PRESERVATION OF DOG SEMEN IN THREE EXTENDERS AT REFRIGERATION TEMPERATURE

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled "**PRESERVATION OF DOG SEMEN IN THREE EXTENDERS AT REFRIGERATION TEMPERATURE**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Dedicated to my beloved parents

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Introduction

INTRODUCTION

Dog was the first animal to be domesticated world over in different places almost at the same time. But only very little work has been done on scientific breeding of dogs to exploit the full genetic potential of pedigree males. Increased emphasis on scientific dog breeding in recent time has encouraged many researchers to explore the possibilities of artificial breeding in dogs.

Apart from exploitation of genetic potential of superior male, AI in dogs would greatly help problem like shyness, inexperience, habitual premature erection, weakness and mating difficulties. Although dog was the first species to be used for artificial insemination as early as 1780 by Spallanzani, very little progress has been made in artificial insemination either by using chilled or frozen semen, probably due to difficulty in evolving suitable medium for preservation of dog semen.

The technique of artificial insemination has gained momentum among dog breeders resulting in increased interaction and exchange of dog semen nationally and internationally. These turn of events necessitated a detailed investigation on semen preservation and A.I.

In India, because of commercialization of dog breeding, scientific kennels have been organized more in urban areas. In Kerala too, because of increased demand of pedigree male dogs, AI has gained importance in recent times.

Although, excellent media for preservation of bovine semen in both chilled and frozen state have been developed, only very few attempts have been made on the environmental conditions required to prolong the viability of canine sperm. The most valuable work on the dilution and preservation of dog semen was reported by Brochart and Coulomb in 1952. They found that equal parts of egg yolk and 2.7 per cent sodium citrate or fructose solution would keep sperm motile for four days.

First successful AI in dogs using chilled stored semen was reported by Harrop in 1954b. He found that the sperms stored at 4°C for four days in pasteurized milk were fertile. Although, stray reports of successful preservation of dog semen at 4°C are available, thorough investigations have been attempted only in a limited number of studies (Foote and Leonard, 1964; Roychoudhury and Dubay, 1974; Bouchard *et al.*, 1990; Rota *et al.*, 1995). Moreover, most of these studies were concentrated only on evaluation of diluents based on motility. Reports on combination of tests necessary to evolve a suitable diluent for preservation are scanty. The present

study was, therefore, undertaken with the following objectives.

1. To study the physical and morphological characteristics of dog semen.

2. To compare the efficacy of Egg yolk tris, Egg yolk citrate glycine glucose and Goat milk extenders on the basis of motility, live and dead sperm, abnormal sperm and acrosomal integrity after preservation at 4°C, with the ultimate object of recommending a suitable diluent for preservation of dog semen at refrigeration temperature.

Review of Literature

REVIEW OF LITERATURE

2.1 Semen collection

Semen can be collected fairly and readily from most of the dogs by using artificial vagina (AV) or digital manipulation. Harrop (1954a) designed a canine AV of different sizes suitable for dogs of different sizes but it was generally considered that semen collection by AV was of poor quality probably due to deleterious effect of latex liner upon canine semen. Boucher *et al.* (1958) observed that semen collection using latex was of inferior quality because it impaired the motility of sperms. Heywood and Sortwell (1971) could not differentiate three fractions of ejaculate from beagle dogs using AV. According to England and Allen (1992) first and third fraction of dog semen adversely affected the semen quality and hence discouraged the use of AV in dogs. But Mickelsen *et al.* (1993) did not find any adverse effect of warm latex rubber lining on dog semen. However, it is generally considered that semen collected by digital manipulation was of superior quality and quantity.

2.1.1 Digital manipulation

Lambert and Mc-kenzie (1940) were first to advocate digital manipulation technique for dogs in order to induce

effective ejaculation. It was recommended that bulbus glandis be rhythmically compressed with exerting moderate pressure behind the bulbus glandis but he also reported that many dogs induce ejaculation by thrust. Gill *et al.* (1970) and Smith (1989) used estrus bitch for semen collection by digital manipulation. According to Hopkins and Evans (1989) some dogs might require additional stimulation rather than digital pressure behind bulbus glandis which was achieved by gentle massage in the tip of the penis. Boucher *et al.* (1958) obtained better collection when teaser bitch was in the vicinity of the donor. It was further reported that with the presence of teaser, dog was less distracted by extraneous sounds and adapted more readily for semen collection. On the contrary, Seager (1969) opined that there was no need for teaser bitch but observed better semen samples when collection was made in quite area familiar to male dog. According to Taha *et al.* (1981) good ejaculate could be procured in the presence of a familiar bitch.

2.1.2 Frequency of semen collection

Perusal of available literature gives scanty information on frequency of semen collection and its effect on semen quality. Boucher *et al.* (1958) could obtain high quality semen by collecting every second day, but Laiblin *et al.* (1978) found that higher sperm output per ejaculate was

achieved when collections were made twice weekly. This was supported by Taha *et al.* (1981) and England and Pon[illegible]o (1996).

However, Foote (1964) found that semen sample obtained after 5 days of sexual rest appeared to be higher in percentage of motile sperm during storage. Olar *et al.* (1983) found an increase in sperm concentration when semen was collected once in every 4 to 5 days. Kumi-Diaka (1993) and Rawlings *et al.* (1994) also recommended semen collection twice a week from dogs. But Taha *et al.* (1983) reported that ejaculation once in a day for 5 days did not affect the sex libido and seminal characteristics but observed that ejaculation twice a day for 5 days reduced sexual libido and spermatozoal output and ejaculation thrice a day dramatically reduced the sex libido and semen quality.

2.1.3 Fractions of dog semen

Semen of dog comprised of three fractions viz. pre sperm, sperm rich and post sperm fraction. Harrop (1960) reported that first fraction was 0.25 ml and was probably secretion of urethral mucous glands. The second fraction varied from 0.5 to 3.5 ml and was the sperm bearing fraction and third was of prostatic origin and the volume varied from 2 to 30 ml. The collection of three fractions was made by quickly changing graduated centrifugal tubes attached to AV. There is consensus of opinion that first and third portion were

deleterious to sperm viability and sperm bearing second fraction was used for insemination. Fougner (1977) found that first fraction even damaged the sperm during freezing. Wales and White (1963), Kibble (1969), Seager and Fletcher (1973) and England *et al.* (1990) have reported that dilution of sperm rich fraction with first and third fractions produced a decline in the number of normal spermatozoa.

2.2 Semen evaluation

2.2.1 Volume

The mean volume of first, second and third fraction was reported to be 0.5 to 2, 0.5 to 4 and 3.5 to 25 ml respectively (Boucher *et al.*, 1958 and Harrop, 1960). In Alsatian dogs the corresponding values were 3.1, 1.3 and 1.4 ml respectively (Deshpande *et al.*, 1970). Average volume of dog semen was reported to be 3.1 ml (Heywood and Sortwell, 1971), 2.57 ml (Daiwadnya and Hukeri, 1993) and 2.2 ml (Dobrinski *et al.*, 1993). RoyChoudhury and Dubay (1974) observed that the volume of sperm rich fraction averaged 3.45 ml, while England and Allen (1989) reported a value of 1.2 ml.

2.2.2 Colour and density

Desphande *et al.* (1970) observed that semen of different breeds of dogs had different colour. Olson (1992) found that pre and sperm rich fraction had a creamy to white colour and a homogenous appearance. He further remarked that cloudy sample might indicate azoospermia or oligospermia. Daiwadnya and Hukeri (1993) found that colour of dog semen was cloudy to thin milky.

2.2.3 Mass activity

Harrop (1960) reported, that fresh dog semen showed rippling movement unlike wave motion of bovine semen. Desphande *et al.* (1970) reported that mass activity of dog semen varied from ++++ to ++ and a scale of + to ++++.

2.2.4 Motility

Gill *et al.* (1970) recorded the mean motility of 79 per cent in Beagle dogs, while RoyChoudhury and Dubay (1974) observed mean sperm motility of 65 per cent. The mean percentage motility was reported to be 89.5 (England and Allen, 1989); 95 (Ferguson *et al.*, 1989); 70 (Olson, 1992); 84.1 (Dobrinski *et al.*, 1993), 67.5 (Mickelsen *et al.*, 1993), 72.8 (Kumi-Diaka, 1993) and 78.6 Rota *et al.* (1995) in different breeds of dogs.

2.2.5 pH

Boucher *et al.* (1958) observed pH of dog semen varied according to the method of semen collection and found that the pH was 6.59, 6.7 and 6.52 respectively when semen was collected using AV without teaser bitch and digital manipulation with and without teaser.

Harrop (1960) reported that the sperm containing second fraction was more acidic than third fraction containing prostatic fluid. He found that the pH of normal dog semen varied from 5.8 to 6.9 and became more acidic when kept longer and pH of first, second and third fraction were 6.37, 6.10 and 7.20 respectively.

RoyChoudhury and Dubay (1974) reported that the averaged pH of sperm-rich fraction of 15 ejaculates was 6.47 ± 0.35 (6.0-7.0)in dog semen. Chatterjee *et al.* (1976) observed an average pH of 6.8 within a range of 6.4 to 6.9 in dog semen collected by digital manipulation.

2.2.6 Sperm concentration

Harrop (1960) recorded sperm concentration of 125 million sperm per ml in variety of dogs while Gill *et al.* (1970) reported an average sperm concentration of 98 million spermatozoa per ml. Heywood and Sortwell (1971) reported that

sperm concentration vary from 18.5 to 78.7 million per ml and opined that total sperm per ejaculate was more appropriate in an unfractionated ejaculate. According to Chatterjee *et al.* (1976) sperm concentration averaged 161.58 million per ml and ranged from 30 to 570 million per ml. Daiwadnya and Hukeri (1993) recorded an average sperm concentration 264 ± 15.0 million per ml in semen of mongrel dogs. The total number of motile sperm per ejaculate was reported to be 241 to 928 million (Drawer, 1979), 200 million (Soderberg, 1986), 332.75 million (England and Allen, 1989), 250 million (Olson, 1992) and 310 million (Dobrinski *et al.*, 1993). However, Rota *et al.* (1995) found that total sperm output per ejaculate averaged 916 million in various breeds of dogs. England and Allen (1989) stated that concentration of second fraction of semen varied 60 to 550 million per ml and averaged 299.6 million per ml.

2.2.7 Live and Dead Sperms

Heywood and Sortwell (1971) found that percentage of live sperm showed considerable monthly variations and recorded between 51 to 89 per cent of total live sperms. Taha *et al.* (1983) remarked that frequency of semen collection had no significant effect on total live sperm output and also found that percentage of dead sperms ranged between 7.6 and 10.4. The normal live sperm per ejaculate averaged 78.2 per cent

(England and Allen ,1989), 97 to 99 per cent (Ferguson *et al.*, 1989), 82.81 per cent (Daiwadnya and Hukeri, 1993) and 85.3 per cent (Chatterjee *et al.*, 1976) for various breeds of dogs.

2.2.8 Abnormal spermatozoa

Chatterjee *et al.* (1976) reported 14.7 per cent of abnormal sperms in normal dog semen while Plummer *et al.* (1987) observed higher percentage of (87 per cent) protoplasmic droplet. Kawakami *et al.* (1988) observed a high percentage of abnormal sperms with coiled tail and attributed the same to testicular, epididymal and ductus deferens dysfunction. On the other hand, a low percentage (12-31 per cent) was recorded by Ferguson *et al.* (1989), (10-11 per cent) by England and Allen (1989) and (10.08 per cent) by Daiwadnya and Hukeri (1993). Oettle (1993) classified abnormal spermatozoa in dog semen as acrosomal, head, mid piece and tail defects and sperm agglutination.

2.2.9 Acrosome integrity

Acrosomal abnormality of canine sperm has been reported by a few workers. The percentage of damaged and missing acrosome was ranging from 2.5 to 7.5 and 0.5 to 1.5 (Ferguson *et al.*, 1989). Kumi-Diaka and Badtram (1994) found that the mean percentage of acrosome defects was 2.43 ± 1.15 in fresh dog semen. The percentage of vesiculated, swollen and lost

acrosomes were 10.7 ± 8.3 and other abnormalities of acrosome constituted 2.7 ± 2.0 per cent in fresh semen of various breeds of dogs (Rota *et al.*, 1995).

2.3 Preservation of dog semen at 4°C

2.3.1 Extenders used for preservation of dog semen

Most valuable work on dilution and preservation of dog semen was reported by Brochart and Coulomb (1952). According to them equal parts of egg yolk and 2.7 per cent of sodium citrate or fructose solution had kept the sperms motile over a period of four days. Harrop (1956) recommended pasteurized milk for preservation of dog semen at 4°C and found that sperms were fertile even after four days of storage. Harrop (1956) reported that second sperm rich fraction diluted in egg yolk sodium citrate was a suitable medium for preservation of dog semen at 4°C. Gutierrez Nales (1957) reported a prolonged survival time of dog semen in yolk citrate, milk and egg yolk milk extenders. According to Foote and Leonard (1964) sodium citrate was superior to phosphate buffer. They also recommended that addition of glucose prolonged the storage period. It was also reported that milk extender was less satisfactory and formulated a new diluent, sodium citrate dihydrate 1.16 per cent, glycine 0.75 per cent, glucose 1 per cent, egg yolk 20 per cent with 10,000 IU of penicillin

and 10,000 μg of dihydrostreptomycin per ml of extender and pH of 6.7. They also found that when 8 per cent glycerol was added in this extender, sperm motility was reduced by 13 per cent over a storage period of 12 days. Gill *et al.* (1970) and RoyChoudhury and Dubay (1974) suggested that tris egg yolk diluent was superior when compared to citrate yolk and milk yolk extenders. Province *et al.* (1984) compared the effect of six extenders (EYT, EYB, beltsville F-3, CUE, Caprogen and Heated skim milk) at three glycerol levels (0, 3 and 6 per cent) on the motility of dog sperm stored at 4°C. They recommended Caprogen as the extender for preservation of canine sperm at 4°C but reported that 6 per cent glycerol level reduced the motility.

Morton and Bruce (1989) reported that the semen extended in Tris-citrate glycerol egg yolk diluent preserved its motility of about 80 per cent even after 4 days. Bouchard *et al.* (1990) opined that motility of canine semen was better preserved in NFDMSG extender than in egg yolk citrate extender when semen was cooled at a medium or fast rate for a storage at 4°C. Insemination with extended dog semen diluted in EYCGG extender obtained successful pregnancy rates (Dabas *et al.*, 1991). Muller (1992) found that there was no significant difference in the preservability of dog semen in the Tris-egg yolk and Skim milk diluent with or without BSA when stored at 5°C for 144 h. Satisfactory sperm motility and viability of

refrigerated dog semen preserved in tris-egg yolk diluent was reported by Sainiz *et al.* (1993).

Rota *et al.* (1995) suggested that egg-yolk Tris extender seemed to be superior to egg yolk milk and egg-yolk cream for preservation of dog semen at 4°C. He also reported that for chilled extended semen used for AI, best results were obtained with egg-yolk Tris (62.5%) than egg-yolk cream (51.1%). England and Ponzio (1996) used non-fat dried milk glucose extender for storage of dog semen at 5°C and compared the keeping quality with that of frozen semen. They suggested that chilling was the most suitable method of semen storage, if the semen samples were used within 4.9 days of collection.

2.3.2 Dilution rate

Harrop (1956) reported that the sperm rich fraction diluted 1:7 times with pasteurized milk resulted in successful conception. Foote and Leonard (1964) observed that sperm motility was higher in semen extended 1:30 (20 million sperm per ml) than that of semen extended 1:3 (200 million per ml). The sperm rich fraction was diluted at the rate of 1:5 in citrate-yolk, milk-yolk and tris-yolk extender, for preservation of dog semen at 4°C without any deleterious effect (RoyChoudhury and Dubay, 1974). Dabas *et al.* (1991) reported successful pregnancy by using extended semen 1:3 times with egg yolk citrate-glycine-glucose extender.

Rota *et al.* (1995) extended semen at concentrations of 30 to 105 million per ml of extender to study the effects of seminal plasma and three extenders on canine semen stored at 4°C and achieved high pregnancy rate with egg yolk tris extender. England and Ponzio (1996) diluted the second fraction of ejaculate 1:4 with a non fat dried milk glucose extender and compared the quality of cooled rewarmed dog semen with the quality of frozen thawed semen in tris yolk extender. They found that chilling was the most suitable method of semen storage if the samples were used approximately within 4.9 days of collection.

2.3.3 Cooling rate

Derin-Bennett *et al.* (1974) stated that sperm susceptibility to cold shock was species dependent. Canine, human, fowl and rabbit spermatozoa were more tolerant to cold shock than those of large domestic animals. Second fraction of ejaculate was diluted at ratio of 1:4 with a non fat dried milk-glucose extender, and was cooled slowly (half rate of cooling = 9 minutes) to 5°C for storage and suggested that chilling is the most suitable method upto 4.9 days (England and Ponzio, 1996). Bouchard *et al.* (1990) reported that the progressive motility and velocity of sperm in semen cooled at 4°C in NFDMS-G were higher at the fast (-1.0°C/min) and medium (-0.3°C/min) cooling rates than at the slow cooling

rate (0.1°C/min). They recommended medium or fast cooling rates for cooling canine spermatozoa. Diluted semen kept at 37°C and subsequently cooled in a refrigerator to about 4°C over a period of 2 to 3 hours was found to be beneficial for storage (Sainiz *et al.*, 1993).

2.3.4 Evaluation of preserved semen

2.3.4.1 Motility

Brochart and Coulomb (1952) reported 50 per cent motility after 4 days of storage by diluting the sperm rich fraction in 2.7 per cent sodium citrate egg yolk extender and stored at 4°C. Harrop (1956) found 50 per cent motility upto 120 hours in pasteurised milk at 4°C. Wales and White (1958) observed that the motility of dog spermatozoa preserved at 4°C was depressed by diluent containing 0.02 M carbonate buffer. They also reported that motility of dog semen was depressed by addition of prostatic fluid. Foote and Leonard (1964) found that the average percentage of motile spermatozoa was 71, 70, 69, 62 and 43 respectively after 1, 2, 4, 8 and 16 days of storage at 4°C extended with yolk citrate-glycine-glucose extender. RoyChoudhury and Dubay (1974) diluted sperm-rich fraction in citrate-yolk, milk-yolk and tris-yolk extender and stored at 5°C. They found that the sperm motility averaged 61, 66 and 67 per cent respectively in the three diluent initially, 55, 62 and 59 per cent after 4 hours, 35, 30 and

45 per cent after 24 hours, 30, 36 and 36 per cent after 30 hours, 12, 17 and 35 after 48 hours and 10, 5 and 35 per cent after 72 hours. Province *et al.* (1984) observed that the mean dog sperm motility was 52 percentage after 240 hours of storage at 4°C in CAP as compared to 47 per cent in CUE although the sperm motility in CUE was equivalent to CAP for the first 72 hours. Mean percentge of motility was 100, 98, 90, 87, 80 and 35 respectively on 0, 24, 48, 72, 96 and 120 hours of storage in chilled semen extended in tris-citrate glycerol egg yolk extender (Morton and Bruce, 1989).

Ekrod (1989) found that the sperm motility of undiluted semen at 0 and 24 hours was 68.4 and 20.4 per cent and after dilution in tris-egg-yolk diluent, motility was 74.8 and 59.2 per cent respectively.

Gunzel-Apel and Ekrod (1991) reported that the progressive motility of the sperm rich fraction of undiluted semen or diluted with prostatic fluid and with Tris-egg yolk extender at 0 hours of storage at 4°C was 64.4, 68.4 and 74.8 per cent and after 24 hours of storage at 4°C was 31.6, 20.4 and 59.2 per cent respectively. Kumi-Diaka and Badtram (1994) obtained mean percentage motility of 82.00 ± 12.93 and 79.25 ± 9.21 in fresh and chilled dog semen diluted in tris egg yolk extender and stored for 24 hours. Rota *et al.* (1995) reported that motility upto day 4 was higher in egg yolk Tris (53.6%)

than in those preserved in egg yolk milk (30.4%) and egg yolk cream (14.1%). The mean motility of the sperm preserved in egg yolk Tris extender on day 0, 1, 2, 3 and 4 were 76.8 ± 14.0, 73.6 ± 15.5, 70.4 ± 15.4, 60.9 ± 18.9 and 53.6 ± 20.1 % respectively.

2.3.4.2 Live and dead sperms

Martin *et al.* (1977) recorded that the percentage of dead spermatozoa in semen immediately after collection, after refrigeration and after freezing was 15.3, 20 and 24.17 respectively. The mean percentage of dead spermatozoa in the fresh semen and chilled semen stored for 24 hours in Tris egg yolk extender was reported to be 9.1 ± 0.9 and 11.7 ± 1.3 respectively (Kumi-Diaka and Badtram, 1994). England and Ponzio (1996) reported 81 to 94 percentage of live spermatozoa on day 0 and 18 to 33 percentage on day 10 after cooling to 5°C and rewarming.

2.3.4.3 Abnormal spermatozoa

Morton and Bruce (1989) reported that as the motility falls, the proportion of abnormal spermatozoa rises. They observed that the defects mainly involved the mid-piece (37%) and tail (50%), while head defects accounted for only a small percentage (13%) of the overall abnormal non-motile spermatozoa. Kumi-Diaka and Badtram (1994) found that the

storage of canine semen at 5°C for 24 hours did not significantly impair the physical and functional integrity of spermatozoa. They opined that the overall mean percentage of motility, hypo osmotic swelling response, acrosome reacted spermatozoa, acrosomal defects and percentage of live spermatozoa did not significantly differ between the fresh and semen chilled for 24 h. England and Ponzio (1996) observed that on cooling and rewarming of dog semen extended in non fat-dried milk-glucose extender, morphologically normal live spermatozoa was found to be 78 to 90 per cent on day 0 and 0 to 8 per cent on day 10.

2.3.4.4 Acrosome integrity

Oettle (1986) reported significant acrosomal damage during dilution and cooling to 5°C and observed that sperm motility did not correlate with acrosomal integrity. Kumi-Diaka and Badtram (1994) found that the mean percentage of acrosome defect in fresh and chilled semen diluted in Tris egg yolk extender and stored for 24 h were 2.43 ± 1.15 and 2.65 ± 1.11. England and Ponzio (1996) diluted semen in non fat dried milk glucose extender and stored at 5°C for 10 days. They found that the normal acrosomal count was 84 to 88 per cent and 6 to 18 per cent respectively on day 0 and day 10. Rota *et al.* (1995) reported significantly lower acrosomal

changes in egg yolk Tris than autologous seminal plasma on day 3 and day 4 after preservation at 4°C. Egg yolk Tris maintained the integrity of acrosome better than egg-yolk milk and egg yolk cream extender (Aalseth and Sacke, 1985).

Materials and Methods

MATERIALS AND METHODS

3.1 Experimental animals

Six healthy, adult male mongrel dogs selected at random formed material for the study. The dogs were maintained under standard identical managemental condition in the kennels located at Veterinary College hospital, Mannuthy. Six ejaculates from each dog were collected at weekly interval and a total of 36 ejaculates were used for the study.

3.2 Semen collection

All the dogs were initially trained for collection by digital manipulation (Allen, 1991) without presenting a teaser bitch for two weeks.

A quiet place with a nonslip floor was chosen for semen collection. After kneeling down on the left side of the dog, the penis in its sheath was encircled firmly between forefinger and thumb, behind the bulbus glandis with a gloved hand. This caused partial erection of penis. The dogs that did not respond to this technique, to and fro massage was given.

When the bulbus glandis had begun to swell up, the whole length of penis was exposed from the sheath. The penis was

again gripped behind the bulbus glandis with forefinger and thumb. Pulsating digital pressure was applied in a rythmic pattern behind the bulbus glandis leading to ejaculation of semen. The three fractions of semen were collected separately in three sterile graduated collection vials, attached with sterile glass funnels. The behaviour of dog during semen collection, the pattern of ejaculation of the various fractions of ejaculate and ejaculation time were observed. The collection vials containing different fractions were kept separately in a water bath at 37°C for evaluation. Care was taken to ensure that the semen was not exposed to unfavourable conditions during and after collection.

3.3 Semen evaluation

3.3.1 Volume

The volume of pre sperm, sperm rich and post-sperm fraction was assessed directly from the graduated semen collection vials.

3.3.2 Colour

The colour of pre sperm, sperm rich and post sperm fraction was assessed directly by viewing against a white background immediately after collection of semen.

3.3.3 Density

Density of the sperm rich fraction was assessed directly by holding the collection vial against a good source of diffused light. Density was graded on a scale of D to DDDD.

3.3.4 Mass activity

A small drop of freshly collected sperm rich fraction was gently spread on a warm slide with a glass rod and examined under low power (x100) of bright-field microscope.

3.3.5 Motility

The progressive motility of sperm rich fraction was assessed by visual evaluation of wet mount preparations under high power of a microscope (x400) on prewarmed glass slide (Thomas *et al.*, 1993).

The percentage of spermatozoa with normal vigorous and forward linear motion was subjectively assessed to the nearest 5 per cent at five different areas of the slide (England and Pon[illegible]o, 1996).

3.3.6 pH

pH indicator strips (Merck) with a range of 5.0 to 7.5 were used for the three different fractions of semen to assess the pH immediately after each collection.

3.3.7 Sperm concentration

The concentration of spermatozoa in the three fractions was assessed separately by Neubaur haemocytometer. Total spermatozoan output was calculated by multiplying the concentration by the volume of each fraction.

3.3.8 Live and dead spermatozoa

The live and dead sperms were assessed by eosin and nigrosin staining technique (Blom, 1950 and Harrop, 1960). A drop of fresh semen was placed on a clean warm glass slide, and mixed with two drops of 5 per cent eosin and five drops of nigrosin 10 per cent. Smears were prepared from this mixture and dried in air. In each slide two hundred spermatozoa were examined under oil immersion (x1000) objective of a microscope. Both fully and partially stained spermatozoa were counted as dead.

3.3.9 Abnormal spermatozoa

The percentage of spermatozoa with abnormal morphology was evaluated using eosin-nigrosin stained smears examined by bright-field microscope under oil immersion. The sperm morphology was classified as head, mid piece and tail abnormalities (Morton and Bruce, 1989).

3.3.10 Acrosome integrity

The acrosome morphology was assessed by Giemsa staining technique. Semen smears were fixed by drying and immersing in a 5 per cent formaldehyde for 30 minutes at 37°C. The smears were washed in running tap water and dried. The slides were then immersed in Giemsa stain (Watson, 1975) and kept for 3 hours. Then the smears were rinsed in distilled water and dried in air. In each smear two hundred spermatozoa were counted and the acrosome integrity was evaluated. Acrosome morphology was classified as swollen, vesiculated, lost and other abnormalities (Rota *et al.*, 1995).

3.3.11 Methylene Blue Reduction Test (MBRT)

The procedure adapted was similar to Salisbury and VanDemark (1961). Egg yolk Tris diluent 0.4 ml, fresh sperm rich fraction 0.1 ml and methylene blue solution (0.05%) 0.05 ml were taken in a 10 ml test tube and mixed gently. The

mixture was covered with 1 cm layer of liquid paraffin. It was kept in water bath at 46.5°C. Time required for disappearance of blue colour was recorded.

3.3.12 Incubation test

Sperm rich fraction was diluted in Tris egg yolk extender at the rate of 1:10 in a 10 ml test tube. The test tube was immediately placed in water bath at 46.5°C and percentage of motility was assessed at 10 minutes interval upto 30 minutes.

3.4 Preparation of diluent

a. Composition of egg yolk citrate-glycine glucose (EYCGG)

(Foote and Leonard, 1964)

Sodium citrate dihydrate	- 1.16 g
Glycine	- 0.75 g
Glucose	- 1.00 g

These ingredients were transferred into a 100 ml volumetric flask containing 50 ml of double distilled water and dissolved completely and the volume was made upto 100 ml. Then 20 ml of buffer was replaced with egg yolk. Benzyl penicillin 1000 IU/ml and dihydrostreptomycin - 1000 μg/ml were added and mixed again. The diluent so prepared was kept in a water bath at 38°C.

b. Egg Yolk Tris (EYT) (Rota *et al.*, 1995)

Tris (hydroxymethyl) amino methane	-	3.025 g
Citric acid	-	1.70 g
Fructose	-	1.25 g

The chemicals were taken in a 100 ml volumetric flask containing of double 50 ml distilled water and dissolved completely. Final volume was made upto 100 ml and 20 ml of Tris buffer was replaced with egg yolk. To this Benzyl penicillin-1000 IU/ml and Dihydro-streptomycin sulphate - 1000 µg/ml were added, mixed and kept in a water bath at 38°C.

c. Goat milk (GM) (Harrop, 1956)

Fresh goat milk was heated to 90°C for 15 minutes in a water bath, cooled and left in the refrigerator overnight. The cream layer was removed and remaining milk was utilized as diluent after addition of the following antibiotics.

Benzyl penicillin - 1000 IU/ml
Dihydro streptomycin sulphate - 1000 µg/ml

3.5 Dilution and cooling of semen

The sperm rich fraction was diluted in three extenders at the rate of 1:4 by split sample technique (England and Ponnio, 1996; Rota *et al.*, 1995).

Diluted semen was filled in 1 ml screw caped serum vials and kept in the water bath at 38°C and diluted semen was gradually cooled to 4°C and stored at that temperature for preservation.

3.6 Evaluation of preserved semen

Motility, live sperm count, abnormal sperm count and acrosomal integrity were performed on preserved semen at 24 hours interval for five days or until total cessation of motility, whichever was earlier as done earlier for undiluted semen.

Results

RESULTS

A study was carried out using semen samples from six mongrel dogs maintained in the kennels at Veterinary College Hospital, Mannuthy to evolve a suitable diluent for preservation of dog semen at 4°C.

4.1 Response of the dogs to digital manipulation technique for semen collection

Six dogs out of seven showed good response to digital manipulation without presenting a teaser bitch following two weeks of training. The erection of penis occurred within 30 to 60 seconds after firm holding of penis behind the bulbus glandis with gloved hand (Plate 1). Pelvic thrust was coincident with the ejaculation of the pre sperm fraction and persisted around 0.5 to 1 minute (Plate 2). The dogs remained steady during which sperm rich fraction was ejaculated over 30 to 60 seconds (Plate 3). Then the dogs tried to turn. During this period the penis was turned back and the emission of prostatic fraction lasted for 3 to 20 minutes (Plate 4). The three fractions of semen were collected separately in three graduated collection vials (Plate 5).

4.2 Semen evaluation

A total number of 36 ejaculates, six ejaculates from each dog was evaluated for physical and morphological

characteristics of dog semen immediately after semen collection. The values are presented in the Table 4.1, 4.2, 4.3 and 4.4.

4.2.1 Volume

The mean volume of first, second and third fraction of semen was (ml) 0.63 ± 0.07, 1.29 ± 0.08 and 4.12 ± 0.23 respectively (Table 4.1). The mean total volume of ejaculate was 6.15 ± 0.75 with a range of 2.2 to 10.0 ml. There was significant difference ($P<0.01$) in volume of semen from different dogs.

4.2.2 Colour and Density

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 (Table 4.1). The mean density of second fraction varied from D to DDD with an average of DD (Table 4.3).

4.2.3 Mass activity

The average mass activity of sperm rich fraction of ejaculate was ++(+) with a range of + to +++(+) (Table 4.3).

4.2.4 Motility

The mean sperm motility was noted to be 86.67 ± 1.07 with a range of 70 to 95 per cent immediately after semen collection (Table 4.3). There was significant difference ($P<0.01$) in sperm motility of different dogs.

4.2.5 pH

The mean pH of first, second and third fraction of semen recorded was 6.24 ± 0.01, 6.36 ± 0.01 and 6.65 ± 0.02 respectively (Table 4.2). Analysis of pH of different fraction revealed that there was no significant difference between first and second fraction. But third fraction was found to have significant ($P<0.01$) difference from first and second fraction. There was no significant difference in the pH of semen of different dogs.

4.2.6 Concentration

The mean spermatozaoal concentration of second fraction was 416.28 ± 22.56 with a range of 220 to 840 million per ml while that of third fraction was 6.11 ± 1.66 within a range of one million to 20 million spermatozoa per ml (Table 4.2). The first fraction hardly contained any spermatozoa. The mean total sperm output of the ejaculate was found to be 527.50 ± 29.46 with a range of 280 to 1092 million. There was highly significant ($P<0.01$) difference in sperm concentration of sperm rich fraction and total sperm output per ejaculate between dogs.

4.2.7 Live and dead sperm

Overall mean of live sperm (Table 4.4) count was 89.44 ± 0.57 with a range of 80 to 96 per cent. There was significant difference in the live sperm count for different dogs.

4.2.8 Abnormal spermatozoa

The mean percentage of head, mid piece and tail abnormalities was 0.77 ± 0.09, 2.85 ± 0.19 and 3.96 ± 0.24 respectively (Table 4.4). The mean total abnormal sperm was 7.59 ± 0.45 with a range of 4 to 15.8 per cent. There was significant ($P<0.01$) difference in the abnormal spermatozoa of the different dogs. Common sperm abnormalities recorded in this study were thick middle piece, coiled tail, bent tail and presence of proximal protoplasmic droplets (Plate 6 & 7).

4.2.9 Acrosome integrity

The average percentage of swollen, vesiculated and ruffled, lost and other acrosomal abnormalities was 2.92 ± 0.15, 1.43 ± 0.06, 0.53 ± 0.05 and 1.74 ± 0.20 respectively. The mean total acrosomal abnormality was 6.63 ± 0.38 with a range of 3.9 to 12.4 per cent (Table 4.4 and Plate 8, 9 and 10). There was significant ($P<0.01$) difference in the acrosomal abnormalities of the different dogs.

4.2.10 Methylene blue reduction test (MBRT)

The overall average time taken for reduction of methylene blue by dog spermatozoa was 26.40 ± 0.86 with a range 20 to 38 minutes (Table 4.5). There was significant ($P<0.01$) difference in time taken for reduction of methylene blue by semen from different dogs.

4.2.11 Incubation test

The overall mean percentage of sperm motility at 0, 10, 20 and 30 minutes of incubation (46.5°C) was 86.38 ± 1.04, 88.33 ± 1.13, 70.56 ± 1.26 and 53.20 ± 2.17 respectively (Table 4.6).

Average percentage of sperm motility on day 1, 2, 3, 4 and 5 of preservation at 4°C was 79.86 ± 1.32, 73.47 ± 1.72, 66.80 ± 1.99, 58.47 ± 2.41 and 49.86 ± 2.74 respectively (Table 4.6). Correlation coefficients were worked out for 30 minutes incubation at 46.5°C and day 1, 2, 3, 4 and 5 preservation at 4°C (Table 4.7). Analysis revealed that there was significant ($P<0.01$) correlation between sperm viability at 30 minutes incubation and sperm motility upto five days of preservation under refrigeration temperature.

4.3 Evaluation of preserved semen

A total of 36 ejaculates were extended in three extenders viz., egg yolk tris (EYT), egg yolk citrate glycine glucose (EYCGG) and goat milk (GM). Each of the extended semen samples was preserved at 4°C and sperm motility, live sperm count, abnormal sperm count and acrosomal integrity were evaluated at 24 h interval for 5 days.

4.3.1 Motility

The average percentage of sperm motility in three extenders at different time intervals on preservation at 4°C are given (Table 4.8 and Figure 4.1). The average percentage of sperm motility on day 0, 1, 2, 3, 4 and 5 was 86.38 ± 1.04, 79.86 ± 1.32, 73.47 ± 1.72, 66.80 ± 1.99, 58.47 ± 2.41 and 49.86 ± 2.74 respectively in EYT. The corresponding values were 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 respectively in EYCGG. In GM the average percentage of sperm motility was 76.25 ± 1.26, 61.94 ± 1.76, 41.66 ± 1.92, 26.11 ± 2.04, 7.50 ± 1.01 and 0.00 respectively on day 0, 1, 2, 3, 4 and 5.

On analysis it was found that the sperm motility did not vary significantly between semen stored in EYT and EYCGG at different intervals of time. But there was a significantly ($P<0.01$) lower percentage of motile sperms in semen preserved

in GM when compared to motility in the corresponding intervals of storage in both EYT and EYCGG.

4.3.2 Live sperm count

The mean percentage of live sperm in three extenders after preservation at 4°C are presented in the Table 4.9 and Figure 4.2 .

The average percentage of live sperm recorded in the study was 89.54 ± 0.76, 86.44 ± 0.84, 80.75 ± 0.90, 74.65 ± 0.99, 68.87 ± 1.13 and 60.87 ± 1.58 respectively on day 0, 1, 2, 3, 4 and 5 in EYT. The mean percentage of live sperm on day 0, 1, 2, 3, 4 and 5 were 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 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 GM respectively.

There was no significant difference in the percentage of live sperm between EYT and EYCGG but it was statistically significant ($P<0.01$) from GM. The live sperm count preserved in GM from day 0 to day 5 was found significantly lower when compared to EYT and EYCGG.

4.2.3 Abnormal sperm count

Average percentage of abnormal sperm in three extenders during storage at 4°C from day 0 to day 5 are presented in the Table 4.10 and Figure 4.3 and Plate 11.

Average percentage of abnormal sperm recorded in the present study was 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 on day 0, 1, 2, 3, 4 and 5 in EYT and 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 and 12.31 ± 0.67, 18.74 ± 1.12, 24.43 ± 1.55, 30.70 ± 1.83, 37.03 ± 1.85, 44.75 ± 2.02 in GM extender on preservation at 4°C. Statistical analysis revealed that there was no significant difference on percentage of abnormal sperm between EYT and EYCGG, but it was significantly ($P<0.01$) different between GM and EYT and GM and EYCGG. There was significantly ($P<0.01$) higher percentage of sperm abnormality in GM on day 0 to 5 when compared to EYT and EYCGG.

4.3.4 Acrosome integrity

The mean percentage of acrosome damage in three extender after perservation at 4°C from day 0 to day 5 are presented in the Table 4.11 and Fig. 4.4 (Plate 12).

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 and 31.54 ± 2.73 respectively in EYT, 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 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 GM. Statistical analysis revealed

that there was no significant difference in the percentage of acrosome damage between EYT and EYCGG but there was significant difference between GM and the other two diluents. There was significantly ($P<0.01$) higher percentage of acrosomal damages from day 0 to day 5 in GM when compared to EYT and EYCGG.

Table 4.1 Physical characteristics of dog semen

Dog	Volume (ml)				Colour		
	First fraction	Second fraction	Third fraction	Total	First fraction	Second fraction	Third fraction
1	0.20± 0.05	0.72± 0.06	2.05± 0.25	2.90± 0.35	Clear, watery	Thick milkly	Clear, watery
2	0.65± 0.18	1.26± 0.18	3.97± 0.28	5.89± 0.22	Clear, watery	Thick milkly	Clear, watery
3	1.27± 0.20	1.88± 0.25	4.77± 0.73	7.92± 0.82	Clear, watery	Thick to thin milkly	Clear, watery
4	0.53± 0.07	0.97± 0.08	4.58± 0.32	6.08± 0.28	Clear, watery	Thick to thin milkly	Clear, watery
5	0.41± 0.07	1.33± 0.06	3.97± 0.07	5.71± 0.29	Clear, watery	Thick milkly	Clear, watery
6	0.68± 0.03	1.56± 0.10	5.38± 0.33	7.63± 0.28	Clear, watery	Thick milkly	Clear, watery
Overall mean	0.63± 0.07	1.29± 0.08	4.12± 0.23	6.15± 0.75	Clear, watery	Thick to thin milkly	Clear, watery
F value				17.624**			

** P<0.01

Table 4.2 Physical characteristics of dog semen

Dog No.	No.of ejaculate	pH			Concentration (million)			
		First fraction	Second fraction	Third fraction	First fraction	Second fraction	Third fraction	Total number of sperms/ ejaculate
1	6	6.27± 0.06	6.30± 0.03	6.66± 0.06	-	641.66± 53.11	-	455.50± 46.65
2	6	6.33± 0.33	6.45± 0.03	6.62± 0.03	-	307.66± 15.38	20.00± 2.58	427.00± 53.77
3	6	6.23± 0.02	6.30± 0.04	6.55± 0.06	-	383.33± 24.68	-	722.83± 103.30
4	6	6.18± 0.03	6.40± 0.05	6.70± 0.07	-	321.66± 34.13	16.66± 4.21	362.33± 28.91
5	6	6.19± 0.01	6.35± 0.02	6.67± 0.07	-	433.33± 21.47	-	574.00± 27.59
6	6	6.22± 0.03	6.33± 0.04	6.72± 0.05	-	410.00± 39.04	-	623.33± 26.17
Overall mean	36	6.24± 0.01	6.36± 0.01	6.65± 0.02		416.28± 22.56	6.11± 1.66	527.50± 29.46
F value		2.401 NS	2.737*	0.957 NS		12.901**		5.874**

* P<0.05
** P<0.01
NS - Non significant

Table 4.3 Physical characteristics of dog semen

Dog No.	Number of ejaculate	Density	Mass activity	Motility (percentge)
1	6	DDD	+++	90.83±1.54
2	6	D	++	76.67±2.47
3	6	DD	++(+)	90.83±1.54
4	6	DD	++(+)	87.50±1.71
5	6	DD	++(+)	88.50±2.14
6	6	DDD	++(+)	86.67±1.67
Overall	36	DD	++(+)	86.67±1.07
F-value				7.769**

**P<0.01

Table 4.4 Morphological characteristics of dog spermatozoa

Dog No.	Live sperm (%)	Abnormal sperms (%)				Acrosomal integrity (%)				
		Head	Mid piece	Tail	Total	Swollen	Vesiculated & ruffled	Lost	Others	Total
1	92.60+ 0.56	0.633± 0.13	2.200± 0.13	4.233± 0.74	7.233± 0.98	2.02± 0.17	1.57± 0.16	0.70± 0.05	1.78± 0.27	6.07± 0.43
2	83.06+ 0.30	1.667± 0.23	4.467± 0.52	5.716± 0.69	11.883± 1.21	4.00± 0.34	1.90± 0.08	1.12± 0.08	4.12± 0.18	11.13± 0.38
3	92.10+ 0.21	0.383± 0.03	2.283± 0.13	3.650± 0.16	6.317± 0.09	2.32± 0.14	1.32± 0.08	0.30± 0.02	1.40± 0.24	5.33± 0.47
4	89.52+ 0.16	0.767± 0.11	2.417± 0.45	4.350± 0.39	7.533± 0.91	2.33± 0.17	1.00± 0.11	0.35± 0.03	1.07± 0.10	4.13± 0.65
5	89.62+ 0.40	0.367± 0.04	3.567± 0.11	2.667± 0.05	6.600± 0.15	3.43± 0.16	1.20± 0.05	0.30± 0.04	1.02± 0.05	5.95± 0.17
6	89.76+ 0.23	0.383± 0.04	2.483± 0.33	3.150± 0.34	5.967± 0.64	3.43± 0.32	1.62± 0.32	0.40± 0.03	1.08± 0.07	6.53± 0.26
Overall mean	89.44+ 0.57	0.77 ± 0.09	2.85 ± 0.19	3.96 ± 0.24	7.59 ± 0.45	2.92± 0.15	1.43± 0.06	0.53± 0.05	1.74± 0.20	6.63± 0.38
F value	5.849**				7.471**					31.718**

** P<0.01

Table 4.5 Methylene blue reduction test by using dog semen

Dog No.	Number of ejaculate	MBRT (minutes)
1	6	23.33 ± 1.15
2	6	36.17 ± 1.94
3	6	24.66 ± 0.30
4	6	24.20 ± 1.57
5	6	24.55 ± 1.48
6	6	25.50 ± 1.48
Overall	36	26.40 ± 0.86
F-value		17.264**

**P<0.01

Table 4.6 Viability of dog spermatozoa

Dog No.	Incubation at 46.5°C (motility in percentage)				Preservation at 4°C (motility in percentage)				
	0 min	10 min	20 min	30 min	day 1	day 2	day 3	day 4	day 5
1	90.83± 1.54	91.67± 2.48	75.83± 1.54	58.33± 4.61	83.33± 1.67	78.33± 1.67	72.50± 1.71	64.16± 2.39	55.00± 3.43
2	76.67± 2.48	80.00± 2.90	60.83± 3.01	35.00± 3.66	65.83± 3.01	54.16± 3.76	44.16± 3.76	33.33± 5.60	21.66± 6.07
3	90.83± 1.54	93.33± 1.06	74.17± 1.54	63.33± 1.67	85.00± 2.24	80.00± 2.24	75.00± 2.24	69.16± 2.01	62.50± 2.82
4	87.50± 1.71	88.33± 2.80	72.50± 3.61	56.67± 4.96	82.50± 1.71	77.50± 1.71	70.00± 2.24	60.83± 3.53	48.33± 4.79
5	86.67± 1.67	89.17± 2.01	70.00± 2.24	52.50± 4.23	81.66± 1.67	75.83± 2.01	70.00± 2.01	65.00± 2.24	56.66± 3.08
6	85.33± 1.54	87.50± 2.15	70.00± 2.59	53.33± 5.30	80.83± 1.54	75.00± 1.83	68.33± 2.80	58.33± 4.61	48.33± 4.79
Overall mean	86.38± 1.04	88.33± 1.13	70.56± 1.26	53.20± 2.17	79.86± 1.32	73.47± 1.72	66.80± 1.99	58.47± 2.41	49.86± 2.74

Table 4.7 Coefficient of correlation (r) of sperm viability on incubation and preservation at 4°C

Number of ejaculate	Correlation between	Coefficient of correlation (r) in EYT
36	30 minutes incubation and day 1 preservation	0.818**
36	30 minutes incubation and day 2 preservation	0.787**
36	30 minutes incubation and day 3 preservation	0.762**
36	30 minutes incubation and day 4 preservation	0.746**
36	30 minutes incubation and day 5 preservation	0.738**

** - Significant at 1% level
* - Significant at 5% level

Table 4.8 Sperm motility (percentage) in three extenders at 4°C

Extender	Days					
	0	1	2	3	4	5
EYT	a 86.38± 1.04	a 79.86± 1.32	a 73.47± 1.72	a 66.80± 1.99	a 58.47± 2.41	a 49.86± 2.74
EYCGG	a 80.83± 1.20	a 75.41± 1.59	a 70.13± 1.85	a 64.44± 1.96	a 55.41± 2.49	a 48.33± 2.62
GM	b 76.25± 1.26	b 61.94± 1.76	b 41.66± 1.92	b 26.11± 2.042	b 7.50± 1.61	b 0.00

Figures having different superscript in column varies significantly ($P<0.01$)

Table 4.9 Live sperm (percentage) in three extenders at 4°C

Extender	Days					
	0	1	2	3	4	5
EYT	a 89.54± 0.76	a 86.44± 0.84	a 80.75± 0.90	a 74.65± 0.99	a 68.87± 1.13	a 60.87± 1.58
EYCGG	a 87.87± 0.87	a 84.78± 0.91	a 78.85± 0.98	a 72.54± 1.32	a 66.24± 1.64	a 57.67± 2.24
GM	b 85.28± 0.88	b 76.48± 1.07	b 56.75± 1.54	b 41.78± 1.972	b 28.87± 2.23	b 18.56 2.87

Figures having different superscript in column varies significantly ($P<0.01$)

Table 4.10 Sperm abnormality (percentage) in three extenders at 4°C

Abnormal sperms	EYT						EYCGG						GM					
	0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3	4	5
Head	0.95± 0.12	1.22± 0.14	1.43± 0.17	1.66± 0.19	1.83± 0.20	2.10± 0.22	1.12± 0.14	1.35± 0.16	1.62± 0.17	1.91± 0.19	2.12± 0.20	2.39± 0.21	1.36± 0.17	1.82± 0.21	2.23± 0.22	2.54± 0.22	2.74± 0.22	2.94± 0.24
Midpiece	3.21± 0.21	4.27± 0.29	5.37± 0.38	6.41± 0.46	7.58± 0.60	8.95± 0.74	3.49± 0.25	4.61± 0.34	5.68± 0.44	6.80± 0.59	8.03± 0.67	9.34± 0.84	4.38± 0.34	6.74± 0.56	8.94± 0.79	11.33± 0.94	13.60± 1.04	15.94± 1.12
Tail	4.59± 0.27	6.08± 0.38	7.22± 0.46	8.54± 0.54	9.89± 0.61	11.18± 0.74	5.22± 0.31	6.71± 0.45	8.04± 0.52	9.36± 0.60	10.87± 0.73	12.58± 0.96	6.72± 0.50	10.15± 0.81	13.22± 1.07	16.79± 1.31	20.47± 1.27	24.87± 1.75
Total	a 8.82± 0.49	a 11.65± 0.64	a 14.17± 0.85	a 16.67± 0.85	a 19.35± 0.99	a 22.28± 1.17	a 9.88± 0.53	a 12.73± 0.67	a 15.39± 0.79	a 18.13± 0.92	a 21.07± 1.04	a 24.99± 1.44	b 12.51± 0.67	b 18.74± 1.12	b 24.43± 1.55	b 30.70± 1.822	b 37.03± 1.85	b 44.75± 2.02

Figures having different superscript in column varies significantly (P<0.01)

Table 4.11 Acrosomal damage (percentage) in three extenders at 4°C

Abnormal sperms	EYT						EYCGG						GM					
	0	1	2	3	4	5	0	1	2	3	4	5	0	1	2	3	4	5
Swollen	4.06± 0.26	5.89± 0.50	7.76± 0.70	9.78± 0.88	12.14± 1.16	14.48± 1.43	4.69± 0.36	6.62± 0.51	8.72± 0.76	11.49± 1.25	13.67± 1.49	15.84± 1.63	6.31± 0.41	11.08± 0.76	16.71± 1.21	23.50± 1.45	30.27± 1.59	33.34± 1.68
Vesiculated & reffled	2.01± 0.15	3.10± 0.30	4.28± 0.46	5.37± 0.51	6.63± 0.55	8.16± 0.68	2.32± 0.17	3.55± 0.35	4.98± 0.58	6.26± 0.75	7.53± 0.80	9.19± 0.89	3.84± 0.36	6.81± 0.67	10.36± 0.88	14.55± 1.72	17.47± 1.18	18.75± 1.19
Lost	0.61± 0.06	0.88± 0.09	1.11± 0.11	1.36± 0.14	1.60± 0.15	18.83± 0.17	0.66± 0.07	0.96± 0.10	1.21± 0.13	1.50± 0.16	1.78± 0.18	2.04± 0.21	1.20± 0.14	1.73± 0.19	2.23± 0.24	2.54± 0.30	2.67± 0.24	2.84± 0.34
Other	2.21± 0.25	3.10± 0.33	4.01± 0.44	4.58± 0.50	5.89± 0.65	7.08± 0.82	2.55± 0.29	3.51± 0.38	4.56± 0.55	5.54± 0.67	6.55± 0.74	7.55± 0.86	3.67± 0.38	5.73± 0.62	8.46± 0.90	10.85± 1.04	13.08± 1.07	14.50± 1.15
Total	a 8.88± 0.58	a 12.96± 1.04	a 17.19± 1.48	a 21.29± 1.76	a 26.19± 2.21	a 31.54± 2.73	a 10.23± 0.71	a 14.69± 1.10	a 19.48± 1.70	a 24.78± 2.42	a 29.58± 2.80	a 34.56± 3.10	b 15.02± 1.07	b 25.36± 1.91	b 38.13± 2.93	b 51.66± 3.49	b 62.93± 3.66	b 69.45± 4.20

Figures having different superscript in column varies significantly (P<0.01)

Fig 4.1 Average Sperm Motility in three extenders at 4°C

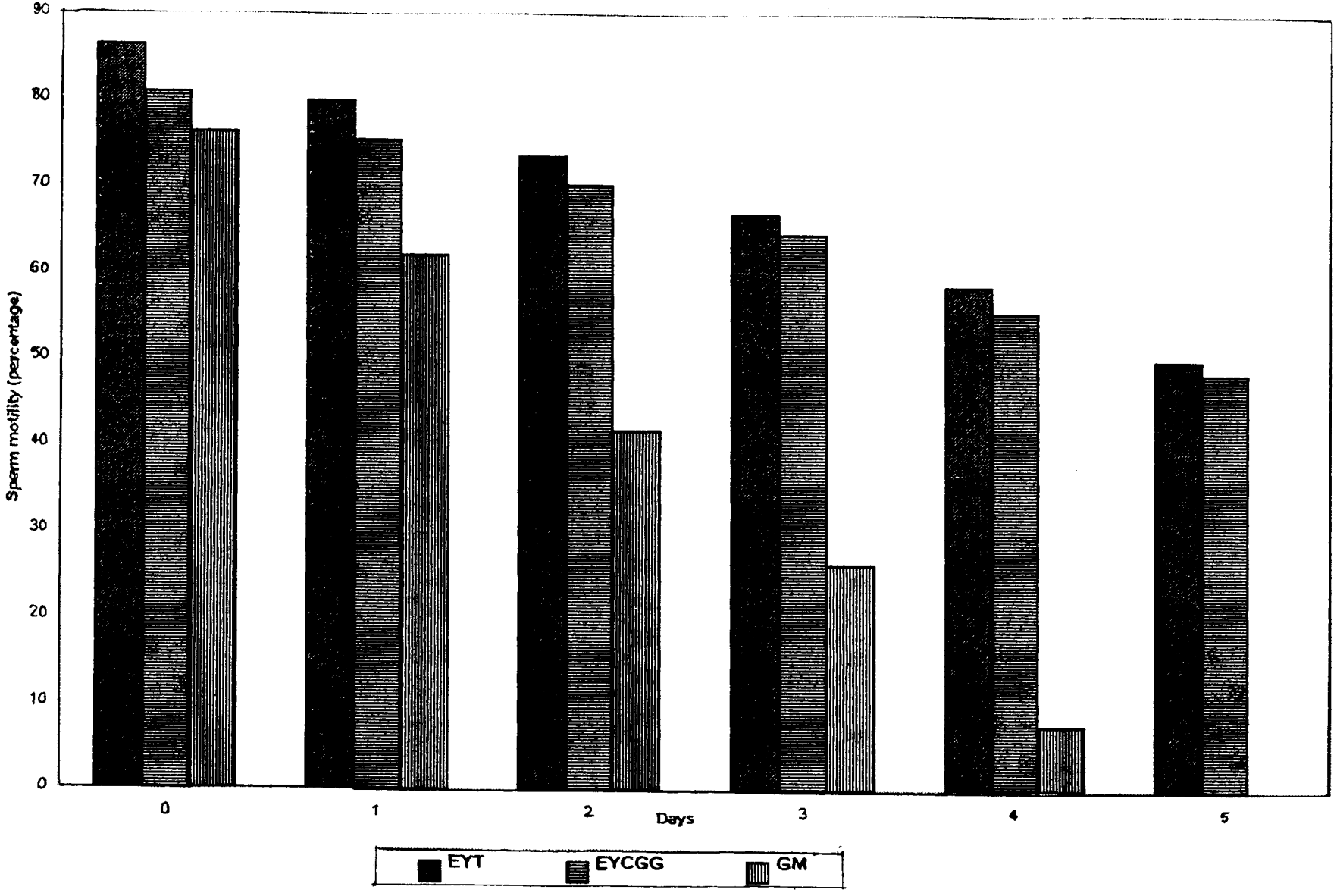


Fig.4.2: Average Live sperm in three extenders at 4°C

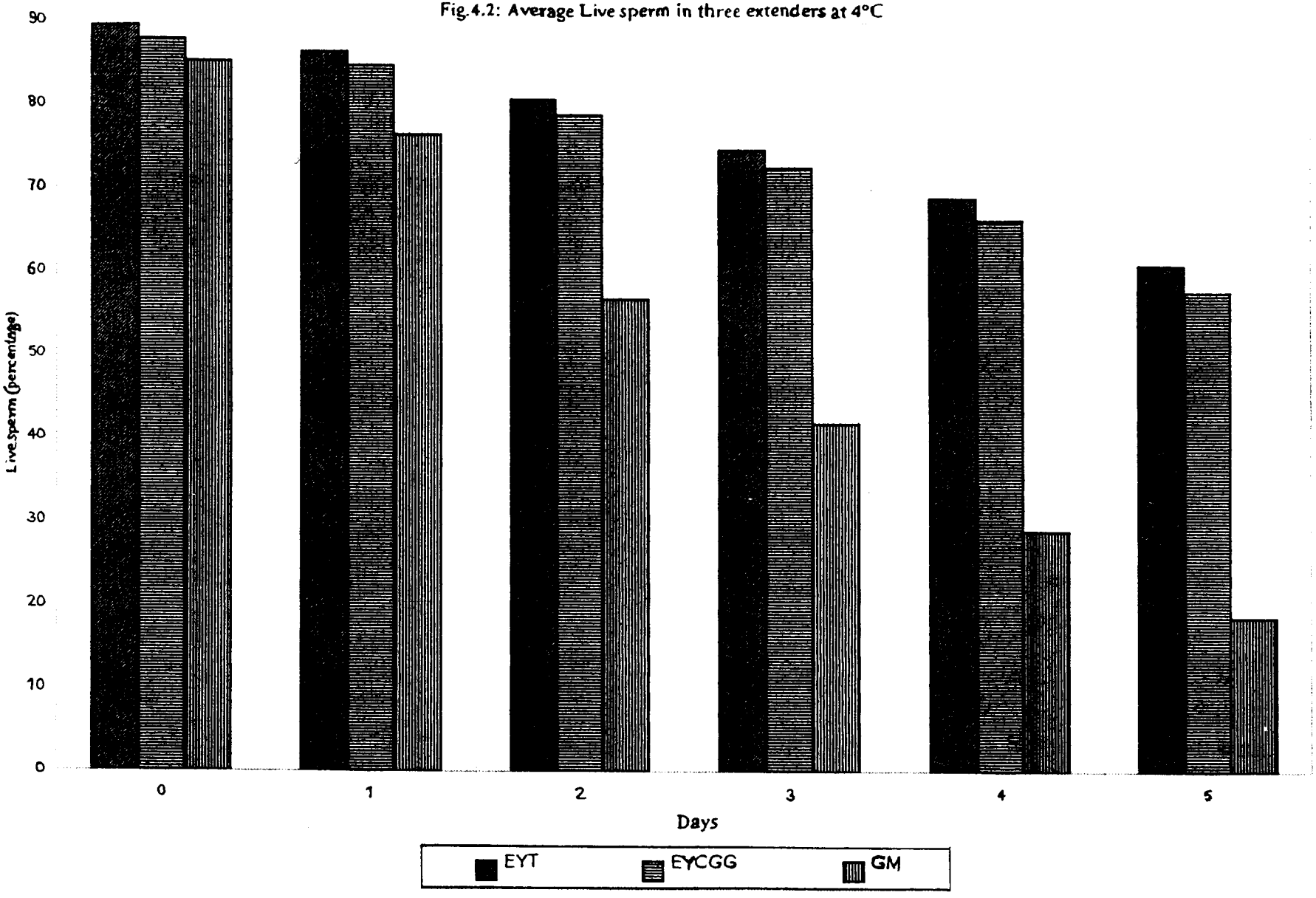


Fig.4.3: Average sperm abnormality in three extenders at 4°C

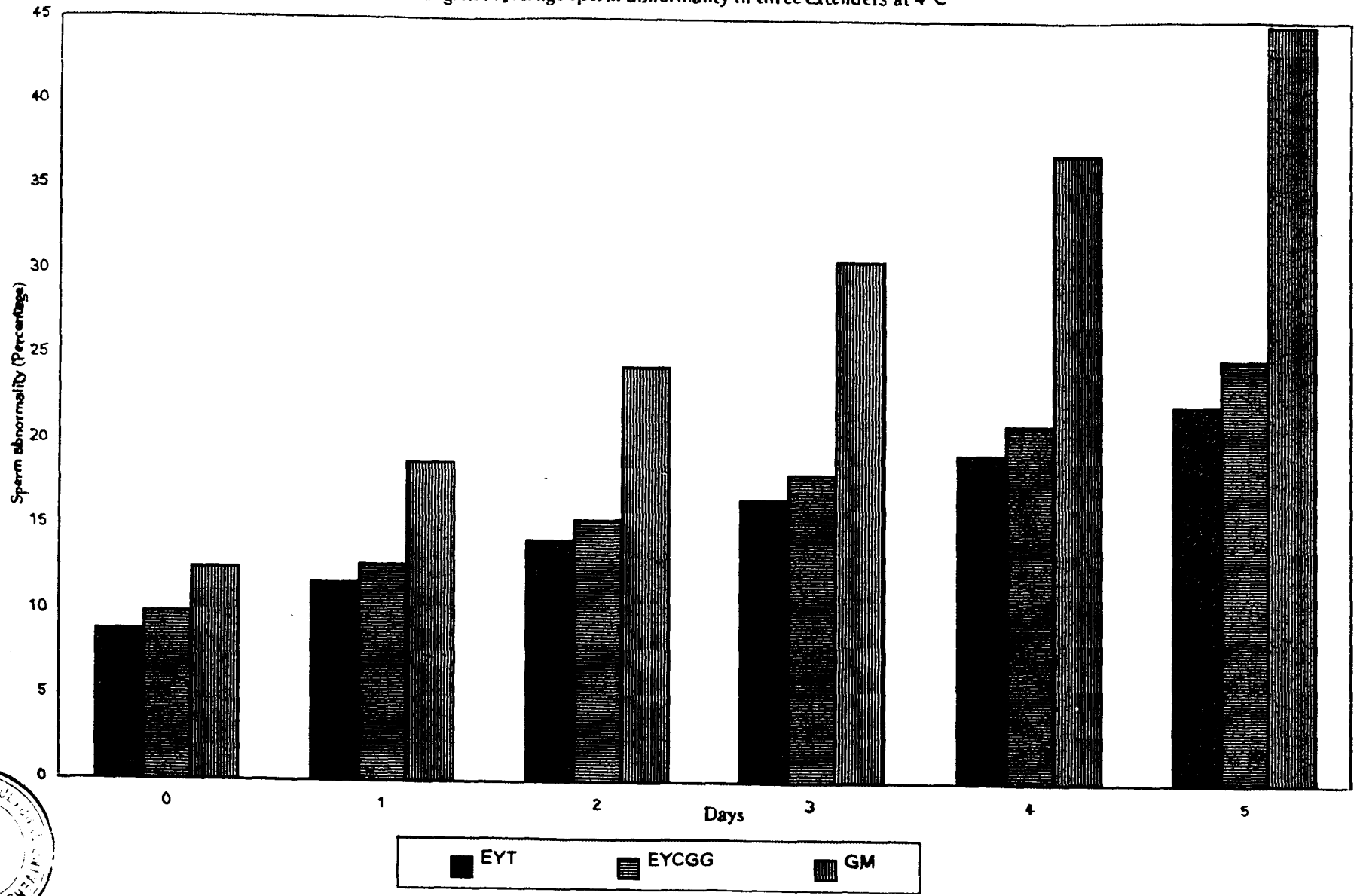


Fig 4.4: Average acrosomal damage in three extenders at 4°C

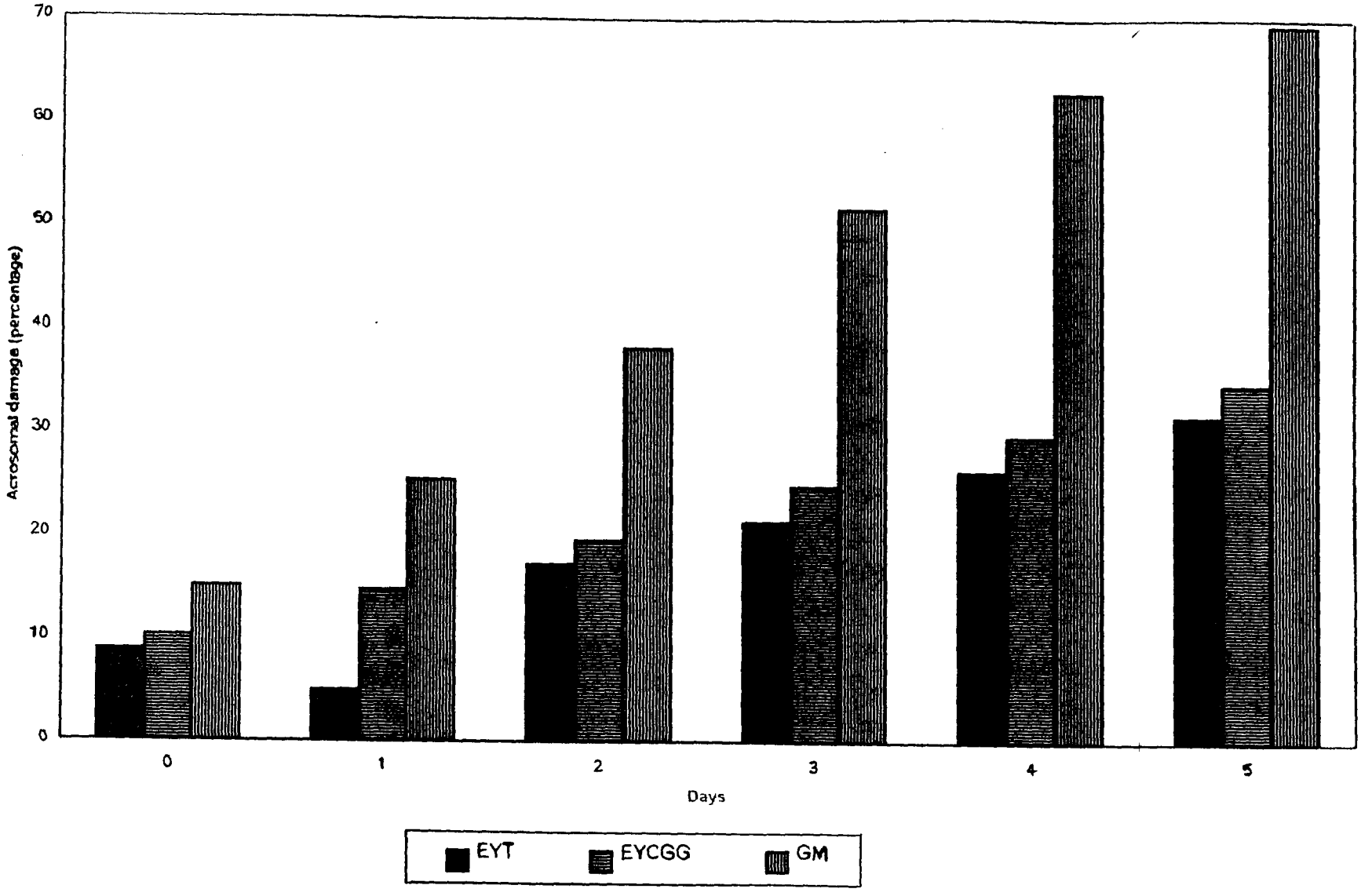


Plate 1. **Semen collection: Stage-I**

Digital pressure using thumb and index finger behind the bulbus glandis of the exposed penis

Plate 2. **Semen collection: Stage-II**

Ejaculation of pre-sperm fraction

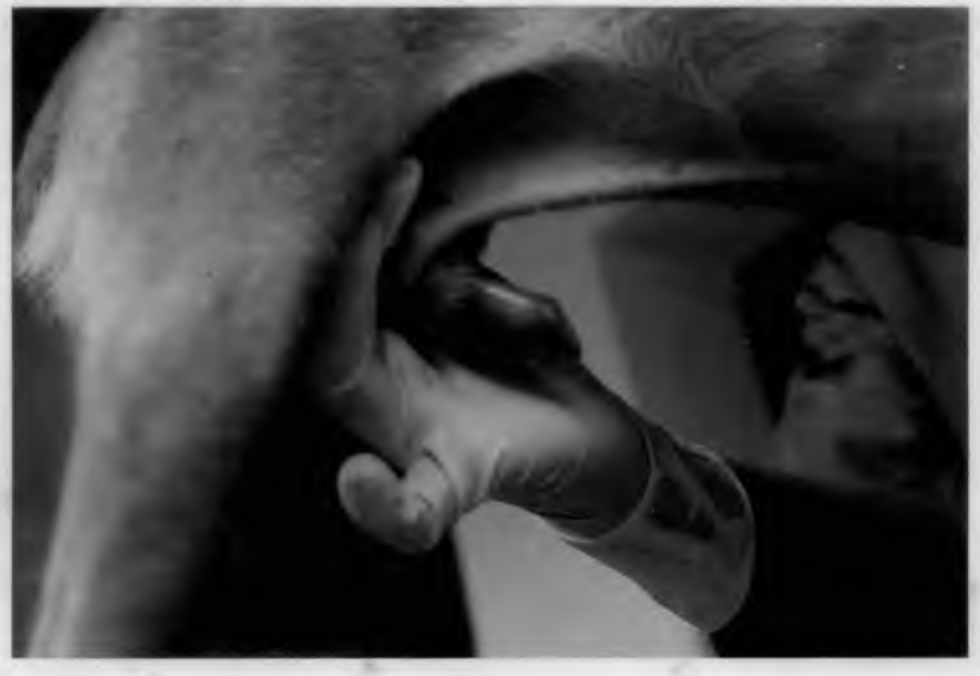


Plate 3. **Semen collection: Stage-III**
Ejaculation of sperm rich fraction

Plate 4. **Semen collection: Stage-IV**
Ejaculation of post sperm fraction

Plate 5. **Different fractions of dog semen**

1. **Pre sperm fraction**
2. **Sperm rich fraction**
3. **Post sperm fraction**

1
2
3

Plate 6. **Sperm morphology**
Normal spermatozoa (1000x)

Plate 7. **Sperm morphology**
Pseudodroplet (1500x)

Plate 8. Normal acrosome (1500x)

Plate 9. Swollen acrosome (1500x)

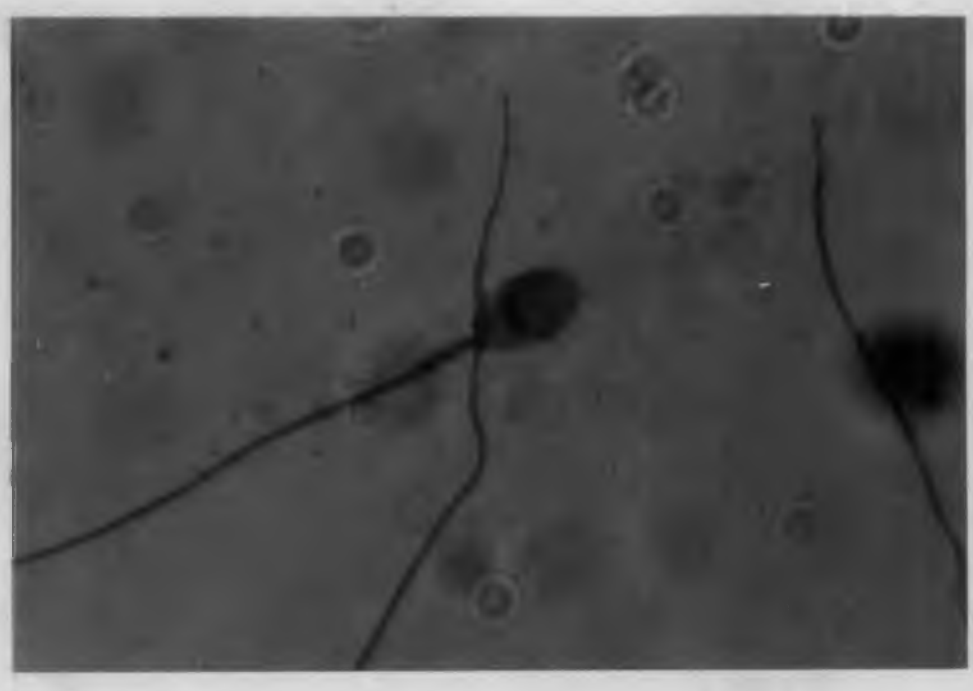


Plate 10. Loosening of acrosome (1500x)

Plate 11. Sperm morphology after preservation at 4°C
Note bent tails and coiled tails

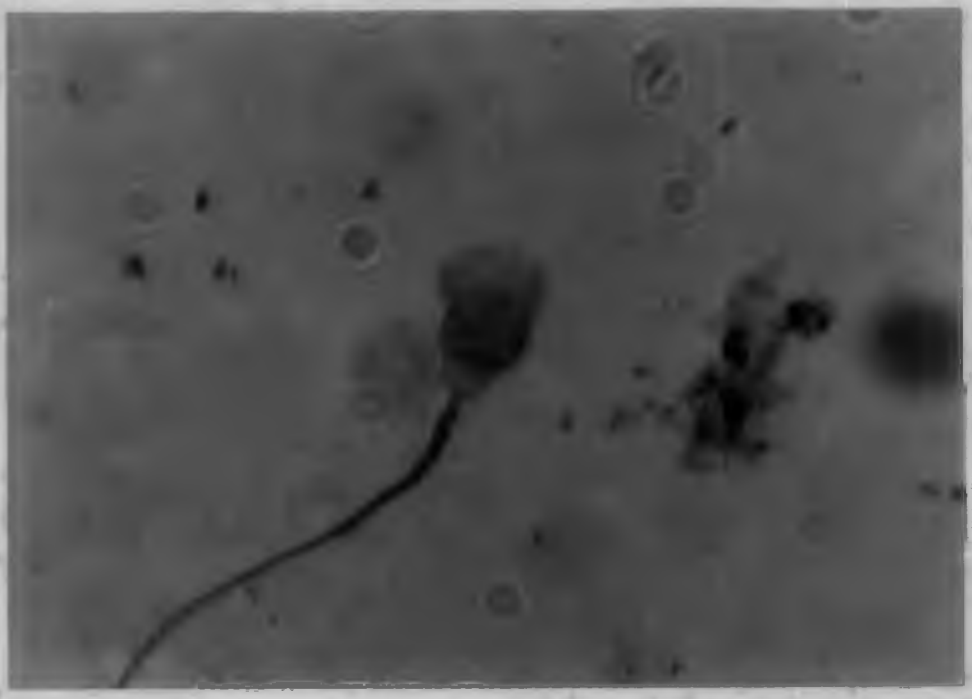
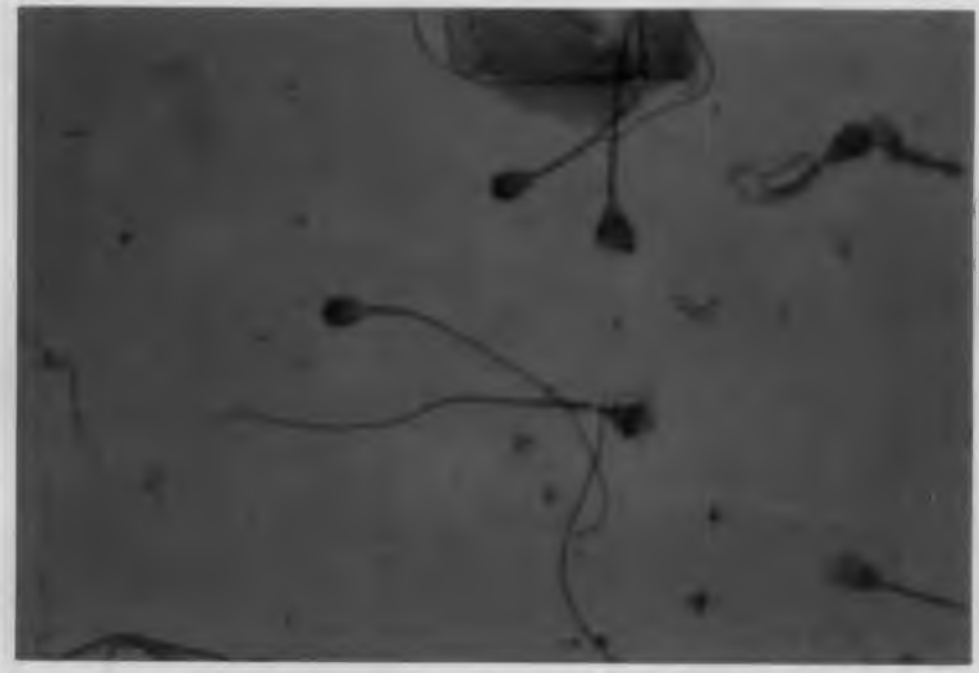


Plate 12. Acrosomal damage after preservation at 4°C
Note swollen, loosening, lost acrosome



Discussion

DISCUSSION

The present study was undertaken with the objective of evolving a suitable extender for preservation of dog semen at 4°C. A total of 36 ejaculates, six ejaculates from each dog were collected by digital manipulation. After evaluation of physical and morphological characteristics, the semen samples were extended in three extenders viz., Egg yolk tris (EYT), Egg yolk citrate glycine glucose (EYCGG) and Goat milk (GM) by split sample technique. Extended semen samples were preserved at 4°C and sperm motility, live sperm count, abnormal sperm count and acrosomal integrity were evaluated at 24 hours interval for five days.

5.1 Response to digital manipulation

Seven dogs were trained for semen collection by digital manipulation on alternate days for two weeks without using a teaser. Six dogs readily responded to the training. The erection of penis occurred within 30-60 seconds after holding the penis behind the bulbus glandis. Mounting and pelvic thrust were followed by emission of pre sperm fraction lasting for 0.5 to 1 minute. The sperm rich fraction was ejaculated over a period of 30 to 60 seconds when animal remained steady. The emission of post sperm fraction proceeded after turning back of the penis and lasted for 3 to 20 minutes. All the six

dogs showed good response and ejaculated good quality semen. The behaviour of dogs during semen collection, the pattern of ejaculation of different fractions of ejaculate and ejaculation time are in general agreement with observations of Boucher *et al.* (1958), Morton and Bruce (1989) and Allen (1991). The dogs responded well after one or two collections with digital manipulations. There was penile erection even at the sight of the operator and collection could be obtained unassisted even without muzzling the dog.

5.2 Semen evaluation

Three fractions were collected separately in different collection vials and only sperm rich fraction was used for preservation.

The mean volume of first, second and third fraction of semen observed is in agreement with that of Harrop (1956) and England and Allen (1989). However, higher volumes of second fraction were recorded by Dobrinski *et al.* (1993). First fraction could be collected separately with difficulty, as the duration of the ejaculation of the fraction was very short. The volume obtained also widely varied (0-2 ml). The fact that the first fraction hardly contained any spermatozoa, also point that probably first fraction was not collected in full in order to avoid mixing the same with second fraction.

Colour and consistency of first and third fractions of semen were clear and watery and the second fraction was thin milky to thick milky. The mean density of second fraction recorded in this study was DD with which varied from D to DDD. Deshpande *et al.* (1970) reported that semen of different breeds of dog had different colour. According to Olson (1992) sperm rich fraction was creamy to white and homogenous in appearance. The cloudy and milky thin consistency of dog semen reported by earlier workers (Deshpande *et al.*, 1970 and Daiwadnya and Hukeri, 1993) might be due to mixing of different fractions of the ejaculate.

The mean mass activity of sperm rich fraction was ++(+) which is in agreement with Daiwadnya . . (1987). Daiwadnya and Hukeri (1993) also recorded mass activity which agrees with the values obtained in the present study.

The sperm motility immediately after collection (86.67 ± 1.07 per cent) is in general agreement with that of Gill *et al.* (1970), England and Allen (1989), Dobrinski *et al.* (1993) and Rota *et al.* (1995) for different breeds of dogs. A higher initial progressive motility was recorded by Ferguson *et al.* (1989) in beagle dogs. However, low initial sperm motility 70 per cent was reported by Olson (1992), 67.5 per cent by Mickelsen *et al.* (1993) and 72.8 per cent by Kumi-Diaka (1993).

The mean pH of first, second and third fraction was 6.24 ± 0.01, 6.36 ± 0.01 and 6.65 ± 0.02 respectively. Harrop (1960) and RoyChoudhury and Dubay (1974) reported pH of dog semen similar to the values obtained in the present study. Boucher *et al.* (1958) observed that pH of dog semen varied according to the method of collection. However, Chatterjee *et al.* (1976) found the average pH of 6.8 within a range of 6.4 to 6.9 in dog semen collected by digital manipulation.

The mean concentration of second fraction was 416.28 ± 22.56 million per ml while that of third fraction was 6.11 ± 1.66 within a range of one to 20 million per ml. The total sperm output per ejaculate was 527.50 ± 29.46 million. The perusal of available literature showed wide variation in the sperm concentration of various breeds of dogs. The total sperm concentration per ml was reported to be 125 million (Harrop, 1960), 98 million (Gill *et al.*, 1970), 18.5 to 78.7 million (Heywood and Sortwell, 1971), 161.58 million (Chatterjee *et al.*, 1976) and 264 ± 15 million (Daiwadnya and Hukeri, 1993). The concentration of sperm rich fraction per ml was reported to be 30-120 million (70 million) (RayChoudhury and Dubey, 1974), 60-550 million (299.6 million), (England and Allen, 1989). The values reported by England and Allen (1989) is in agreement with the present finding. According to Heywood and Sortwell (1971), total sperm per ejaculate was more meaningful in an unfractioned

ejaculate. The total sperm output was reported 89.42 to 269.8 million (Heywood and Sortwell, 1971), 24 to 928 million (Drawer, 1979), 36 to 530 million (England and Allen, 1989). However much higher values of 4751.95 million by Chatterjee *et al.* (1976), 916 millions by Rota *et al.* (1995), have been reported in various breeds of dogs. The sperm concentration in the present study widely varies from the above reports which might be attributed to the method of collection, circumstances in which the animal was made to ejaculate, climatic variation of different places and breed of dogs. Use of teaser bitch might also have contributed for this.

The mean percentage of live sperm was found to be 89.44 ± 0.57 is within the range of 62 to 90 per cent reported by England and Allen (1989), 51 to 89 per cent (Heywood and Sortwell, 1971), and 36 to 99 per cent (Chatterjee *et al.*, 1976). However, the higher range (97-99 per cent) was reported by Ferguson *et al.* (1989) in beagle dog semen. The percentage of dead sperm ranged between 4 to 20 which agrees with that of Taha *et al.* (1983).

The mean percentage of abnormal sperms was 7.59 ± 0.45 (4 to 15.8) which was lower than the values reported 14.7 per cent by Chatterjee *et al.* (1976), 11.6 per cent by England and Allen (1989) and 12 to 31 per cent by Ferguson *et al.* (1989), but is in agreement with that Daiwad nya and Hukeri (1993).

The acrosome abnormalities recorded in the present study (6.63 ± 0.38) was similar to that of Ferguson *et al.* (1989) and Rota *et al.* (1995). But lower value (2.43 ± 1.15) was recorded by Kumi-Diaka and Badtram (1994).

Perusal of the available literature provides no information regarding MBRT in dog semen. The average time taken for reduction of methylene blue by dog semen recorded in this study was higher compared to bull semen. This may be due to low sperm concentration of dog semen. This is in agreement with Salisbury and VanDemark (1961) who reported methylene blue reduction time become progressively longer with each unit decrease in sperm number in the semen.

Perusal of the available literature provides no information regarding incubation test using dog semen. The mean percentage of sperm motility at 10 minutes of incubation was slightly higher than 0 minutes of incubation. Salisbury and VanDemark (1961) reported that the motility and metabolic activity of spermatozoa were higher at a temperature of few degree above body temperature. From 10 to 30 minutes of incubation motility gradually decreased. This agrees with Salisbury and VanDemark (1961) who reported that above body temperature the total life expectancy of the spermatozoa was reduced. This may be due to the increased rate of metabolic activity and exhaustion of nutrients in the semen.

Joseph (1983) reported significant correlation between sperm viability at 30 minutes of incubation and sperm motility at 96 hours of preservation under refrigeration temperature in buck semen. The present study recorded significant ($P<0.01$) correlation between sperm viability at 30 minutes of incubation and sperm motility upto 5 days. Dog semen maintained significantly higher motility at 30 minutes of incubation (46.5°C) and also at 96 hours of preservation than buck semen.

5.3 Evaluation of preserved semen

Analysis revealed that there was no significant difference on sperm motility between EYT and EYCGG extender from day 0 to day 5. But significantly ($P<0.01$) lower sperm motility was recorded in GM in comparison with other two extenders. The sperm motility in EYT is in accordance with the findings of Rota *et al.* (1995) who reported that average percentage of sperm motility was 76.8 ± 14.0, 73.6 ± 15.5, 70.4 ± 15.4, 60.9 ± 18.9 and 53.6 ± 20.1 respectively on day 0, 1, 2, 3 and 4. But slightly higher values (100, 98, 90, 87, 80 and 35) was recorded by Morton and Bruce (1989) in tris-citrate glycerol-egg yolk extender on day 0, 1, 2, 3, 4 and 5. Slightly lower motility of 74.8 per cent at 0 hours and 59.2 per cent at 24 hours was reported by Ekrod (1989).

The sperm motility in EYCGG extender is in accordance with the finding of Foote and Leonard (1964). Sperm motility reduced immediately after dilution in EYCGG than that in EYT. But the rate of reduction of motility per day was slightly lower as compared to EYT. These observations are in agreement with Gabriel (1955) in bull semen.

The average percentage of sperm motility in GM in this study was significantly lower, which is in agreement with that of Foote and Leonard (1964). They suggested that milk extender was less satisfactory.

EYT and EYCGG extenders were maintaining significantly ($P<0.01$) higher motility than GM from day 0 to day 5. Though EYT and EYCGG did not differ significantly in sperm motility during preservation, a trend of higher values was observed in EYT extender. As the Goat milk (GM) has given lower motility, the use of GM as an extender was not satisfactory.

Average percentage of live sperm on day 0 to day 5 did not vary significantly between EYT and EYCGG after storage at 4°C. But significantly ($P<0.01$) lower number of live sperm was noted in GM in comparison with other extenders. The rate of decrease of live spermatozoa per day was significantly ($P<0.01$) higher in GM. Perusal of the available literature provides no information regarding live sperm after preservation at 4°C in these three extenders.

The mean percentage of abnormal spermatozoa on day 0 to day 5 did not significantly ($P<0.01$) differ between EYT and EYCGG after storage at 4°C. But significantly ($P<0.01$) higher number of abnormal sperm was obtained in GM in comparison with other extenders. The rate of development of sperm abnormalities per day was significantly ($P<0.01$) higher in GM when compared to other extenders. Perusal of the available literature provides no information regarding abnormalities of dog spermatozoa after preservation at 4°C in these three extenders.

From this study average percentage of acrosomal damage on day 0 to day 5 did not differ significantly in EYT and EYCGG extender after preservation at 4°C. But significantly ($P<0.01$) higher damage of acrosome occurred during storage at 4°C in GM in comparison with EYT and EYCGG. The rate of acrosomal damage per day was significantly ($P<0.01$) higher in GM than in other two extenders.

Though, acrosome damage did not differ significantly in EYT and EYCGG extender, a trend of less damage was recorded in EYT. This is in accordance with the observation of Rota *et al.* (1995). He reported that egg yolk tris maintained the integrity of acrosome better than egg-yolk milk and egg yolk cream extender. This may be due to capability to maintain the spermatozoa alive and motile longer, there by preventing the

acrosome reaction that occur in association with sperm death or irreversible damage (Aalseth and Sacke, 1985). Perusal of the available literature provides no information, regarding acrosome integrity after preservation at 4°C in EYCGG and GM.

The above finding clearly shows that EYT and EYCGG maintains higher percentage of sperm motility and live sperm, lower percentage of abnormal sperms and acrosomal damage than in GM. Eventhough, the values are not statistically significant among EYT and EYCGG, EYT was found to have higher percentage of sperm motility and live sperms, lower percentge of abnormal sperms and acrosomal damage when compared to EYCGG. Besides EYT was also found to have better clarity for microscopical examination when compared to EYCGG. Hence it could be inferred that Egg yolk tris is superior to Egg yolk citrate glycine glucose and Goat milk for preservation of dog semen at 4°C.

Summary

SUMMARY

A study was conducted with the object of evolving a suitable extender for preservation of dog semen at 4°C. A total of 36 ejaculates, six ejaculates from each dog was collected by digital manipulation from six mongrel dogs maintained in the kennels at Veterinary College Hospital, Mannuthy. Immediately after semen collection, physical and morphological characters were evaluated and good quality semen was extended in three extenders viz., Egg Yolk Tris (EYT), Egg Yolk Citrate Glycine Glucose (EYCGG) and Goat milk (GM) by split sample technique. Extended semen samples were preserved at 4°C and sperm motility, live sperm count, abnormal spermatozoa and acrosomal integrity were evaluated at 24 hours interval for five days.

Six out of seven dogs showed good response to digital manipulation without teaser bitch and ejaculated good quality semen. The overall mean volume of first, second and third fraction of semen was 0.63 ± 0.07 ml, 1.29 ± 0.08 ml and 4.12 ± 0.23 ml respectively. The colour and consistency of the first and third fraction was clear and watery and second fraction was thick milkly to thin milkly. The average mass activity of sperm rich fraction was ++(+) and the density was DD. The mean initial sperm motility was 86.67 ± 1.07 per cent. The mean pH of first, second and third fraction of

semen was 6.24 ± 0.01, 6.36 ± 0.01 and 6.65 ± 0.02 respectively.

The overall mean spermatozoal concentration of second fraction was 416.28 ± 22.56 million per ml and that of third fraction was 6.11 ± 1.66 million per ml. The average total sperm output per ejaculate was 527.50 ± 29.46 million and the overall mean live sperm was 89.44 ± 0.57 per cent. The percentage of abnormal sperm and acrosomal abnormality were 7.59 ± 0.45 and 6.63 ± 0.38 respectively.

The average time taken for reduction of methylene blue by dog semen was 26.40 ± 0.86 minutes. The mean percentage of sperm motility at 0, 10, 20 and 30 minutes of incubation (46.5°C) was 86.38 ± 1.04, 88.33 ± 1.13, 70.55 ± 1.26 and 53.2 ± 2.17 respectively. There was significant ($P<0.01$) correlation between sperm viability at 30 minutes of incubation and sperm motility upto 5 days of preservation under refrigeration temperature.

The average percentage of sperm motility after preservation at 4°C on day 0, 1, 2, 3, 4 and 5 was 86.38 ± 1.04, 79.86 ± 1.32, 73.47 ± 1.72, 66.80 ± 1.99, 58.47 ± 2.41 and 49.86 ± 2.74 respectively in EYT; 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 respectively in EYCGG and 76.25 ± 1.26, 61.94 ± 1.76, 41.66 ± 1.92, 26.11 ± 2.04, 7.50 ± 1.61 and 0.0 respectively

in GM. The percentage of sperm motility was significantly higher in EYT and EYCGG than that preserved in GM.

The mean percentage of live sperm on day 0, 1, 2, 3, 4 and 5 was 89.54 ± 0.76, 86.44 ± 0.84, 80.75 ± 0.90, 74.65 ± 0.99, 68.87 ± 1.13 and 60.87 ± 1.58 respectively in EYT; 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 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 GM. Significantly higher percentage of live sperm in EYT and EYCGG was noted in comparison to that in GM.

Average percentage of abnormal spermatozoa was 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 on day 0, 1, 2, 3, 4 and 5 after preservation in EYT, 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 and 18.74 ± 1.12, 24.42 ± 1.55, 30.70 ± 1.82, 37.02 ± 1.85 and 46.75 ± 2.02 in GM extenders.

The mean percentage of acrosomal damage on day 1, 2, 3, 4 and 5 was 8.88 ± 0.58, 12.96 ± 1.04, 17.19 ± 1.48, 21.29 ± 1.76, 26.19 ± 2.21 and 31.54 ± 2.73 respectively in EYT, 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 and 15.02 ± 1.07, 25.36 ± 1.91, 38.13 ± 2.93, 51.66 ± 3.50, 62.92 ± 3.66 and 69.45 ± 4.20 in GM. A significantly lower percentage of abnormal spermatozoa

and acrosomal damage was observed upto day 5 in EYT and EYCGG than in GM.

The above finding clearly showed that EYT and EYCGG maintain higher percentage of sperm motility and live sperm, lower percentage of abnormal sperms and acrosomal damage than in GM. Eventhough, the values were not statistically significant among EYT and EYCGG, EYT was found to have higher percentage of sperm motility, and live sperms, and lower percentge of abnormal sperms and acrosomal damage when compared to EYCGG. Besides EYT was also found to have better clarity for microscopical examination when compared to EYCGG. Hence it could be inferred that Egg yolk tris is superior to Egg yolk citrate glycine glucose and goat milk for preservation of dog semen at 4°C.

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PRESERVATION OF DOG SEMEN IN THREE EXTENDERS AT REFRIGERATION TEMPERATURE

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ABSTRACT

With the ultimate objective of evolving a suitable diluent for preservation of dog semen at 4°C, semen was collected from six mongrel dogs maintained in kennels at Veterinary college hospital, Mannuthy. A total of 36 ejaculates, six from each dog was collected by digital manipulation and physical and morphological characters were evaluated. Three extenders viz., Egg Yolk Tris (TYT), Egg Yolk citrate glycine glucose (EYCGG) and Goat milk (GM) were used for preservation of semen. Sperm motility, percentage of live sperm, abnormal spermatozoa and acrosomal integrity were evaluated at 24 hours interval for five days after preservation at 4°C in the above extenders.

Six out of seven dogs showed good response to digital manipulation and ejaculated good quality semen without teaser bitch. The overall mean volume of first, second and third fraction of semen was 0.63 ± 0.07 ml, 1.29 ± 0.08 ml and 4.12 ± 0.23 ml respectively. The colour and consistency of the first and third fraction was clear, watery and second fraction was thick milkly to thin milkly. The average mass activity of sperm rich fraction of semen was ++(+) and the density was DD. The mean initial sperm motility was 86.67 ± 1.07 per cent.

The mean pH of first, second and third fraction of semen was 6.24 ± 0.01, 6.36 ± 0.01 and 6.65 ± 0.02 respectively.

The overall mean spermatozoal concentration of second fraction was 416.28 ± 22.56 million per ml and that third fraction was 6.11 ± 1.66 million per ml. The average total sperm output per ejaculate was 527.50 ± 29.46 million. The overall mean live sperm and abnormal sperm was 89.44 ± 0.57 and 7.59 ± 0.45 per cent. The percentage of acrosomal abnormality was 6.63 ± 0.38. The average time taken for reduction of methylene blue by dog semen was 26.40 ± 0.86 minutes. The mean percentage of sperm motility at 0,10,20 and 30 minutes of incubation (46.5°C) was 86.38 ± 1.04, 88.33 ± 1.13, 70.55 ± 1.26 and 53.2 ± 2.17 respectively. There was significant ($P<0.01$) correlation between sperm viability at 30 minutes of incubation and sperm motility upto 5 days of preservation under refrigeration temperature.

The percentage of sperm motility upto day 5 was significantly higher in Egg Yolk Tris (49.86 per cent) and Egg Yolk citrate Glycine Glucose (48.33 per cent) than in Goat milk (0 per cent).

There was significantly higher percentage of live sperms and lower percentage of abnormal sperms and acrosomal damage in EYT and EYCGG than in GM. Eventhough the values are not statistically significant among EYT and EYCGG, EYT was found to have higher percentage of sperm motility and live sperm,

lower percentage of abnormal sperms and acrosomal damage when compared to EYCGG. Besides EYT was also found to have better clarity for microscopical examination. Hence it could be inferred that Egg yolk tris is superior to Egg yolk citrate glycine glucose and goat milk for preservation of dog semen at 4°C.