Theriogenology 82 (2014) 844–850

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Comparison of endoscopic-assisted transcervical and laparotomy insemination with frozen-thawed dog semen: A retrospective clinical study

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ARTICLE INFO

Article history:
Received 6 July 2013
Received in revised form 12 June 2014
Accepted 18 June 2014

Keywords:
Intrauterine
Frozen-thawed
Semen
Dog
Laparotomy
Transcervical

ABSTRACT

The objective of this retrospective clinical study was to compare pregnancy rates obtained after the use of endoscopic-assisted transcervical catheterization (EIU) or laparotomy (SIU) for insemination of frozen-thawed dog semen. Healthy bitches from various breeds were inseminated with semen from multiple donors processed by different freezing centers. Data from 118 inseminations (78 EIU and 40 SIU) performed between 2009 and 2011 were analyzed. Insemination timing was based on vaginal cytology, serum progesterone concentrations, and vaginoscopy. A ureterorenoscope and a CH-5 Transcervical insemination catheter were used for EIU; 28 of the bitches in this group were inseminated twice with the second insemination less than 12 hours after the first. The numbers of live morphologically normal sperm (LMNS) were determined to characterize insemination doses. Overall, pregnancy rate was greater ($P < 0.05$) in the EIU group (65%) than in the SIU group (45%). Pregnancy rates were greater ($P \leq 0.06$) when more than 100×10^6 LMNS were inseminated regardless of insemination method; the greatest pregnancy rate was observed in the EIU group when this insemination dose was used (38/49; 78%). There was no significant difference in pregnancy rate whether one (69%) or two inseminations (64%) were performed in the EIU group. Complications in the SIU group included anesthetic-induced bradycardia during surgery, significant postsurgery pain, seroma formation over the abdominal incision, and delayed wound healing. No complications were noted during or after insemination in the EIU group. In conclusion, these results support the use of EIU as a noninvasive alternative to laparotomy for insemination of frozen-thawed dog semen. In addition, use of more than 100×10^6 LMNS is also recommended for insemination.

1. Introduction

Since the first reported successful pregnancy from frozen-thawed dog semen in 1969 [1], pregnancy rates and insemination methods have improved dramatically. Although initial reports adopted vaginal insemination [1,2], it is now well accepted that intrauterine deposition yields superior results [3–5]. Andersen [2] reported early success of laparotomy (SIU) and later developed a technique for nonsurgical intrauterine insemination using the Norwegian Elk Catheter, a technique that is still commonly used in many European Countries [6,7]. The catheter was developed "to adapt the technique for practical conditions" [6], thus providing an alternative to surgical intrauterine insemination in the bitch. The registration bodies of some countries have restricted the use of SIU, notably the United Kingdom and some European countries, for ethical concerns over the need for anesthetics and surgery to inseminate the bitch. Despite the lack of data supporting SIU over other nonsurgical methods of intrauterine insemination, SIU is still a

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0093-691X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2014.06.014

common practice in many countries. Although there are data to confirm that SIU can result in pregnancy in the bitch, there are few studies documenting its success [8,9], with only one containing clinical retrospective data [9].

The reported insemination techniques using frozen-thawed dog semen comprise intravaginal insemination and four intrauterine techniques (Norwegian Elk Catheter, laparoscopic assisted, endoscopic-assisted transcervical catheterization [EIU], and SIU). A higher pregnancy rate has been well reported with inseminations using Norwegian Elk Catheters compared with vaginal insemination with both fresh semen [10] and frozen-thawed dog semen [3,10], whereas laparoscopic techniques have been reported to have a similar pregnancy rate to vaginal inseminations with both fresh [5,11] and frozen semen [11]. Although there are a number of published retrospective studies using Norwegian Elk Catheter [3,12–16], EIU [3], SIU [9], or vaginal insemination [17] for artificial insemination in the bitch [3,9,12–17], no reports have compared the success of EIU and SIU. Most published studies on intrauterine inseminations performed using frozen-thawed semen have been undertaken using the Norwegian Elk Catheter [3,12–16]. Reports on EIU have either used small sample sizes (19/327; [3]) or were skewed toward one particular breed [18] or highly fertile dogs [19,20]. Of the few reports on SIU, most are experimental [2,21] or descriptive in nature [22]. There is one clinical retrospective study on SIU reporting success using fresh, chilled, or frozen semen [9], which reported similar overall pregnancy rates to other retrospective studies using noninvasive techniques [3,13–16].

A major influence on the pregnancy rate is the total number of live morphologically normal sperm (LMNS) inseminated [7]. In clinical practice, the insemination dose of frozen-thawed dog semen is usually determined considering only the progressive motility alone and not taking into account the morphologic characteristics of the sample [3]. Although motility is known to be correlated to fertility, the percentage of morphologically normal spermatozoa in the sample is also correlated to fertility [23]. The reported number of progressively motile spermatozoa required to attain pregnancy varies from 100 to 200 $\times$ 10^6 [3,12,24]. This number dates back to the earliest reports on use of frozen-thawed dog semen [6] and has changed little. There is some debate in the literature as to whether the insemination dose should be calculated on progressive motility alone, or whether morphologic characteristics of the spermatozoa should also be considered as in more recent reports [7,16].

The objective of this study is to compare the success rates of SIU and EIU, correlate them to LMNS inseminated, and evaluate the need for anesthetics and invasive procedures to attain pregnancy using frozen-thawed dog spermatozoa.

2. Materials and methods

2.1. Bitches

A total of 118 inseminations were performed on 115 bitches presenting to Monash Veterinary Clinic for routine insemination of frozen-thawed dog semen from November 2009 to March 2011. All bitches were clinically healthy, between the ages of 1.25 and 8.25 years (3.9 $\pm$ 1.63 years; mean $\pm$ SD) and of varying parity ranging from 0 to 4 litters, with most bitches having had either zero or one litter before presenting. Bitches that had a history of uterine disease or known infertility were not included in the study. German Shepherds (10.2%), Border Collies (7.6%), Newfoundlands (6.8%), Irish Setters (6.8%), and Bernese Mountain Dogs (5.1%) were the most represented breeds. Breeds considered to have higher fertility such as Greyhounds [25] and dogs from commercial working facilities (observed unpublished data) were not included in the study.

2.2. Insemination timing

Insemination timing was based on vaginal cytology, serum progesterone concentrations, and vaginoscopy [26–28]. All bitches were presented to the clinic for their first assessment 5 to 7 days after the onset of vaginal swelling or discharge being noted by the owner. 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 [29,30]. 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. Serum progesterone concentration was determined at the first visit and every 3 to 4 days until the LH surge was detected (progesterone concentration >2 ng/mL). Subsequent to the LH surge, serum progesterone concentration was determined every 1 to 2 days until ovulation was deemed complete [31]. Once ovulation was determined (progesterone concentration of 5–8 ng/mL), vaginoscopic examinations were commenced and insemination was not performed unless ovulation was deemed complete (progesterone concentration >10 ng/mL). Serum progesterone assays were not continued subsequent to a concentration of greater than 10 ng/mL.

Vaginoscopy was performed using a Sigmoidoscope (32020 Fibreoptic Sigmoidoscope; WelchAllyn, Skaneateles, NY, USA) daily from the time of ovulation (progesterone concentration of 5–8 ng/mL) until the time of insemination [30]. Insemination was performed on the first day in which maximal crenulation (vaginal folds at their most shrunken and angular state) of the anterior vagina was detected [30] in conjunction with a progesterone concentration of greater than 10 ng/mL.

2.3. Semen handling

Frozen semen used in this study was obtained from a variety of sources both within Australian and international freezing centers and had been stored for varying periods of time (3 months to 22 years). Semen had been frozen in pellets using Camelot farms or International Canine Semen

Bank extenders or frozen in straws using Clone, International Canine Genetics , Uppsala, Minitube, or Triladyl extenders. The freezing center's instructions for thawing semen were followed when available. Semen pellets were transferred into a small plastic bag containing 1 mL 0.9% sodium chloride or thaw media, as provided by the freezing center, and thawed in a water bath at 37 °C for 60 seconds. Semen straws were transferred directly to a water bath at 37 °C for 60 seconds. The straws were then emptied into a small plastic bag containing warmed thaw media provided by the freezing center; recommendations were followed in relation to the volume of thaw media used per straw. The total volume of semen used ranged from 1 to 3 mL, as determined appropriate for the size of the bitch.

All insemination doses were assessed after thawing to determine the number of LMNS inseminated. The percentage of progressively motile spermatozoa was determined subjectively under phase-contrast microscopy and ×100 magnification on a 37 °C warmed stage. Spermatozoa concentration was determined using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) after 10% dilution of the original sample using 5% formal-buffered saline solution. Spermatozoa morphology was assessed by examining a minimum of 100 spermatozoa under phase-contrast microscopy and ×400 magnification [32,33]. The number of LMNS inseminated was determined by multiplying the total number of spermatozoa by the percentage of progressively motile spermatozoa and the percentage of morphologically normal spermatozoa. The recommended insemination dose for frozen-thawed canine semen is 100 to 200 $\times$ 10^6 motile spermatozoa [6,24,34]. For statistical analysis of the pregnancy rate in relation to the number of spermatozoa, the insemination results were classified into over and under 100 $\times$ 10^6 LMNS, because this value is considered the minimum industry standard [34].

2.4. Insemination procedures

All inseminations were performed by registered veterinarians employed by Monash Veterinary Clinic. Determination of insemination technique (SIU or EIU) was made by the client after a discussion about both insemination techniques with one of the authors. The clients were informed that there was no comparative study of the pregnancy rates of SIU and EIU, and from the results published of each technique individually, it would be assumed that pregnancy rates were comparable. A total of 40 SIU and 78 EIU were performed. Two inseminations were performed in 28 (36%) of the bitches in the EIU group, with the second insemination less than 12 hours after the first. The insemination dose was determined to be the total number of LMNS from both inseminations when more than one was performed. Two inseminations were used when either elected by the client or in cases in which the number of LMNS inseminated were less than 100 $\times$ 10^6 and more semen was available to be used.

2.4.1. Surgical insemination via laparotomy

Bitches were premedicated either with 1 to 2 mg/kg of xylazine and 0.04 mg/kg of atropine and induced with 10 to 35 mg/kg of thiopentone (XATI) or with 0.05 mg/kg of acetylpromazine and 0.02 mg/kg of buprenorphine and induced with 2 mg/kg of alfaxalone (ABAI). The choice of anesthetic protocol between was random and based on surgeon preference. Anesthesia was maintained using a mixture of isoflurane and oxygen. Bitches were placed in dorsal recumbency with their hindquarters elevated, the lower abdomen was clipped and prepared aseptically, and a midline incision was made to identify and exteriorize the uterus. Both ovaries and the uterus were examined for the presence of obvious pathologies. A 22-ga intravenous catheter was introduced into the lumen of each horn when the uterine body was manually clamped tightly. The syringe containing the semen was then attached to one of the catheters and half of the dose was inseminated over a period of 1 minute. The process was then repeated with the other horn with the remaining semen. Slight pressure was placed over the entry points of the catheters to avoid hemorrhage and the uterine body was kept clamped for another 2 to 5 minutes. Routine abdominal closure was performed using 2-0 Polydioxanone (PDS II; Ethicon, OH, USA) absorbable suture material in the linea-alba and subcutaneous tissues and Supramid nonabsorbable skin sutures. Bitches were monitored for 2 hours post-operatively until fully recovered for complications and pain. Telephone contact was maintained with owners until sutures were removed 10 to 14 days after surgery; bitches were clinically assessed by when deemed necessary.

2.4.2. Endoscopic-assisted transcervical catheterization

Bitches were placed on a table in a standing position or were restrained manually on the floor. If required, bitches were sedated with 0.02 mg/kg of acetylpromazine, iv. 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). A CH-5 Transcervical catheter (Minitube of America, Verona, USA) was passed through the cervix into the uterine body and semen was inseminated slowly over a period of 10 to 15 minutes. This technique of EIU uses the same principles as that described by Wilson [20], differing only in the use of air insufflation to allow improved visualization, and a longer and thinner endoscope allowing adaptability to greater size ranges of bitches. Bitches were monitored for 30 minutes after procedure for potential complications and phone contact was maintained with owners for the first 2 weeks after the procedure.

2.5. Pregnancy diagnosis

Pregnancy was diagnosed 21 to 35 days after LH surge by ultrasonography in 26 bitches in the SUI group and 57 bitches in the EIU group. Bitches were examined with a B-mode ultrasound equipped with a 7.5 MHz probe (MyLab 30VetGold; The Esaote Group, Genova, Italy) when standing with the probe placed on the ventral and/or lateral abdomen. Insemination success (pregnancy rate) was defined by whelping for the remaining bitches.

2.6. Statistical analysis

Statistical analysis was performed using Prism (Graphpad Software Inc., CA, USA). Differences in bitch age and parity, age of donor dog, length of time semen was frozen, and insemination time postovulation among groups were analyzed using one-way ANOVA. The effects of insemination technique and number of LMNS on pregnancy rates were analyzed using the Fisher exact test.

3. Results

No significant differences were noted between groups for mean bitch age (SIU = 4.4 years and EIU = 3.7 years), parity (SIU = 0.74 and EIU 0.52), age of donor dog (SIU = 4.9 years and EIU = 5.5 years), age of frozen semen (SIU = 3.7 years and EIU = 5.6 years), and insemination time postovulation (SIU = 3.47 days and EIU = 3.46 days).

Overall, pregnancy rate was greater ($P < 0.05$) in the EIU group (51/78; 65%) than in the SIU group (18/40; 45%). The insemination doses used in this study ranged from 20 to 308×10^6 LMNS ($115.3 \pm 62.0 \times 10^6$; mean ± SD). There was no significant difference in insemination dose between the groups (SUI = $94.4 \pm 47.6 \times 10^6$ and EIU = $126.1 \pm 65.9 \times 10^6$ LMNS). Pregnancy rates were greater ($P \leq 0.06$) when more than 100×10^6 LMNS were inseminated regardless of the insemination method (Fig. 1). There was no significant difference in pregnancy rate whether one (36/52; 69%) or two inseminations (18/28; 64%) were performed in the EIU group.

Post-thaw spermatozoa motility ranged from 10% to 80%. Seven semen doses had post-thaw motility less than 40%, four of which resulted in pregnancy. Normal spermatozoa morphology ranged from 20% to 96%. Five semen doses had less than 40% normal sperm, one of which resulted in pregnancy.

In the SIU group, no significant difference ($P = 1$) in pregnancy rates was observed when either XATI (18; 44%) or ABAI (22; 45%) anesthetic protocol was used. One bitch in this group suffered anesthetic-induced bradycardia, which responded to sympathomimetic therapy, 10 bitches suffered from significant postsurgery pain, one bitch had seroma formation over the abdominal incision, and one bitch suffered from poor/delayed wound healing. Sedation was required for insemination in two bitches in the EIU group, but no complications were noted during or after insemination in this group.

4. Discussion

The results of this study indicate that greater pregnancy rates with frozen-thawed dog semen can be achieved with noninvasive endoscopic-assisted transcervical insemination than with laparotomy. Pregnancy rates were greater when more than 100×10^6 LMNS were inseminated using EIU.

Before 2012, studies related to SIU were either experimental with small sample sizes or descriptive in nature only [22]. A recent study using SIU of fresh, chilled, or frozen semen [9] found a comparable pregnancy rate (70.3%) to this study and previously published pregnancy rates using noninvasive techniques [3,12,19,35]. However, no direct comparison with less invasive techniques was conducted. It is also difficult to draw a direct comparison with the SIU results of the present study, because fresh, chilled, and frozen semen were used. There is also limited published data pertaining to the use of EIU with frozen-thawed dog semen, with the most commonly reported device for noninvasive intrauterine insemination being the Norwegian Elk Catheter [3,7]. There have been two large recent studies in which over 300 bitches in each were inseminated using the Norwegian Elk Catheter [3,12]. Thomassen et al. [12] achieved a pregnancy rate of 75% with inseminations of approximately 200×10^6 LMNS. Linde-Forsberg [3] inseminated on average 185×10^6 LMNS and achieved a pregnancy rate of 84.4%; however, pregnancy success was assessed in a well-controlled setting wherein a single inseminator, extender, and freezing center were used for all inseminations; this likely accounts for the lower pregnancy rates observed in the clinical setting in the

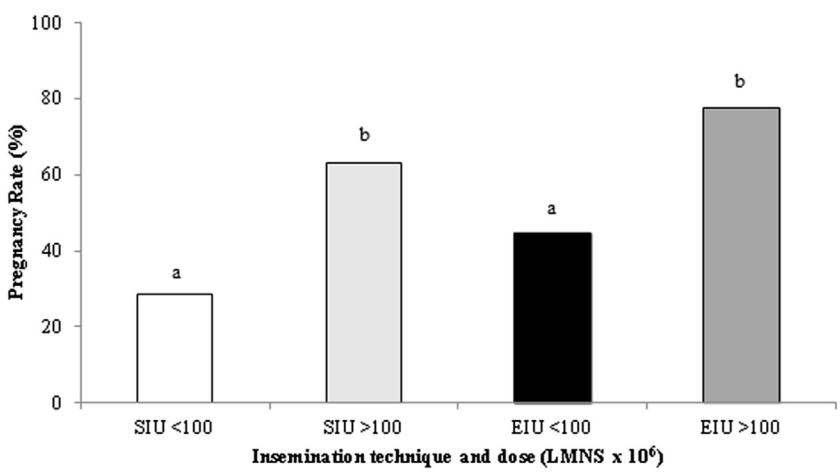

Fig. 1. Pregnancy rates after intrauterine insemination using frozen-thawed dog semen according to the insemination method (SIU, laparotomy; EIU, endoscopic-assisted transcervical catheterization) and dose. LMNS, live morphologically normal sperm. Columns with different superscripts a and b differ ($P \leq 0.06$).

present study with multiple inseminators, semen donors, extenders, and freezing centers. Both studies reported that LMNS, age of bitch, and semen quality influenced pregnancy rate [3,12]. In the present study, fewer spermatozoa were inseminated using EIU (average 115 $\times$ 10^6 LMNS), resulting in an overall pregnancy rate of 65%. No bitches were excluded based on age, nor were inseminations of poor quality semen excluded, which could also help to explain the reduced success. Comparisons of the present results with other studies using exclusively EIU are also complicated because of the fact that those have used breeds that are known to have high fertility (Greyhounds, commercial working dogs) or have used single stud dogs [18,19]. However, these studies do report that very high pregnancy rates can be achieved using EIU (89%; [18]), and that pregnancy can be obtained with insemination doses as low as 30 $\times$ 10^6 spermatozoa [19].

Some features of natural breeding are worth considering when discussing success of EIU and SIU. During natural breeding, the engorged penis and bulbus glandis of the male stretches the vagina of the bitch causing release of oxytocin, which stimulates myometrial contractions [36]. This suggests that myometrial contractions are an important component of achieving pregnancy in the bitch most probably by assisting passage of spermatozoa to the oviduct. Stimulation of the cervix and vagina, as occurs during EIU, has been shown to increase myometrial contractions [36]. In contrast, SIU may result in reduced myometrial contractions as anesthetic agents have been shown to reduce myometrial contractions in other species [37], and it would be expected that this would concur in the bitch. This is a significant limitation of inseminating a bitch under general anesthesia as it must be questioned how much of the insemination dose remains in the uterus after these procedures. In published descriptions of laparoscopy [5] and SIU [22], both of which require general anesthesia, it is commonly noted that semen can be found in the vagina immediately after or during the procedure. This would help to explain why the currently reported success of vaginal and laparoscopic insemination is similar, and lower than those reported with transcervical techniques [3,5,11,19,20]. The choice of anesthetic protocol, XATI or ABAI, did not appear to have an effect on the outcome of pregnancy in the SIU group. Sedation was required only for two bitches in the EIU group. Although both became pregnant, this sample size is too small to draw any clear conclusions on the true effect of sedation on uterine motility and pregnancy rates.

Stimulation of the endometrium after transcervical catheterization has led to the proposal that EIU may cause irritation of the endometrium, subsequent cystic endometrial hyperplasia, or endometritis and/or pyometra. Although it has been shown that stimulation of the endometrium during diestrus can induce the aforementioned reactions [38], this has not been shown in estrus when the insemination is occurring. The catheter is placed in the body of the uterus just cranial to the cervix, close to the same place as catheters are placed during SIU. If the clinician has adequately assessed the timing of the insemination to be performed during estrus, there is no reason to believe that EIU will increase the risk of uterine disease. In the 78 EIU procedures undertaken in this study, none resulted in any clinical uterine disease. Additionally, there is no published data to support an increased risk of uterine disease subsequent to EIU versus SIU.

Since its development, EIU has been a slowly evolving procedure in veterinary medicine. The technique has been dismissed by many for being considered too difficult to learn [39] and for requiring equipment deemed too expensive (personal communications). However, these excuses are fast losing their validity because of the increasing number of veterinarians able to perform the technique, the availability of training courses, and the relatively low equipment leasing costs available today. In recent years, EIU has gained increased popularity over the Norwegian Elk Catheter and has a number of advantages. Firstly, obesity and stomach distension of the bitch have little influence over the degree of difficulty. The Norwegian Elk Catheter technique is often rendered too difficult in these bitches and an alternative technique has to be adopted [3,7]. Secondly, the endoscope allows visualization of the cervical os and catheterization can be confirmed. The technique does have a steep learning curve but as with any other technical procedure, competency and efficiency is easily attained through repeated use and experience. The original endoscope used by Wilson [19] was limited to use in medium-sized breeds. However, the endoscope used in this study overcomes this limitation by being thinner and longer, thus allowing the technique to be available in all sized breeds. To our knowledge, this is the first published use of this type of endoscope.

In contrast to EIU, SIU has been widely practiced for many years in Australia, USA, and to a lesser extent in Europe, whereas being deemed illegal in many other countries [7,40]. There is increasing public scrutiny as to the ethical treatment of animals, with the increasing presence of animal welfare groups. The Royal College of Veterinary Surgeons (UK) requires veterinarians to record why transcervical insemination is not an option and performing invasive SIU requires justification [25]. Although SIU allows for assessment of gross abnormalities of the uterus and ovaries, the authors believe there would be more benefit in ultrasound examination of the bitch's reproductive tract should this be a priority of the clinician. Additional to anatomical assessment, SIU is often promoted for allowing the clinician to place semen at the tip of the uterine horns, close to the oviducts. However, it has been shown that irrespective of the uterine location of semen deposition during SIU, the semen will be distributed through the whole uterus [41,42] As professionals trained to improve the welfare of animals, it is contradictory to promote invasive surgical procedures when other less invasive procedures that produce similar outcomes are available. In spite of the lack of published data on the risks associated with the use of both EIU and the Norwegian Elk Catheter, some of which include vaginal or uterine perforation, passage into the bladder, and cervical trauma, the authors believe these to be minimal subsequent to adequate training, and minimally detrimental to the bitch when compared with anesthetic complications and the invasiveness of a laparotomy.

The value in comparing pregnancy rates between studies can be affected by the methods of assessing semen and reporting insemination dose. In general, canine semen

samples are assessed based on progressive motility alone. However, it has been suggested that the total number of progressively motile and morphologically normal spermatozoa may be more appropriate for fertility assessments of canine semen samples [16,35]. Oettlé [43] noted that it is inadvisable to base an assessment of semen quality on spermatozoa motility alone, and later suggested that dog semen samples with less than 60% normal morphology spermatozoa had reduced fertility [23]. To reduce the impact of including semen of all quality, and therefore optimize the reliability of results from this study, sperm morphology was also taken into consideration for calculating insemination doses. Because of the impact of spermatozoa numbers on pregnancy rates, results in this study were further divided into under and over 100×10^6 LMNS to make them relevant to the minimum industry standards [34]. When over 100×10^6 LMNS were inseminated in this study, a pregnancy rate of 78% was achieved, which is similar to the larger studies [3,12]. These results are also in agreement with previous studies reporting that optimal pregnancy rates are achieved when more than 100×10^6 LMNS are used for insemination [6,24,34].

Previous reports using the Norwegian Elk Catheter recommend two inseminations with a minimum of 150×10^6 LMNS per insemination [3,35]. Many of the bitches in this study received two inseminations because of there being less than 100×10^6 LMNS in the insemination dose from the freezing center. Bitches receiving one EIU insemination did so either because the semen contained in the insemination dose was over 100×10^6 LMNS or there was a lack of availability of further semen. The pregnancy rate of the bitches receiving two inseminations in this study was no greater than with those receiving one insemination. The time of insemination postovulation in this study was comparable with that described in previous studies, which found a second insemination is not required to improve pregnancy success if timing of insemination is optimal [12]. Because of the fact that there is no significant difference in pregnancy rates between bitches receiving one or two EIU, the authors believe that the increased success of EIU in this study is not because of the ability to repeat the procedure. A large retrospective comparison of pregnancy rates from one versus two inseminations using EIU and the Norwegian Elk Catheter technique would assist clarification of the impact of this variable.

Five samples used in this study had more than 40% abnormal spermatozoa and one of which resulted in pregnancy. The accepted minimal motility value for satisfactory fertility when using frozen-thawed semen is 40% [3,12]; seven of the semen samples used in this study had progressive motility values less than 40% and four of which resulted in pregnancy. The method for assessing spermatozoa numbers used in this study was a Makler counting chamber, because it allows quick assessment of semen samples making it practical for use in a clinical setting. Although it has been shown that the Makler has reduced reliability compared with hemocytometers [44], all analyses were conducted using the same device by the same operator to minimize variations.

In addition to factors affecting pregnancy rate, accurate assessment of pregnancy success is important. Reporting pregnancy success can be done through early detection methods (ultrasound) or whelping. Of the 13 bitches confirmed pregnant on ultrasound after SIU, one failed to whelp. Of the 39 bitches confirmed pregnant on ultrasound after EIU, six failed to whelp. On the basis of these observations, it is probable that failure to whelp was also the result of embryonic death in a small percentage of the 19 bitches that were not examined by ultrasonography (nine in each group) and that failed to whelp. Although, this is a limitation of this study, the likely reduction in the presented success rates would be very minor.

4.1. Conclusions

The EIU technique for insemination of frozen-thawed dog semen resulted in greater pregnancy rate when compared with the SIU technique. In addition, greater pregnancy rates were obtained when more than 100×10^6 LMNS were inseminated using EIU. These results support the use of EIU as a noninvasive alternative to laparotomy for insemination in bitches.

Acknowledgments

We wish to thank Dr. Jennifer Clulow and Dr. Peter Chenoweth for their assistance in preparing the final manuscript.

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