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Successful pregnancy following surgical intrauterine insemination using frozen semen in Afghan hound dog

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Abstract : A 3 year-old female Afghan hound came to the Veterinary Referral Hospital of the College of Veterinary Medicine, Seoul National University for artificial insemination (AI) with frozen semen. In order to inseminate, semen was frozen in USA 3 years ago. Frozen semen was sent by air from Santiago to Seoul for AI. The stud died 2 years ago, so we could only use a limited amount of frozen semen in that estrus cycle. The number of total motile spermatozoa was 59.4×10^6 and the total volume was 1.2 ml. The frozen spermatozoa were thawed in 70°C water for 8 sec, which were then deposited at the bilateral uterine horns by a surgical method. The number of corpus luteum was 6. Sixty days after artificial insemination resulted in the birth of 4 puppies, all of which are alive and healthy.

Key words : artificial insemination, frozen dog semen, intrauterine insemination

Artificial insemination (AI) with fresh and frozen-thawed ejaculated spermatozoa has been used widely and successfully in canine [1, 4, 5, 11]. Insemination of bitches with frozen-thawed semen has become a widely accepted means of introducing new bloodlines, often between countries, to breeding colonies. Cryopreservation of spermatozoa plays an important role in preserving the genetic diversity and assisting in the reproduction technique of canine. In 1969, Seager [9] had adapted the pellet-freezing method to dog sperm, and the first canine pregnancies resulted from AI of bitches with cryopreserved spermatozoa that had been frozen as pellets on dry ice. For 4 years after that experiment, this method had been used to produce 21 litters of pups from frozen-thawed semen, some of the semen had been stored in LN2 for > 1 year [8]. AI of domestic bitches with frozen thawed semen has been offered as a routine clinical service as a part of the assisted reproductive technique (ART).

AI using frozen semen is used to continue breeding from an outstanding stud long after he is dead. For the young stud showing great promise, his semen can be collected and stored in the event of his untimely death or infertility.

Another benefit of AI is its preciseness. Semen quality and quantity is determined. In natural mating, people hope for the best and deal with anything less afterward. Since AI allows dogs to be bred without coming in contact with each other sexually, the risk of transmitting sexual diseases is also minimized.

However, the pregnancy rates with frozen-thawed semen vary highly and are generally lower than those with fresh semen with whelping rates averaging from 0% to 80% and litter sizes being around 30% smaller compared with those obtained using fresh semen [3-5, 10].

Surgical insemination allows for direct insemination of the semen into the uterus. Similar to the technique used in the process of ovariohysterectomy in bitches, it involves injecting semen into the exposed uterus through a needle. Surgical insemination provides an opportunity to examine the bitches suspected of having uterine or ovarian diseases. In addition, surgical insemination can improve the conception rates in toy

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breeds with historically poor conception rates by natural breeding. The technique also benefits males with low sperm-cell counts who have difficulty in impregnating bitches through natural breeding.

The ideal timing of intrauterine insemination is 3-4 days after ovulation because oocyte maturation is completed [2, 12].

A 3 year-old intact female Afghan hound with a weight of 23 kg was presented to the Veterinary Referral Hospital of the College of Veterinary Medicine, Seoul National University for the process of AI using frozen semen that was frozen in the USA 3 years ago and imported for AI. The bitch has never experienced parturition. Any sexual behavior was not observed when the dog was presented in the hospital. The bitch was observed natural reproductive cycle and the estrus cycle was the second. The first estrus cycle occurred 10 months ago. The vaginal discharge was detected 7 days ago in this reproductive cycle. Any pseudo pregnancy sign including mammary gland swelling or nesting was not observed. The vaginal cytology test revealed many superficial cells with a few intermediated cells. The serum progesterone concentration was 1.2 ng/ml on hospitalization day, which means that the bitch was revealed at the proestrus stage.

For successful AI, it is critical to determine the exact day of ovulation. Thus, in order to determine the exact day of ovulation, we measured serum progesterone concentration on a daily basis. Blood samples (3-5 ml) were collected everyday by cephalic venupuncture into a 5-ml syringe with a 23 G needle, centrifuged at 3,000 rpm for 20 min and stored at −20°C until assayed. The samples were analyzed via DSL-3900 ACTIVE Progesterone Coated-Tube Radioimmunoassay Kit (Diagnostic Systems Laboratories, USA) with the assistance of Neodine VetLab, Korea. The day of ovulation was estimated from peripheral blood progesterone concentrations as described by Johnston *et al*. [9]. The day on which the progesterone concentration initially reached 4.0-7.5 ng/ml was regarded as the day of ovulation.

The stud died 2 years ago, so we could use a limited amount of frozen semen from that estrus cycle.

Three straw of frozen semen was imported and the volume of each straw was 0.4 ml. The information on the number of spermatozoa in each straw was not provided. Semen was thawed in 70°C water for 8 sec [7]. The total volume of thawed semen was 1.2 ml and the total sperm count was 132×10^6. Sperm motility and concentration were examined with the Markler's counting chamber (ZDL, USA). Motility was 45%, so 59.4×10^6 motile spermatozoa were inseminated at the bilateral uterine horns. The frozen semen was providing for use in surgical insemination can be used without thawed buffer. We examined only sperm motility due to the limited volume of semen. The sperm motility test was performed once due to the limitation.

Intrauterine insemination was performed 3 days after ovulation. AI was performed under general anesthesia. The bitch was pretreated with atropine sulfate (0.05 mg/kg) and acepromazine maleate (0.025 mg/kg) and anesthesia was induced using propofol and maintained with isoflurane. The ventral abdomen was clipped and the bitch was placed on the operating table in dorsal recumbency. The abdomen was draped in preparation for surgery. A 5 cm incision was made midway between the pubis and the umbilicus. The incision was made in the skin subcutaneous fat through the linea alba. The uterus was identified and elevated to the surface through the incision. The uterus was draped with saline moistened sterile gauze as the semen was prepared for the injection procedure. The semen to be injected was gently drawn into a 3-ml syringe. 24 G IV catheter was inserted into the lumen of the uterine horn at a 45-degree angle with the bevel of the needle upwards. A syringe was attached to the end of the catheter, and then the semen was slowly injected into the uterine horns. A saline moistened gauze was held over the injection sites after the catheter was withdrawn. After 1 min, the gauze was removed and the uterus was replaced into the abdomen. Closure of the fascia, muscle, subcutaneous tissue, and skin was completed with a routine process [4]. Post operative treatment was given with long acting penicillin G benzathine 50,000 U/kg IM.

Corpus luteum was counted in the right and left ovaries at the time of insemination. It was regarded as the number of ovulated ova. In this case, the numbers of corpus luteum were 3 and 3 in the right and left ovary, respectively.

Pregnancy was determined by the detecting fetal sac 25 days (Fig. 1) after insemination using an ultrasonographic imaging diagnostic system SONOACE 9900 (Medison, Korea). We detected 3 fetal sacs and the diameter of each sac ranged from 35.2 to 36.5 mm. The number of newborn was 4 (2 male and 2 female) dogs that were counted on the delivery day. The puppies

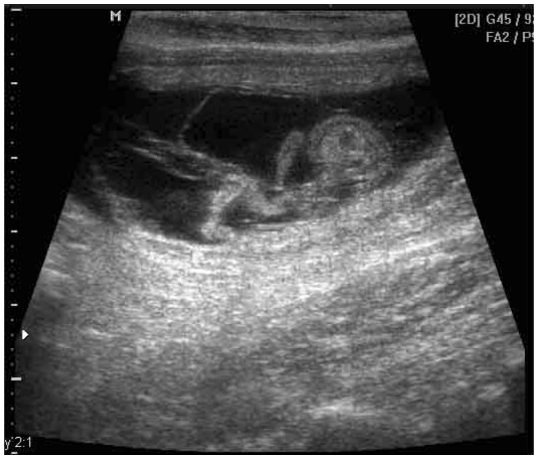

Fig. 1. Ultrasonographic image of a pregnant bitch 25 days after the artificial insemination, showing the fetus in longitudinal section from the head (right) to caudal abdomen (left). The head shows evidence of the shape of the snout.

body weight ranged from 460 to 520 g.

Generally, the success of pregnancy in AI with frozen semen depends on semen quality, thawing, insemination timing, insemination methods and many other factors. It has been reported that conception rate using AI twice with chilled semen contained 4×10^8 spermatozoa was 70% [13]. The conception rates of AI with frozen semen contained 4×10^8 spermatozoa with a Norwegian catheter, fiber optic endoscope and vaginal insemination rod were 84.4%, 58.9%, and 57.9%. The average litter sizes were 5.4, 4.0, and 6.0 [6]. In other studies, $150\text{-}200 \times 10^6$ spermatozoa used for AI is recommended [10, 14], but pregnancies have been produced with considerably fewer spermatozoa. In one study, pregnancies were achieved through insemination with live, intact 50×10^6 frozen-thawed spermatozoa by intrauterine deposition twice during estrus [15]. In this case, we had to use the limited number of spermatozoa of a dead stud that was imported from USA. The amount of that semen and number of spermatozoa were not enough for conventional intravaginal insemination. Considering all situations, we thought the surgical intrauterine AI was the most suitable method for this case.

We inseminated 3 days after ovulation to elevate the efficacy of pregnancy because oocyte maturation becomes complete 3-4 days after ovulation. The total number of inseminated spermatozoa was 59.4×10^6. Four puppies were delivered 60 days after surgical insemination. The ratio of the number of newborns to the number of corpora lutea was 66% (4/6).

In conclusion, the frozen semen that was imported had a low sperm count, however it was used to perform surgical AI that resulted in the birth of puppies.

If AI is used to genetically eliminate undesirable characteristics or to improve the potential of desirable traits, it will have a positive effect whenever it is used correctly.

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