EFFECT OF DIFFERENT FREEZING RATES ON CANINE SPERMATOZOA

By

R. GEETHA

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences
Kerala Agricultural University

Department of Animal Reproduction
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR - 680651
KERALA, INDIA

2000

DECLARATION

I hereby declare that this thesis entitled "EFFECT OF DIFFERENT FREEZING RATES ON CANINE SPERMATOZOA" is a bonafide record of research work done by me during the course of work and this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

16-12-2000
Mannuthy

R. Geetha
R.GEETHA

CERTIFICATE

Certified that thesis entitled "EFFECT OF DIFFERENT FREEZING RATES ON CANINE SPERMATOZOA" is a record of research work done independently by Ms. R. GEETHA, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

Mannuthy.
16-12-2000.

Dr. T. Sreekumaran,
(Chairman, Advisory Committee),
Associate Professor & Head,
Department of Animal Reproduction
College of Veterinary and Animal Sciences,
Mannuthy.

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Ms. R. Geetha**, a candidate for the degree of Master of Veterinary Science in Animal Reproduction, agree that this thesis entitled "**EFFECT OF DIFFERENT FREEZING RATES ON CANINE SPERMATOZOA**" may be submitted by **Ms. R. Geetha**, in partial fulfilment of the requirement for the degree.

Dr. T. Sreekumaran
(Chairman, Advisory Committee)
Associate Professor & Head
Department of Animal Reproduction
College of Veterinary and Animal Sciences,
Mannuthy.

Dr. P.P.Balakrishnan
Special officer
College of Veterinary & Animal Sciences
Pookot, Wayanad.
(Member)

Dr. V.Vijayakumaran
Associate Professor & Head
Cattle Breeding Farm
Thumbrumuzhi.
(Member)

Dr. K.V.Raghunandannan
Associate Professor
Centre for Advanced studies in Animal
Breeding & Genetics
Mannuthy.
(Member).

17/02/

External Examiner

Dr. C. VEERAPPAN

To my beloved parents and Appachi

ACKNOWLEDEMENTS

I wish to express my deep sense of gratitude to ***Dr. T. Sreekumaran****, Associate Professor and Head, Department of Animal Reproduction, for his scholastic advice, valuable suggestions, constructive criticism, constant encouragement and parental affection shown towards me during my course of work as the chairman of the advisory Committee. It has been a privilege to work under his guidance.*

I am deeply indebted to ***Dr. P.P.Balakrishan****, Special officer, College of Veterinary and animal Sciences, Pookot, Wayanad, as a member of the Advisory Committee, for his unstinted support, incessant encouragement and interest shown during the study.*

I express my profound gratitude to ***Dr. V. Vijayakumaran****, Associate Professor and Head, Cattle Breeding Farm, Thumbrumuzhi, and* ***Dr. K.V. Raghunandanan****, Associate Professor, Centre for Advanced studies in Animal Breeding and Genetics, for their valuable help and guidance rendered me during various stages of the study as a members of the Advisory Committee.*

Words fail to express my heartfelt gratitude to Late. ***Dr. E. Madhavan****, for his timely help, valuable suggestions and meticulous guidance rendered me during my correction of thesis.*

I am thankful to Dr. K. Prabhakaran Nair, Dr. K.V.Athman, Dr.Suresan Nair, Dr. K.N. Aravinda Ghosh, Dr. K. Ramachandran, Dr. Metilada Joseph, Dr. A.M. Vahida, Dr. G. Aijt kumar, Dr. C.Jayakumar, Dr. K. Pramod for their technical guidance, encouragement and timely advice.

I would like to thank Smt. Narayanikutty, Asstistant Professor and Head, Smt. Santa bai, Computer Programmer, Department of Statistics for their valuable help rendered me during result analysis.

With great pleasure I express my heartful thanks to Dr.Bibinbecha, Dr.Liz simon, Dr.Lydia Priscilla, Dr.Gopikrishnan, Dr.Kantharaj, and Dr.Senthilkumar.

The invaluable help and advice rendered by my friends Latha, Bindiya, Sreevidya, Sindhu, Priya, Princy, Manju, Kamala, Mumtaz, Bindu, Sofia, Methai, Gautam and Kumaresan were utilised by me and I am very much thankful to them. I would also like to record my thanks to Raji, Bincy, Sabiha, Sangeetha, and Lakshmi for their help and moral support.

I wish to express my thanks to Mr. Peter for takjng Photographs, Mohanan, Dasan, and other non-teaching staffs, Department of Animal Reproduction, Mannuthy, for their great help rendered me during my course of work.

I acknowledge the dog owners, as without their help there would have been no thesis at all.

I am thankful to **Dr. Sulochana,** *Dean, College of Veterinary and animal Sciences for the facilities provided and help rendered during the study.*

I am grateful to **ICAR** *for awarding me the Junior Research Fellowship for the period of study.*

I would acknowledge M/s Delta Computers, Mannuthy for the timely and successful completion of the thesis.

I sincerely indebted to my beloved family members for their affection, moral support and continuous encouragement for the successful completion of thesis.

Above all I bow my head before God Almighty who blessed me with good health, wealth and confidence for the successful completion of thesis.

R. Geetha

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Introduction

1. INTRODUCTION

Artificial insemination in dog was first reported by Spallanzani in 1780 (Harrop, 1956), which resulted in the birth of three pups. Progress, however in this area was slow, and subsequent developments included mainly procurement of artificial insemination equipments and methods for short term preservation of fresh and later chilled semen which had their own limitations. Commercialization of dog breeding necessitated methods for long term preservation of dog semen. During the last two decades interest was generated among dog breeders for preservation of superior germplasm which resulted in the World's first litter using frozen dog semen (Seager, 1969). Since then dog breeders worldwide put increasing pressure on the scientific community for progress in canine reproductive biotechnology.

Frozen thawed semen is being used widely for artificial insemination in dogs for its convenience in preservation and the transportation of genetic material. Although there have been many reports of successful freezing and thawing of dog

semen, majority adopted methodology designed for other species. (Foote, 1964; Andersen, 1972).

The technique of semen collection and processing for frozen semen production has been elaborated in the literature, but the information regarding the fertility of frozen semen has been scanty. The major lacunae has been with regard to the ability of dog spermatozoa to survive after thawing which is considered as an indicator of fertility. There are several reports indicating that poor fertility of frozen thawed dog semen was due to reduced penetration into oocyte by the spermatozoa. (Froman *et al.*, 1984). However, this has not been proved conclusively. The unsatisfactory whelping rates and litter size obtained by using frozen semen necessitate a thorough investigation on the diluents to be recommended for cryopreservation of dog semen to maintain normal fertility after thawing.

Furthermore, the marked individual variation in the post thaw survivability of spermatozoa of different breeds suggests that selection of donors would exert a marked effect on fertility of frozen semen. Moreover, the nature and character of dog semen with its different fractions and its effect on various diluents might

also influence the success of freezing techniques and post thaw motility. It is therefore felt necessary to have a detailed study on preservation of dog semen with a view to long term storage for use locally or thousands of miles away.

Many of the above aspects that prevent successful use of frozen semen have resulted in the limited use of this technique in the cryopreservation of dog semen. There is a general agreement that insemination with frozen thawed spermatozoa is short-lived, compared with that of natural mating. The delayed maturation of dog ovum is an additional problem to complicate the issue.

In Kerala very limited experiments have been carried out in artificial insemination for dogs. Kadirvel (1998) has reported that Tris diluent can be successfully used for the preservation of chilled semen. However suitability of the above diluent for freezing dog semen is yet to be studied. The rate of freezing and post thaw motility of frozen dog semen using Tris diluent have to be subjected to a detailed investigation for recommending it for freezing of dog semen.

The present study was therefore aimed to standardize the freezing technique of semen in Dachshund breed

a) by evaluating the effect of different freezing rates on the quality of dog spermatozoa and
b) by developing a freezing method which would improve the post thaw motility.

Review of Literature

2. REVIEW OF LITERATURE

Dog breeding has been recognized as a commercial preposition and therefore an international exchange of various aspects of dog breeding with specific emphasis to artificial insemination has received increased attention with special reference to preservation of semen. Effective and commercially viable methods of semen collection and preservation must be an essential feature in the near future.

Several methods of collection and preservation of dog semen have been reviewed in the past.

2.1 Collection of semen

Collection of semen has been successfully done by using artificial vagina (AV), by electro ejaculation and by digital manipulation.

2.1.1a. Artificial Vagina Method

Boucher *et al.* (1958) successfully collected semen by artificial vagina but found that slow flow of semen over the warm latex immobilized the spermatozoa. Harrop (1960) designed an artificial vagina composed of an outer rubber lining 7.5" long and 2.33" in diameter. Hot water was filled between the outer covering and the liner. One bulb was used for adjusting the size of the lumen and the other for making pulsation and recommended that this could be used for any size of dog because of its design. Seager and Fletcher (1973) used a conical rubber sheath attached to a centrifuge tube for collection of semen.

England and Allen (1992a) opined that the liner and the lubricants used in the AV affected the motility and morphology of canine spermatozoa. They also warned that short time contact with the rubber had deleterious effect on motility and long time contact even changed the sperm morphology.

England and Allen (1992b) also suggested that the pre sperm and post sperm fractions affected the quality of semen and mixing of fractions occurs while using artificial vagina. Arthur *et al.* (1996) reported that the latex lining of the artificial vagina

caused deleterious effect on canine sperm and also opined that better quality of semen was obtained by digital manipulation.

2.1.1b. Electro ejaculation Method

Though electro ejaculation method has been tried in the past for semen collection in the dog, Christensen and Dougherty (1955) opined that the semen so obtained was contaminated with urine and the ejaculates were of poor quality and hence found unfit for routine collection. A similar observation was made by Harrop (1960).

2.1.1c. Digital manipulation

Boucher *et al.* (1958) described the digital manipulation for semen collection with or without a teaser bitch. Pressure was applied behind the bulbus glandis with thumb and forefinger to induce erection. The pressure was continued behind the bulb with additional massage when the dog was not readily stimulated. Pulsations usually were noted almost immediately and ejaculation was completed within 15 to 20 Minutes. Kibble (1969) collected semen using an oestrus bitch as teaser or gentle

stimulation within the prepuce resulting in the exposure of penis with the urethral bulb outside the prepuce. Light digital pressure behind the bulb resulted in venous return and erection.

Gill *et al.* (1970) and Taha *et al.* (1981) also described semen collection by digital manipulation in the presence of a bitch. Hopkins and Evans (1989) and Dabas *et al.* (1991) also described the same procedure and obtained semen by intermittent massage of penis using thumb and forefinger from back to forth behind the bulbus glandis leading to penile erection and ejaculation. Digital manipulation for semen collection has been described by Rota *et al.* (1995) and Linde-Foresberg *et al.* (1999).

2.1.2 Periodicity of semen collection

Boucher *et al.* (1958) opined that the concentration of semen sample decreased when the collection frequency was more while volume was not affected. According to Seager *et al.* (1975) collection frequency should be once in a week and should not be more than thrice in a week. But Taha *et al.* (1981) and Dobrinski *et al.* (1993) reported successful ejaculation every second day in a week which was found to be suitable for freezing. Taha *et al.*

(1983) recorded that the ejaculation twice a day for five days reduced sexual libido and spermatozoal output and ejaculation thrice a day severely reduced sex libido and semen quality but once in a day collection did not affect the above characters.

Thomas *et al.* (1993) however could carry out semen collection at four days interval for cryopreservation studies. Rawlings *et al.* (1994) suggested twice a week collection from dogs. Similarly Rodriguez-Gil *et al.* (1994) reported successful semen collection suitable for freezing once or twice weekly even without an oestrus female. England (1999) studied the influence of a short time second ejaculation in semen quality in dogs and collected semen with a mean interval of 63 minutes. He found that lower values for the volume and sperm concentration in the second fraction.

2.1.3 Fractions of semen

Detailed studies have been carried out on the nature of the dog semen and time required for ejaculation of each fraction. According to Harrop (1955) the time required for ejaculation of each fraction was 30 to 50 seconds for the first, 50 to 80 seconds

for the second and 3 to 30 minutes for the last fraction. Morton and Bruce (1989) found that behaviour of pelvic thrust was coincident with ejaculation of pre sperm fraction, which took 1 to 2 minutes. They also found that the dog become steady when the sperm rich fraction was ejaculated over the next 30 to 60 seconds. It was also be reported that the dog would turn when the post sperm fraction was ejaculated over a period of 10 to 75 minutes.

Arthur *et al.* (1996) observed that production of the pre sperm fraction occured during the quiescent phase over a period of upto 50 seconds after vigorous pelvic thrust. Then the ejaculation of the sperm rich fraction followed immediately within few seconds and post sperm fraction was ejaculated over a period of three to 30 minutes. Kadirvel (1998) noted that the erection of penis occurred within 30 to 60 seconds after firm holding of penis behind the bulbus glandis and pelvic thrust was coincident with the ejaculation of pre sperm fraction over a period of 0.5 to one minute. The sperm rich fraction was ejaculated over 30 to 60 seconds during steady period, and thereafter the penis of the dog was turned back and the ejaculate was collected for the next three to 20 minutes as post sperm fraction.

Fougner (1977) reported that the admixture of pre sperm fraction with sperm rich fraction of the ejaculate affected the freezing quality of semen.

2.2 Evaluation of semen

2.2.1 Volume

Harrop (1960) found variation in volume of semen ejaculated due to size and breed of the dog. The difference was mainly in volume of the third fraction of the ejaculate. The larger breeds produced larger amounts and vice-versa. The volume of three different fractions measured were 0.25 to 5 ml, 0.5 to 3.5 ml and two to 30 ml respectively. Kibble (1969) observed the volume of three portions of ejaculate as 2 ml of clear fluid, 2 to 3 ml of sperm rich fraction and 20 ml of post sperm fraction. According to Deshpande *et al.* (1970) the volume of semen of Dachshund and Afghan hound was found to be 1ml.

Dubiel (1974) remarked that ejaculate volume was dependent on age, size and breed of dog. Taha *et al.* (1981) reported that ejaculate volume of Beagle dogs averaged 6.74±0.31, the first, second and third fractions being 1.17±0.09ml,

0.69±0.03ml and 4.89±0.26 ml respectively. Morton and Bruce (1989) found that in some dogs sperm rich fraction was only two to three drops and ejaculated in a short period of 30 seconds, 1-2 minutes after the start of collection. The mean volume of 2.2±0.2ml of sperm rich fraction was obtained by Dobrinski *et al.* (1993). Kadirvel (1998) recorded the average volume of pre sperm, sperm rich and post sperm fractions as 0.63± 0.07 ml 1.29±0.08 and 4.12±0.23 ml respectively.

2.2.2 Colour and density

Boucher *et al.* (1958) described the colour and consistency as clear when the graduation in the collection vial was readable easily, cloudy when the graduation was faintly visible and milky when the graduation blended with the semen. Deshpande *et al.* (1970) noted that the semen colour varied in different breeds of dogs. The creamy to white colour for the pre sperm and sperm rich fraction of the ejaculate was reported by Olson (1992).

Daiwadnya *et al.* (1995) observed the colour of the sperm rich fraction of semen as cloudy or milky in nature. Kadirvel (1998) recorded the colour of the first and third fraction of semen

as clear and watery and second fraction varied from thin milky to thick milky and density ranged from D to DDD in mongrel dogs.

2.2.3. Initial motility

The average sperm initial motility in Beagle dogs was reported to be 79per cent (Gill *et al.*, 1970) and 100per cent (Morton and Bruce, 1989). England and Allen (1989) recorded the average percentage motility of 89.5±7.6 among twenty eight fertile dogs of different breeds. Daiwadnya and Hukeri (1993) observed an average initial motility of +3.600.

The mean percentage of sperm motility was reported to be 70 (Olson, 1992) and 67.5 (Mickelsen *et al.*, 1993) in different breeds of dogs. Kumi-Diaka (1993) noted the percentage of initial motility as 72.8±20.1 in certain mixed breeds. Rota *et al.* (1995) recorded an average initial motility of 78.6±13.6 per cent for canine spermatozoa for various breeds of dogs. The average initial motility was reported to be 87.30±0.91 per cent (Nair, 1996), 78.61 per cent. (Strom *et al.*, 1997) and 86.67±1.07 per cent (Kadirvel, 1998) in mongrel dogs.

2.2.4 pH

Harrop (1956) reported the average semen pH as 6.75 with a wide range of 5.8 to 6.9 in different dog breeds. Boucher *et al.* (1958) observed the pH of the semen collected by digital manipulation with or without teaser as 6.52 and 6.57 respectively and remarked that collection procedure did not affect the pH of semen.

Wales and White (1958) found the pH of sperm rich fraction was 6.3 as against a mean pH of 6.8 for the post sperm fraction. Chatterjee *et al.* (1976) noted a mean pH of 6.8 in dog semen. Dobrinski *et al.* (1993) reported that the pH of semen immediately after collection was 6.2 ± 0.02, while England (1993) and Kadirvel (1998) reported the pH of second fraction ejaculate as 6.27± 0.03 and 6.36±0.03 respectively.

2.2.5 Concentration

It is generally accepted that increasing frequency of collection reduces the sperm concentration. Harrop (1955) recorded the average concentration of spermatozoa as 125 millions per ml in

100 ejaculates belonging to several breeds of dogs. The average sperm concentration was reported to be 70 millions per ml (Roychoudhury and Dubay, 1974), 4.1±1.8 X 10^8 per ml within a range of 1.2 to 8.5 X 10^8 per ml (Tsutsui *et al.*, 1988), 299.6 millions per ml (England and Allen, 1989), 264 millions per ml (Daiwadnya and Hukeri, 1993) and 310 millions per ml (Silva *et al.*, 1996).

Rodriguez-Matrinez *et al.* (1993) obtained a total sperm concentration of approximately 150X10^6 spermatozoa per ml from eight fertile dogs. Guerin *et al.* (1999) assessed the sperm concentration of five different breeds of dogs and found that it exceeded 150X10^6 per ml.

Taha *et al* (1981), England and Ponzio (1996) and Pena *et al.* (1999) found seasonal influence on sperm concentration.

2.2.6 Dead and Live spermatozoa

Taha *et al.* (1981) reported that the mean percentage of dead sperms was 10.48±0.67. He also observed a two fold increase

in the number of dead but morphologically normal sperms in June when compared with that of March. Yubi *et al.* (1987) recorded 9.5±0.71 and 9.33±7.64 per cent dead spermatozoa from donor and stud dogs respectively immediately after collection.

Daiwadnya and Hukeri (1993) observed that the percentage of live sperm was 82.81 in forty eight ejaculates collected from four mongrel dogs. Kumi-Diaka and Badtram (1994) found that the mean percentage of dead spermatozoa was 9.1±0.9 in fresh semen of mature dogs of various breeds. The average live sperm percentage was recorded to be 84.06±1.27 (Nair, 1996), 71.5±13.5 (Nothling *et al.*, 1997) and 89.44±0.57 (Kadirvel, 1998).

2.2.7 Spermatozoan abnormality

Kawakami *et al.* (1988) reported high percentage of abnormal acrosomes when semen was obtained from the testis and high percentage of abnormal sperm tails in semen from the epididymis and ductus deferens. They also reported a folded tail abnormality in one dog. Morton and Bruce (1989) found that the mean percentage of head, mid piece and tail abnormalities was 5.62, 13.4 and 9.7 respectively. They also found that the major

primary sperm abnormalities were in the mid piece. Dobrinski *et al.* (1993) reported that sperms with morphological defects of head, mid piece and principal piece, detached head, proximal and distal protoplasmic droplets were 10.4±0.9, 6.9±0.7, 2.2±0.8, 1.3±0.2, 9.2±1.1 and 1.0±0.2 per cent respectively.

Oettle (1993) opined that semen with less than 60 per cent normal sperm morphology would adversely affect the fertility of dog. According to Keskin *et al.* (1996) the percentage of defects in the spermatozoan head, mid piece and tail abnormalities to the extent of 0.1, 10.3 and 6.9 in fresh semen were within the permissible limits. The recorded percentage of sperm abnormalities in fresh semen was found to be 2.16±0.13 (Nair, 1996) and 6.63±0.38 (Kadirvel, 1998) in mongrel dogs.

2.2.8 Acrosome damage

Aguirre *et al.* (1987) reported that the percentage of spermatozoa with intact acrosome and acrosome score were 35q16mu/ml and 0.80q 0.18mu/ml respectively in cold shocked semen when compared with 82.5q10mu/ml and 0.33q 0.20mu/ml respectively in fresh semen 30 minutes after collection. Kumi-

Diaka and Badtram (1994) recorded the mean percentage of acrosomal defects to be 2.43±1.15 in fresh semen. However England and Ponzio (1996) have reported that the percentage of normal acrosome ranged from 88 to 95 per cent.

2.3 Semen Processing

2.3.1 Semen Extenders

The choice of a semen extender is an important factor influencing the success of AI. The most commonly used extender for freezing canine semen is Tris-fructose-citric acid extender containing egg yolk and glycerol (Foote, 1964; Oettle, 1986; Yubi *et al.*, 1987; Olar *et al.*, 1989). Yubi *et al.* (1987) obtained better post thaw motility by using the diluent contained Tris 2.9g, Fructose 1.25g, Citric acid 1.32g, distilled water 100ml, glycerol 8ml and egg yolk 20 per cent. Ferguson *et al.*, (1989) used Tris-fructose- citric acid diluent with 8per cent glycerol for freezing dog semen and obtained a conception rate of 67 per cent. Extenders containing lactose (Olar *et al.* 1989) have also been used.

Olar *et al.* (1989) and England (1993) found that two to four per cent (v/v) of glycerol in the final dilution protected the

sperm cells during rapid freezing with the minimum risk of profound toxic effects and 10per cent egg yolk (v/v) gave better post thaw motility than 20per cent. Thomas *et al.* (1993) observed that the post thaw progressive motility was greater in Tris citric acid diluent than that in BES lactose diluent. Nair (1996) used Tris fructose citric acid diluent for freezing of dog semen. In contrast to the above Ivanovo- Kicheva *et al.* (1997) found that semen diluted in Tris- lactose diluent and frozen in aluminium tubes had the highest motility and higher percentage of intact acrosomes when compared to Tris- glucose and Tris- fructose diluent. According to Rota *et al.* (1997) addition of 0.5 per cent Equex STM paste in Tris citrate glucose extender increased longivity of thawed spermatozoa and prolonged the maintenance of both motility and plasma membrane integrity.

Kadirvel (1998) compared three different extenders for preservation of dog semen under refrigeration temperature and found that egg yolk tris diluent was superior to egg yolk citrate glycine glucose diluent and goat milk diluent. Rota *et al.* (1998) reported that five per cent glycerol in egg yolk tris diluent resulted in better post thaw motility of spermatozoa than three per cent glycerol level. Farstad (2000) modified the tris egg yolk extender

by the addition of 0.5 per cent Equex STM paste and showed improved post survival rate during incubation at 38°C.

2.3.2. Dilution rate

Foote (1964) found that sperm motility was higher during storage when semen was diluted 1:30 with 20 million sperms per ml than diluted 1:3 with 200 million sperms per ml in mature Beagle dogs. Yubi *et al.* (1987) diluted the semen sample at a ratio of 1:5 with Tris citric acid fructose egg yolk glycerol diluent for freezing of dog semen. Yurdaydin and Kotzab (1987) suggested a dilution ratio of 1:3 with Tris citric acid egg yolk glucose glycerol diluent.

Linde-Forsberg *et al.* (1999) resuspended the semen pellete in an extender CLONE –A at room temperature as an initial dilution and second dilution was done at 5°C with the same volume of second extender CLONE–B with a final concentration of 100 to $300X10^6$ normal spermatozoa.

2.4. Freezing of dog semen

Although there have been many reports of successful freezing and thawing of dog semen (Foote, 1964; Andersen, 1972), there has been only a limited use of frozen dog semen mainly due

to poorer fertility. All the earlier workers had adopted methodology designed for other species although a thorough investigation has been attempted regarding comparison between diluents (Smith, 1985). The first success in freezing dog semen was that of Rowson (1954). Seager (1969) reported first canine pregnancy using frozen semen. Recent studies on cryopreservation of dog semen (Olar, 1985; Yubi *et al.*, 1987) were aimed to improve the diluents and freezing methods. Yu-Xueli *et al.* (1997) developed a freezing method in which semen was dropped directly into liquid nitrogen for five to eight seconds and then the semen pellets were frozen 2cm above the liquid nitrogen surface for six minutes which resulted in better post thaw motility when compared to common method using plastic plate over the liquid nitrogen.

2.4.1 Cooling, Glycerolisation and Equilibration

In freezing of dog semen only arbitrary values have been used for cooling rate and equilibration time before freezing. Seager (1969) suggested that equilibration time was not important. Later studies (Olar, 1985) suggested that there should be at least one hour cooling and one hour equilibration or two hour cooling and two hour equilibration period before freezing.

Farstad and Andersen-Berg (1989) and Wilson (1993) also reported that equilibration period of two hour at 5°C for freezing of dog semen. Hopkins and Evans (1989) recommended two step dilution of dog semen in which an extender (Pipes citrate glycine) containing glycerol was added dropwise to obtain a dilution ratio of 1:1. The extended semen was cooled in a refrigerator at 5°C for 30 minutes and an additional aliquot of extender cooled to 5°C was added dropwise to obtain a dilution of 1:2. England (1993) suggested that spermatozoa of several species required a pause of several hours of cooling, before freezing to develop maximal resistance to the effects of freezing.

Ravazova *et al.* (1996) have observed that 4per cent glycerol was most suitable concentration for cryopreservation of dog ejaculates and also found that with increasing glycerol concentration semen showed a reduction in the percentage of progressively motile spermatozoa. Rodriguez- Martinez *et al.* (1998) diluted semen sample with Tris fructose citric acid extender with 8per cent glycerol and then cooled to 4°C over 45 minutes and was given the equilibration period of two hours at 4°C. Nair (1996) found that the maximum post thaw motility was recorded for semen samples processed with 9per cent glycerol and four hour

equilibration period for cryopreservation of dog semen. Rota *et al.* (1997) reported that there was no significant effect of equilibration period on motility and plasma membrane integrity of dog spermatozoa.

2.4.2 Packaging of semen

The pellet method of semen freezing was adopted in many earlier studies. (Seager, 1969; Seager and Fletcher,1973). Seager *et al.* (1975) utilized pellet method of freezing and obtained better recoveries than that following storage in straws. However Davies (1982) did not find any difference between storage either by pellet or straw. Strom *et al.* (1997) recorded more than 70 per cent post thaw motility when 0.5ml of straws were used for cryopreservation of semen.

2.4.3 Rate of freezing

Andersen (1972), Farstad (1984) and Fontbonne and Badinand (1993) recommended rapid freezing by placing the straws on the rack above the liquid nitrogen. However attempts have been made to control freezing rates by placing straws at different heights

above the level of dry ice (Yubi *et al.*, 1987), above the liquid nitrogen (Battista *et al.*, 1988) and in forced vapour freezer, (Olar *et al.*, 1989). The effect of rate of freezing has not been systematically evaluated in the case of dog semen.

Farstad and Andersen-Berg (1989) described the fast freezing method and reported 60per cent post thaw motility with Tris, in contrast to this Olar *et al.* (1989) observed moderate freezing rate with 51 per cent post thaw motility.

Dobrinski *et al.* (1993) compared the three different freezing rates on post thaw viability of canine spermatozoa. The freezing rate was accomplished by placing the straws 20 cm above the liquid nitrogen for 30 minutes as slow freezing, 12 cm above the liquid nitrogen for 20 minutes as intermediate freezing and four cm above the liquid nitrogen for 10 minutes as fast freezing and found that the average percentage of progressively motile spermatozoa were 31.8±1.5, 27.5±1.5 and 24.2±1.5 respectively.

Nair (1996) recorded the higher percentage of post thaw motility of dog semen for slow freezing rates (46.5±1.9) than fast freezing rates (14.23±0.67) in a programmable freezer. Strom

et al. (1997) used Andersen method of freezing dog semen in which the 0.5 ml straws were placed four cm above the liquid nitrogen for 10 minutes and obtained 70 per cent post thaw motility.

2.4.4 Rate of thawing

It is well established that optimum cell survival requires an appropriate rate of thawing in addition to freezing rates. It is suggested that fast freezing requires fast thawing to reverse osmotic gradient, rehydrate and restore lipid protein configuration of membrane in a manner similar to events induced during freezing. (Mazur, 1985).

Accordingly there are reports of moderate to fast rates of thawing (Mazur, 1985). Andersen (1972) suggested thawing of dog semen in 75°C water bath for 6.5 seconds. Davies (1982) found a higher motility score when the samples were thawed at 70°C than at 37°C. Olar (1985) obtained higher post thaw motility when the samples were thawed at 75°C than at 37°C. Ivanova-Kicheava *et al.* (1995) observed the percentage of spermatozoa manifesting strong intensity or the reaction was comparatively higher after thawing at 55°C than at 37°C.

Hay *et al.*(1997) thawed the 0.5ml straws in water bath temperature of 70°C for 6 seconds while Strom *et al.* (1997) recommended thawing rate at 70°C for 8 seconds. However Farstad (1996) suggested the use of thawing rates recommended by the person or the laboratory providing the frozen semen since thawing rates are closely connected with the freezing protocol. Rota *et al.* (1998) found that thawing semen straws in 70°C water bath increased the spermatozoan recovery and longivity during incubation compared with thawing at 88°C.

2.4.5 Post thaw evaluation

2.4.5.1 Motility

Farstad (1984) suggested that the ultimate success of semen processing technique for frozen thawed semen is evaluation of fertility in terms of whelping rates. However a post thaw motility of 30 to 70 per cent for the samples stored in liquid nitrogen for the period of two months to eight years have also been recommended. Morton and Bruce (1989) recorded a post thaw motility of 61.2 per cent for freezing of dog semen. Wilson (1993) reported the percentage of motile spermatozoa ranged from 10 to

80. However England and Ponzio (1989) obtained a post thaw motility of 45 to 70 per cent in Beagle dog semen.

Kim and Kim (1995) found that post thaw motility of semen preserved at –196°C was significantly higher than that of semen preserved at –80°C on days 15 and 30 after freezing. The post thaw motility and viability of semen frozen with glycerol was significantly higher than that of semen frozen with DMSO. Silva and Verstegen (1995) recorded the post thaw motility of 80per cent in Tris egg yolk glycerol extender. Nair (1996) recorded the higher percentage of post thaw motility of dog semen for slow freezing rates (46.5±1.9per cent) than fast freezing rates (14.23±0.67) in a Programmable freezer. Strom *et al.* (1997) found the average post thaw progressive motility of 70 ± 5.1per cent and 75.3 ± 4 per cent with Andersen and CLONE method of freezing respectively .

2.4.5.2 Dead and abnormal spermatozoa

Yurdaydin and Kotzab (1987) found that the percentage of dead spermatozoa and morphologically abnormal spermatozoa were 33.12 and 27.91 respectively after thawing.

England and Ponzio (1996) observed that the percentage of normal live spermatozoa ranged from 20 to 32 after freeze-thaw process. Nair (1996) reported higher percentage of live spermatozoa and reduced percentage of abnormal spermatozoa at slow freezing rate compared to fast freezing rate. Nair *et al.* (1998) recorded the average live sperm percentage was 67.5 and abnormal sperm percentage was 26.92 after freeze-thaw procedure of semen sample obtained from mongrel dogs.

2.4.5.3 Acrosomal damage

Oettle (1986) observed that there was 31.75±10.18 per cent decrease in intact acrosome after freezing and thawing process. Strom Holst *et al.* (1998) found that the major acrosomal damage of spermatozoa occurred during freezing and thawing process rather than during the initial dilution and cooling. Rodriguez–Martinez *et al.* (1993) reported that spermatozoa subjected to freezing and thawing showed major changes in their morphology particularly in the acrosomal region and along the mid piece of sperm.

Ivanano-Kicheva *et al.* (1995) noted that thawing procedure could itself influence the canine acrosome with higher thawing rates resulting in a lower proportion of damaged acrosomes. Strom *et al.* (1997) observed that acrosome defects such as vesiculated and swollen acrosomes, acrosomal rupture and acrosomal loss were 45.7±5.3 and 44±9.2 per cent respectively under Andersen and CLONE methods. They further reported that proportion of spermatozoa with primary acrosomal abnormalities were 4.7±2.5 and 5.1±2.5 for the above two methods. Nair (1996) recorded the percentage of spermatozoa with damaged acrosomes as 36.27±0.6 per cent for slow freezing rate and 46.18±0.67 per cent for fast freezing rate.

Materials and Methods

3.MATERIALS AND METHODS

3.1 Animals

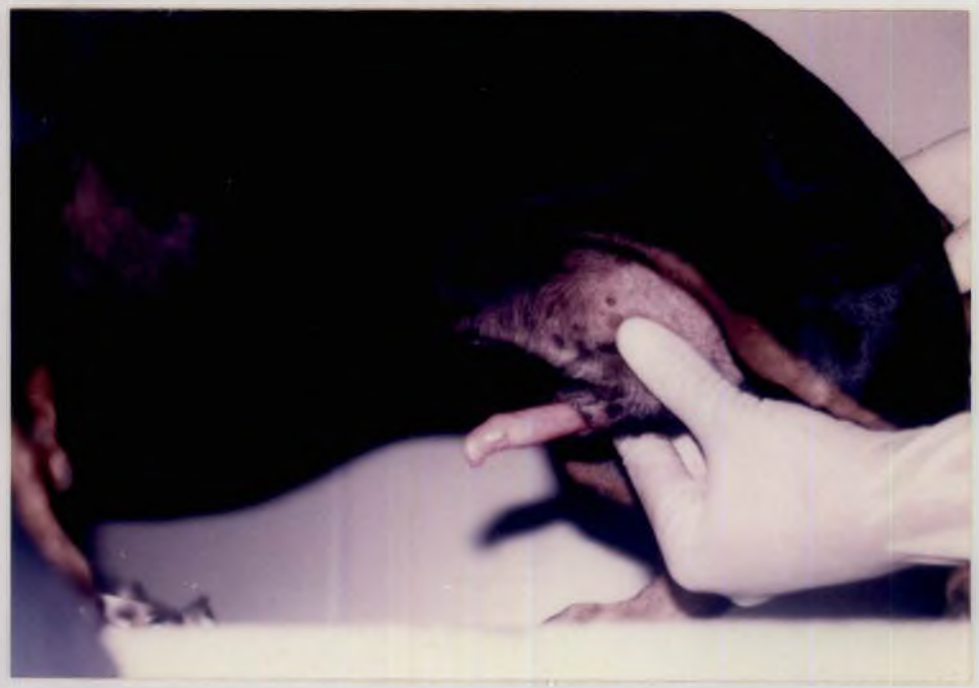
Six apparently healthy adult male Dachshunds aged between two to six years in and around Thrissur area, formed material for the study. Semen collection was made under strict hygienic conditions in the same environment. A total of 36 ejaculates, six ejaculates from each dog were collected at weekly intervals.

3.2 Collection of semen

Initially all the dogs were trained for semen collection by digital manipulation without the presence of a bitch. The dogs were allowed to stand on a platform while taking collection. By standing on one side of the dog the prepuce was gently massaged with a gloved hand to bring partial erection of penis (Plate 3.1). The prepuce was pushed backwards behind the bulbus glandis and continuous rhythmic pressure was applied behind the bulbus glandis by encircling with thumb and index finger. This caused full

Plate 3.1 **Stages of semen collection – Phase I**
Partial erection of penis

Plate 3.2 **Phase II**
Full erection of penis



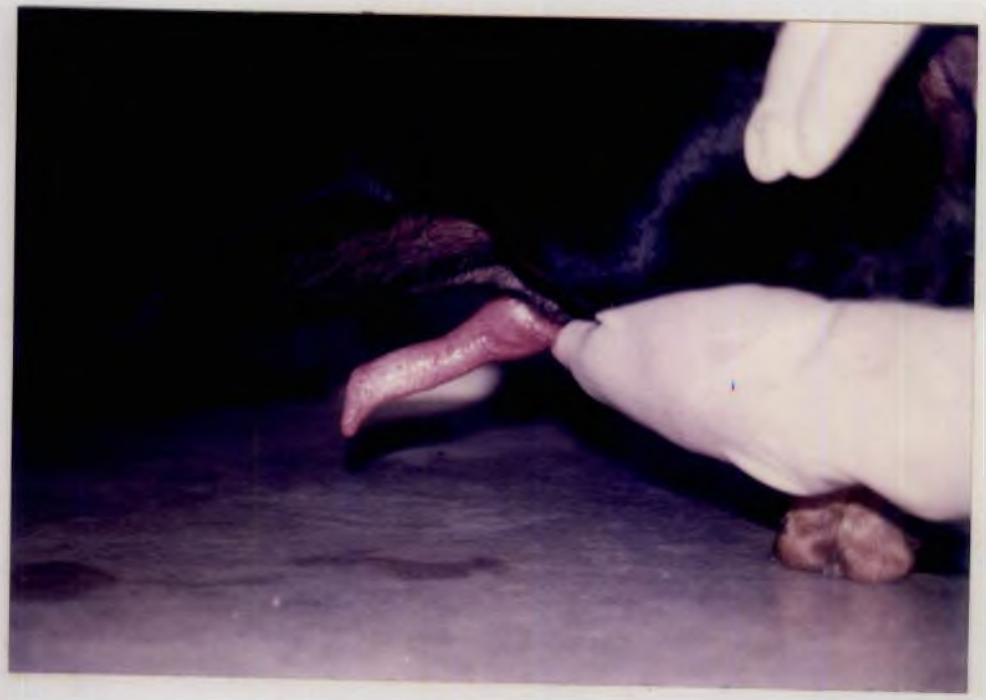
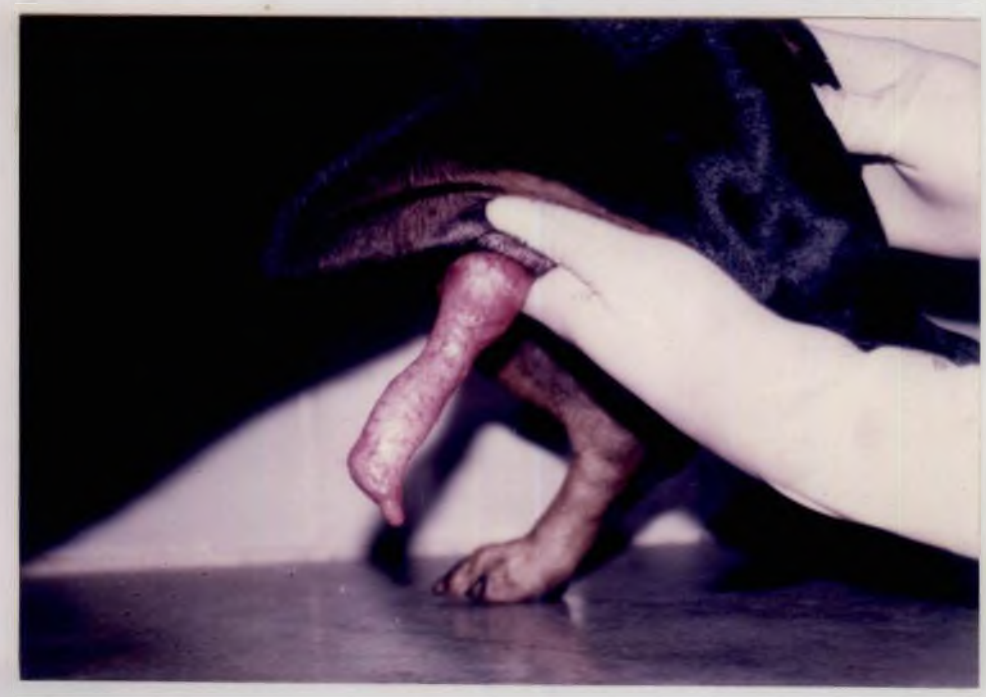


Plate 3.3 Phase III
Slight engorgement of penis

Plate 3.4 Phase IV
Full engorgement of penis



erection, (Plate 3.2) swelling and engorgement of bulbus glandis and pars longa glandis (Plate 3.3 & 3.4) associated with vigorous pelvic thrust resulting in ejaculation of pre sperm fraction. The animal then remained steady and sperm rich fraction was ejaculated followed by turning back by lifting the hind leg. At that time penis was bent downwards and backwards between the dog's hind legs and collected the post sperm fraction of the semen sample (Allen, 1991). All three fractions were collected in separate sterile graduated collection tubes maintained at 37°C.

3.3 Evaluation of semen

Immediately after semen collection sperm rich fraction of semen was evaluated for volume, colour, density, initial motility, pH, concentration, dead and abnormal spermatozoa and acrosomal integrity.

3.3.1 Volume

The volume of sperm rich fraction of the semen sample was recorded directly from the graduated semen collection tube.

3.3.2 Colour

The colour of the semen sample was assessed directly by visual observation against a white background.

3.3.3 Density

Density was recorded by keeping the collection tube against a source of diffused light.

3.3.4 Initial motility

Initial motility was assessed by placing a drop of semen sample in the pre-warmed clean glass slide over which a cover slip was gently placed and examined under high power of a microscope (400X).

3.3.5 pH

pH of the semen sample was estimated immediately after semen collection by using pH indicator paper (MERCK) having a range of five to 7.5.

3.3.6 Concentration

Concentration was assessed by using Neubaur Haemocytometer.

3.3.7 Dead and Live spermatozoa

The dead and live spermatozoa were identified by Vital staining technique (Ataman *et al.*, 1998). Two drops of five per cent Eosin and five drops of 10 per cent Nigrosin were placed in the pre-warmed glass slide, near to this a drop of semen sample was placed. All the drops were gently mixed and smear was prepared in a clean grease free glass slide and air-dried. The dead and live spermatozoa were counted microscopically under oil immersion (x 1000).

3.3.8 Sperm abnormalities

Sperm abnormalities in Eosin- Nigrosin stained smears were counted under oil immersion objective of microscope (x 1000).

3.3.9 Acrosome integrity

The acrosome integrity of spermatozoa was assessed by Giemsa staining technique (Sarma, 1995). A thin smear of semen was made on clean,grease free glass slide and air-dried. The dried smear was immersed in a staining jar containing methanol for 15 minutes at room temperature. The slide was then air dried and transferred to another jar containing staining solution consisting of stock Giemsa stain 6cc, 0.2M phosphate buffer 4cc and Millipore water 90cc. The stock Giemsa stain was prepared by dissolving one gram of Giemsa powder in 66ml of methanol and 60ml of glycerol. Then it was filtered and preserved in airtight containers. The slide was left for staining at 37°C for three to four hours and were taken out, washed in tap water, air dried and examined under oil immersion.

3.4 Semen Processing

3.4.1 Preparation of diluent

The extender used for freezing dog spermatozoa was Tris citric acid fructose egg yolk glycerol extender. The extender was prepared as Extender I and Extender II. (Rota *et al.*, 1998).

3.4.1.1 Composition of Extenders

	Extender I	Extender II
Tris hydroxy methyl amino methane (MERCK)	2.42 g	2.42 g
Citric acid (MERCK)	1.34g	1.34 g
Fructose(MERCK)	1.0 g	1.0 g
Distilled water	80 ml	80 ml
Dihydro streptomycin	1000µg	1000µg
Benzyl penicillin	1000IU	1000IU
Hen's egg yolk	10 ml	10 ml
Glycerol	-	8%

3.4.1.2 Egg yolk and Glycerol levels

Ten percent egg yolk and four per cent glycerol were used in freezing dog semen in this study (England, 1993).

3.4.2 Methods of dilution

The rate of dilution was fixed according to concentration of spermatozoa. The dilution rate of 1:2 to 1:4 was used so as to adjust the concentration between 50 to 100 million sperms per ml. The sperm rich fraction was half diluted with

extender I at room temperature. Then the semen sample was evaluated for motility, dead and live spermatozoa, abnormal spermatozoa and acrosomal damage.

3.4.3 Chilling

The tubes containing initially diluted semen sample and extender II were kept in water bath at 37°C and transferred to cold handling unit maintained at a temperature of 5°C for 1½ hours. At 5°C the semen was assessed for sperm motility, dead and live spermatozoa, sperm abnormalities and acrosomal damage.

3.4.4 Glycerolisation

After cooling to 5°C the extender II was added to half diluted semen in three steps at 10 minutes interval to obtain a final glycerol concentration of four percent.

3.4.5 Equilibration

The extended semen sample was equilibrated for a period of three hours before freezing. Then the semen sample was

examined for motility, dead and live sperm count, sperm abnormalities and acrosomal damage.

3.4.6 Packaging of semen

Packaging was done in 0.5ml French medium straws of different colours to code the various freezing protocols. For packaging of semen sample straws of different colours, filling comb, bubbler comb, bubbler dish, straw clip, freezing rack, freezing ramp, towel, cotton, poly venyl alcohol powder etc were kept in the cold handling unit and pre-cooled. Filling of straw was carried out after 1½ hour of equilibration and straws were allowed to equilibrate another 1½ in the cold handling unit. Thereafter straws were arranged in straw clip and it was attached to a filling comb at the factory seal end. The comb was connected to a vacuum pump. After gently mixing, the semen was transferred to the bubbler dish and the open ends of the straws attached to the comb were dipped in it and semen was filled using negative pressure. Air space was created in the straw by using bubbler comb. Then the open end of the straws were sealed with poly venyl alcohol powder of 5mm thickness. The excess powder sticking to the straw were removed by sterile cotton. After removing from the clip and comb,

the straws were immersed in water bath kept at 5°C. Then the straws were taken out and dried by using towel and were arranged in freezing rack.

3.5 Technique of freezing and storage

Freezing was performed by using a wide mouthed liquid nitrogen freezer (Plate 3.5). The level of liquid nitrogen maintained upto the level of grill in the freezer. Then the straws arranged in the freezing rack were transferred to the freezer (Plate 3.6) from the cold handling unit with quick motion. Freezing was done under three different protocols as 4 cm height above the liquid nitrogen level for 10 minutes (fast freezing), 8 cm for 15 minutes (moderate freezing) and 12 cm for 20 minutes (slow freezing rates). The filled straws along with freezing rack were placed above the grill level and exposed the straws to liquid nitrogen vapour for different freezing rates. After subjecting the straws to each freezing rates the lid was opened and frozen straws were collected quickly by gloved hand and loaded into the pre-cooled goblets and plunged into the liquid nitrogen. A label showing the freezing date, dog number, rate of freezing was tied on the handle of the canister for identification of the straws.

Plate 3.5 Wide mouthed liquid nitrogen freezer

Plate 3.6 Straws arranged above the grill for freezing


M V E
Cryogenics

3.5.1 Thawing rate

Fifteen days after freezing straws were thawed out in water bath temperature of 70°C for eight seconds.

3.5.2 Post thaw evaluation

The straws for thawing was picked up from the storage container with pre-cooled forceps within seconds. By simple jerking of the straw the liquid nitrogen drop was removed. The straws were then placed in a water bath at 70°C for eight seconds. Then they were dried with sterile cotton and by cutting both ends of the straw, the semen was transferred to sterile test tube for evaluation. Motility was assessed by visual examination of wet mount preparations using high power objective of microscope on pre-warmed glass slide. Semen smears were made for evaluation of dead and live sperm count, sperm abnormalities and acrosomal damage.

The results were statistically analyzed as per standard procedures (Snedecor and Cochran, 1967).

Results

4.RESULTS

A study was carried out to compare the effect of three different freezing rates on post thaw motility, live sperm count, sperm abnormalities and acrosomal damage of canine spermatozoa. Semen samples from six Dachshund dogs in and around Thrissur area were collected and utilized for freezing.

A total of 36 ejaculates comprising of six ejaculates from each Dachshund dog were used for the study.

4.1 Collection of semen

The semen collection was done by digital manipulation. The behaviour of pelvic thrust was coincident with the ejaculate of pre sperm fraction within 15 to 30 seconds. The animals became steady when the sperm rich fraction was ejaculated over ½ to one minute. Then the penis was bent backward and the post sperm fraction of the semen was collected over a period of five to 10 minutes.

4.2 Preliminary evaluation of fresh semen

4.2.1 Semen volume

The mean volume of pre sperm ,sperm rich and post sperm fraction of semen samples were 0.49±0.05ml, 0.68±0.03ml and 2.32±0.09 ml respectively (Table 4.1).There was highly significant ($p<0.01$) difference in the volume of pre sperm fraction of semen between dogs. There was no significant difference between volume of sperm rich and post sperm fraction of semen ejaculates between dogs.

4.2.2 Colour and consistency

The colour and consistency of the sperm rich fractions were thin milky and that of pre sperm and post sperm fractions were clear and watery (Table 4.1).

4.2.3 Density

The overall mean density of sperm rich fraction of semen was DD (D) with a range of DD to DDD (Table 4.1).

4.2.4 Initial motility

The average initial sperm motility was observed to be 75±0.93 per cent with a range of 70 to 80 per cent. (Table 4.2 and Figure 4.1). There was no significant difference in the initial motility of semen between dogs.

4.2.5 pH

The mean pH of sperm rich fraction of semen sample was 6.63±0.02 with a range of 6.4 to 6.9 (Table 4.1). There was significant ($P<0.01$) variation in pH of semen between dogs.

4.2.6 Concentration

The average spermatozoal concentration of sperm rich fraction of semen sample was recorded to be 221.50±7.36 millions per ml with a range of 105 to 312 millions per ml (Table 4.1). There was highly significant ($P<0.01$) difference in the sperm concentration of semen from different dogs.

4.2.7 Live sperm count

The mean live sperm count in semen sample was found to be 81.17±0.73 per cent with a range of 72.36 to 88.24 per cent. (Table 4.2 and Figure 4.2). There was significant ($P<0.05$) variation in live sperm count in semen of different dogs.

4.2.8 Sperm abnormalities

The mean percentage of head, mid piece and tail abnormalities were 1.83±0.52, 1.20±0.04 and 2.99±0.05 respectively. The mean percentage of total abnormal sperm count was 5.23±0.29 with a range of 2.04 to 5.55. (Table 4.2 and Figure 4.3). The mean percentage of proximal and distal protoplasmic droplets in semen were 1.01±0.18 and 1.16±0.09 respectively (Table 4.2). The sperm abnormalities encountered in this study were micro head, detached head, thick middle piece, coiled tail, bent tail and presence of proximal and distal protoplasmic droplets (Plate 4.2 to 4.4). The percentage of sperm abnormalities between dogs was found to be highly significant ($P<0.01$).

4.2.9 Acrosomal damage

The average percentage of acrosomal damage recorded in fresh semen was 2.32+-0.25 with a range of 0.8 to 6.48 (Table 4.2 and Figure 4.4).

4.3 Effect of dilution, chilling and equilibration period on motility and morphological characteristics of canine spermatozoa

4.3.1 Dilution effect

The mean percentage of sperm motility, live sperm count, abnormal sperm count and acrosomal damage of semen immediately after dilution were 70.41±1.22, 75.63±0.65, 7.28±0.43 and 5.34±0.31 respectively. (Table 4.3 and Figure 4.1 to 4.4). Significant ($P<0.01$) reduction in percentage of motility and livability and increase in sperm abnormalities and acrosomal damage were observed after dilution of semen sample (Table 4.6). A highly significant ($P<0.01$) variations in percentage of motility, livability, sperm abnormalities and acrosomal damage were found between dogs (Table 4.3).

4.3.2 Chilling effect

The average percentage of sperm motility and live sperm count were significantly (P<0.01) reduced to 58.75±1.34 and 63.61±0.89 respectively. The mean percentage of sperm abnormalities and acrosomal damage were significantly (P<0.01) increased to 10.04±0.32 and 10.13±0.41 respectively (Table 4.6 and Figure 4.1 to 4.4). There was highly significant (P<0.01) variation between dogs in the percentage of motility, livability, sperms abnormalities and acrosomal integrity (Table 4.4).

4.3.3 Equilibration effect

The average spermatozoal motility, live sperm count, sperm abnormalities and acrosomal damage of semen samples were 47.78±1.59, 50.65±1.31, 11.79±0.36 and 16.20±0.57 per cent respectively (Table 4.5 and Figure 4.1 to 4.4). There was a highly significant (P<0.01) difference in motility, live sperm count, sperm abnormalities and acrosomal damage of semen between dogs. The motility and live sperm count of semen samples were significantly (P<0.01) reduced and the sperm abnormalities and acrosomal

damage were significantly (P<0.01) increased after equilibration period (Table 4.6).

4.4 Effect of different freezing rates on motility and morphological characteristics of canine spermatozoa, 15 days after freezing

4.4.1 Post thaw motility

The mean percentage of post thaw motility of semen obtained from six Dachshund dogs in fast, moderate and slow freezing rates were 34.31±1.66, 25.83±1.40 and 24.44±1.27 respectively. (Table 4.7 and Figure 4.5). There was a highly significant (P<0.01) variation in post thaw motility of semen between fast and moderate freezing rates and fast and slow freezing rates but no significant difference between moderate and slow freezing rates (Table 4.7). There was a highly significant (P<0.01) difference in percentage of motility between dogs (Table 4.7).

4.4.2 Live sperm count

The average percentage of live sperm count of semen for fast, moderate and slow freezing rates were 38.26±1.52, 29.03±1.31 and 27.89±1.35 respectively(Table 4.8 and Figure 4.6). There was a highly significant (P<0.01) variation in percentage of

live sperm count of semen between fast and moderate freezing rates and fast and slow freezing rates but no significant variation between moderate and slow freezing rates for all five dogs except one dog where there was no significant difference between three methods (Table 4.8). The mean percentage of live sperm count of semen samples significantly ($P<0.01$) varied between dogs (Table 4.8).

4.4.3 Sperm abnormalities

The mean percentages of abnormalities of head, mid piece and tail were 5.35±0.29, 1.43±0.25 and 9.30±0.38 for protocol I, 6.19±0.30, 1.55±0.13 and 10.14±0.47 for protocol II and 6.34±0.29, 2.07±0.32 and 10.83±0.52 for protocol III respectively. The overall mean percentage of total sperm abnormalities of semen in fast, moderate and slow freezing rates were 15.3±0.73, 17.25±0.55 and 18.55±0.52 respectively (Table 4.9). There was a highly significant ($P<0.01$) difference in percentage of sperm abnormalities in fast and moderate freezing rates and fast and slow freezing rates but no significant difference between slow and moderate freezing rates but in one dog no significant variation was noticed between three freezing rates (Table 4.9).

There was highly significant ($P<0.01$) variation in percentage of total sperm abnormalities between dogs (Table 4.9).

4.4.4 Acrosomal damage

The mean percentage of acrosomal damage of semen samples in fast, moderate and slow freezing rates were 27.39±0.73, 29.06±0.69 and 29.96±0.69 respectively (Table 4.10 and Figure 4.8). The acrosomal damagesencountered in this study were swollen, loosening and lost acrosomes (Plate 4.5 to 4.7). There was a significant increase in the percentage of acrosomal damage after freezing in all three freezing protocols. But no significant difference was observed between three freezing protocols in five dogs. But significant ($P<0.05$) variation was found between fast and moderate freezing rates and fast and slow freezing rates in one dog (Table 4.10). The overall mean percentages of acrosomal damage of semen samples between dogs were found to be significant (Table 4.10).

Table 4.1 Physical characteristics of fresh semen

Dog No.	Volume (ml)(Mean ± SE)			Colour			Density (sperm rich)	pH (sperm rich)	Concentration (sperm rich) millions / ml
	Pre sperm	Sperm rich	Post sperm	Pre sperm	Sperm rich	Post sperm			
I.	0.23±0.06	0.68±0.03	2.17±0.31	Clear & watery	Milky	Clear & watery	DD(D)	6.55±0.04	200.67±9.09
II.	0.23±0.03	0.60±0.09	2.67±0.21	Clear & watery	Milky	Clear & Watery	DD(D)	6.63±0.04	239.83±12.31
III.	0.67±0.13	0.78±0.09	2.33±0.21	Clear & watery	Milky	Clear & Watery	DD	6.77±0.05	165.17±12.76
IV.	0.68±0.08	0.67±0.06	2.08±0.20	Clear & watery	Milky	Clear & Watery	DD	6.67±0.02	248.67±15.69
V.	0.48±0.11	0.68±0.06	2.08±0.27	Clear & Watery	Milky	Clear & Watery	DD(D)	6.63±0.04	210.00±7.54
VI.	0.65±0.11	0.68±0.07	2.58±0.15	Clear & watery	Milky	Clear & watery	DDD	6.55±0.04	264.67±15.51
Mean	0.49±0.05	0.68±0.03	2.32±0.09				DD(D)	6.63±0.02	221.50±7.36
F value	5.11**	0.73 NS	1.22 NS					3.65*	8.52**

* Significant (P< 0.05)
** Highly significant (P<0.01)
NS Non significant

Table 4.2 Motility and Morphological characteristics of fresh semen (Mean ± SE)

Dog No.	Initial motility (%)	Live sperm Count (%)	Sperm abnormalities (%)				Proximal protoplasmic droplets (%)	Distal protoplasmic droplets (%)	Acrosomal damage (%)
			Head	Mid piece	Tail	Total			
I.	75.00±2.34	80.29±2.15	1.54±0.09	1.23±0.03	1.94±0.12	4.09±0.58	3.24±0.31	1.45±0.31	2.06±0.65
II.	76.67±3.33	83.63±1.32	1.35±0.04	0.81±0.01	2.75±0.01	3.96±0.49	0.70±0.07	1.56±0.23	1.60±0.26
III.	70	75.50±1.39	1.90±0.04	1.38±0.09	4.24±0.03	6.75±0.44	1.05±0.22	1.27±0.19	4.36±0.69
IV.	75.00±2.24	82.59±1.91	2.48±0.08	1.89±0.01	3.88±0.11	7.31±0.55	0.79±0.99	1.25±0.12	1.24±0.12
V.	76.67±2.11	81.67±0.78	2.14±0.03	0.95	3.05±0.03	5.35±0.39	0.91±0.03	1.11±0.16	3.09±0.45
VI.	76.67±2.10	83.32±0.77	1.55±0.03	0.92	2.05±0.02	3.96±0.35	0.85±0.52	0.89±0.46	1.59±0.21
Mean	75.00±0.93	81.17±0.73	1.83±0.52	1.20±0.04	2.99±0.05	5.23±0.29	1.01±0.18	1.16±0.09	2.32±0.25
F Value	1.33^{NS}	4.18*				9.855**			6.80*

* Significant (P< 0.05)
** Highly significant (P< 0.01)
NS Non significant

Table 4.3 Motility and Morphological characteristics of spermatozoa after dilution (Mean± SE)

Dog No.	Motility	Live sperm Count (%)	Total Sperm abnormalities (%)	Proximal protoplasmic droplets (%)	Distal protoplasmic droplets (%)	Acrosomal damage (%)
I	69.17±2.71	73.14±1.42	4.56±0.18	1.29±0.69	1.43±0.42	6.15±0.75
II	75.83±2.39	76.57±1.45	5.32±0.39	0.75±0.17	1.84±0.37	4.83±0.44
III	60.83±0.83	71.26±1.19	11.11±0.98	1.44±0.39	1.19±0.33	6.92±0.70
IV	73.33±3.07	78.62±1.48	8.59±0.72	1.77±0.14	1.92±0.41	3.93±0.38
V	68.33±1.67	75.70±0.79	7.87±0.31	0.93	1.79±0.28	6.58±0.61
VI	75.00±2.24	78.48±0.87	6.20±0.44	1.08±0.08	1.02±0.08	3.65±0.46
Mean	70.41±1.22	75.63±0.65	7.28±0.43	1.24±0.15	1.58±0.15	5.34±0.31
F Value	6.07**	5.678**	17.84**			6.026**

* Significant (P< 0.05)
** Highly significant (P< 0.01)

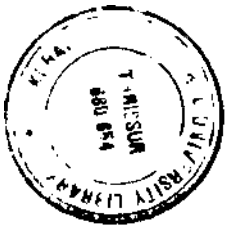


Table 4.4 Motility and Morphological characteristics of spermatozoa after chilling (Mean ± SE)

Dog No.	Motility (%)	Live sperm Count (%)	Sperm abnormalities (%)	Proximal protoplasmic droplets (%)	Distal protoplasmic droplets (%)	Acrosomal damage (%)
I.	53.33±1.67	60.26±1.39	9.52±0.40	0.85	1.34±0.28	12.07±0.82
II.	65.00±1.83	64.93±1.99	6.99±0.59	-	1.39±0.44	8.79±0.44
III.	48.33±1.05	56.94±1.24	10.15±0.61	0.61±0.12	0.80±0.07	10.79±0.41
IV.	62.50±3.10	67.23±2.04	11.69±0.43	1.18±0.10	1.79±0.16	8.06±0.56
V.	57.50±1.71	64.12±1.09	11.41±0.51	1.38±0.23	1.83±0.18	13.33±0.48
VI.	65.83±2.71	68.20±1.31	10.47±0.31	1.10±0.08	1.26±0.17	7.75±0.38
Mean	58.75±1.34	63.61±0.89	10.04±0.32	1.14±0.10	1.40±0.11	10.13±0.41
F value	10.754**	7.582**	12.029**			18.252**

* Significant (P< 0.05)
** Highly significant (P< 0.01)

Table 4.5 Motility and Morphological characteristics of spermatozoa after equilibration period (Mean ± SE)

Dog No.	Motility (%)	Live sperm Count (%)	sperm abnormalities (%)	Proximal protoplasmic droplets (%)	Distal protoplasmic droplets(%)	Acrosomal damage (%)
I	39.17±3.00	44.56±1.56	11.02±0.82	0.81±0.03	1.41±0.31	16.37±0.69
II	55.83±2.39	54.51±4.01	8.43±0.61	0.98	0.89±0.05	13.43±0.99
III	37.50±1.71	42.93±1.58	11.72±0.34	1.42±0.51	1.36±0.20	19.35±0.74
IV	52.50±3.09	54.51±2.39	13.11±0.55	1.12±0.11	1.96±0.39	12.84±0.91
V	45.83±2.01	48.14±1.48	12.90±0.25	1.04±0.13	2.25±0.28	19.86±1.13
VI	55.83±2.71	59.28±1.46	13.58±0.46	1.09	1.23±0.15	15.37±0.93
Mean	47.78±1.59	50.65±1.31	11.79±0.36	1.08±0.10	1.54±0.13	16.20±0.57
F value	10.43**	7.989**	12.448**			10.386**

* Significant ($P< 0.05$)
** Highly significant ($P< 0.01$)

Table 4.6 Effect of dilution, chilling and equilibration period on motility and morphological characteristics of spermatozoa (Mean ± SE)

Semen	Motility (%)	Live sperm count (%)	Sperm abnormalities (%)	Acrosomal damage(%)
Fresh	75.00±0.93[a]	81.17±0.73[a]	5.23±0.29[a]	2.32±0.25[a]
Diluted	70.41±2.24[b]	75.63±0.65[b]	7.28±0.43[b]	5.34±0.31[b]
Chilled	58.75±1.34[c]	63.61±0.89[c]	10.04±0.32[c]	10.13±0.41[c]
Equilibrated	47.78±1.59[d]	50.65±1.31[d]	11.79±0.36[d]	16.20±0.57[d]
F Value	89.367**	212.72**	84.914**	250.814**
CD	3.627	1.616	0.1614	0.1944

** Highly significant (P<0.01)
Figures having different superscript differs significantly.

Table 4.7 Percentage of post thaw motility of spermatozoa in three different freezing protocols (Mean ± SE)

Freezing Protocol	Dog I	Dog II	Dog III	Dog IV	Dog V	Dog VI	Mean	F value	CD
I	23.33 ± 1.67^{aB}	43.33± 2.11^{aA}	25.00± 1.83^{aB}	42.50± 3.10^{aA}	29.17± 1.54^{aB}	42.50± 1.71^{aA}	34.31± 1.66	21.208**	5.94
II	17.50± 1.12^{bC}	35.00± 2.58^{bA}	20.00± 1.29^{bC}	25.83± 2.01^{bB}	20.83± 1.54^{bBC}	35.83± 2.01^{bA}	25.83± 1.40	18.750**	5.27
III	17.50± 1.12^{bC}	32.50± 1.71^{bA}	18.33± 1.67^{bC}	25.00± 2.24^{bB}	20.00± 1.29^{bBC}	33.33± 2.11^{bA}	24.44± 1.27	16.585**	5.01
F value	6.477**	6.88**	4.643**	15.709**	12.065**	5.915 **			
CD	3.99	6.52	4.85	7.51	4.39	5.87			

* Significant (P< 0.05)

** Highly significant (P< 0.01)

Mean bearing different superscripts (small letters) in a row differs significantly

Mean bearing different superscripts (capital letters) in a column differs significantly.

Table 4.8 Percentage of live sperms in three different freezing protocols (Mean ± SE)

Freezing protocol	Dog I	Dog II	Dog III	Dog IV	Dog V	Dog VI	Overall mean	F value	CD
I	31.17± 1.19^{aB}	49.25± 1.65^{aA}	29.65± 1.33^{aB}	44.20± 2.58^{aA}	30.18± 1.37^{B}	44.59± 2.43^{aA}	38.26± 1.52	21.962**	3.18
II	22.92± 1.88^{bCD}	38.54± 2.01^{bA}	20.86± 1.07^{bD}	28.13± 2.37^{bB}	26.65± 1.29^{BC}	37.07± 1.71^{bA}	29.03± 1.31	16.34**	3.29
III	22.86± 1.29^{bBC}	37.18± 2.33^{bA}	18.83± 1.14^{bC}	25.50± 2.56^{bB}	25.90± 1.59^{B}	37.12± 1.29^{bA}	27.89± 1.35	17.83**	3.32
F value	9.681**	10.394**	23.284**	15.299**	2.535**	5.341**			
CD	3.03	3.6	2.41	4.76	2.78	3.3			

* Significant (P< 0.05)

** Highly significant (P< 0.01)

Mean bearing different superscripts (small letters) in a row differs significantly

Mean bearing different superscripts (capital letters) in a column differs significantly.

Table 4.9 Percentage of Sperm abnormalities in three different freezing protocols (Mean± SE)

Freezing Protocol	Sperm abnormalities	Dog I	Dog II	Dog III	Dog IV	Dog V	Dog VI	Over all mean	F Value	CD
I	Head	4.65±0.27	3.45±0.20	5.05±0.24	4.98±0.27	7.07±0.47	6.92±0.32	5.35±0.29		
	Mid Piece	1.77±0.23	1.18±0.19	1.17±0.12	1.64±0.48	-	1.39±0.22	1.43±0.25		
	Tail	8.60±0.41	6.35±0.35	9.24±0.30	10.47±0.32	11.06±0.47	10.05±0.45	9.30±0.38		
	Total	14.14 ± 0.61^{bD}	10.39 ± 0.49^{bE}	15.27 ± 0.27^{bCD}	16.24 ± 0.67^{bBC}	18.13 ± 0.83^{bA}	17.66 ± 0.24^{AB}	15.30±0.73	27.089**	0.21
II	Head	6.06±0.27	4.04±0.35	5.38±0.20	6.56±0.49	8.14±0.27	6.99±0.24	6.19±0.30		
	Mid Piece	1.37±0.05	1.51±0.03	2.06±0.24	1.56±0.28	1.40	1.37±0.05	1.55±0.13		
	Tail	9.68±0.46	6.90±0.54	10.66±0.57	11.41±0.48	12.06±0.34	10.12±0.43	10.14±0.47		
	Total	16.43 ± 0.56^{aC}	11.44 ± 0.71^{abD}	17.75 ± 0.74^{aBC}	19.28 ± 0.83^{aAB}	20.39 ± 0.41^{aA}	18.2 ± 0.65^{BC}	17.25±0.55	24.502**	0.24
III	Head	6.48±0.37	4.11±0.33	6.23±0.32	5.74±0.36	8.46±0.15	7.27±0.26	6.34±0.29		
	Mid Piece	1.87±0.43	1.81±0.27	1.74±0.29	2.56±0.47	2.78	1.65±0.13	2.07±0.32		
	Tail	10.16±0.34	8.34±0.71	11.70±0.47	11.78±0.29	12.51±0.31	10.49±0.67	10.83±0.47		
	Total	17.58 ± 0.66^{aC}	13.55 ± 0.84^{aD}	19.09 ± 0.63^{aBC}	20.02 ± 0.35^{aBC}	21.94 ± 0.69^{aA}	19.13 ± 0.98^{BC}	18.55±0.52	16.129**	0.25
F Value		8.349**	5.296*	11.475**	9.783**	8.139**	1.085^{NS}			
CD		0.23	0.30	0.21	0.23	0.23	0.24			

* Significant (P< 0.05)

** Highly significant (P< 0.01)

NS Non significant

Mean bearing different superscripts (small letters) in a row differs significantly.

Mean bearing different superscripts (capital letters) in a column differs significantly.

Table 4.10 Percentage of acrosomal damage of spermatozoa in three different freezing protocols (Mean ± SE)

Freezing protocol	Dog I	Dog II	Dog III	Dog IV	Dog V	Dog VI	Overall mean	F value	CD
I	30.16± 1.53^{A}	23.89± 1.48^{C}	31.67± 1.62^{A}	23.87± 1.23^{C}	29.10± 0.83^{AB}	25.66± 1.18^{bBC}	27.39± 0.73	6.267**	3.88
II	31.22± 1.46^{AB}	26.19± 1.39^{CD}	33.05± 1.82^{A}	24.98± 1.13^{D}	29.81± 1.33^{BC}	29.08± 0.75^{aBC}	29.06± 0.69	5.025**	3.91
III	31.10± 1.18^{AB}	28.25± 1.00^{AB}	34.33± 1.76^{A}	25.36± 1.45^{C}	31.38± 1.52^{AB}	29.22± 0.95^{aBC}	29.96± 0.69	5.284**	3.87
F Value		2.784^{NS}	0.585^{NS}	0.369^{NS}	0.835^{NS}	4.281*			
CD						2.94			

* Significant (P< 0.05)

** Highly significant (P< 0.01)

Mean bearing different superscripts (small letters) in a row differs significantly

Mean bearing different superscripts (capital letters) in a column differs significantly.

Fig.1 Percentage of sperm motility in fresh, diluted, chilled and equilibrated semen samples

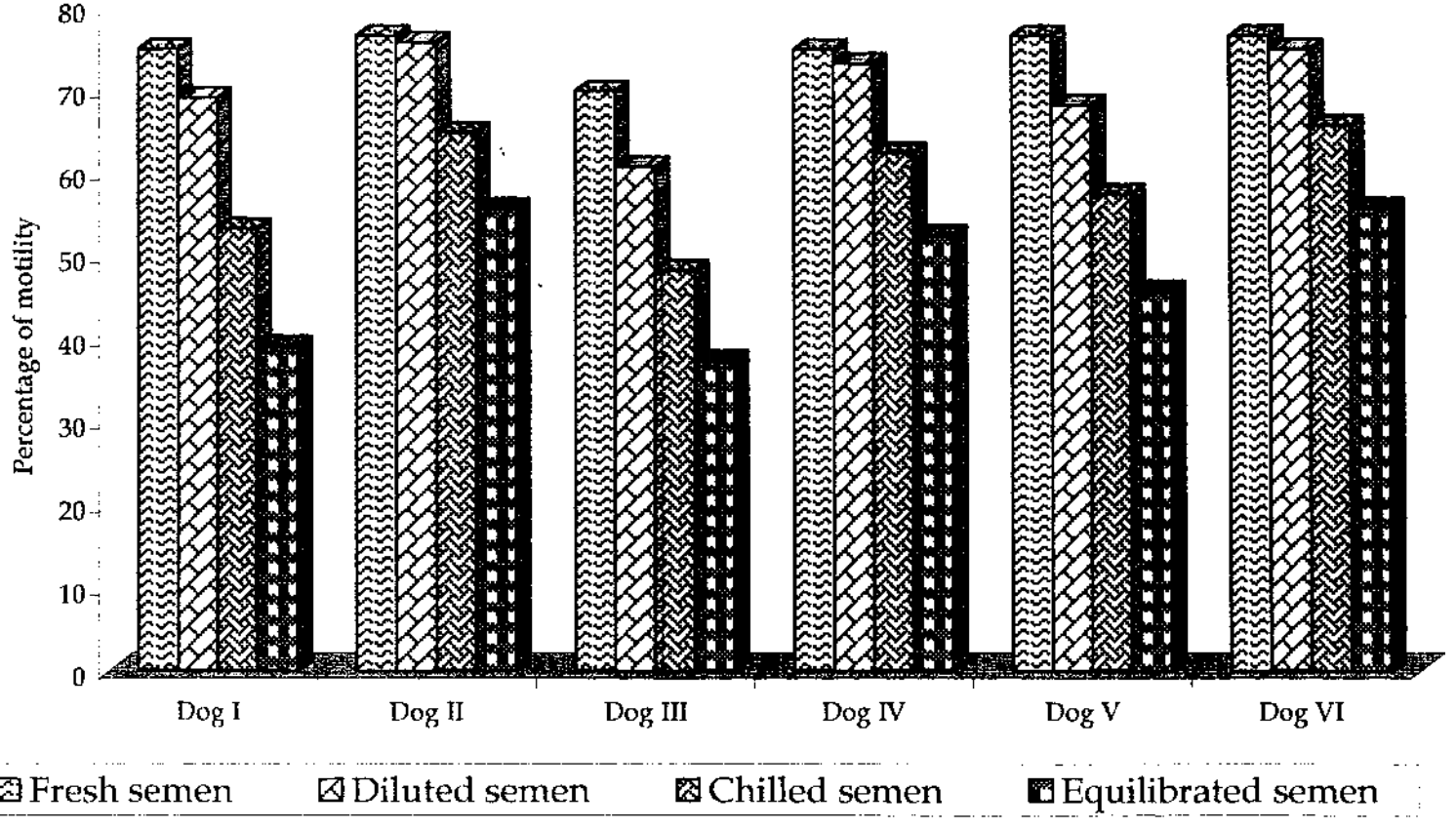


Fig.2 Percentage of live sperms in fresh, diluted, chilled and equilibrated semen samples

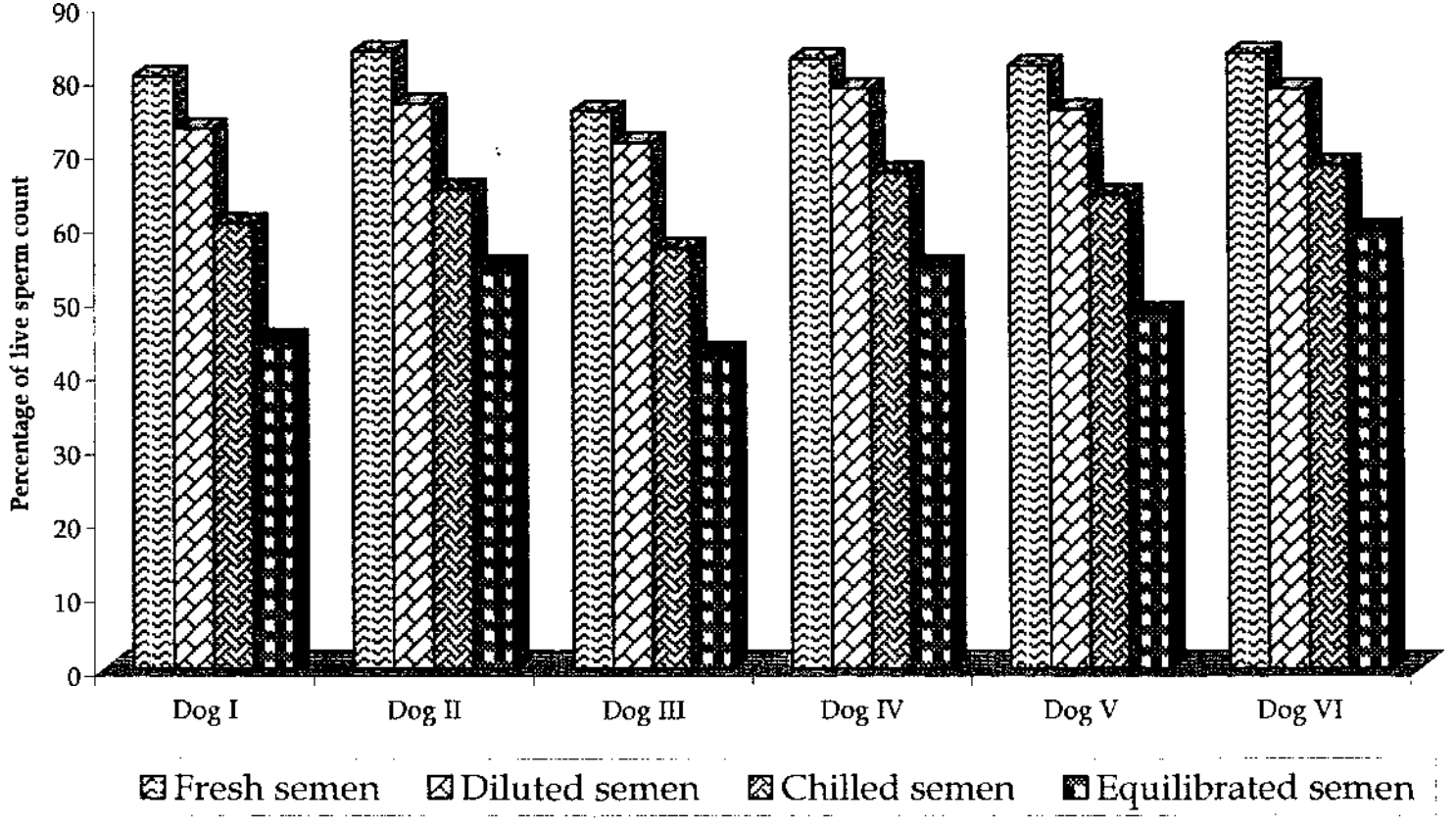


Fig.3 Percentage of sperm abnormalities in fresh, diluted, chilled and equilibrated semen samples

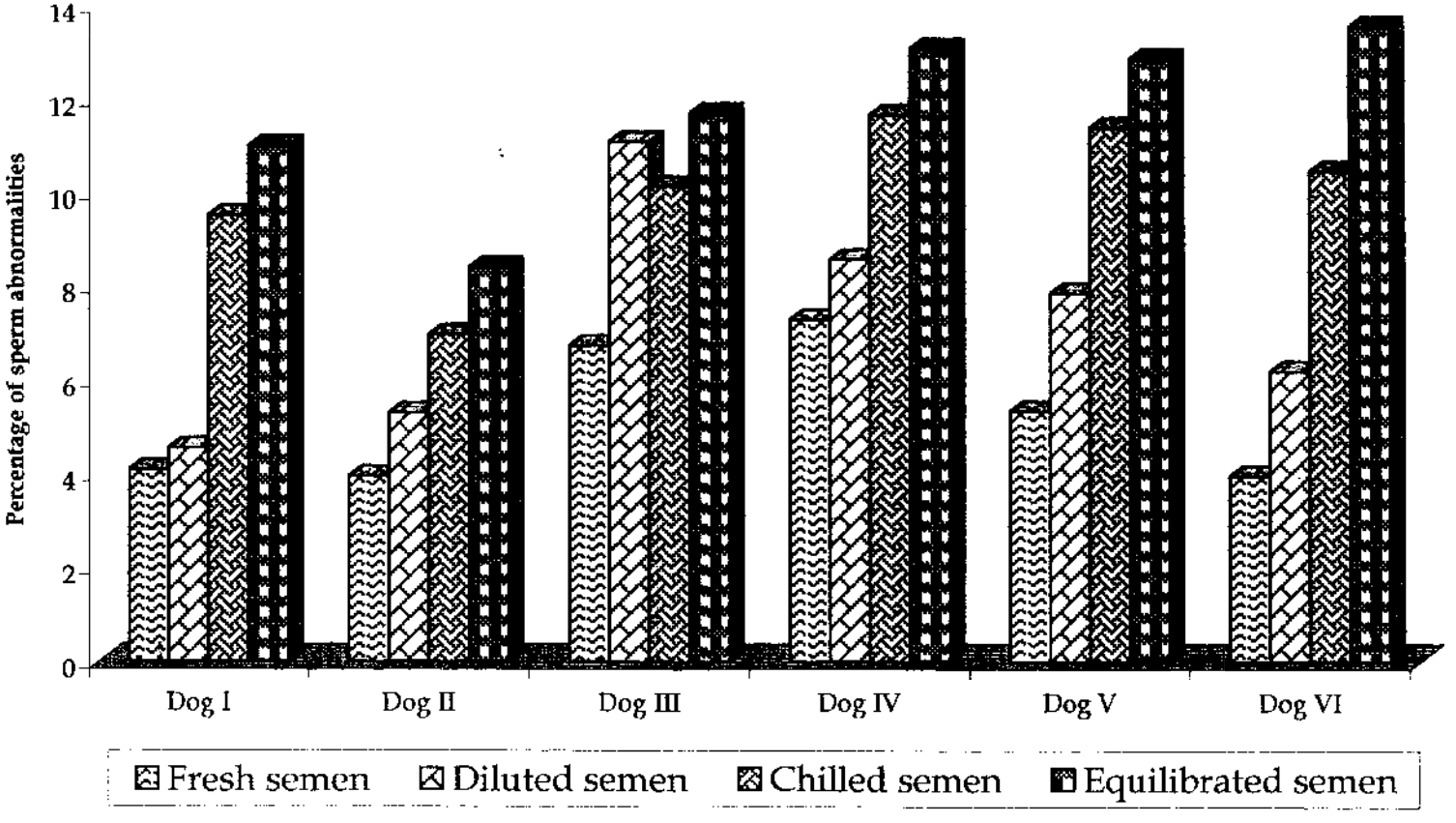


Fig.4 Percentage of ascrosomal damange in fresh, diluted, chilled and equilibrated semen samples

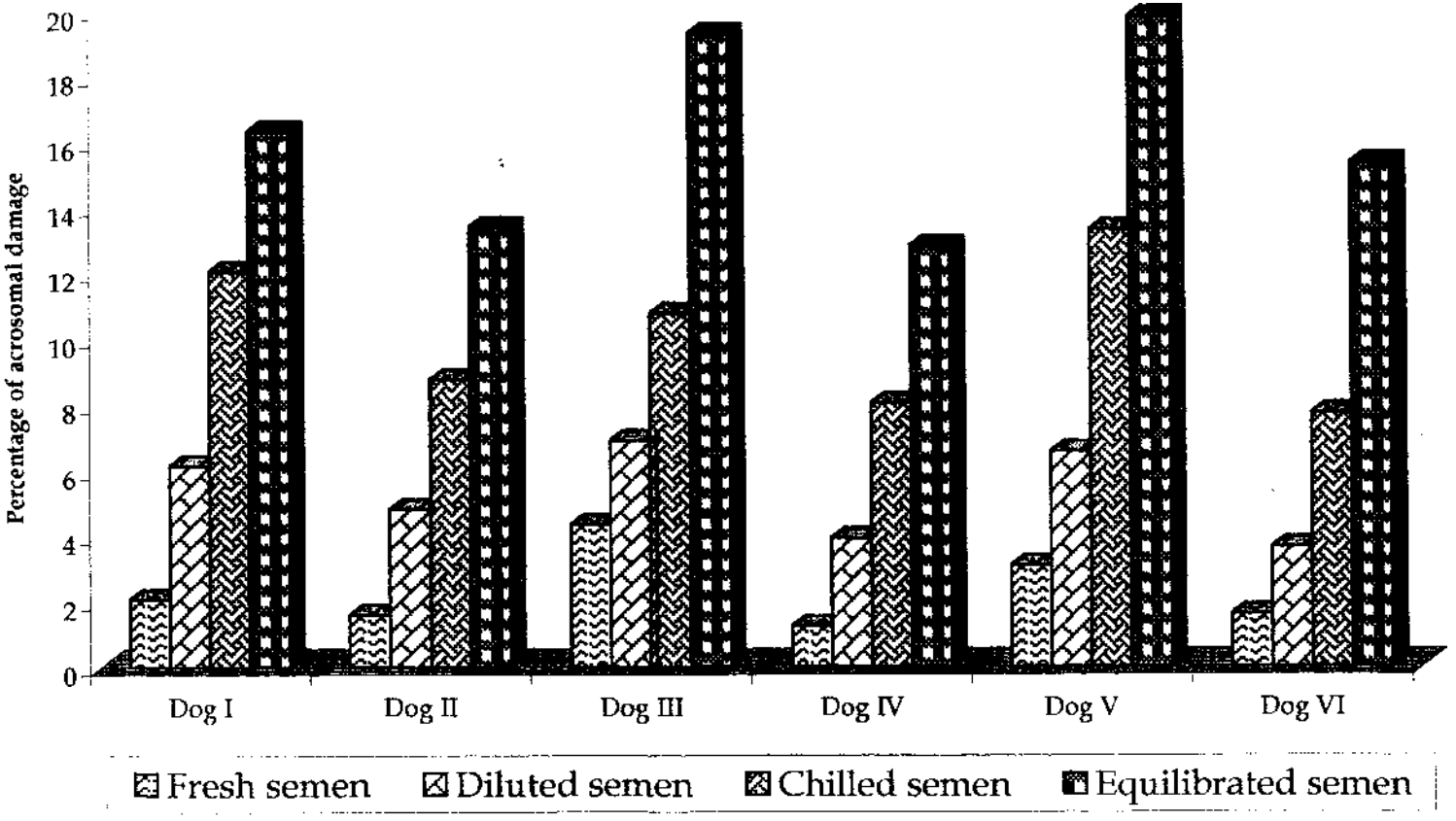


Fig.5 Percentage of post thaw motility of spermatozoa in three different freezing protocols

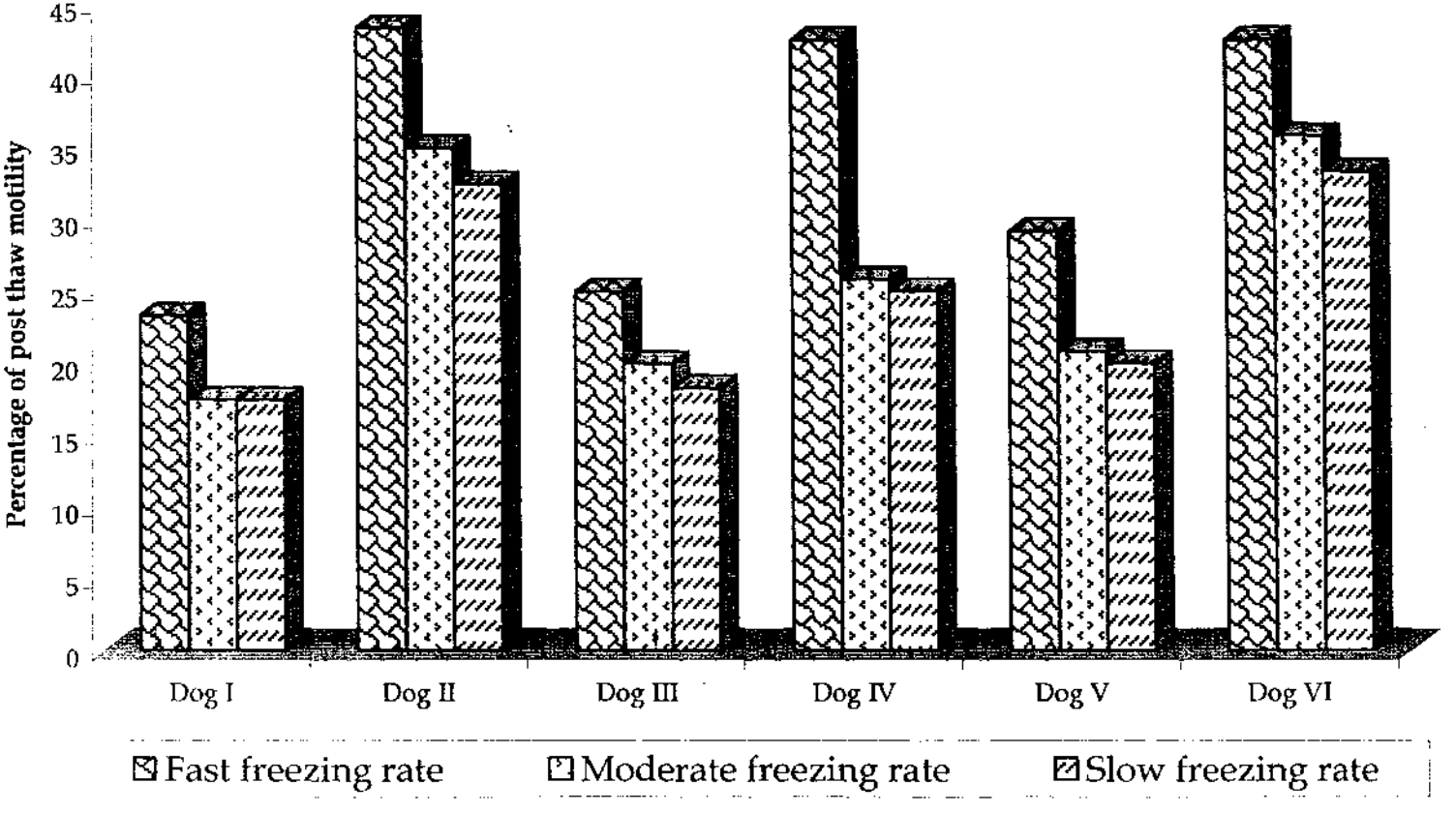


Fig.6 Percentage of live sperms in three different freezing protocols

Fig.7 Percentage of sperm abnormalities in three different freezing protocols

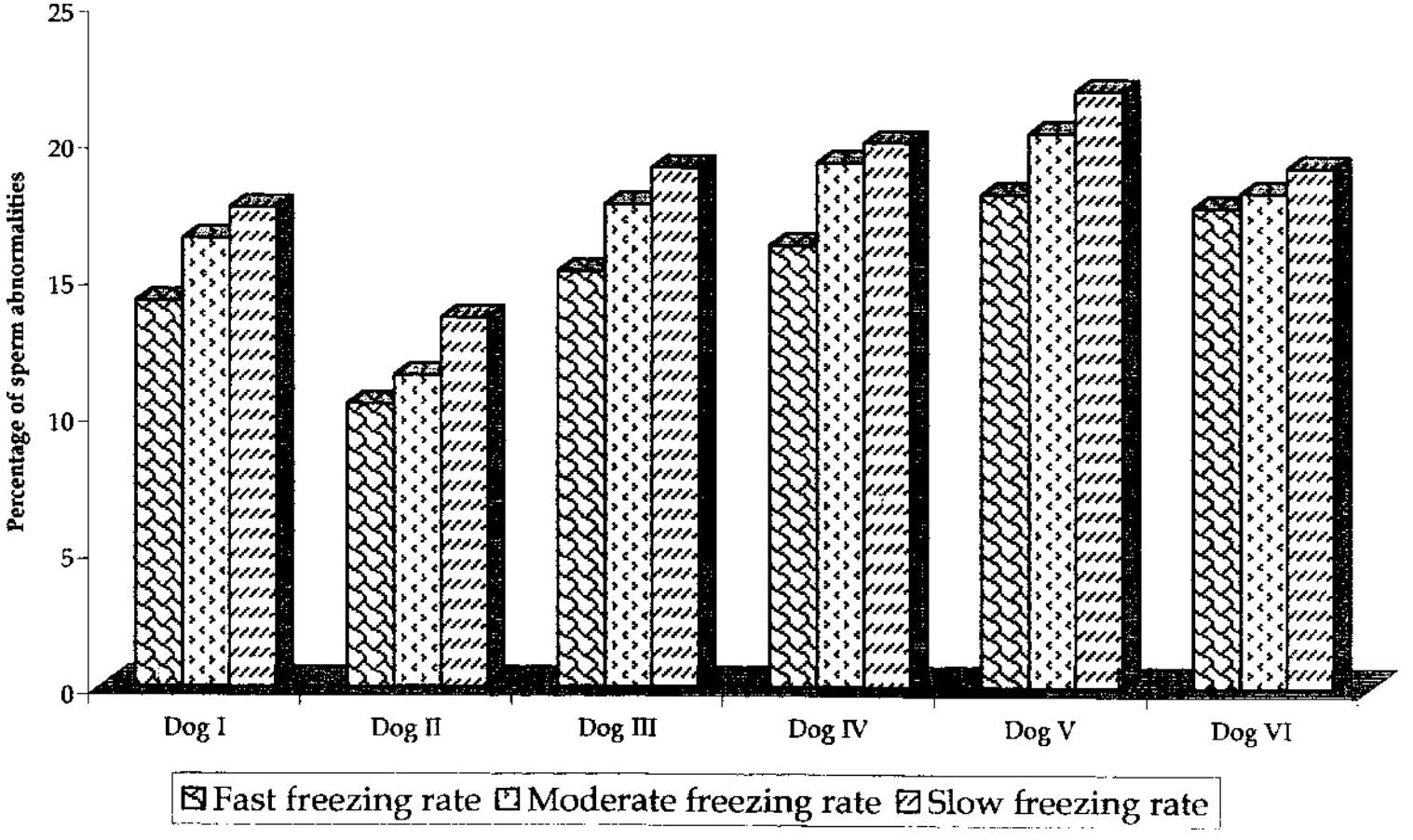


Fig.8 Percentage of acrosomal damage in three different freezing protocols

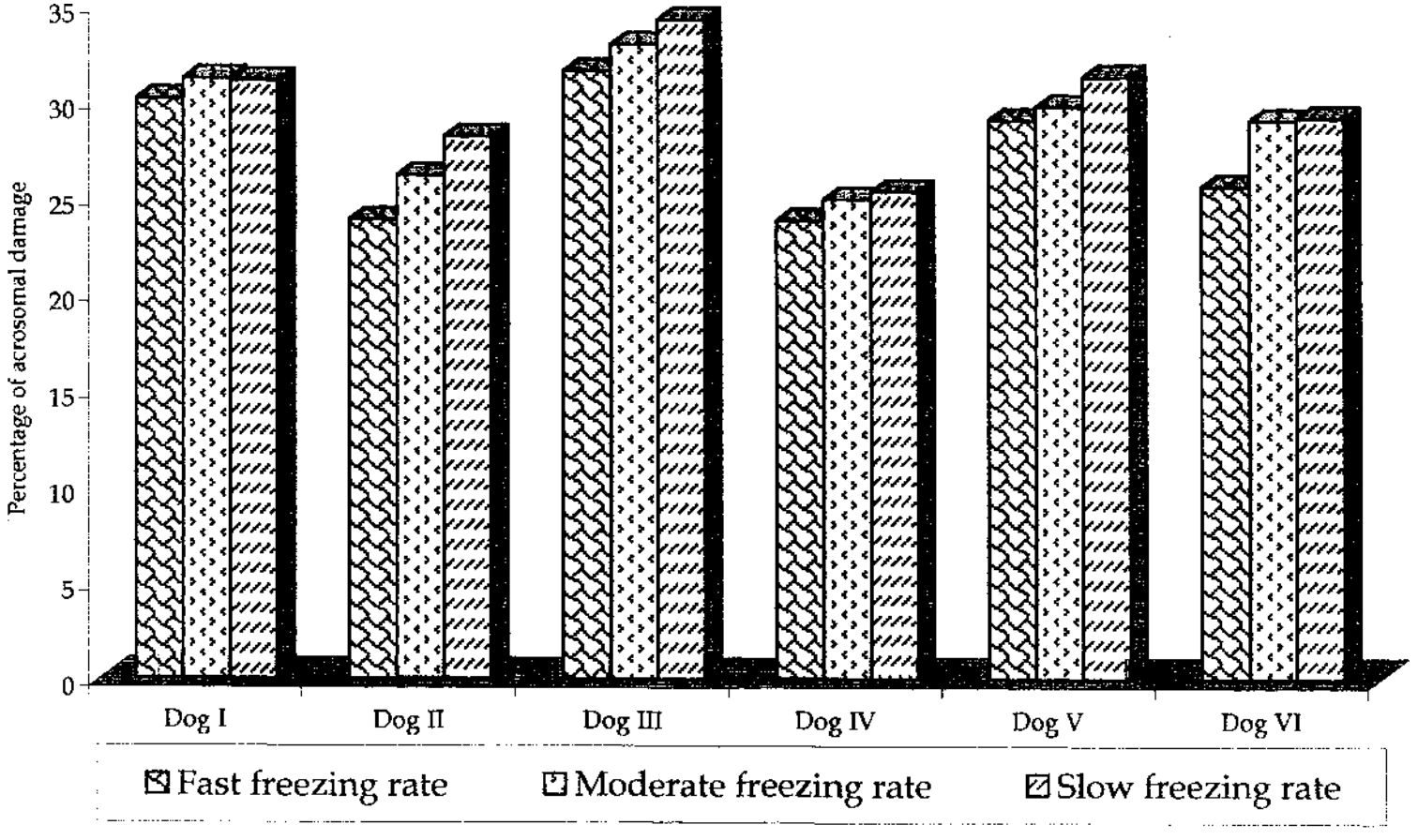


Plate 4.1 Dead and live spermatozoa
(E & N Staining, x 1000)

Plate 4.2 Sperm abnormalities - Bent tail
(E & N Staining, x 1000)

Plate 4.3 Detached head
(E & N Staining, x 1000)

Plate 4.4 Distal protoplasmic droplet
(E & N Staining, x 1000)

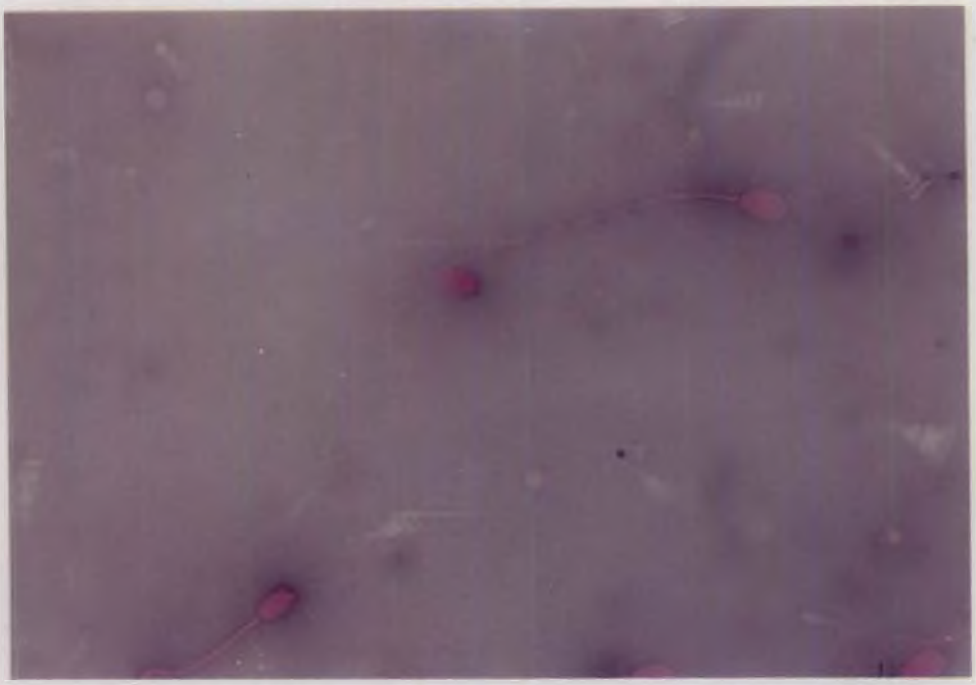
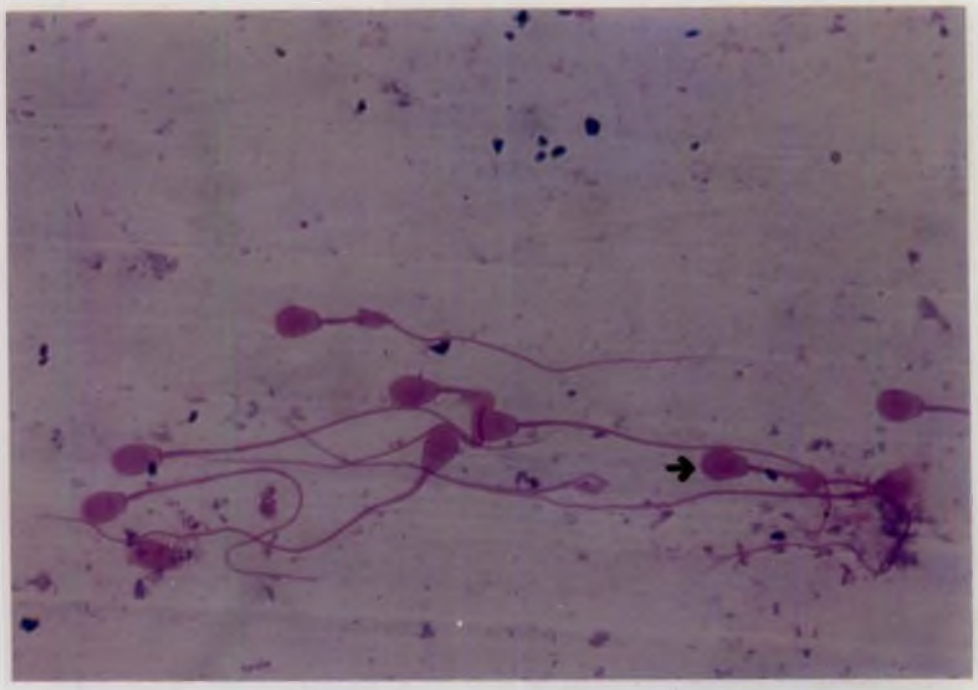


Plate 4.5 Normal acrosomes
→ Swollen acrosome
(Giemsa Staining, x 1000)

Plate 4.6 Loosening acrosome
(Giemsa Staining, x 1000)



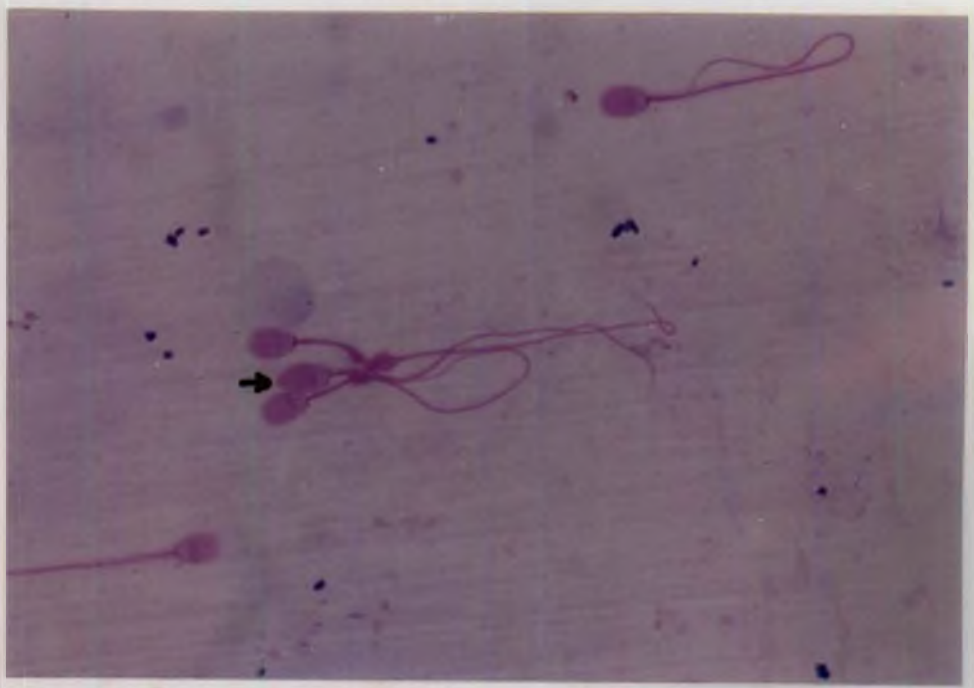
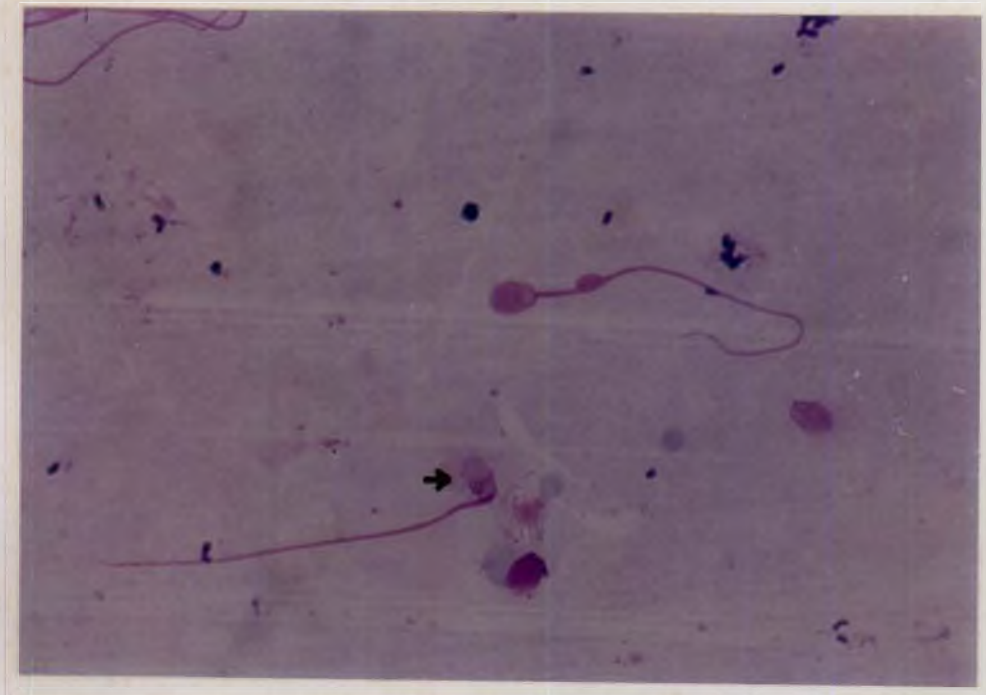


Plate 4.7 Lost acrosome – Broken neck
(Giemsa Staining, x 1000)



Discussion

5.DISCUSSION

Semen samples from six Dachshund dogs were subjected to freezing for assessing the effect of different freezing rates on canine spermatozoa.

5.1 Collection of semen

Semen collection was done by digital manipulation described by Hopkins and Evans (1989), Dabas *et al.* (1999) and Rota *et al.* (1995). The admingling of various fractions of the ejaculate affected the freezing quality of semen sample as reported by Fougner (1977). Hence collection of three fractions were obtained in three separate collection vials and sperm rich fraction was used for freezing. The semen from each dog was collected at weekly intervals. This is in accordance with the study carried out by Rodriguez–Gil *et al.* (1994).

The time required for emission of each fraction of semen from dogs obtained in this study was almost similar to that reported by Harrop (1955); Boucher *et al.* (1958); Arthur *et*

al.(1996) and Kadirvel (1998). The behaviour of pelvic thrust was coincident with ejaculation of pre sperm fraction and the animal became steady when the sperm rich fraction was ejaculated. This is akin to the observation of Morton and Bruce (1989).

5.2 Evaluation of semen

The seminal characteristics like volume, colour, density, pH, concentration and initial motility of sperm rich fraction of the ejaculate were assessed.

The average volume of pre sperm, sperm rich and post sperm fraction of semen from Dachshund dogs recorded as 0.49±0.05ml, 0.68±0.03ml and 2.32±0.09ml were almost in accordance with the values reported by Deshpande *et al.* (1970). The volume of sperm rich fraction recorded in this study is almost similar to observation recorded by Taha *et al.* (1981) in Beagle dogs. However higher values for all the three fractions were recorded by Dobrinski *et al.* (1993) and Kadirvel (1998). This could be probably due to difference in size and breed of the dog.

Colour and consistency of pre sperm and post sperm fraction of the semen was found to be clear and watery and sperm rich fraction was milky in nature. This is consistent with the findings of Daiwadnya *et al.* (1995) and Kadirvel (1998). The density of sperm rich fraction recorded was in accordance with Kadirvel (1998).

The initial motility of fresh semen was found to be 75±0.93 per cent with a range of 70 to 80 per cent. Almost similar values were recorded by Gill *et al.* (1970); Rota *et al.* (1995) and Strom *et al.* (1997) in different breeds of dogs. Higher values were reported by Morton and Bruce (1989); Nair (1996) and Kadirvel (1998). However lower initial sperm motility was reported by Olson (1992) and Mickelsen *et al.* (1993). The mean pH of sperm rich fraction of semen noted in this study was 6.63±0.02 within a range of 6.4 to 6.9. This is in agreement with the value recorded by Harrop (1956) and Boucher *et al.* (1958). Lower values are reported by Wales and White (1958), Dobrinski *et al.* (1993) and England (1993).

The average concentration of sperm rich fraction of semen was found to be 221.50±7.36 millions per ml with a range of

105 to 312 millions per ml. This is almost akin to the values recorded by England and Allen (1989) and Daiwadnya and Hukeri (1993). But Roychoudhury and Dubey (1974), Rodriguez-Martinez *et al.* (1993) and Guerin *et al.* (1999) have reported lower values and Nair (1996) and Kadirvel (1998) have recorded higher sperm concentration. Persual of available literature showed wide variation in the sperm concentration of various breeds of dogs. The low concentration recorded in the present study may be attributed to the small size of Dachshund dogs.

The percentage of live sperm count obtained was 81.17±0.73, which is comparable with the value reported by Daiwadnya and Hukeri (1993). The lower percentage of dead sperm count was reported by Taha *et al.* (1981); Yubi *et al.* (1987) and Kumi-Diaka and Badtram (1994). However higher values of live sperm percentage was recorded by Nair (1996) and Kadirvel (1998) and lower percentage was noted by Nothling *et al.* (1997).

The mean percentage of sperm abnormalties was observed to be 5.23±0.29 in fresh semen. Which is lower than the values obtained by Morton and Bruce (1989); Dobrinski *et al.*

(1993) and Kadirvel (1998) and higher than the values were reported by Nair (1996).

The mean percentage of damaged acrosome was 2.32±0.25 in fresh semen. A similar value was noted by Kumi-Diaka (1993) and higher values were reported by England and Ponzio (1996), Nair (1996) and Kadirvel (1998).

5.3 Effect of dilution, chilling and equilibration period on motility and morphological characteristics of dog spermatozoa

5.3.1 Dilution effect

The mean percentages of motility, live sperm count, sperm abnormalties and acrosomal damage of semen samples immediately after dilution were 70.41±1.22, 75.63±0.65, 7.28±0.43 and 5.34±0.31 respectively. (Table 4.3) There was significant ($P<0.01$) reduction in motility and live sperm count and significant ($P<0.01$) increase in sperm abnormalties and acrosomal damage after dilution (Table 4.6). This could be probably due to effect of diluent and its composition used in this study.

5.3.2 Chilling effect

Average percentage of motility, live sperm count, sperm abnormalities and acrosomal damage of semen sample after chilling was found to be 58.75+1.34, 63.61+0.89, 10.04+0.32 and 10.13+0.41 respectively. There was significant ($P<0.01$) reduction in motility and livability and an increase in sperm abnormalties and sperms with acrosomal damage after the process of chilling (Table 4.6). The damage can be attributed to the expected cold shock at refrigeration temperature (Kadirvel, 1998).

5.3.3 Equilibration effect

The mean percentages of motility, live sperm count, sperm abnormalities and acrosomal damage of spermatozoa after equilibration period were 47.78±1.59, 50.65±1.31, 11.79±0.36 and 16.20±0.57 respectively (Table 4.5). The spermatozoan motility and live sperm count were significantly ($P<0.01$) decreased after equilibration period (Table 4.6). This is not in agreement with the study conducted by Rota *et al.* (1997). Higher percentage of sperm abnormalities and acrosomal damages were also recorded in this study. This could be probably due to the deleterious effect of glycerol on canine spermatozoa.

5.4 Effect of freezing rates on motility and morphological characteristics of spermatozoa 15 days after freezing

5.4.1 Post thaw motility

The post thaw motility is considered to be a prime factor in determining the fertility of dog semen. The mean percentagesof motility obtained in fast, moderate and slow freezing rates were 34.31±1.66, 25.83±1.40 and 24.44±1.27 respectively (Table 4.7). There was highly significant decrease in sperm motility after freezing. This may be due to temperature change during freezing and thawing resulting in sperm death. In fast freezing, the post thaw motility obtained in this study was higher when compared to moderate and slow freezing rates. A similar trend was reported by Wilson (1993), England and Ponzio (1996) and Strom *et al.* (1997). But Dobrinski *et al.* (1993) and Nair (1996) were recorded higher post thaw motility in slow freezing rate than fast freezing rate.

5.4.2 Live and abnormal sperm count

The average percentages of live spermatozoa after thawing recorded in fast, moderate and slow freezing rates were 38.26±1.52, 29.03±1.31 and 27.89±1.35 respectively. (Table 4.8).

This is akin to the observation reported by England and Ponzio (1996). But higher values were reported by Yurdaydin and Kotzab (1987), Nair (1996) and Nair *et al.* (1998). A similar finding was reported for abnormal sperm count. The mean percentages of sperm abnormalities in three freezing protocols were 15.30±0.73, 17.25±0.55 and 18.55±0.52 respectively. Higher percentages were reported by Yurdaydin and Kotzab (1987) and Nair *et al.* (1998).

5.4.3 Acrosomal damage

Post thaw acrosomal integrity may also be a useful indicator of semen quality and has been shown to decrease significantly during the freeze-thaw process. Acrosomal damage in three freezing protocols were recorded as 27.39±0.73, 29.06±0.69 and 29.96±0.69 per cent respectively (Table 4.9). Higher percentage of acrosomal damage (36.27+0.6) was recorded by Nair (1996) in fast freezing rates. A highly significant increase in acrosomal damage was recorded after freezing and thawing process. Though there was no significant difference found between three freezing protocols, a lower percentage of acrosomal damage was noted in fast freezing rate compared to moderate and slow freezing rates.

In fast freezing, the straws exposed to critical temperature is minimum when compared to moderate and slow freezing. This could be the reason for high post thaw motility and livability of spermatozoa in fast freezing.

In conclusion, the result of this study indicated that fast freezing rate maintain higher percentage of post thaw motility and livability and lower percentage of sperm abnormalities when compared to moderate and slow freezing rates. Eventhough the percentage of acrosomal damage did not differ significantly in fast, moderate and slow freezing rates, higher values were recorded in moderate and slow freezing rates when compared to fast freezing rates. Among the three protocols of freezing canine semen, protocol I where the straws were subjected to freezing at a height of 4cm above the liquid nitrogen level for 10 minutes was found to be superior when compared to the protocols II and III.

Summary

6. SUMMARY

A study was conducted to find out the effect of different freezing rates on post thaw motility, livability and acrosomal damage of spermatozoa in Tris diluent. Semen samples collected from six Dachshund dogs in and around Thrissur were utilized for this study. A total of 36 ejaculates, six ejaculates from each dog were collected at weekly intervals. The semen collection was done by digital manipulation. The good quality semen samples extended in Tris citric acid fructose egg yolk diluent with four per cent glycerol were subjected to three different freezing protocols as 4cm height above the liquid nitrogen level for 10 minutes (Fast freezing), 8cm for 15 minutes (Moderate freezing) and 12cm for 20 minutes (Slow freezing). The semen was evaluated for motility, live sperm count, abnormal sperm count and acrosomal damage after initial dilution, chilling, equilibration and fifteen days after freezing.

The mean volume of sperm rich fraction was observed to be 0.68±0.03 ml. The colour and consistency of sperm rich fractions were thin milky in nature. The mean density of sperm rich

fraction was DD (D) and mean pH was observed to be 6.63±0.02. The average initial motility of sperm rich fraction was 75±0.93 per cent. The average spermatozoal concentration of sperm rich fraction was 221.0±7.36 millions per ml. The mean live sperm count, sperm abnormalities and acrosomal damage of spematozoa in the sperm rich fraction was found to be 81.17±0.73 per cent, 5.23±0.29 per cent and 2.32± 5per cent respectively. Significant ($P< 0.05$) difference in pH, concentration, live and abnormal sperm count and acrosomal damage of spermatozoa in sperm rich fraction was found between dogs.

The mean percentages of motility, live sperm count, abnormal sperm count and acrosomal damage of spermatozoa immediately after dilution were 70.41±1.22, 75.63±0.65, 7.28±0.43 and 5.34±0.31 respectively. After chilling, the percentages of motility and livability were reduced to 58.75±1.34 and 63.60±0.89 respectively and abnormal sperms as well as acrosomal damage were increased to 10.04±0.32 and 10.13±0.41 respectively. The mean percentages of motility, live and abnormal sperm count and acrosomal damage of spermatozoa after equilibration was found to be 47.78±1.59, 50.65±1.31, 11.79±0.36 and 16.20±0.57 respectively. Significantly ($p<0.01$) high reduction in motility and

live sperm count and increase in sperm abnormalities and acrosomal damage of sperms were observed after dilution, chilling and equilibration period. There was significant ($P< 0.01$) variation in motility, livability, abnormal sperms and acrosomal damage of spermatozoa on dilution, chilling and equilibration period between dogs.

The mean percentages of post thaw motility of spermatozoa in fast, moderate and slow freezing rates were 34.31±1.66, 25.83±1.40 and 24.44±1.27 respectively. The percentage of sperm motility was significantly ($P< 0.01$) higher in fast freezing rate compared to moderate and slow freezing rates between dogs.

The average percentages of live sperm count recorded in fast, moderate and slow freezing rates were 38.26±1.52, 29.03±1.31 and 27.89±1.35 respectively. Significantly ($P< 0.01$) higher percentage of live sperms in fast freezing rate was noted when compared to moderate and slow freezing rates.

The mean percentages of head, mid piece and tail abnormalities were 5.35±0.29, 1.43±0.25 and 9.30±0.38 for freezing protocol I, 6.19±0.30, 1.55±0.13 and 10.14±0.47 for

protocol II and 6.34±0.29, 2.07±0.32 and 10.83±0.52 for protocol III respectively. The over all mean percentages of sperm abnormalities found in fast, moderate and slow freezing rates were 15.30±0.73, 17.25±0.55 and 18.55±0,52 respectively. There was highly significant ($P<0.01$) difference in percentage of sperm abnormalities between fast and moderate freezing rates and fast and slow freezing rates and between dogs.

Average percentages of acrosomal damage of spermatozoa in fast, moderate and slow freezing rates were 27.39±0.73, 29.06±0.69 and 29.96±0.69 respectively. There was significant increase in the percentage of acrosomal damage after freezing in all three freezing protocols. There was slight increase in acrosomal damage in moderate and slow freezing rates compared to fast freezing rates. Significant ($P< 0.01$) variation in acrosomal damage of spermatozoa was found between dogs.

The above study revealed that fast freezing where the straws were frozen at 4cm height above the liquid nitrogen level for 10 minutes maintained higher percentage of sperm motility and livability and lower percentage of sperm abnormalities and acrosomal damage and hence it is recommended for freezing of dog semen.

References

REFERENCES

*Aguirre, S. M., Capaul, E. and De-Luca, L. (1987). Morphology and biochemical evaluation of fresh and cold shocked dog semen. *Veterinaria-Argentina*. **35** : 433-439.

Allen, W.E. (1991). Semen collection. In. Boden. E. (ed) 1991. Canine Practice 1st Ed., Bailliere, Tindal.

*Andersen, K. (1972) Fertility of frozen dog semen. *Acta-Veterinaria-Scandinavica*. **13**: 128-130.

Arthur, G. H., Noakes, D. E., Pearson, H. and Parkinson, T. J. (1996). Fertility and infertility in male animals. Veterinary Reproduction and Obstetrics.7th Ed.WB Saunders company Ltd. London. pp 572-633.

*Ataman, M. B., Yildiz, C., Kaya, A., Aksay, M. and Lehimcioglu, N. (1998). Effectiveness of various staining methods for detecting abnormal sperm types and their incidence in fresh and frozen thawed dog semen. *Veteriner-Bilimleri- Dergisi.* **14** :121-129.

*Battista, M., Parks, J. and Concannon, P. (1988). Canine sperm survival following freezing in straws or pellets using PIPES, Lactose, Tris or TEST extenders. 11th International Congress on Animal Reproduction and Artificial insemination. University College Dublin, Ireland. June 26-30. Brief Communications. Vol. 33. Paper No. 229. pp3.

Boucher, J. H., Foote, R.H. and Kirk, R.W. (1958). The evaluation of semen quality in the dog and the effects of frequency of ejaculation upon semen quality, libido and depletion of sperm reserves. *Cornell. Vet.,* **48**: 67-86.

Chatterjee, S. N. , Meenakshi Sharma, R. N. and Kar, A. B. (1976) Semen characteristics of normal and vasectomised dogs. *Indian. J. Exp Biol.,* **14**: 411-414.

Christensen, G.C. and Dougherty, R.W. (1955). A simplified apparatus for obtaining semen from dogs by electrical stimulation. *J. Am. Vet. Med. Asso.,* **127**: 50-52.

Dabas, Y. P. S., Atheya, U.K., Prakash, P. and Verma, M.C. (1991). A note on artificial insemination in dogs. *Indian. Vet. Med. J.,* **15** : 64.

Daiwadnya, C. B and Hukeri,V. B. (1993). A note on evaluation of dog semen. *Indian. J. Anim. Reprod.,* **14** : 62-63.

*Daiwadnya, C. B and Hukeri,V. B. and Sonawane, S. A. (1995). Studies on evaluation of dog semen. *Livestock Adviser.* **20** : 34-37.

Davies, P. R. (1982). A Study of spermatogenesis, rates of sperm production, and methods of preserving the semen of dogs. Ph.D. Thesis. Cited in: England, G. C. W. (1993). Cryopreservation of dog semen. a review. *J. Reprod. Fert. Suppl.*, **47** : 243-255.

Deshpande, B.R., Velhankar, D.P., Hadi, M.A., Gujarathi, K.G., Kaikini, A.S. and Sane, C.R. (1970). Artificial insemination in dogs. A record of 30 cases. Bombay Veterinary College Magazine. **17**: 27-30. In: Sane, C.R., Luktuke,S.N., Deshpande,B.R., Kaikini, A.S., Velhankar,D.P., Hukeri,.V.B. and Kodagali,S.B. (1982). Reproduction in farm animals. 1st Ed. Varghese Publishing House, Bombay. pp. 522-530.

Dobrinski, I., Lulai, C., Barth, A.D. and Post, K. (1993). Effects of four different extenders and three different freezing rates on post thaw viability of dog semen. *J. Reprod. Fert. Suppl.*, **47** : 291-296.

*Dubiel, A. (1974). Studies on semen collection by the masturbation method and on the ejaculatory reflex in dogs. *Anim. Breed. Abstr.* **42** : 2356.

England, G. C. W. (1993). Cryopreservation of dog semen a review. *J. Reprod. Fert. Suppl.*, **47** : 243-255.

England, G. C.W. (1999). Semen quality in dogs and the influence of a short interval second ejaculation. *Theriogenology,* **52** : 981-986.

England, G.C.W. and Allen, W.E. (1989). Seminal characteristics and fertility in dogs. *Vet. Rec.*,**125** : 399.

England, G. C. W. and Allen, W. E. (1992a). Factors affecting the viability of canine spermatozoa. I. Potential influences during processing for artificial insemination. *Theriogenology,* **37** : 362-371.

England, G.C.W. and Allen, W.E. (1992b). Factors affecting the viability of canine spermatozoa, effects of seminal plasma and blood. *Theriogenology,* **37** : 373-381.

England, G. C. W. and Ponzio, P. (1996). Comparison of the quality of frozen thawed and cooled rewarmed dog semen. *Theriogenology,* **46** : 165-171.

Farstad, W. (1984). Bitch fertility after natural mating and artificial insemination with fresh or frozen semen. *J. Small. Anim. Pract.,* **25** : 561-565.

Farstad, W. (1996). Semen cryopreservation in dogs and foxes. *Anim. Reprod. Sci.,* **42** : 251-260.

Farstad, W. (2000). Assisted reproductive technology in canid species. *Theriogenology,* **53** : 175-186.

Farstad, W. and Anderson- Berg, K. (1989). Factors influencing the success rate of artificial insemination with frozen semen in the dog. *J. Reprod. Fert. Suppl.*, **39**: 289-292.

Ferguson, J.M., Renton, J.P., Farstad, W. and Douglas, T.A. *(1989). Insemination of* Beagle bitches with frozen semen. *J. Reprod. Fert. Suppl.*, **39** : 293- 298.

Fontbonne, A. and Badinand, F. (1993). Canine artificial insemination with frozen semen; Comparison of intravaginal and intrauterine insemination of semen. *J. Reprod. Fert. Suppl.* , **47**: 325-327.

Foote, R. H. (1964). The influence of frequency of semen collection, fractionation of the ejaculate and dilution rate on the survival of stored dog sperm. *Cornell. Vet.*, **54** : 89-96.

Fougner, J. A. (1977). Nordic symposium on artificial insemination in dogs and deep freezing of dog semen. Uppasla. In: Christiansen, I.B.J. (ed), 1984. Reproduction in dog and cat. 1st Ed. Bailliere Tindal, London. pp: 18.

Froman, D.P., Amann, R.P., Rick, P.M. and Olar, T.T. (1984). Acrosin activity of canine spermatozoa as an index of cellular damage. *J. Repro. Fert.*,**70** : 301-308.

Gill, H. P., Kaufman, C.F., Foote, R.H. and Kirk, R. W. (1970). Artificial insemination of Beagle bitches with freshly collected, liquid stored and frozen thawed semen. *Am. J. Vet. Sci.*,**31** : 1807-1813.

Guerin, P., Ferrer, M., Fontbonne, A., Benigi, L., Jacquet, M. and Menezo, Y. (1999). *Invitro* capacitation of dog spermatozoa as assessed by chlortetracycline staining. *Theriogenology,* **52** : 617-628.

Harrop, A.E. (1955). Some observations on canine semen. *Vet. Rec.,* **61** : 771-779.

Harrop,A.E. (1956). A review of canine artificial insemination. *J. Am. Vet. Med. Asso.,***15** : 564-567.

Harrop,A.E. (1960). Reproduction in the dog. 1st Ed. Baillere, Tindall and Cox. London. pp: 66-69.

Hay, M.A., King, W.A., Gartley, C.J., Leibo, S.P. and Goodrowe, K. L. (1997). Canine spermatozoa. Cryopreservation and evaluation of gamete interaction. *Theriogenology,* **48** :1329-1342.

Hopkins, S.M. and Evans, L.E. (1989). Artificial insemination. In: McDonald, L. E. and Pineda, M. H.(eds). Veterinary Endocrinology and Reproduction. 4th Ed. Lea & Febiger, Philadelphia. pp : 366-367.

Ivanova- Kicheva, M.G., Bobadov, N. and Somlev,B. (1997). Cryopreservation of canine semen in pellets and in 5ml aluminum tubes using three extenders. *Theriogenology,* **48** : 1343-1349.

Ivanova-Kicheva, M.G., Subev, M.B., Bobadov, N.D., Dacheva, D.P. and Rouseva, I.A. (1995). Effect of thawing regimens on the morpho functional state of canine spermatozoa. *Theriogenology,* **48** :565-569.

Kadirvel, G. (1998). Preservation of dog semen in three extenders at refrigeration temperature. M.V.Sc. Thesis. Kerala Agricultural University, Thrissur.

Kawakami,E., Tsutsui,T. and Ogasa,A. (1988). Two cases of acrosome and folded tail abnormality in dog spermatozoa. *Japn. J. Vet. Sci.,***50**: 1274-1276.

*Keskin, O., Tekin, N., Yurdaydin, N. and Gurcan, I. S. (1996). Morphological changes in dog spermatozoa frozen in different diluents. *Lalahan- Hayvancilile- Arastirma-Enstitusu- Dergisi*. **36**: 86-100.

Kibble, R. M. (1969). Artificial insemination in dogs. *Aust. Vet. J.,* **45** : 194-199.

*Kim, B.J. and Kim, Y.J. (1995). Studies on artificial insemination with frozen semen using methanol and preserved in liquid nitrogen. *Korean. J. Vet. Clin. Med.,***12**:207-214.

Kumi-Diaka, J. (1993). Subjecting canine semen to hypo-osmotic swelling test. *Theriogenology,* **39** : 1279-1289.

Kumi-Diaka, J. and Badtram, G. (1994). Effects of storage on sperm membrane integrity and other functional characteristics of canine spermatozoa. *Invitro* bio assay for canine semen. *Theriogenology,* **41**: 1355-1366.

Linde-Forsberg, C., Strom-Holst, B. and Govetta, G. (1999). Comparison of fertility data from vaginal Vs intra uterine insemination of frozen thawed dog semen. A retrospective study. *Theriogenology,* **52** : 11-23.

Mazur, P. (1985). Freezing of living cells: mechanism and implications. *Am. J. Physiol.*,**247** : c125- c142.

Mickelsen, M. D., Memon, M. A., Anderson, P. B. and Freeman, D. A. (1993). The relationship of semen quality to pregnancy and litter size following artificial insemination in the bitch. *Theriogenology,* **39**:353-360.

Mortan, D. B. and Bruce, S. G. (1989). Semen evaluation, cryopreservation and factors relevant to the use of frozen semen in dogs. *J. Reprod. Fert. Suppl.*, **39**: 311-316.

Nair, S.R.M. (1996). Viability and membrane integrity of cryopreserved dog semen. M.V.Sc. thesis. Tamilnadu Veterinary and Animal Sciences University. Chennai.

Nair, S.R.M., Kalatharan, J., Raman, K.S. and Rajasekaran, J. (1998). Evaluation of canine frozen semen by hypo osmotic swelling test. *Indian. J. Anim. Sci.*, **68**: 1179-1180.

Nothling, J.O., Gerstenberg,C. and Volkmann, D.H. (1997). Semen quality after thawing ; correlation with fertility and fresh semen quality in dogs. *J. Reprod. Fert. Suppl.*, **51**: 109-116.

Oettle,E.E. (1986). Changes in acrosome morphology during cooling and freezing of dog semen. *Anim. Reprod. Sci.*, **12**: 145-150.

Oettle,E.E. (1993). Sperm morphology and fertility in the dog. *J. Reprod. Fert. Suppl.*, **47** : 257-260.

*Olar, T.T. (1985). Cryopreservation of dog spermatozoa. *Dissertation- Abstracts- International-B- Sciences and Engineering*. **45** : 283.

Olar,T.T., Bowen,R.A. and Pickett,B.W. (1989). Influence of extender, cryopreservative and seminal processing procedures on post thaw motility of canine spermatozoa frozen in straws. *Theriogenology*, **31**: 451-461.

Olson, P. N. (1992). Collection and evaluation of canine semen. In: Kirk. (1992). Current Veterinary Therapy. Small Animal Practice 11th Ed. W.B. Saunders Company, London, pp : 938-943.

Pena, A., Johannisson, A. and Linde- Forsberg, C. (1999). Post thaw evaluation of dog spermatozoa using new triple fluorescent staining and flow cytometry. *Theriogenology,* **52** : 965-980.

*Ravazova, O., Mesaros, P., Cigankova, V. and Lukacinova, M. (1996). A study of the properties of dog ejaculate during long term storage. *Folia- Veterinaria.* **40**:95-99.

Rawlings, C.A., Caudle, A.B. and Crowell, W.A. (1994). Semen evaluation after partial intra capsular Prostatectomy in normal dogs. *Theriogenology,* **42**: 1323-1328.

Rodriguez-Gil, J. E., Montserrat, A. and Rigan, T. (1994). Effects of hypo osmotic incubation on acrosome and tail structure on canine spermatozoa. *Theriogenology,* **42**: 815-829.

Rodriguez-Martinez, H., Ekwall,H. and Linde-Forsberg, C. (1993). Fine structure and elemental composition of fresh and frozen spermatozoa. *J. Reprod. Fert. Suppl.,* **47**: 279-285.

Rota, A., Linde-Forsberg, C., Vannozzi, J., Romagnoli, S. and Rodriguez-Martinez, H. (1998). Cryo-survival of dog spermatozoa at different glycerol concentration and freezing thawing rates. *Reproduction in Domestic Animals.* **33** : 355-361.

Rota, A., Strom, B. and Linde-Forsberg, C. (1995). Effects of seminal plasma and three extenders on canine semen stored at 4°C. *Theriogenology,* **44** : 885-900.

Rota, A., Strom, B., Linde-Forsberg, C. and Rodriguez-Martinez, H. (1997). Effects of Equex STM paste on viability of frozen thawed dog spermatozoa during *invitro* incubation at 38°C. *Theriogenology,* **47** : 1093-1101.

Rowson, L.E.A. (1954). Infertility of cow, sow and bitch. *Irish Veterinary Journal.* 8 : 216-221. Cited in : England, G. C. W. (1993). Cryopreservation of dog semen. a review. *J. Reprod. Fert. Suppl.,* **17** : 243-255.

* Roychoudhury, P. N. and Dubay, M. I. (1974). Observation in dog semen. 1. Dilution and conservation in three extenders. *Zootechica-Veternaria.* **29** : 117-121.

Sarma, P. V. (1995). A simplified staining technique for evaluation of acrosomal status of sperm cells. *Indian J. Anim. Reprod.,***16** : 127.

*Seager, S. W. J. (1969). Successful Pregnancies utilising frozen dog semen. *AI Digest.* **17** : 6-7.

Seager, S.W.J. and Fletcher, W.S. (1973). Progress on the use of frozen semen in the dog. *Vet. Rec.*,**92** : 6-10.

Seager, S.W.J., Platz, C.C. and Fletcher,W.S. (1975). Conception rates and related data using frozen dog semen. *J. Reprod. Fert.*,**45** : 189-192.

Silva, L. D., Onclin, K., Lejeune, B. and Verstegen, J. P. (1996). Comparison of intra-vaginal and intrauterine insemination of bitches with fresh or frozen semen. *Vet. Rec.*,**138** : 154-157.

Silva, L.D.M. and Verstegen, J.P. (1995). Comparison between three different extenders for canine intrauterine insemination with frozen thawed spermatozoa. *Theriogenology,* **44** : 571-579.

*Smith, F. O. (1985). Cryopreservation of canine semen : technique and performance. *Dissertation-B-Sciences and Engineering*. **45** : 3441.

Snedecor, G.W. and Cochran, W.G. (1967). Statistical Methods. 6th Ed. Oxford and IBH company, Calcutta.

Strom, B., Rota, A. and Linde-Forsberg, C. (1997). *In vitro* characteristics of canine spermatozoa subjected to two methods of cryopreservation. *Theriogenology,* **48** : 247-256.

Strom Holst, B., Rota, A., Andersen-Berg, K. and Linde-Forsberg, C. (1998). Canine sperm head damage after freezing thawing: Ultra structural evaluation of selected elements. *Reproduction in Domestic Animals.* **33**: 77-82.

Taha, M. B., Noakes, D.E. and Allen, W.E. (1981). The effect of season of the year on the characteristics and composition of dog semen. *J. Small. Anim. Pract.,***22** : 177-184.

Taha, M. B., Noakes, D.E. and Allen, W. E. (1983). The effect of the frequency of ejaculation on seminal characteristics and libido in the Beagle dog. *J. Small. Anim. Pract.,* **24** : 309-315.

Thomas, P.G.A., Larsen, R.E., Burns, J.M. and Hahr, C.N. (1993). A comparison of three packaging techniques using two extenders for the cryopreservation of canine semen. *Theriogenology,* **40** : 1199-1205.

Tsutsui, T., Tezuka, T., Shimizu, T., Murao, I., Kawa-Kami, E. and Ogasa, A. (1988). Artificial insemination with fresh semen in Beagle bitches. *Jap. J. Vet. Sci.,***50**:193-198.

Wales, R.G. and White, I.G. (1958). The interaction of pH, tonicity and electrolyte concentration on the motility of dog spermatozoa. *J. Physiol.*,**141** : 273-280.

Wilson, W.S. (1993). Non-surgical intrauterine artificial insemination in bitches using frozen semen. *J. Reprod. Fert. Suppl.*, **47** : 307-311.

Wong, W.T. and Dhaliwal, G. K. (1985). Observations on semen quality of dogs in the tropics. *Vet. Rec.*,**116** : 313-314.

Yubi, A. C., Ferguson, J. M., Renton, J.P., Harvey, M. J. A., Bagyenji, B. and Douglas, T. A. (1987). Some observations on the dilution, cooling and freezing of canine semen. *J. Small. Anim. Pract.*,**28** : 753-761.

*Yurdaydin, N. and Kotzab, E. (1987). Studies on the deep freezing of dog semen. *Ankara-universittesi-Veteriner-Fakultesi-Dergisi.* **34** : 534-540.

*Yu-Xueli, L., Pang-Youzi, Z., Li-Yingang, H. and Wang-Zhanbin, B. (1997). Study on freezing canine pellet semen by the new method of frying in liquid nitrogen. *Chinese. J. Vet. Sci.*,**17**: 289-291.

* Original articles not consulted.

EFFECT OF DIFFERENT FREEZING RATES ON CANINE SPERMATOZOA

By

R. GEETHA

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Veterinary Science

Faculty of Veterinary and Animal Sciences

Kerala Agricultural University

Department of Animal Reproduction

COLLEGE OF VETERINARY AND ANIMAL SCIENCES

MANNUTHY, THRISSUR - 680651

KERALA, INDIA

2000

ABSTRACT

The objective of the study was to find out the effect of different freezing rates on post thaw motility, livability and acrosomal damage of dog spermatozoa. A total of 36 ejaculates of good quality collected from six Dachshund dogs by digital manipulation were processed for freezing in Tris citric acid fructose egg yolk diluent containing four per cent glycerol. The processed semen samples were subjected to three different freezing protocols such as 4cm height above the liquid nitrogen level for 10 minutes (Fast freezing), 8cm for 15 minutes (Moderate freezing) and 12cm for 20 minutes (Slow freezing).

The mean volume of sperm rich fractions was 0.68±0.03ml. The colour and consistency of sperm rich fractions were thin milky. The mean density of sperm rich fraction was DD(D) and mean pH was 6.63±0.02. The mean concentration of sperm rich fraction was 221±7.36 millions per ml and the average initial motility was found to be 75±0.93 per cent. The mean percentage of live sperm count, sperm abnormalities and acrosomal damage of spermatozoa was 81.17±0.73, 5.23±0.29 and 2.32±0.25

respectively. Significant ($P<0.05$) variation in livability, sperm abnormalities and acrosomal damage of spermatozoa was found between dogs.

The average percentage of motility, live sperm count, sperm abnormalities and acrosomal damage of spermatozoa was 70.41±1.22, 75.63±0.65, 7.28±0.43 and 5.34±0.31 after dilution, 58.75±1.34, 63.60±0.89, 10.04±0.32 and 10.13±0.41 after chilling and 47.78±1.59, 50.65±1.31, 11.79±0.36 and 16.20±0.57 after equilibration period respectively. There was significant ($P<0.01$) reduction in sperm motility and livability and increase in sperm abnormalities and acrosomal damage of spermatozoa after dilution, chilling and equilibration period. Significant ($P<0.01$) difference was found between dogs for the above parameters.

The percentage of post thaw motility of spermatozoa was significantly ($P<0.01$) higher in fast freezing rate (34.31±1.66) when compared to moderate (25.83±1.66) and slow (24.44±1.27) freezing rates. There was significantly ($P<0.01$) higher percentage of live sperms and lower percentage of sperm abnormalities in fast freezing rate than in moderate and slow freezing rates. Eventhough the percentage of acrosomal damage was not statistically

significant among fast, moderate and slow freezing rates, lower percentage of acrosomal damage was recorded in fast freezing rate.

From this study it could be inferred that fast freezing in which the straws were frozen at to 4cm height above the liquid nitrogen level for 10 minutes was superior to moderate (8cm for 15 minutes) and slow (12 cm for 20 minutes) freezing rates.