TO STUDY THE EFFECT OF PROSTATIC FLUID ON SEMEN PRESERVATION AT REFRIGERATION TEMPERATURE IN MONGREL DOGS

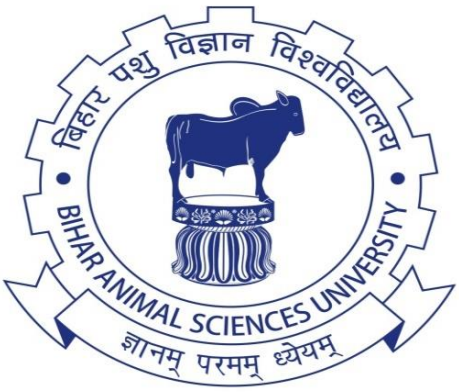
THESIS

By

Dr. PRAVEEN KUMAR

(VM0043/2018-19)

SUBMITTED TO



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PATNA, BIHAR

In Partial fulfilment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE
IN
VETERINARY GYNAECOLOGY & OBSTETRICS
2020

DEPARTMENT OF VETERINARY GYNAECOLOGY & OBSTETRICS

Bihar Veterinary College, Patna-800014

(Bihar Animal Sciences University Patna, Bihar)

CERTIFICATE- I

This is to certify that the thesis entitled, "**TO STUDY THE EFFECT OF PROSTATIC FLUID ON SEMEN PRESERVATION AT REFRIGERATION TEMPERATURE IN MONGREL DOGS"** submitted in partial fulfilment of the requirements for the award of the degree of Master of Veterinary Science in the discipline of **Veterinary Gynaecology & Obstetrics** of the faculty of Post-Graduate Studies, Bihar Animal Sciences University, Patna, Bihar is the bonafide research work carried out by **Dr. PRAVEEN KUMAR, Registration No- VM0043/2018-19,** son of Shri. **GANESH PRASAD SINGH** under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

Place: Patna

Date:

Dr. Ankesh Kumar

(Major Advisor)

DEPARTMENT OF VETERINARY GYNAECOLOGY & OBSTETRICS

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CERTIFICATE- II

This is to certify that the thesis entitled, "**TO STUDY THE EFFECT OF PROSTATIC FLUID ON SEMEN PRESERVATION AT REFRIGERATION TEMPERATURE IN MONGREL DOGS"** submitted by **Dr. PRAVEEN KUMAR, Registration No-VM0043/2018-19,** son of Shri **GANESH PRASAD SINGH** to the Bihar Animal Sciences University, Patna, Bihar in partial fulfilment of the requirements for the degree of Master of Veterinary Science in the discipline of **Veterinary Gynaecology & Obstetrics** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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Place: Patna

Date:28-09-2020 (*Praveen Kumar)*

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Composition of Eosin- Nigrosin staining

Eosin Y : 1.67 gram

Nigrosin : 10 gram

Distilled water : up to 100 ml

Composition of Hypo-osmotic solution (100 mOsm/L osmolarity)

Tri-sodium citrate – 0.49 gram

Fructose – 0.99 gram

Distilled water – ad up to 100 ml.

Sorensen's phosphate buffer

Sorensen's phosphate buffer was prepared by mixing 17 ml of 0.1 M potassium phosphate monobasis (KH_2PO_4) solution and 33 ml of 0.1 M sodium phosphate anhydrous (Na_2HPO_4) solution. The pH of buffer was adjusted at 7.0.

0.1 M potassium phosphate solution

Potassium phosphate monobasic (KH_2PO_4) - 13.609 gm

Triple glass distilled water ad - 1000 ml

0.1 M sodium phosphate solution

Sodium Phosphate anhydrous (Na_2HPO_4) - 14.198 gm

Triple glass distilled water ad - 1000 ml

Modified Hancock's fixative

Sodium chloride (NaCl) - 1.5 gm

Sodium bicarbonate ($NaHCO_3$) - 0.1 gm

Formalin 41 % (HCHO) - 12.5 ml

Triple glass distilled water ad - 100 ml

Egg yolk tris citrate fructose extender (EYTCFE) composition

TRIS : 2.4 gm,

Citrate acid monohydrate :1.3 gm,

Fructose : 1 gm,

Egg yolk : 20 ml,

Distilled water : up to 100 ml,

Penicillin G potassium : 100000 IU,

Streptomycin : 100 microgram

ABBREVIATIONS

et al	: All others
%	: Percentage
/	: Per
@	: at the rate
AI	: Artificial insemination
i.e	: that is
viz	: Namely
^{0}C	: Degree Celsius
Sec	: Second
Kg	: Kilogram
Gm	: Gram
ml	: Mililiter
Fig.	: Figure
ad lib	: as necessary
EYTCF	: Egg yolk tris citrate fructose extender
EYT	: Egg yolk tris
Min.	: Minute
mOsm	: Milliosmole
Mm	: Millimoles
N.D	: Not determind
Sec	: Second
SE	: Standard error.

≤	: Lesser than or equals to
≥	: Greater than or equals to
µl	: Micro Liter
µg	: Micro gram
ANOVA	: Analysis of Variance
Eosin Y	: Eosin yellow
HOS	: Hypo-osmotic solution
IU	: International Units
Kg	: Kilogram
S. No.	: Serial Number
P Value	: Level of significance
pH	: Hydrogen ion concentration
±	: Plus or Minus
&	: And
etc	: Et cetera

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DEPARTMENT OF VETERINARY GYNAECOLOGY & OBSTETRICS
Bihar Veterinary College, Patna-800014.
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Name of student: **Dr. Praveen Kumar** Reg. no.: VM0043/2018-19

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Major Advisor: **Dr. Ankesh Kumar**

ABSTRACT

The present research work was conducted with the objective to study the physico-morphological characteristics of mongrel dog semen and the effect of removal of prostatic fluid on semen preservation at refrigeration temperature (4^0C). A total of 18 ejaculates were collected from 3 mongrel dogs, 6 from each dog by digital manipulation. All the collected samples were divided into three Groups viz. Group I (Non-extended semen), Group II (Semen extended with egg yolk tris citrate fructose extender) and Group III (Sperm pellet extended with egg yolk tris citrate fructose extender) and kept at refrigeration temperature (4^0C) for individual progressive motility, live dead percent, sperm morphological abnormality, HOST (Hypo-osmotic swelling test) and acrosomal integrity test, at each day of preservation till progressive motility goes down less than 40 per cent(%). In Group III semen was centrifuged at 720 x g for 5 minutes by centrifuge machine for making of sperm pellet. We found that sperm motility was reduced to below 40 per cent (%) on day 2 in group I and on day 4 in group II and group III. The group II and III maintained sperm motility, sperm live percent, HOST responses, acrosome integrity and lower morphological abnormalities of spermatozoa up to days 4 of preservation. The higher ($P<0.05$) mean individual progressive motility, sperm live percent, HOST and acrosome integrity and lower morphological abnormalities of spermatozoa were found in Group II. In conclusion, removal of prostatic fluid is beneficial for sperm preservation at refrigeration temperature in mongrel dogs.

Dr. Praveen Kumar **Dr. Ankesh Kumar**
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1 INTRODUCTION

Since stone age, dogs (*Canis familiaris*) are the companion animals and surprisingly the first animal species domesticated because of its faithfulness and excellent hunting qualities. With progress of industrialization from agriculture, change in social life from joint family to nuclear family there is increased trend to keeping pets as companion animals and also dogs are well known for their honesty, because of these qualities of the dogs attracted the human for their domestication. It is known that dogs are of much importance not only to growing children but also they render physiological benefits to adults. Dog is reliable, affectionate having a sense of routine life. In recent times dog is used for racing, sports, watch dog, blind persons guide and number of other purposes that suit the characteristics of a particular breed. Apart from psychological benefits, people are also kept dogs to protect their property or to guard as this animal can protect human being at the cost of their life.

Very little experimental work has been done on semen preservation and artificial insemination (AI) in canine in India. Dogs are neither used for production of meat or neither milk nor they are apparently able to boost up the rural economy and for that reason scientist and researcher always ignored works on dogs. Nowadays there is growing interest in dog breeders in breeding with pure and registered breed dogs. Dog breeding is a multimillion-dollar industry worldwide and lack of successful breeding is generally multifactorial. Thus, the dogs are gradually becoming one of the biggest income generating animals for dog lovers and dog breeders. In dog breeder, natural mating is used most commonly for conception but many a time there is problem in transportation of animal to long distance due to shyness of male animal, differences in size of male and female animal, moreover, inability of male animal to mount because of muscular skeletal diseases or other congenital conditions, non-availability of stud male at proper mating time result in loss of valuable breeding season. To overcome the above obstacles artificial insemination (AI) can be used as an alternative. So for artificial insemination (AI) we need to preserve the semen at refrigerating temperature (4^0C) or freezing temperature (-196^0C). To get optimum quality dog breed, pure breed with physical characteristics and its offspring, AI is most important technique. Successful dog breeding is

based on the determination of the optimal mating time or insemination time and selection of healthy and fertile male and females (Johnston *et al.,* 2001).

Very little work has been done till date for the evaluation and preservation of dogs semen to fully exploit the genetic potential of the male animals. Dogs were the first species of animals in which semen was collected, artificial insemination performed and off springs obtained successfully by Spallanzani in 1776 and in year 1954 the first successful AI using chilled dog semen was reported by Harrop and that the use of frozen-thawed semen was reported by Seager in 1969 (Rijsselaere *et al*., 2011). The widespread dissemination of semen from a small number of dogs with superior genetic merit to remote areas in order to inseminate a large number of bitches requires preservation of semen under artificial environment. AI in dogs help to alleviate psychological problems like shyness, inexperienced male, habitual premature erection and mating difficulties like female dominance, unequal size of dog and bitch and low libido in male dogs and long distance between stud dog and bitch, semen can be transported and bitch can be inseminated using artificial insemination.

For successful application of AI technique, the most important step is preservation of semen. In case of dogs, the semen can be preserved at chilling (4^0C) and freezing temperature (-196^0C). Preservation of semen generally requires a reduction or arrest of the metabolism of sperm cells there by prolonging their fertile life. Preservation of semen at refrigerating temperature has many advantages over the freezing temperature such as cost effectiveness, efficient and practical method to preserve semen over a period of 4- 5 days requires less equipment as compared to frozen semen and also conception rates are found higher in AI using chilled semen (Gill *et al.,* 1970; Farstad 1984; Linde-Forsberg 1991; Linde-Forsberg and Forsberg 1993). Cryopreservation of dog semen is complicated and time consuming process which is not practically possible in field condition due to unavailability of laboratories and equipment while using of chilling method to preserve the semen is comparatively easier. Success of cooled or stored semen use depends on number of factors such as temperature of storage, composition of extender, number of sperm inseminated and number of insemination. In our study, we have focused on temperature of storage and composition of extender. To chill a sperm sample, the second sperm-rich fraction can be diluted with Tris- citric acid-fructose diluent with 20% egg yolk in a proportion of 1:3 to 1:4 (Rijsselaere *et al.,* 2011).

In order to preserve the fertilizing ability of spermatozoa from their own toxic byproducts, cold shock and osmotic shock during the chilling semen extender are added into the semen as well as also acts as a source of nutrition (Zorinkimi *et al.,* 2017a). Extender should protect the spermatozoa from cold shock during cooling by adding of 20% egg yolk, it provides energy substrate (fructose or glucose) and maintain constant pH and osmolarity by adding buffer (England 1993). To prevent growth of bacteria, semen extender should also contain antibiotics mostly penicillin or streptomycin.

The physico-morphological characteristics of semen viz. colour, volume, pH, mass motility, sperm concentration (million/ ml), individual progressive motility, sperm live-dead percent, HOST (Hypo-osmotic swelling test), sperm morphological abnormality and acrosome integrity to assess the quality of dog semen. Semen assessment is a key of the evaluation of fertility in males and it should be performed/ completed before artificial insemination or semen preservation.

Dog semen is normally ejaculated in three fractions. The first and third fraction originate from prostate gland and are not collected because prostatic fluid was shown to be unsuitable for preserving canine semen at 4^0C and has harmful effects on spermatozoa during preservation (Rijsselare *et al.,* 2002). Only the second sperm rich fraction is collected, however it is not always possible to avoid the admixture of other fractions. So centrifugation immediately after sperm collection is one of the method to remove prostatic fluid that is unsuitable for preserving dog semen at 4^0C. Proper centrifugation protocol (CP) have an important role in the semen quality to preservation. The duration of centrifugation may be more important than the centrifugation force for causing sperm membrane injuries which results in an increased formation of reactive oxygen species (Shekarriz *et al.,* 1995). By increasing centrifugation time and force, the number of sperm cells can be maximized but also physical damage is exerted on the sperm cells at the same time. The negative results of centrifugation on semen quality are determined by the decrease in motility and velocity in comparison to uncentrifuged controls (Jasko *et al.,* 1991). Sperm parameters can be better by low-speed centrifugation of canine spermatozoa in a single density gradient (Dorado *et al.,* 2013). Spermatozoa from different mammals showed different sensibility to centrifugation process. While the rat, human (Makler and Jakobi 1981) and mouse (Schreuders *et al.,* 1996)

spermatozoa may shows very sensitive to mechanical and centrifugal forces, spermatozoa from equine and bovine (Pickett *et al.,* 1975; Crockett *et al.,* 2001) are slightly less sensitive to centrifugation process. This information shows that species specificity is very important with respect to spermatozoa injury caused by centrifugation process (Carvajal *et al.,* 2004). This seems that it is essential to establish an ideal centrifugation regime for sperm preservation techniques associated to the species. Furthermore, species, breeds and individual variation are also important factors because the compositions of seminal plasma and sperm membrane varies greatly between species and individuals. Dilution of semen or removal of seminal plasma is believed to be necessary for prolonged storage (Pickett *et al.* 1975).

Therefore, keeping in views of the above mentioned facts the present study has been designed with following objectives

OBJECTIVES

1. To study the physico-morphological characteristics of mongrel dog semen.

2. To study the effect of removal of prostatic fluid on semen preservation at refrigeration temperature (4^0C).

2 REVIEW OF LITERATURE

2.1 SEMEN COLLECTION

Gill *et al.* (1970) used estrus bitch as teaser for semen collection by digital manipulation. Dog semen is generally collected by digital manipulation and stimulation of the *bulbus glandis* (Linde-Forsberg 1991). During semen collection by digital manipulation method the massage of the prepuce is initiated in bulbus glandis area. Following massage of the prepuce, partial erection of penis developed. After that quick retraction of the prepuce and penile expose is essential for successful semen collection in dog. A circular, fixed pressure should be maintained with the left hand to simulate the copulatory lock or tie (Freshman 2001; Johnston *et al.,* 2001). When a clear prostatic fluid starts to flow, then the semen collector can smoothly divert the collection cone out of the penis (Linde-Forsberg 2005; Farstad 2010). The semen collection in dog is commonly achieved by massage of the penis and the use of a cone or sleeve made of plastics or any special semen collecting vials (Linde-Forsberg 2005). Ideal intervals between semen collections are 2 to 5 days, and if interval exceed more than 10 days may result in an increased number of morphological abnormalities and decreased motility (Johnston *et al.,* 2001; Freshman 2002). In most dogs, twice semen collection can be made by an interval of about 30 minutes gap (Farstad 2010), however, the second semen sample is generally diluted as compared to previous one. The volume of the whole ejaculate varies between breeds and size of the dogs (Farstad 2010). Only the spermatic and prostatic fractions were assessed and semen having mass motility +3 or more and concentration >200 million spermatozoa/ml should be consider to be suitable for preservation (Bencharif *et al.,* 2013).

2.2 SEMEN EXTENDER

Semen is the liquid cellular suspension containing spermatozoa, the male gametes and secretions from the accessory organs of the male reproductive tract. The fluid portion of this suspension, which is formed at ejaculation, is known as seminal plasma (Hafez and Hafez 2000). The extenders are used commonly with the choice of extender being based on semen storage tests conducted with dogs. During chilling, a certain number of phenomena occur in

which some are not fully understood, this is commonly known as 'cold shock' and results in a reduction in sperm motility, metabolism and an alteration in membrane permeability, resulting in disrupted cellular exchanges these changes which are occurring during cooling are irreversible, however, the uses of a semen extenders can limit this phenomenon, besides the storage temperature also help to improve the preservation of chilled semen (Rota *et al.,* 1995 and Rota *et al.,* 1997). It is evident that at 4^0C egg yolk extender was superior in protecting sperm motility parameters and the optimal results were obtained using egg yolk supplemented Tris glucose extender, which protects motility and acrosome integrity in chilled dog semen for up to 10 days (Iguer- ouada and Verstegen 2001). Semen extenders are diluents of the semen which preserve fertilizing abilities of the spermatozoa and also acts as buffer which help in protecting the spermatozoon from toxic byproducts produce itself, cold shock and osmotic shock during the chilling and also provide nutrition to them (Zorinkimi *et al.,* 2017a) besides these antibiotics are also added to semen extenders to prevent the growth of bacteria. From many decades different types of semen extenders or dilutors have been tested for their competence for better keeping qualities of sperm motility in chilled dog semen (Province *et al.,* 1984; Bouchard *et al.,* 1990). Egg yolk used in extenders of chilled semen to protect sperm cells from cold- shock (Beccaglia *et al.,* 2008). Extenders included Citrate, TRIS, Phosphate or Glycine buffers, heated or dried milk, sterilized cream, with or without of sugars, egg yolk and antibiotics (Rota *et al.,* 1995). Sugar is one of the main constituents in semen diluters or extenders. Tsutsui *et al*. (2003) examined three stock solutions as diluters or extenders viz. Egg yolk- Citrate -Glycine- Glucose solution, Egg yolk Tris– Fructose- Citrate solution (EYT-FC), and Egg yolk sodium citrate di-hydrate solution (EYCD) and observed that the EYT-FC group have better sperm motility and viability. The Tris-citric acid-egg-yolk-fructose extenders are mostly used extenders for the preservation of dog sperm (Rijsselaere *et al.,* 2011). Mammalian spermatozoa require exogenous substrates for a variety of functions i.e to preserve intracellular energy reserves, cell components and most notably to support motility (Salisbury 1978).

Santos *et al. (*1999) studied the comparative studies on five extenders (Tris- fructose-citric acid, glycine, lactose, skim milk and tris-fructose-citrate extenders) in dog semen freezing and found that the extender tris-fructose-citric acid showed the best post-thaw progressive motility and forward progressive velocity as compared to the other diluters or

extenders. Both egg yolk and milk are used to protect against cold shock of the sperm cells as they are cooled from body temperature to 5^0C. Egg yolk protects cell membranes of spermatozoon against cold shock, and also prevents or restores phospholipid loss from the cell membrane. Egg Yolk has been added to Tris–Glucose buffer and used for canine semen cooling up to 27 days with exchange of extender (Verstegen *et al.*, 2005). There is strong action of Glucose and fructose on motility and patterns of movement of chilled canine semen and the fructose maintained higher sperm motility as compared to glucose and the mixture (Ponglowhapan *et al.*, 2004) and they observed that Egg yolk tris extender or dilutors supplemented with fructose (@ 70 mM concentration) was found best among the tested extenders for canine semen preservation for long-term at chilling temperature. Two major system of sperm storage (liquid and frozen) have been used for preservation. Preservation of sperm generally requires reducing or arresting the metabolism of sperm cells, thereby prolonging their fertile life (Maxwell and Salamon 1993; Yoshida 2000). The ultimate goal of semen preservation is to obtain pregnancies after artificial insemination as effectively as natural mating. This depends on several factors apart from semen quality.

Artificial Insemination (AI) is a reproductive technique in which semen is collected from a fertile male manually and introduces into the female vagina so that fertilization can be achieved in the absence of natural mating by the male. Dog artificial insemination (AI) provides convenience for owners and breeders. Semen from precious dogs can be preserved and stored into semen banks to be used in further generations in spite of their death or senility. In addition, the benefits associated with artificial insemination (AI) are avoiding direct contact between the male and female and prevents sexually transmitted diseases viz. Brucellosis, Herpes virus infection, etc. (Linde-Forsberg 2005 and Farstad 2010). Spallanzani in 1776 semen was collected from the dog, artificial insemination performed and off springs obtained successfully. The first successful Artificial insemination (AI) with chilled dog semen was reported in 1954 by Harrop and use of frozen-thawed semen in 1969 was reported by Seager (Zorinkimi *et al.*, 2017a). Artificial insemination (AI) in dogs is accomplished by involving semen collection, evaluation, preservation and insemination techniques. Artificial insemination (AI) with chilled and frozen- thawed semen makes it possible to use the genetic material at future (after dog death) and has many economic and sanitary advantages.

2.3 SEMEN PRESERVATION AT 4^0C

Success in cooled/stored semen use depends on a lot of confounding factors such as temperature of storage, composition of the extender, number of sperm inseminated, number of inseminations etc. The widespread dissemination of semen from a small number of dogs with superior genetic merit in remote locations to inseminate large number of bitches is only possible through preservation of semen under the field condition. The duration for the storage of canine semen is a very important consideration when shipping chilled semen over ever-greater distances. Two major systems of sperm storage are liquid and frozen preservation. Under chilled condition spermatozoa are kept at refrigerator temperature i.e. 4^0C. In preservation of sperm there is reduction or arrest of the metabolism of sperm cells, which prolongs their fertile life. The chilled semen was first used for artificial insemination in dog in the year 1954 by Harrop. Bouchard *et al.* (1990) compared three storage temperatures (+35^0C, +22^0C and +4^0C) and demonstrated that the best results was obtained at +4°C. Long-term chilled storage of semen, however, may lead to acrosomal damage or total destruction of sperm (White 1993). The uses of chilled semen is increasing in dog because it easy for preparation and inseminations and also for transportation within countries and it's fertility is greater than with frozen thawed semen (Rota *et al.*, 1995). The transport of chilled semen is easier as compared to frozen semen stored in liquid nitrogen, and easy to inseminate (Intravaginal Vs Intrauterine insemination). The conception rates of chilled semen are very high in comparison to frozen semen (Rota *et al.*, 1997). On the basis of semen evaluation chilling of semen is most suitable method of semen storage in bitches if semen samples are used within approximately 4-5 days of collection (Province *et al.*, 1984; Rota *et al*., 1995). The principal advantage of using liquid-preserved semen is that fertility is maintained even with low numbers of spermatozoa in the insemination (Zou and Yang 2000). Metabolites generated during preservation decrease the pH of the semen, and reactive oxygen species (ROS) damage the cell membrane and damage the sperm enzymatic activity (Tsutsui *et al.*, 2003). Artificial insemination with cooled- shipped semen has become a routine procedure also in the equine breeding industry because of having good fertility rates and relatively low costs (Contri *et al.*, 2010). Chilled semen can be inseminated in the bitch either at 4^0C or after warming the semen sample to 37^0C (Rijsselaere *et al.*, 2011). The storage of dog semen at 5^0C

for 24 h did not significantly impair the physical and functional integrity of spermatozoa (Srivastava and Mathur 2011).

2.4 CENTRIFUGATION OF SEMEN

The effect of Centrifugation may exert both beneficial and harmful effects on sperm motility (Martin *et al.,* 1979). Many Evidences have suggested that the recovery rate of spermatozoa after centrifugation is closely related to the g-force applied. On the other hand, an excessive centrifugation force reduced the viability of spermatozoa, probably because of the physical pressure against the wall of the tube (Pickett *et al.,* 1975; Jasko *et al.,* 1991; Hoogewijs *et al.,* 2010). A small amount of seminal plasma in stallion semen improves sperm motility during preservation (Jasko *et al.,* 1991). Centrifugation of the semen immediately after semen collection removes prostatic fluid which helps in semen preservation at 4^0C in dog (Rijsselare *et al.,* 2002). Rijsselaere *et al.* (2002) observed that after centrifugation of dog semen for 5 min at 720 x g removed prostatic fluid in a best manner because the loss of sperm cells was acceptable and other functional parameters of the spermatozoa were well preserved, even after 3 days of storage. Violeta and Pana (2007) studied that the centrifugation force and time affects the number of spermatozoa loss by supernatant. Centrifugation at 1500 x g showed less loss of spermatozoa as compared to centrifugation at 650 x g, at 2 minute and 5 minute and Centrifugation regime 1500 x g for 5 minutes determined the best recuperative rate of spermatozoa on centrifugation semen (Violeta and Pana 2007). Love *et al*. (2005) and Kozdrowski *et al.* (2007) reported that there is positive effect of removing seminal plasma on the frozen semen in the buck. The removal of seminal plasma had beneficial effect on semen freezing and thawing quality in buck (Purdy 2006 and Kozdrowski *et al.,* 2007). However, High-speed centrifugation of dromedary cammel (*Camelus dromedarius*) semen, decreased sperm motility by about 12.7 per cent (El- Bahrawy 2010). In Angora buck sperm frozen with or without centrifugation showed higher percentages of subjective motility (Sariozkan *et al.,* 2010). Daramola and Adekunle (2017) observed that semen washing improved sperm quality in West African dwarf (WAD) goats when cryopreserved in Tris egg yolk extender.

Density gradient centrifugation performed in colloidal solution is one of the procedures recommended by the WHO (World Health Organization 1999) for human spermatozoa preparation used in assisted reproductive technologies. This method has been suggested as

effective tools for improving the quality of sperm after cryopreservation in animals (Rodriguez-Martinez *et al.*, 1997; Hallap *et al.*, 2004). Recently a modified gradient centrifugation technique called single layer centrifugation (SLC) was proposed to enhance the semen quality before conservation and artificial insemination (AI) (Morrell 2006; Morrell and Rodriguez- Martinez 2009). This technique provides high quality sperm, with an increased number of motile and membrane-intact spermatozoa after processing.

2.5 SEMEN EVALUATION

Immediately after collection, the sperm-rich fraction is evaluated macroscopically (volume, color, mixtures and homogeneity) and microscopically (motility, concentration, morphology and membrane integrity) especially when the sample is to be chilled or frozen and transported. The main goal of semen evaluation is to predict the fertilizing capacity of a semen sample. An ejaculate should contain a minimal concentration of around 200x 10^6 spermatozoa per milliliter of the mixture of the spermatic and prostatic fractions. Below this concentration, it is considered to be unsuitable for cooling. Sperm motility is critical for passing through the female genital tract and barriers around the oocyte for subsequent fertilization (Bucak *et al.*, 2012). Apart from other parameters, intactness of sperm chromatin is essential for oocyte fertilization and future embryo development (Tuncer *et al.*, 2010). Plasma membrane integrity and functions are essential for cell viability, as the selective permeability of the plasma membrane maintains intracellular metabolic activities, pH and ionic composition.

2.5.1 COLOUR

Thin milky colour of the semen ejaculate in a single Doberman Pinscher dog was noted by Ahmed *et al*. (1990). In mongrel dogs colour and consistency of semen ejaculates varied from cloudy to milky (Daiwadnya and Hukeri 1993). Kadirvel (1998) observed 36 ejaculates from 6 mongrel dogs the colour of the first and third fraction of semen was found to be clear watery and second fraction varied from thin milky to thick milky. Silva *et al.* (2002) observed that the fresh sperm fraction was an opalescent white color. Silva *et al.* (2006) collected semen by manual stimulation twice from each of 5 dogs and the sperm-rich fraction was analyzed. They observed that fresh dog semen was milky-white in color. Filho *et al.* (2011) also collected 15 ejaculates from five adult Boxer dogs in his experiment by manual

stimulation and found that the sperm-rich fraction was white-opalescent in color with a milky viscosity. Zorinkimi *et al.* (2017b) collected 42 ejaculates of semen by digital manipulation from 3 dogs and observed that the semen color was clear and watery in the first fraction while sperm-rich fraction was milky white and opaque.

2.5.2 SEMEN VOLUME

The semen volume is not an indicator of semen quality in dogs. Takeishi *et al.* (1980) collected semen from 7 dogs (6-16 months of age) at monthly interval and reported that ejaculate volume increased with age from 1.5 ml to 3.50 ml. Wildt *et al*. (1982) recorded semen ejaculate volume of 1.9 ± 1.0 and 3.2 ± 0.4 ml in inbred and outbred Foxhound dogs. In German Shepherd dogs an average volume of semen ejaculates was recorded as 7.17 ml by Souza (1987). The canine semen consists of 3 fractions of which the first and third fractions have mainly prostatic fluid while the second fraction is being rich in spermatozoa. The volume of pre sperm fraction or first fraction ranges from 1ml to 5 ml and is colourless, while the second fraction, the sperm-rich portion is grayish-white in colour with a volume of 1-3 ml (England *et al.,* 1990) is also rapidly completed (1- 2 minutes). However, third fraction volume ranges between 30 ml to 40 ml is also contributed by prostate (Johnston *et al.,* 2001). In 28 fertile Labrador retriever, Golden retriever and German Shepherd dog breeds an average volume of semen ejaculate was recorded as 1.2 ml with the range of 0.4 to 3.4 ml by England and Allen (1989). Daiwadnya *et al.* (1995) collected 48 ejaculates of 4 mongrel dogs at 3 days interval and they reported average semen volume to be 2.57 ±0.14 ml. Kadirvel (1998) observed 36 ejaculates from 6 mongrel dogs the mean total volume of ejaculate was recorded 6.15±0.75 with a range of 2.2 to 10 ml. Silva *et al.* (2002) observed the mean volume of 1.54 ± 0.64 ml for dog semen. During an experiment on six proven stud dogs of 1 to 6 years of age, Cardoso *et al.* (2005) observed the volume of sperm-rich fraction was 1.2±0.5 ml. Silva *et al.* (2006) collected semen by manual stimulation twice from each of 5 dogs and the sperm-rich fraction was analyzed and they they observed that mean (±S.D.) volume of the sperm-rich fraction was 0.9 ± 0.4 ml. Depending on how much prostate fluid was collected and the size of the dog, Blendinger (2007) recorded that the dog semen volume varies between < 2 to > 20 ml but is typically 5 ml. Biswas (2010) observed from 3 dogs of Alsatian breed the mean total volume of ejaculate was recorded 5.51±0.16 ml. The volume of ejaculate differ between

breeds in dogs (Farstad 2010) mainly with size of the animal and is dependent on the volume of the third fraction collected, which is highest among three fraction which constitute about 95% of the ejaculate volume (Farstad 2010). Filho *et al.* (2011) collected 15 ejaculates by manual method from 5 adult Boxer dogs and recorded that the average volume was 0.9 ± 0.3 ml. Zorinkimi *et al.* (2017b) collected 42 ejaculates of semen by digital manipulation from 3 mongrel dogs and observed that the over-all mean semen volume of first and second fractions was 2.95±0.41 ml in his experiment.

2.5.3 pH

The pH of the second fraction (sperm rich) of domestic canine ejaculates is approximately *6.2* (Whales and White 1958 and Rota *et al.,* 1995) and the third fraction or prostatic fluid is slightly more basic at a pH of 6.8 (Whales and White 1958). Tekin *et al*. (1987) evaluated semen from five males of kangal and German shephard dogs at interval of two days and semen pH was 6.1and 6.2 respectively. Daiwadnya *et al*. (1995) reported pH of dog semen to be 6.57± 0.04 in mongrel dogs. Jain (2004) observed 34 ejaculates from 6 mongrel dogs the overall mean pH of ejaculate was recorded 6.39±0.03 with a range from 6.23 to 6.55. Biswas (2010) observed from 3 dogs of Alsatian breed the mean pH of ejaculate was recorded 6.38±0.03. Zorinkimi *et al.* (2017b) in his experiment collected 42 ejaculates by digital manipulation and found that the over-all mean value of pH was 6.59 ± 0.76.

2.5.4 MASS MOTILITY

Despandey *et al.* (1970) reported that the mass activity of dog semen varied from ++ to ++++ on a scale of ++++. Daiwadnya (1987) evaluated 24 ejaculates from mongrel dogs and observed the mass motility as ++ and the initial motility as 3.79± 0.08 on a grade of 1 to 5. Daiwadnya *et al.* (1995) collected 48 ejaculates from 4 mongrel dogs at 3 days interval and they reported average mass activity of semen sample to be 2.05 ±0.10 and initial motility to be 3.60± 0.09 on a grade of 1 to 5. Kadirvel (1998) observed 36 ejaculates from 6 mongrel dogs the mean mass activity of sperm rich fraction of ejaculate was ++ (+) with a range of + to +++ (+). Raut (2009) observed that the average mass activity of sperm rich second fraction was 3.32 ± 1.07 and 3.5 ± 0.98 in Group I (Younger) and in Group II (Older) German Shepherd dogs respectively. Thapak (2009) collected sperm rich fraction of dog semen by massage

method of eight German shephard dogs for 8 weeks and observed mean mass motility of dog spermatozoa as 3.54 ± 0.04 and range from 3 to 4.

2.5.5 SPERM CONCENTRATION

Concentration is inversely related to volume of semen collected. The wide variation in the sperm concentration of different fraction depends on method of semen collection, frequency, environmental factor, breed and size of dog and presence of teaser bitch. The spermatozoon concentration in each ejaculate also varies with age, testicular size, sexual activity and the size of the dog (Amann 1986). Takeishi *et al.* (1980) collected semen from 7 dogs (6-16 months of age) at monthly interval and reported that the sperm concentration as 0.23 to 2.68 x 10^8. Farstad and Andersen-Berg (1989) using vaginally inseminated fresh semen, stated that at least 200×10^6 motile spermatozoa were needed for fertility to be comparable to that obtained in Beagles after natural mating and for frozen-thawed dog semen deposited into the uterus, an insemination dose of around 150 × 10^6– 200 ×10^6 motile spermatozoa was recommended. The number of motile spermatozoa for successful artificial insemination should be greater than 150 x10^6 (Linde-Forsberg 1991). Concentration of sperm has very less value as an indicator of semen quality (Robert *et al.,* 2016). Gunzel (1986) studied 74 ejaculates from various breed of dogs and reported the mean concentration of spermatozoa per ejaculate as 1024.4 x 10^6 ± 557.6x 10^6. Daiwadnya *et al*. (1995) collected 48 ejaculates of 4 mongrel dogs at 3 days interval and they reported average sperm concentration to be 264 ±15 x 10^6 per ml. Hay *et al.* (1997) observed the mean sperm concentration of 18 ejaculate to be 277±56.4 x 10^6 spermatozoa/ml, with a range of 200 to 387 x 10^6 spermatozoa/ml. Kadirvel (1998) observed 36 ejaculates from 6 mongrel dogs the mean total sperm output of the ejaculate was found to be 527.50± 29.46 with a range of 280 to 1092 million. Jain (2004) observed 34 ejaculates from 6 mongrel dogs sperm concentration of ejaculate was 313.61±13.82 million per ml with a range of 200 to 390 million per ml. Verstegen *et al.* (2005) recorded mean ± S.D sperm concentration 455 ± 32x10^6 spermatozoa/ml in fresh canine semen. Silva *et al.* (2005) evaluated the semen collected from six dogs and extended in tris with egg-yolk and glycerol in two different dilution rates. The average sperm concentration was 1420.8± 228.3x 10^6 spermatozoa / ml. Schafer-Somi *et al*. (2006) found that the sperm-rich fractions of ejaculates from 12 male beagles and noted the

average concentration of spermatozoa to be 225.4 ± 44.1x 10^6/ml. Silva *et al*. (2006) collected semen by manual stimulation twice from each of 5 dogs and the sperm-rich fraction was analyzed and they observed mean ±S.D. concentration 1030.3± 600.2 x 10^6 sperm/ml in the ejaculate. Michael *et al.* (2009) collected single semen ejaculate from five dogs and observed average sperm concentration of 503±25 x 10^6/ ml. Raut (2009) characterized the German Shepherd dog semen and observed that the concentration of spermatozoa was more in Group I (younger dog) 189.23±25.32 million/ml than in Group II (older dogs) 163.66±20.66 million/ml. Zorinkimi *et al.* (2017b) collected 42 ejaculates of semen by digital manipulation from 3 dogs and observed the over-all mean concentration of spermatozoa recorded in mongrel dog semen in the study was recorded as 298.73 ± 35.46 million per ml.

2.5.6 INDIVIDUAL PROGRESSIVE MOTILITY

Sperm motility is critical for passing through the female genital tract and barriers around the oocyte for subsequent fertilization (Bucak *et al.*, 2012). The direction of sperm in the the reproductive tract of female to catch the egg is important for fertilization. A motility of 70% or greater is rated very good, 30% - 50% motility is fair and 10% - 30 % is poor. Sperm should be moving rapidly in forward direction, not in circles. Motility is an indicator of structural and functional ability of spermatozoa; therefore the percentage of progressively motile spermatozoa is generally positively correlated with that of plasma membrane integrity (Kumi- Diaka 1993; Rodriguez- Gil *et al.*, 1994) and of normal morphology (Ellington *et al.*, 1993). Normal motility is usually described as rapid, progressive, forward motion (Feldman and Nelson 1996). Larsen (1980) proposed that fertile dogs should have at least 70% of total sperm motility. Speed or quality of motility also may be assessed; a canine spermatozoon with normal motility should traverse the microscopic field of view in 2–3 seconds (Threlfall 2003). The optimal temperature for assessment of dog sperm motility is 39^0C. The normal dog semen contains at least 70% of progressively motile spermatozoa (Feldman and Nelson 1996). It is generally found that motile spermatozoa decrease may results from temperature shock, contamination with water, urine, blood or any lubricants and also from long sexual rest and systemic or infectious diseases. Chatterji *et al.* (1976) studied individual motility in 86 ejaculates collected from 69 dogs, the average sperm motility noted by them was 85.3 percent. However Dubiel (1976) reported sperm motility ranging from 60 to 100 percent in 181

ejaculates from 102 dogs. Takeishi *et al*. (1980) collected semen from 7 dogs (6-16 months of age) at monthly interval and found the percentage of motile spermatozoa ranging from 52.5 to 80.01 percent. Wong and Dhaliwal (1985) collected one ejaculates by digital manipulation from 8 mongrel and 17 pure breed dogs ageing 2.3 to 4.8 years and reported progressive motility of spermatozoa to be 91± 3.6 percent and 85 ±13.6 percent respectively. Gunzel (1986) studied 74 ejaculates from various breed of dogs and reported 71±9 percent forward motility of spermatozoa. Similarly Souza (1987) reported 68.84 % motility in semen of 25 German Shepherd dogs ageing 1 to 7 years. Rota *et al.* (1995) reported that motility up to 4 days was higher in egg yolk tris (53.6%), egg yolk milk (30.4%) and egg yolk cream (14.1%) extender. Kadirvel (1998) observed 36 ejaculates from 6 mongrel dogs the mean sperm motility recorded 86.67±1.07 with a range of 70 to 95 percent immediately after semen collection. Kadirvel (1998) observed 36 ejaculates from 6 mongrel dogs the average percentage of sperm motility recorded on day 0, 1, 2, 3, 4 and 5 days of preservation were 86.38±1.04, 79.86±1.32, 73.47±1.72, 66.8±1.99, 58.47±2.41 and 49.86±2.74, respectively in EYT (Egg yolk tris), 80.83±1.20, 75.41±1.59, 70.13±1.85, 64.44±1.96, 55.41±2.49, and 48.33±2.62 in EYCGG (Egg yolk citrate glycine glucose) and 76.25±1.26, 61.94±1.76, 41.66±1.92, 26.11±2.04, 7.50±1.01 and 0.00 in goat milk extender preserved at 4^0C. Jain (2004) observed 34 ejaculates from 6 mongrel dogs the average percentage of sperm motility recorded at 0, 24, 48, 72 hours of preservation were 84.54±0.54, 73.24±0.5, 63.91±0.69, 52.37±1.06 percent respectively, in EYC(Sodium citrate egg yolk) dilutor, 81.49±0.51, 70.05±0.54, 58.05±0.30, 40.16±0.88 in TFC (Tris fructose citric acid) dilutor preserved at 5^0C. Verstegen *et al.* (2005) recorded average percentage of motility ranging from 85 to 95% (mean ± S.D. 93 ± 3) in fresh canine semen. Silva *et al.* (2006) collected semen by manual stimulation twice from each of 5 dogs and the sperm-rich fraction was analyzed and they observed that mean (±S.D.) sperm motility was 97.5 ± 3.5%. Santos *et al.* (2007) studied the semen parameter of twenty dogs (Six Boxers, three Collies, three Rottweiler, two German shepherd and six Mongrel dogs) and assessed the vigor scoring as 0 – 4 and observed that the mean percentage of motility in dog is 86.316%. Biswas (2010) observed from 3 dogs of Alsatian breed the mean value of progressive motility of the sperm was 76.33±2.72. Biswas (2010) observed 18 ejaculates from 3 dogs of Alsatian breed the mean value of progressive motility recorded on day 3 and day 7 of preservation were 59.666 and 23.833 in Egg yolk tris fructose extender, respectively and 61 and 41.333 percent in

EYTG (Egg yolk tris glucose) extender, respectively. Zorinkimi *et al.* (2017b) collected 42 ejaculates of semen by digital manipulation from 3 dogs and observed the over-all mean progressive motility of mongrel dog semen obtained in the study was 95.30 ± 1.65 %.

2.5.7 LIVE DEAD PERCENT

Eosin–nigrosine is one of the best methods to determine the percentage of live-dead sperm in given sample which is most widely used in all domestic species. Good canine semen sample should contain at least 80% morphologically normal and viable spermatozoa (Johnston *et al.*, 2001). The dead spermatozoa possess disintegrated plasma membrane allowing eosin penetration thus, looks pinkish as compared to intact white normal spermatozoa. The normal dog semen consists of maximal percentage of 30% of dead sperm cells. When the proportion of normal spermatozoa was below 60 percent, fertility was found to be adversely affected (Oettle 1993). Daiwadnya *et al*. (1995) collected 48 ejaculates of 4 mongrel dogs at 3 days interval and they reported average live sperm percentage to be 82.81 ± 0.93. Kadirvel (1998) observed 36 ejaculates from 6 mongrel dogs the overall mean live sperm count was 90.43±1.24 with a range of 84.25 to 94.89 percent. Kadirvel (1998) observed 36 ejaculates from 6 mongrel dogs the average percentage of live sperm recorded on day 0, 1, 2, 3, 4 and 5 days of preservation were 89.54±0.76, 86.44±0.84, 80.75±0.90, 74.65±0.99, 68.87±1.58 and 60.87±1.58 respectively in EYT (Egg yolk tris), 87.87±0.87, 84.78±0.91, 78.85±0.98, 72.54±1.32,66.24±1.64, and 57.67±2.24 in EYCGG (Egg yolk citrate glycine glucose) and 85.28±0.88, 76.48±1.07, 56.75±1.54, 41.78±1.97, 28.87±2.23 and 18.56±2.87 in goat milk extender preserved at 4^0C. Jain (2004) observed 34 ejaculates from 6 mongrel dogs the overall mean live sperm count was 89.44±0.57 with a range of 80 to 96 percent. Jain (2004) observed 34 ejaculates from 6 mongrel dogs the average percentage of live sperm count at 0, 24, 48, 72 hours of preservation were 87.15±0.48, 76.56±0.5, 66.19±0.59, 55.09±0.67 percent, respectively in EYC(Sodium citrate egg yolk) dilutor, 83.92±0.39, 73.10±0.51, 60.86±0.47, 43.15±0.61 percent in TFC (Tris fructose citric acid) dilutor preserved at 5^0C. Biswas (2010) observed from 3 dogs of Alsatian breed the mean overall mean live sperm count was 84.166±1.990 percent. Biswas (2010) observed 18 ejaculates from 3 dogs of Alsatian breed the mean value of live sperm percentage recorded on day 3 and day 7 of preservation at 4^0C were 71.666 and 59.00 in Egg yolk tris fructose extender respectively and 74.666 and 61.50

percent in EYTG (Egg yolk tris glucose) extender respectively. Zorinkimi *et al.* (2017b) collected 42 ejaculates of semen by digital manipulation from 3 dogs and observed the over-all mean percentage of live spermatozoa in mongrel dog semen in the study was 87.84 ± 1.8.

2.5.8 SPERM MORPHOLOGY

Morphology is the examination of the structure of the individual spermatozoa. Any abnormality in the structure in any part may affect sperm movement and its function. For a good sample the minimum 80% of the sperm should have normal in morphology. Abnormality is evaluated by counting about 100 to 200 spermatozoa in a stained semen slide under 100 × magnifications. Count only free heads and not free tails (Feldman and Nelson 1996). The types of abnormalities can be classified as primary (occurring during spermatogenesis) or secondary (occurring during maturation or sample preparation) or as major or minor abnormalities (Oettle and Soley 1988). The normal sperm morphology in the canine semen should be greater than 70% (Gunzel- Apel 1994). Chatterji *et al.* (1976) studied 86 ejaculates collected from 69 dogs and reported that the abnormal spermatozoa percentage was 14.7. Wong and Dhaliwal (1985) collected semen ejaculates from 8 mongrel and 17 purebred dogs ageing 2.3 to 4.8 years by massage method and they recorded the percentage of abnormal spermatozoa to be 9 ±4.3 and 18 ±17.2 respectively. Gunzel (1986) reported percentage of abnormal spermatozoa as 17.6 ± 8.1 in 74 ejaculates in dogs. Souza (1987) evaluated semen of 25 German shepherd dogs, ageing 1-7 years, he reported that the incidence of major sperm defects was 9.92 percent and minor defects was 7.62 percent. Tekin *et al.* (1987) evaluated semen from five males of kangel and German shephard dogs, they were ejaculated five times at interval of two days and reported percentage of abnormal spermatozoa was 7.9 and 6.2 respectively. Daiwadnya *et al*. (1995) collected 48 ejaculates of 4 mongrel dogs at 3 days interval and they reported to be 10.08 ± 0.95% abnormal spermatozoa. Kadirvel (1998) found that the average percentage of morphological abnormalities of sperm in 36 ejaculates from 6 mongrel dogs on day 0, 1, 2, 3, 4 and 5 days of preservation were 8.82±0.50, 11.65±0.64, 14.17±0.75, 16.67±0.85, 19.35±0.99 and 22.28±1.17, respectively in EYT (Egg yolk tris), 9.88±0.53, 12.73±0.67, 15.39±0.79, 18.13±0.92, 21.07±1.04 and 24.99±1.44 in EYCGG (Egg yolk citrate glycine glucose) and 12.31±0.67, 18.74±1.12, 24.43±1.55, 30.70±1.83, 37.03±1.85 and 44.75±2.02 in goat milk extender preserved at 4^0C. Kadirvel (1998) found in

his experiment that the mean total abnormal sperm was 7.59±0.45 with a range of 4 to 15.8 percent in 36 ejaculates from 6 mongrel dogs. Rijsselaere *et al.* (2002) reported that in Anglo – Normand and crossbred German Shepherd, that after 3 days of storage at 4^{O}C the mean percentage and S.D. of sperm cells with normal morphology 87.8± 96, 85.3.0±18.7, 89.7±4.8 and 86.6±8.4 after centrifugation at 180x g, 720xg, 1620x g, and 2880x g respectively. Biswas (2010) observed that the mean total abnormal sperm was 14.83±2.38 percent in 3 dogs of Alsatian breed. Biswas (2010) observed 18 ejaculates from 3 dogs of Alsatian breed the mean value of total abnormal sperm percentage recorded on day 3 and day 7 of preservation were 25.16 and 32.83 in Egg yolk tris fructose extender respectively and 22.67 and 29.167 percent in EYTG (Egg yolk tris glucose) extender, respectively.

2.5.9 HOST (HYPO-OSMOTIC SWELLING TEST)

The functional integrity of spermatozoa membranes are evaluated by hypo-osmotic swelling test (HOST). In this test when sperm exposed to hypo-osmotic solution, there are swelling of sperm tail. This phenomenon indicates normal water transport across the sperm plasma membrane. Thus, the swelling phenomenon in hypo-osmotic swelling test can be considered as a sign of functional integrity of the sperm plasma membrane (Jeyendran *et al.*, 1984). Motility is a manifestation of structural and functional competence of spermatozoa thus, the percentage of progressively motile spermatozoa is considered positively correlated with that of plasma membrane integrity. (Kumi- Diaka 1993). In the hypo-osmotic swelling test incubation of spermatozoa into a hypo-osmotic solution is essential and those spermatozoa that have intact plasma membranes will swell as fluid moves into the sperm cells, this will cause swelling and coiling of the tail (Pena-Martinez 2004). Rota *et al*. (1995) found that the effect of seminal plasma and three different extenders on canine semen stored at 4^{0}C and noted that the intact plasma lemma (HOS- test) in fresh semen was 93.6 percent which declined to 86 percent after 4 days of preservation. Michael *et al.* (2009) collected single semen ejaculates from five dogs and observed average of 92.7±0.6 percent plasma membrane intact spermatozoa with HOST. Raut (2009) characterized the German Shepherd dog semen and noted percentage of plasma membrane intact spermatozoa in the fresh ejaculate to be 66.35±2.35 % (Group I younger) and 66.22±5.65 % (Group II older GSD). Michael *et al*. (2009) collected single semen ejaculates from five dogs and observed average of 92.7±0.6 %

plasma membrane intact spermatozoa with HOST. Biswas (2010) observed from 3 dogs of Alsatian breed the mean value of HOSS positive sperm percentage was 80.166±2.522. Biswas (2010) observed 18 ejaculates from 3 dogs of Alsatian breed the mean value of HOSS positive sperm percentage recorded on day 3 and day 7 of preservation were 66.833 and 52.50 in egg yolk tris fructose extender respectively and 68.833 and 56.00 percent in EYTG (Egg yolk tris glucose) extender respectively. Zorinkimi *et al.* (2017a) collected 21 ejaculates of semen by digital manipulation in his experiment from 3 dogs and found that the mean percentage of HOSST-reacted sperm was 91.95±3.57, 92.80±3.51 and 92.28±4.35 per cent at 0 hour; 87.77±4.57, 89.93±3.89 and 90.15±4.24 per cent at 24 hours; 80.68±7.30, 85.52±3.37 and 86.66±3.92 per cent at 48 hours; and 66.51±24.26, 81.66±3.61 and 83.37±3, 97% at 72 hours of preservation in Beltsville Thawing Solution (BTS), Citrate Dextrose Egg Yolk Extender (CDE) and Tris- buffered egg yolk extender (TRIS) extenders respectively. Zorinkimi *et al.* (2017b) collected 42 ejaculates of semen by digital manipulation from 3 mongrel dogs and found that the over-all mean HOST – reacted sperm was 93.27± 0.53 percent.

2.5.10 ACROSOMAL INTEGRITY

The spermatozoa could be highly motile but not fertile, owing to acrosomal damage. The assessment of acrosomal integrity should therefore always be a major part of the assessment of spermatozoa. Abnormal acrosome affected sperm renders unfit for fertilization. The various physical interventions such as centrifugation regimes, cold shock, osmotic stress, improper sperm-handling procedure etc. lead to acrosomal abnormalities and due to this the acrsomal enzymes (mainly acrosin and hyaluronidase) responsible for fertilization events are depleted. Oettle (1986) reported significant acrosomal damage during dilution and cooling to 5^0C and observed that sperm motility did not correlate with acrosomal integrity. Rota *et al*. (1995) reported significantly lower acrosomal changes in egg yolk tris extender than autologous seminal plasma on day 3 and day 4 after preservation at 4^0C. Kumi- Diaka and Badtram (1994) studied that the mean percentage of acrosome defect in fresh and chilled semen diluted in Tris egg yolk dilutor and preserved for 24 hours were 2.43±1.15 and 2.65±1.11. Kadirvel (1998) observed 36 ejaculates from 6 mongrel dogs the mean total acrosomal abnormality was 6.63±0.38 with a range of 3.9 to 15.8 percent. Kadirvel (1998) observed 36 ejaculates from 6 mongrel dogs the average percentage of acrosome damage

recorded on day 0, 1, 2, 3, 4, and 5 days of preservation were 8.88±0.58, 12.96±1.04, 17.19±1.48, 21.29±1.76, 26.19±2.21, 31.54±2.73 respectively in EYT (Egg yolk tris), 10.23±0.71, 14.69±1.10, 19.48±1.70, 24.78±2.42, 29.59±2.80 and 34.56±3.10 in EYCGG (Egg yolk citrate glycine glucose) and 15.02±1.07, 25.36±1.91, 38.13±2.93, 51.65±3.50, 62.93±3.66 and 69.45±4.20 in goat milk extender preserved at 4^0C. Rijsselaere *et al.* (2002) reported in pooled semen of Anglo–Normand and crossbred German Shepherd that after 3 days of storage at 4^0C mean percentage and S.D. of sperm cells with intact acrosome as determined by a fluorescent PSA Staining were 93.6± 4.6, 96.0±3.5, 94.3±2.3 and 95.7±1.5 after centrifugation at 180x g, 720xg, 1620x g and 2880 x g respectively. Zorinkimi *et al.* (2017b) collected 42 ejaculates of semen by digital manipulation from 3 dogs and observed the overall mean incidence of intact acrosome in the present study was 97.37 ± 0.77 per cent in mongrel dogs.

3 MATERIALS AND METHODS

The present research work entitled "To study the effect of prostatic fluid on semen preservation at refrigeration temperature in mongrel dogs" was carried out in the Department of Veterinary Gynaecology and Obstetrics, Bihar Veterinary College, Patna Bihar in the month of February 2020 to July 2020.

3.1 Selection of Experimental Animals

Experimental animals include 3 (Three) mongrel dogs of more than one year of age with normal libido having average body weight of 20- 30 kg. The dog had housed in Kennel of Bihar Veterinary College, Bihar Animal Sciences University Patna and were kept on uniform diet (Two times in day with ad lib access to drinking water) and management during the entire study. All animals were dewormed orally with Tab Skyworm^{R} (Praziquantel, Pyrantel Emboate and Febantel) 1 tab per 10 kg b.wt and also vaccinated for Rabies (Rabigen Mono^{R} 1 ml s/c) 15 days before the start of the experiment.

Semen from each dog was collected by digital manipulation method without harming or giving stress to the animal in a calibrated plastic vial connected with funnel after proper cleaning of prepuce and perineal region with antiseptic solution following the method described by Christiansen (1984) with slight modifications with using an estrous bitch as a teaser.

3.2 Method of Semen Collection

Dog semen is generally collected by digital manipulation and stimulation of the bulbus glandis. Semen collection process can be started by massage of the dog prepuce at the level of the bulbus glandis until partial erection developed, followed by the quick retraction of the prepuce and penile exposure. A circular, fixed pressure was maintained with the left hand to simulate the copulatory lock or tie. When a crystal clear prostatic fluid begins to flow into the collection tube, a circumferential pressure was applied posterior to the bulbus glandis till ejaculation complete. The pre-sperm, sperm rich and post-sperm fractions of semen were collected into a single 15 ml graduated transparent centrifuge tube attached to the funnel.

3.3 Samples collection

All three dogs were trained for semen collection by mechanical stimulation. Semen was collected twice a week from all the 3 mongrel dogs by digital manipulation in the morning hours. Thus a total of 18 ejaculates were collected, 6 from each dog. All the collected samples were divided into three Groups viz. Group I (Non-extended semen), Group II (Semen extended with egg yolk tris citrate fructose extender) and Group III (Sperm pellet extended with egg yolk tris citrate fructose extender) and kept at refrigeration temperature (4^0C).

3.4 Evaluation of semen

After semen collection, each individual semen sample was immediately assessed for volume, colour, pH and then transfer immediately to the laboratory and stored at 37^0C in a water bath for further evaluation of seminal attributes.

3.4.1 Colour

Colour of semen ejaculates was observed/ recorded by visual observation immediately after collection in graduated transparent centrifuge tube in natural day light and recorded as watery, milky white and watery clear. Deviation from normal if any was also recorded.

3.4.2 Volume (ml)

Immediately after semen collection the total ejaculate volume (ml) obtained from each collection was recorded directly from the transparent graduated centrifuge tube.

3.4.3 pH

pH of each semen sample was recorded after collection using pH paper.

3.4.4 Mass motility:

Mass motility of the each semen ejaculate was assessed immediately after the collection on a scale of 0 to 5 by Herman and Madden (1953). A drop of neat semen was spread uniformly on a clean dry glass slide and observed without cover slip under low power magnification of microscope (10 x).

The mass activity was expressed as

Appearance	Numerical scale
No motility	0
10 to 20 percent motile with slight undulating movement, mostly weak and oscillatory	+
20 to 40 percent motile sperm, no waves or eddies, many inactive spermatozoa.	++
40 to 60 percent progressively motile sperm, slow eddies or waves, few inactive spermatozoa.	+ ++
60 to 80 percent progressively motile sperm, waves or eddies of great rapidity	+ + ++
80 to 100 percent motile sperm, extremely rapid eddies or waves.It is difficult to trace the origin and disappearance of the waves.	+ + + ++

3.4.5 Sperm concentration

Sperm concentration was determined with the help of a Neubauer hemocytometer after a dilution with a diluting fluid and expressed in million per milliliter of semen. Composition of diluting fluid: Eosin yellow- 0.05 gram, Sodium chloride (NaCl)- 1.00 gram, Formalin - 1ml, Distilled water add up to 100 ml. Diluting fluid should be capable of killing and dispersing the sperm cells evenly.

Procedure

- The neat semen is drawn carefully upto mark (0.5) of RBC pipette.
- Tip of pipette is removed and wiped.
- The diluting fluid is then drawn carefully into the mixing chamber up to calibrated mark (101) which result in standard dilution of 1:200.
- The content of pipette was thoroughly mixed by rolling it between palms.
- Discard first 1-2 drops from pipette, a small drop of diluted semen was placed near the edge of the cover slip. The fluid was immediately drawn by capillary action and spread evenly.

- The charged counting is allowed to settle for few minutes and then number of heads lying within the two sides of subunits are counted under 40x microscope.

The central square of Neubauer counting chamber can be used for counting sperm. The central square (Primary square) is divided into 25 (5x5) small secondary squares. Each secondary squares is further divided into 16 (4x4) tertiary squares. Thus each Primary square is divided into 400 (25x16) small tertiary squares, which measures 1 mm^2 in area. After charging semen, these squares under cover slip became 0.1 mm thickened film. Thus a total volume of semen in 400 smaller tertiary squares was 0.1 mm^3.

Method of Calculation:

Volume of counting chamber = 1 mm^2 x 0.1 mm

= 0.1 mm^3

Let the number of sperm counted in 5 squares be 'n'

Therefore, nx 5 = The number of sperm in 0.1 mm^3 of diluted semen.

200 times dilution in RBC pipette.

Therefore, n x 5 x10 x 200 = The number of sperm in 1 mm^3 of diluted semen.

Thus, n x5 x 10x 200 x 1000 = The number of sperm in 1 ml of semen sample.

3.4.6 Individual progressive motility

Individual progressive motility of each semen sample was assessed as described by Herman and Madden (1953). A fine drop of diluted semen was placed on pre-warmed clean, dry glass slide (37^0C) and covered with cover slip. The percentage of progressively motile sperm was observed by examined under high power microscope (40 x) and sperm motility was recorded from 0 to 100 on visual appraisal based on the percentage of progressive motile spermatozoa.

$$\text{Motile sperm percentage} = \frac{\text{Total sperm – non motile/dead sperm}}{\text{Total sperm}} \times 100$$

3.4.7 Live dead percent

The percentage of live spermatozoa was estimated by using Eosin- Nigrosin staining technique described by (Mukherjee and Dott 1960).

Composition of Eosin- Nigrosin staining: Eosin Y – 1.67 gram, Nigrosin - 10 gram, Distilled water - up to 100 ml

Procedure:

- 2-3 drops of staining solution was placed on clean grease free slide.
- One drop of fresh semen was added and mixed with above staining solution.
- Allowed to stand for 30-60 seconds.
- A thin smear was then prepared out of the mixture on a clean grease- free slide with the help of another slide with smooth edge.
- Smear was allowed to air dry and 200 spermatozoa were examined in different fields of the smear under oil immersion objective of the microscope at 100 x magnification.
- Spermatozoa which were stained or partially stained were considered as dead and those were not stained were considered as live sperm.

Microscopic examination revealed head of live spermatozoa as white and dead spermatozoa as pink.

3.4.8 Sperm morphology

The percentage of Sperm morphology was estimated by using Eosin- Nigrosin staining technique.

Procedure:

- 2-3 drops of staining solution was placed on clean grease free slide.
- One drop of fresh semen was added and mixed with above staining solution.
- Allowed to stand for 30-60 seconds.
- A thin smear was then prepared out of the mixture on a clean grease- free slide with the help of another slide with smooth edge.
- Smear was allowed to air dry and 200 spermatozoa should be counted in different fields of the smear under oil immersion objective of the microscope at 100 x magnification.

  The percentages of cells with particular morphological defects and of normal cells are calculated.

3.4.9 HOST (Hypo-osmotic swelling test)

The functional integrity of the sperm membrane was determined by using a Hypo-osmotic solution test as per the method described by Jeyendran *et al.* (1984).

Composition of Hypo-osmotic solution (100 mOsm/L osmolarity)

Tri-sodium citrate – 0.49 gram, Fructose – 0.99 gram, Distilled water – add up to 100 ml.

Procedure

- 100µl (0.1 ml) of semen sample was taken and mixed with one ml of HOS solution.
- The mixture was incubated at 37^0C for 1 hrs.
- After incubation the mixed solution was shaken gently and one drop of it was placed on slide and covered with cover slip.
- A total of 200 spermatozoa were counted in different field under 40 x microscope.
- The result was recorded as coiled tail (intact plasma membrane) and uncoiled tail (non-intact / reacted plasma membrane) spermatozoa.

3.4.10 Acrosome integrity

The acrosome integrity was studied following the technique of staining with Giemsa stain.

Acrosome was simply classified as normal or damaged as below

Normal: Either the acrosomal surface is completely smooth or slightly roughened.

Abnormal acrosome: Either the acrosome surface is severe to moderate roughened or completely detached acrosome.

Preparation of Giemsa stain

Giemsa stain powder (3.80 gm) was mixed with 375 ml of absolute methanol. A total of 125 ml of glycerol was added in it and the stain mixture was then allowed to be ripening/ maturation for about one week by keeping it in an incubator at 37^0C. During the period of maturation it was shaken daily for a few minutes. The stocked Giemsa stain was ready to use then transferred to the refrigerator at 5^0C.

Sorensen's phosphate buffer

Sorensen's phosphate buffer was prepared by mixing 17 ml of 0.1 M potassium phosphate monobasis (KH_2PO_4) solution and 33 ml of 0.1 M sodium phosphate anhydrous (Na_2HPO_4) solution. The pH of buffer was adjusted at 7.0.

0.1 M potassium phosphate solution

Potassium phosphate monobasic (KH_2PO_4) - 13.609 gm

Triple glass distilled water add up to - 1000 ml

0.1 M sodium phosphate solution

Sodium Phosphate anhydrous (Na_2HPO_4) - 14.198 gm

Triple glass distilled water add up to - 1000 ml

Giemsa working solution

Giemsa stock solution - 3 ml

Sorensen's phosphate buffer (pH 7) - 2 ml

Triple glass distilled water - 35 ml

Modified Hancock's fixative

Sodium chloride (NaCl) - 1.5 gm

Sodium bicarbonate ($NaHCO_3$) - 0.1 gm

Formalin 41 % (HCHO) - 12.5 ml

Triple glass distilled water add up to - 100 ml

Procedure

- A drop of semen was placed on a clean grease free slide.
- A smear was prepared and allowed to dry in air.
- The smear was then fixed in modified Hancock's fixative for 30 minutes.
- The slide was rinsed in slow running tap water for 20 minutes.
- Then slide was allowed to air dry.
- The slide was stained in Giemsa working solution overnight.

- It was then rinsed in distilled water, air dried and mounted using DPX mountant.
- 200 spermatozoa were counted to determine the percentage of acrosome integrity in different fields of the smear under oil immersion objective (100 x) of the microscope.

3.5 Semen preservation

Egg yolk tris citrate fructose extender (EYTCFE) was used to dilute semen samples to be chilled.

Composition

TRIS	: 2.4 gm,
Citrate acid monohydrate	: 1.3 gm,
Fructose	: 1 gm,
Egg yolk	: 20 ml,
Distilled water	: up to 100 ml,
Penicillin G potassium	: 100000 IU,
Streptomycin	: 100 microgram
pH adjusted to	: 6.8.

Egg yolk tris citrate fructose extender (EYTCFE) was made different for each semen sample. Egg yolk and antibiotics were added immediately before use. The semen was diluted 1:1 and preserved at refrigeration temperature (4^0C). Semen examination will be done on every days of preservation till progressive motility goes down less than 40 per cent (%).

3.6 Semen centrifugation

Immediately after semen collection and primary evaluation, semen is selected for centrifugation.

The semen is centrifuged at 720 x g for 5 minutes by centrifuge machine Tarsons Spinwin MC05-R. After centrifugation of semen, remove the supernatant from sample and re-suspended the semen pellet with an Egg yolk tris citrate fructose extender at room temperature, the tube containing the re-diluted semen samples were placed into beaker filled with water at room temperature. The beaker with the tubes was kept immediately at refrigeration temperature (4^0C) for further semen evaluation on daily basis.

The preserved semen were examined for individual progressive motility, live dead percent, Sperm morphological abnormality, HOST (Hypo-osmotic swelling test) and

acrosome integrity at each day of preservation till progressive motility goes down less than 40 per cent (%).

3.7 Statistical analysis

The data generated for different semen parameters was compiled and analyzed using SPSS (version 20.0 for Windows; SPSS, Chicago. 111 (USA). The data was subjected to Analysis of Variance (2-way ANOVA for different semen parameters) and Tukey's Honest Significant Difference tests for comparing the means to find out the difference between groups. The smallest difference for two means was reported as significantly different ($P<0.05$).

4 RESULTS AND DISCUSSION

The different physical characteristics of semen observed during this study are presented in table - 1.

Table 1: The mean ± S.EM of Physical characteristics of semen in dogs (N=18)

S. No	Parameters	Observations
1	Volume (ml)	3.55±0.31
2	pH	6.56±0.03
3	Mass motility (Scale 0-5)	3.75±0.12
4	Sperm concentration (10^6/ml)	307±19.38

4.1 Colour

The colour of majority of semen samples (N=18) collected from mongrel dogs in our study was observed as milky white (N=13) and remaining samples (N= 05) was thin milky.

Silva *et al.* (2006) and Kadirvel (1998) reported milky white color of dog fresh semen in his study which supports our findings. The colour of the semen was clear and watery in the first fraction and milky white and opaque in the sperm-rich fraction observed by Zorinkimi *et al.* (2017b) in 3 mongrel dogs during different ejaculates. The variation in the colour of different ejaculate of semen is due to different spermatozoa concentrations in the semen.

4.2 Volume

The mean volume of the semen samples (N=18) collected from mongrel dogs in this study was presented in the table 1. The mean volume of semen collected from mongrel dogs in this study was 3.55±0.31 ml and ranges from 2.0 ml to 7.0 ml in a single ejaculation.

The present results corroborated with the finding of Daiwadnya *et al.* (1995) who reported that the average semen volume to be 2.57 ±0.14 ml collected from 4 mongrel dogs at 3 days interval in 48 ejaculates. However, Zorinkimi *et al.* (2017b) collected 42 ejaculates of semen by digital

manipulation from 3 mongrel dogs and observed the over-all mean volume of first and second fractions of the ejaculate in the study was 2.95±0.41 which is slight lower than our observed value. Kadirvel (1998) observed 36 ejaculates from 6 mongrel dogs the mean total volume of ejaculate was recorded 6.15±0.75 with a range of 2.2 to 10 ml which is higher than our observed value.

These variations in the semen volume have been due to variation in size of dog, animals age, body weight, and breeds of dog, size of prostate gland, frequency of semen collection, volume of 3rd fraction collection (Dubiel 1976).

4.3 pH

The mean value of pH of the semen samples (N=18) collected from mongrel dogs in this study was presented in the table 1. The mean pH value of mongrel dogs semen was observed 6.56±0.03 with a range from 6.4 to 6.8.

Our observed pH value of semen was similar to the values reported by Daiwadnya *et al*. (1995) and Zorinkimi *et al.* (2017b) who reported pH values of 6.57± 0.04 and 6.59 ± 0.76 in mongrel dog. However, Biswas (2010) observed from 3 dogs of Alsatian breed and found that the mean pH of ejaculate was 6.38±0.03 which was lower than our observed value. Jain (2004) observed 34 ejaculates from 6 mongrel dogs the overall mean pH of ejaculate was recorded 6.39±0.03 with a range from 6.23 to 6.55.

Variation in the pH of dog semen (6.23- 7.01) has been reported due to the different method of semen collection (with an artificial vagina without a teaser, by hand manipulation with a teaser and by hand manipulation without a teaser in dogs (Boucher *et al.*, 1958).

4.4 Mass motility

The mean value of mass motility of the semen samples (N=18) collected from mongrel dogs in this study was presented in the table 1. The mass motility in the fresh semen immediately after collection from dog showed tremendous activity and a peculiar wave movement. The mean mass motility value of mongrel dogs semen was observed to be 3.75±0.12 on a scale of 0 to 5 and it ranges from 3.0 to 4.5.

Average mass activity as observed in the present study was higher with the earlier findings of Daiwadnya and Hukeri (1993) who recorded 2.05 mass activity in mongrel dogs semen and accordance with the findings of 3.32±1.07 and 3.5±0.98 in the sperm rich second fraction of German shepherd dogs as recorded by Raut (2009). Daiwadnya *et al*. (1995) collected 48 ejaculates from 4 mongrel dogs at 3 days interval. They reported average mass activity of semen sample to be 2.05 ±0.10 and initial motility to be 3.60± 0.09 on a grade of 0 to 5.

4.5 Sperm concentration

The mean value of sperm concentration of the semen samples (N=18) collected from mongrel dogs in this study was presented in the table 1. The mean concentration of spermatozoa calculated in this study was 307±19.38 x 10^6/ml with the range of 210 to 574x 10^6 spermatozoa /ml.

The present findings are in agreement with the findings of Zorinkimi *et al.* (2017b) who reported the sperm concentration of 42 ejaculates of semen by digital manipulation from 3 mongrel dogs and observed the over-all mean concentration of spermatozoa was 298.73 ± 35.46 million per ml. However, Daiwadnya *et al.* (1995) reported average sperm concentration in mongrel dogs to be 264± 15 million/ml, which is lower than present findings. Biswas (2010) observed from 3 dogs of Alsatian breed the mean of sperm concentration of ejaculate was 274.33±2.66 million per ml. The number of spermatozoa per ejaculate also varies according to age, testicular weight, sexual activity and the size of the dog (Amann 1986).

4.6 Individual progressive motility

The mean value of individual progressive motility of different groups is presented in the Table 2 and Fig.1.

Table: 2 Individual motility of spermatozoa at different days of interval (N=06) preserved at 4^0C (Mean±S.EM)

S. No	Group	Day 0	Day 1	Day 2	Day3	Day 4
1	Group I	88.33±2.78^{aA}	70.00±2.88 ^{bB}	36.33±1.20 ^{cB}	N.D.	N.D.
2	Group II	92.50±1.70 ^{aA}	84.16±2.50 ^{aA}	72.83±3.96 ^{abA}	60.00±5.62 ^{abA}	39.16±13.19 ^{bcA}
3	Group III	88.00±3.10 ^{aA}	80.33±4.63 ^{abAB}	71.66±4.22 ^{abA}	58.33±6.91 ^{bcA}	35.00±11.97 ^{cAB}

Means bearing different superscripts differed significantly (P<0.05) row wise a, b, c
Means bearing different superscripts differed significantly (P<0.05) column wise A, B

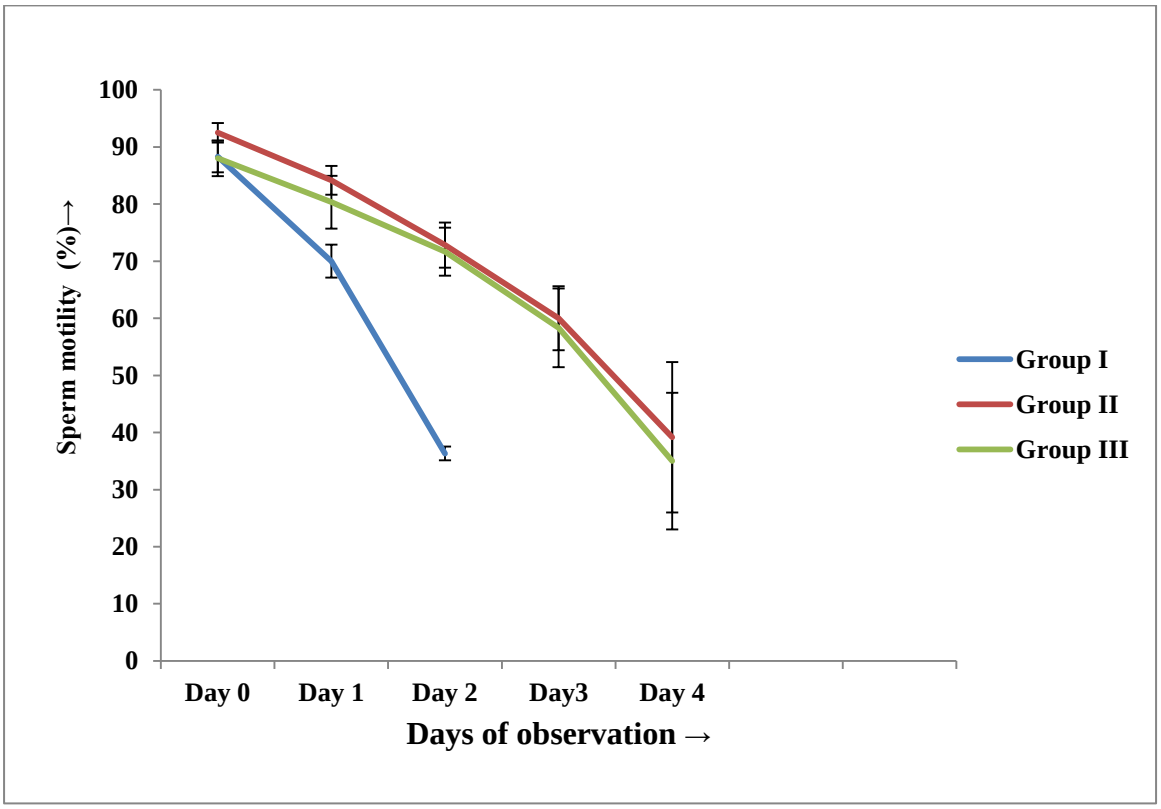

Fig. 1. Individual motility of spermatozoa at different days of interval (N=06) preserved at 4^0C.

In the present study, it was observed that the mean individual progressive motility reduced below 40 per cent (%) on day 2 in group I (Non extended semen), day 4 in group II (extended with EYTCF extender) and group III (Sperm pellet extended with EYTCF extender) respectively. There were non-significant differences found in sperm motility percentage at day 0 (Day of semen collection). Significant difference (P<0.05) found at day 1 of semen

evaluation between group I (Non extended semen) and Group II (extended with EYTCF extender) and mean value is higher in group II. On day 2 of semen evaluation, there were significant difference ($P<0.05$) in sperm motility found in between group II and Group III from group I and non-significant in between group II and Group III. On day 3 and day 4 of semen evaluation there were non-significant difference found in sperm motility between group II and group III.

In group I there were significant difference ($P<0.05$) found in sperm motility on day 0, day 1 and day 2 of semen evaluation which reduces below 40 per cent (%) on day 2. In group II there were significant difference ($P<0.05$) found in sperm motility on day 0, day 1, day 2 and day 3 from day 4 and non-significant in other days within group. In group III there were significant difference ($P<0.05$) found day 0, day 1, day 2 from day 3 and day 4 and also day 3 from day 4 within the group.

In present study sperm motility ranges between 88 to 92.5 per cent (%) at day 0 of semen evaluation which is in agreement with the findings of Zorinkimi *et al.* (2017b) who reported the progressive motility of mongrel dog semen of 42 ejaculates of semen by digital manipulation from 3 mongrel dogs and observed the over-all mean of progressive motility of mongrel dog semen was 95.30±1.65%. Kadirvel (1998) also observed 36 ejaculates from 6 mongrel dogs and the mean sperm motility recorded 86.67±1.07 with a range of 70 to 95 percent immediately after semen collection with also corroborate our findings. Our findings that in EYTCF extender motility reduces close to 40% at 4^{th} day of semen evaluation are in close similarity with the record of Rota *et al*. (1995), who reported that the mean motility of sperm preserved in egg yolk tris extender reduces gradually on preservation at 4^0C and drop to about 53% on day 4 of evaluation.

Kadirvel (1998) observed 36 ejaculates from 6 mongrel dogs the average percentage of sperm motility reduces from 86.38% to 49.86% in day 0 to day 5 of semen preservation in Egg yolk tris (EYT) extender which is in close proximity with our finding i.e. sperm motility reduces from 92.50 % to 39.16 % in day 0 to day 4 of semen preservation in EYTCF. Biswas (2010) observed that in 18 ejaculates from 3 dogs of Alsatian breed the mean value of progressive motility recorded on day 3 of preservation were 59.666 in Egg yolk tris fructose extender and 61 percent (%) in EYTG (Egg yolk tris glucose) extender. It is found that

progressive motility also decreases with EYTCF and centrifugation with EYTCF samples preserved at 4^0C but slower than normal preservation at 4^0C in refrigerator.

4.6 Percentage live spermatozoa

The mean value of percentage live spermatozoa of different groups is presented in the Table 3 and Fig. 2.

Table 3: Percentage live spermatozoa on different days of semen evaluation preserved at 4^0C (Mean±S.EM)

S. No.	Group	Day 0	Day 1	Day 2	Day3	Day 4
1	Group I	93.83±2.13 ^{aA}	80.66±3.86 ^{bA}	63.33±2.47 ^{cB}	N.D.	N.D.
2	Group II	94.91±1.20 ^{aA}	89.75±1.97 ^{aA}	82.83±2.77 ^{abA}	76.66±3.94 ^{abA}	48.50±15.65 ^{bA}
3	Group III	90.33±1.33 ^{aA}	87.83±2.88 ^{aA}	80.33±3.74 ^{aA}	73.83±3.70 ^{aA}	25.66±16.33 ^{bAB}

Means bearing different superscripts differed significantly (P<0.05) row wise a, b, c
Means bearing different superscripts differed significantly (P<0.05) column wise A, B

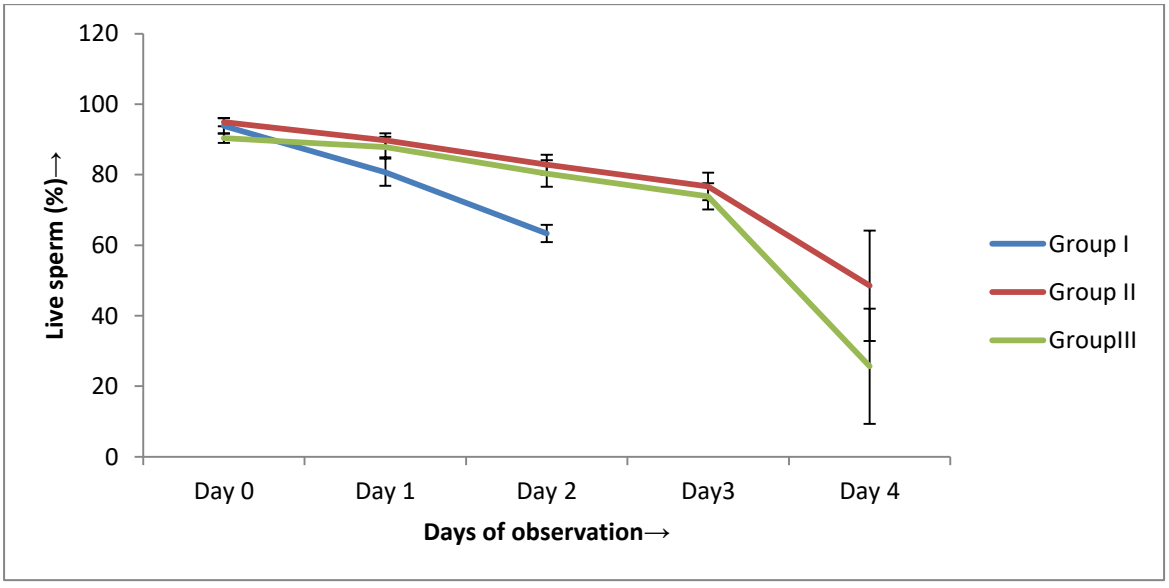

Fig. 2. Percentage live spermatozoa on different days of semen evaluation preserved at 4^0C.

In the present study, it was observed that the mean percentage live spermatozoa reduced to 63.33±2.47 percent (%) on day 2 in group I (Non extended semen), 48.50±15.65 percent (%) on day 4 in group II (extended with EYTCF extender) and 25.66±16.33 percent (%) in group III (Sperm pellet extended with EYTCF extender) respectively. There were non-significant differences found in percentage live spermatozoa at day 0 (Day of semen collection) and day 1 in group I, group II and group III. Further there were non-significant differences found in sperm live percent at day 2, day 3, day 4 between group II and group III but significant difference ($P<0.05$) found on day 2 of semen evaluation between group I from Group II and group III and mean value is higher in group II (extended with EYTCF extender).

In group I there were significant difference ($P<0.05$) found in sperm live percent on day 0, day 1 and day 2 of semen evaluation which reduced 63.33% on day 2. In group II there were significant difference ($P<0.05$) found in sperm live percent on day 0, day 1, day 2, day 3 from day 4 and non-significant in other days within group. In group III there was non-significant difference found on day 0, day 1, day 2, day 3 and day 4.

The present findings are in agreement with the findings of Kadirvel (1998) and Zorinkimi *et al.* (2017) who reported that the mean percentage of live spermatozoa in mongrel dog semen was 90.43±1.24% and 87.84±1.8% in freshly collected semen (Day 0). Kadirvel (1998) reported that the average percentage of live sperm recorded on day 0, 1, 2, 3, 4 and 5 days of preservation were 89.54±0.76, 86.44±0.84, 80.75±0.90, 74.65±0.99, 68.87±1.58 and 60.87±1.58, respectively in EYT extender. These findings are also in agreement with our findings. However, the sperm live percent reduces to 48.50±15.65% at 4^{th} day in group II but, Kadirvel (1998) reported 68.87±1.58% in EYT extender. These finding suggest that EYTCF extender is better for preservation of semen at 4^0C in comparison to without extender in group I and extender with centrifugation in group III.

4.7 Morphological abnormalities of sperm

The mean values of morphological abnormalities of spermatozoa of different groups are presented in the Table 4 and Fig.3.

Table 4: Morphological abnormalities of sperm at different days of semen evaluation (N=6) preserved at 4^0C (Mean±S.EM)

S. No.	Group	Day 0	Day 1	Day 2	Day3	Day 4
1	Group I	9.50±0.65 ^{bAB}	11.66±0.76 ^{bAB}	15.00±0.68 ^{aA}	N.D.	N.D.
2	Group II	8.25±0.77 ^{cB}	9.58±0.89 ^{cB}	11.16±1.07 ^{abA}	12.66±1.17 ^{abA}	13.00±2.77 ^{aAB}
3	Group III	11.50±0.88 ^{cA}	13.33±1.14 ^{bA}	14.83±1.35 ^{abA}	15.25±1.16 ^{aA}	16.00±0.87 ^{aA}

Means bearing different superscripts differed significantly (P<0.05) row wise a, b, c
Means bearing different superscripts differed significantly (P<0.05) column wise A, B

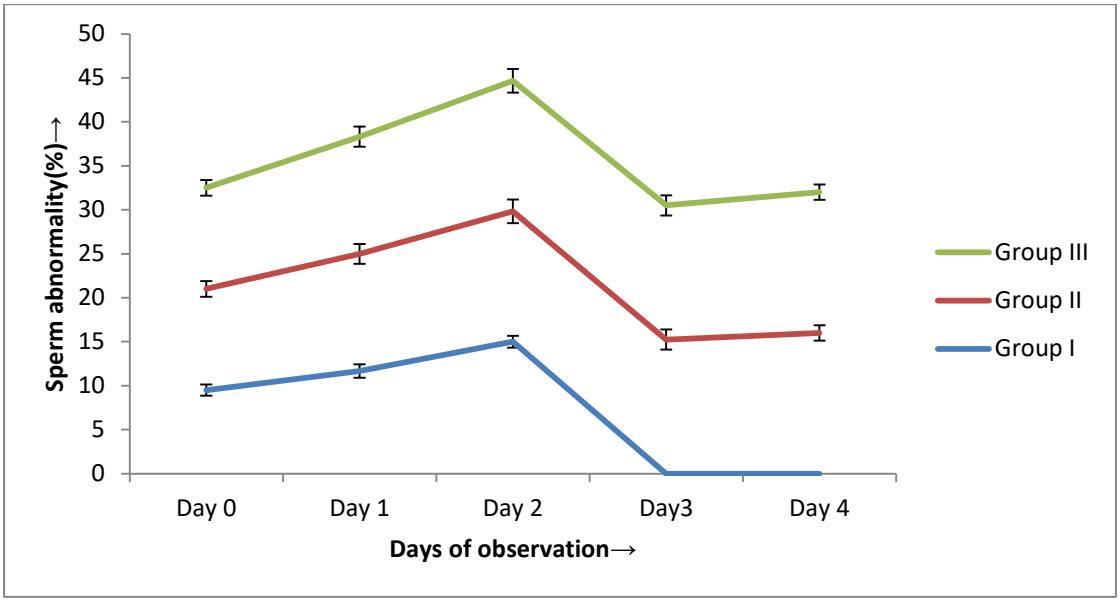

Fig. 3. Morphological abnormalities of sperm at different days of semen evaluation (N=6)

Morphological abnormalities found during study were abnormal head, middle piece defects, proximal protoplasmic droplets, tail abnormalities. In the present study, it was observed that the mean morphological abnormalities of spermatozoa reached 15±0.68 % on day 2 in group I (Non extended semen), 13±2.77 % and 16.00±0.87 % on day 4 in group II (extended with EYTCF extender) and group III (Sperm pellet extended with EYTCF extender) respectively. There were non-significant differences found in morphological abnormalities of spermatozoa percent at day 0 (Day of semen collection) and day 1 in group I, group II but significance difference (P<0.05) found in group III from group I and group II.

There were non-significant differences found in morphological abnormalities of spermatozoa percent at day 2 between group I, group II and group III. On day 3 and day 4 of semen evaluation were non-significant differences found in morphological abnormalities of spermatozoa between Group II and group III.

In group I there were significant difference ($P<0.05$) found in sperm morphological abnormalities of spermatozoa on day 2 from day 0 and day 1 of semen evaluation. In group II there were significant difference ($P<0.05$) found in sperm morphological abnormalities of spermatozoa on day 0, day 1 from day 2, day 3 from day 4 and non-significant differences in other days within group. In group III there were significant difference ($P<0.05$) found on day 0 from day1, day 2, day 3 and day 4 and non-significant differences on day 2, day 3 and day 4 within group. It has been observed that the percentage of morphological abnormalities increases with increasing days of preservation in all the three groups.

The present findings are in agreement with the report of Kadirvel (1998) who observed that 36 ejaculates from 6 mongrel dogs the average percentage of morphological abnormalities sperm recorded on day 0, 1, 2, 3, 4, and 5 days of preservation were 8.82±0.50, 11.65±0.64, 14.17±0.75, 16.67±0.85, 19.35±0.99 and 22.28±1.17 respectively in EYT extender. However, in our experiment the rate of increase of abnormal spermatozoa with increasing days of semen preservation up to 4^{th} day is comparatively slower. The present findings are also in agreements with the reports of Rijsselaere *et al*. (2002), who reported that the mean normal sperm morphology in dog after 3 days of storage at 4^0C the percentage of spermatozoa with a normal morphology was 85.3± 18.7 after centrifugation at 720x g. In group III there is also increase in abnormal sperm morphology with increasing days of semen preservation after centrifugation in EYTCF extender. However, our present findings are not in agreement with the findings of Biswas (2010) who reported higher percentage of sperm abnormalities at day 3 of semen preservation in both egg yolk tris fructose and egg yolk tris glucose extenders.

4.8 HOST (Hypo-osmotic swelling test)

The hypo-osmotic swelling test (HOST) was developed to evaluate the functional integrity of the membranes of spermatozoa. The ability of the sperm tail to swell when

exposed to hypo-osmotic conditions is a sign that water transport across the membrane occurs normally. Thus, the swelling phenomenon can be considered as a sign of functional integrity of the sperm membrane (Jeyendran *et al.*, 1984). Spermatozoa with intact plasma lemma become swollen and show coiled tails. In dog spermatozoa, the hypo-osmotic swelling was found to be positively correlated with sperm motility.

The mean value of HOST (Hypo-osmotic swelling test) of different groups is presented in the Table 5 and Fig. 4.

Table 5: Hypo-osmotic swelling test percentage at different days of semen evaluation (N=6) preserved at 4^0C (Mean±S.EM)

S. No.	Group	Day 0	Day 1	Day 2	Day3	Day 4
1	Group1	93.83±1.88 ^{aA}	82.00±3.31 ^{bB}	76.66±2.55 ^{bB}	N.D	N.D.
2	Group2	95.66±0.66 ^{aA}	91.83±1.75 ^{aA}	87.00±1.91 ^{aA}	81.33±2.44 ^{aA}	52.00±16.54 ^{abA}
3	Group3	95.83±0.14 ^{aA}	93.00±0.81 ^{aA}	88.66±1.40 ^{aA}	56.50±17.91 ^{abA}	28.50±18.03 ^{bAB}

Means bearing different superscripts differed significantly (P<0.05) row wise a, b, c
Means bearing different superscripts differed significantly (P<0.05) column wise A, B

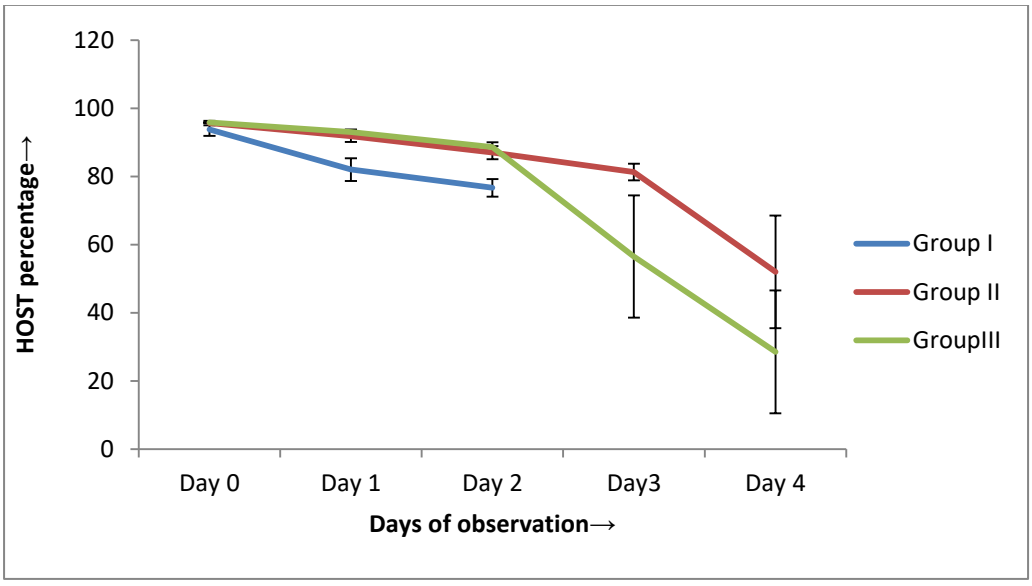

Fig. 4. Hypo-osmotic swelling test percentage at different days of semen evaluation (N=6) preserved at 4^0C.

In the present study, it was observed that the mean HOST (Hypo-osmotic swelling test) reduced 76.66±2.55 % on day 2 in group I (Non extended semen), 52.00±16.54 % and 28.50±18.03 % on day 4 in group II (extended with EYTCF extender) and group III (Sperm pellet extended with EYTCF extender) respectively. There were non-significant differences found in HOST (Hypo-osmotic swelling test) at day 0 (Day of semen collection) in group I, group II and group III. On day 1 and day 2 there were non- significant differences found in HOST (Hypo-osmotic swelling test) between group 2 and group III but significant difference ($P<0.05$) found on day 1 and day 2 of semen evaluation group I from Group II and group III.

In group I there were significant difference ($P<0.05$) found in HOST (Hypo-osmotic swelling test) on day 0 from day 1 and day 2 of semen evaluation. In group II there were non-significant difference found in HOST (Hypo-osmotic swelling test) on day 0, day 1, day 2, day 3 and day 4 within group. In group III there were significant difference ($P<0.05$) found on day 4 from day 0, day 1, day 2 and day 3 and non- significant differences found in day 0, day 1, day 2 and day 3 within group.

The present findings are in agreement with the findings of Rota *et al*. (1995) and Zorinkimi *et al.* (2017). Zorinkimi *et al.* (2017b) reported that the mean HOSTT– reacted sperm in mongrel dog semen was 93.27±0.53% in freshly collected semen. However, our present findings are inconsistent with the findings of Biswas (2010) who reported lower percentage of host reacted sperm at day 3 of semen preservation in both egg yolk tris fructose and egg yolk tris glucose extenders. Although, the percentage of HOST reacted spermatozoa decreases with increasing days of preservation in all the three groups, but comparatively slower in group II. Thus group II is better for future fertility of sperm preservation. The swelling phenomenon in HOST is considered as a sign of functional integrity of the sperm membrane (Jeyendran *et al.*, 1984). Thus motility is an indicator of structural and functional competence of spermatozoa; therefore, the percentage of progressively motile spermatozoa is usually positively correlated with that of sperm plasma membrane integrity. (Kumi- Diaka 1993).

4.9 Acrosomal integrity

The mean value of acrosomal integrity of different groups is presented in the Table 6 and Fig. 5.

Table 6: Acrosomal integrity percentage at different days of semen evaluation (N=6) preserved at 4^0C (Mean±S.EM)

S. No	Group	Day 0	Day 1	Day 2	Day3	Day 4
1	Group I	94.00±1.03 ^{aA}	88.50±1.15 ^{bA}	80.66±1.45 ^{cB}	N.D	N.D.
2	Group II	94.50±0.61 ^{aA}	89.50±0.50 ^{aA}	85.66±1.05^{aAB}	80.00±1.91 ^{aA}	52.16±9.64 ^{abA}
3	Group III	95.33±1.14 ^{aA}	91.66±1.74 ^{aA}	87.16±1.77 $^{a A}$	56.50±17.92b ^{aA}	28.50±18.02 ^{bAB}

Means bearing different superscripts differed significantly (P<0.05) row wise a, b, c
Means bearing different superscripts differed significantly (P<0.05) column wise A, B

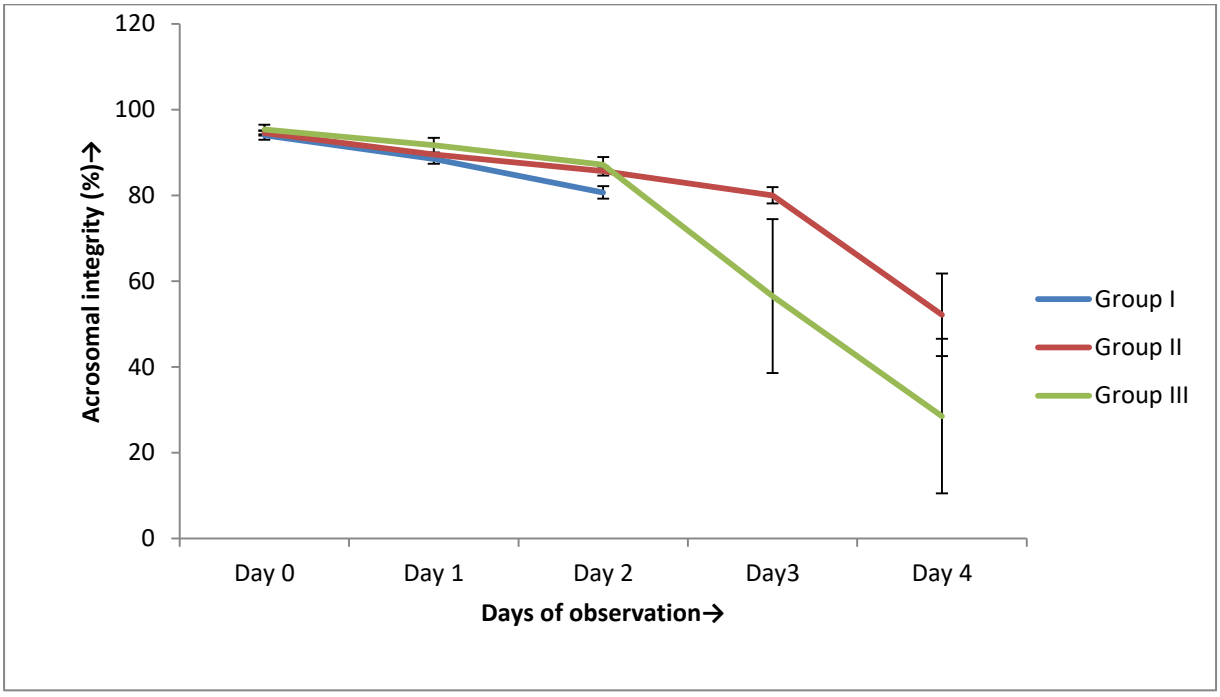

Fig. 5. Acrosomal integrity percentage at different days of semen evaluation (N=6) preserved at 4^0C

In the present study, it was observed that the mean acrosomal integrity reduced to 80.66±1.45 % on day 2 in group I (Non extended semen), 52.16±9.64 % and 28.50±18.02 %

on day 4 in group II (extended with EYTCF extender) and group III (Sperm pellet extended with EYTCF extender) respectively. There were non-significant differences found in acrosomal integrity at day 0 (Day of semen collection) and day 1 in group I, group II and group III. On day 2 there were significant differences ($P<0.05$) found in acrosome integrity group I and group II from group III. On day 3 and day 4 of semen evaluation non-significant difference found between group II and group III.

In group I there were significant differences ($P<0.05$) found in acrosome integrity on day 0, day 1 and day 2 of semen evaluation. In group II there were non-significant difference found in acrosome integrity on day 0, day 1, day 2, day 3 and day 4 within group. In group III there were significant difference ($P<0.05$) found on day 4 from day 0, day 1, day 2, and day 3 and non-significant differences found in day 0, day 1, day 2 and day 3 within group.

The present findings are in agreement with the findings of Zorinkimi *et al.* (2017b) at day 0, who reported that the percentage of mean acrosomal integrity in mongrel dog semen was 97.37±0.77 per cent (%) in fresh semen. The present findings are also in agreement with the reports of Kadirvel (1998) who observed that the average percentage of acrosome damage recorded on day 0, 1, 2 and 3 days of preservation were 8.88±0.58, 12.96±1.04, 17.19±1.48, 21.29±1.76 respectively in EYT extender preserved at 4^0C. But we found that the acrosomal integrity severely reduces at day 4 in group II (extended with EYTCF extender). The mean acrosomal integrity reported in the present study at day 3 in group III was 56.50±17.92 per cent (%) which was lower than the value reported by Rijsselaere *et al*. (2002), who reported 96.0± 3.5 per cent (%) after 3 days of storage at 4^0C and centrifugation at 720 x g. However, good percentage of acrosomal integrity reported up to day 2 (87.16±1.77%) in group III.

5 SUMMARY AND CONCLUSIONS

The present research work was conducted with the objective to study the physico-morphological characteristics of mongrel dogs semen and the effect of removal of prostatic fluid on semen preservation at refrigeration temperature (4^0C). A total of 18 ejaculates were collected from 3 mongrel dogs, 6 from each dog by digital manipulation. Immediately after semen collection, colour, volume, pH, mass motility, sperm concentrations were evaluated. Mass motility (≥3) and sperm concentrations (≥200x10^6 per ml) were selected to further processing and preservation of semen at refrigeration temperature (4^0C).

All the collected samples were divided into three Groups viz. Group I (Non-extended semen), Group II (Semen extended with egg yolk tris citrate fructose extender) and Group III (Sperm pellet extended with egg yolk tris citrate fructose extender) and kept at refrigeration temperature (4^0C). The preserved semen samples were examined for individual progressive motility, live dead percent, sperm morphological abnormality, HOST (Hypo-osmotic swelling test) and acrosomal integrity at each day of preservation till progressive motility goes down to 40%.

The colour of semen samples collected from mongrel dogs was observed as milky white to thin milky. The Mean±S.EM volume, pH, mass motility and sperm concentration were 3.55±0.31 ml, 6.56±0.03, 3.75±0.12, 307±19.38x10^6 per ml respectively.

The Mean±S.EM of the individual progressive motility preserved at refrigeration temperature (4^0C) at day 0 were 88.33±2.78, 92.50±1.70, 88.00±3.10, on day 1 were 70.00±1.15, 84.16±2.50, 80.33±4.63, on day 2 were 36.33±1.20, 72.83±3.96, 71.66±4.22 on day 3 were 00±00, 60.00±5.62, 58.33±6.91, on day 4 were 00±00, 39.16±13.16, 35.00±11.97 percent for Group I (Non-extended semen), Group II (Semen extended with egg yolk tris citrate fructose extender) and Group III (Sperm pellet extended with egg yolk tris citrate fructose extender) respectively.

The Mean±S.EM of the percentage live spermatozoa preservation at refrigeration temperature (4^0C) at day 0 were 93.83±2.13, 94.91±1.20, 90.33±1.33, on day 1 were 80.66±3.86, 89.75±1.97, 87.83±2.88, on day 2 were 63.33±2.47, 82.83±2.77, 80.33±3.74, on

day 3 were 00±00, 76.66±3.94, 73.83±3.70, on day 4 were 00±00, 48.50±15.65, 25.66±16.33 percent for Group I (Non-extended semen), Group II (Semen extended with egg yolk tris citrate fructose extender) and Group III (Sperm pellet extended with egg yolk tris citrate fructose extender) respectively.

The Mean±S.EM of the morphological abnormalities of spermatozoa preservation at refrigeration temperature (4^0C) at day 0 were 9.5±0.65, 8.25±0.77, 11.50±0.8, on day 1 were 11.66±0.76, 9.58±0.89, 13.33±1.14, on day 2 were 15.00±0.68, 11.16±1.07, 14.83±1.35, on day 3 were 00±00, 12.66±1.17, 15.25±1.16, on day 4 were 00±00, 13.00±2.77, 16.00±0.87 percent for Group I (Non-extended semen), Group II (Semen extended with egg yolk tris citrate fructose extender) and Group III (Sperm pellet extended with egg yolk tris citrate fructose extender) respectively.

The Mean±S.EM of the hypo-osmotic swelling test (HOST) preservation at refrigeration temperature (4^0C) at day 0 were 93.83±1.88, 95.66±0.66, 95.83±0.14, on day 1 were 82.00±3.31, 91.83±1.75, 93.00±0.81, on day 2 were 76.66±2.55, 87.00±1.91, 88.66±1.40, on day 3 were 00±00, 81.33±2.44, 56.50±17.91, on day 4 were 00±00, 52.00±16.54, 28.50±18.03 percent for Group I (Non-extended semen), Group II (Semen extended with egg yolk tris citrate fructose extender) and Group III (Sperm pellet extended with egg yolk tris citrate fructose extender) respectively.

The Mean±S.EM of the acrosomal integrity preservation at refrigeration temperature (4^0C) at day 0 were 94.00±1.03, 94.50±0.61, 95.33±1.14, on day 1 were 88.50±1.15, 89.50±0.50, 91.66±1.74, on day 2 were 80.66±1.45, 85.66±1.05, 87.16±1.77, on day 3 were 00±00, 80.00±1.91, 56.50±17.92, on day 4 were 00±00, 52.16±9.64, 28.50±18.02 percent for Group I (Non-extended semen), Group II (Semen extended with egg yolk tris citrate fructose extender) and Group III (Sperm pellet extended with egg yolk tris citrate fructose extender) respectively.

In the present research work different seminal attributes (Individual progressive motility, live dead percent, sperm morphological abnormality, HOST (Hypo-osmotic swelling test) and acrosomal integrity) of mongrel dogs semen observed were comparable with the finding of other research works.

Conclusions

- Sperm motility had gone down below 40 per cent (%) on day 2 in group I (Non-extended semen) and on day 4 in group II (Semen extended with egg yolk tris citrate fructose extender) and group III (Sperm pellet extended with egg yolk tris citrate fructose extender). Therefore, removal of prostatic fluid is beneficial for sperm preservation at refrigeration temperature (4^0C) in mongrel dogs.

- The group II and group III were maintained sperm motility, sperm live percent, HOST responses, acrosome integrity and lower morphological abnormalities of spermatozoa up to days 4 of preservation at refrigeration temperature (4^0C).

- The present research work revealed higher mean individual progressive motility, sperm live percent, HOST and acrosome integrity and lower morphological abnormalities of spermatozoa in Group II as compared to Group I and Group III.

- Egg yolk tris citrate fructose extender is suitable for short term storages/ preservation of mongrel dogs sperm at refrigeration temperature (4^0C).

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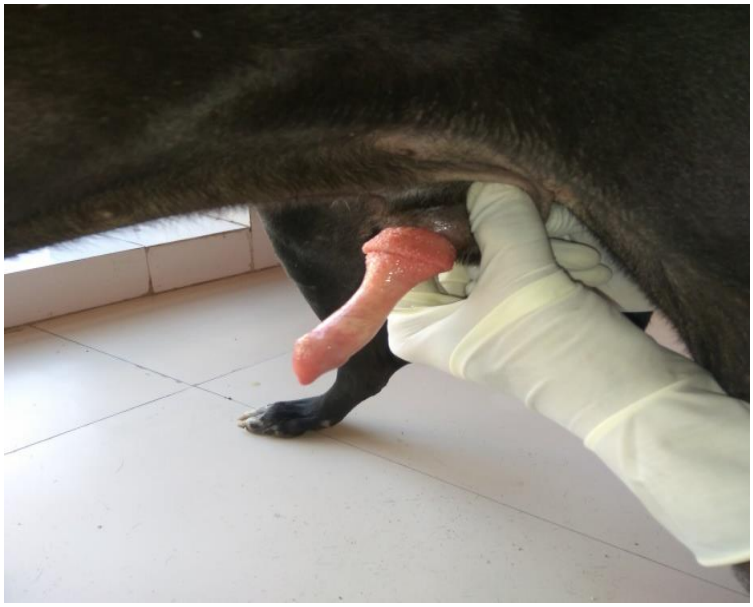

Image 1: Erected penis of dog showing pars longa glandis and bulbus glandis

Image 2: Semen collection by digital manipulation of penis of dog

Image 3: Collected semen (milky white)

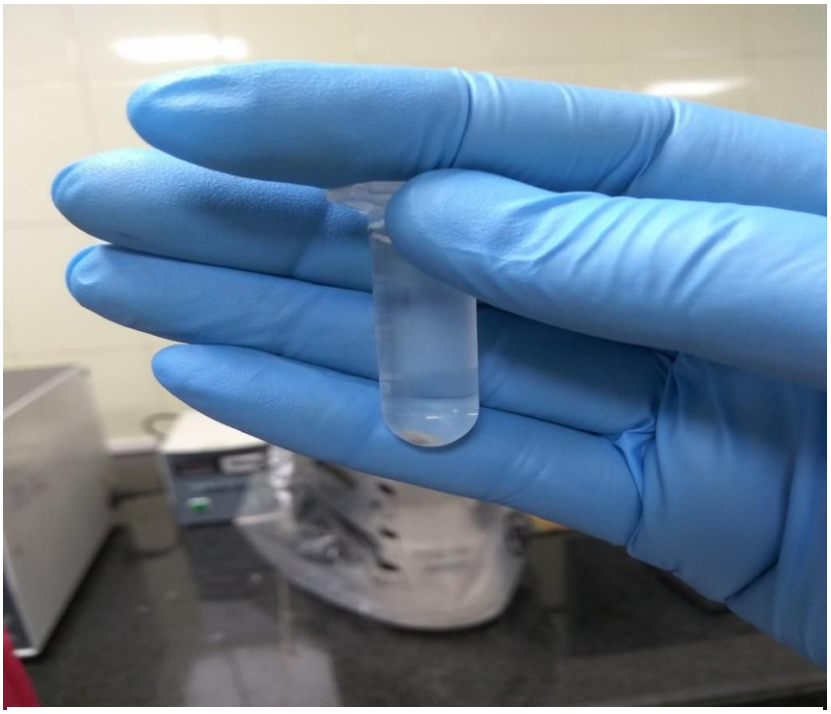

Image 4: Sperm pellet after centrifugation of semen

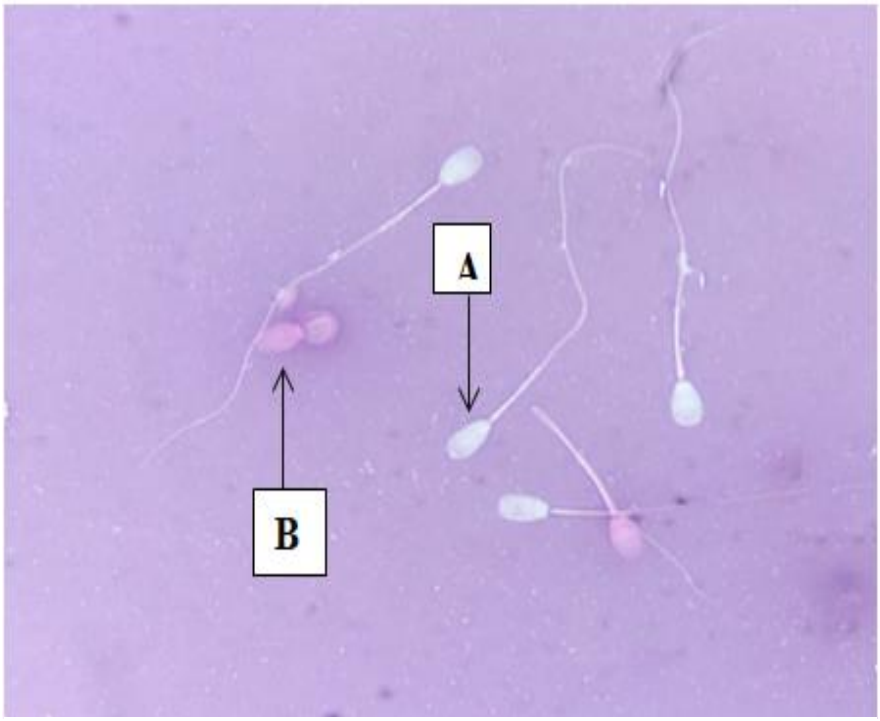

Image 5: Eosin-Nigrosin staining (A- Live; B- Dead Sperm)

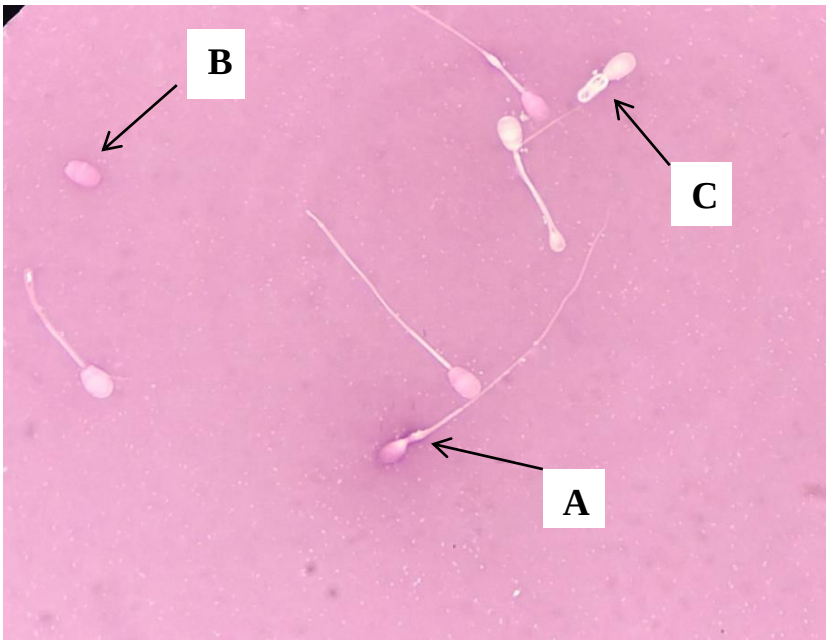

Image 6: Morphological abnormalities of sperm
A- Proximal cytoplasmic droplet, B- Free head,
C- Coiled tail

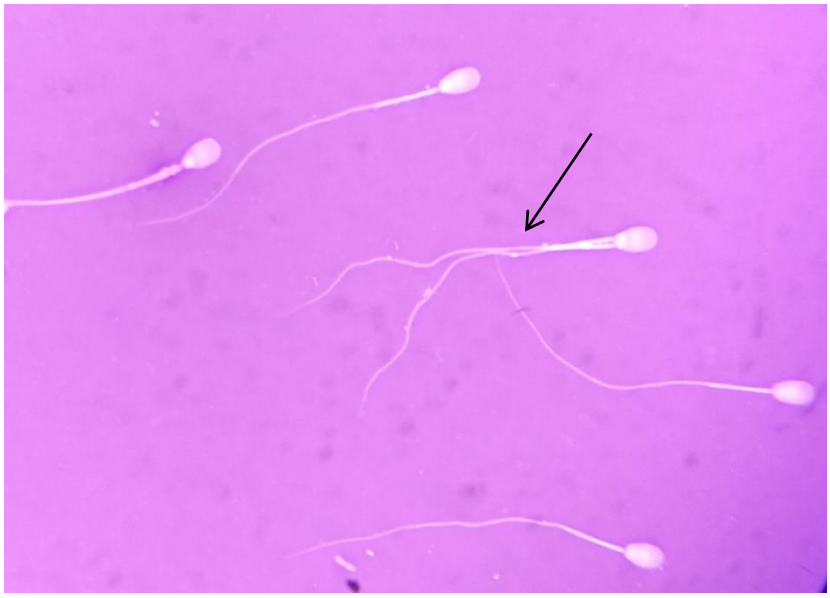

Image 7: Morphological abnormalities of sperm
(Double tail)

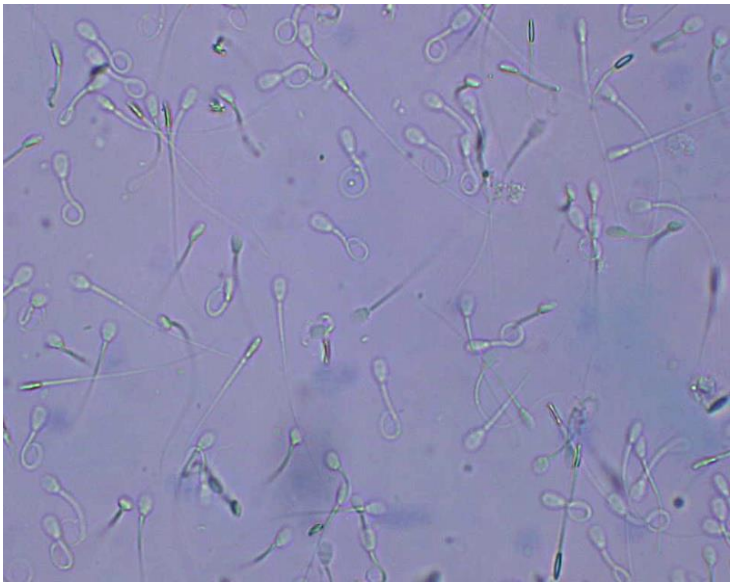

Image 8: Hypo-osmotic sperm swelling test showing coiled tail

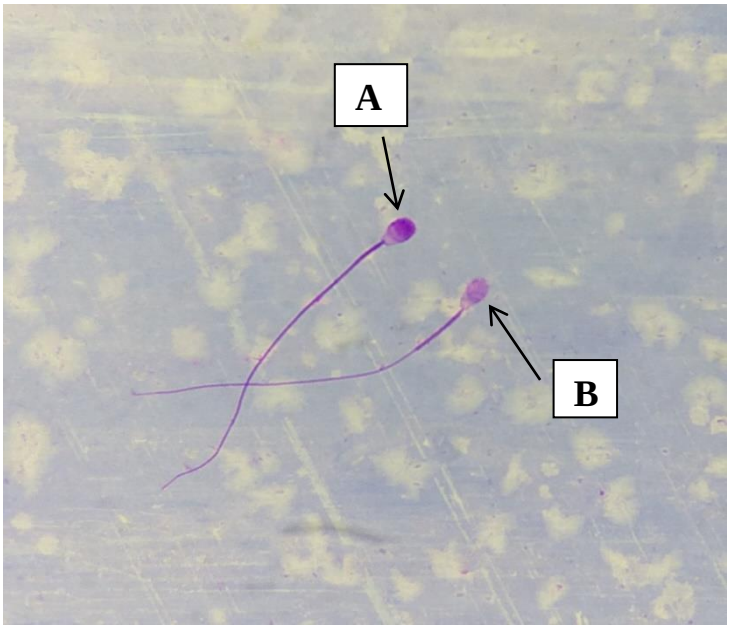

Image 9: Giemsa staining for acrosomal integrity of sperm
A- Normal intact acrosome
B- Abnormal acrosome

RESUME

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03.	B.V.Sc& AH	College of Veterinary & Animal Sciences Pantnagar	G.B.P.U & AT Pantnagar, Uttrakhand	2018	7.549/10
04.	M.V.Sc.	Bihar Veterinary College, Patna	Bihar Animal Sciences University, Patna	2020	8.427/10

Title of the M.V.Sc Thesis: TO STUDY THE EFFECT OF PROSTATIC FLUID ON SEMEN PRESERVATION AT REFRIGERATION TEMPERATURE IN MONGREL DOGS.

Scholarship : NTS

Publications

Research Paper : 03

Clinical Article : 03