Archives of Veterinary Science , v 11, n.2. p.47-54, 2007
Printed in Brazil
ISSN 1517-784X

CRYOPRESERVATION OF DOG SPERMATOZOA: EFFECT OF BOVINE SERUM ALBUMIN ON ACROSOMAL INTEGRITY AND PREGNANCY RATES AFTER ARTIFICIAL INSEMINATION

(Criopreservação de sêmen canino: efeito da albumina sérica bovina sobre a integridade do acrossoma e taxa de prenhez após a inseminação artificial)

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Abstract - The objective of the present study was to compare the in vitro and in vivo profile of frozen dog semen with Tris-bovine serum albumin (TB) and Tris-egg yolk (TE) extenders. Twenty dogs were used as donors. Each dog was stimulated by penile massage and only the sperm-rich fraction was collected weekly until 40 ejaculates were obtained. After macroscopic and microscopic analysis, equal parts of each ejaculate were diluted with TB and TE by the one-step method at 37 ºC. The semen was added to 0.5-mL French straws which presented normal characteristics before freezing and after thawing. Acrosomal integrity was evaluated by double Trypan blue-Giemsa staining, in which alive intact (LI), alive reacted (LR), dead intact (DI) and dead reacted (DR) spermatozoa, were identified by the time of thawing and up to 4 h of incubation at 39 °C, the TE being significantly superior to TB (P<0,01) in the LI and LR variables. The TB being significantly superior to TE (P<0,01) in the DR variable. Female dogs in natural heat were submitted to artificial insemination, 20 receiving TE-semen and 20 receiving TB-semen with the Osiris probe (IMV, L'Aigle, France) and the numbers indicate that TE was significantly better than TB (P<0,01) to pregnancy rate and number of puppies/delivery. We concluded from this study, that TE was better than TB, because this, induced an early acrossome reaction in dog's sperm.

Key–Words: Bitch; oestrus; extenders; semen.

Resumo - Objetivo do presente trabalho foi comparar a performance de sêmen de cachorro in vitro e in vivo com soro de albumina bovina – tris (TB) e diluente de gema de ovo com Tris (TR). Para isso, foram usados vinte cães como doadores. O sêmen dos cães foi colhido via massagem peniana. Semanalmente a fração rica espermática foi colhida até quarenta ejaculados. Após o exame macroscópico e microscópico, as amostras foram diluídas em partes iguais de cada ejaculado com (TB e TE), pelo método one-step a 32º C. O sêmen foi embalado em palhetas francesas de 0,5 mL, as quais apresentaram características normais antes e após o congelamento. Foi avaliado a integridade cromossômica pelo método de coloração azul tripan giemsa, observando-se espermatozóides vivos intactos (LI), vivos com reação (LR), mortos intactos (DI), mortos com reação (DR). Os espermatozóides foram identificados pelo tempo de descongelamento e após 4 horas de incubação a 39ºC, o (TE) foi significativamente superior ao TB (P < 0,01) nas variáveis LI e LR. O TB foi significativamente superior ao TE (P < 0,01) na variável DR. Cadelas no estro natural foram submetidas à inseminação artificial, vinte delas recebendo sêmen com TE e vinte recebendo sêmen com TB mediante o uso de uma pipeta de Osiris (IMV, L'Aigle, France). Os resultados indicaram que o grupo TE foi significativamente melhor que TB (P < 0,01) em relação à taxa de prenhez e ao número de filhotes. Concluiu-se neste estudo que o grupo TE foi melhor que TB, porque isto induziu a uma reação acrossômica mais precoce no sêmen canino.

Palavra-Chave: cadela; estro; diluidor; semen.

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INTRODUCTION

The use of frozen semen permits the planning of artificial insemination since semen can be transported over large distances. Frozen semen banks favor the improvement of breeds and preserve wild species in extinction, in addition to permitting the insemination of several female dogs with semen from the same male.

The objective of a new cryopreservation technique is to minimize damage to the spermatozoa in an attempt to improve the capacity of fertility of the oocyte. *In vitro* methods which are generally used to evaluate sperm viability are motility, vigor, membrane integrity, acrosome, and morphology. Therefore, we have little knowledgement of the degree of different types of damage that can be induced in the dog's spermatozoa without rendering them incapable for fertilization (STRÖM and LINDE-FORSBERG, 1997; FARSTAD, 2000).

The vital double Trypan blue-Giemsa staining method (KOVACS and FOOTE, 1992), was used to determine the acrosome condition of sperm since it permits to distinguish between true and false acrosome reactions, i.e., the difference between alive and dead spermatozoa. These aspects make this staining technique the most adequate parameter for the *in vitro* assessment of post-thaw spermatozoa during routine assisted reproduction in dogs.

Freezing of dog semen using Tris-fructose-egg yolk as extender resulted in 40% progressively motile sperm and 38.5% acrosomal integrity of spermatozoa stained with Spermac (NÖTHLING and SUTTLEWORTH, 2004). Evaluation of the acrosomal integrity of fresh and post-thaw spermatozoa originating from the epididymis and bull ejaculate by double staining (Trypan blue-Giemsa) permitted their classification into four classes: live intact, live acrosome-reacted, dead intact, and dead acrosome-reacted spermatozoa and could be used for prognosis of the potential fertility of bovine semen samples used for IVF or AI (DIAS, 2002; TARTAGLIONE and RITTA, 2004).

RODRIGUES (1997), determined *in vitro* the influence of Tris-bovine serum albumin (BSA) extender on sperm viability during freezing of dog semen using three different BSA concentrations (0.25, 0.5 or 1%) compared with each other and with Tris-egg yolk extender. The Author found a significant reduction in motility and vigor for semen diluted in Tris-egg yolk compared to semen diluted in Tris-BSA regardless of the BSA concentration. No significant differences in acrosomal integrity were observed among the three BSA concentrations tested and the Tris-egg yolk diluent. The best fertility outcomes using frozen semen are obtained when insemination is performed by the intrauterine route (LINDE-FORSBERG, 1991). Vaginal artificial insemination is an easy procedure, but pregnancy rates are lower than those obtained with intrauterine insemination when frozen-thawed semen is used (ROTA *et al*., 1999). Artificial insemination by the Norwegian technique (intrauterine deposition of semen through a cervical catheter) using 150 to 200 million thawed spermatozoa at a 48-h interval resulted in 10 pregnancies among 11 female dogs inseminated in natural heat and the litter size ranged from 1 to 7 puppies (ANDERSEN, 1975; SZASZ *et al*., 2000).

GUÉRIN (1998), has been recommended the Osiris probe for the insemination of female dogs, even when thawed semen is used. In this case, a 10 to 15% drop in the results was observed compared to those obtained with chilled or fresh collected semen.

During proestrus, serum progesterone increases from 0.5 ng/mL (anestrus) to 1 ng/mL 24 to 48 h before the preovulatory LH surge, reaching a concentration of 2 to 4 ng/mL during the LH surge, and ovulation initiates 2 days later when the progesterone concentration ranges from 4 to 10 ng/mL. However, in the case of frozen-thawed semen, the first insemination should be performed as close to oocyte maturation as possible, which in most dogs occurs by the fourth day after the LH peak (day 0) when the progesterone levels reach 10 to 13 ng/mL, and repeated, usually 24 to 36 h later (ENGLAND and LOFSTEDT, 2000; FELDMAN and NELSON, 2004).

The objective of the present study was to determine the *in vitro* and *in vivo* efficiency of frozen dog semen with Tris-BSA (TB) and Tris-egg yolk (TE).

MATERIALS AND METHODS

1.1. Animals

Thirty-two dogs of different breeds weighing 20 to 40 kg and ranging in age from 2 to 5 years were submitted to andrologic examination. Twenty dogs (six boxers, three collies, three rottweilers, two German shepherds and six mongrel dogs) fulfilled the minimum requisites of the andrologic test (CBRA, 1998). After conditioning for semen collection, these dogs served as donors for the experiment.

Forty 3- to 6-year-old female dogs of different breeds (six Brazilian Fila, four collies, eight boxers, four rottweilers, six German shepherds and twelve mongrel dogs) with a known reproductive history and

gynecologically healthy, weighing 20 to 40 kg, were inseminated artificially.

The animals were fed with commercial ration (Nutron® Alimentos Ltda, Toledo, Brazil) twice a day and were vaccinated against rabies. The animals were kindly provided by the owners living in Palotina city and kept at their residence. Collection of material for vaginal cytology and observation of the female's behavioral signs were carried out at the residence of the dog's owner. Semen collection and artificial insemination were performed at the Laboratory of Animal Reproduction, Federal University of Paraná, Palotina, Brazil.

1.2. Preparation of the dogs and semen collection

In the semen collection room, the prepuce and penis of the dogs were cleaned using a No. 16 urinary catheter coupled to a syringe containing 20 mL physiological saline at 35°C. The catheter was introduced through the preputial ostium which was obstructed manually. After injection of the content, the catheter was removed, the prepuce was massaged, and the content was collected into a transparent container. The process was repeated until the content was clear.

After cleaning the prepuce, the dog was stimulated by penile massage and only the sperm-rich fraction of the ejaculate was collected into a graded tube coupled to a plastic funnel sec RISOPATRÓN *et al.*(2000). Semen was collected once a week from each dog until a minimum of 40 ejaculates were obtained.

1.3 *Evaluation of freshly collected semen*

Immediately after collection, the volume of the semen was determined. The material was mounted on a slide and coverslipped, and vigor scoring (0 = immobile, 1= without progressive motility, 2= slow progressive motility, 3= moderate progressive motility and 4= fast progressive motility) and progressive motility (0 to 100%) were analyzed under a phase-contrast microscope (Olympus BX41TF, Tokyo, Japan). Sperm concentration was determined in a Neubauer chamber with the diluted semen 1:100 with formol saline, and the final count was calculated using the formula of KRAUSE (1966). Major and minor defects were analyzed and classified according to OETTLE and SOLEY (1988).

1.4. Extender preparation

The stock solution consisted of 2.9 g Tris (Merck, Darmstadt, Germany), 1.226 g glucose (Merck), 1.735 g citric acid (Merck), 0.06 g penicillin G (Sigma, St. Louis, MO, USA), 0.1 g streptomycin (Sigma), and 100 mL qsp twice-distilled water. The TE consisted of 72 mL stock solution, 8 % glycerol for final concentration (Merck) and 20 mL egg yolk. TB consisted of 90 mL stock solution, 8 % glycerol for final concentration and 2 g BSA.

1.5. Dilution and freezing

The semen was prepared for cryopreservation by one-step dilution by dividing each ejaculate into two equal parts, one diluted with TE and the other with TB.

Before one-step dilution, the semen was adjusted based on KRAUSE' formula at a concentration of 50 x 10^6 viable spermatozoa and introduced into previously coded 0.5-mL straws and the same were left to stabilize for 1 h at 4 °C. The straws were then placed in nitrogen vapor for 15 min and the temperature was monitored with a digital thermometer (Cryogenetic Tech., Inc., Yoncalla, OR, USA) according to the freezing curve of 6°C/min from +4°C to –20°C followed by 15°C/min from –20°C to –80°C, by 8°C/min from –80°C to –100°C and at 5°C/min from –100°C to –115°C, then the straws were immersed in liquid nitrogen. After this step, the straws were placed on a rack and stored in a cryobiological cylinder.

1.6. Thawing and incubation semen

The samples were thawed in a water bath at 37 °C for 30 sec (ROTA *et al.*2001 and CARDOSO *et al.* 2003). One aliquot of semen was mounted between a slide and coverslip and analyzed for motility and vigor under a phase-contrast microscope at x 100 magnification. The remaining sample was incubated in a water bath at 39 °C for up to 4 h for the incubation (BURGESS *et al*., 2001; SILVA *et al*., 2003).

1.7. Acrosomal integrity

Forty ejaculates of frozen semen with TE and frozen semen with TB after thawing were submitted to double Trypan blue-Giemsa staining as described by DIAS (2002). One 20-µL sample was transferred to a 1.5-mL microtube, 20 µL 0.4% Trypan blue (Sigma, T 8154 - USA) was added, and the mixture was incubated in a water bath for 20 min at 37 ºC. Next, 1 mL physiological saline was added and the sample was centrifuged at 700 x *g* for 5 min to remove excess dye. Each sample was washed twice, the supernatant was discarded and the sample was resuspended in 0.5 mL physiological saline. Three smears were

then prepared, rapidly dried in an air flow and fixed in methanol for 5 min, followed by drying. The smears were then stained with 10% Giemsa for 18 to 20 h, removed from the dye and analyzed regarding acrosomal integrity by counting 100 spermatozoa under a phase-contrast microscope equipped with a x 1000 immersion objective.

Spermatozoa were classified as follows:

- dead acrosome-intact (DI) spermatozoon: blue or purple head with a pink acrosome;
- dead acrosome-reacted (DR) spermatozoon: blue or purple head with a colorless acrosome;
- alive acrosome-intact (LI) spermatozoon: pink head with a pink acrosome;
- alive acrosome-reacted (LR) spermatozoon: pink or white head with a colorless acrosome.

1.8. ARTIFICIAL INSEMINATION

Twenty-five female dogs showing a natural estrous cycle monitored on the basis of clinical signs, vaginal cytology and progesterone measurement were submitted to artificial insemination. Twelve females were inseminated with frozen semen with TE and thirteen with frozen semen with TB. The insemination dose was 4 straws (50 x 10^6 viable spermatozoa /straw) administered with the Osiris probe (IMV, L'Aigle, France).

1.8.1. Vaginal cytology (acidophilic index)

Vaginal cytology was performed every 48 h during proestrus and every 24 h during estrus. After cleaning the vulva, a swab was introduced into the vagina without touching the vulvar lips, removed after some rotational movements and spread over a slide. The material was fixed, stained and interpreted by the Harris-Shorr technique as described by MIALOT (1988).

1.8.2. Estrous behavioral score

The female kept on a dog leash was presented daily to three different males, which were also properly confined, from the day of bleeding on (proestrus). This method was used to observe the individual preference of each female for certain male. Based on the signs shown by the female, the following scores were established: 1 = physical signs (discharge and vulvar edema), 2 = physical signs + approximation of the male, 3 = physical signs + approximation of the male + mounting acceptance.

1.8.3. Insemination technique

After cleaning the vulva, the Osiris probe was introduced into the vagina up to the cranial third and the balloon was inflated until it firmly adhered to the wall of the vagina. The syringe containing the insemination dose was then coupled to the mobile catheter of the probe and the catheter was moved cranially to release the orifice of the tip. The semen was immediately injected and the catheter was returned to its initial position to obstruct the orifice. The probe remained in place for 15 min and the balloon was then disinflated and removed from the vagina (GUÉRIN, 1998; FELDMAN and NELSON, 2004).

The first artificial insemination was performed 4 days after vaginal cytology which indicated the presence of estrus (at least 80% of cells with a pyknotic or absent nucleus) and the animal showed an estrous behavioral score of 3 and the serum progesterone reach a minimum of 10 ng/ml. The second insemination was performed 24 h after the first one.

Serum progesterone was measured daily by chemiluminescence (IMMULITE® 2000 L2KPW6, DPC® Los Angeles, USA) at Laboratório Álvaro, Cascavel, Brazil.

1.9. Diagnosis of pregnancy

Twenty to 30 days after the last artificial insemination, the female was submitted to ultrasonography using an ultrasound apparatus (Scanner 485, Pie Medical Inc., Maastricht, Holland) equipped with a 5-MHz convex transducer for external use and a printer. The images were obtained and saved for later printing. The litter size was confirmed after delivery.

1.10. Statistical analysis

The statistical analyses were performed at Statistical Department of University of Paraná, Brazil. Two treatments were carried out (TE, TB). The data of the variables motility and vigor of extended semen and acrosomal integrity were analyzed by the Fisher Exact Test. The variables pregnancy rates and the number of puppies were analyzed by the χ^2 Test. For all tests, 95% confidence level was considered using The STATISTIC program.

RESULTS

Evaluation macroscopy and microscopy of the 40 dog ejaculates are shown in Table 1.

The values of means and standard deviation presented in table 1, has shown a good uniformity and quality of the ejaculates rich-fraction.

There is no significant difference between semen extended in TE and TB to motility and vigor before freezing as shown in table 2.

The values of acrosomal integrity of dog sperm stained by double Trypan blue-Giemsa derived from frozen ejaculates with TE and TB, as well as their variation among the spermatozoa class result are shown in Table 3.

Analysis of Table 3 showed that the most important data for sperm viability were those of LI with TE being significantly superior to TB (P< 0,01).

The interaction among incubation time, diluents (TE and TB) and spermatozoa class show that acrosomal integrity was preserved by TE in the dead spermatozoa as shown by the data means of the DI variable. Dead spermatozoa presented a high percentage of damaged or reacted acrosomes, with TB being responsible for the data means of the DR variable. Evaluation of acrosomal integrity up to 4 h of incubation of thawed semen showed that TB induced an early acrosome reaction in dog sperm.

The results of artificial insemination with semen diluted in TE and TB are shown in Table 4.

Artificial insemination of frozen semen by the Osiris method showed a significant difference which was observed in the number of puppies/delivery, with TE being superior to TB (P < 0.01). With respect to pregnancy rate, the numbers indicate that TE was significantly better than TB (P < 0.01).

TABLE 1 - MEANS AND STANDARD DEVIATION OF THE DOG'S EJACULATES SPERM-RICH FRACTION FRESH COLLECTED SEMEN (N = 40).

Variables	Means	Standard Deviation
Volume (mL)	1.547	±0.126
Motility (%)	86.316	±0.924
Vigor score (0 –5)	4.579	±0.116
Concentration (mm)	292.842	±3.592
Minor defects (%)	12.316	±0.502
Major defects (%)	5.737	±0.274

TABLE 2 MEANS AND STANDARD DEVIATION OF THE DOG'S SEMEN EXTENDED USING TRIS-EGG YOLK (TE) AND TRIS-BSA (TB) BEFORE FREEZING (N = 40).

Extenders	Variables	(means ± sd)
	Motility (%)	Vigor (0 –5)
TE	85.897^{NS} ± 3.155	3.946^{NS} ±0.520
TB	85.897^{NS} ± 3.155	4.135^{NS} ± 0.481

^{NS} (P>0,05) BETWEEN TREATMENTS.

TABLE 3 - VALUES OBTAINED *IN VITRO* OF THE ACROSOMAL INTEGRITY OF DOG SPERMATOZOA FROZEN WITH TRIS-EGG YOLK (TE) AND TRIS-BSA (TB) EXTENDERS, THAWED AT 37 °C AND SUBMITTED TO 4 HOURS AT 39 °C INCUBATION. (N = 40)

Extenders	Spermatozoa class (means ± sd)			
	LI	LR	DI	DR
TE	12,032^{a} ±3,144	51,694^{a} ± 2,831	5,137^{NS} ±0,603	30,794^{b} ± 1,731
TB	0,936^{b} ±0,915	19,862^{b} ± 1,963	4,691^{NS} ± 0,582	74,805^{a} ± 3,223

^{A,B} DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES IN SAME COLUMN (P<0.01) BETWEEN TREATMENTS.

^{NS} (P>0,05).

LI = ALIVE ACROSOME-INTACT;

LR= ALIVE ACROSOME-REACTED;

DI= DEAD ACROSOME-INTACT;

DR=DEADACROSOME-REACTED

TABLE 4 - VALUES OF THE VARIABLES OBTAINED DURING THE EXPERIMENT FOR THE *IN VIVO* ASSESSMENT OF FROZEN SEMEN WITH TRIS-EGG YOLK AND TRIS-BSA (*N* = 40)

Semen	n	Pregnant bitches (%)	Nonpregnant bitches (%)	No. of puppies	Puppies / delivery
TE	20	16[a]	4[b]	68[a]	4.25[a]
TB	20	6[b]	14[a]	12[b]	2.00[b]

[A,B] – VALUES WITH DIFFERENT LETTERS IN SAME COLUMN SHOW ARE SIGNIFICANTLY DIFFERENCES (*P*< 0.01) BETWEEN TREATMENTS.

DISCUSSION

The analysis of the Table 1, show the uniformity of the donors dogs ejaculates. This is very important on the final results of the semen technology.

The results showed in the table 2 agree in part with those reported by RODRIGUES (1997). The author found reduction in motility and vigor for semen diluted in Tris-egg yolk compared with Tris-BSA. This partial agreement is due to the concentration of the extenders components.

The results of cryopreservation of dog semen with TE and TB obtained in the present study are different from those reported by RODRIGUES (1997) who, using BSA concentrations of 0.25, 0.5 and 1%, did not observe a significant difference in acrosomal integrity between dog spermatozoa cryopreserved with TE and TB. This disparity might be due to the higher BSA concentration (2%) used in the present study.

The acrosomal integrity of LI spermatozoa cryopreserved with TE was higher than that observed for spermatozoa cryopreserved with TB during 4 h of incubation. In the case of LR spermatozoa, TE exceeded TB from thawing up to 4 h. Regarding DR spermatozoa, TB exceeded TE from thawing up to 4 h, showing that BSA induces an early acrosomal reaction in dog sperm (Table 3). The acrosome is an important part of the spermatozoon and is essential for the mechanism of fecundation. However, the acrosome is very sensitive to aggressions caused by the process of cryopreservation. Although many factors can cause acrosome damage, we believe that the extender is fundamental for preserving acrosomal integrity.

Assessment of the acrosomal integrity of dog sperm permitted the comparison of the efficiency of egg yolk and BSA as protein components of the Tris-based extender. In the present study, TE was significantly superior than TB in terms of acrosomal integrity of LI and LR spermatozoa ($P < 0.01$), while TB showed higher significance than TE in the case of DR spermatozoa ($P < 0.01$), (Table 3).

The results regarding acrosomal integrity of dog sperm frozen with TE are similar to those described previously (KOVACS and FOOTE, 1992; DIAS, 2002; TARTAGLIONE and RITTA, 2004). Although these authors studied bovine spermatozoa, the technique used was double Trypan blue-Giemsa staining which permitted the classification into live intact, live reacted, dead intact and dead reacted spermatozoa. On the other hand, our results disagree with the 38.5% post-thaw acrosomal integrity reported by (NÖTHLING and SUTTLEWORTH, 2004). These authors used the Spermac dye which does not differentiate between live and dead spermatozoa. LI and LR spermatozoa are considered to be those responsible for fertility.

Artificial insemination with thawed semen by the Osiris method show that TE extender was significantly better than TB in terms of the number of puppies per delivery ($P < 0.01$), with larger litters being obtained for female dogs inseminated with TE and smaller litters for animals inseminated with TB. These results agree in part with those reported by ANDERSEN (1975) who obtained litter sizes ranging from 1 to 7 puppies born from female dogs inseminated by the intrauterine route with frozen-thawed semen using TE. This partial agreement is due to the fact that in the present study vaginal insemination through the Osiris probe was used.

Significantly higher pregnancy rates were obtained with TE compared to TB ($P < 0.01$). Comparison of the data for the pregnancy item in Table 4 showed 16 pregnancies among 20 female dogs inseminated with TE versus 6 pregnancies among 20 animals inseminated with TB. The results obtained with TE are similar to those reported by ANDERSEN (1975) who obtained 10 pregnancies among 11 inseminated animals. Although no report regarding the artificial insemination of female dogs with semen frozen with BSA is available in the literature, based on the present results regarding acrosomal integrity obtained after up to 4 h of incubation of semen at 39 °C and after double Trypan blue-Giemsa staining we believe that the difference in pregnancy rates and litter size between TE and TB can be explained by an early acrosome reaction of spermatozoa processed with TB as shown in Table 3, specifically in terms of the LI and LR variables.

The serum progesterone concentrations observed on the occasion of first insemination of female dogs with score 3 ranged 10 ng/mL, value that agree with

those reported by ENGLAND and LOFSTEDT (2000), ranging from 10 to 13 ng/mL, and by FELDMAN and NELSON (2004), ranging from 4 to 10 ng/mL.

We agree that the other techniques have their advantages described by KOVACS and FOOTE, 1992); however, none of them stains live spermatozoa for the identification of reactions or damage possibly occurring in the acrosome of thawed spermatozoa that are inherent to freezing and not to the preparation of the sample for analysis, predicting fecundating ability and thus permitting the release of a semen lot to be used for artificial insemination.

In the incubation of thawed dog semen, BSA did not preserve acrosomal integrity, a fact resulting in low pregnancy rates accompanied by small litter sizes when compared to the use of egg yolk. Thus, BSA cannot replace egg yolk as a protein source in Tris-based diluents used for cryopreservation of dog semen. We recognize that this conclusion is based on data from a relatively small number of female dogs (i.e., 20 per group). However, our conclusion about artificial insemination with TE is consistent with the data in ANDERSEN (1975). Therefore, more research will be conducted to determine other dog's semen extenders to improve the vaginal artificial insemination fertility.

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