in fraction I, II and III and total volume of semen recorded as 0.69 ± 0.06 , 1.60 ± 0.11 , 2.33 ± 0.13 and 4.62 ± 0.25 ml respectively. In present study, the average semen volume was comparatively lower than the other reports, Filho *et al.*, (2011). The sperm concentration of sperm rich fraction of dog semen was 369.94 ± 13.45 million/ml which is in accordance with the findings of Hermansson and Linde, (2006) but is lower than mentioned by Prinosilova *et al.*, (2012).

Encapsulation of dog semen

The post freeze semen sample showed arrested motility for first 10–15 minutes followed by resumption of progressive motility in the same semen sample. Solution 1.5% Sodium Alginate, 1% Calcium Chloride and 0.1% Poly-L-lysine concentration was used for the encapsulation of dog semen had

yield spherical shape capsules. During the trial the formed capsules were stabilized in 0.1% 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.

Pre and post freeze motility of dog semen

Freshly collected sperm rich fraction of dog semen showed an average percent initial motility 82.89 ± 1.05 . After addition of Tris-Citrate-Glucose dilutor in Group I and 1.5 % Sodium Alginate in Group II semen, the motility reduces significantly to 59.31 ± 1.53 and 71.25 ± 1.15 percent respectively. Analysis of variance for average motile spermatozoa showed significantly higher percentage of motile spermatozoa in Group – II than in Group I. Following freeze – thawing of dog semen significantly higher average post thawed motility was observed in Group – II compared to Group I. Similar progressive motility of microencapsulated dog sperm was reported by Shah *et al.*, (2010) in Beagle dogs after microencapsulated dog semen in 1.0% alginate which was stored at 4 °C for 0, 1, 4, and 7 days and then cultured at 38.5 °C for 0, 6, and 24 hours than those of unencapsulated spermatozoa. Post thawed sperm motility observed in Group – I and II was 13.06 ± 0.64 and 22.36 ± 1.28 percent, respectively. These observations are in agreement with the earlier findings of Shah *et al.*, (2011). Significant reduction in dog sperm motility in Group – I and II at different stages of semen dilution, cryopreservation and thawing. Similar reports of dog cryopreserved semen by conventional method are by Bencharif *et al.*, (2012). The differences may be due to species, climate and difference in dilutor, as a cryoprotectant dimethyl sulphoxidase was used in the study. The residues of alginate matrix could interfere with sperm kinetic activity, which reduced the motility of spermatozoa.

Pre and post freeze viability of dog semen

Eosin staining method was used to differentiate live and dead spermatozoa in

dog semen sample from Group I and II. Freshly collected sperm rich dog semen fraction showed, an average initial viability of $91.65 \pm 0.35\%$. Dilution with Tris-Citrate-Glucose dilutor in Group I $73.71 \pm 0.83\%$ and 1.5 % Sodium alginate in Group II $78.11 \pm 0.75\%$ shows significant reduction in sperm viability. The post thawed dog semen sample in Group I and Group II $54.36 \pm 1.06\%$ and $59.04 \pm 1.53\%$ respectively showed significant reduction in viable spermatozoa. Analysis of variance for average viable spermatozoa showed significantly higher per cent viable spermatozoa in Group II than in Group I. Gradual significant reduction observed in the percent viable spermatozoa in dog semen in the fresh sperm rich fraction to post thaw viability, in Group I and Group II. At 0 to 24 hours of culture after 4 and 7 days of chilling storage viability of microencapsulated sperm were higher than unencapsulated spermatozoa. Our findings were in accordance with Shah *et al.*, (2011) who recorded initial viability after exposure of glycerol in dog sperm.

Pre and post freeze plasma membrane integrity of dog semen

Initial plasma membrane integrity $81.64 \pm 1.25\%$ was observed. After dilution of canine semen in Tris-Citrate-Glucose dilutor significant reduction was observed in Group I- 51.46 ± 1.91 and in 1.5 % Sodium alginate containing extender in Group II- 63.40 ± 1.49 . Immediately after capsules prepared, it was dissolved in sodium citrate solution and release semen showed an average plasma membrane intact spermatozoa $51.54 \pm 1.19\%$. In Group II ($31.64 \pm 2.31\%$) significantly higher plasma membrane intact spermatozoa was observed in the freeze – thawed canine semen than in Group I (24.13 ± 1.41). Encapsulation enhances semen preservation by providing protection against membrane damage on dilution.

Conception rate

The freeze – thawed semen sample at a concentration of at least 200×10^6 sperm/ml, having progressive motility of at least 20 % were used for intra vaginal insemination in

bitches. The whelping rate was calculated in the conceived bitches. From Group I an average conception rate was 50.00 % which was non significant with the conception rate of 83.33% as observed in Group II. Similar reports of conventionally frozen dog semen comparatively were of Silva *et al.*, (2002) who noted 60% pregnancy rates in bitches after vaginal and intrauterine insemination with dog semen frozen at a concentration of 200×10^6 spermatozoa/ml. Comparatively lower conception and whelping rate was observed in present study than previous study as it may vary between breed to breed and depend on nutritional status of individual bitch as all bitches were maintained differently.

Conclusions

Significantly higher motility, viability and plasma membrane integrity was observed in the cryopreserved dog semen from Group II than in Group I with significantly higher conception and whelping rate by in intra vaginally inseminated bitches with frozen thawed semen. Therefore encapsulation of dog semen can be used as an alternative method for the cryopreservation of dog semen.

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