大韓獸醫學會誌(2009) 第49卷 第4號
Korean J Vet Res(2009) 49(4) : 285~290

Superfecundation induction by intrauterine insemination with different frozen-thawed canine semen and parentage test using microsatellite analysis

Ji Hye Lee[1], Keun Jung Kim[1], Seon A Choi[1], Xiaoxia Li[1], Eun Young Kim[1], Hyun Ju Oh[2], Byeong Chun Lee[2], Hye Jin Kim[3], Byung Kwon Park[4], Min Kyu Kim[1,*]

[1]*College of Agriculture and Life Sciences, Chungnam National University, Daejeon 305-764, Korea*
[2]*College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea*
[3]*Haemaru Small Animal Referral Hospital, SungNam 463-050, Korea*
[4]*College of Industrial Science, Kongju National University, Yesan 340-702, Korea*
(Accepted: December 2, 2009)

Abstract : This study was performed to investigate the possibility of superfecundation by surgical intrauterine artificial insemination in dogs of confirmed genetic pedigree. Artificial insemination was performed on 3 days after ovulation with 1.3×10^8 spermatozoa. Five puppies were delivered on 60 days after insemination. The ratio of the number of newborns to the number of corpora lutea was 83.3% (5/6). Parentage analysis with 10 canine-specific microstatellite markers demonstrated that one puppy was genetically relative to the sire-A family and four puppies were genetically relative to the sire-B. The present study demonstrated that two kinds of puppies with different genetic pedigree can be produced by surgical uterine insemination of semen of individual dog into each uterine horn of a bitch.

Keywords : frozen-thawed semen, intrauterine insemination, microsatellite, superfecundation

Introduction

Cryopreservation of spermatozoa is an important part to preserve genetic diversity and to assist the reproduction technique of dogs. Since the first offspring was produced from the canine frozen semen in 1969 [22], artificial insemination (AI) of domestic bitches with frozen-thawed semen has been offered as a routine clinical service as a part of assisted reproductive technique. For AI in dogs, the character of fresh semen [3, 11, 29], cooled semen [11, 30] and frozen semen [3, 11, 27] have been investigated. However, the pregnancy rates with frozen-thawed semen are highly variable and generally lower than those with fresh semen with whelping average rates from 0 to 80% and litter sizes being around 30% smaller compared with those obtained using fresh semen [10, 12, 13, 23]. To promote the efficacy of AI with frozen-thawed semen, several studies have been proceeded with intravaginal insemination, intrauterine insemination or intratubal insemination in dogs [21, 24, 27, 28]. It is generally considered that only a small number of spermatozoa inseminated into the uterus reaches the uterine tubes and fertilizes the ova. Most of the spermatozoa inseminated into the vagina were not involved in fertilization [19]. The intrauterine deposition was initially used to increase poor conception rates in the use of canine frozen semen. Since then, surgical deposition of semen into the uterus has become a routine technique used in numerous situations encountered in canine reproductive medicine. It has been reported that the number of spermatozoa required to obtain conception by intrauterine horn insemination is 1/10 of that required by intravaginal insemination in dogs, i.e. 1.0×10^8 *vs.* 1.0 to 4.0×10^9 spermatozoa [27]. Thawed spermatozoa have short life span time, thus insemination timing is critical to obtain a large litter size. The ideal timing of intrauterine insemination is 3 to 4 days after ovulation because oocyte maturation is completed on 3-4 days after

*Corresponding author: Min Kyu Kim
College of Agriculture and life sciences, Chungnam National University, Daejeon 305-764, Korea
[Tel: +82-42-821-5773, Fax: +82-42-825-9754, E-mail: kminkyu@cnu.ac.kr]

ovulation [8, 25].

Superfecundation most commonly happens within hours or days of the first instance of fertilization with ova released during the same cycle. Heteropaternal superfecundation is very rare in humans, though more common in animals [8]. Especially, superfecundation is reported to more commonly occur in cats. For superfecundation to happen, conception must occur after tertiary follicles have been induced to ovulatory behavior in a pregnant female [20]. In cats, periods of follicular growth do occur during the luteal phase [32]. However, the bitch reproductive cycle is mono-oestrus with spontaneous ovulation. In the natural mating of dogs, the spermatozoa of first mating sire may fertilize all the oocytes and for many reasons, the natural mating between two selected partners is impossible. Thus, the breeder may take the alternative route of using AI for superfecundation in order to have puppies with good genetic pedigree in a short period of time.

Accordingly, the present study was performed to investigate the possibility of superfecundation by surgical uterine AI of frozen-thawed semen in dogs of confirming genetic pedigree. The fertility rate of this intrauterine insemination was monitored and the parentage analysis was performed with microsatellite assay.

Materials and Methods

Animals

A four year-old female Afghan hound came to the veterinary referral hospital of the college of veterinary medicine, Seoul national university for AI using frozen semen from two same breed male. The sire-A was a pedigreed dog to championship in dog show, the sire-B have a distinguished and ancient lineage. The bitch has never experienced parturition and her estrus cycle was the third.

Chemicals

All chemicals were purchased from Sigma-Aldrich (USA) unless otherwise specifically indicated.

Semen collection and semen extenders

Semen was collected from each dog by digital manipulation with no female present. For the semen collection, a modified disposable artificial vagina was placed over the protruded penis and circumferential pressure was applied posterior to the bulbus glandis to stimulate erection and ejaculation. Only the sperm rich fraction of each ejaculate was used. Total volume of collected semen was 1.2 and 1.5 mL from sire-A and sire-B, respectively. Sperm motility and concentration were examined with the Markler's counting chamber (ZDL, USA). The extender was used to dilute the samples of semen to be frozen. Tris buffer contained 2.4 g Tris, 1.4 g citric acid, 0.8 g glucose, 0.06 g Na-benzylpenicillin and 0.1 g streptomycin sulphate in 100 mL distilled water (pH 6.5, 249 mOsm). The first freezing buffer (1st buffer) was made of Tris buffer with 20% (v/v) egg-yolk (pH 6.4, 843 mOsm). The second freezing buffer (2nd buffer) was made of 1st buffer with 6% (v/v) glycerol (pH 6.4, 1685 mOsm).

Sperm dilution and freezing

The collected semen was centrifuged at 750 × g for 5 min and the supernatant was removed. The resultant sperm pellet was resuspended and diluted with the 1st buffer at concentration of 4.0×10^8 sperm/mL at room temperature. The extended semen was then immediately to equilibrate and placed in cold room at 5°C (1 h). The semen was then divided into 5 equal aliquots. The second dilution was carried out with the same volume of the 2nd buffer with 6% glycerol at 5°C (1 h). The final sperm concentration was 2.0×10^8 sperm/mL. After equilibration, the extended semen was placed in 0.5 mL straws sealed at 5°C and horizontally exposed to nitrogen vapor (6 cm above the surface of the liquid nitrogen) for 10 min. Finally, straws were immediately plunged into the liquid nitrogen at −196°C and stored until thawing and AI.

Detection of ovulation day

To determine the ovulation day in the dog, blood was collected from the cephalic vein daily starting 7 days after the beginning of bleeding in estrus and the ovulation day was estimated from the blood progesterone level. Sera were analyzed using a DSL-3900 Active Progesterone Coated-Tube Radioimmunoassay Kit (Diagnostic Systems Laboratories, USA). The day on which the progesterone concentration initially reached 4.0 - 7.5 ng/mL was regarded as the day of ovulation [9].

Intrauterine insemination using surgery and pregnancy diagnosis

Semen was thawed in 70°C water for 8 sec and evaluated on the Markler counting chamber [16]. Total

Table 1. Conception result of artificial insemination with different frozen-thawed semen in each uterine horn

Site of insemination (Sire)	No. of corpus luteum	No. of inseminated spermatozoa	No. of delivered puppy	Conception rate	Total whelping rate*
Right (A)	2	1.2×10^8	1	50%	83.3%
Left (B)	4	1.5×10^8	4	100%	

*Total whelping rate was the ratio of the number of newborns to the number of corpora lutea.

Fig.1. Four puppies born from a bitch inseminated with two male dogs' spermatozoa. Black color puppy is genetically related to the sire-A and the remaining three puppies are genotypically related to the sire-B. One puppy died at 20 days after birth.

volume of inseminated semen was adjusted to 1 mL in dilution with Tris-citrate-glucose buffer and inseminated at the each uterus horn. Intrauterine insemination was performed at 3 days after ovulation. For surgical intrauterine insemination, anesthesia was induced with 6 mg/kg propofol (Pofol; Hana Pharm, Korea), and general anesthesia was maintained with 2% isoflurane (Ifran; Hana Pharm, Korea). While in dorsal recumbency, the recipients were aseptically prepared for surgery and a caudal ventral incision was made to expose the uterus. The prepared different semen was slowly injected into the each uterine horns by 3 mL syringe through 24 G IV catheter was inserted into the lumen of the uterine horn at 45-degree angle with the bevel of the needle up. Corpus luteum was counted in the right and left ovary at the time of insemination. Pregnancy was diagnosed using a SONOACE 9900 (Medison, Korea) ultrasound scanner with an attached 7.0 MHz linear probe 26 days post AI.

DNA extraction and microsatellite analysis for genotyping

Parentage analysis was performed in the dam, sire-A, sire-B and puppies to confirm the genetic identity. Genomic DNA from the blood of each dog was extracted according to instruction of G-spin Genomic DNA Extraction Kit (Intron, Korea). The following 10 markers were selected for analysis: PEZ1, PEZ3, PEZ5, PEZ6, PEZ8, PEZ12, PEZ20, FH2010, FH2054, and FH2079 [9, 18]. The isolated genomic DNA samples were dissolved in 50 µL TE and used for microsatellite assay with 10 canine-specific markers. Length variations were assayed by polymerase chain reaction (PCR) amplification with fluorescently labeled (FAM, HEX, and NED) locus-specific primers and PAGE on an automated DNA sequencer (ABI 373; Applied Biosystems, USA). Proprietary software (GeneScan and Genotyper; Applied Biosystems, USA) was used to estimate PCR product size in nucleotides.

Table 2. Parentage test with canine-specific microsatellite markers among sires and puppies

	PEZ-1		PEZ-3		PEZ-5		PEZ-6		PEZ-8		PEZ-12		PEZ-20		FHC-2010		FHC-2054		FHC-2079	
	peak1	peak2	peak1	peak2	peak1	peak2	peak1	peak2	peak1	peak2	peak1	peak2	peak1	peak2	peak1	peak2	peak1	peak2	peak1	peak2
Dam	110	117	115	118	115	118	200	200	234	234	261	275	179	179	229	229	178	178	275	290
Sire-A	117	117	115	118	115	118	187	187	234	234	261	275	175	179	229	233	174	178	271	275
Puppy-A1	110	117	115	118	115	118	187	200	234	234	261	275	179	179	229	233	178	178	275	290
Sire-B	117	122	118	128	105	118	200	200	222	234	270	275	171	175	229	229	171	175	270	275
Puppy-B1	110	117	115	118	115	118	200	200	234	234	270	275	171	179	229	229	171	178	270	275
Puppy-B2	117	117	118	118	105	118	200	200	234	234	261	275	171	179	229	229	171	178	275	275
Puppy-B3	117	122	118	128	118	118	200	200	234	234	270	282	171	179	229	229	171	178	270	290
Puppy-B4	110	122	115	128	105	118	200	200	234	234	270	275	171	179	229	229	171	178	275	275

Alleles are named for the total length of the segment amplified.

Results

Total volume of thawed semen was 0.7 and 0.8 mL from sire-A and sire-B, respectively. Total sperm count was 2.0×10^8 or 2.1×10^8, respectively. Motility was 62.0 and 69.0%, respectively, thus 1.2×10^8 or 1.5×10^8 motile spermatozoa were inseminated at the each uterus horn. Pregnancy was determined by the detecting fetal sac 26 days after insemination using an ultrasonographic imaging diagnostic system.

As shown in Table 1, the numbers of corpus luteum were 2 and 4 in the right and left ovary, respectively. The number of newborn counted on the delivery day was 5 (Fig. 1). The ratio of the number of newborns to the number of corpora lutea was 83.3%.

As a result of parentage analysis, one puppy was genetically relative to the sire-A family and 4 puppies were genetically relative to the sire-B blood (Table 2).

Discussion

Most breeders desire to have puppies with good genetic pedigree in a short period of time. We can think AI is an ideal means to produce puppies from two superior male dogs in one trial of AI. The possibility of superfecundation by natural breeding is low because spermatozoa from the first mated male fertilized all the oocytes of bitch. In contrast, the probability of superfecundation can be increased if the spermatozoa from each male dog are deposited into each uterine horn artificially and fertilized with oocytes from each ovary.

In the present study, we performed AI for superfecundation. AI with fresh or frozen-thawed ejaculated spermatozoa has been used widely and successfully in dogs [3, 4, 12, 13, 15, 24]. Insemination of bitches with frozen-thawed semen has become a widely accepted means of introducing new bloodlines, often between countries, to breeding colonies. Various methods of insemination are presently available to the inseminator including intrauterine insemination with laparotomy [5, 17], the Norwegian catheter [1] or fiberoptic endoscopy [2] or intravaginal insemination. The present study demonstrates a higher superfecundation rate following surgical intrauterine AI using frozen thawed semen in a bitch. Superfecundation in dogs was performed by AI per vaginal using frozen semen [25] or fresh semen and naturally by serial mating with two or more sires [26]. However, intravaginal insemination usually has low conception rate, and in natural mating the spermatozoa of first mating sire may fertilize all the oocytes. Intrauterine insemination has advantage over both the intravaginal or natural method. Thomassen *et al.* [24] reported a higher whelping rate after intrauterine insemination (71%) than after simple intravaginal insemination (29%). Likewise, the present study also reported 83.3% whelping rate following intrauterine insemination.

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 2 times AI with chilled semen containing 4.0×10^8 spermatozoa was 70% [30]. The conception rates of AI with frozen semen containing 4.0×10^8 spermatozoa with Norwegian catheter, fiber optic endoscope

and vaginal insemination rod were 84.4%, 58.9% and 57.9%, respectively and liter size were 5.4, 4.0 and 6.0, respectively [14]. In other studies, $1.5\text{-}2.0 \times 10^8$ spermatozoa was recommended for AI [23, 31]. However, pregnancies have been produced with considerably fewer spermatozoa [28]. In one study, pregnancies were achieved through insemination with 0.5×10^8 live normal frozen-thawed spermatozoa by intrauterine deposition twice during estrus [33]. In our study, the time of insemination was 3 days after ovulation to elevate the efficacy of pregnancy because oocyte maturation is completed on 3 to 4 days after ovulation. Average number of inseminated spermatozoa was 1.3×10^8. Five puppies were delivered on 60 days after insemination. The ratio of the number of newborns to the number of corpora lutea was 83.3% (5/6). As result, our study is the first report of delivery of newborns obtained by each intrauterine insemination using two different frozen-thawed canine semen. For the parentage testing of pups, DNA fingerprinting using 10 different canine microsatellite markers was performed. Each puppy was confirmed genetically to be an offspring of either sire-A or sire-B. In order to confirm the superfecundation, various methods such as blood typing, hair specimens, puppy size and microsatellite markers have been used [6, 7, 31]. However, DNA fingerprinting using microsatellite markers was the most useful tool for discrimination of individual sires involved in superfecundation.

In conclusion, the present study demonstrated that two kinds of puppies with different genetic pedigree can be produced by surgical uterine insemination of individual dog semen into each uterine horn of a bitch.

Acknowledgments

This study was financially supported by NRF (Grant # R01-2009-000-21076-0) and the Korean MEST, through the BK21 program for creative research in animal biotechnology.

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