FERTILITY IN BITCHES FOLLOWING INTRAVAGINAL AND INTRAUTERINE INSEMINATION WITH FRESH AND FROZEN SEMEN

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CERTIFICATE

This is to certify that the thesis entitled **"FERTILITY IN BITCHES FOLLOWING INTRAVAGINAL AND INTRAUTERINE INSEMINATION WITH FRESH AND FROZEN SEMEN"** submitted in partial fulfillment for the requirements of the degree of **DOCTOR OF PHILOSOPHY in ANIMAL REPRODUCTION, GYNAECOLOGY AND OBSTETRICS** to the Tamilnadu Veterinary and Animal Sciences University, Chennai, is a record of bonafide research work carried out by T.**SATHIAMOORTHY** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

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ABSTRACT

Title	:	**FERTILITY IN BITCHES FOLLOWING INTRAVAGINAL AND INTRAUTERINE INSEMINATION WITH FRESH AND FROZEN SEMEN**
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Year	:	2007

This study was conducted to compare the conception rate and litter size in bitches inseminated following intravaginal and intrauterine route with fresh and frozen semen and to study the effect of commercial semen extender on post thaw characters of dog semen. To study the efficacy of vaginoscopy to predict fertile insemination time in bitches was also taken as one of the objectives. Sixty bitches were randomly divided into six groups viz., Group I, II, III, IV, V and VI 10 each. The time of insemination was based on vaginoscopy in group I to V and on vaginal exfoliative cytology in group VI bitches.

Semen was collected from seven male dogs of different breeds, evaluated and used for cryopreservation either with Tris or Tes-tris extender.

Group I and II bitches were subjected to intravaginal insemination while Group III and IV bitches were subjected to intrauterine insemination using fresh and frozen semen, respectively. Group V and VI bitches were inseminated intravaginally using frozen semen supplemented with prostatic fluid and with fresh semen using vaginal exfoliative cytology to determine time of insemination, respectively. The conception rate obtained in this study was 50, 20, 50, 30, 30 and 40 per cent in group I, II, III, IV, V and VI bitches respectively. The mean post thaw motility was significantly higher for semen cryopreserved with Tris when compared to Tes-tris extender. The percentage of mean abnormality was significantly lower in semen cryopreserved with Tris when compared to Tes-tris extender. The mean percentage of plasma membrane integrity was significantly higher for Tris when compared to Tes-tris extender. Hence, it is concluded from this study that the conception rates were similar for both intravaginal and intrauterine insemination with fresh semen and higher conception rates were obtained when frozen semen was deposited intrauterine rather than intravaginal. Semen cryopreserved with Tris extender was found to gave better post thaw characters compared to Tes-tris extender. Vaginoscopy was found to be effective in assessing the optimum insemination time compared to vaginal exfoliative cytology. Addition of prostatic fluid with frozen semen had significant influence on increasing the conception rate with intravaginal insemination.

Key words : Fertility , Artificial insemination, Bitches

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RESEARCH FINDINGS

Degree	**:**	**Ph.D.**
Title	**:**	**Fertility in bitches following intravaginal and intrauterine insemination with fresh and frozen semen**
Name	**:**	**T. SATHIAMOORTHY**
Department	**:**	**Animal Reproduction, Gynaecology and Obstetrics**

- A conception rate of 50 per cent was achieved for both intravaginal and intrauterine insemination using fresh semen.
- Intravaginal and intrauterine insemination of frozen semen resulted in 20 and 30 per cent conception rates, respectively.
- Addition of prostatic fluid with frozen semen increased the conception rate upto 10 per cent over intravaginal insemination of frozen semen without the prostatic fluid.
- Vaginoscopy was found to assess the optimum insemination time more accurately compared to vaginal exfoliative cytology and higher conception rate was achieved when insemination was done based on vaginoscopy rather than vaginal exfoliative cytology.
- Intrauterine insemination through endoscopic transcervical technique could be employed effectively for artificial insemination in bitches without undue stress to the bitches.
- Tris extender was found to give best post thaw functional and morphological parameters in freezing dog semen than Tes-tris extender.
- The mean litter size ranged from 5.25 to 6.25 in bitches inseminated with fresh semen compared to 2.0 to 3.0 in bitches inseminated with frozen semen.

CHAPTER I

INTRODUCTION

Artificial insemination in dogs has become an increasingly accepted and successful method of producing offspring under circumstances where natural mating is not possible due to anatomical, psychological or physical problems, or not practical due to geographical constraints. Artificial insemination is indicated when physical defects in either the male or female prevent mounting and intromission. Artificial insemination is requested by breeders because of weakness or pain in the hindquarters of the male that inhibits mounting, congenital defects in the vestibule or vagina that cause discomfort during intromission, a short or abnormal attachment of the os penis that prevents intromission, or behavioral unwillingness of a male or female to accept their selected mate. Intensive use of the stud dog during a short period of time may exhaust his willingness to mate or to produce an acceptable ejaculate. Dividing one ejaculate to artificially inseminate more than one bitch reduces the number of ejaculates that the stud dog would be forced to produce during a short period of time (Soderberg, 1986).

The semen for artificial insemination may be used immediately (fresh semen artificial insemination), diluted with an extender that nourishes and protects spermatozoa, chilled and used for insemination within 24 h (extended chilled semen) or extended with a cryoprotectant, frozen and stored in liquid nitrogen (frozen semen artificial insemination) (Root and Johnston, 2000)

In dogs, the first successful artificial insemination using frozen thawed semen was reported by Seager in 1969. Since then, different studies have been reported testing various freezing techniques and extenders (Gill *et al*.,

1970). However, pregnancy rates following AI with using frozen thawed semen in this species remains unsatisfactory, particularly under clinical conditions (Linde-Forsberg and Forsberg, 1993).

Greater accuracy in determining the ovulation time is essential when artificial insemination using frozen semen is adopted because the viability of the frozen semen is poor. Frozen thawed semen has an estimated intrauterine fertile lifespan of less than 24 hours (Concannon and Batista, 1989) when compared to fresh semen which has a fertile intrauterine lifespan of more than 4 days (Concannon *et al*., 1983)

The duration of proestrus in a bitch on an average is 9 days but it may be as short as 3 to as long as 25 days (Feldman and Nelson,1996 and Root and Johnston, 2000). The duration of estrus in a bitch is 9 days but again it may vary from 1 to 2 to as long as 18 to 20 days (Feldman and Nelson,1996). Hence, ovulations may also occur any time between 5 and 21 days of the estrous cycle (Wright,1991 and Feldman and Nelson,1996)

Thus, the development of a rapid and reliable method for predicting ovulation in dogs would have a large impact on canine reproduction. Such a method would improve the fertility and the cost efficiency of semen preservation techniques. It would also find application in breeding and infertility management and diagnostic, research and embryo transfer (Bouchard *et al*., 1991).

Vaginal cytology has been commonly used to monitor the canine estrous cycle. This technique defines adequately the most fertile period but it estimates ovulation time only retrospectively (Holst and Phemister,1975).

Serial evaluation of vaginal mucosa by vaginoscopy for evidence of crenulation (wrinkling) is one of the methods to estimate ovulation time and has been reported as an excellent adjunct to vaginal cytology and hormonal assays (Lindsay,1990).

Several important aspects with regard to the successful use of frozen semen in the dog have yet to be researched in detail. As with other species, it seems there are as yet undefined qualities in the ejaculate that determine whether the freezing will be successful, as evidenced by the fact that the semen from some fertile males do not freeze well. Surprisingly, even information on semen quality in relation to fertility is sparse. Furthermore, there is little information on the minimum number of spermatozoa that are needed to ensure successful conception rates, or on sperm motility or morphology, site and volume of the inseminate.

Although the first reported success of frozen thawed semen AI in the dog was after intravaginal insemination, deposition of semen into the uterus has been shown to be of great importance to the success of AI. Intra uterine semen deposition is an essential part of the successful use of frozen canine semen (Andersen, 1975 and Farstad and Andersen – Berg ,1989).

A transcervical approach to the canine uterus has always been desirable, but only recently has it become technically feasible. Endoscopic transcervical insemination technique provides visualization of the cervix using a rigid endoscope, and passage of a plastic catheter through the cervical canal (Wilson, 2001). This technique provides intra uterine deposition of semen, which is a vital part of frozen semen technology. Insemination using the endoscopic transcervical insemination technique involves minimal stress to the bitch and repeat inseminations are possible. The ability to do repeat

inseminations has been reported to increase conception rates or litter size (Farstad and Anderson Berg,1989 and Wilson, 1993).

The addition of prostatic fluid to frozen thawed dog semen resulted in a significantly higher fertility after intravaginal insemination when compared to frozen-thawed semen to which no fluid was added after thawing (Nothling and Volkmann, 1993).

In India, till now only very few work has been undertaken on the methods to predict fertile breeding time in bitches, fertility trials after fresh and frozen semen artificial insemination and on the methods of insemination especially transcervical insemination and the effect of commercial extenders on freezing and post thaw motility of dog semen. Hence, the present study was undertaken with following objectives

Objectives

1. To study the effect of commercial semen extender on freezing and post thaw motility of dog semen.

2. To study the efficacy of vaginoscopy to predict fertile insemination time in bitches.

3. To compare the conception rate and litter size in bitches inseminated following intravaginal and intrauterine route with fresh, frozen semen and intravaginal insemination of frozen semen supplemented with prostatic fluid.

CHAPTER II

REVIEW OF LITERATURE

Several factors affect the outcome of Artificial Insemination in bitches. Prediction of fertile breeding time and site of deposition of semen are the factors that decide the success rate of Artificial Insemination. Timing of the inseminations is of great importance, particularly when frozen thawed semen is used. Post thaw semen quality is depending on the quality of ejaculated semen, its freezability and techniques used for the freezing-thawing process.

2.1 DETERMINATION OF FERTILE BREEDING TIME IN BITCHES

Indications for determining the day of ovulation for managed breeding in the bitch included (1) AI with fresh, extended or frozen semen, when cost of each insemination was high (2) managed breeding of bitches with historical failure to show standing behaviour, historical conception failure or historical small litter size and (3) use of natural breedings which were difficult or expensive, so that a single breeding or high chance of conception was desired (Johnston, 1995).

In the bitch the ova were released as primary oocytes over a 2-3 day period (Wildt *et al*., 1978) and needed at least another 2-3 days to mature before being fertilized (Anderson and Simpson, 1973). Mature ova were therefore be expected to be present when peripheral plasma progesterone concentration value was 10 ng/ml. Fresh semen had a survival time of at least 4-6 days in the uterus of the bitches (Doak *et al*., 1967). Therefore, it was theorized that the optimal time for mating and artificial inseminations was, when all ova have been shed and mature 4-7 days after the LH peak (Linde-Forsberg and Forsberg, 1989).

2.1.1 Vaginal Exfoliative Cytology

Examination of the cells on the surface of the vaginal epithelium would give information about the stage of estrous cycle (Linde and Karlson, 1984, Olson *et al*.,1984 and Wright and Parry, 1989).

Under the influence of rising estrogen levels, the number of layers making up the vaginal epithelium increased dramatically, presumably to provide protection to the mucosa during copulation. Therefore, as estrogen rose during proestrus, the maturation rate of the epithelial cells seen on a vaginal smear was increased (Goodman, 2001).

The changing cellular types within the bitches vaginal smear had been investigated by many workers (Evans and Cole, 1931, Christiansen 1984, Wright,1990 and England, 1992). Standard classifications of cell types were now in general use (Feldman and Nelson, 1996) and examination of vaginal cytology was commonly used to monitor the bitches estrous cycle and to suggest the optimal time for mating or insemination (Hewitt and England, 2000).

The vaginal exfoliate cells were collected either by swab technique (Feldman and Nelson 1996) or direct aspiration (England,1992, Hewitt and England, 2000 and Thomassen *et al*. 2006). However, proper technique was important so that the cells obtained were representative of the hormonal changes. The sample was collected from the anterior vagina because cells from the clitoral fossa and/or caudal vagina were not an indicative of the stage of the cycle (Goodman, 2001).

2.1.1.1 Staging of the estrous cycle with vaginal exfoliative cytology

Proestrus

Feldman and Nelson (1996) reported that during early and mid proestrus the vaginal exfoliative cytology showed a mixture of epithelial cells including parabasal, small and large intermediate and superficial cells. Neutrophils and RBCs were usually present. During late proestrus, neutrophils usually decreased in number and large intermediate and superficial cells were predominant. Bacteria were often present in large numbers.

Estrus

Brukes (1986) reported that when partially or fully cornified superficial cells comprised of 95 to 100 per cent in a smear with concomitant loss of leucocytes and non- superficial epithelial cells, it was an indication that estrogen secretions and follicle maturation had reached a point at which LH surge resulted in ovulation. Neutrophils were absent during estrus. However, RBC's might or might not be present (Thrall and Olson, 1993 and Feldman and Nelson, 1996).

Diestrus

Holst and Phemister (1975) reported that the change from intermediate and parabasal cells during proestrus to superficial cells was more gradual than the dramatic decrease in superficial cells as seen at the onset of diestrus.

Thrall and Olson (1993) reported that day 1 of diestrus occurred 8 days (6 to 10) after LH peak in most cycles and was cytologically characterized by an abrupt change in relative number of superficial epithelial cells.

Feldman and Nelson (1996) reported that within a 24 to 48 h period at the end of estrus, the percentage of superficial cells fell to approximately 20 per cent, with the majority of cells being intermediate and/or parabasal cells.

2.1.1.2 Fertile breeding time based on vaginal exfoliative cytology

Vander Holst and Best (1976) noted that fertility improved with increasing numbers of cornified epithelial cells and Feldman and Nelson (1996) suggested that breeding should be attempted throughout the period when more than 80 per cent of epithelial cells were superficial in nature.

The cytological scoring method had practical value and was easy to apply, however, vaginal smears gave only a rough indication of whether the bitch was ready to be inseminated or not. Maximal cornification appeared as early as 4 days before the time of ovulation (Concannon *et al*., 1975) or as late as 3 days afterwards (Linde and Karlson, 1984)

Brukes (1986) studied the vaginal cytology in different phases of the estrous cycle and correlated it with LH surge. The parabasal cells decreased from 5 to 30 per cent at initial stage to less than 5 per cent within 4 to 5 days before the LH peak and were almost absent by 2 to 3 days before the peak. Small intermediate cells disappeared in the same pattern. Large intermediate cells initially increased until they reached a peak incidence of 5 to 25 per cent by 5 to 6 days before the LH peak and then decreased in numbers to nil at the LH peak. During the week prior to the LH peak, superficial cells increased from 30 to 60 per cent at 1 to 5 days before the LH peak. Before 6 days of the LH peak, the vaginal smear was dominated entirely by superficial epithelial cells.

Mestre *et al.* (1990) studied the correlation between the exfoliative vaginal cytology and plasma concentrations of progesterone, LH and estradiol – 17 β during estrus in nine German Shepherd bitches and reported that estrogen levels rose to a maximum of 75 pg/ml one day before the LH peak and then decreased abruptly. The eosinophilic index began to change five days before estrogen had reached peak values. The index increased to a maximum of 76 per cent, two days after the estrogen peak. The LH concentration reached maximal one day after the maximum estrogen concentration.

Olson *et al*. (1984) reported that the distinct decrease in the abundance of superficial cells in vaginal smears obtained in late estrus usually began 7 to 9 days after LH peak but occurred as early as 6 or as late as 11 days. Maximum cornification based on the percentage of superficial cells in the vaginal smear occurred as early as 8 or more days before the LH peak or 2 to 3 days after LH peak.

England and Russo (2006) reported that breeding should normally be attempted throughout the period when more than 80 per cent of epithelial cells were anuclear. Neutrophils were generally absent from the vaginal smear during this period.

The value of the vaginal cytology was often over estimated in breeding management but one absolute finding in vaginal cytology was the onset of diestrus. The vaginal cytology changed abruptly and dramatically over 24 to 48 hours from a predominantly non-cornified pattern. Neutrophils were present in large numbers. The first day of this dramatic cytologic change was referred to as diestrus day 1. This information was valuable because it could be used to predict gestation length (Purswell and Parker, 2000)

The use of vaginal cytology was not without problems as some bitches reach peak values of only 60 per cent cornification while in others there might be two peaks of cornification (Vander Holst and Best, 1976). Some bitches demonstrated poor cellular changes in the vaginal smear (Tstusui, 1975). Occasional discovery of polymorphonuclear leucocytes during the fertile period in some bitches and the low peak of cornification (only 60 per cent anuclear cells) in others (Hewitt and England, 2000) were some of the inaccuracies in vaginal exfoliative cytology.

Although the examination of vaginal smears taken from bitches at regular intervals was useful for monitoring the estrous cycle, the variations that occurred between and within bitches indicated that its own vaginal cytology was not reliable enough to allow the optimal time for mating to be determined (Hewitt and England, 2000).

Linde-Forsberg and Forsberg (1989) concluded that vaginal cytology smears were helpful in determining the correct time for insemination with fresh semen. For bitches to be inseminated with frozen thawed semen, however more exact timing was necessary. Thus quick and inexpensive assay methods for LH or progesterone would be of great benefit.

Day of ovulation cannot be determined prospectively using vaginal cytology. Vaginal cytology indicated that complete cornification had occurred or not the cornified smear would persist throughout the stage of estrus without indicating the day of ovulation or best days to achieve breeding. Detection of first day of cytologic diestrus, determined by examining daily cytology smears, permitted retrospective determination of ovulation, as the diestrus smear followed ovulation by about 6 days.

2.1.2 Vaginoscopy

The vaginal mucosa, as a target organ for reproductive hormones, reflected changes in their levels as the estrous cycle progressed. The observation of these changes via vaginoscopy was a quick and useful tool to predict the time of ovulation (Lindsay, 1983, Jeffcoate and Lindsay, 1989 and England and Russo, 2006).

Lindsay (1983) staged the different phases of estrous cycle based on observations of the mucosal fold contours and profiles, mucosal colour and the character and color of any fluid that was present.

Vaginoscopy was a useful tool in determining when a bitch should be bred. This was especially true when only one or two breedings were allowed by the owner of the male or when using fresh extended or frozen semen (Lindsay and Concannon, 1986 and Cain, 1991)

The rate and extend of vaginal changes seen varied between dogs. Maximal crenulation in one bitch might be moderate in another. In spite of these limitations, this relatively non invasive procedure gave useful information when used as an adjunct tool for timing the breeding (Goodman, 2001).

2.1.2.1 Staging of the estrous cycle with vaginoscopy

Proestrus

In proestrus, the vaginal mucosa became markedly edematous as estrogen level increased. The vaginal lumen was obliterated from view, and the mucosal surface was smooth and shiny. Vaginal folds were pink and billowed out into the lumen as a result of fluid retention (Goodman, 2001).

Lindsay (1983) studied 45 bitches of various breeds vaginoscopically and reported that during proestrus two stages of mucous membrane changes were observable endoscopically. An early proliferative / edematous stage, during which new mucous membrane folds developed and all folds become edematous; this stage was succeeded by a 'shrinking' phase, in which all became less edematous and more complex. All mucous membrane folds appeared edematous, soft rounded and even in outline. A clear bright red fluid was seen among the folds. About mid-proestrus the 'shrinking' stage became evident. Towards the end of proestrus, transversely disposed complex rugal adjacent to the para cervix emphasized the crumbled 'concertina-like' picture.

Estrus

As estrus approached declining estrogen and rising progesterone levels caused edema to subside. The vaginal tissue became stretched and did not rebound quickly enough to accommodate the loss of fluid. As a result, vaginal mucous membrane folds collapsed and subtle surface wrinkling / crenulations became apparent on the vaginal mucosa at the time of the LH surge. The mucosa became progressively more crenulated, the lumen more distinguishable and the vaginal folds more flattened as the edema diminished. Maximal effects were seen during the fertile period (4-7 days) after the LH surge (Lindsay, 1983).

Lindsay (1983) reported that shrinkage of vaginal fold intensified during estrus so that a sharp 'peaked' or 'angulated', profile of all general vaginal mucous membrane folds became the outstanding feature of estrus. During the total or maximum angulated / peaked period of estrus the vaginal folds appeared as a series of pale angular peaks, diminishing in height cranially to low angular mounds or tubercles.

Lindsay (1990) detected four consecutive periods of cyclic vaginoscopic mucosal changes. The cycle, which started with proliferative edematous period, was typified by massive edema of the vaginal mucosa. Two consecutive periods of progressive shrinkage followed and were defined by the character of their mucosal profiles. In the first shrinkage period, mucosal shrinkage without angulation, there was shrinkage of mucosal folds but their vaginoscopic profiles remained rounded. In the second shrinkage period, mucosal shrinkage with angulation, mucosal folds profile become sharp edged. A period of decline and cessation of shrinkage when the mucosa thinned and sharp profiles became round marked the end of the cycle.

The simple, radiating vestibular folds had sharp, even profiles. In many bitches when viewed during the last 48 hours of estrus, the rugose area adjacent to the para cervix had a very wide lumen lined by a characteristic 'crepe paper', wrinkled, mucosa of very low, very fine folds. The scanty, clear, rather straw colored fluid typical of estrus in many bitches, or less frequently a clear bright red fluid was observed.

During estrus, the luminal surface of the vagina became progressively more wrinkled or "crenulated". This effect often visible within 24 hours of the LH surge maximal crenulation and seen as development of angulated folds of vaginal mucosa with sharp edges, was noted to occur in the interval between ovulation and oocyte maturation, 2 to 5 days later (Feldman and Nelson, 1996).

Diestrus

By diestrus, the mucosa was flat and variegated. Since the protective layers of epithelium had diminished, the mucosa was friable, and so patches of superficial hemorrhages were often seen (Goodman, 2001).

Vaginoscopically, the most characteristic feature of diestrus was the widespread patchy or banded hyperemic appearance of the general vaginal mucous membrane giving it an overall tigroid appearance; the dorsal median fold and plicate vaginal folds were low, simple, soft and rounded in outline. The change over from estrus to diestrus was signaled by the initial rounding out of the tubercles of the dorsal median fold, the rapid and almost total disappearance of folds from the vestibular and rugose area. This changeover was rapidly followed usually 24 hours, by a rounding out of all folds (Lindsay, 1983).

2.1.2.2 Fertile breeding time based on vaginoscopy

Feldman and Nelson (1996) and Jeffcoate (1998) reported that obvious and maximum angulations of the vaginal mucosa were striking vaginoscopic features and therefore very useful indicators of peak fertility and were noted to occur in the interval between ovulation and oocyte maturation, 2 to 5 days later. This visible creunulation continued as long as 4 to 9 days after the LH surge and natural matings were recommended at this stage because it indicated the fertilization period.

Lindsay (1990) reported that a high rate of successful conceptions were achieved and could be expected from single matings of bitches with vaginal mucosal scores of S_4 or S_5, which consistency fell within the peak fertilization period. Selection of the shrinkage with mucosal angulation period for natural mating was particularly important when for management reasons mating opportunities were restricted or when fertility of the dog or bitch was unknown or suspected. When only a single mating was possible, peak fertility was most likely to be achieved by delaying mating until the peak A period. Inseminations in peak fertilization stages, S_4 and S_5 might be essential to maximize conception rates when using frozen semen, which had a very short post thaw viability.

Sridevi (2001) concluded that vaginal mucosal shrinkage with angulation corresponding to fertile period could be accurately estimated based on vaginoscopy and breeding during this period could result in high conception rates.

During proestrus and estrus vaginal mucosal folds were enlarged and edematous or pink / white epithelial folds followed by a progressive shrinking of these folds and pallor during the preovulatory decline in estrogen. Subsequently, the shrinkage was accompanied by wrinkling of the folds with angulated borders and dense cream to white in colour. This change normally occurred around the time of ovulation, and breeding was therefore planned to commence two days later. Interestingly, the wrinkling continued in a progressive manner and hence daily examination of the bitch was not essential to document when breeding should occur (England and Russo, 2006).

2.1.3 Progesterone Assay

The fact that peripheral plasma progesterone concentrations increased before estrus and ovulations occurred meant that monitoring the initial rise in this hormone was an excellent way of predicting estrus and ovulation.

Pre ovulatory follicular luteinization resulted in plasma progesterone concentrations showing a detectable increase from baseline anoestrus values (0.5 mg/ml) to between 1 and 2 ng/ml during late proestrus, 2 to 4 ng/ml approximately two days before ovulation and between 5 and 10 ng/ml during the fertile period. (Hewitt and England, 2000). Mating or inseminations were to be planned between 4 and 6 days after the plasma progesterone concentration exceeded 2 ng/ml, at the time of LH surge, or preferably should commence one day after values exceed 8 to 10 ng/ml which indicated the beginning of the fertilization period (Hewitt and England, 2000 and England and Russo, 2006).

2.1.4 LH Assay

Measurement of the peripheral plasma concentration of LH provided an accurate method for determining the optimal time for mating (Hewitt and England, 2000).

Shimatsu *et al*. (2003) performed ovulation timing test by using LH assay in twenty Beagle bitches and inseminated them with frozen semen three to seven days after the LH surge. At present, LH assay could not be recommended as the most accurate and practical method of ovulation prediction because LH peak did not occur before ovulation in all the dogs and some dogs exhibited LH peak of short duration so that the peak was not identified even with daily serum sampling.

2.1.5 Detection of Ovulation by Ultrasonography

The ovaries of a bitch could be identified using real-time diagnostic B-mode ultrasound and, with careful and repeated examination, it was possible to monitor follicular growth and to detect the time of ovulation (Hewitt and England, 2000).

England *et al*. (2003) opined that currently, the detection of ovulation using ultrasound had limited use for the detection of the optimal breeding time, especially when compared with the more simple method of measurement of plasma progesterone concentration as it increased immediately before ovulation and remained elevated.

2.2 METHODS OF INSEMINATION

2.2.1 Intravaginal Insemination

Vaginal insemination was easy, required simple, cheap, readily available equipments, and could be repeated over a number of days if necessary to ensure insemination at the time when oocytes were fertilizable (Nothling and Volkmann, 1993).

Vaginal insemination yielded lower pregnancy rates than intrauterine insemination when frozen thawed semen was used for artificial insemination. Deposition of semen in the anterior vagina could be performed using a disposable bovine uterine catheter, the Norwegian catheter, or the Osiris catheter with a balloon on its tip, which could be inflated to prevent the reflex of semen.

2.2.2 Intrauterine Insemination

Uterine insemination posed practical problems because it either required one to pass a pipette through the cervix, which was sometimes impossible to achieve (Farstad, 1984 and Lindey-Forsberg and Forsberg 1989); to possess expensive fiber-optic equipment to catheterize the cervix under visual control (Wilson, 1993) or surgical (or) laparoscopic exposure of the uterus (Silva *et al*., 1996 and Farstad, 2000)

Although the first reported success of frozen – thawed semen AI in the dog was after intravaginal insemination, deposition of semen into the uterus was shown to be of great importance to the success of AI. Intra uterine semen deposition was an essential part of the successful use of frozen canine semen (Fougner *et al*., 1973 and Andersen, 1975).

2.2.2.1 Norwegian technique

Fougner *et al*. (1973) developed a transcervical insemination technique for the fur industry in Norway. This technique was later shown to be equally feasible in the bitch (Andersen, 1975). This technique involved palpation of the cervix through the abdominal wall and passage of a rigid metal catheter through the cervical canal.

Farstad and Andersen Berg (1989) deposited semen into the uterus by means of catheterization of the cervix using a specially designed catheter and fixing the cervix through abdominal palpation to ease catheterization. Morton and Bruce (1989) observed that uterine insemination although more difficult to achieve, was far preferable to surgical AI with its attendant risks and overtones.

2.2.2.2 Endoscopic intrauterine insemination technique

A transcervical approach to the canine uterus was always desirable, but only recently it became technically feasible. Endoscopic transcervical insemination was developed as an alternative technique to the Norwegian catheter (Wilson, 2001). The technique provided visualization of the cervix using a rigid endoscope, and passage of a plastic catheter through the cervical canal. This technique provided intra uterine deposition of semen, which was a vital part of frozen semen technology. Insemination using the endoscopic transcervical insemination technique involved minimal stress to the bitch and repeat inseminations were possible. The ability to do repeat inseminations has been reported to increase conception rates or litter size (Farstad and Anderson-Berg, 1989, Wilson, 1993 and Linde-Forsberg and Forsberg, 1989). Where the semen was of poor quality, repeat inseminations allowed more semen to be inseminated over an extended period (Wilson, 2003).

Transcervical intrauterine insemination could also be accomplished with the aid of a rigid fibroptic endoscope with urinary or angiographic catheter, in the standing bitch, and without sedation (Linde-Forsberg, 2001).

The anatomy of the vagina and cervix in the bitch presented particular obstacles to the successful catheterization of the cervix. Access to the cervical os was restricted by the dorsal median fold, narrow paracervical area and position of the external orifice of the cervical canal, which was directed towards the vaginal floor (Lindsay, 1983).

Fibroptic endoscopes allowed direct visualization of the cervix and also provided a means of presenting a catheter at the external os. However, the technique used to pass the flexible catheter through the cervix involved manipulation of the scope and catheter and required a great deal of practice. Some bitches were easy to catheterize and others extremely difficult, depending on the length of the vagina, width of the paracervical area and position of the os. The technique appeared less stressful to the bitch than the Norwegian method and any risks were virtually eliminated (Wilson, 1993).

Wilson (1993) used a rigid cysto-urethroscope, 30 cm in length and 4 mm in diameter with an oblique viewing angle of 25˚ together with 23 Fr gauge stainless steel sheath. A dog urinary catheter, 6-8 Fr gauge was passed through the operating channel of sheath. The endoscope was introduced into the vagina and advanced until the external os of the cervix could be visualized and the urinary catheter was manipulated into the cervical opening and further into the uterus and the semen was infused into the uterus. After, the intra-uterine semen deposition, the bitches hindquarters were elevated and the AI catheter was removed.

Rota *et al*. (1999) did transcervical insemination in ten bitches with rigid endoscope and uterine catheterization was performed with semi rigid sterile male dog urinary catheter and after insemination the hindquarter of the bitch was elevated for 10 minutes.

The New Zealand method utilized a rigid cystourethroscope to visualize and catheterize the cervix. The equipment consisted of 36-41 cm length and 3.5-4 mm diameter cystourethroscope with 30° viewing angle. A protective sheath that contained a channel for the catheter, ports for infusion or suction, a bridge containing an additional port for passage of the insemination catheter, a cold light source and an optional camera and monitor. An 8 Fr polypropylene urinary catheter was inserted into the channel within the sheath (Davidson and Bruce Eilts, 2006).

2.2.2.3 Laparotomy

Laparotomy was often objectionable to clinicians and breeders, especially with respect to elective procedures such as artificial insemination, because it required general anesthesia and was invasive. Additionally, the invasiveness of the procedure limited certain diagnostic tests and intrauterine insemination to a single event during each estrus (Davidson and Bruce Eilts, 2006).

2.2.2.4 Laparoscopy

Laparoscopic approaches to the canine uterus had been used infrequently as they required special equipments and expertise with laparoscopy. Diagnostic tests and insemination were also limited to a single event during estrus, because of the need for general anesthesia. Laparoscopy was less invasive than laparotomy, but surgical incisions were still involved.

Silva *et al*. (1995) adopted laparoscopic method of insemination in five Beagle bitches with fresh semen and obtained 100 per cent pregnancy rate with a litter size of 5 ±1.8.

2.3 NUMBER OF SPERMS USED FOR INSEMINATION

The minimum number of sperms required for acceptable fertility had not been established in the dog. In farmed foxes, intrauterine inseminations with as little as 20 X 10^6 fresh spermatozoa gave good fertility results (Wilson, 1993).

Linde-Forsberg and Forsberg (1989) used a insemination dose of <150 X 10^6 live spermatozoa per dose for frozen semen.

Farstad and Andersen Berg (1989) and Shimatsu *et al*. (2003) suggested that 100 X 10^6 live spermatozoa gave acceptable conception rate and litter size. But, Morton and Bruce (1989) recommended 150-200 X 10^6 motile spermatozoa in 2-3 ml on 2 or 3 occasions for acceptable fertility in artificially inseminated bitches with fresh semen. In a study using intravaginal insemination with fresh semen (Tsutsui *et al.* 1988), it was found that a total sperm dose of at least 200 X 10^6 was required in order to achieve a pregnancy rate similar to natural mating (89 vs 95 per cent, respectively). Reducing the total sperm dose to 100 X 10^6, with similar motility scores, resulted in a pregnancy rate of 33 per cent.

Nothling and Volkmann (1993) used mean doses of 101.20 X 10^6 and 100.90 X 10^6 progressively motile frozen thawed sperm per insemination in the two groups in their study to achieve an overall mean pregnancy rate of 80 per cent. This study used multiple intravaginal inseminations once daily, the timing of which was based on vaginoscopy.

Wilson (1993) obtained a pregnancy rate of 85 per cent in bitches inseminated by transcervical insemination technique with two inseminations of only 30-35 X 10^6 live frozen thawed spermatozoa. Silva *et al.* (1996) used total sperm concentrations of 200 X 10^6 with motility greater than 60 per cent to achieve a 60 per cent pregnancy rate using intravaginal insemination (Osiris gun) with frozen thawed semen.

In an extensive retrospective study of frozen semen inseminations, Linde-Forsberg *et al.* (1999) noted an apparent increase in whelping rate and litter size with increasing total sperm dose per intravaginal insemination.

Shimatsu *et al*. (2000) used 200 X 10^6 sperms in two straws for inseminating the bitches intravaginally and reported 100 per cent conception rate.

Recently, a study was conducted by Hye Jin Kim *et al*. (2007) to assess the least number of post thaw spermatozoa necessary to achieve pregnancy by intrauterine or intratubal artificial insemination (both by surgical method) and obtained a pregnancy rate of 80 and 20 per cent through intrauterine and intratubal insemination using just 5 X 10^6 and 4 X 10^6 spermatozoa, respectively.

2.4 NUMBER OF INSEMINATIONS

The number of inseminations seemed to be an important determinant for fertility. Both with fresh and frozen semen, there was a clear tendency for the pregnancy rate to increase with the number of inseminations performed. The number of days between first insemination and the whelping increased with increasing number of inseminations. This was considered to mirror the fact that the bitches inseminated several times were early in estrus at the first insemination.

Linde-Forsberg and Forsberg (1989) reported that there was a clear tendency for a better pregnancy rate with repeated inseminations after performing a total of 470 inseminations in various breeds of bitches

Fontbonne and Badinand (1993) inseminated 38 bitches one to three times with frozen semen both intravaginally and transcervically and reported 52.6 per cent of conception rate with a mean litter size of 4.2 and 73.6 per cent conception rate with a mean litter size of 5.5, respectively.

Linde-Forsberg and Forsberg (1993) reported that pregnancy rate was significantly higher in bitches inseminated twice with fresh semen than in those inseminated once only. Litter size and gestation length increased with the number of artificial inseminations. Shimatsu *et al*. (2000) inseminated the bitches with frozen semen consecutively 3 days ie 4, 5 and 6 after the LH surge and reported good fertility rate.

2. 5 PREGNANCY DIAGNOSIS BY ULTRASONOGRAPHY

Toal *et al*. (1986) and England and Allen (1990) reported that 3 mode ultrasonography had been reported to be 94-98 per cent accurate for pregnancy diagnosis when used after 24-25 days of gestation, and 99 per cent accurate at 28 days from the last breeding in bitches.

England and Allen (1990) reported that measurement of head diameter of fetuses with or without measurement of dorso-ventral trunk diameter has been demonstrated to be accurate within 2-4 days for estimation of gestational age.

Yeager and Concannon (1990) reported that the anechoic gestation sac was first detected at 17 to 20 days after the LH surge (15 to 21 days after the first breeding) when it was 1-2 mm in diameter and 1-4 mm in length. The gestational sacs were initially either spherical or oblate.

England and Allen (1990) stated that the ultrasonic detection of canine pregnancies was first achieved 21 to 23 days after the calculated LH peak. The earliest structures of pregnancy that could be imagined were spherical fluid filled anechoic conceptuses with hyper echoic walls. From day 30 of gestation the contours of the fetuses became discernible. The head and rump could be distinguished and the limbs were visible as echoic buds.

Yeager *et al*. (1992) found that limb buds and the choroidal plexus of the brain would be imagined from day 32 after the LH peak. Fetal motion was detected at day 34 to 36, while hypoechoic skeleton casting acoustic shadows was detected on days 33 to 39. The anechoic bladder and stomach were the first abdominal viscera detected on day 35 to 39.

2.6 CONCEPTION RATE

2.6.1 Intravaginal Insemination

2.6.1.1 Fresh semen

Many factors that contributed to the result of insemination with fresh and frozen semen were site of semen deposition, timing of insemination, semen quality, sperm dose and frequency of insemination.

Feldman and Nelson (1996) reported that the success of artificial insemination depended upon several variables, which included the use of a fertile stud dog with normal semen, proper handling of the semen during collection and insemination and the deposition of semen in to the anterior vaginal vault of the bitch at the appropriate time of estrus

Mickelsen *et al*. (1993) obtained a conception rate of 71 per cent in artificially inseminated bitches with fresh, undiluted semen deposited intravaginally.

Linde- Forsberg and Forsberg (1989) have reported a pregnancy rate of 65.70 per cent following insemination of 405 bitches with fresh semen. Root and Johnson (2000) reported 62.30 to 100 per cent pregnancy rate following intravaginal artificial insemination with fresh semen.

2.6.1.2 Frozen semen

Conception rates with frozen thawed dog semen had generally been poor when compared with those of other species. This was probably related to the poor penetration of oocytes by frozen thawed (compared with fresh) dog spermatozoa, although the problem was not entirely the result of poor semen quality after thawing, but also due to the difficulty in identifying the optimal time for insemination (England *et al*., 1996). An additional problem was the marked variation, in individual dogs, of post thaw survival of spermatozoa (Davies, 1982).

Farstad (1984) reported a pregnancy rate of 25 per cent following intravaginal insemination of frozen semen. Linde-Forsberg and Forsberg (1989) obtained a conception rate of 33.3 per cent in 6 bitches in which the frozen semen was deposited in the cranial vagina.

Font Bonne and Badinand (1993) obtained a conception rate of 52.6 and 73.6 per cent insemination through intravaginal and intrauterine deposition of frozen semen respectively. However, Rota *et al*. (1999) reported 73 per cent conception rate in bitches when the frozen semen was deposited intravaginally.

2.6.2 Intrauterine Insemination

2.6.2.1 Fresh semen

Farstad (1984) reported that birth rate after natural service was 92 per cent compared with 84 per cent when fresh semen was deposited in the uterine body.

Silva *et al.* (1996) and Concannon *et al.* (2001) reported that pregnancy rates up to 100 and 90 per cent respectively could be achieved with artificial insemination using fresh semen in fertile dogs.

Linde-Forsberg and Forsberg (1989) reported a pregnancy rate of 65.70 per cent following insemination of 405 bitches with fresh semen.

2.6.2.2 Frozen semen

Farstad (1984) reported that when frozen semen was inseminated intrauterine a birth rate of 67 per cent was obtained. Rota *et al*. (1999) achieved 100 per cent conception rate in bitches inseminated by endoscopic transcervical technique using frozen semen.

Devendra Pathak *et al*. (2005) transcervically inseminated 5 bitches of different breeds using frozen semen and obtained 60 per cent conception rate.

Thomassen *et al*. (2006) reported a conception rate of 75 per cent out of 665 bitches inseminated transcervically with frozen semen.

Battista *et al*. (1988) reported 25 per cent of whelping rate (3 of 12) in bitches artificially inseminated by using endoscopic method with frozen thawed semen.

Wilson *et al*. (1993) obtained 80 per cent of whelping rate in artificially inseminated bitches with endscopic method using frozen thawed semen.

Linde-Forsberg and Forsberg (1989) concluded that there was no significant difference in fertility between primiparous and multiparous bitches after performing 470 inseminations in both the animals. Linde-Forsberg and Forsberg (1993) reported that the fertility of 33 bitches with a previous history of reproductive problems was lower than that of the normal bitches.

2.7 GESTATION LENGTH

Apparent length of gestation, considered as the interval from a fertile mating to parturition, varied greatly in dogs. It ranged from 56 to 68 days with an average of 64 days. In some instances, parturition did not occur until 71 or 72 days after the first of a series of matings. In contrast, the real gestation length, defined endocrinologically, showed very little variation. For nearly all normal pregnancies, the interval from the preovulatory LH peak to parturition was 64 to 66 days (Holst and Phemister, 1974 and Concannon, 1983).

Linde-Forsberg and Forsberg (1993) reported that most bitches whelped 62 days after a single AI with fresh or frozen semen, but the gestation period varied between 55 and 69 days.

Linde-Forsberg and Forsberg (1993) reported that gestation length averaged 61.5 days after a single AI with fresh semen, while the bitches that were inseminated twice whelped on average of 62.7 days after the first and 60.7 days after the second AI.

Tsutsui *et al*. (2006) studied the effect of day of mating and litter size on gestation length in 36 Beagle bitches and reported that the gestation length from the day of ovulation was 63.90 ± 0.20 days and concluded that the duration of gestation was not significantly affected by the timing of mating

or the number of fetuses. In contrast, Okkens *et al*. (2001) reported that the number of fetuses and the duration of gestation were negatively correlated.

2.8 WHELPING RATE

Linde-Forsberg and Forsberg (1989) reported an overall pregnancy rate of 62.3 per cent and a whelping rate of 61.5 per cent after performing 470 inseminations on 400 bitches with both fresh and frozen semen. Linde-Forsberg *et al*., (1999) obtained a whelping rate of 84.4 per cent in bitches inseminated intrauterine route using frozen semen while, Shimatsu *et al*. (2000) reported a 100 per cent whelping rate in six Beagle bitches inseminated with frozen semen intravaginally.

2.9 LITTER SIZE

Seager *et al*. (1975) showed that primiparous bitches produced smaller litters than multiparous bitches. Fontbonne and Badinand (1993) obtained an average litter size of 4.8 and 5.5 upon intravaginal and transcervical insemination with frozen semen, respectively.

Linde-Forsberg and Forsberg (1989) reported mean litter size of 5.67 for 290 litters. For the 263 litters produced with fresh semen the litter size was 5.79 ± 0.18 while, the mean size of the litters from frozen semen was 4.44 ± 0.50 puppies.

Shimatsu *et al*. (2000) obtained a litter size of 4.00 ± 3.00 in six beagle bitches inseminated intravaginally with frozen semen. Shimatsu *et al*. (2003) studied the relationship between the timing of insemination based on LH surge and litter size and reported a mean litter size of 4.2, 4.3, 2.5 and 4.0 in bitches inseminated 4, 5 and 6, 3 and 5, 4 and 6 and 5 and 7 days after LH surge, respectively.

2.10 SEMEN COLLECTION

Semen could be collected from the dog by digital manipulation with or without a teaser bitch, with the use of artificial vagina, by electrical stimulation and by pharmacological methods

2.10.1 Semen Collection by Digital Manipulation

Lambert and Mackenzie (1940) were the first to advocate digital manipulation technique in dogs for semen collection. It was recommended that bulbus glandis should be rhythmically compressed with exerting moderate pressure behind the bulbus glandis.

Wong and Dhaliwal (1985) opined that there was no need for teaser bitch and observed better samples when collection was made in a quiet area familiar to the male dog.

Morton (1988) collected semen into a glass beaker by digital manipulation and opined that this method avoided the deleterious effect of rubber lining of artificial vagina.

Arthur *et al*. (1989) reported that the quality and quantity of semen of dogs collected by digital manipulation were much better and suggested that latex of artificial vagina had a deleterious effect on spermatozoa.

Fontbonne and Badinand (1993) collected semen by digital manipulation in the presence of a teaser bitch. While, England and Allen (1989), Linde-Forsberg *et al*. (1999) and Rota *et al*. (1999) collected semen from male dogs by digital manipulation in the absence of teaser bitch.

2.10.2 Semen Collection by Artificial Vagina

England and Allen (1992a) described that the first and third fraction of ejaculates adversely affected the semen quality and hence they discouraged the use of artificial vagina in dogs. Guerrin *et al*. (1999) collected semen by masturbation using an artificial vagina and a sterile disposable collection tube in the absence of teaser bitch.

2.10.3 Pharmacologic Methods

Pharmacological methods of semen collection were helpful in fearful or painful dogs that were unable to be collected by digital manipulation and in cases in which electro ejaculation was not an option (Kutzler, 2005).

Trass and Kustritz (2004) administered oxytocin (10 IU, i/m) or prostaglandin F_2 α (2.5 mg, i/m) 10 min before semen collection to three Samoyed dogs and found no significant improvement in the number of sperms in the ejaculate.

Root Kustritz and Milan Hess (2007) studied the effect of $PGF_2\alpha$ administration on increasing the number of spermatozoa 15 min prior to semen collection in the presence or absence of an estrus bitch and concluded that treatment with $PGF_2\alpha$ and presence of a teaser bitch had an additive effect on the number of spermatozoa. Further they suggested that this was a useful technique to increase the number of spermatozoa in a single canine ejaculate.

2.11 FREQUENCY OF SEMEN COLLECTION

Dobrinski *et al*. (1993) obtained high quality semen by collecting every second day. However, higher sperm output was obtained by collecting semen twice a week from dogs (England and Ponzio, 1996).

2.12 FRACTIONS OF DOG SEMEN

Semen of dog comprised of three fractions viz pre-sperm, sperm rich and post-sperm fractions (Amentea, 1914). Harrop (1960) reported that the first fraction was 0.25 ml and was probably secretion of urethral mucous glands, the second fraction was 0.5 to 3.5 ml of sperm rich fraction and the third of prostatic origin that varied from 2 to 30 ml.

Root and Johnston (2000) recommended that a minimal amount of prostatic fluid be collected so as to yield a final semen volume adequate for insemination. Estimates of these amounts were 1.5 to 3 ml for bitches weighing 4.5 kg or less, 3 to 5 ml for bitches weighing 4.5 to 22.7 kg and 5 to 8 ml for bitches weighing 22.7 kg or more.

2.13 SEMEN EVALUATION

2.13.1 Volume

Kadirvel (1998) reported clear and watery colour for first and third fraction, creamy and milky colour for second fraction of Mongrel dog semen.

Kurien (2000) reported a mean volume of 1.30 ± 0.02 ml of second fraction of semen from mongrel dogs.

Silva *et al*. (2005) reported that the average volume of second fraction of semen from six dogs of different breeds was 1.40 ± 0.30 ml.

2.13.2 Colour and Consistency

Olson (1992) was of the opinion that the pre-sperm and sperm rich fraction had a creamy to white colour and homogenous appearance.

Nair (1996) and Kadirvel (1998) recorded clear and watery colour for first and third fraction, creamy and milky colour for second fraction of Mongrel dog semen.

Kurien (2000) and Uma (2005) observed white to milky white colour and medium to thin consistency for the second fraction of dog semen.

2.13.3 Motility

2.13.3.1 Initial motility

The average motility of the normal dog semen sample varied from 70 (Olson, 1992), 78.60 (Rota *et al.,* 1995), 89.50 (England and Allen, 1989) and 99.20 (Cardso *et al*., 2003) per cent. It has been proposed that fertile dogs should have atleast 70 per cent initial motility (Larson, 1980). Prinosilova *et al*. (2006) recorded an average of initial motility of 78.00 ± 11.79 per cent in thirty fresh ejaculates from fifteen breeds of dogs.

Concannon and Battista (1989) suggested that at least 40-50 per cent sperm motility was necessary for success in artificial insemination with canine frozen semen. However, pregnancies had been obtained with frozen semen with a post thaw motility between 20 and 30 per cent (Linde-Forsberg and Forseberg, 1989).

2.13.3.2 Post thaw motility

Rota *et al*. (1999) reported 47 per cent of mean post thaw motility of dog semen cryopreserved with Tris-egg yolk extender.

Cardso *et al*. (2003) observed post thaw motility of 35 to 49 per cent after diluting with coconut water and different levels of glycerol for dilution and freezing.

Prinosilova *et al*. (2006) recovered an average post-thaw motility of 41.50 per cent in thirty ejaculates collected from fifteen dogs and frozen in Tris-fructose-citric acid-egg yolk extender with a glycerol content of 6 per cent.

2.13.4 Spermatozoal Concentration

Total sperm concentration in normal ejaculates ranged from 300 to 2000 millions however, it varied between breeds, as sperm production depended on testicular size (Johnston, 1991). Rota *et al*. (1995) reported that the average total sperm output per ejaculate was 916 million in different breeds of dogs.

Feldman and Nelson (1996) observed that the total number of spermatozoa in the ejaculate of a normal adult dog was two million to greater than a billion.

Prinosilova *et al*. (2006) reported an average sperm concentration of 276.3 millions/ml in thirty ejaculates collected from fifteen dogs of various breeds.

2.13.5 Live Spermatozoa

2.13.5.1 Live spermatozoa in fresh semen

The normal live sperm percentage was 99 in Cocker Spaniel (Ferguson *et al*., 1989) and 82.81 (Daiwadnya and Hukeri, 1993), 89.40 (Kadirvel, 1998) and 83.80 (Prinosilova *et al*., 2006) in Mongrels for fresh semen.

2.13.5.2 Live spermatozoa in post thaw semen

Pefia *et al*. (1998) observed a post thaw live sperm percentage of 36.00 ± 11.10 and 57.00 ± 12.90 in 6 and 8 per cent glycerol levels, respectively.

2.13.6 Abnormal Spermatozoa

2.13.6.1 Abnormal spermatozoa in fresh semen

Normal canine ejaculates should have at least 80 per cent morphologically normal and viable spermatozoa (Johnston *et al*., 2001). When a proportion of morphologically normal spermatozoa was below 60 per cent, fertility was found to be adversely affected (Oettle, 1993). Yubi *et al*. (1987) and Cardoso *et al*. (2003) reported 10 to16 percent of abnormal sperms in fresh semen.

2.13.6.2 Abnormal spermatozoa in post thaw semen

Feldman and Nelson (1996) reported that normal male dogs generally had greater than 70 per cent morphologically normal spermatozoa. The primary and secondary abnormalities should constitute less than 10 and 20 per cent of the defective sperm, respectively.

Cardso *et al*. (2003) observed 39.50 ± 7.70, 33.20 ± 3.70 and 32.80 ± 8.20 per cent of sperm abnormalities after freezing canine semen with coconut water with 4, 6 and 8 per cent glycerol, respectively. The major sperm abnormalities reported by them were detached head, coiled tail and bent tail.

Santos *et al*. (2006) reported 8 to 44 per cent of sperm with knobbed acrosome defect in four closely related infertile miniature Schnauzer dogs and opined that this might be due to genetic origin

Prinosilova *et al*. (2006) reported both 57.50 and 12.92 per cent of abnormal spermatozoa in cryopreserved dog semen.

2.13.7 Acrosomal Integrity in Fresh Semen

Kurien (2000) and Rijsselaere *et al*. (2002) reported 92 and 97 per cent of acrosome intrgrity in fresh dog semen.

Ponglowhapan *et al*. (2004) and Nothling and Shuttleworth (2005) reported 4.2 and 10.5 per cent of acrosomal damage in fresh semen.

2.13.8 Intact Acrosome in Post Thaw Semen

Yildiz *et al*. (2000) recorded 44.60 and 47.90 per cent damaged acrosome in frozen thawed semen using fructose and glucose, respectively.

Uma (2005) observed 42.30 per cent of intact acrosome in dog semen cryopreserved with Tris-egg yolk diluent under slow freezing.

Prinosilova *et al*. (2006) recorded 41.30 ± 11.34 per cent of sperm with secondary defects of acrosome in dog semen frozen with Tris-egg yolk extender.

2.13.9 Hypo Osmotic Swelling Test (HOST) for Fresh Semen

The capacity of sperm tail to swell in the presence of hypo osmotic solution indicated that transport of water across the membrane occured normally when the sperm had intact membrane function (Drevius and Ericksson, 1966).

Jeyendran *et al*. (1984) observed significant correlation between percentage of hypo osmotic swelling response and percentage of spermatozoa to undergo capacitation and penetrate hamster oocyte.

Rota *et al*. (1995) reported that hypo osmotic swelling response of spermatozoa in fresh dog semen was 93.6 ± 3.7 per cent whereas, Kurien (2000) reported an average hypo osmotic swelling response of 91.30 ± 0.92 per cent in fresh semen of Mongrel dogs.

2.13.10 Hypo Osmotic Swelling Test for Post Thaw Semen

Kurien (2000) reported 49.88 ± 0.71 per cent of Hypo osmotic swelling response for dog semen cryopreserved with Tris extender in a slow freezing rate.

2.14 SEMEN PROCESSING TECHNIQUE

2.14.1 Extenders for Freezing of Canine Sperm

The most commonly used diluent for freezing dog semen was Tris-fructose-citric acid extender containing egg yolk and glycerol (Oettle, 1986, Yubi *et al*., 1987 and Morton and Bruce, 1989).

Dog semen frozen well in an extender containing Tris-egg yolk-glycerol-citric acid-fructose had high fertility (Andersen, 1976, Farstad, 1984

and Ferguson *et al.,* 1989). Andersen (1976) found that 8 per cent glycerol in Tris-egg yolk extender was optimal for deep-freezing of dog semen. Christiansen and Schmidt (1980) used 20 per cent egg yolk in Tris-egg yolk extender for cryopreservation of dog semen.

Yubi *et al*. (1987) recorded a pre freeze abnormality of 19.00 ± 5.60 and 24.90 ± 20.90 per cent after diluting with Tris extender in the semen of Beagle and Boxer breeds, respectively. They also observed a pre freeze abnormality of 38.50 ± 0.70 and 29.75 ± 23.90 per cent after diluting with Lactose extender in the semen of Labrador Retriever and German Shepherd dogs, respectively.

Dobrinski *et al*. (1993) used Tris, Pipes, IMV Universal and Triladyl extenders for cryopreservation of dog semen and observed no difference in the percentage of post thaw motility between the extenders. Fontbonne and Badinand (1993) used Tris-Fructose-Citric acid extender with 6.4 per cent glycerol and 20 per cent egg yolk.

Silva and Verstegen (1995) compared the efficacy of Laciphos-egg yolk- glycerol, Tris-egg yolk glycerol and Biociphos-egg yolk-glycerol extenders on pregnancy rates in bitches and reported a pregnancy rate of 60, 60 and 100 per cent, respectively.

Yildiz *et al*. (2000) studied the cryoprotective ability of sugars on dog sperm cells after dilution in Tris based extender and concluded that sugars added to Tris- citric acid extender did not improve motility and viability.

2.14.1.1 Commercial extenders

Silva and Verstegen (1995) compared three extenders for deep-freezing dog semen. Biociphos-egg yolk extender and Tes / Tris egg yolk

extender resulted in 65 per cent post thaw sperm motility, 80 per cent live spermatozoa and 60 per cent pregnancy rate. The Biociphos-egg yolk extender resulted in 50 per cent motile spermatozoa, 78 per cent live spermatozoa following thawing and 100 per cent pregnancy rate.

More recently, prolonged post thaw maintenance of motility and plasma membrane integrity was observed by Rota *et al*. (1997) by addition of Equex paste (a commercial paste containing sodium and triethylnolamine lauryl sulphate) to canine semen. The addition of detergent to egg yolk extenders was thought to act through alteration of the egg yolk possibly increasing its protective effects during the cryopreservation process.

Nothling *et al*. (2007) studied the effects of homologous prostatic fluid added prior to cooling, after thawing, or at both times to epididymal spermatozoa and also compared Biladyl with Equex STM paste extender and with Andromed extender for freezing of epididymal spermatozoa and concluded that Biladyl with Equex STM paste extender was more suitable than Andromed and moreover, prostatic fluid improved the freezability and post thaw longevity of epididymal spermatozoa frozen in Biladyl with Equex STM paste.

Nothling and Volkmann (1993) reported that dog sperm frozen in Triladyl extender with the surfactant Equex STM paste and to which homologous prostatic fluid was added after thawing resulted in better fertility of vaginally inseminated bitches than bitches inseminated with similar semen but to which no fluid was added after thawing.

2.14.2 Dilution Rate

England and Ponzio (1996) and Kadirvel (1998) recommended 1:4 dilution rate for better cryopreservation of dog semen while, Kurien (2000) diluted the semen at the rate of 1:3 and got better post thaw parameters in dog semen.

Silva *et al*. (2005) compared two dilution rates for freezing dog semen based on sperm concentration and volume:volume extension and observed that no significant differences were observed between treatments after thawing regarding sperm motility and vigor, normal sperm morphology rate (or) longevity and concluded that both dilution rates were effective in canine semen freezing.

2.14.3 Egg Yolk

Egg yolk, particularly the phospholipids component, had been shown to protect cell membranes from cold shock and was regularly incorporated in semen diluents. For the preservation of dog semen a range of egg yolk concentrations was utilized. Christiansen and Schmidt (1980) and Davies (1982) used 20 per cent egg yolk in Tris-egg yolk extender for cryopeservation of dog semen.

2.14.4 Glycerol Level

Andersen (1976) found that 8 per cent glycerol in Tris-egg yolk extender was optimal for deep-freezing dog semen. Davies (1982) and Olar *et al*. (1989) reported that after freezing and thawing, the survival of dog spermatozoa was best when the glycerol concentration in the extender ranged from 2 to 8 per cent.

Olar (1984) found that post thaw motility was highest when 3-4 per cent glycerol was used in Tris-egg yolk based diluent, although Smith (1984) found that 9 per cent glycerol in an egg-yolk-pipes extender was optimal.

Pena and Linde-Forsberg (2000), Seager and Fletcher (1973), Fontbonne and Badinand (1993) and Somi *et al*. (2006) reported that in canine semen addition of 3 to 5 per cent glycerol in the diluent during equilibration period did not decrease post thaw motility and longevity.

Pena *et al*. (1998) reported that for freezing of dog semen addition of 8 per cent glycerol in a Tris-fructose-citric acid diluent was superior to 2, 4 and 6 per cent glycerol.

The optimal glycerol concentration represented a compromise between protective and toxic effects. Shimatsu *et al*. (2000) used 7 per cent glycerol and Tris-egg yolk extender for freezing of dog semen and reported 49.60 ± 3.60 per cent post thaw motility.

Cardso *et al*. (2003) concluded that coconut water extender with the addition of 20 per cent egg yolk and 4, 6 or 8 per cent glycerol could be used successfully in cryopreservation of canine semen.

The longevity of sperm and number of intact acrosome after thawing was significantly higher using 5 per cent glycerol in the extender when compared with 3 per cent glycerol (Rota *et al.,* 1998). This finding was supported by conception rates of 75-91 per cent in bitches that had been inseminated with frozen thawed semen extended at 35˚C with an extender containing 8 per cent glycerol (Andersen, 1975 and 1976).

Somi *et al*. (2006) concluded that the combination of semen centrifugation, two-step dilution with 10 per cent glycerol concentration and the addition of Equex STM paste shortly before freezing were superior when compared to all the other combinations. However, post thaw longevity and progressive motility were clearly improved by the use of a computer assisted freezing machine and a two step freezing curve with a faster decrease in temperature during the first step than given with the Styrofoam box.

2.14.5 Equilibration Time

Varying cooling and equilibration times, ranging from 45 to 54 min have been reported for canids (Farstad and Andersen Berg, 1989, Nothling *et al*., 1995 and Silva *et al*., 1996).

England (1992b) found that an equilibration time of 4 h at 5°C resulted in the greatest post thaw motility and live sperm when compared to 2 and 6 h equilibration times using Tris-egg yolk extender with 6.5 per cent glycerol.

Olar (1984) suggested that for storage in straws, either there should be a 1h cooling and 1h equilibration (or) 2 h cooling and 2 h equilibration period before freezing. Fontbonne and Badinand (1993) equilibrated the semen for 120 min at 5ºC and Thomassen *et al*. (2006) equilibrated the semen straws for about 2-3 h at 5˚C. Shimatsu *et al*. (2000) equilibrated the semen straws for 3 h at 5˚C in a programmable freezer and reported 49.60 ± 3.60 per cent post thaw motility.

2.15 Semen Packing

Dog spermatozoa had been frozen in ampoules (Gill *et al*., 1970), straws (Olar *et al*., 1989 and Pena *et al*., 2003) and pellets. Salisbury *et al*. (1978) opined that permanent labeling of pellets was difficult and there was a possibility of bacterial contamination in the freezing process.

The straw method of semen packing was adopted in many studies and a volume of 0.25 ml (Linde-Forsberg and Forsberg, 1989, Kurien 2000 and Uma, 2005) or 0.5 ml (Farstad and Andersen Berg, 1989, Dobrinski *et al*., 1993 and Pena *et al*., 2003) was adopted for packing of dog semen cryopreservation.

A trial to investigate the effect of straw size, freezing rate and thawing rate on the post thaw motility of sperm was carried out by Nothling *et al. (*2005) who reported that semen frozen 8 cm above liquid nitrogen, in 0.5 ml straws and thawed for 8 seconds at 70˚C had the best post thaw motility.

2.16 SEMEN FREEZING

The earliest reports of cryopreservation of dog semen on rapid freezing rates involved placing pellets of semen on a solid carbon dioxide (dry ice) block (Platz and Seager, 1977) while, others froze samples in ampoules (Gill *et al*., 1970) and straws in liquid nitrogen vapour (Oettle, 1986 and Morton, 1988). Attempts were made to control the freezing rate by placing straws at different heights above liquid nitrogen (Battista *et al*., 1988), on dry ice (Yubi *et al*., 1987) or placing them in forced vapour freezers (Olar *et al*., 1989).

Ponglowhapan *et al*. (2006) studied the freezing ability of canine epididymal spermatozoa after cool storage at 5˚C for 2 or 4 days and reported that storing the dog epididymal sperm at 5˚C for upto 4 days before freezing did not have any detrimental effect on post thaw motility and acrosome integrity, but storage might have decreased post thaw motility.

2.16.1 Freezing Rate

Andersen (1972 and 1976) and Christiansen (1984) reported that dog spermatozoa could be frozen in liquid nitrogen vapour.

Olar (1984) compared three rates for freezing dog spermatozoa and found that a moderate rate (5ºC min^{-1} from 5 to –15ºC followed by 20ºC min^{-1} from –15 to -100ºC) was superior to either a fast rate (75ºC min^{-1}) or a slow rate (2ºC min^{-1} from 5 to - 15ºC, followed by 10ºC min^{-1} from -15 to -100ºC)

Smith (1984) used different extenders and found greatest post thaw motility when straws were frozen 8 inches above the surface of liquid nitrogen compared with 1.5 or 4 inches

Smith and Graham (1984) reported that a very slow freezing rate (1.89ºC / min from 3 to -100ºC) yielded best post thaw motility

Rota *et al.* (1998) compared slow and fast freezing rates using a programmable freezer. In slow rate of 3ºC min^{-1} from 4 to -6ºC, a stop for 1 min at -6ºC and 10ºC min^{-1} from -6 to -40ºC and 50ºC min^{-1} from -40 to -140ºC. In fast freezing rate 3ºC min^{-1} from -4 to -6ºC, a stop for 1 min at -6ºC and 15ºC min^{-1} in the range of -6 to - 40ºC and 50ºC min^{-1} from -40 to -140ºC and concluded that there was no significant difference between two methods.

Kurien (2000) reported that fast freezing rate using programmable freezer (3ºC min^{-1} from -5 to -15ºC, 10ºC min^{-1} from -15 to -40ºC and 50ºC min^{-1} from -40 to -100ºC) was better than slow freezing rate on post thaw motility, live sperm and hypo osmotic swelling response.

Uma (2005) studied the freezing of dog semen using both programmable freezer and conventional freezing method with different cooling rates and concluded that the post thaw motility, live spermatozoa, morphologically normal spermatozoa, acrosomal and plasma membrane integrity was higher in slow cooling rate of both programmable and conventional method of freezing than with medium and fast cooling rates of programmable freezing.

2.17 THAWING RATE

Dog semen had been successfully thawed in a 75ºC water bath for 6.5 sec (Andersen, 1975) and in a 37-40ºC water bath for 1 min (Morton, 1988).

Davies (1982) found a higher motility score when samples were thawed at 70ºC than at 37ºC, although there were no differences in the percentage of motile spermatozoa. Similarly, Olar (1984) demonstrated higher post thaw motility when samples were thawed at 75ºC than at 35ºC.

Yubi (1984) compared rapid (75˚C for 6 sec) thawing with slow (37˚C for 2 min) thawing and concluded that rapid thawing method was best to thaw the dog semen. Fontbonne and Badinand (1993) thawed semen straws at 37ºC for 45 sec and achieved 73 per cent conception rate upon transcervical insemination.

Nair (1996) found that thawing the semen straws at 37˚C to 40˚C for one min was the best for dog semen. Rota *et al.* (1998) and Thomassen *et al*. (2006) found that a faster thawing rate (8 sec at 70ºC) resulted in increased sperm longevity and a greater number of intact acrosomes.

2.18 ADDITION OF PROSTATIC FLUID

Practically, intravaginal insemination of bitches was preferable to intrauterine insemination because it was often cheaper and easier to perform. Intrauterine insemination required very skilled inseminators and, for some methods, expensive equipment such as endoscopes or invasive techniques such as surgery was required. These invasive techniques may also raise welfare issues. The use of invasive techniques also meant that, for humane and practical reasons, only one or two inseminations could be carried out on any one bitch during a single estrus period. This makes accurate timing

essential, which may be costly as numerous progesterone assays were required. Little skill was required to inseminate a bitch intravaginally and the procedure was to be repeated daily without compromising the bitch. Achieving an acceptable fertility rate through intravaginal insemination using frozen semen would make artificial insemination techniques in the bitch very simple, easily adoptable and widely practiced, The addition of prostatic fluid to the frozen semen had proved to be helpful to increased the fertility rate when intravaginal insemination was performed (Nothling and Volkmann, 1993).

2.18.1 Effect of Addition of Prostatic Fluid on Fertility of Frozen Thawed Semen

The addition of prostatic fluid to frozen thawed dog semen resulted in a significantly higher fertility after intravaginal insemination when compared to frozen thawed semen to which no fluid was added after thawing (Nothling and Volkmann, 1993). Nothling and Volkmann (1993) found that the beneficial effect of prostatic fluid upon intravaginal insemination of frozen semen could not be achieved by replacing the prostatic fluid with albumin free TALP and suggested that prostatic fluid promoted the fertility of vaginally inseminated bitches.

Nizanski (2006) reported that the pregnancy rate, whelping rate and litter size were reduced when frozen thawed, prostatic fluid supplemented semen was vaginally deposited.

Nothling *et al*. (2007) studied the effects of homologous prostatic fluid added prior to cooling, after thawing or at both times to epididymal spermatozoa and frozen with Biladyl with Equex STM paste (or) Andromed

and concluded that Biladyl with Equex STM paste was more suitable for freezing of epididymal spermatozoa than Andromed and that prostatic fluid improved the freezability and post thaw longevity of epididymal spermatozoa frozen in Biladyl with Equex STM paste.

CHAPTER III

MATERIALS AND METHODS

3.1 SOURCE AND SELECTION OF ANIMALS

Sixty healthy intact cycling bitches of different breeds between 2 to 6 years of age brought to the Small Animal Gynaecology and Obstetrics unit of Madras Veterinary College Teaching Hospital for breeding advice were utilized for this study. Particulars such as date of spotting, number of previous estrous cycles, number of previous crossing and whelping were recorded. The stage of the reproductive cycle of the bitch was determined based on the clinical signs, vaginal exfoliative cytology and vaginoscopy. Two stud dogs maintained at the Department of Animal Reproduction, Gynaecology and Obstetrics, Madras Veterinary College, Chennai and five male dogs of different breeds maintained by the pet owners were used as semen donors.

3.1.1 REPRODUCTIVE HISTORY OF MALE DOGS USED FOR SEMEN COLLECTION

The age and reproductive history of male dogs used for semen collection are presented in table 1. Seven sexually mature adult male dogs of different breeds, aged between 2 to 4.5 years were used for the study. Reproductive history revealed that 5 out of 7 dogs had sired a litters before and the remaining two dogs had not been used for breeding.

3.1.2 REPRODUCTIVE HISTORY OF BITCHES USED FOR ARTIFICIAL INSEMINATION

Reproductive history of bitches such as breed, age, number of previous estrous cycles, number of cycles mated and whether or not the bitches

previously had a litter are presented in table 2 and 3. Bitches subjected to intravaginal (30) and intrauterine insemination (30) using fresh semen were within the age group of 1.0 to 5.5 years. Sixteen out of 30 bitches included for fresh semen artificial insemination had failed to conceive previously following mating with proven males on different occasions including two bitches which were inseminated in the first estrous cycle. However, none of these bitches were mated after assessing the fertile breeding time and most commonly breedings were done on 9th, 11th and 13th day since proestrual bleeding. Due to varying length of proestrus and estrus,breeding on predetermined dates could have led to conception failure in these bitches which were included in this study. Similarly, out of 30 bitches included for frozen semen artificial insemination 20 bitches had not littered before.

Table 1

Age and reproductive history of male dogs used for semen collection

S.No.	Breed	Age (yrs)	Reproductive history
			Proven/not proven/not mated previously
1	Rajapalayam	3.5	Not mated previously
2	Doberman	2.5	Proven
3	German shepherd	3	Proven
4	Boxer	4	Proven
5	Spitz	2	Not mated previously
6	Labrador	4.5	Proven
7	Lasa aphso	2.5	Proven

Table 2

Breeding details of the bitches included for fresh semen artificial insemination

S.No.	Breed	Age (Years)	Previous estrous cycles		Number of previous whelpings
			Number of cycles	Number of cycles mated	
1	Rajapalayam	2	3	Nil	Nil
2	Doberman	2.5	2	1	Nil
3	German shepherd	1.5	1	Nil	Nil
4	German shepherd	1.5	1	1	1
5	Doberman	2	4	2	2
6	Boxer	3	2	1	1
7	Doberman	3.5	3	2	1
8	German shepherd	2	4	2	1
9	German shepherd	1	1	Nil	Nil
10	Boxer	3	3	1	Nil
11	German shepherd	2.5	2	1	Nil
12	German shepherd	3	3	1	1
13	Spitz	1.8	2	1	1
14	German shepherd	3	3	1	1
15	German shepherd	3.5	4	4	2
16	Doberman	5.5	4	4	2
17	German shepherd	2	1	1	1
18	Doberman	3	3	1	Nil
19	Lasa apsho	1.5	1	Nil	Nil
20	Doberman	2	1	1	Nil
21	Doberman	1.5	2	Nil	Nil
22	Doberman	4	4	2	2
23	Doberman	5	7	6	3 (Not conceived since last 3 matings)

24	Doberman	3	2	2	Nil
25	Doberman	2.5	2	1	Nil
26	Doberman	1	-	-	First cycle
27	German shepherd	1.5	-	-	First cycle
28	Dalmation	5	8	4	4
29	German shepherd	2	2	1	Nil
30	Doberman	2	2	Nil	Nil

Table 3

Breeding details of the bitches included for frozen semen artificial insemination

S.No.	Breed	Age (Years)	Previous estrous cycles		Number of previous whelpings
			Number of cycles	Number of cycles mated	
1	Doberman	2.5	2	1	1
2	German shepherd	2	1	1	Nil
3	German shepherd	4	3	1	Nil
4	Doberman	2	2	1	1
5	Spitz	2	1	Nil	Nil
6	German shepherd	2	2	Nil	Nil
7	German shepherd	2	2	2	Nil
8	German shepherd	4	3	2	2
9	German shephered	5	4	3	1
10	German shepherd	1.5	2	Nil	Nil
11	Doberman	1.5	1	Nil	Nil
12	Labrador	4	3	1	Nil
13	German shepherd	2.5	4	Nil	Nil
14	German shepherd	4	3	2	Nil
15	German shepherd	4.5	4	2	1
16	German shepherd	1.5	1	Nil	Nil
17	German	1.5	2	1	1

	shepherd				
18	German shepherd	3	3	Nil	Nil
19	German shepherd	2.5	2	2	Nil
20	German shepherd	4	4	2	Nil
21	Doberman	2	1	1	Nil
22	German shepherd	2	2	Nil	Nil
23	Boxer	3.5	3	2	Nil
24	Doberman	3	4	2	1
25	German shepherd	2	1	Nil	Nil
26	Spitz	3	3	1	1
27	Boxer	1.5	1	Nil	Nil
28	German shepherd	2	2	1	1
29	Doberman	1.8	2	1	Nil
30	Doberman	5	6	4	2

3.2 EXPERIMENTAL DESIGN

The study consisted of two experiments, I and II

3.2.1 Experiment I

In experiment I the effect of Tes-tris, a commercial extender and Tris on freezing and post thaw functional and morphological characters of spermatozoa was studied.

Under this experiment semen was collected from the stud dogs and was subjected to freezing using both Tes-tris and Tris extenders. The semen frozen with the diluent that had the best functional and morphological post thaw characters was used for carrying out artificial insemination in Group II, Group IV and Group V bitches of experiment II.

3.2.2 Experiment II

Experiment II involved the determination of optimum insemination time using vaginoscopy / vaginal exfoliative cytology and intravaginal /intrauterine artificial insemination using fresh and frozen semen.

Sixty bitches of different breeds brought to the Small Animal Gynaecology and Obstetrics unit of Madras Veterinary College Teaching Hospital were randomly divided into six groups, each consisting of 10 bitches. In Group I to Group V bitches both vaginal exfoliative cytology studies and vaginoscopy were performed. However, the timing of insemination was based on vaginoscopy alone. In Group VI bitches the timing of insemination was determined based on vaginal exfoliative cytology only.

Group I (n=10): Intravaginal artificial insemination using fresh semen

Group II (n=10): Intravaginal artificial insemination using frozen semen

Group III (n=10): Intrauterine artificial insemination using fresh semen

Group IV (n=10): Intrauterine artificial insemination using frozen semen

Group V (n=10): Intravaginal artificial insemination using frozen semen mixed with prostatic fluid

Group VI (n=10): Intravaginal artificial insemination using fresh semen based on vaginal exfoliative cytology alone.

3.3 DETECTION OF OPTIMUM INSEMINATION TIME

3.3.1 Vaginoscopy

Group I to Group V bitches were subjected to vaginoscopy at 2 days interval starting from day 5 of proestrus till day 1 of diestrus. Vaginoscopy was performed using rigid vaginoscope (Karl-Storz, GmbH, Tiuttlingen, Germany). The sterilized vaginoscope (Plate 1) was introduced into the vagina in a craniodorsal direction after everting the vulval lips, as far cranially as possible. The changes in the mucosal fold contours, colour of mucosa and fluid present, were assessed vaginoscopically (Lindsay, 1983).

VAGINOSCOPIC MUCOSAL SCORE SYSTEM (Lindsay, 1990**)**

Period	Symbol	Score and range
Edematous	O	O – Edematous
Shrinkage without angulations of mucosal surface	S	S1 – Onset of shrinkage S2 – Obvious shrinkage S3 – Maximum shrinkage
Shrinkage with angulations of mucosal surface	S	S3 – Onset of angulation S4 – Obvious angulation S5 – Maximum angulation
Declining shrinkage and rounding out of mucosal profiles	D	D5 – Onset, extreme angulation with thinning of mucosa D3 – Initial rounding out D1 – Obvious rounding out
Diestrus onset	D	D1 – Obvious rounding out

3.3.1.1 Proestrus

In vaginoscopy, onset of proestrus was identified by obvious edematous enlargement of the vaginal folds (Plate 2). The folds of the mucus membrane appeared soft, rounded and even in outline. About mid proestrus the folds showed little shrinkage and towards the end of proestrus, more shrinkage without angulation (Plate 3) was observed.

3.3.1.2 Estrus

The presence of sharp, angulated mucosal folds (Plate 4) with crenulation and scanty clear straw coloured fluid indicated estrus.

3.3.1.3 Diestrus

The mucus membrane showed wide spread patchy or banded hyperemic appearance with rounding out of profiles.

3.3.1.4 Insemination time

Based on vaginoscopy inseminations were performed either intravaginally or intrauterine on alternate days throughout the period of mucosal shrinkage with angulation which corresponded to fertile period.

3.3.2 Vaginal Exfoliative Cytology

Vaginal smears were obtained from all sixty bitches at alternate days starting from day 5 of proestrual bleeding until day 1 of diestrus by cotton swab technique as described by Feldman and Nelson (1996). The swab was then rolled gently from one end of the microscopic glass slide to the other. The slides were air dried, stained with Leishman's stain (Hi Media®) and viewed under oil immersion. Total of 100 cells in different fields were counted and the stage of cycle was interpreted according to the type and percentage of cells present.

The cells were classified based on the classification of Feldman and Nelson (1996). Cell pattern during proestrus, estrus and diestrus were as follows

3.3.2.1 Proestrus

Early proestrus was determined by the presence of parabasal, intermediate, red blood cells (RBC) and neutrophils. The late proestrus was marked by the appearance of plenty of superficial cells along with few intermediate, cornified cells and sometimes red blood cells (Plate 5).

3.3.2.2 Estrus

Estrus was determined by the presence of more than 80 per cent of superficial or cornified cells (Plate 6).

3.3.2.3 Diestrus

Sudden appearance of numerous large intermediate, few parabasal and small intermediate cells with reappearance of neutrophils characterized the day 1 of diestrus (Plate 7).

3.3.2.4 Insemination time

Based on Vaginal Exfoliative Cytology studies the day of estrus and day 1 of diestrus were determined for bitches of all six groups. In Group V bitches inseminations were started when smears showed more than 80 per cent of superficial plus cornified cells and were continued to be performed on alternate days until day 1 of diestrus.

3.4 SEMEN COLLECTION

Semen was collected by digital manipulation (Plate 8) without the presence of teaser bitch as described by Kutzler (2005). A total of 22 collections were made in each German Shepherd and Doberman dogs and each one collection was done in Labrador, Rajapalayam, Spitz, Lasa aphso and Boxer breeds of dogs. The pre sperm, sperm rich and post sperm fractions were collected separately in clear, graduated sterile semen collection cups (Plate 9). For insemination with fresh semen, along with first and second fraction enough prostatic fluid was collected to bring the total volume between 3 to 5 ml so that large number of sperms was not lost in the insemination process.

3.5 SEMEN EVALUATION

After collection, semen samples were immediately examined for their fitness for cryopreservation and intravaginal artificial insemination based on the sperm motility, sperm concentration and percentage of sperm abnormality.

3.5.1 Volume

The volume of sperm rich ejaculation was assessed directly from the graduated semen collection cup.

3.5.2 Colour and Consistency

The colour was assessed from the collection cup and classified as white to milky white. The consistency was assessed by holding the semen collection cup against a good source of light and graded as thin to medium.

3.5.3 Motility

A drop of semen was kept on a pre warmed glass slide and covered with a cover slip and examined under phase contrast microscope (20X) to assess the percentage of progressive motile spermatozoa. Semen samples containing a minimum of 70 per cent motility were taken for cryopreservation.

3.5.4 Spermatozoa Concentration

The concentration of spermatozoa in the sperm-rich fraction was assessed by Neubauer haemocytometer.

3.5.5 Live Spermatozoa

Total live and dead spermatozoa were assessed by Nigrosin-Eosin staining technique (Christiansen, 1984).

3.5.6 Abnormal Spermatozoa

The percentage of spermatozoa with abnormal morphology was evaluated using 3 per cent Rose Bengal stained smear and examined under phase contrast microscope (100X) (Plate 10).

3.5.7 Acrosomal Integrity

Acrosomal integrity of spermatozoa was assessed by preparing semen smears and staining them with spermac stain (Fertipro, Belgium) as per the manufactures protocol, with slight modification.

3.5.8 Hypo Osmotic Swelling Test

The hypo osmotic swelling test to assess the plasma membrane integrity of spermatozoa (Plate 11) was performed by the method described by Jeyendran *et al*. (1984).

3.6 CRYOPRESERVATION OF SEMEN

3.6.1 Diluents used for Freezing

In this study, Tris-fructose-citric acid extender and, a commercial extender (Tes-tris) (Plate 12) were used to dilute the semen samples for freezing.

Composition of Tris extender (Yubi *et al*., 1987).

Contents	**Quantity**
Tris	2.9 g
Fructose	1.25 g
Citric acid	1.32 g
Distilled water	100 ml
Penicillin	1,00,000 IU
Streptomycin	100 mg
Egg yolk	20%
Glycerol	9% w/v

Composition of Tes-tris extender (Silva and Verstegen, 1995).

Contents	**Quantity**
Tes	4.4 g
Tris	2.3 g
Distilled water	100 ml
Egg yolk	20 ml
Glycerol	8 ml

3.6.2 Dilution of Semen

The sperm rich fraction of semen was diluted with the extenders in the ratio of 1:2 or 1:3 depending upon the concentration of sperms in the ejaculate so as to ensure an average sperm concentration of 200 million sperm/ml of diluted semen. Collection cups containing the diluted semen samples were placed in a flask containing ice.

3.6.3 Semen Packaging

The semen samples kept in a flask were immediately transferred into cold handling chamber at 5°C. French mini straws (0.25 ml) of different colours were used to load the semen samples. After loading, the straws were sealed with poly vinyl alcohol powder and immersed in a water bath at 5°C to enable proper sealing and equilibration.

3.6.4 Equilibration Period

Semen samples were equilibrated at 5°C for 2 h.

3.6.5 Racking

After equilibration, the straws were dried with pre-cooled towel. Then the straws were arranged on racking platform inside the cold handling cabinet at 5°C.

3.6.6 Freezing of Semen Straws

The semen straws were frozen with conventional freezing method at slow cooling rate using a styrofoam box containing liquid nitrogen to a level of 5 to 6 cm and the straws were arranged on a freezing rack at 7 to 8 cm above the liquid nitrogen for 30 min.

3.6.7 Storage of Semen Straws at -196°C

After freezing, the straws were immediately transferred into goblets filled with liquid nitrogen and stored at -196°C in liquid nitrogen container, until further use.

3.7 POST THAW EVALUATION

After 24 h of storage the frozen semen straws were thawed at 37°C for one minute to assess the post thaw characteristics.

Post thaw evaluation of semen for motility, live spermatozoa, abnormal spermatozoa, acrosomal integrity and plasma membrane integrity was done as per the procedure followed for fresh semen samples.

3.8 PREPARATION OF PROSTATIC FLUID

The post sperm fractions of the ejaculate were collected in semen collection cups and transferred into centrifuge tubes and centrifuged at 1000 rpm for 10 min and the supernantant was transferred into 2 ml serum vials. The vials were labeled with details such as breed and date of collection and stored in freezer until use.

3.9 ARTIFICIAL INSEMINATION

Fresh semen inseminations were performed by collecting the semen from male dogs, evaluated and used as such.

Based on cryopreservation studies it was found that semen frozen with Tris extender showed better post thaw functional and morphological characteristics and hence, all frozen semen inseminations in the present study were carried out using semen cryopreserved with Tris extender.

3.9.1 INSEMINATION TECHNIQUE

3.9.1.1 Intravaginal Insemination Technique

The bitch in estrus was restrained standing on a table or the floor in such a way that no upward pressure was placed on her caudal abdomen. The semen was drawn into a sterile 5 cc syringe. A sterile sheath (used for artificial insemination in bovines) was introduced at the dorsal commissure of the vulva in a dorso-cranial direction and then horizontally as far as possible. The sheath was then cut to the level of the vulva and was fitted with syringe containing semen. The semen sample was injected into the anterior vagina followed by a bolus of air to clear all semen inside the sheath. A gloved finger was used to gently stimulate (feathering) the vagina for several minutes, which induced contractions of vaginal musculature. The gloved finger was withdrawn and hind quarter of bitch was elevated for 5 to 10 min to promote pooling of the semen at the external cervical os (Plate 13).

3.9.1.2 Intrauterine Insemination Technique

All intrauterine inseminations in the present study were performed using the endoscopic transcervical technique. The equipment used for transcervical insemination technique consisted of rigid cystourethroscope with a 30° viewing angle, a protective sheath that contained a channel for the catheter, ports for infusion or suction, a bridge containing an additional port for passage of the insemination catheter, a cold light source, camera and monitor. An infant feeding tube (size 8) was inserted into the channel within the sheath. The bitch was made to stand on a hydraulic table. The cystourethroscope was inserted into the vulva and vestibule and was directed dorsally to avoid the urethral papilla and clitoral fossa, then it was directed over the pelvic brim into the vagina. The vaginal mucosal folds were viewed through a monitor and the cystourethroscope was directed cranially within the

vaginal lumen until the dorsal median postcervical fold was visualized. The dorsal median postcervical fold was recognized as a semicylindrical fold oriented longitudinally in the dorsal cranial vagina. The dorsal median post cervical fold was followed until the cervix was visualized as a small, reddish rosette or cauliflower like structure (Plate 14) on a tubercle. Once the cervix was visualized, an infant feeding tube was passed cranially into the vaginal cervical os (Plate15) and gently rotated through the cervical canal. Lowering the operator end slightly helped to position the cystouretroscope so that it paralleled the direction of the cervical canal thereby facilitating passage of the catheter into the uterus. In certain situations when the passage of the catheter into the uterus was found difficult, the angle of the catheter was slightly adjusted by either lowering the catheter or by adjusting the angle of the scope. Once the catheter was inserted through the cervical os, 1 ml of semen was injected through the catheter (Plate 16).

3.10 INSEMINATION DOSE

A minimum of 150 to 200 x 10^6 spermatozoa was used for insemination of fresh or frozen semen.

3.11 CONCEPTION RATE

On day 30 following last insemination, pregnancy was confirmed by using ultrasonography (Plate17) to determine conception rate.

3.12 WHELPING RATE AND LITTER SIZE

The whelping rate and litter size were estimated following whelping.

3.13 STATISTICAL ANALYSIS

Statistical analysis was performed as per the standard technique described by Snedecor and Cochran (1989).

3.4 SEMEN COLLECTION

Semen was collected by digital manipulation (Plate 8) without the presence of teaser bitch as described by Kutzler (2005). A total of 22 collections were made in each German shepherd and Doberman dogs and each one collection was done in Labrador, Rajapalayam, Spitz, Lasa aphso and Boxer breeds of dogs. The pre sperm, sperm rich and post sperm fractions were collected separately in clear, graduated sterile semen collection cups (Plate 9). For insemination with fresh semen, along with first and second fraction enough prostatic fluid was collected to bring the total volume between 3 to 5 ml so that large number of sperms was not lost in the insemination process.

3.5 SEMEN EVALUATION

After collection, semen samples were immediately examined for their fitness for cryopreservation and intravaginal artificial insemination based on the sperm motility, sperm concentration and percentage of sperm abnormality.

3.5.1 Volume

The volume of sperm rich ejaculation was assessed directly from the graduated semen collection cup.

3.5.2 Colour and Consistency

The colour was assessed from the collection cup and classified as white to milky white. The consistency was assessed by holding the semen collection cup against a good source of light and graded as thin to medium.

3.5.3 Motility

A drop of semen was kept on a pre warmed glass slide and covered with a cover slip and examined under phase contrast microscope (20X) to assess the percentage of progressive motile spermatozoa. Semen samples containing a minimum of 70 per cent motility were taken for cryopreservation.

dorsal median postcervical fold was recognized as a semicylindrical fold oriented longitudinally in the dorsal cranial vagina. The dorsal median post cervical fold was followed until the cervix was visualized as a small, reddish rosette or cauliflower like structure (Plate 14) on a tubercle. Once the cervix was visualized, an infant feeding tube was passed cranially into the vaginal cervical os (Plate15) and gently rotated through the cervical canal. Lowering the operator end slightly helped to position the cystouretroscope so that it paralleled the direction of the cervical canal thereby facilitating passage of the catheter into the uterus. In certain situations when the passage of the catheter into the uterus was found difficult, the angle of the catheter was slightly adjusted by either lowering the catheter or by adjusting the angle of the scope. Once the catheter was inserted through the cervical os, 1 ml of semen was injected through the catheter (Plate 16).

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On day 30 following last insemination, pregnancy was confirmed by using ultrasonography (Plate17) to determine conception rate.

3.12 WHELPING RATE , LITTER SIZE AND GESTATION LENGTH

The whelping rate, litter size and gestation length were estimated following whelping.

3.13 STATISTICAL ANALYSIS

Statistical analysis was performed as per the standard technique described by Snedecor and Cochran (1989).

CHAPTER IV

RESULTS

4.1. SEMEN COLLECTION BY DIGITAL MANIPULATION

The semen was collected by digital manipulation without using a teaser bitch and was used for artificial insemination either as fresh or frozen semen.Good quality semen was obtained in most occassions from almost all the dogs by digital manipulation without using teaser bitch. If the quality of semen was not acceptable, the samples were discarded. Depending on the length of the estrus period minimum of one to a maximum of four collections were made on alternate days. It was found that alternate day semen collection did not affect the libido of the male. However, as the frequency of semen collection increased the sperm concentration declined.

4.2 PHYSICAL AND MORPHOLOGICAL CHARACTERISTICS OF FRESH DOG SEMEN

The physical and morphological characteristics of semen from 7 male dogs of different breeds were examined for volume, colour, consistency, percentage of gross motility, spermatozoal concentration, percentage of live and abnormal sperms, percentage of acrosomal integrity and plasma membrane integrity are presented in table 4.

4.2.1. Volume

The mean volume of second fraction of semen was 1.30 ±.08 ml with a range of 0.6 -2.5 ml

4.2.2. Colour

The first and third fraction of semen were clear and watery while the second fraction or the sperm rich fraction varied from white to milky white in colour.

4.2.3. Consistency

The consistency of semen varied from thin to medium.

4.2.4. Initial Motility

The gross motility of fresh semen ranged from 60 to 90 percentage with an overall mean value of 76.79 ± 1.78 per cent.

4.2.5. Spermatozoal Concentration

The mean sperm concentration was 434 ± 19.00 millions/ml of semen. It ranged from 180-540 millions/ml of semen.

4.2.6. Percentage of Live Sperms

The mean percentage of live spermatozoa was 79.71 ± 1.12 with a range of 68 to 93 per cent.

4.2.7 Percentage of Abnormal Sperms

The mean percentage of abnormal spermatozoa was 19.39 ± 0.68 and it ranged from 14 to 26 per cent.

4.2.8 Percentage of Acrosomal Integrity

The mean percentage of acrosomal integrity was 71.46 ± 0.98 and ranged from 64 to 80 per cent.

4.2.9. Percentage of Plasma Membrane Integrity

The mean percentage of plasma membrane integrity was 76.14 ± 1.18 and ranged from 64 to 88 per cent.

Table 4

Characteristics of fresh dog semen

	Volume (ml) (n=49)	**Colour and consistency** (n=49)	**Motility (%)** (n=49)	**Sperm concentration (10^6/ml)** (n=49)	**Live spermatozoa (%)** (n=49)	**Abnormal spermatozoa (%)** (n=49)	**Acrosomal Integrity (%)** (n=49)	**Plasma Membrane integrity (%)**

Mean	1.30 ± 0.08	White to milky white and thin to medium	76.79 ± 1.78	434 ±19.0	79.71±1.12	19.39 ± 0.68	71.46 ± 0.98	76.14 ± 1.18
Range	0.6-2.5		60-90	180-540	68-93	14-26	64-80	64-88

4.3 CRYOPRESERVATION OF SEMEN

The semen was cryopreserved with two extenders using Tris and Tes-tris.

The functional and morphological characteristic of cryopreserved semen are presented in the table 5 and figure 1

4.3.1 Percentage of Post Thaw Motility

The mean post thaw motility of semen cryoperserved with Tris and Tes-tris extenders was 42.14 ± 0.81 and 32.86 ± 2.21 per cent and ranged from 30 to 60 and 20 to 50 per cent, respectively. The post thaw motility was significantly higher (P<0.01) for Tris when compared to Tes-tris extender.

4.3.2. Percentage of Live Spermatozoa

The mean percentage of live spermatozoa in the semen cryopreserved with Tris and Tes-tris diluents was 59.0 ± 1.70 and 49.71 ± 1.62 and ranged from 52 to 70 and 42 to 60 percentage, respectively. The percentage of live spermatozoa was significantly higher (P<0.01) for Tris than Tes-tris extender.

4.3.3. Percentage of Abnormal Spermatozoa

The percentage of abnormal spermatozoa ranged from 24 to 34 and 33 to 44 per cent in semen cryopreserved with Tris and Tes-tris extenders respectively. The mean percentage of abnormality was 29.14 ± 1.10 and 36.50 ± 1.17 per cent respectively. The percentage of abnormal spermatozoa was significantly higher (P<0.01) when semen was cryopreserved with Tes-tris than Tris.

4.3.4. Percentage of Acrosomal Integrity

The percentage of acrosomal integrity ranged from 40 to 56 and 40 to 56 per cent in semen cryopreserved with Tris and Tes-tris extenders, respectively. The mean percentage of acrosomal integrity was 49.42 ± 1.15 and 46.14 ± 1.60 per cent respectively. There was no significant difference between the two extenders on the percentage of acrosomal integrity.

4.3.5. Percentage of Plasma Membrane Integrity

The percentage of plasma membrane integrity of spermatozoa ranged from 49 to 61 and 42 to 54 per cent in semen cryopreserved with Tris and Tes-tris extenders, respectively. The mean percentage of plasma membrane integrity was 53.79 ± 0.96 and 49.00 ± 0.98 per cent, respectively. The percentage of plasma membrane integrity was higher for semen cryopreserved with Tris than Tes-tris extender.

Table 5

Effect of semen diluents on characteristics of cryopreserved dog semen

Semen extender	Post thaw motility (%) (n=14)	Live spermatozoa (%) (n=14)	Abnormal spermatozoa (%) (n=14)	Acrosomal integrity (%) (n=14)	Plasma membrane integrity(%) (n=14)
Tris	42.14 ± 0.81[a] (30 to 60)	59.00 ± 1.70[a] (52 to 70)	29.14 ± 1.10[a] (24 to 34)	49.42 ± 1.15[a] (40 to 56)	53.79 ± 0.96[a] (49 to 61)
Tes-tris	32.86 ± 2.21[b] (20 to 50)	49.71 ± 1.62[b] (42 to 60)	36.50 ± 1.17[b] (33 to 44)	46.14 ± 1.60[a] (40 to 56)	49.00 ± 0.98[b] (42 to 54)

Figures in parenthesis indicates range

Different superscript between rows differ significantly (P<0.01)

4.4 DETECTION OF OPTIMUM INSEMINATION TIME

4.4.1 Vaginoscopy

Mean length (days) of vaginoscopic mucosal related changes in groups I toV are presented in the table 6

Throughout the period of proestrus, vaginal mucosal folds appeared rounded and edematous. The mucosal profiles appeared round and the colour of the mucosa varied from pink to cream white. The serosanguinous discharge appeared as reddish fluid in between the furrows of mucosal folds.

Late proestrus or early estrus was characterized by the onset of shrinkage without angulation in the vaginal mucosa which progressed to shrinkage with angulation period which corresponded to the fertile period in bitches.

The mean length of proliferative edematous period was 7.80 ± 0.62 days with a range of 6 to 10.

The mean non-angulated shrinkage period was 5.12 ± 0.37 days with a range of 4 to 7 days.

The mean angulated shrinkage period (peak fertile period) was 4.40 ± 0.81 with a range of 3 to 6.

4.4.2 Vaginal cytology

Details on the day of onset of cytological estrus and day of onset of cytological diestrus for all the sixty bitches included in the study are presented in the table 7.

Table 6

Mean length (days) of vaginoscopic mucosal related changes in group I to V bitches

	Proliferative edematous period	**Non-angulated shrinkage period**	**Angulated shrinkage period**
Mean ± S.E	7.80 ± 0.62	5.12 ± 0.37	4.40 ± 0.81
Range	6 to 10	4 to 7	3 to 6

Table 7

Assessment of onset of estrus and diestrus based on vaginal cytology bitches of group I to VI bitches

	Day of onset of cytological estrus (days)	**Day 1 of diestrus (days)**
Mean ± S.E	9.11 ± 3.14	15.58 ± 3.73
Range	4 to 15	6 to 24

Table 8

Mean (±SE) percentage of superficial keratinized cells in vaginal smears of bitches in relation to day 1 of diestrus

Days of estrous cycle									
Proestrus				**Estrus**					**Diestrus**
-18	**-16**	**-14**	**-12**	**-10**	**-8**	**-6**	**-4**	**-2**	**D1**
8.40 ± 0.32	12.36 ± 0.86	26.45 ± 0.85	48.34 ± 1.03	82.05 ± 1.50	88.19 ± 0.83	92.23 ± 2.40	94.52 ± 3.40	94.63 ± 1.80	37.49 ± 2.20

The mean day of onset of cytological estrus occurred 9.11 ± 3.14 days from the day one of proestrual bleeding and it ranged from day 4 to 15 in all the bitches included for the study

Day 1 of diestrus occurred 15.58 ± 3.73 days from day 1 of proestrual bleeding and ranged from day 6 to 24.

Pattern of occurrence of vaginal exfoliative cells(mean) in relation to day 1 of diestrus (d1) were presented in figure 2.

The percentage of parabasal cells at the onset of proestrus was 18 ± 2.04 and it decreased to 4.00 ± 0.64 per cent 4 days later. Similarly, the intermediate cells was 42.00 ± 3.46 per cent at the onset of proestrus and started decreasing progressively to 9.00 ±1.40 per cent 2 days prior to the onset of estrus.

Superficial intermediate cells were 4.00 ± 2.40 per cent at the onset of proestrus and after 2 days its level increased to 64.00 ± 3.26 per cent. After that it started decreasing and at the onset of estrus it was 18.00 ± 2.0 per cent. Superficial cells gradually increased from nil percentage to 6.14 ± 0.78, 10.00 ± 1.00, 24.06 ±1.26, 56.1 ± 4.26, per cent on -12 ,-10, -8, -6 and -4 day before day 1 of diestrus.

Similarly, the percentage of cornified cells was nil during the onset to mid proestrus and it started to appear to 6.14 ± 0.78 per cent and 56.10 ± 4.26 per cent 2 days prior to the day 1 of diestrus.

Mean (± S.E) percentage of superficial keratinized cells in vaginal smears of bitches in relation to day 1 of diestrus are presented in the Table 8.

The first day of diestrus was marked by a sudden decrease in superficial and cornified cells to <37.49 per cent and parabasal and intermediate cells increased to 28.00 ± 1.42 and 39.00 ± 4.42 per cent, respectively.

RBC's, neutophils and occasionally metestrum cells were other cell types observed in vaginal exofoliative cytology during estrous cycle. RBC's and neutrophils were large in number during early proestrus reducing gradually during late proestrus and were nil during estrus. Day 1 of diestrus was identified by reappearance of neutrophils. Bacteria were identified in large numbers during proestrus and estrus.

The shift from parabasal to intermediate cells in proestrus to superficial and cornified cells in estrus was gradual. Throughout the period of estrus, smears were predominated by superficial and cornified cells. The day 1 of diestrus was marked by a sudden decrease in superficial and cornified cells and increase in parabasal, intermediate cell types and reappearance of neutrophils.

The significant exception with regard to predominance of cell types in this study was that in four bitches, the percentage of superficial and cornified cells did not increase above 52 per cent even though they were found to be in estrus through vaginoscopy. Similarly, in two bitches two peaks of cornifcation were identified.

Although vaginal exfoliative cytology studies were carried out in all the sixty bitches included in the study, only in Group VI bitches vaginal exfoliative cytology was used to assess the time of insemination. In all other groups, inseminations were based on vaginoscopy.

4.5 NUMBER OF INSEMINATIONS

In Group I to V bitches inseminations were performed on alternate days throughout the fertile period as determined by vaginoscopy until day 1 of diestrus while in Group VI bitches inseminations were performed on alternate days throughout the period of cytological estrus until day 1 of diestrus.

The number of inseminations ranged from 1 to 3, 1 to 3, 1 to 4, 1 to 4, 2 to 3 and 2 to 4 in group I, II, III, IV, V and VI, respectively.

The relation between the number of inseminations and conception rate are presented in the figure 3.The number of inseminations did not correlate positively with percentage of conception.

4.6 CONCEPTION RATE FOLLOWING ARTIFICIAL INSEMINATION

The conception rate, whelping rate and litter size following artificial insemination using fresh, frozen and frozen semen with the addition of prostatic fluid are presented in the table 9 and figure 4.

The conception rate in bitches inseminated intravaginally with fresh and frozen semen (Group I and II) was 50 and 20 per cent, respectively. The

Table 9

Conception rate,whelping rate and litter size in bitches inseminated with fresh and frozen semen

Method of insemination	Groups	Type of semen	Conception rate(per cent)	Whelping rate(per cent)	Litter Size
Intravaginal	Group I	Fresh	50	80	5.50 ±1.30 (2 to 8)
	Group II	Frozen	20	100	2.00 ± 0.00 (2 to 3)
Intrauterine	Group III	Fresh	50	80	6.25 ± 0.10 (6 to 8)
	Group IV	Frozen	30	100	3.00 ± 1.00 (2 to 5)
Intravaginal	Group V	Frozen semen with prostatic fluid	30	66	3.00 ± 0.57 (2 to 4)
	Group VI	Fresh semen – Insemination time based on vaginal cytology alone	40	100	5.25 ±1.70 (3 to 7)

conception rate of 50 and 30 per cent was obtained in bitches(Group III and IV) inseminated transcervically with fresh and frozen semen,respectively.The conception rate in bitches inseminated intravaginally with frozen semen supplemented with prostatic fluid (Group V) was 30 per cent. A 40 per cent conception rate was achieved in bitches inseminated with fresh semen intravaginally without performing vaginoscopy (Group VI).

4.7 WHELPING RATE AND GESTATION LENGTH

A whelping rate of 80, 100, 80,100, 66 and 100 per cent was obtained in group I, II, III,IV and V and VI bitches respectively.

Representative pictures of pups born through artificial insemination are presented in plates 18 - 21.

The percentage of bitches that whelped on 54, 55, 56, 57, 58, 59 and 60 days after day 1 of diestrus were 5.2, 10.5, 21.0, 26.3, 21.0, 10.5 and 5.2, respectively.

The mean length of gestation obtained in the present study was 57 days with a range of 54 to 60 days from the day 1 of diestrus. The range of potential due dates of parturition was 56 to 70 days from the day of first breeding.

Whelping days in relation to day 1 of diestrus are presented in the figure 5.

4.8 LITTER SIZE

The litter size obtained in group I, II, III,IV ,V and VI bitches was 5.50 ± 1.30, 2.00 ± 0.00, 6.25 ± 0.10, 3.00 ± 1.00, 3.00 ± 0.57 and 5.25 ± 1.70, respectively with a range of 2 to 8, 2 to 3, 6 to 8, 2 to 5, 2 to 4 and 3 to 7.

CHAPTER V

DISCUSSION

Artificial insemination of bitches with fresh and frozen semen has had mixed success and is still the subject of controversy. It is generally accepted that the deposition of fresh semen in the uterus or vagina gave results as good as natural mating. Artificial insemination with frozen semen has been less successful and the results have varied greatly from one study to another due to variation in protocols used, route of insemination, quality of semen before freezing and after thawing, volume or concentration, the number and timing of insemination and the method for freezing and thawing the semen.

In this study, the objectives were to evaluate the effects of the technique of insemination on conception rate and litter size using both fresh and frozen semen.

5.1 SEMEN COLLECTION BY DIGITAL MANIPULATION

Digital manipulation technique was adopted to collect semen from male dogs. Good quality semen was obtained in most occassions in almost all the dogs without the presence of teaser bitch. However,in one Spitz male dog repeated attempts were required before an ejaculate was obtained which could be due to unfamiliar location or first experience. One Doberman male dog had a tight penile sheath which made it difficult to push the sheath behind the bulbus glandis before it became fully engorged. In such situations one had to wait for the bulbus glandis to return to normal size before another attempt was made. To alleviate this problem, a slight modification in the digital manipulation technique as described by Feldman and Nelson (1996) and Kutzler (2005) was adopted. In this technique, after cleaning the

prepuce the sheath was pushed behind the bulbus glandis even before erection of penis started and then rhythmic compression was exerted behind the bulbus glandis resulting in full engorgement of bulbus glandis and complete ejaculation. On two other occasions during semen collection in the Doberman male a few drops of bleeding occurred after complete erection which got mixed with semen and hence, was not used for artificial insemination.

A few drops of blood at the end of the semen collection may occur in some normal healthy male dogs due to prolonged sexual anticipation before being allowed to copulate as suggested by the Feldman and Nelson (1996). Since the mixing of blood with semen might affect the motility and viability of sperm it was not used for insemination. It was frequently reported that the presence of blood in the ejaculate lead to infertility since blood was said to be spermicidal (Feldman and Nelson, 1987).

The three fractions could be collected separately in a glass semen collection vials. Wong and Dhaliwal (1985) made similar observation regarding semen collection in a dog with digital manipulation technique.

5.2 FREQUENCY OF SEMEN COLLECTION

Semen was collected on alternate days and was used for fresh semen artificial insemination. A maximum of 4 collections were made on alternate day and it was found that alternate day semen collection had little effect on the sexual libido of the male. However, a reduction in the spermatozoal concentration was noticed when the alternate day semen collection exceeded three. Similar finding was reported by Dobrinski *et al*. (1993) who also advocated alternate day semen collection. Previous studies by Boucher *et al*. (1958) and Taha (1981) and Olar *et al*. (1983) have shown that when dogs

ejaculated every day there was depletion of the extragonadal sperm reserves within few days, which was not observed to occur when dogs ejaculated every second day.

5.3 PHYSICAL AND MORPHOLOGICAL CHARACTERISTICS OF FRESH DOG SEMEN

5.3.1 Volume

The mean volume of the sperm rich fraction of semen observed in the present study was 1.30 ± 0.08 ml with the range of 0.6 to 2.5 ml which was similar to the volume of 1.30 ± 0.02 and 1.40 ± 0.30 ml recorded by the Kurien (2000) and Silva *et al*. (2005) respectively. But Dobrinski *et al*. (1993) recorded a higher mean volume of 2.20 ± 0.20 ml. This could be due to difference in the breed, age, size of the dog and frequency of collection as suggested by Feldman and Nelson (1996).

5.3.2 Colour and Consistency

The colour of the sperm rich fraction of semen in this study varied from white to milky white, while the colour of the first and third fraction of semen was clear and watery. The colour observed was in accordance with Kurien (2000) and Uma (2005). However, Olson (1992), Nair (1996) and Kadirvel (1998) reported a creamy to milky white colour for the second fraction and they attributed it for the higher sperm concentration.

The consistency of second fraction of semen was thin to medium. This observation was in agreement with the report of Nair (1996), Kurien (2000) and Uma (2005), who also observed similar consistency in various breeds of dogs.

5.3.3 Initial Motility

The initial motility of fresh semen in the present study ranged from 60 to 90 per cent with a mean value of 76.79 ± 1.78 per cent. The range and mean values obtained in this study were similar to the values reported by Olson (1992), Rota *et al*. (1995), Uma (2005) and Prinosilova *et al*. (2006). On the contrary, Cardso *et al*. (2003) reported 99.2 per cent initial motility.

5.3.4 Spermatozoal Concentration

The mean spermatozoal concentration in the present study was 434 ± 19.0 millions per ml with the range of 180 to 540. These values were similar to the findings of Uma (2005). But lower values were reported by Kurien (2000) (319 ± 11.79 millions / ml) and Prinosilova *et al*. (2006) (276.3 millions/ml). Higher sperm concentrations of 916 millions/ml were reported by Rota *et al*. (1995).

Comparatively higher values of mean sperm concentration obtained in the present study might be due to the fact that most of the breeds used for semen collection were large breeds such as German shepherd, Doberman, Rajapalayam and Boxer. This was in accordance with the findings of Olar *et al*. (1983) and Rijsselaere *et al*. (2007) who opined that the sperm production was directly related to the testicular size and large breeds might have larger testicular diameter and higher sperm production.

Feldman and Nelson (1996) suggested that the variation in sperm concentration was influenced by the breed, age, sexual activity or frequency of collection and season of the year.

Takeishi et al. (1975) found evidence of a seasonal effect on seminal characteristics with a larger total volume of ejaculate and a greater number of abnormal spermatozoa in summer and the greatest sperm concentration and sperm output in spring. The authors considered that the poor seminal characteristics obtained in the summer months was due to higher environmental temperatures.

5.3.5 Live Spermatozoa

The mean percentage of live spermatozoa in the present study was 79.71±1.12 with a range of 68 to 93 per cent which was similar to the finding of Daiwadnya and Hukeri (1993) and Prinosilova *et al*. (2006) but lower than the values reported by Ferguson *et al*. (1989) (99 per cent) in Cocker Spaniel dogs.

5.3.6 Abnormal Spermatozoa

In this study the mean value of abnormal spermatozoa recorded was 19.39 ± 0.68 and ranged from 14 to 26 per cent. These values were higher than the values reported by Yubi *et al*. (1987), Chatdarong *et al*. (1997) and Cardso *et al*. (2003), who observed 10 to 16 per cent of abnormal sperms.

The major sperm abnormalities observed were coiled tail, bent tail, coiled mid piece and detached head which were similar to the reports of Yubi *et al*. (1987) and Ferguson *et al*. (1989). Causes of abnormal sperm morphology in the dog may be heat stress associated with local inflammatory conditions (Oettle and Soley, 1986) or hyperthermia, infections of reproductive tract (Christiansen, 1984), decreased LH and testosterone secretion (Kawakami *et al*., 1998) or iatrogenic (Freshman, 1989). Pena Martinez (2004) also suggested that healthy dogs transferred to a new environment or to a new owner may show a transient increase in sperm abnormalities probably due to increased endogenous corticoid secretion.

5.3.7 Acrosomal Integrity

The mean percentage of acrosomal integrity recorded in the present study was 71.46 ± 0.98 with the range of 64 to 80 per cent. These values were lower than the earlier reports of Kurien (2000) (92 per cent), Rijsselaere *et al*. (2002) (97 per cent), Ponglowhapan *et al*. (2004) (95.2 per cent) and Nothling and Shuttleworth (2005) (89.5 per cent) in fresh dog semen. This could be due to the difference in staining technique and individual variation in the assessment of acrosomal damage.

5.3.8 Plasma Membrane integrity

In the present study, the plasma membrane integrity was found to be 76.14 ± 1.18 per cent with the range of 64 to 88 which was lower than values reported by Rota *et al*. (1995) (93.60 ± 3.70 per cent) and Kurien (2000) (91.30 ± 0.92 per cent).

5.4 EFFECT OF SEMEN EXTENDERS ON DIFFERENT CHARACTERISTICS OF CRYOPRESERVED DOG SEMEN

The conception rate following artificial insemination using frozen semen was mainly dependent on the functional and morphological characteristics of the cryopreserved semen,which in turn was influenced by the semen extender, optimum freezing protocol and thawing rate.

In the present study, Tris-fructose-citric acid extender with 20 per cent egg yolk and 9 per cent glycerol and Tes-tris extender with 20 per cent egg yolk and 8 per cent glycerol were used as the semen diluent and the semen was frozen following conventional slow freezing method.

Perusal of available literature has shown that although much work has been carried out on cryopreservation of dog semen using Tris extender, only two researchers have worked with Tes-tris as an extender. Hence, effective comparision of results of the present study could not be carried out.

5.4.1 Post Thaw Motility

A wide range of post thaw motility percentage was reported by several authors, ranging from 33 per cent (Pena *et al*., 1998) to 70 per cent (Strom *et al*., 1997) using different types of extenders, varying level of glycerol concentration and different freezing protocols.

The post-thaw motility of 42.14 ± 2.81 obtained in this study using Tris extender was similar to the values reported by Rota *et al*. (1999) (47 per cent), and Prinosilova *et al*. (2006) (41.5 per cent).

However, Dobrinski *et al.* (1993) recorded a low post thaw motility of 30.30 ±1.40 in Tris extender following slow freezing rate. On the contrary, Silva and Verstegen (1995) recorded a higher post thaw motility of 60 per cent in a similar study. The differences in post-thaw motility between the present study and other findings were attributed to the difference in freezing protocols used and breed variation. A large number of other factors viz., initial sperm motility, sperm concentration and individual variation would also be a cause for differences in post thaw motility as reported by Pena and Linde-Forsberg (2000).

The post thaw motility of 32.86 ± 2.21 per cent obtained for Tes-tris extender in the present study was lower than the 65 per cent reported by Silva and Verstegen (1995). The difference in values might be caused by the different freezing and thawing protocol adopted in the above study. Another major contributing factor for the higher values in their study could be due to the use of semen sample with high prefreeze values of more than 95 per cent.

5.4.2 Live Spermatozoa

The mean percentage of live spermatozoa in the semen cryopreserved with Tris diluent was 59.00 ± 1.70 and ranged from 52 to 70 percent. This value was lower than the earlier reports of Kurien (2000).

The mean percentage of live spermatozoa obtained with Tes-tris was 49.71± 1.62. This value was lower than the report of Silva and Verstegen (1995) who reported 80 per cent of live spermatozoa after freezing the semen with Tes-tris extender compared to pre-freeze motility.

5.4.3 Abnormal Spermatozoa

Sperm abnormalities was one of the main critieria used to asses the quality of the frozen semen and its fitness to be used for artificial insemination. The mean percentage of abnormal spermatozoa obtained in this study was 32.14 ±1.10 and 41.71±1.22 in Tris and Tes-tris extenders, respectively.

The percentage of sperm abnormalities obtained in this study with Tris extender was lower than the earlier reports of Cardso *et al*. (2003), Prinosilova *et al*. (2006) and Santos *et al*. (2006)

5.4.4 Post Thaw Acrosomal Integrity

The mean percentage of acrosomal integrity in the present study was 49.42 ± 1.15 and 46.14 ± 1.60 per cent for Tris and Tes-tris extenders respectively. The results concurred with the finding of Uma (2005) who obtained 49.60 ± 7.10 and 46.38 ±1.46 per cent acrosomal integrity respectively but were lower than values reported by Kurien (2000) and Yildiz *et al*. (2000) with tris extender. The lower values obtained in the present study

could be due to highly precise spermac staining technique used as it provided better resolution and accurate assessment of the membrane changes as reported by Hay *et al*. (1997). Pena and Linde-Forsberg (2000) reported that the proportion of acrosomal reaction increased with increasing number of spermatozoa per straw and acrosomal enzymes and toxic products released from dead spermatozoa might have contributed to the destabilization of acrosomal membranes in live spermatozoa.

5.4.5 Post Thaw Plasma Membrane Integrity

The integrity of the sperm membrane was obviously an important parameter in the assessment of sperm viability, since it was essential for general cell function (Jeyendran *et al*., 1984). In the present study, the mean plasma membrane integrity or tail curling percentage was 53.79 ± 0.96 and 49.00 ± 0.98 per cent for the Tris and Tes-tris extenders, respectively.

The percentage of plasma membrane integrity obtained in this study with Tris extender was comparable with the results of Uma (2005) with slow freezing. Cytoplasmic membrane of the spermatozoa would be disrupted by cryopreservation procedure,leading to loss of intracellular solute. This accounted for the ability of spermatozoa to curl because the damaged membrane would be unable to support osmotic swelling (Kumi-Diaka,1993).

Bueno *et al*. (2001) reported that comparative analysis between the Tris- citrate and Tes-Tris extenders showed that Tris-citrate was superior as compared to Tes-Tris based on the results of the hypo osmotic swelling test, thermo resistance test, sperm motility, forward progression and acrosome integrity after cryopreservation with all values higher by 15 per cent for the Tris-citrate.

The results of the present study also showed that semen cryopreserved with Tris extender was superior to the Tes-tris extender in terms of post thaw functional and morphological characteristics, an important attribute for achieving optimum conception rate. This difference may be related to difference in the viscosity of the Tris –tes extender. Hence, in order to exclude this difference, semen that was frozen with Tris was used for artificial insemination in the present study.

5.5 ASSESSMENT OF OPTIMUM TIME OF INSEMINATION

5.5.1 Vaginoscopy

Vaginal mucosal folds were edematous with rounded profiles during proestrus due to fluid retention in the mucosa in response to higher estrogen level in the plasma.

The onset of estrus was marked by withdrawal of fluid retention in the mucosal folds consequent to the decrease in estradiol level. As a result, vaginal mucosal folds collapsed and subtle surface wrinkling (or) crenulations became apparent.

Vaginoscopic mucosal characteristics and changes observed during proestrus and estrus were similar to the observations of Lindsay (1993) Feldman and Nelson (1996) and Jeffcoate (1998).

In the present study, mean length of proliferative edematous period was 7.8 ± 0.62 days from the day of proestrual bleeding with a range of 6 to 10 days. Similarly, Sridevi (2001) also reported mean length of proliferative period was 7.15 ± 0.68 days and opined it corresponded to the early ovulatory phase.

The mean length of non- angulated shrinkage period observed in this study was 5.22 ± 0.37 days with a range of 4 to 7 days. This finding was concurred with the earlier reports of Lindsay (1983), Lindsay (1990) and Sridevi (2001) who correlated this period with the preovulatory phase. .

The mean length of angulated shrinkage period corresponding to vaginal mucosal score S3-S5 recorded in this study was 4.4 ± 0.81 with a range of 3 to 5 days which was similar to the findings of Lindsay (1983) , who reported the length and range of early ovulatory, periovulatory and fertile periods to be 5 to 7, 4 to 6, and 1 to 11, 4 to 7 and 1 to 10 days respectively.

Vaginal mucosal scores S3 to S5 were distinct vaginoscopic features as suggested by Goodman (2001) and therefore were useful indicators of peak fertile period.

High fertilization rates could be achieved from single matings of bitches with VMS S4 or S5, which consistently fell within peak fertilization period (Lindsay, 1983)

The result of the present study concurred with the report of Lindsay (1983) since only single artificial insemination was done in one bitch in group I, II and III each and they conceived.

5.5.2 Vaginal Exfoliative Cytology

The mean percentage of parabasal, intermediate and superficial intermediate cells decreased from 18.00 ± 2.04, 42.00 ± 3.46 and 34.00 ± 2.40 to nil, nil and 18.00 ± 2.00 at the onset of proestrus to onset of estrus, respectively. Superficial intermediate cells continued to gradually decline further and reached its lowest per cent of 4.20 ± 0.12 days before day 1 of diestrus. Superficial and cornified cells were at 42.32 ± 1.03 and 6.14 ± 0.78 percent during late proestrus and reached 72.05 ± 0.86 and 10.00 ± 0.10 per

cent, respectively at the onset of estrus. Day 1 of diestrus was marked by a sudden drop in superficial and cornified cells to 25 per cent and increase in the parabasal and intermediate cells to 28 and 39 per cent respectively and reappearance of neutrophils. The changing cell patterns observed in this study during proestrus, estrus and diestrus were similar to the earlier reports of Holst and Phemister (1975), Thrall and Olson (1993) and Feldman and Nelson (1996). Brukes (1986) reported that when partially or fully cornified superficial cells comprised of 95 per cent in a smear and with concomitant loss of leucocytes and non-superficial epithelial cells, it was an indication that estrogen secretion and follicle maturation had reached a point at which LH surge resulted in ovulation.

In this study, the change from intermediate and parabasal cells during proestrus to superficial cells was more gradual than the sudden decrease in superficial cells as seen at the onset of diestrus as reported by Holst and Phemister (1975), Feldman and Nelson (1996) and Sridevi (2001).

Day of onset of estrus was characterized by the predominance of anuclear cells in some bitches and superficial cells in others. The difference in maximum keratinization between individual bitches was probably due to difference in maximum levels of estradiol as suggested by Sridevi (2001).

The percentage of superficial and cornified cells exceeded 80 per cent on the day of estrus in all bitches included in this study except in four in which the superficial and cornified cells did not exceed 52 per cent. Similar observations were also made by Hewitt and England (2000) who reported that in some bitches the peak values of cornification was only 60 per cent. Similarly, two bitches in the present study had two peaks of cornification which was similar to the observation made by the Vander Holst and Best (1976).

Hewitt and England (2000) reported that causes for inaccuracies in vaginal cytology include the occasional discovery of polymorphonuclear leucocytes during fertile period in some bitches and low peak of cornification in others. Because of these variations that occured between bitches and different cycles within the same bitch, vaginal cytology alone could not be relied on to predict the optimal time for mating or artificial insemination as suggested by Hewitt and England (2000).

In the present study although vaginal smears were obtained and evaluated for the bitches of all six groups, it was not used as the method of timing of insemination except in Group VI bitches as it determined ovulation time only retrospectively. Since, vaginoscopy was considered to be far superior than vaginal cytology in predicting ovulation time and fertile period it was used to time the inseminations. Further, to prove the efficacy of vaginoscopy in predicting fertile breeding time, group VI bitches were inseminated based on vaginal cytology alone.

5.6 CONCEPTION RATE

The conception rate and litter size following artificial insemination was dependent on method of deposition or the use of fresh or frozen semen.

In the present study, the conception rate following intravaginal or transcervical artificial insemination using either fresh or frozen semen or intravaginal insemination of frozen semen with the addition of prostatic fluid was in the range of 20 to 50 per cent.

5.6.1 Intravaginal Insemination

5.6.1.1 Fresh Semen

In the present study, a conception rate of 50 and 40 per cent was achieved after intravaginal deposition of fresh semen.

The conception rate obtained in this study was lower than the earlier reports of Linde Forsberg and Forsberg (1989), Mickelsen *et al*. (1993) and Root and Johnston (2000), who reported conception rates ranging from 62 to 71 per cent. However, when compared to 25 per cent conception rate reported by Farstad (1984), the results obtained in this study was higher. One reason for the comparatively lower conception rate after intravaginal deposition of fresh semen could be due to backflow of semen through the vagina or through the sheath, despite the elevation of the bitch's hindquarters during and after insemination. Similar observations were also made by Farstad (1984).

The backflow of semen could be prevented by the use of balloon catheter instead of Cassou sheath for intravaginal deposition of semen as suggested by Root and Johnston (2000).

Mickelsen *et al*. (1993) suggested that the low conception rate after inseminating with good quality semen at fertile period might be due to individual female related factors.

5.6.1.2 Frozen Semen

The conception rate obtained after intravaginal deposition of frozen semen was 20 per cent. The conception rate obtained in this study was higher than the report of Thomassen *et al*. (2006) who obtained a 10 per cent

conception rate in a similar study. However, higher conception rates of 25 per cent and 33.3 per cent were reported by Olar *et al*. (1989) and Linde-Forsberg and Forsberg (1989). However, still better conception rates of 52.6 and 73.6 were obtained by the Fontbonne and Badinand (1993) and Rota *et al*. (1999), respectively.

Although the first reported success of frozen thawed semen artificial insemination in the dog was after intravaginal deposition almost all further studies clearly demonstrated that intrauterine deposition was essential for the success of frozen semen artificial insemination (Foughner *et al*., 1973, Andersen, 1975 and Farstad and Andersen Berg, 1989) because the post thaw viability of dog semen was very short (12 h) when compared to other species.

Further, the lowest conception rate in bitches inseminated intravaginally with frozen semen may be due to the difference in the volume of the inseminate. For bitches inseminated with fresh semen, mean inseminate volume was 3.2 ml compared to 1.0 ml for frozen semen. Another factor could be the number of spermatozoa used for insemination. The mean number of spermatozoa per inseminate for bitches inseminated with fresh semen would be much higher (minimum of 150-200 X 10^6) when compared to frozen semen.

An alternative explanation was that spermatozoal transport was altered due to the freeze thaw process (Olar *et al*., 1989). These findings showed that if frozen thawed dog semen was to be deposited in the vagina more artificial inseminations with higher total number of spermatozoa were needed to obtain results on par with those achievable using trancervical artificial insemination.

5.6.2 INTRAUTERINE INSEMINATION

All intrauterine inseminations in the present study were performed using the endoscopic transcervical technique. A rigid cystourethroscope having a 29 cm length and 22 French diameter along with rigid telescope with a 30° viewing angle together with a bridge, cold light source with cable and monitor was used in this study. In some bitches, vaginal discharge caused poor visibility of the cervix and this was overcome by drawing the fluid using infant feeding tube (size 10). Another problem faced in the present study was that in some bitches the cervix could not be reached because of the narrow paracervical diameter. Wilson (2003) stated that this problem occured in some maiden bitches of small or medium sized breeds and in some toy breeds and this problem was solved by using a cystourethroscope of smaller diameter. Another limiting factor was that in some bitches the successful catheterization of the cervix could not achieved and in such cases the semen was deposited near the cervical Os. It might be due to the use of infant feeding tube (size 8) with a stylet as against the 8-french polypropylene urinary catheter recommended by Wilson (2003) and Davidson and Bruce Eilts (2006). Yet another problem faced in this technique was the backflow of semen through the cystourethroscope due to unsuccessful cervical catheterization. Davidson and Bruce Eilts (2006) recommended that if the cervix could not be catheterized and deep intravaginal insemination was to be performed, it was best to withdraw the cystourethroscope and perfom routine intravaginal artificial insemination via a rigid infusion pipette, because semen would lack out along the sheath of the cystourethroscope. It was observed from this study that bitches in estrus co-operated well for this technique of artificial insemination. None of the bitches needed any sedation. However, a specially designed artificial insemination table with a provision of tie point to the collar and abdominal band to limit sideways movement and attempts to sit would make transcervical technique much easier.

5.6.2.1 Fresh semen

The conception rate of 50 per cent was obtained in this study following intrauterine insemination by endoscopic transcervical insemination technique using fresh semen. A higher conception rate of 65.7 (Linde Forsberg and Forsberg, 1989), 84 (Farstad, 1984), 90 (Concannon *et al*., 2001) and 100 per cent (Silva *et al*., 1996) was reported in different studies.

Unsuccessful catheterization of cervix prevented intrauterine deposition of semen on few occasions in some bitches might have contributed to the lower conception rate when compared to earlier studies. Similar findings have been reported by Thomassen *et al*. (2006).

5.6.2.2 Frozen Semen

A 30 per cent conception rate was achieved in the present study following intrauterine deposition of frozen semen by endoscopic transcervical insemination technique. The conception rate obtained was higher than the report of Battista *et al.* (1988) in a similar study. However, still better conception rates were reported by Devendra Pathak et al.(2005) (60 per cent), Farstad (1984) (67 per cent); Thomassen et al.(2006) (75 per cent) and Rota *et al*. (1998) (100 per cent).

Linde-Forsberg *et al.* (1999) reported that the intrauterine artificial insemination significantly improved the whelping rate and litter size which was in agreement with the findings of the present study. The conception rate by intra-uterine artificial insemination significantly increased to 50 per cent from 36 per cent for frozen-thawed semen inseminated intrauterine compared to vaginal artificial insemination.

Pretzer *et al*. (2005) obtained a conception rate of 89.4 per cent following single transcervical insemination with frozen semen in Greyhound bitches and concluded that this technique was suitable for the breeding of Grehound bitches.

5.7 EFFECT OF ROUTE OF INSEMINATON ON CONCEPTION RATE

The conception rate obtained in this study in bitches inseminated intravaginally with fresh and frozen semen (Group I and II) was 50 and 20 per cent, respectively. The conception rate of 50 and 30 per cent was obtained in bitches (Group III and IV) inseminated transcervically with fresh and frozen semen, respectively. The conception rate in bitches inseminated intravaginally with frozen semen supplemented with prostatic fluid (Group V) was 30 per cent. A 40 per cent conception rate was achieved in bitches inseminated with fresh semen intravaginally without performing vaginoscopy (Group VI).

Silva *et al.* (1996) and Rota *et al. (*1999) compared the effect of insemination route (intravaginal vs intra-uterine) upon the fertility of frozen thawed dog sperm and found no significant difference. However, a study by Linde-Forsberg *et al.* (1999) showed significantly higher pregnancy rate and litter size for intrauterine insemination using the Norwegian catheter. However, Linde-Forsberg *et al.* (1999) achieved the same pregnancy rate for intravaginal and intrauterine (via endoscopy) inseminations (58.9 per cent and 57.9 per cent, respectively) similar to the findings of Silva *et al.* (1996) who also obtained 60 per cent conception rates for intravaginal insemination and intrauterine insemination via laparotomy (60 per cent for each technique).

It is interesting to note that the dog is considered to be a species with intra-uterine deposition of semen at natural mating, because by the time the copulatory tie is over, the spermatozoa have reached the oviducts. The sperm

rich fraction that is ejaculated at intromission and during the early stage of the copulatory tie, is followed by a large volume of prostatic fluid which immediately flushes the spermatozoa from the narrow cranial vagina through the cervix into the uterine body and horns.

The vagina appears to offer an unfavorable environment for canine spermatozoa, since a large proportion loose their tails within minutes after deposition in the cranial vagina (Linde-Forsberg, 1995). It has also been shown that around 10 times as many spermatozoa are required to obtain similar results by vaginal artificial insemination as by intra-uterine artificial insemination with fresh semen (Tsutsui, 1988) as well as with frozen-thawed semen (Linde-Forsberg, 1991). There fore many factors contribute to the poorer results obtained by intravaginal semen deposition compared to intra-uterine deposition.

5.8 EFFECT OF ADDITION OF PROSTATIC FLUID ON CONCEPTION RATE

The conception rate obtained in this study following intravaginal insemination of frozen semen with the addition of prostatic fluid was 30 per cent. Nothling and Volkman (1993) obtained a higher conception rate of 100 per cent in a similar study. The lower conception rate obtained in this study might be due to the volume of prostatic fluid used. Nothling and Volkmann (1993) added 7 to 10 ml of prostatic fluid as against 3 ml of prostatic fluid used in the current study. The higher volume might have influenced the conception rate. Nizanski (2006) also obtained a conception rate of 57.1 per cent following intravaginal deposition of frozen semen supplemented with prostatic fluid using Osiris catheter.

5.9 LITTER SIZE

The average litter size obtained in this study in bitches inseminated intravaginally with fresh and frozen semen was 5.5 and 2.0, respectively.

Litter size obtained in this study with fresh semen inseminated intravaginally was similar to the litter size of 5.8 reported by Linde-Forsberg and Forsberg (1989) (5.6) and Linde-Forsberg (2000).

Fontbonne and Badinand (1993), Linde Forsberg *et al*. (1999), Shimatsu *et a*l. (2000), Linde-Forsberg (2001) and Thomassen *et al*. (2006) reported a higher litter size of 4.8, 5.4, 4.0, 5.8 and 5.7, respectively than the 2.0 litter size obtained in this study following intravaginal insemination using frozen semen.

Intrauterine inseminations with fresh and frozen semen yielded 6.25 and 3.0 litter size following, respectively in this study.

The results obtained in this study was similar to the earlier reports of Linde-Forsberg (2001), who obtained a litter size of 6.5 in a similar study.

Higher litter sizes of 5.5 (Fontbonne and Badinand, 1993) 5.4 (Linde-Forsberg *et al*., 1999), 5.0 (Linde-Forsberg, 2001) and 5.7 (Thomassen *et al*., 2006) were reported when compared to the 3.0 litter size obtained following intrauterine insemination by transcervical method using frozen semen. Shimatsu *et al.* (2003) studied the relationship between the timing of insemination based on LH surge and litter size and stated that when insemination was done on 3 and 5 days after LH surge the litter size was high.

Mickelson *et al*. (1993) reported a litter size of 5.6 in bitches inseminated with fresh semen deposited intravaginally. He also compared the functional and morphological parameters of spermatozoa with litter size and concluded that litter size was not affected by the percentage of motility, total number of motile sperm cells, percentage of morphologically normal sperm cells per ejaculate.

Linde-Forsberg (1995) reported that the hostile nature of vaginal micro environment was reflected in the observation that detached sperm head were often found in vaginal smears only minutes after AI was performed.

5.10 GESTATION LENGTH

Apparent length of gestation, considered as the interval from a fertile mating to parturition, varied greatly in dogs. It ranged from 56 to 68 days with an average of 64 days. The mean length of gestation obtained in the present study was 57 days with a range of 54 to 60 days from the day 1 of diestrus. The range of potential due dates of parturition was 56 to 70 days from the day of first breeding (Feldman and Nelson, 1996).

Variation in the timing of ovulation, multiple breeding dates and the in consistent length of estrus made it difficult to identify the day of fertilization or exact date for a litter. Despite these variations, the actual pregnancy length was constant and parturition occurred on an average 56 to 58 days from day 1 of diestrus as determined from vaginal cytology (Brukes, 1996 and Feldman and Nelson, 1996). Tsutsui *et al*. (2006) found that the duration of gestation was not significantly affected by the timing of mating or the number of fetuses, whereas Okkens *et al*. (2001) reported that the number of fetuses and duration of gestation were negatively correlated.

5.11 NUMBER OF SPERMS USED FOR INSEMINATION

The minimum number of sperms required for acceptable fertility had not been established in the dog.

Farstad and Andersen Berg (1989), Nothling and Volkmann (1993) and Stimatsu *et al*. (2003) suggested that 100 X 10^6 live spermatozoa gave acceptable conception rates and litter sizes. However, Morton and Bruce (1989) and Linde- Forsberg and Forsberg (1989) recommended 150-200 X 10^6 motile spermatozoa in 2-3 ml on 2 or 3 occasions for acceptable fertility in artificially inseminated bitches with fresh semen.

Similarly, in the present study 150 to 200 X 10^6 spermatozoa were used and 20 and 30 per cent conception rates were achieved following intravaginal and intrauterine insemination using frozen semen, respectively. However, Wilson (1993) obtained a pregnancy rate of 85 per cent in bitches inseminated intrauterine by transcervical insemination technique with two inseminations of only 30-35 X 10^6 frozen thawed spermatozoa.

In an extensive retrospective study of frozen semen inseminations, Linde-Forsberg *et al.* (1999) noted an apparent increase in whelping rate and litter size with increasing total sperm dose per intravaginal insemination.

Recently, a study was conducted by Hye Jin Kim *et al*. (2007) to assess the least number of post thaw spermatozoa necessary to achieve pregnancy by intrauterine or intratubal artificial insemination (both by surgical method) and obtained a pregnancy rate of 80 and 20 per cent through intrauterine and intratubal insemination using just 5 X 10^6 and 4 X 10^6 spermatozoa, respectively. The recent studies showed that optimum conception rate is possible following artificial insemination with frozen semen

In conclusion, the results of the present study have shown that the conception rates for artificial insemination with fresh semen were similar irrespective of the site of deposition. However, for frozen semen artificial insemination intrauterine deposition of semen was an important contributing factor in increasing pregnancy rates when compared to intravaginal insemination. Intrauterine insemination in the present study were performed by the transcervical endoscopic method, a technique that provided intrauterine deposition of semen, a vital part of frozen semen technology. A significant advantage of the technique was that it allowed direct visualization of the cervical opening. Still, it involved manipulation of the scope and catheter and required some practice. Another added advantage was the ability to do repeated inseminations without undue stress to the bitch without the need and risk of general anesthesia and surgery. Equally important to the successful use of fresh or frozen semen are the timing of insemination, semen quality and bitch fertility.

In the present study conception rates were higher when timing of insemination was done based on vaginoscopy than vaginal exfoliative cytology indicating the efficacy of vaginoscopy as a means of predicting fertile breeding time in bitches.

The lower conception rates with frozen semen when compared to other studies could be attributed to the following factors. Inseminations were performed on alternate days throughout the fertile period as against daily inseminations reported by most authors. Since freezing altered the post thaw survival of frozen semen to less than 12 hours, daily inseminations would have helped to achieve higher conception rates. The thawing temperature of 37° C for I minute as against the 70° C for 8 seconds followed in the recent studies could also be a contributing factor for the lowered fertility.

The inability to catheterize the cervix in some bitches could also be a factor for reduced fertility with frozen semen artificial insemination.

The results of the present study showed that semen cryopreserved with Tris extender was superior to the Tes-tris extender in terms of post thaw functional and morphological characteristics an important attribute for achieving optimum conception rate. Addition of prostatic fluid to frozen semen helped to improve the conception rates when compared to intravaginal insemination of frozen semen without prostatic fluid.

CHAPTER VI

SUMMARY AND CONCLUSION

Sixty healthy intact cycling bitches of different breeds between 2 to 6 years of age brought to the Small Animal Gynaecology and Obstetrics unit of Madras Veterinary College Teaching Hospital for breeding advice were randomly divided into six groups viz., Group I to VI of 10 bitches each. Seven male dogs of different breeds were used for semen collection. This study consisted of two experiments viz., I and II. In experiment I, the efficacy of Tes-tris, a commercial extender and Tris on freezing and post thaw functional and morphological characters of spermatozoa was studied. Since, the semen cryopreserved with Tris extender gave better post thaw functional and morphological characters, this semen was used for artificial inseminations in Group II, IV and V bitches of Experiment II. Experiment II involved the determination of optimum insemination time using vaginoscopy / vaginal exfoliative cytology and intravaginal / intrauterine artificial insemination using fresh and frozen semen.

Group I to Group V bitches were subjected to vaginoscopy at 2 days interval starting from day 5 of proestrus till day 1 of diestrus. Vaginal smears were obtained from bitches of all groups at alternate days starting from day 5 of proestrual bleeding until day 1 of diestrus.

In Group I to Group V bitches, the timing of insemination was based on vaginoscopy alone. Inseminations were started when vaginal mucosal folds showed shrinkage with angulation. In Group VI bitches the timing of insemination was determined based on vaginal exfoliative cytology only and inseminations were started when vaginal exfoliative cytology showed more than 80 per cent of superficial and cornified cells. Inseminations were continued at alternate days in all the bitches during fertile period until diestrus.

Group I and II bitches were inseminated intravaginally using fresh and frozen semen respectively. Group III and IV bitches were inseminated with fresh and frozen semen via intrauterine route using the transcervical endoscopic technique. Group V bitches were inseminated intravaginally using frozen semen supplemented with prostatic fluid and Group VI bitches were inseminated intravaginally with fresh semen wherein insemination time was determined based on vaginal cytology. Pregnancy was confirmed in all bitches 30 days after last insemination. Gestation length,whelping rate and litter size were determined after whelping.

The mean post thaw motility and percentage of live spermatozoa of semen cryopreserved with Tris and Tes-tris extenders was 42.14 ± 0.81 and 32.86 ± 2.21 and 59.0 ± 1.70 and 49.71 ± 1.62 per cent respectively. The mean percentage of abnormality was 29.14 ± 1.10 and 36.50 ± 1.17 per cent in semen cryoperserved with Tris and Tes-tris extenders, respectively. The mean percentage of acrosomal integrity and plasma integrity were 49.42 ± 1.15 and 46.14 ± 1.60 and 53.79 ± 0.96 and 49.00 ± 0.98 per cent in semen cryoperserved with Tris and Tes-tris extenders, respectively.

The mean day of onset of cytological estrus and day 1 of diestrus occurred 9.11 ± 3.14 and 15.58 ± 3.73 days from the day one of proestrual bleeding and it ranged from day 4 to 15 and 6 to 24, respectively in all the bitches included for the study.

Vaginal exfoliative cytology showed a shift in parabasal and intermediate cells in proestrus to superficial and cornified during estrus.The superficial keratinized cells significantly increased from proestrus to 82.05 ±1.5 per cent at the onset of estrus. Throughout the period of estrus, smears were predominated by superficial keratinized cells reaching 94 per cent 2 days prior to day 1 of diestrus. The first day of diestrus was marked by a

sudden decrease in superficial and cornified cells to <37 per cent and increase in parabasal and intermediate cells to 28 ± 1.42 and 39 ± 4.42 per cent, respectively.

Throughout the period of proestrus, vaginal mucosal folds appeared rounded and edematous. Late proestrus or early estrus was characterized by the onset of shrinkage without angulation in the vaginal mucosa which progressed to a more obvious shrinkage with angulation period which corresponded to the fertile period in bitches. The mean length of proliferative edematous period, non-angulated shrinkage period and angulated shrinkage period was 7.80 ± 0.62, 5.12 ± 0.37 and 4.40 ± 0.81 days with a range of 6 to 10, 4 to 7 and 3 to 5 days, respectively.

The conception rate obtained in this study was 50, 20, 50, 30, 30 and 40 per cent in group I, II, III, IV, V and VI bitches respectively. A whelping rate of 80, 100, 80,100, 66 and 100 per cent was obtained in group I, II, III,IV and V and VI bitches respectively. The percentage of bitches that whelped on 54,55,56,57,58,59 and 60 days after day 1 of diestrus and the mean litter size were 5.2, 10.5, 21.0, 26.3, 21, 10.5 and 5.2 and 5.50 ± 1.30, 2.00 ± 0.00 , 6.25 ± 0.10, 3.00 ± 1.00, 3.00 ± 0.57 and 5.25 ± 1.70 in group I, II, III, IV and V and VI bitches, respectively.

From the present study the following conclusions were derived.

- The conception rates were similar for both intravaginal and intrauterine insemination with fresh semen.
- The conception rate was higher when frozen semen was deposited intrauterine rather than intravaginal.

- Transcervical endoscopic technique could be successfully employed for repeated intrauterine insemination without undue stress to the bitch.
- Tris extender was found to give better post thaw characters after freezing compared to Tes-tris extender
- Vaginoscopy was found to be effective in assessing the optimum insemination time compared to vaginal exfoliative cytology.
- Addition of prostatic fluid with frozen semen had significant influence on increasing the conception rate on intravaginal insemination.
- Number of inseminations did not positively correlate with conception rate.
- Vaginal exfoliative cytology alone could not be employed for prediction of optimum insemination time because of its inaccuracy.
- The litter size of the artificially inseminated bitches with fresh semen were higher than frozen semen.

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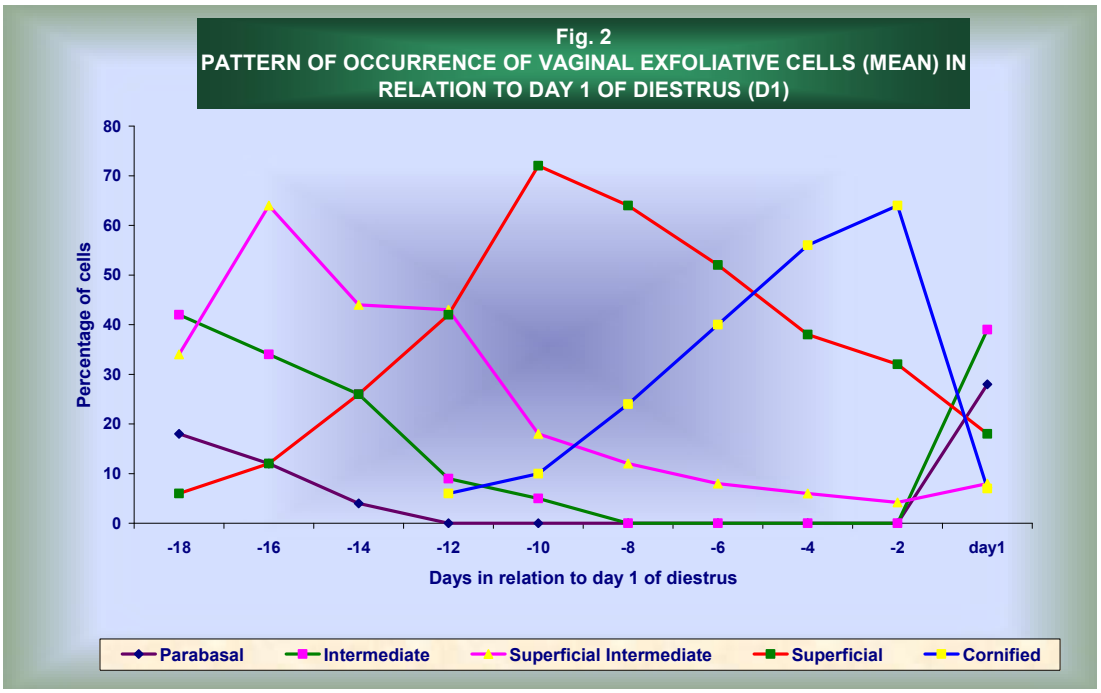
Fig. 2
PATTERN OF OCCURRENCE OF VAGINAL EXFOLIATIVE CELLS (MEAN) IN RELATION TO DAY 1 OF DIESTRUS (D1)
Percentage of cells
80
70
60
50
40
30
20
10
0
-18
-16
-14
-12
-10
-8
-6
-4
-2
day1
Days in relation to day 1 of diestrus
Parabasal
Intermediate
Superficial Intermediate
Superficial
Cornified

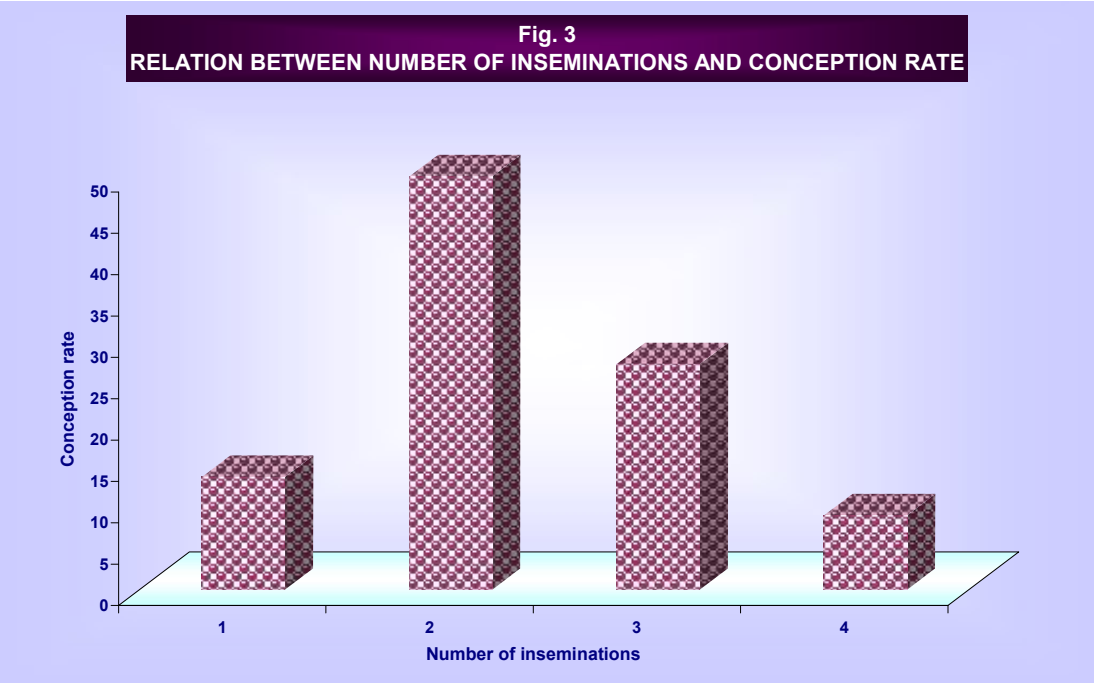
Fig. 3
RELATION BETWEEN NUMBER OF INSEMINATIONS AND CONCEPTION RATE
Conception rate
50
45
40
35
30
25
20
15
10
5
0
1
2
3
4
Number of inseminations

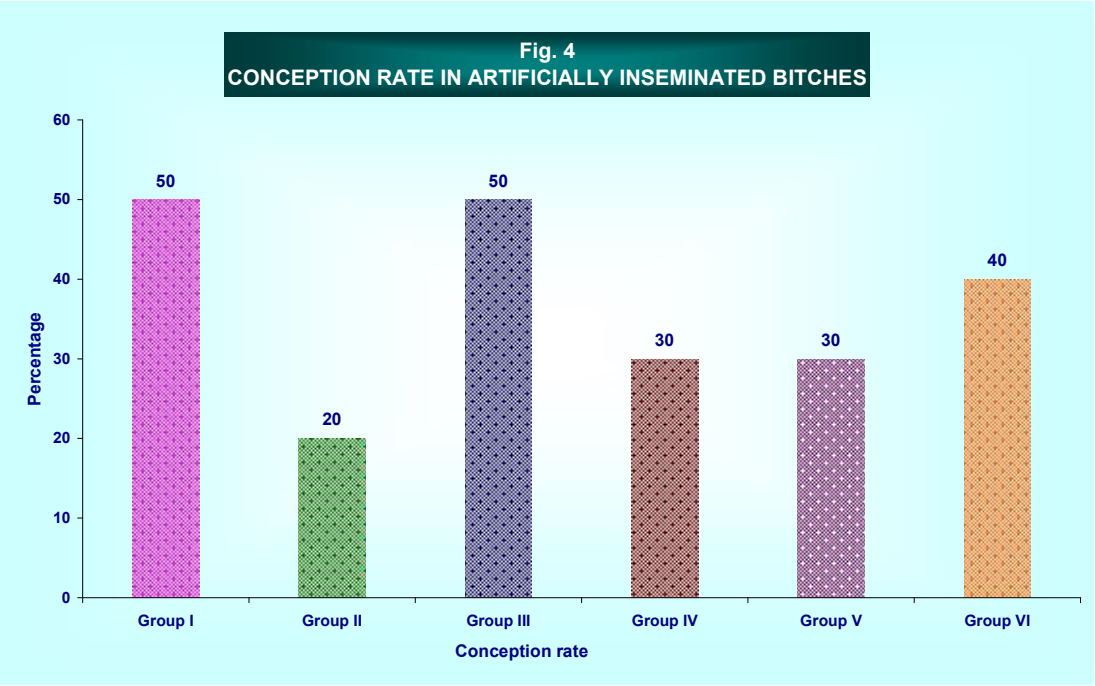
Fig. 4
CONCEPTION RATE IN ARTIFICIALLY INSEMINATED BITCHES
Percentage
60
50
40
30
20
10
0
50
20
50
30
30
40
Group I
Group II
Group III
Group IV
Group V
Group VI
Conception rate

Fig. 5
WHELPING DAYS IN RELATION TO DAY 1 OF DIESTRUS
Per cent of bitches
30
25
20
15
10
5
0
54
55
56
57
58
59
60
Days of whelping

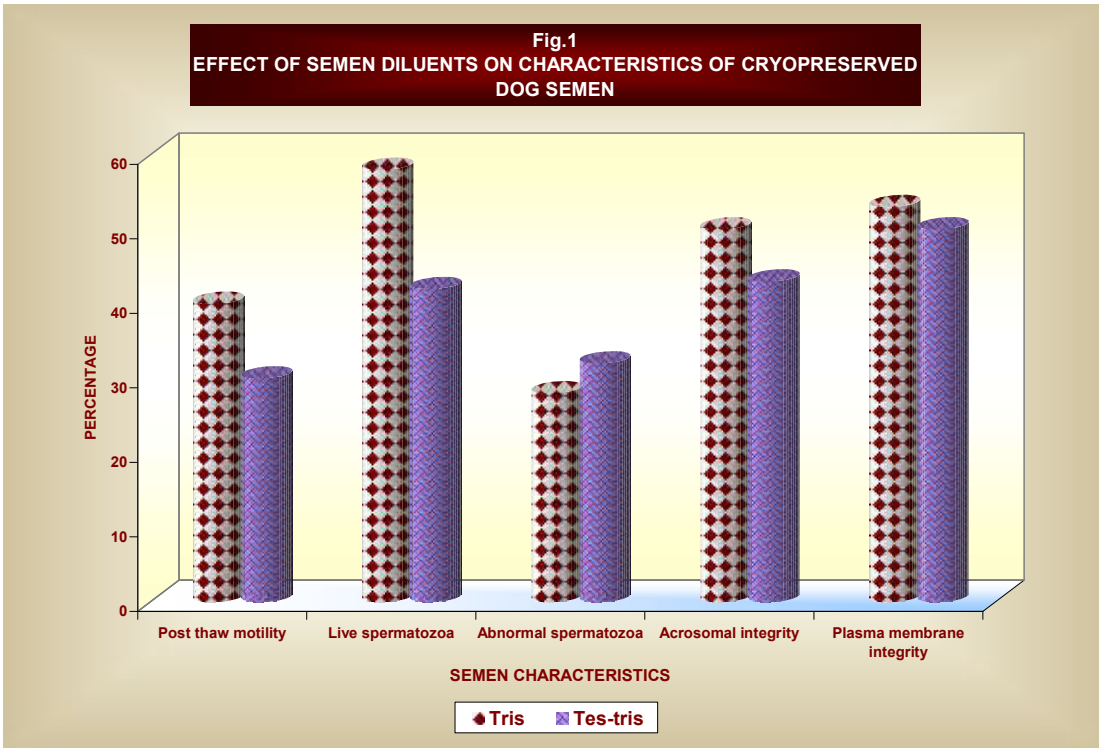
Fig.1
EFFECT OF SEMEN DILUENTS ON CHARACTERISTICS OF CRYOPRESERVED DOG SEMEN
PERCENTAGE
60
50
40
30
20
10
0
Post thaw motility
Live spermatozoa
Abnormal spermatozoa
Acrosomal integrity
Plasma membrane integrity
SEMEN CHARACTERISTICS
Tris
Tes-tris