

Evaluation of Transcervical Insemination using Frozen Semen by Flexible Endoscope in Dogs

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ABSTRACT. We evaluated transcervical insemination (TCI) using frozen semen by flexible endoscopy in dogs. Eight female and eight male beagles were used in this study. A flexible endoscope and a washing tube were used for TCI. A tracheal tube was used as an alternative to the penis and was an auxiliary for inserting the flexible endoscope. The mean time required to insert the washing tube into the external os of the uterus after inserting the endoscope into the tracheal tube was 7.5 min. Slight or mild pain was observed in all bitches during TCI. However, TCI could be easily performed with retention in all bitches and without sedation anesthesia. The tracheal tube was useful to ensure the visual field using air sufflation. Clinical signs suspicious of infection were not observed in any bitches from the TCI to the pregnancy diagnosis. The conception rate was 87.5%, and the mean number of fetuses was 6.3. TCI using a flexible endoscope in bitches was performed quickly with minimal invasiveness. We present a new method of TCI in dogs. This method should be studied in small and large breeds to obtain more detailed results.

KEY WORDS: canine, flexible endoscope, frozen semen, transcervical insemination.

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Artificial insemination (AI) using frozen semen in dogs has been increasing in recent years. However, AI using cryopreserved semen generally yields lower pregnancy rates with vaginal deposition than with uterine deposition because of the short life span of frozen-thawed spermatozoa [5, 19]. Therefore, AI methods usually use uterine deposition with frozen semen. Surgical insemination is highly invasive and requires general anesthesia, whereas transcervical insemination (TCI) using metal catheterization is minimally invasive but requires a skilled technique [24]. In contrast, TCI using a rigid endoscope is easy and less invasive [13, 30]. TCI with a flexible endoscope is also less invasive because the scope is soft, and it has an air supply and instrument channel, which endows it with good operability [21, 29]. Therefore, a flexible endoscope might be useful as a new tool for TCI.

The purpose of this study was to evaluate TCI using frozen semen by flexible endoscope in dogs.

MATERIALS AND METHODS

Eight female beagles aged 1–8 years (mean \pm standard deviation: SD, 4.1 ± 2.5 years) and weighing 10.7–15.1 kg (mean \pm SD, 12.8 ± 1.3 kg) were used in this study; 4 were parous, and the others were nulliparous. Eight male beagles aged 1–7 years (mean \pm SD, 3.6 ± 1.8 years) and

weighing 11.4–15.8 kg (mean \pm SD, 13.1 ± 1.4 kg) were used as sperm donors. Unrelated dogs were used to prevent inbreeding. This study was performed in accordance with the Guide for the Experimentation of Animals of the College of Bioresource Sciences, Nihon University (AP11B018, 2010).

The sperm-rich fraction of a single ejaculate was collected from each dog by digital manipulation [11]. The collected semen was centrifuged at $400 \times g$ for 5 min. The supernatant was discarded, and the pellets were gently suspended in 1 ml with egg-yolk Tris-fructose citrate extender supplemented with 1% Orvus ES paste (OEP) [26–28]. Sperm concentration was measured 3 times in each sample with an automated blood cell counter (MEK-5257, Nihon Koden, Tokyo, Japan), and the average value was used. Finally, the prepared semen was adjusted to 200×10^6 sperm/ml in a glass vial with the same extender. The adjusted semen was cooled from 25°C to 4°C for 60 min using a programmed freezer (ET-1N type, Fujihira Industry, Tokyo, Japan). The semen was subsequently diluted to 100×10^6 sperm/ml with egg-yolk Tris-fructose citrate extender supplemented with 1% OEP and 10% glycerol in the programmed freezer, and a glycerol equilibration was performed for 60 min at 4°C. The final concentrations of OEP and glycerol were 0.5% and 5%, respectively. After equilibration, the semen was packed into 0.5-ml straws. The semen straws were horizontally placed on a 7-cm high stainless steel rack from the surface of the liquid nitrogen in a styrene foam box ($27.5 \times 23.4 \times 14.0$ cm and 1.8 cm thick) for 10 min and stored in a liquid nitrogen tank [17, 18, 20]. Frozen semen was thawed by immersing the straws in a water bath at 70°C for 8 sec just before AI [16]. Sperm progressive motility and sperm malformation

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rate were evaluated by direct microscopic examination.

The timing of insemination in relation to the luteinizing hormone (LH) surge was determined by monitoring serum progesterone (P_4) concentration using an automated chemiluminescence immuno analyzer (Spotchem Vidas SV-5020, Arkray, Kyoto, Japan) [1, 2, 4, 7, 8, 14]. The start date of proestrus was confirmed by daily observation of vulvar swelling and estrous bleeding. Serum P_4 concentrations were measured daily after 5 days from confirming the beginning of proestrus or the appearance of >80% cornified cells in the vaginal smear. In principle, the estimated day of the LH surge was determined when serum P_4 concentrations first reached 2–4 ng/ml. However, among bitches in which serum P_4 concentration was initially <2 ng/ml and then rapidly increased to >4 ng/ml the next day, the estimated day of the LH surge was determined when the serum P_4 concentration was >4 ng/ml. The serum P_4 concentration was measured on the fifth day after the LH surge day (day 0) when the TCI was performed.

The bitch was retained in the prone position on an operating table that was angled at about 30 degrees so that the tail was high and the head was low. A flexible endoscope (VQ-6092-A: outside diameter, 6 mm; Olympus, Tokyo, Japan) was used for TCI. A tracheal tube (inside diameter: 10.0 mm, outside diameter: 13.7 mm, Portex, Smiths Medical, St. Paul, MN, U.S.A.) was inserted into the vulva as a guide tube for the flexible endoscope. Then, the tracheal tube cuff was injected with air to prevent inflow of air into the bladder. The cuff air volume in the tracheal tube (30–45 ml) was adjusted according to the physique of the bitch. The flexible endoscope was inserted into the tracheal tube, and the position of the external os of the uterus was confirmed (Fig. 1). A washing tube for bronchial lavage (PW-2L1: outside diameter, 1.8 mm; Olympus) filled with egg-yolk

Tris-fructose citrate was inserted into the external os of the uterus from the channel in the endoscope, and 1 ml/(100 × 10⁶ sperm) of thawed semen was injected (Fig. 2). After the semen was injected, the posture of the bitch was maintained for 10 min to prevent semen reflux. The necessary time for the procedure was considered to be the time to insert the washing tube into the external os of the uterus after inserting the endoscope into the tracheal tube.

The presence of infection was confirmed by clinical symptoms for 30 days after AI. Fetuses and the gestational sac were confirmed by ultrasonography (Sonosite180, SonoSite Inc., Seattle, WA, U.S.A.) at 30 days after AI, and the number of fetuses was confirmed [3, 10, 23]. All data were expressed in means ± SD.

RESULTS

Progressive motility of thawed sperm was $58.8 \pm 9.2\%$, and the sperm malformation rate was $8.6 \pm 2.4\%$. The serum P_4 concentration was 3.7 ± 0.9 ng/ml on the LH surge day and 31.3 ± 10.2 ng/ml on the day of TCI (Table 1). Ovulation was assumed to have occurred when serum P_4 concentration was >5 ng/ml, and the AI day was confirmed [1, 2, 4, 14]. Serum P_4 concentrations on the day of TCI in this study were >15 ng/ml.

The TCI procedural time was 7.5 ± 4.6 min. The bronchial lavage washing tube was inserted approximately 1 cm into the external os of the uterus within 15 min in all bitches (Table 1). The tracheal tube was useful as a guide tube for inserting the endoscope into the vagina to ensure a visual field using the air inflated cuff. The amount of cuff air volume in the tracheal tube was 36.7 ± 5.6 ml (Table 1). Slight or mild pain was observed in all bitches during AI. However, AI was easily performed without any sedation anesthesia.

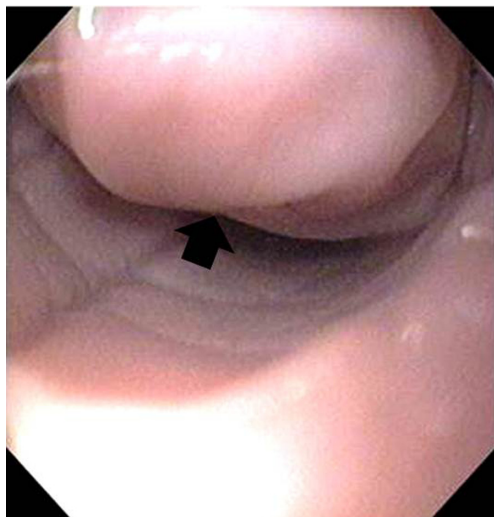


Fig. 1. Findings of the external os of the uterus using a flexible endoscope. Arrowhead shows the external os of the uterus.



Fig. 2. Findings of the washing tube that was inserted into the external os of the uterus from the flexible endoscope channel.

Table 1. Summary of results in intrauterine TCI using flexible endoscopy

Bitches	Age (year)	BW (kg)	Parity	Sperm progressive motility (%)	Sperm malformation rate (%)	P ₄ concentrations (ng/ml) LH surge day	TCI day	Cuff volume (ml)	Procedure time (min)	Number of fetuses
Bitch 1	1	13.2	nulliparous	65	5	5.0	32.4	45	12	9
Bitch 2	6	13.0	parous	45	12	2.4	24.0	45	5	5
Bitch 3	5	12.0	parous	50	9	3.9	43.1	40	2	5
Bitch 4	2	15.1	nulliparous	70	8	2.9	15.4	30	8	5
Bitch 5	6	13.6	parous	60	8	3.2	26.7	35	3	Nonconception
Bitch 6	2	10.7	nulliparous	70	6	3.2	24.0	35	15	9
Bitch 7	8	12.5	parous	55	11	4.6	39.0	30	10	6
Bitch 8	3	12.4	nulliparous	55	10	4.4	45.4	35	5	11
Mean \pm SD	4.1 \pm 2.5	12.8 \pm 1.3	N/A	58.8 \pm 9.2	8.6 \pm 2.4	3.7 \pm 0.9	31.3 \pm 10.2	36.9 \pm 5.9	7.5 \pm 4.6	6.3 \pm 3.4

TCI: Transcervical insemination, BW: Body weight, P₄: Progesterone, LH: luteinizing hormone, N/A: not applicable, SD: standard deviation

No clinical signs suspicious of infection were observed in any bitch during the pregnancy diagnosis. Seven bitches became pregnant, and the conception rate was 87.5%. The number of fetuses was 6.3 ± 3.4 (Table 1). Because non-conception occurred in one bitch, a statistical analysis was not performed. However, no characteristic differences in age, presence of parity, serum P₄ concentrations on the days of LH surge and TCI, or sperm quality were observed between the bitches that conceived and those that did not conceive.

DISCUSSION

The collection of sperm and the preparation of frozen semen were performed as reported previously [11, 16–18, 20]. Sperm motility was 50%–70% in previous studies, which was similar to the rates we obtained, and no abnormalities were observed in sperm malformation rate [9, 27]. In addition, the conception rate was 87.5% in this study. Thus, the methods for preparing the frozen semen in this study were sufficient for TCI.

The P₄ assay method we used has been previously used to estimate the LH surge day [1, 2, 4, 7, 8, 14]. Since the measurement methods for chemiluminescence immunoassay, radioimmunoassay and enzyme immunoassay were almost perfectly correlated, the reference value of the P₄ assay method in this study was fixed [2, 4, 14]. Subsequently, the LH surge and TCI days were also estimated by measuring serum P₄ concentrations. As a result, the conception rate was 87.5%. Conception rates were 70–90% in a previous report; thus, TCI timing using this method was appropriate [9, 12].

Our study is the first report of TCI performed using a flexible endoscope in dogs. Among the many TCI reports, the method using a rigid endoscope is relatively easy and non-invasive [13]. However, flexible endoscopes are widely used in the field of gastroenterology, and are less invasive than a rigid cystoscope because they are soft [21, 22, 29]. In this study, slight pain was observed, but TCI could be easily performed without anesthesia in all bitches. In addition, procedural time was approximately 8 min, which was shorter than a previous report using a rigid endoscope [13]. This result was considered, because of a good view obtained by

air insufflation and higher operability by using an instrument channel as well as increased mobility of the endoscope tip. Therefore, the flexible endoscopy method was considered to be useful for TCI in dogs. However, it is necessary to verify this method on smaller and larger dogs, because we targeted medium-sized dogs with a body weight of about 10 kg.

We used a tracheal tube as a guide tube for the endoscope and as an alternative to the penis [15]. An Osiris catheter has been used as an alternative to the penis [15]; however, these catheters cannot be used as a guide tube for inserting an endoscope. The tracheal tube was useful for inserting the endoscope and securing the field of view. We observed no signs of infection after TCI, suggesting that a tracheal tube is useful as an auxiliary TCI tool with a flexible endoscope.

The conception rate was 87.5%, with six fetuses in this study. The conception rate in a TCI study that used a rigid endoscope was 90% with 5 fetuses [13]. In addition, the conception rate and number of fetuses are about the same for intravaginal AI with fresh semen [6, 15]. However, TCI is recommended, because the survival time of sperm is short in frozen semen [6, 25]. As frozen semen was used in this study and both conception rate and the number of fetuses were similar to previous reports using a rigid endoscope, our method appears more useful for TCI with frozen semen. In the other hand, since one dog that was not pregnant in this study showed no difference in age, presence of parous, serum P₄ concentrations and sperm quality, the cause for nonconception was unclear.

This study had one major limitation. This study results were confirmed using ultrasonography of fetuses and the gestational sac, but not confirmed grossly the number of puppies. Therefore, it was unclear whether this method results in a normal gestation period and normal delivery. In addition, since the number of fetuses was confirmed by ultrasonography, it may not be as accurate as those observed in the delivery. Therefore, it is necessary to examine the gestation period, delivery, and the number of fetuses by delivery in the future.

In summary, TCI using a flexible endoscope in dogs was performed quickly with minimal invasiveness. The tracheal tube was useful for inserting the endoscope and to prevent

infection. In addition, the conception rate and the number of fetuses were similar to previous reports. This method should be studied in small and large breeds to obtain additional detailed results and verify the methodology.

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