ORIGINAL ARTICLE

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A retrospective clinical study of endoscopic-assisted transcervical insemination in the bitch with frozen–thawed dog semen

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Contents

Since the conclusion of data collation from previously published work, a further 352 inseminations using frozen–thawed dog semen by endoscopic-assisted transcervical insemination (EIU) have been performed by the author. Insemination was performed on the second day in which crenulation of the anterior vagina was detected in conjunction with a progesterone concentration of >10 ng/ml. All semen samples were analysed for total number of sperm, total motility and progressive motility using computer-assisted semen analysis (CASA). The insemination dose was based on the progressively motile normal spermatozoa (PMNS). Insemination was performed on all bitches as previously described using a ureterorenoscope. Additional extender was inseminated subsequent to the semen to expand and fill the uterus. The semen and additional extender were inseminated slowly over a period of 15–20 min. Pregnancy was determined by B-mode ultrasound equipped with a 7.5-MHz probe whilst standing and/or via the whelping rate. The number of sperm inseminated ranged from 9×10^6 PMNS to 519×10^6 PMNS, with progressive motility values ranging between 20% and 80%. The overall pregnancy rate was 68% (238/352). When stratified by PMNS, pregnancy rates were as follows: $>150 \times 10^6$ PMNS - 76% (110/145), $100–150 \times 10^6$ - 68% (87/128) and $<100 \times 10^6$ PMNS - 52% (41/79). Pregnancy rate was significantly higher when $>150 \times 10^6$ PMNS ($p = .003$) or $100–150 \times 10^6$ PMNS ($p = .027$) were inseminated compared to $<100 \times 10^6$ PMNS. These data are concordant with previous reports recommending the insemination of $>150 \times 10^6$ PMNS to maximize pregnancy rate. These results indicate that one optimally timed EIU insemination results in similar pregnancy rates to previous publications of one optimally timed, or two or more non-optimally timed inseminations using the Norwegian catheter.

1 | INTRODUCTION

Intrauterine insemination is well established as the superior method for frozen–thawed dog semen insemination (Fontbonne & Badinand, 1993; Linde-Forsberg, Strom Holst, & Govette, 1999; Silva, Onclin, Snaps, & Verstegen, 1995). There are many published studies showing the results of large numbers of transcervical insemination using the Norwegian catheter (Linde-Forsberg & Forsberg, 1989, 1993; Linde-Forsberg et al., 1999; Thomassen, Farstad, Krogenaes, Fougner, & Berg, 2001; Thomassen et al., 2006); however, there are few clinical studies of EIU with frozen–thawed dog semen (Linde-Forsberg et al., 1999; Mason & Rous, 2014; Pretzer, Lillich, & Althouse, 2006; Wilson, 1993). EIU with frozen–thawed dog semen has been shown to be superior to intrauterine insemination via surgery (Mason &

Rous, 2014). In some countries, insemination of bitches via surgery is deemed illegal or unethical (England & Millar, 2008; Linde-Forsberg et al., 1999), yet in many countries, the procedure is still performed (Mason & Rous, 2014). Previously published reports on EIU in a clinical setting still comprise small sample sizes (Linde-Forsberg et al., 1999; Mason & Rous, 2014) and a highly fertile breed (Pretzer et al., 2006) or appear to be skewed towards one highly fertile dog (Wilson, 1993).

The total number of progressively motile normal spermatozoa (PMNS) is well correlated to fertility of frozen–thawed dog sperm (Linde-Forsberg, 2000; Linde-Forsberg & Forsberg, 1989; Mickelsen, Memon, Anderson, & Freeman, 1993). Additionally, previous publications pertain to the use of a minimum of 150×10^6 PMNS on one or two occasions (Linde-Forsberg & Forsberg, 1989, 1993; Linde-Forsberg et al., 1999; Thomassen et al., 2001, 2006), yet it is commonplace to inseminate 100×10^6 PMNS on one occasion via surgery (Burgess, Mitchell, & Thomas, 2012). Thus, the aim of this study was to present the pregnancy results of 352 EIU inseminations performed at one facility and relate the results to the number of PMNS inseminated.

2 | MATERIALS AND METHODS

2.1 | Bitches

A total of 352 inseminations were performed on 329 bitches presenting to Monash Veterinary Clinic for routine insemination of frozen–thawed dog semen from April 2011 to December 2015. All bitches were clinically healthy, between the ages of 1.35 and 7.65 years (3.2 ± 1.42 years; mean ± SD) and of varying parity ranging from 0 to four litters, with the majority of bitches having had either zero (86) or one litter (134) prior to presenting. Bitches with a history of uterine disease or known infertility were not included in the study. German Shepherds (8.2%), Labrador Retrievers (8%), British Bulldogs (5.7%), Bullmastiffs (4.5%) and Rottweilers (4.3%) were the most represented of the 70 breeds presented. Breeds considered to have higher fertility such as Greyhounds (Thomassen & Farstad, 2009), and dogs from commercial working facilities (observed unpublished data, Mason and Rous) were not included in the study.

2.2 | Insemination timing

Insemination timing was based on vaginal cytology, serum progesterone concentrations, and vaginoscopy (Badinand, Fontbonne, Maurel, & Siliart, 1993; England, 1993; Goodman, 1992). Most bitches were presented to the clinic for their first assessment 5–7 days after the onset of vaginal swelling and/or discharge being noted by the owner (344/352), with the remainder presenting 1 day after a progesterone value of 2–3 ng/ml was detected with their local veterinarian. Vaginal smears were collected by introducing a moistened cotton swab into the caudal vagina. Swabs were then gently rolled onto glass microscope slides and stained using Diff Quick® (Australian Biostain, Pty/Ltd, Victoria, Australia). Vaginal smears were evaluated during the first visit to help stage the cycle (Lindsay & Concannon, 1986; Olson,

Thrall, Wykes, & Nett, 1984). Further evaluation of vaginal smears was not performed unless there was concern about the cycle not progressing as expected based on progesterone changes.

Blood was collected via jugular or cephalic venipuncture into tubes with no additives and submitted to a commercial laboratory for analysis of serum progesterone concentrations using chemiluminescence (Siemens ADVIA Centaur XPT, Germany). Serum progesterone concentration was determined at the first visit and every 3–4 days until the initial rise in progesterone (progesterone concentration >2 ng/ml), and subsequently every 1–2 days until ovulation was deemed complete (Jeffcoate & Lindsay, 1989). Insemination was not performed unless ovulation was deemed complete (progesterone concentration >10 ng/ml) (Jeffcoate & Lindsay, 1989). Serum progesterone assays were not continued subsequent to a concentration of >10 ng/ml.

Vaginoscopy was performed using a Sigmoidoscope (32020 Fibreoptic Sigmoidoscope, WelchAllyn, Skaneateles, NY) daily from the time of ovulation (progesterone concentration 5–8 ng/ml) until the time of insemination (Lindsay & Concannon, 1986). Insemination was performed on the second day in which crenulation of the anterior vagina was detected (Lindsay & Concannon, 1986).

2.3 | Semen handling

Frozen semen used in the study was obtained from a variety of sources both within Australian and international freezing centres and had been stored for varying periods of time (1 month–27 years). Frozen semen from a total of 337 different dogs were used, with no one dog being used more than twice. Semen had been frozen in pellets using Camelot farms or ICSB extenders or frozen in straws using Clone, Synbiotics/ICG, Uppsala, MOFA Global or Tris extenders. Semen pellets were thawed using a "step-thaw" protocol; pellets were transferred into a small plastic bag containing 1 ml pre-warmed AndroPRO AI® (MOFA Global, Verona, WI, USA) thaw media in a water bath at 37°C for 60 s, and then, a further 2 ml of AndroPRO AI® thaw media (pre-warmed) was slowly added to the thawed, extended semen. Semen straws were transferred directly to a water bath at 37°C for 60 s. The straws were then emptied into a small plastic bag containing warmed thaw media; the volumes used per straw are provided in Table 1. The total volume of semen used ranged from 1.3 to 16 ml, as determined by the number of straws used.

All insemination doses were assessed post-thawing to determine the number of PMNS inseminated. The percentage of progressively motile spermatozoa and concentration was determined by CASA

TABLE 1 Volume of AndroPRO AI® thaw media used per straw for different freeze extenders

Extender	Volume of extender
Uppsala	1.5 ml per straw
MOFA CaniPRO	0.5 ml per straw
Synbiotics/ICG	1–1.5 ml per straw
CLONE	1.5 ml per straw
Tris	0.5 ml per straw

analysis (Spermvision SAR®, MOFA Global) on a 37°C warmed stage. Five μL of the thawed extended semen was removed and used to fill a single chamber in a 20-μm leja four-chamber slide (Leja Products B. V. Nieuw-Vennep, The Netherlands). Excess fluid was blotted off the well with a tissue to arrest sperm drift. All samples had a sperm concentration of less than 50×10^6 sperm/ml. The recommended insemination dose for frozen–thawed canine semen is $100–200 \times 10^6$ motile spermatozoa (Andersen, 1975; Farstad & Berg, 1989). For statistical analysis of the pregnancy rate in relation to the number of spermatozoa, the insemination results were classified into $>150 \times 10^6$ PMNS, $100–150 \times 10^6$ PMNS and $<100 \times 10^6$ PMNS, as this value is considered the minimum industry standard (Farstad & Berg, 1989). Morphology assessments were not formally performed post-thawing for each insemination as per previous studies (Linde-Forsberg et al., 1999; Thomassen et al., 2006), with the assumption that progressively motile sperm are normal like in the bovine (Barth & Oko, 1989). In all samples, seven fields of view were reviewed for morphological abnormalities and if cytoplasmic droplets or spermatozoa head defects were noted on gross examination, a formal morphological assessment was performed with a formal-buffered saline sample (Mason & Rous, 2014) as these defects result in motile spermatozoa. All samples used in the study contained less than 20% abnormal spermatozoa. Semen thawing was performed before catheter placement in primiparous and multiparous bitches, and after catheter placement in nulliparous bitches.

2.4 | Insemination procedure

All inseminations were performed by one of two registered veterinarians employed by Monash Veterinary Clinic as per the authors' previous publication (Mason & Rous, 2014). Only one insemination was performed on each bitch. Bitches were placed onto a table standing or were restrained manually on the floor. No bitches were sedated for the procedure. Visualization of the cervix was performed using a ureterorenoscope (Karl Storz, Tuttlingen, Germany) equipped with a xenon cold light source and camera with images displayed on a monitor (Sony Trinitron CRT Monitor, PVM 2053MD, Sony, Tokyo, Japan). Insufflation of the vagina was achieved using a rectal insufflation bulb (30200 rectal insufflation bulb WelchAllyn, Skaneatele, NY). A CH-5 TCI catheter (MOFA Global; Verona, USA) was passed through the cervix into the uterine body and semen was inseminated slowly and then followed by additional extender (AndroPRO AI®) until the uterus was deemed full by fluid appearance at the cervical os. The semen and additional extender were inseminated over a period of 15–20 min in all bitches. The vulva of the bitch was massaged by the operator throughout the insemination. This technique of EIU uses the same principles as that described by Wilson (Wilson, 2001), differing only in the use of air insufflation to allow improved visualization, a longer and thinner endoscope allowing adaptability to greater size ranges of bitches, and a greater volume of total inseminate. Bitches were monitored for 30 min post-procedure for potential complications (none observed), and phone contact was maintained with owners for the first 2 weeks following the procedure. Catheterization of the cervix was achieved in all (100%) bitches.

2.5 | Pregnancy diagnosis

Pregnancy was diagnosed 21–35 days after the initial rise in progesterone (>2 ng/ml) by ultrasonography in 208 bitches. Bitches were examined with a B-mode ultrasound equipped with a 7.5-MHz probe (MyLab 30VetGold, The Esaote Group, Genova, Italy) whilst standing with the probe placed on the ventral and/or lateral abdomen. Insemination success was determined by whelping alone for the remaining 144 bitches.

2.6 | Statistical analysis

Statistical analysis was performed using Prism (Graphpad Software Inc, CA, USA), and the results are given as percentage (pregnancy and whelping rate). The effects of the number of previous pregnancies of the bitches, number of PMNS inseminated and veterinarian performing the insemination on pregnancy rates were analysed using Fisher's exact test—two tailed. No data were excluded from the statistical analyses. p-values <.05 were considered statistically significant.

3 | RESULTS

The overall pregnancy rate was 68% (238/352). When $>150 \times 10^6$ PMNS were inseminated, the pregnancy and whelping rates were 76% (110/145); when $100–150 \times 10^6$ PMNS were inseminated, the pregnancy and whelping rates were 68% (87/128); and when $<100 \times 10^6$ PMNS were inseminated, the pregnancy rate was 52% (41/79) and the whelping rate 42% (32/79). The pregnancy rate was significantly higher when $>150 \times 10^6$ PMNS ($p = .003$) or $100–150 \times 10^6$ PMNS ($p = .027$) were inseminated compared to $<100 \times 10^6$ PMNS. There was no statistically significant difference between the insemination of $>150 \times 10^6$ PMNS and insemination of $100–150 \times 10^6$ PMNS ($p = .176$). Nine of 153 confirmed pregnant bitches (via ultrasound) underwent complete resorption (early embryonic death) resulting in an overall whelping rate of 65% (229/352). There was no difference in the pregnancy rate of nulliparous bitches 71% (61/86) and primiparous/multiparous bitches 66% (178/266) ($p = .51$). The pregnancy rate resulting from inseminations performed by veterinarian one (67%; 141/211) was not significantly different ($p = .642$) from that of veterinarian two (70%; 98/141).

TABLE 2 For each group: the average number of PMNS inseminated (range of PMNS) and average motility values of samples (range of motility)

Group	Sperm numbers × 10^6	Progressive motility %
$<100 \times 10^6$ PMNS	61.1 (9–99)	38.8 (20–78)
$100–150 \times 10^6$ PMNS	121 (100–149)	48.3 (23–80)
$>150 \times 10^6$ PMNS	249 (150–519)	58.67 (28–80)

4 | DISCUSSION

Results of this study show that one optimally timed insemination of frozen–thawed dog semen via EIU results in similar pregnancy or whelping rates to published reports of both one (Linde-Forsberg & Forsberg, 1993; Linde-Forsberg et al., 1999; Thomassen et al., 2006) and two inseminations using the Norwegian catheter (Linde-Forsberg & Forsberg, 1989, 1993; Linde-Forsberg et al., 1999; Thomassen et al., 2001, 2006). The results attained are also similar to those previously published by the author (Mason & Rous, 2014), with no significant difference in pregnancy rates attained relative to the number of previous pregnancies of the bitches, nor the veterinarian performing the insemination.

Compared to insemination using the Norwegian catheter, EIU has the advantage to the operator of clearly seeing where the catheter is placed and 100% confidence that the insemination is intrauterine. Non-surgical intrauterine insemination (EIU and Norwegian catheter) of frozen–thawed dog semen is the preferred method of insemination, not only due to ethical issues of surgery (England & Millar, 2008; Linde-Forsberg et al., 1999), but also as EIU has previously been shown to have a significantly higher pregnancy rate than SIU (Mason & Rous, 2014).

In contrast to the authors' previous study, the EIU catheter was placed in the uterus after the semen was thawed in primiparous and multiparous bitches and before the semen was thawed in nulliparous bitches (ensuring normal reproductive tract anatomy). The thawed semen was stored at room temperature until insemination. In all cases with primiparous and multiparous bitches, the catheter was placed adequately through the cervix within two minutes ensuring the semen was not stored for long before placement in the uterus. Performance of EIU in this manner requires confidence and skill of the procedure and may not be appropriate for all operators. In nulliparous bitches, the catheter was also placed adequately through the cervix within two minutes.

Slow insemination of the thawed semen and extender is required to enable containment of the large volumes used in this study within the uterus (observed unpublished data by author). The larger than previously published volumes used in this study are believed to maximize litter size and pregnancy rate (unpublished data by author). Massaging the vulva during insemination is believed to stimulate uterine contractions as per previous reports of digital manipulation of the dorsocaudal vagina, which is believed to aid in pregnancy (England, Burgess, Freeman, Smith, & Pacey, 2006; England, Moxon, & Freeman, 2012b).

Results of both pregnancy rate and whelping rate are presented here. Previous studies of frozen semen insemination via transcervical insemination have presented data on either pregnancy rate (Linde-Forsberg & Forsberg, 1993), whelping rate (Linde-Forsberg, 2000; Linde-Forsberg & Forsberg, 1989; Linde-Forsberg et al., 1999; Thomassen et al., 2001) or a combination of both pregnancy and whelping rates (Thomassen et al., 2006). All of these previously presented reports contain no methods of ultrasound examination of bitches for pregnancy, so it would be assumed that pregnancy rate

in these reports actually pertains to whelping rate. With nine of the 153 (6%) confirmed pregnant bitches by ultrasound resulting in early embryonic death, it is pertinent to believe that of the remaining bitches that did not undergo ultrasound examination and subsequently failed to whelp, a similar percentage are likely to have undergone early embryonic death. To the authors' knowledge, this is the first clinical study presenting both pregnancy and whelping results of EIU with frozen–thawed dog semen, providing an insight into probable rates of early embryonic death, although data are small and further work is required in this area. Of the bitches to undergo confirmed early embryonic death, they were all contained in the $<100 \times 10^6$ PMNS group. This may pertain to poorer quality semen samples or poorer quality processing by collecting veterinarians which may have resulted in DNA damage whilst having adequate motility and morphology values. All samples provided for use came with paperwork from the collecting veterinarian ascertaining the samples to contain $>100 \times 10^6$ PMNS; however, in many cases, this was not the case, and if available, more semen was inseminated.

As with previous studies, maximum pregnancy rates have been attained with insemination of $>150 \times 10^6$ PMNS, albeit via one insemination only in this study. Previous studies with the Norwegian catheter showed no significant increase in pregnancy rate with the increase in numbers of inseminations (Linde-Forsberg & Forsberg, 1989, 1993; Linde-Forsberg et al., 1999); however, Thomassen showed an increased pregnancy rate with two versus one insemination with the Norwegian catheter when timing of insemination was not optimal (Thomassen et al., 2001, 2006). This concurs with the authors' previous study wherein two inseminations via EIU did not significantly increase the pregnancy rate (Mason & Rous, 2014), albeit all inseminations were optimally timed. It has been shown that two inseminations via the Norwegian catheter will increase litter size significantly from one insemination (Thomassen et al., 2001, 2006); yet, other studies show a trend to increased litter size with no significant increase in pregnancy rate (Linde-Forsberg & Forsberg, 1989, 1993; Linde-Forsberg et al., 1999). Litter size data, whilst collated, were beyond the scope of this study.

During a natural mating, it is commonly believed the male urethral opening aligns with the cervix of the bitch, resulting in intrauterine insemination of both the semen-rich fraction and third, prostate, fraction of the ejaculate (Verstegen J, personal communication). It is commonly accepted that to achieve maximal pregnancy rates and litter size, the mating "tie" should last for a minimum of 10–15 min. During this time, there will be a large volume of prostate fluid entering the uterus of the bitch which is likely to help attain the maximal pregnancy rate and litter size. There is increasing evidence of post-mating endometritis in bitches subsequent to natural mating and intrauterine and vaginal inseminations (England, Moxon, & Freeman, 2012a). The reproductive cycle of the bitch is dissimilar to any of the other domestic species, so it is possible that there may be a beneficial effect of post-mating endometritis in the bitch perhaps enhancing clearance of the large volume of unwanted seminal plasma, bacteria, debris and prostate fluid (England et al., 2012; England et al., 2012a). Whilst this

phenomenon requires further investigation, the author has followed similar principles of a natural mating during the EIU process, by using large volumes of synthetic extender in place of prostate fluid after the semen insemination. It would be believed that this would aid in attainment of maximal pregnancy rates and litter size from one optimally timed frozen semen insemination; however, litter size data are yet to be published by the author.

Whilst there was no significant difference between the insemination of $>150 \times 10^6$ PMNS and $100–150 \times 10^6$ PMNS, there is a trend towards greater pregnancy rate with more PMNS inseminated; this concurs with previous studies (Farstad & Berg, 1989; Linde-Forsberg & Forsberg, 1989, 1993; Linde-Forsberg et al., 1999; Thomassen et al., 2001, 2006). More cases need to be assessed to ascertain whether there is ultimately a significant difference in pregnancy rate between these groups.

All semen samples were thawed using one type of thaw media (AndroPRO AI®). This was chosen due to difficulties of obtaining like-branded thaw media for imported semen due to Australia's quarantine regulations (failure of appropriate paperwork when importing semen with thaw media), or failure of the collecting veterinarian to supply thaw media. Additionally, the author has previously received a number of semen samples from collecting veterinarians provided with of freeze media instead of thaw media (unlabelled or poorly identified receptacles) which has resulted in poor post-thaw motility results (<5%) and failed pregnancies due to the high glycerol concentration. For this reason, the author decided to use the same commercially available thawing media for all samples, meaning all samples could be thawed in thawing media rather than some samples in thaw media and some samples in saline (which is likely to affect post-thaw motility values). The volumes of thaw media used in this study differ from manufacturers' recommendations; however, the author has found that these volumes both adequately dilute the glycerol content of the frozen semen whilst minimizing agglutination of the thawed semen to particles in the extender (observed, unpublished data by author). The volumes of thaw media used also allow for accurate assessment of the semen samples due to the sperm being dispersed and not concentrated.

Computer-assisted semen analysis has been shown to provide lower sperm concentration values compared to the haemocytometer due to the viscus formation of diluted semen in the leja chamber (Douglas-Hamilton, Smith, Kuster, Vermeiden, & Althouse, 2005; Hansen et al., 2006). All samples were analysed equivalent to each other, and so, this effect would be consistent across all groups. A downfall of CASA is that inadequately diluted samples will give inaccurate concentration values and motility values (Hansen et al., 2006). This effect was not seen in this study as the thaw media volumes used resulted in an evenly distributed, single sperm layer in the analysis chamber, with adequate room for the sperm to move without colliding, maximizing accuracy of semen assessments. The CASA system used in this study allowed for rapid semen assessment compared to a haemocytometer or macler chamber, and should 150×10^6 or more PMNS have not been attained from the recommended number of straws or pellets (via the semen freeze report), then additional semen

(if available) could be thawed and rapidly assessed to add to the initial thawed sample, maximizing PMNS to be inseminated. The rapidity of assessment using this CASA system allowed for minimum time for the nulliparous bitches to be waiting with the catheter in the uterus. To the authors' knowledge, this is the first clinical paper describing the use of EIU with CASA to maximize PMNS numbers for insemination to allow maximal pregnancy rates to be attained.

In conclusion, the EIU technique described here with frozen–thawed dog semen provided similar results to the authors' previous study, and to previously published data via Norwegian catheter. As per previous studies, these results agree that higher pregnancy rates are attained with the use of more PMNS. As is published in other studies, these results indicate that similar pregnancy rates may be obtained with one well-timed insemination via EIU using $>100 \times 10^6$ PMNS as with the Norwegian catheter. These results support the use of EIU as an optimal technique for insemination of frozen–thawed dog semen.

CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

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