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Artificial insemination with frozen semen in dogs: A retrospective study of 10 years using a non-surgical approach

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Abstract

From 1994 to 2003, a total of 526 bitches of 99 different breeds were artificially inseminated in 685 estrus cycles with domestic ($n = 353$) or imported ($n = 332$) frozen-thawed semen from 368 males. The overall whelping rate was 73.1% and mean (±S.E.M.) litter size 5.7 ± 0.1 pups. The whelping rate was higher after intrauterine insemination (75.0%; $n = 665$) than after intravaginal insemination (10.0%, $n = 20$; $P < 0.05$). Insemination at the optimal time resulted in a higher whelping rate (78%, $n = 559$; $P < 0.01$) and larger litter size (5.8 ± 0.2; $P < 0.05$) than inseminations performed late or too late (55.7% and 4.5 ± 0.5, $n = 61$). Two inseminations ($n = 384$) yielded a higher whelping rate ($P < 0.05$) and mean litter size ($P < 0.01$) than one insemination ($n = 241$), 78.1% and 6.0 ± 0.2 and 70.5% and 5.1 ± 0.2, respectively. For inseminations performed at the optimal time, however, the whelping rate was not significantly different for bitches inseminated twice (79.3%, $n = 358$) versus once (76.8%, $n = 168$), but the litter size was larger (6.0 ± 0.2 and 5.3 ± 0.3). Semen classified as of poor quality (progressive motility <50% or percentage abnormal sperm >20%) resulted in a lower whelping rate ($P < 0.01$) than semen classified as of good quality (progressive motility ≥50% and percentage abnormal sperm ≤20%), 61 and 77%, respectively. Small breeds ($n = 50$) had a smaller litter size (3.9 ± 0.3; $P < 0.01$) than larger breeds (medium [5.7 ± 0.3, $n = 94$], large [5.9 ± 0.2, $n = 295$] or giant breeds [6.1 ± 0.5, $n = 62$] [$P < 0.01$]). Bitches older than 6 years had a lower whelping rate (68.2%) than younger ones (77.0%; $P < 0.05$). The duration of pregnancy was longer ($P < 0.01$) for bitches with a litter size of <3 pups (61.7 ± 0. 4 days, $n = 30$) than for bitches with larger litters (60.5 ± 0.1 days, $n = 177$). These results show the potential of transcervical intrauterine insemination for routine artificial insemination in dogs. The results with frozen semen inseminations were optimised by inseminating bitches ≤6 years old 2 and 3 days after ovulation with semen of good quality from males ≤8 years old.

Keywords: Dog; Artificial insemination; Frozen semen; Transcervical insemination

1. Introduction

More than 30 years have elapsed since the first transcervical insemination technique for dogs was developed in the early 1970s [1,2]. This technique is often referred to as the Norwegian or Scandinavian method and has proven to be very successful in canine breeding [2–9]. The technique enables semen to be deposited non-surgically into the uterus in standing, usually non-sedated bitches. Thus, frozen-thawed semen, in which both the number of fertile sperm and the longevity of the sperm are reduced (compared to

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freshly ejaculated semen), may be deposited closer to the site of fertilisation. During the last 10 years, we have inseminated 685 bitches with frozen semen from a variety of sources; to our knowledge, this represents the largest database of AI with frozen semen so far published. The aims of this retrospective study were to review the results of AI with frozen-thawed semen in dogs at our clinic over a 10-year interval (1994–2003), and to discuss factors that may influence the success rate with frozen-thawed canine semen.

2. Material and methods

2.1. Animals

A total of 526 bitches were inseminated in 685 estrus cycles: 476 in one, 41 in two and nine bitches in three estrus cycles each. These cases are referred to as 685 bitches, and they were inseminated with frozen-thawed semen from 368 males of 99 different breeds at our clinic from 1994 to 2003. The inseminations were performed by experienced personnel (>5 years practice with the technique), and all inseminations with frozen-thawed semen performed during this period are included in the material. The population of bitches was unselected, since the owners wanted frozen semen to be used in accordance with their breeding plans. A total of 353 bitches were inseminated with semen frozen at our clinic, and 332 bitches were inseminated with imported semen from a variety of sources. The results of the inseminations were obtained by personal contacts with the bitch owners. The animals were grouped according to the weight of the female into small, medium, large and giant breeds. Small breeds weighed up to 12 kg, medium breeds ranged from 13 to 25 kg, large breeds from 26 to 40 and giant breeds weighed >40 kg.

2.2. Semen evaluation and processing

The domestic semen was collected by digital manipulation in the presence of a bitch in estrus. Only the sperm-rich fraction was used for evaluation and processing. Motility was evaluated subjectively at 37 °C under a light microscope and an aliquot of the semen sample was evaluated for morphology. The evaluation of morphology was done on fixed semen samples using Hayems fixative. Several fields (10–15) in the microscope were viewed, estimating the percentage of abnormal spermatozoa. Sperm with primary defects [10] and proximal droplets were counted as abnormal. Semen with a motility of ≥90% and a proportion of abnormal sperm of ≤20% was regarded suitable for freezing. Semen with motility ≥75 and ≤30% abnormal sperm was frozen, but if post-thaw motility was <40%, the owner of the dog was strongly encouraged to allow us to dispose of the semen. Semen of poorer quality (motility <75% or abnormal sperm >30%) was not frozen. The sperm-rich fraction was diluted in a prewarmed (35–37 °C) Tris–fructose–citric acid extender containing 8% (v/v) glycerol and 20% (v/v) egg yolk to a final concentration of approximately 1.0×10^8 spermatozoa/mL [1]. The diluted semen was wrapped in paper to ensure slow cooling and placed in a refrigerated room at 5 °C. After an equilibration time of 2–3 h, the semen was filled in 0.5 mL medium PVC straws (Minitüb, Tiefenbach, Germany), frozen in N_2 vapour [1] and stored in liquid nitrogen containers. The volume of each insemination dose was 2–2.5 mL with a total of approximately 2.0×10^8 spermatozoa. Imported semen was received from various private or university clinics, or from commercial agencies whose cryopreservation procedures were proprietary. The insemination dose, therefore, may differ from the ones using domestic semen.

2.3. Semen thawing and post-thaw evaluation

The domestic semen was thawed in a 70 °C water bath for 8 s, and the imported semen according to recommendations from the veterinarian or agency that had processed the semen; either in water bath at 70 °C for 8 s, or at 37 °C for at least 30 s. Before insemination semen was evaluated subjectively for sperm motility and morphology (at 37 °C using a light microscope). A small drop of semen was placed on a slide and evaluated for motility. Morphology was assessed from the same sample either at the borders of the coverslip or after the motility had ceased. Progressive motility ≥50% was classified as ''good'', and <50% as ''poor'' semen quality. If the percentage of morphologically abnormal spermatozoa was >20%, semen quality was classified as ''poor'', regardless of semen motility [11]. If the semen was of poor quality, the insemination dose may have been increased, for example, by adding an extra straw.

2.4. Timing of the insemination

For all bitches, the timing of the AI was based on a clinical evaluation, including a vaginal smear and serum progesterone concentration. Cells for the vaginal smears were collected with a plastic catheter (Bovivet®, Kruse, Marslev) inserted deeply into the vagina. Cells that

stuck to the catheter were transferred to a prestained slide, Testsimplets® (Boehringer, Mannheim) by placing a small drop of saline on the slide and gently tapping the slide with the tip of the catheter. A coverslip was placed on the slide, and the smear was evaluated for cornification of the epithelial cells, presence and number of erythrocytes and leucocytes [12]. Progesterone assays were performed on one or more occasions in all bitches. The progesterone analyses were performed using an IMMULITE® progesterone kit (Diagnostic Products Corporation, Los Angeles, CA, USA), with an intra- and inter-assay coefficient of variation at 20 nmol/L of 6.3 and 5.8%, respectively, and a detection limit of 0.6 nmol/L. The intra- and inter-assay coefficients of variation at 5 nmol/L were 7.9 and 10%, and at 65 nmol/L, 5.0 and 8.6%, respectively. Time of AI was based on estimated time of ovulation determined from serum progesterone analysis. Whenever possible, two inseminations were performed, one on the second and one on the third day after estimated ovulation, as this is regarded as the optimum time for fertilisation [13]. However, some bitches were inseminated only once, either because the bitch first appeared late in estrus ($\geq$3 days after estimated ovulation) at our clinic or if a limited amount of semen was available. The mean serum progesterone concentration at ovulation was approximately 15–20 nmol/L [14]. Therefore, a progesterone concentration of 15–25 nmol/L indicated insemination 2 and 3 days later, 25–35 nmol/L 1 and 2 days later, 35–60 nmol/L AI on the same day and the day after and a concentration of >60 nmol/L as a single AI on that day. Timing of insemination was classified as optimal if at least one AI was performed 2 or 3 days after the estimated time of ovulation, late insemination was when the concentration of serum progesterone was 60 nmol/L or more, and too late when in addition to high concentration of serum progesterone (>60 nmol/L) the vaginal smear indicated metestrus.

2.5. Artificial insemination technique

Inseminations were performed on standing bitches, and the semen was deposited in the uterus by fixing the cervix abdominally with one hand and inserting the stainless steel catheter transcervically into the uterus with the other [2]. If insertion of the catheter through the cervix was not possible ($n = 20$), semen was deposited deep into the vagina. Care was then taken to avoid backflow of the semen by: (i) the plastic sheath was pulled backwards and pressed against the base of the catheter as the semen was deposited into the vagina and (ii) the hind quarters of the bitch were then kept elevated for 10–15 min (with concurrent stimulation of the area around the vulva).

The bitches were not usually sedated, but a few individuals that were sensitive to deep abdominal palpation were given an IV injection of 2–6 mg xylazine (Rompun; Bayer, Leverkusen). Also, a few bitches of large and giant breeds were sedated to enable abdominal fixation of the cervix. In total, <10% of females were sedated, and sedation was not considered necessary as a standard procedure with this technique.

The length of pregnancy was calculated in bitches inseminated between 1999 and 2003 and was counted from the day of insemination to parturition. If the bitch was inseminated twice, for example, 60 and 61 days before parturition, the pregnancy length was counted as 60.5 days.

2.6. Statistical analysis

The statistical analyses were performed in Excel (2000) and Epiinfo 6.03 (1996), and the results are given as percentage (whelping rate) and means $\pm$ S.E.M. (litter size). Comparisons of all whelping rates were evaluated using Chi square analysis and comparison of litter sizes and pregnancy length was done using a Student's *t*-test. When calculating the difference in pregnancy length for different litter sizes, the material was grouped and litter size <3 pups was compared to litter size of three pups or more. For comparison of site of deposition of the semen (intravaginal versus intrauterine), all inseminations were included in the calculations. For length of pregnancy, all bitches that were inseminated during 1999–2003 and that whelped are included. For the rest of the comparisons, intravaginal inseminations were excluded from the calculations. The level of significance was set at $P < 0.05$.

3. Results

Of the 685 bitches that were inseminated, 501 (73.1%) gave birth to a litter, and the mean litter size was 5.7 ± 0.1 pups. Of the 685 bitches, 665 (97.1%) were inseminated into the uterus, with a pregnancy rate of 75.0% and a mean litter size of 5.7 ± 0.1 pups. Twenty bitches (2.9%) were inseminated deeply into the vagina after the attempt to pass the catheter through the cervix had failed. Only two of these bitches whelped (10%), each giving birth to six pups.

The mean ($\pm$S.E.M.) serum progesterone concentration 2 days prior to insemination was 20.7 ± 0.5 nmol/L ($n = 180$), 35.0 ± 0.9 nmol/L ($n = 171$) the day before

insemination, 43.5 ± 0.8 nmol/L ($n = 177$) on the first day of insemination and 66.8 ± 1.4 nmol/L ($n = 140$) on the last day of insemination.

Two inseminations ($n = 384$) yielded a higher whelping rate ($P < 0.05$) and mean (±S.E.M.) litter size ($P < 0.01$) than one AI ($n = 241$) in the overall material, 78.1% compared to 70.5% and 6.0 ± 0.2 pups and 5.1 ± 0.2 pups, respectively. For inseminations where the timing was classified as optimal, however, the whelping rate was not different for two (79.3%, $n = 358$) versus one (76.8%, $n = 168$) insemination. Litter size, however, differed (6.0 ± 0.2 and 5.3 ± 0.3 pups; $P < 0.05$).

Domestic and imported semen yielded similar results; 76.0% whelping rate and litter size of 5.8 ± 0.2 pups, versus 74.0% and 5.5 ± 0.2 pups.

Semen that we classified as having poor post-thaw quality ($n = 93$) resulted in a lower pregnancy rate ($P < 0.01$) than semen classified as being of good quality ($n = 566$), 61.3% versus 77.4%. Litter size, however, was not significantly different in the two groups: 6.1 ± 0.5 versus 5.7 ± 0.1.

Timing of insemination classified as optimal ($n = 559$) resulted in a higher ($P < 0.01$) whelping rate than timing classified as late or too late ($n = 61$), 78.2 and 55.7%, respectively. The litter size was 5.8 ± 0.2 compared to 4.5 ± 0.5 ($P < 0.05$). Small breeds had a smaller litter size (3.9 ± 0.3, $n = 50$) than medium (5.7 ± 0.3, $n = 94$), large (5.9 ± 0.2, $n = 295$) or giant (6.1 ± 0.5, $n = 62$) breeds ($P < 0.01$). The fertility results for the six numerically largest breeds are listed in Table 1.

Older bitches, >6 years of age ($n = 148$), had lower fertility than younger ones ($n = 517$). The whelping rate was lower (68.2% versus 76.9%, $P < 0.05$) and the litter size tended to be smaller (5.3 ± 0.3 versus 5.8 ± 0.2, $P = 0.12$). Males >8 years tended to have poorer fertility than younger ones; insemination with frozen-thawed semen from males >8 years ($n = 206$) had a whelping rate of 70.9%, compared to 76.9% for the younger males ($n = 459$; $P = 0.1$) and the litter sizes were 5.6 ± 0.3 and 5.7 ± 0.2, respectively (not significantly different).

For bitches inseminated in the period 1999–2003, the duration of pregnancy was longer ($P < 0.05$) for bitches pregnant with one or two pups ($n = 30$) compared to larger litters ($n = 177$), 61.7 ± 0.4 days versus 60.5 ± 0.1 days.

Table 1
Breed-related whelping rate and mean (±S.E.M.) litter size after intrauterine insemination with frozen-thawed semen

Breed	No.	Whelping rate (%)	Litter size
Labrador Retriever	57	84.2	6.4 ± 0.4
Boxer	37	91.8	5.3 ± 0.5
Golden Retriever	32	65.6	5.0 ± 0.7
Bull Mastiff	29	82.8	6.7 ± 0.9
Rhodesian Ridgeback	28	92.9	7.9 ± 0.7
English Setter	24	62.5	5.2 ± 0.8

Results comprise the six breeds with the largest number of individuals ($n = 207$, from a total of 665).

4. Discussion

Although the first reported success of frozen-thawed semen AI in the dog was after intravaginal insemination [15], deposition of semen into the uterus has been shown to be of great importance to the success of AI [5–9]. In the present study, only two out of 20 bitches (10%) that were inseminated deeply into the vagina after the attempt to pass the catheter into the uterus had failed, whelped. In contrast, a 75% success rate was achieved after intrauterine semen deposition. The failure to pass the catheter through the cervix was mainly due to large and obese bitches, making cervical fixation difficult. Other authors [5,8,9] although achieving better results after intravaginal deposition of the frozen-thawed semen than the present team, yet obtained significantly better whelping rates with intrauterine semen deposition. Nöthling et al. [16] reported a whelping rate (88%) after intravaginal AI, comparable to that by intrauterine deposition of the semen. However, these bitches were inseminated on average 5.6 times during estrus, which is less convenient under practical conditions.

Timing of the insemination is of great importance to achieve good results with AI with frozen-thawed semen. In the present material, inseminations carried out at the time regarded as optimal yielded a significantly higher whelping rate (78% versus 55.7%) and significantly larger litter size (5.8 ± 0.2 versus 4.5 ± 0.5), compared to inseminations at a time classified as late or too late. Theoretically, two inseminations 24 h apart, would give a greater probability of inseminating at the time of fertilisation, especially if the estimation of the ovulation time was less accurate. It has previously been shown [6–9] that one insemination yields lower fertility results than two, and in the present material that was confirmed. If, however, only inseminations where the timing was evaluated as optimal are included, there was no difference in the whelping rates (79.3 and 76.8%) between one and two inseminations. Mean litter size

was, however, still significantly different, with 0.8 pups less on average when bitches were inseminated only once. For obvious reasons, surgical insemination, which is an alternative to the transcervical methods of inseminating bitches, usually can only be performed once. When comparing this with the present method, this should ideally be made on a one to one basis, i.e. comparing results after one insemination only. However, the possibility of multiple inseminations represents an advantage on behalf of the non-surgical transcervical techniques [1–9,17,18] in cases where an accurate estimation of the time of ovulation is not possible, or when the semen quality is poor. A whelping rate of 92% ($n = 157$) after surgical insemination in racing greyhounds has been reported (Boland P, personal communication). Further, high whelping rates (87.5%) and a litter size of 6.9 ± 2.7 pups have been obtained following endoscopic insemination in Greyhounds [19]. Two of the numerically larger breeds in our material, Boxers ($n = 37$) and Rhodesian Ridgebacks ($n = 27$), also yielded 92–93% whelping rates. These figures may serve as an example of results that are obtainable in breed-standardised groups of fertile animals both with the surgical and the transcervical approach.

Insemination with poor-quality semen (according to the standards used in the present study) resulted in lower whelping rates ($P < 0.01$) than good-quality semen, in agreement with earlier studies [8,9]. However, the mean litter size in the bitches inseminated with poor-quality semen was not significantly different from that in bitches inseminated with good-quality semen, in agreement with previous studies [8,9]. Although one might expect a reduced litter size, there are two explanations to the contrary. Firstly, the classification of semen quality was based on subjective evaluations of post-thaw motility and morphology and was only divided in two groups (good and poor quality); perhaps some samples were judged to have slightly <50% motility and hence were miscategorised, or the collecting veterinarian compensated for the poor semen quality by using a higher number of spermatozoa.

It is generally assumed that small breeds have smaller litter sizes than larger breeds, consistent with the present results. There was however, no difference in whelping rate for the different breed categories when the semen was deposited in the uterus. If the vaginal inseminations are included, the giant breeds had a lower whelping rate than the rest of the breeds. In our experience, although giant breeds do not have poorer fertility than other breeds, their size makes them more difficult to inseminate with our method. It is imperative to urge the breeders to present these bitches in a slim condition if we are to achieve good results with the Norwegian method in these breeds. Endoscope-aided transcervical insemination [17–19] where large size of the bitch is not a critical factor, may be a good alternative in these cases.

If bitches are older than 6 years and have not had a previous litter, we generally discourage the owner to breed the bitch. In our material, some bitches, especially hunting dogs such as English Setters, were older than 6 years, yet nulliparous, but the majority of the bitches that were older than 6 years had previously had at least one litter. In the present study, bitches older than 6 years had reduced fertility after AI with frozen-thawed semen, perhaps due to age-related changes in the uterus.

In our experience, semen quality and freezability may be reduced in dogs older than 8 years. When we evaluate semen for freezing, semen of poor quality is usually not frozen, especially if we consider that the chances of getting the bitches pregnant are small. In that sense, our material is biased regarding the age of the male dogs. Nevertheless, there was still a tendency towards lower whelping rate if the semen donor was older than 8 years (70.9% versus 76.9%, $P = 0.1$) and the semen was frozen. Litter size, however, was not significantly influenced.

It is generally assumed that bitches pregnant with one or two pups tend to have a longer pregnancy than bitches with larger litters. In our study, this was supported; the duration of pregnancy was longer in bitches pregnant with one or two pups (61.7 ± 0.4 days) compared to bitches with larger litters (60.5 ± 0.1 days).

Our goal is to inseminate 2–3 days after ovulation. The pregnancy length of the bitch has been shown to be 65 days from the LH-peak [20] or first detected rise in serum progesterone [21], which is equivalent to 63 days relative to ovulation. Insemination 2 days after ovulation would therefore give an expected pregnancy length of 61 days and AI 3 days after ovulation an expected pregnancy length of 60 days. The fact that the mean length of pregnancy in our material is 60.6 ± 0.1 days should indicate that our estimation of the ovulation time, in retrospect, was accurate.

In conclusion, in the present study, non-surgical transcervical insemination was a very valuable tool in canine breeding. Results with insemination of frozen-thawed semen were optimised by inseminating bitches $\leq$6 years old, 2 and 3 days after ovulation with semen of good quality from males $\leq$8 years of age.

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