EFFECT OF PROCESSING AND FREEZING PROCEDURES ON THE ACROSOME MORPHOLOGY OF BUCK SPERMATOZOA

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled " **EFFECT OF PROCESSING AND FREEZING PROCEDURES ON THE ACROSOME MORPHOLOGY OF BUCK SPERMATOZOA**" is a bonafide record of research work done by me during the course of my 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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CERTIFICATE

Certified that this thesis entitled "**EFFECT OF PROCESSING AND FREEZING PROCEDURES ON THE ACROSOME MORPHOLOGY OF BUCK SPERMATOZOA**" is a record of research work done independently by Smt. **RANJINI , A.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Dedicated to my family

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Introduction

INTRODUCTION

Small ruminants are numerically and economically important livestock in the developing countries. Our country has vast genetic resources of small ruminants as reflected from 40 breeds of sheep and 20 breeds of goat. Nearly 94 per cent of world population of 557 million goats are in developing countries.

India has 43.92 million sheep and 112 million goats. They are the principal livestock contributing much to the sustenance of small and marginal farmers. This population produces about 8.57 per cent of world's goat and sheep meat and 18.07 per cent goat milk. Goats contribute about Rs.10,800 million from meat, Rs.4500 million from milk, Rs.200 million from hair and Rs.1050 million from manure. Their present contribution to gross national production and employment avenues in rural areas, merits adequate attention in the propagation of superior germplasm. Identification of superior bucks and the maximum utilization of their semen for breeding of does are the methods for promoting milk and meat production. Several are the constraints encountered by small farmers in rearing a buck along with the does for breeding purposes alone. Goat husbandry is one of the most important livestock enterprises. In Kerala backyard goat husbandry is very common. Goat forms the

principal source of milk, meat and supplementary income to small and marginal farmers and agricultural labourers. One of the bottlenecks in improving the local stock of goats continue to be the lack of effective methods for large scale scientific breeding with sires of improved genetic potential. The effective remedy for this problem is to develop frozen semen from superior sires and to implement methods for large scale use of artificial insemination with frozen buck semen in different regions.

Bovine semen preservation progressed in a spectacular way, became and remained the absolute model for preservation of semen of other species irrespective of interspecies differences in seminal plasma composition. Major problems in goat semen preservation resulted from the solvenliness of the seminal plasma due to the presence of egg yolk coagulating enzyme in the seminal plasma or more precisely in the cowper's gland secretion. Iritani and Nishikawa (1961) studied the properties of egg yolk coagulating enzyme and found that the removal of seminal plasma from the goat sperm environment improved the sperm survival at 4^0 C, which is a prerequisite for goat sperm preservation. From experiments it was also clear that washing sperm could be detrimental to sperm cells especially the acrosome. Thus it becomes essential that the extent of damage to the acrosome at every stage of processing and freezing procedures is monitored and the stage at which maximum

damage occurs is pointed out , so that corrective measures can be adopted for effective freezing of buck semen. The present study is aimed at elucidating the effect of processing and freezing procedures on the acrosome morphology of buck spermatozoa.

Review of Literature

REVIEW OF LITERATURE

2.1 Semen evaluation

2.1.1 Volume

The normal ejaculate volume was reported to be one ml in White German Improved bucks (Knoblauch, 1962), 0.4 - 1.2 ml in Malabari bucks (Kurian and Raja, 1965 ; Patil, 1970) and 0.67± 0.03 ml and 1.34 ± 0.05 ml in Zambian and Boer bucks respectively (Igboeli, 1974).

Normal ejaculate volume was reported to be 0.98 ml in Angora goats (Cetinkaya *et al.*, 1980) as against 0.77 ± 0.26 ml in African Dwarf goats (Mann, 1980). Ejaculate volume of Pashmina goats averaged 0.62 ± 0.02 ml and there was significant difference in the ejaculate volume between bucks and between collections (Mohan *et al.*, 1980). Saxena and Thripathi (1980) reported the ejaculate volume to be 0.37 ± 0.03 ml in Jamnapari bucks. Sinha and Singh (1982) reported the ejaculate volume as 0.45 ml and 0.72 ml in Black Bengal and Saanen bucks respectively. Singh *et al.* (1982) studied the semen characteristics of Jamnapari and Barbari bucks and reported the volume as 0.86 ± 0.09 ml and 1.01 ± 0.04 ml respectively. Dundar *et al.* (1983) found that the ejaculate volume in Angora goats averaged 0.94 ± 0.11 ml. The normal ejaculate volume was reported to be 0.55 ± 0.01 ml and 0.72 ± 0.01 ml in Malabari and Alpine X Malabari crossbred bucks respectively

(Sarmah, 1983). Pandey *et al.* (1985) reported that the volume of semen in Saanen bucks (0.96 ml) was comparatively more than that of Barbari buck (0.79 ml).

Sevinc *et al.* (1985) studied the sperm characteristics of Angora goats reared in the Cifteler State farm and reported that the ejaculate volume averaged 1.07 ± 0.1 ml. Singh *et al.* (1985) reported the average ejaculate volume to be 0.55 ml and 0.46 ml in Jamnapari and Black Bengal bucks. Ali and Mustafa (1986) in Sudan studied the semen characteristics of Nubian goats and found the average ejaculate volume to be 1.5 ml. The normal ejaculate volume of Saanen, Barbari and cross bred bucks was found to average 0.70 ± 0.022 ml, 0.72 ± 0.032 ml and 0.69 ± 0.024 ml respectively (Prasad *et al.*, 1986). The ejaculate volume was reported to be 0.95 ± 0.30 ml in Angora goats (Bakshi *et al.*, 1987), 0.80 ± 0.06 ml and 0.84 ± 0.07 ml in Cheghu and Changthangi bucks respectively (Mahmood *et al.*, 1988), 0.94 ± 0.24 ml in Ganjam bucks (Pattnaik *et al.*, 1991), 0. 73 ± 0.28 ml in West African Dwarf goats (Wuschko and Seifert, 1991), 0.79 ± 0.02 ml in Beetal bucks (Das and Rajkonwar, 1993) and 0.71 ± 0.02 ml in Alpine X Malabari cross-bred bucks (Prasanth, 1995).

2.1.2 Colour

Knoblauch (1962) reported that semen of White German improved bucks was ivory in colour with creamy consistency. Patil (1970) noted milky yellow to thick creamy yellow colour for Malabari buck semen.

Semen colour of Zambian buck and Boer buck was ranging from creamy white to yellow (Igboeli, 1974), and it varied considerably between breeds and between ejaculates of the same buck. Varying colours have been attributed to the semen of bucks belonging to different breeds with the African dwarf goat semen being ivory coloured and creamy in consistency (Mann, 1980), Saanen goat semen creamy (Pandey *et al.*, 1985) Barbari goat semen yellowish to creamy (Pandey *et al.*, 1985), and Nubian goat semen white to creamy white (Ali and Mustafa, 1986). Prasanth (1995) reported the colour of semen from Alpine X Malabari cross-bred buck to be creamy.

2.1.3 Density

Knoblauch (1962) reported that the density of semen from White German improved bucks to be five by five. Normal semen density of Malabari and Alpine X Malabari crossbreds was reported to be 3.52 $\pm$ 0.03 (Sarmah, 1983). A semen density of 3.3611 $\pm$ 0.3438 was reported in Alpine Malabari cross-breds (Joseph, 1983). There was significant difference in the density between bucks and between ejaculates. (Joseph, 1983). Prasanth (1995) reported the density of semen from Malabari cross bred bucks as DDDD.

2.1.4 Mass activity

The mass activity of buck semen from different breeds of goats was reported to be 3.52 $\pm$ 0.05 in Malabari bucks, 3.80 $\pm$ 0.03 in Alpine X Malabari

crossbred bucks (Sarmah, 1983), 4.19 ± 0.04 in Pashmina bucks (Mohan *et al.*, 1980), 4.44 and 4.75 in Black Bengal bucks (Sinha and Singh, 1982 ; Singh *et al.*, 1982), 4.51 in Saanen bucks (Sinha and Singh, 1982), 3.15 ± 0.06 and 0.72 ± 0.01 in Cheghu and Changthangi bucks respectively (Mahmood *et al.*, 1988), 4.12 ± 0.05 in Ganjam bucks (Pattnaik *et al.*, 1991) and ++++ in Alpine X Malabari crossbred bucks (Prasanth, 1995).

2.1.5 Hydrogen ion concentration

Normal semen pH was reported to be 6.6 for White German improved bucks (Knoblauch, 1962), 6.47± 0.16 (Patil, 1970), 6.93 in African dwarf bucks (Mann, 1980), 6.84 ± 0.02 in Pashmina bucks (Mohan *et al.*, 1980), 6.72 in Saanen bucks (Sinha and Singh, 1982), 6.79 in Black Bengal bucks (Sinha and Singh, 1982), 6.74 ± 0.02 and 6.74 ± 0.01 in Malabari and Alpine X Malabari crossbred bucks (Sarmah, 1983). Dundar *et al.* (1983) reported semen pH of Angora goats to be 6.685 ± 0.05. There was significant difference in semen pH between bucks (Mohan *et al.*, 1980). Sinha *et al.* (1983) reported that semen pH was significantly higher for Black Bengal bucks than for Saanen bucks. Sevinc *et al.* (1985) reported that the semen pH averaged 6.68 ± 0.17 in Angora goats. Normal semen pH was reported to be 6.60 ± 0.002, 6.78 ± 0.002, 6.68 ± 0.002 respectively in Saanen, Malabari and Saanen X Barbari cross breeds (Prasad *et al.*, 1986). A semen pH of 6.43 ± 0.006 was reported in Cheghu bucks as against 6.52 ± 0.07 in

Changthangi bucks (Mahmood *et al.*, 1988). The semen pH was reported to be 6.70 ± 0.03 in Beetal bucks (Das and Rajkonwar, 1993) and 6.85 ± 0.01 in Alpine X Malabari crossbred bucks (Prasanth, 1995).

2.1.6 Sperm concentration

Normal sperm concentration per ml of buck semen was reported to be two-three billion (Kurian and Raja, 1965) and 1.1 to 7.4 billion (Patil, 1970) in Malabari goats as against 1.65 ± 0.02 and 2.70 ± 0.03 billion in Zambian and Boer goats respectively (Igboeli, 1974), 3.67 billion in Angora goats (Cetinkaya *et al.*, 1980) and 3.22 ± 1.22 billion in West African dwarf goats. (Mann, 1980). Sperm concentration of Pashmina goats averaged 3.521 ± 1.18 billion and there were significant differences between bucks and between collections (Mohan *et al.*, 1980).

Normal concentration per ml of semen from Saanen bucks was reported to be 4795 ± 292.97 million in Jamnapari bucks (Saxena and Tripathi, 1980), 2293 ± 728 million and 1920 ± 720 million in Jamnapari and Barbari bucks respectively (Singh *et al.*, 1982), 2.440 billion and 2.780 billion in Black Bengal and Saanen bucks respectively (Sinha and Singh, 1982), 3.31 ± 0.24 billion in Angora goats (Dundar *et al.*, 1983), 3.49 ± 0.09 billion and 3.28 ± 0.07 billion in Malabari and Alpine X Malabari bucks respectively (Sarmah, 1983), 3.11± 0.45 billion in Angora goats (Sevinc *et al.*, 1985), 2619.58 million in Jamnapari goats (Singh *et al.*, 1985) and 1.77 billion in Nubian

goats (Ali and Mustafa, 1986). Prasad *et al.* (1986) reported a sperm concentration of 2820.96 ± 7.427 million, 2117.65 ± 32.445 million and 2375 .47 ± 7.095 million in Saanen, Barbari and Saanen X Barbari cross breds. The sperm concentration was reported to be 2.597 ± 86.34 million in Angora goats (Bakshi *et al.*, 1987), 3.25 ± 0.13 billion and 2.94 ± 0.16 billion in Cheghu and Changthangi goats respectively (Mahmood *et al.*, 1988), 2309.17 ± 95.89 million in Ganjam bucks (Pattnaik *et al.*, 1991), 3.22 ± 0.28 billion in West African dwarf goats (Wuschko and Seifert, 1991), 4401.05 ± 112.07 million in Beetal bucks (Das and Rajkonwar, 1993) and 2843.33 ± 153.93 million in Alpine X Malabari crossbreds (Prasanth, 1995) .

2.1.7 Motility

Normal percentage of sperms having progressive motility were reported to be 40-80 in White German improved bucks (Leiss and Ostrowskii, 1960 ; Knoblauch, 1962), 60-90 in Malabari bucks (Kurian and Raja, 1965 ; Patil, 1970), 52.00 ± 1.3 and 53.20 ± 1.2 in Zambian and Boer goats respectively (Igboeli, 1974), 80 in Angora goats (Cetinkaya *et al.*, 1980), 77.28 ± 7.75 in West African dwarf bucks (Mann, 1980), 60.62 ± 0.04 in Pashmina goats (Mohan *et al.*, 1980) and 72.62 ± 1.06 in Jamnapari bucks (Saxena and Tripathi, 1980). Singh *et al.* (1982) reported the normal percentage of progressively motile sperms to be 74.00 ± 0.40 and 78.30 ± 2.48 in Jamnapari and Barbari bucks respectively.

The normal percentage of progressive forward motility of sperms were reported to be 79.18 ± 0.55 and 82.10 ± 1.32 in Malabari and Alpine X Malabari crossbred bucks (Sarmah, 1983), 83.49 ± 3.63 in Angora goats (Sevinc *et al.*, 1985 ; Bakshi *et al.*, 1987), 75-80 in Ganjam bucks (Pattnaik *et al.*, 1991), 71.00 ± 10 in West African dwarf goats (Wuschko and Seifert, 1991) and 89.70 in Beetal bucks (Das and Rajkonwar, 1993). Prasanth (1995) opined that the motility percentage varied significantly between bucks.

2.1.8 Live sperms

Campbell *et al.* (1956) described experiments on the usefulness and accuracy of Nigrosin-Eosin as a differential stain when measuring the proportion of living and dead spermatozoa in samples of ram, boar or bull semen. The normal live sperm percentage was reported to be 80 (Knoblauch, 1962) in White German improved bucks, 61.38 ± 2.58 in Malabari bucks (Patil, 1970), 87.20 ± 1.00 and 87.70 ± 1.00 in Zambian and Boer bucks respectively (Igboeli, 1974), 80.63 ± 0.29 in Pashmina goats (Mohan *et al.*, 1980), 77. 65 ± 1.04 in Jamnapari bucks (Saxena and Tripathi, 1980), 80.90 ± 2.32 and 83.80 ± 0.26 in Jamnapari and Barbari bucks respectively (Singh *et al.*, 1982), 85.45 ± 0.414 and 85.21 ± 0.402 in Black Bengal and Saanen goats respectively (Sinha and Singh, 1982). Bordoloi and Sharma (1983) reported significant difference on the percentage of live spermatozoa in Beetal, Saanen and non-descript local goats of Assam. The highest average

percentage of live sperms was recorded in Beetal breed followed by Assam local goat and Saanen.

The normal percentage of live sperms was reported to be 89.97 ± 0.58 and 91.31 ± 0.35 in Malabari and Alpine X Malabari cross breds (Sarmah, 1983) and 83.227 and 71.443 in Saanen and Barbari goats respectively (Pandey *et al.*, 1985). Sinha *et al.* (1985) reported the live sperm percentage to be 91.07 ± 1.103, 86.37 ± 1.000, 90.33 ± 0.608 and 78.33 ± 1.76 respectively in Black Bengal, Black Bengal X Jamnapari, Jamnapari and Barbari bucks (Singh *et al.*, 1985). They opined that the effect of breed on percentage of live spermatozoa was not significant.

The normal live sperm percentage were reported to be 86 (Ali and Mustafa,1986) in Nubian goats, 91.13 ± 0.986, 92.53 ± 0.291 and 91.07 ± 0.544 in Saanen, Barbari and Saanen X Barbari goats (Prasad *et al.*, 1986), 76.30 ± 1.980 and 71.73 ± 2.090 in Cheghu and Changthangi bucks respectively (Mahmood *et al.*, 1988). Deshpande and Mehta (1991) opined that the live sperm count in Surti bucks was significantly higher with four per cent glycerol level in all the three dilutors as Egg yolk citrate fructose glycerol, Tris egg yolk citric acid fructose glycerol and Goat milk glycerol. Pattnaik *et al.* (1991) reported the normal live sperm per cent to be 84.83 ± 1.020 in Ganjam bucks which is in close proximity with the observations made in other breeds of goats.

The normal live sperm percentage was reported to be 90.25 in Beetal buck semen (Das and Rajkonwar, 1993). Tuli and Holtz (1994) reported that the percentage of live spermatozoa after freezing of washed sperms were higher than that of whole semen. Prasanth (1995) reported the normal live sperm percentage of Malabari X Alpine cross breds bucks was 91.03 $\pm$ 0.560.

2.1.9 Sperm abnormalities

The percentage of abnormal sperms in Malabari buck semen were reported to be 1 to 18.62 (Patil, 1970). Baicoinau and Patrascu (1977) recorded that the proportion of abnormal bull spermatozoa were 14, 20 and 18 after freezing and storage for eight, seven, and six years. The percentage of abnormal sperms were reported to be 2.3 in Angora goats (Cetinkaya *et al.*, 1980) and 3.45 $\pm$ 8.77 in African dwarf bucks (Mann, 1980).

Deka and Rao (1984 b) recorded the morphological abnormalities of buck semen during processing and freezing in Egg yolk citrate, Tris, Skim milk, and Raffinose extenders. They concluded that the mean percentage of abnormalities of head, mid piece coiled tail and free normal head in different stages of processing and freezing did not differ significantly from that of fresh semen in any extender.

The mean percentage of abnormal sperms were reported to be 2.06 $\pm$ 0.420 in Angora goats (Sevinc *et al.*, 1985), 2.12 $\pm$ 0.155, 2.00 $\pm$ 0.178 and 2.11 $\pm$

0.11 in Black Bengal, Jamnapari and Jamnapari X Black Bengal bucks (Singh *et al.*, 1985), 6.7 per cent primary and 15.3 per cent secondary abnormalities in Nubian goats (Ali and Mustafa, 1986), 7.54 ± 0.449, 5.94 ± 2.520 and 7.63 ± 0.442 respectively in Saanen, Barbari and Saanen X Barbari crossbreds (Prasad *et al.*, 1986), 33.17 ± 3.020 and 31.40 ± 2.650 in Cheghu and Changthangi bucks respectively (Mahmood *et al.*, 1988), 5.42 ± 0.310 in Ganjam bucks (Pattnaik *et al.*, 1991), and 5.89 ± 0.06 in Beetal bucks (Das and Rajkonwar, 1993).

2.1.10 Acrosome morphology

a) Acrosome abnormalities

The higher glycerol level in the diluent and the increased thawing temperature interacted to achieve maximum motility and acrosomal integrity. Using the average rate of 26.3^0 C per minute from -10 to -80^0 C minimal cell injury of bovine spermatozoa was achieved with 8.5 per cent glycerol and thawing water temperature of 65^0 C (Robbins *et al.* , 1976). Becker *et al.* (1977) recorded significant reduction in the percentage of intact acrosomes in frozen semen using Tris yolk diluent with 11 per cent glycerol when thawing was done at one, 20 and 37^0 C for three minutes. The proportion of acrosome deformities of fresh ram semen of 2.9 per cent, increased after glycerolisation to 20.7 per cent but there was no relationship between the proportion of deformed spermatozoa and motility (Gokcen and Asti, 1980).

Berndtson *et al.* (1981) studied the correlation between post thaw motility and acrosomal integrity of bovine sperm and concluded that the correlation between these is only 0.33 and that they varied independently of each other. Curiel and Mendez (1981) opined that glycerol was a better cryoprotective agent than Dimethyl sulphoxide (DMSO) with respect to the acrosome integrity, during freezing of ram spermatozoa.

Sokolovskaya *et al.* (1981) classified a scheme of abnormal spermatozoa as swollen, exfoliated, lost and complete loss of head and suggested that thawed semen is suitable for insemination if 30 per cent of the spermatozoa have, a normal acrosome. The percentage of normal acrosomes was better in semen of rams centrifuged at 5^0 C (Marios, 1982). Centrifugation at 22^0 C increased the incidence of abnormal spermatozoa. Papa (1982) reported that thawing at 70^0 C increased the per cent intact acrosomes in bull semen. Bhosrekar *et al.* (1984) reported that the incidence of loose and detached acrosome was higher when semen was thawed at 25^0 C and 4^0 C when compared to thawing at 37^0 C.

Aalseth and Saacke (1985) observed the swelling of apical ridge and anterior acrosome of motile bovine spermatozoa using differential interference contrast optics. They concluded that storing spermatozoa in seminal plasma at 4^0 C for one day was most conducive to the swelling of apical ridge. Replacing seminal plasma with Egg yolk citrate inhibited

swelling. The percentage of total damaged acrosomes was reported to be 1.500 ± 0.300, 2.100 ± 0.400, 5.730 ± 0.910,18.600 ± 2.740 in fresh semen, after dilution and cooling to 5^0 C, after equilibration and after freezing and thawing in Egg yolk citrate fructose glycerol diluent and 2.500 ± 0.230, 5. 070 ± 0.520, 12.370 ± 1.840 in diluted and cooled sample, after equilibration and after freezing and thawing in Tris egg yolk citric acid fructose glycerol diluent (Deka and Rao, 1985).

Deka and Rao (1985) studied the effect of extenders on the acrosomal integrity of buck spermatozoa and found that sperms stored in Raffinose egg yolk glycerol extender had significantly higher damaged acrosomes. Memon *et al.* (1985) found a significantly higher percentage of normal acrosomes in washed semen in Tris extender than Lactose egg yolk glycerol. The incidence of swollen and total damaged acrosomes did not differ significantly between egg yolk levels before freezing, but after freezing and thawing of buck spermatozoa they were lowered with 20 per cent and 10 per cent egg yolk than with seven per cent egg yolk in the extender (Deka and Rao, 1986 a). They (Deka and Rao, 1986 b) also reported that the percentage of damaged acrosomes was lower with four per cent glycerol and one hour equilibration period while freezing buck semen. Deka and Rao (1986 c) also confirmed that while freezing buck semen the different forms of damaged acrosomes did not differ between thawing methods. The percentage of total

damaged acrosomes was 15.50 ± 0.81 for rapid thawing and 17.05 ± 1.10 for slow thawing.

The incidence of swollen acrosomes increased significantly during storage (Deka and Rao, 1987a) and contributed to the percentage of total damaged acrosomes which increased gradually with increase in storage period. Deka and Rao (1987b) found that the quality of semen during post thawing preservation at 5^0 C was better when buck semen was frozen in Tris egg yolk citric acid fructose glycerol than in Skim milk egg yolk fructose glycerol, Raffinose egg yolk glycerol and Egg yolk citrate fructose glycerol extenders. It was further reported that rapid thawing at 37^0 C for 12-15 seconds was better than slow thawing at 5^0 C for two minutes. Rao *et al.* (1989) studied the normal alterations in deep frozen buffalo spermatozoa and reported that the incidence of damaged acrosomes increased from 16.3 per cent in fresh semen, 25.5 per cent after equilibration, 44.5 per cent after thawing and 47.5 per cent after three hour incubation at 37^0 C.

Pontbriand *et al.* (1989) suggested that the damage of loosening of acrosomal cap was markedly influenced by the cryoprotective properties of the diluent. Ahmed *et al.* (1990) reported the mean percentage of acrosomal abnormalities as 6.122 ± 0.350 and 53.350 ± 2.650 in fresh and frozen semen respectively. They also found that freezing did not affect the dimensions of sperm head significantly but caused significant damage to

acrosomal integrity of buffalo spermatozoa. No significant correlation between acrosome integrity and sperm motility was recorded in fresh and thawed goat semen, by Haunhorst (1990).

Sinha *et al.* (1991) reported the mean percentage of intact acrosomes as 79.01 $\pm$ 0.44, 55.80 $\pm$ 0.38, 51.90 $\pm$ 0.50 in Tris, Skim milk and Egg yolk citrate extenders respectively. They concluded that Tris extender was superior to Skim milk and Egg yolk citrate extender for freezing buck spermatozoa. Austin *et al.* (1991) assessed the influence of cryoprotectives such as glycerol and DMSO on acrosomal damage of deep frozen buck spermatozoa and reported that a combination of six per cent glycerol and two per cent DMSO reduced the incidence of detached and separated acrosomes.

Sinha *et al.* (1992a) reported a decrease in the percentage of intact acrosomes as the glycerol level in the extender and equilibration period were decreased. They concluded that a Skim milk extender with six per cent glycerol equilibrated for four hours gave better quality frozen buck semen. Sinha *et al.* (1992 b) reported that the percentage of intact acrosomes decreased as the equilibration period increased from two to six hours and the glycerol in the extender increased from five to seven per cent. Their study revealed that a combination of four hours equilibration period and six per cent glycerol in Tris extender resulted in better quality frozen buck semen.

Misra *et al.* (1993) reported the percentage of total acrosomal changes in unwashed and washed semen to be 26.85 ± 0.59 and 17.29 ± 0.47 respectively and confirmed that washing of goat semen improved its quality during preservation at 5^0 C. The best rating of per cent intact acrosomes was observed for buffalo semen equilibrated for six hours and thawed at 37^0 C for 30 seconds. (Rao *et al.*, 1993). The lowest percentage of swollen, separating and entirely lost acrosome was recorded to be 11.97 ± 0.82, 0.45 ± 0.18 and 0.29 ± 0.20 respectively at three hours equilibration period in Fructose egg yolk glycerol extender with seven per cent glycerol (Das and Rajkonwar, 1994 d).

Das and Rajkonwar (1994a) reported the percentage of swollen, separating and detached acrosomes after freezing and thawing of buck spermatozoa in Skimmed milk egg yolk glycerol extender to be 9.44, 0.37 and 0.16 respectively for one hour equilibration period with seven per cent glycerol and 14.4, 0.81 and 0.39 for three hours equilibration period and seven per cent glycerol. In Raffinose egg yolk glycerol extender the percentage of spermatozoa with damaged acrosome or without an acrosome increased with increased time of equilibration (Das and Rajkonwar, 1994 c).

Equilibrating for one hour in seven per cent glycerol resulted in least sperm damage before freezing, but after freezing damage was least among spermatozoa that had been equilibrated for three hours. Sinha *et al.* (1994)

found no significant effect on acrosomal damage due to addition of caffeine in the extender. Mendez *et al.* (1995) reported that goat semen frozen and thawed at 55^0 C had a higher percentage of sperm head abnormalities than that thawed at 37^0 C in 15 seconds.

Das and Rajkonwar (1996) studied the acrosomal changes of buck spermatozoa after equilibration and freezing in Egg yolk citrate glycerol extender and reported the lowest percentage of swollen, separating and entirely lost acrosomes for at one hour equilibration with seven per cent glycerol and after freezing it was found to be the lowest at three hours of equilibration with the same percentage of glycerol.

Singh and Purbey (1996) reported that the percentage of intact acrosome (PIA) in buck spermatozoa frozen in liquid nitrogen declined from 88.76 $\pm$ 0.43 in pre freeze to 68.90 $\pm$ 0.82 in post thaw semen when diluted in Tris extender (Tris egg yolk fructose citric acid glycerol). The percentage of swollen, ruffled, fractured, separating and entirely lost acrosomes were estimated as 14.68 $\pm$ 0.21, 7.94 $\pm$ 0.35,1.82 $\pm$ 0.12, 3.12 $\pm$ 0.16 and 3.98 $\pm$ 0.20 .

b) Acrosome staining

Acrosomal structures of ram spermatozoa were prominently stained when air dried smear of diluted semen was fixed for 15 minutes in buffered formol

saline and stained for 90 minutes in six per cent buffered Geimsa solution (Watson,1975). Johnson *et al.* (1977) suggested an improved method using differential interference contrast microscopy for evaluating acrosomes of bovine spermatozoa. They fixed the semen smears in solutions of 0.2 per cent formaldehyde in Phosphate buffered saline. A higher percentage of fixed smears possessed intact acrosomes than unfixed smears. Chenoweth *et al.* (1984) reported that optimal results in acrosomal integrity assessment were obtained by fixing the semen in isotonic formol saline at 25^0 C in fresh bovine semen.

Benjamin (1985) compared the various acrosomal integrity tests including staining methods, differential interference and electron microscopy. Aalseth and Saacke (1985) evaluated the acrosomes using vitally stained smears for determining simultaneously sperm viability and acrosomal morphology. Non staining of the acrosome was highly correlated with percentage of intact acrosomes. Absence of eosinophilic staining in acrosome was used as an indication of sperm viability.

Oettle (1986) tested 'Spermac' stain on fresh and extended semen from bull, ram, goat, dog, horse, boar, cheetah and man. The spermac, stained the acrosome, midpiece and tail region, green, the equatorial region, pale green and the post acrosomal region, red. Detjen (1986) used the Spermac stain for evaluating the quality of fresh and frozen horse semen.

Garde *et al.* (1992) described a triple staining technique capable of simultaneous assessment of sperm viability and acrosomal integrity. Kovacs and Foote (1992) used the Trypan blue or Congo red and Neutral red for determining viability and acrosomal state of bull, boar and rabbit spermatozoa and distinguished 10 classes of spermatozoa.

Tamuli and Watson (1994) combined the two common staining techniques, Nigrosin-Eosin and Geimsa to distinguish acrosomal change in live sperm sub population and reported that this novel staining technique was capable of determining four categories of spermatozoa, live-acrosome intact, live acrosome damaged, dead acrosome intact, dead acrosome damaged.

A simplified staining technique for evaluation of acrosomal status of sperm cells (Sarma, 1995) yielded good results with improved clarity and contrast with respect to acrosome morphology in undiluted, extended and frozen thawed semen. This technique differed from the original method of Hancock (1952) in deletion of pre treatment of semen smears with five per cent formaldehyde for 30 minutes at 37^0 C and washing the smear with water. Instead methanol was used for pre -treatment for 15 minutes.

Kutty *et al.* (1996) reported a more simplified Nigrosin-Eosin-Geimsa staining technique to distinguish acrosome damaged live and dead sperms. The staining technique required very short time and no fixative was needed.

2.2 Processing and freezing of semen

2.2.1 semen extenders

Dauzier *et al.* (1954) reported that the bovine spermatozoal motility could be preserved longest in a 3 part 3 per cent citrate solution to one part egg yolk, but the conception rate was found to be low with the same diluent. Davis *et al.* (1963) found that optimum sperm survival was in the 0.2 mM Tris hydroxy methyl amino methane having a pH 6.75. The sperm motility averaged 42 per cent after eight days storage at 5^0 C compared to Cornell University Extender and Citrate yolk glycerol extender used in freezing bovine spermatozoa.

In a comparative study Gonen (1970) reported a conception rate of 93.87 per cent, 91.66 per cent and 87.75 per cent on insemination with fresh semen, equal parts of fresh semen and semen diluted with 20 per cent egg yolk and 2.9 per cent sodium citrate, and diluted semen respectively. Paggi (1971) tested five modifications of a Raffinose egg yolk glycerol diluent with different concentrations of raffinose and found that the one with 89 parts 13 per cent raffinose, seven parts egg yolk and four parts glycerol gave the best concentration and 65 per cent motility for deep freezing of buck semen.

Sahni and Roy (1972) reported 50-60 per cent post thaw motility for deep frozen (-79^0 C) goat semen with Egg yolk citrate and heated cow's milk. Samoulidis and Hahn (1972) reported that goat semen diluted with laiciphos

had a motility of 60-65 per cent following storage. Cow's milk is a better diluent for preservation of buck semen than Egg yolk citrate, Egg yolk glycine citrate and Goat's milk. (John and Raja, 1973)

Corteel (1974) observed that addition of glucose in skim milk diluent for buck semen freezing increased the post thaw motility. Stagnaro (1975) reported that using semen diluted 1:4 to 1:8 with laiciphos 271 plus four per cent glycerol, 68.6 per cent conceived in the first insemination with an overall conception rate of 82.8 per cent. Westhuysen (1978) diluted semen in Tris based diluent with egg yolk and glycerol for freezing of Angora goat semen.

Cetinkaya *et al.* (1980) reported that semen diluent of Yolk sodium citrate diluent is best for freezing of goat semen. Deka and Rao (1980) observed Tris egg yolk glucose and milk extenders to be superior to Egg yolk citrate extender in maintaining sperm motility on preservation up to 72 hours.

Haranath *et al.* (1982) reported 60 per cent post thaw motility for buck semen frozen in a Tris based diluent containing fructose, citric acid, egg yolk and glycerol. Survival of washed spermatozoa was better when the re-suspending medium contained 1.5 to 12 per cent v/v egg yolk. Egg yolk concentration higher than 12 per cent depressed the post thaw survival of unwashed spermatozoa (Ritar and Salamon, 1982).

Salamon and Ritar (1982) examined the effects of Tris hydroxy methyl amino methane, the type and concentration of sugar in the diluent and rate and method of dilution on the survival of goat spermatozoa after freezing. They concluded that spermatozoal survival depended on the type of sugar included in the diluent. Glucose and fructose were most suitable components than lactose or raffinose. The best result was obtained with a four per cent glycerol concentration in diluted semen.

Singh *et al.* (1982) found egg yolk citrate to be superior for preserving buck spermatozoa at 5^0 C for 96 hours. Corteel (1983) obtained 35 $\pm$ 2 and 27 $\pm$ 2 per cent post thaw motility in goat semen frozen with a 75 per cent milk plus 25 per cent saline and milk alone respectively.

Deka and Rao (1984 a) found Tris egg yolk citric acid fructose glycerol extender was superior in terms of percentage motile sperms. Mathew *et al.* (1984) reported that Tris diluent containing 20 and 25 per cent egg yolk was superior for the preservation of buck spermatozoa at 3 to 5^0 C and Tris diluent with five per cent egg yolk with pH 7.0 was superior at 6 to 8^0 C. Zheltobryukh and Ashurbegov (1984) found that there was an increase of 20-25 per cent in the post thaw motility of goat semen frozen in Lactose egg yolk diluent.

Borgohain *et al.* (1985) reported higher motility when buck semen was preserved for 96 hours in milk diluter than in Egg yolk citrate. Deka and Rao

(1985) reported post thaw motility and percentage of live sperms were significantly higher when buck semen was preserved in Tris egg yolk citric acid fructose glycerol than in Egg yolk citrate fructose glycerol. The incidence of swollen and total damaged acrosomes were apparently lower in Tris egg yolk citric acid fructose glycerol than in Egg yolk citrate fructose glycerol.

Deka and Rao (1985 a) studied the comparative efficacy of four extenders (Egg yolk citrate fructose glycerol, Tris egg yolk citric acid fructose glycerol, Skim milk egg yolk fructose glycerol and Raffinose egg yolk glycerol) and concluded that Tris egg yolk citric acid fructose glycerol and Skim milk egg yolk fructose glycerol were superior for freezing buck spermatozoa. Deka and Rao (1985 b) opined that the post thaw motility for buck semen after equilibration and freezing was significantly higher in Tris extender with glycerol than with DMSO.

Perez (1985) reported better post thaw motility for buck semen in Tris yolk diluent than in Kreb's Ringer solution or in Kreb's Ringer Phosphate glucose solution. Memon *et al.* (1985) found out that there was no significance of extender on post thaw motility of buck semen and the percentage of normal acrosomes was significantly higher in Tris extender than in Lactose egg yolk glycerol.

Deka and Rao (1986) showed that the mean motility in fresh semen preserved in Egg yolk citrate fructose was significantly lower than Tris egg

yolk citric acid fructose diluent. Deka and Rao (1986 a) also found that the incidence of total damaged acrosomes was significantly lower with 20 per cent and 10 per cent egg yolk than with seven per cent egg yolk in the Tris citric acid fructose egg yolk glycerol extender.

The percentage of motile spermatozoa as well as the non return rate was significantly higher with buck spermatozoa frozen in Tris egg yolk citric acid fructose glycerol diluent than in Egg yolk citrate fructose glycerol diluents (Choudhury *et al.*, 1987). Deka and Rao (1987 b) reported that the semen frozen in Tris egg yolk citric acid fructose glycerol extender maintained the highest sperm motility and lowest acrosomal damage. Banu *et al.* (1988) compared the effect of goat milk with Egg yolk citrate and powdered milk as a buck semen diluter and found Egg yolk citrate to be superior for keeping buck semen for five days . Pontbriand *et al.* (1989) reported that the use of BFSF (containing the surfactant component sodium and triethanol amine lauryl sulphate) as cryoprotectant for freezing ram spermatozoa, decreased the percentage of spermatozoa with normal apical ridges.

Chauhan and Anand (1990) reported egg yolk Tris extender with seven per cent glycerol as the best extender for buck semen freezing. Elgaffary (1990) reported the sperm swimming speed to be significantly higher when Tris diluent was used for freezing buck spermatozoa.

Arauja and Nunes (1991) found that the post thawing sperm viability of buck semen diluted in coconut milk containing egg yolk was 44 per cent as against 13 per cent in diluent without egg yolk. The mean sperm motility and live sperm count were significantly higher in Egg yolk citrate fructose glycerol and Tris egg yolk citric acid fructose glycerol than Goat milk glycerol at different stages of buck semen freezing (Deshpande and Mehta, 1991). Melo and Nunes (1991) reported that the average sperm motility was higher in cow's milk than in coconut milk five and 120 minutes after freezing. Sinha *et al.* (1992) opined that Tris extender was superior to Skim milk and Egg yolk citrate for freezing buck semen. The mean sperm motility and percentage of intact acrosomes were higher in semen diluted in Tris extender than in Egg yolk citrate and Skim milk.

Austin *et al.* (1992) reported that Tris diluent containing a combination of six per cent glycerol and two per cent DMSO was superior to six per cent glycerol or two per cent DMSO for the two traits of post thawing percentages of detached and separated acrosomes. Sinha *et al.* (1992 a) found that sperm motility after freezing was significantly higher in semen equilibrated for four hours than two and six hours. The percentage of intact acrosomes had decreased as the glycerol level and equilibration period increased when extended in skim milk diluent with six per cent glycerol. Azawi *et al.* (1993) studied the effect of different diluents on the Shami goat semen and found that the best values for motility, percentage of live spermatozoa and sperm

damage after 120 hours of storage were for Egg yolk tris fructose and Illinoi Variable Temperature extender than for Egg yolk citrate, Egg yolk fructose phosphate, Egg yolk fructose glycine and Egg yolk skimmed cow's milk.

In a study of freezing of buck semen in amine organic buffers, Dunner, (1993) found that N,N-bis (2hydroxy ethyl)-2-ethane sulphonic acid (BESKOH) gave better results at the time of dilution and after chilling at 5^0 C than N-Tris (hydroxy methyl) 2- amino ethane sulphonic acid (TESTRIS), but after freezing no significant difference was found in any of the variables as motility, normal acrosomal ridge and normal swelling between these buffers.

Azawi (1994) observed that the percentage of motile and viable spermatozoa significantly decreased at the higher dilution rates with both diluents, Tris 0.2 M as well as 0.85 per cent sterile sodium chloride. Best results were obtained with Tris diluent at a dilution rate of 1:2 to 1:6. Das and Rajkonwar (1994) reported that the lowest percentage of swollen, separating and entirely lost acrosome after freezing of buck semen in Fructose egg yolk glycerol extender was recorded with three hours equilibration period and seven per cent glycerol levels. These equilibration periods and glycerol levels remained the same for Raffinose egg yolk glycerol extender and Skimmed milk egg yolk glycerol extender.

Molinia *et al.* (1994) found that there was no effect of Tris glucose ratios on the post thaw motility, acrosome integrity or fertility of spermatozoa following

intra uterine insemination. They also studied the effect of polyols on the post thawing motility of pellet frozen ram spermatozoa and found that no combination of polyols and glycerol was superior in terms of post thawing motility and acrosome integrity than six per cent glycerol alone.

Molinia *et al.* (1994) examined the cryoprotectant effects of DMSO, ethylene glycol, glycerol or propanediol alone and concluded that glycerol was the single most effective cryoprotectant and there was no improvement of its cryoprotectant action by the addition of other compounds. They also evaluated *in vitro* the zwitterion buffers in diluents for freezing ram spermatozoa and found them to be superior to Tris egg yolk citrate in semen freezing diluents. Sinha *et al.* (1994) found that fertility rate with Tris + 2 mM caffeine extender was higher than that with Tris diluted semen. Das and Rajkonwar (1995) reported that the highest percentage of motile buck spermatozoa after freezing in Maltose egg yolk glycerol extender and Lactose egg yolk glycerol diluent was recorded with three hours of equilibration and seven per cent glycerol.

Kakadiya and Kavani (1995) reported that Tris citric acid fructose yolk diluent was better for prolonged refrigeration of ram semen than egg yolk citrate glycerol and egg yolk phosphate. Moura *et al.* (1995) studied the efficacy of addition of trehalose into extenders for freezing ram semen and the results indicated that trehalose was not a suitable sugar to incorporate in diluents for

freezing of ram semen. Singh and Purbey (1996) reported that the total acrosomal damage increased from 11.38 per cent to 24.04 per cent in Tris diluent and from 13.98 per cent to 42.32 per cent in egg yolk dilutor due to freezing of buck spermatozoa. Molinia *et al.* (1996) found that compared with Tris diluents, Zwitterion diluents improved the post thawing quality of spermatozoa, however, the fertility of spermatozoa after freeze thawing was similar in Zwitterion and Tris citrate diluents.

Freezing of semen

The criteria adopted for acceptability of a semen sample for freezing of buck semen involves a minimum volume of 0.3 ml, mass activity of ++++, minimum sperm concentration of 3000 X 10^6, live sperm percentage of 80 and a maximum of 10 per cent abnormal sperms. (Morrow *et al.*, 1980).

2.2.2 Washing of spermatozoa

Corteel (1995) evidenced in goat seminal plasma the Egg Yolk Coagulating (EYC) enzyme. In the presence of calcium the EYC hydrolyses egg yolk lecithin to lysolecithin and fatty acid. Lysolecithin are released in quantities that are highly toxic to spermatozoa. Iritani *et al.* (1961) studied the egg yolk coagulating factor in goat semen, its localisation and decline of pH following coagulation. They reported that a thermolabile egg yolk coagulating factor was found in goat semen but not in bull, ram, boar and rabbit semen. The

factor was present in the seminal plasma and was derived from the secretion of cowper's gland. They also found that spermatozoa washed once did not coagulate egg yolk and remained motile for 10 to15 days in egg yolk citrate at 4^0 C ; twice washed spermatozoa were motile for one month .

Iritani *et al.* (1961) studied the properties of the coagulating factor and the conditions such as temperature, yolk concentration and pH and that are found to influence coagulation. Iritani and Nishikawa (1963) studied the egg yolk coagulating property of goat semen and concluded that there is the release of some acids accompanied by the coagulating phenomena .They also tried the purification of the egg yolk coagulating enzyme. Aamdal (1965) reported that the lack of motility of goat sperms on addition of 10 per cent egg yolk was caused by the formation of lysolecithin. Jones and Martin (1973) found that incubation of diluted semen at 35^0 C did not any significant changes in acrosome whereas incubation and storage at 5^0 C changed the structure of acrosomes and middle pieces. The acrosome changes involved swelling and vacuolation and mid pieces showed condensation of mitochondria and loss of material. Corteel (1974) concluded that motility and percentage motile spermatozoa were higher in the samples without seminal plasma both before and after freezing. Corteel and Baril (1975) reported that the post thawing motility of goat semen one-two days after freezing averaged 44.4 and 43.7 per cent in unwashed and washed spermatozoa respectively, but after storage for three to 90 days, the additional loss in motility was 16.6

per cent for unwashed spermatozoa and zero for washed spermatozoa and after storage for 91 to 180 days the corresponding figures were 22.0 and 1.3 per cent. Washing of buck semen before freezing in tris based diluent with egg yolk and glycerol significantly increased post thaw sperm motility (Westhuysen,1978).

Removal of semen plasma by centrifugation of goat semen was beneficial for survival of spermatozoa after thawing (Ritar and Salamon, 1982). Double washing was more effective than single washing. Survival of washed spermatozoa was better when the re suspending freezing medium contained 1.5 to 12 per cent egg yolk than no egg yolk. Egg yolk concentration higher than 1.5 per cent depressed the post thawing survival of non washed spermatozoa.

The percentage of motile sperms in semen frozen with and without seminal plasma was 48.31 $\pm$ 3.11 and 52.38 $\pm$ 3.06 respectively in Egg yolk citrate fructose glycerol diluent and 63.25 $\pm$ 1.83 and 64.15 $\pm$ 2.85 respectively in Tris egg yolk citric acid fructose diluent (Deka and Rao, 1984a). The corresponding values of damaged acrosomes (in percentage) were 5.97 $\pm$ 1.87 , 21.77 $\pm$ 2.47 , 10.73 $\pm$ 1.16 and 15.27 $\pm$ 2.55. Memon *et al.* (1985) reported that the sperm motility and acrosome morphology were significantly reduced in the presence of seminal plasma for the milk extender. Washing of buck semen with Ringer's solution and removing the fluid by centrifugation at

950 g gave a significant effect on motility. Perez (1985) reported that ejaculates from goats washed twice had higher sperm motility and a higher percentage of acrosomal defects than non washed semen after freezing.

Deka and Rao (1987a) diluted buck semen at a rate of 1:5 with Tris buffer and centrifuged at 3000 rpm for five minutes. They reported that the motility was found to be higher when seminal plasma was removed prior to dilution. They concluded that the percentages of progressively motile and live sperm did not vary significantly in unwashed and washed semen. The frozen semen without seminal plasma showed significantly higher percentage of total damaged acrosome.

Deka and Rao (1989) studied the effect of washing of goat semen on the fertility and reported that the sperm motility and total number of sperms per straw varied from 65 to 75 per cent and 155-265 million respectively, the difference between washed and unwashed being negligible. There was also no significant difference in the conception rate between washed and unwashed semen. Haunhorst (1990) reported that buck sperm motility was not significantly affected by centrifugation.

Misra *et al.* (1993) evaluated the effect of washing on the motility and other changes at zero, 24, 48 and 72 hours of preservation of goat semen at 5^0 C and reported that washing improved the quality of semen during preservation at 5^0 C. Tuli and Holtz (1994) reported that progressive motility and

percentage of live spermatozoa were higher after freezing of whole semen than freezing of washed spermatozoa. Glutamic oxaloacetic transaminase (GOT) level in the extracellular fluid was lower in washed spermatozoa before freezing but after thawing washed spermatozoa released more GOT.

From experiments of Corteel (1995), it was also clear that preservation of goat sperm would be simplified by the definition of a diluent which does not necessitate the elimination of seminal plasma.

2.2.3 Glycerolisation

Paggi (1971) reported that the diluted semen samples showing 65 per cent motility after thawing had 89 parts 13 per cent raffinose, seven parts egg yolk and four parts glycerol. Sahni and Roy (1972) has done freezing of buck semen using Egg yolk citrate and heated cow milk containing three, six and nine per cent glycerol as diluents. The result of post thaw recovery with six per cent glycerol level was better than at the other two levels.

Salamon and Ritar (1982) reported that there was interaction between method of semen dilution, holding time at 5^0 C and glycerol concentration in freezing of buck semen. Deka and Rao (1986b) studied the effect of glycerol level in Tris based extender on the motility and acrosomal integrity of frozen goat semen. They concluded that the optimum glycerol level in Tris based

extender and equilibration period were not the same for maintaining maximum sperm motility and acrosomal integrity.

The best freezing regime for Korean native goat semen was 12^0 C per minutes using nine per cent glycerol. (Park *et al.*, 1989). Chauhan and Anand (1990) reported that for buck semen, optimal results for motility, percentage of live spermatozoa and acrosomal integrity after freezing were obtained with 20 per cent dilution rate and seven per cent glycerol.

Deshpande and Mehta (1991) studied the effects of four, five and six per cent glycerol levels on the pre freeze and post freeze sperm motility and live sperm count in Egg yolk citrate fructose glycerol, Tris egg yolk citric acid fructose glycerol and Goat milk glycerol and found them to be higher in all the three dilutors.

The sperm motility and per cent intact acrosomes were higher at six per cent glycerol levels in the Skim milk extender and Tris extender (Sinha *et al.*, 1982a ; Sinha *et al.*, 1982 b) Purohit *et al.* (1992) reported that five per cent glycerol was superior to three per cent and four per cent levels while freezing Sangamneri buck semen in that it yielded best results of sperm motility and live sperm count while freezing buck semen. Tuli and Holtz (1994) concluded that stepwise addition of glycerol to semen at 37^0 C gave a higher score for progressive motility and percentage of live spermatozoa compared to one-step addition.

2.2.4 Equilibration

Deka and Rao (1986 b) reported that progressive motility as well as per cent intact acrosome after equilibration was not affected by the equilibration period, but after thawing they were higher with five hours equilibration period than with one or three hours equilibrated semen.

Sinha *et al.* (1992a) also concluded that four hours equilibration period gave best results with respect to progressive motility and per cent intact acrosomes during freezing of buck semen in Skim milk glycerol extender and Tris extender (Sinha *et al.*, 1992 b).

Purohit *et al.* (1992) in his studies on deep freezing of buck semen, concluded that five hour equilibration period showed significantly better post thaw semen quality .

2.2.5. Freezing methods and effects of freezing and thawing

Sahni and Roy (1972) reported that the mean percentage of abnormalities of head, mid piece, coiled tail and free normal head in different stages of processing and freezing of buck semen did not differ significantly from that of fresh semen in any extender (Deka and Rao, 1984b).

Deka and Rao (1986b) reported freezing of buck semen in 0.5 ml straws by suspending the straws five cm above liquid nitrogen for 10 minutes. Optimal freezing was attained by holding straws in vapour four cm above liquid

nitrogen for 30 seconds, followed by plunging into liquid nitrogen. Initiation of ice crystallisation and the pattern of cooling of semen depended on the size of straws and the type of rack used to hold the straws in vapour during freezing. Cooling curves differed greatly for semen frozen in straws in liquid nitrogen vapour and pellets in dry ice (Ritar *et al.*, 1990).

Addition of caffeine during deep freezing preservation of goat semen in Tris medium was found to have a stimulating effect as sperm motility (Sinha *et al.*, 1994).

2.2.6. Thawing

In frozen bull semen thawed at 75^0 C for 12 seconds, the percentage of unstained spermatozoa after staining with Eosin-Nigrosin was considerably higher than that after thawing at 35^0 C for 30 seconds (Aamdal and Anderson, 1968).

Significantly higher percentages of progressively motile and live sperms were recorded after rapid thawing (37^0 C for 12-15 seconds) than slow thawing (5^0 C for two minutes) of frozen goat semen. This may be due to short period of exposure of sperms to increased solute concentrations during rapid thawing (Deka and Rao,1986 c ; Deka and Rao,1987 a)

The post thaw motility of frozen buck semen was reported to be 56.15, 54.58 and 47.85 per cent in Jamnapari, Barbari and Black Bengal bucks

respectively by thawing frozen goat semen at 70^0 C for 10 seconds (Sinha *et al.*, 1987).

Straws with deep frozen buck semen were thawed in a water bath at 40^0 C for 30 seconds (Torre *et al.*, 1990) at 40^0 C for 10 seconds (Haunhorst, 1990) and at 37^0 C for 12-15 seconds (Sinha *et al.*, 1993). The best temperature for thawing of frozen buck semen was found to be 37^0 C for 10 seconds in a water bath (Das and RajKonwar, 1994 b).

2.2.7. Live sperms during processing and after freezing

Aamdal and Anderson (1968) reported a higher percentage of unstained spermatozoa when straws were thawed at 75^0 C for 12 seconds.

The live sperm count of frozen thawed Jersey bull semen was higher at 37^0 C than at other thawing temperatures (Bhosrekar *et al.*, 1984). Deka and Rao (1985) reported that the percentage of live sperms after thawing was significantly higher in Tris egg yolk citric acid fructose glycerol diluent.

Deka and Rao (1986b) reported that the percentage of live sperms after rapid (37^0 C for 12-15 seconds) and slow thawing (5^0 C for two minutes) were 65.63 $\pm$ 0.98 and 55.47 $\pm$ 1.11 respectively.

The percentage of live sperms in frozen goat semen in different storage periods of zero day, three months, six months, nine months and 12 months are 69.6 $\pm$ 1.96, 68.06 $\pm$ 1.78, 66.67 $\pm$ 2.12, 68.13 $\pm$ 2.01, 68.91 $\pm$ 1.74

respectively with seminal plasma and 71.18 ± 2.15, 70.67 ± 1.29,70.07 ± 1.25, 62.31 ± 1.34 and 70.91 ± 0.25 respectively without seminal plasma (Deka and Rao, 1986c).

Deshpande and Mehta (1991) reported the percentage of live sperms immediately after dilution (P1), five hours after dilution (P2), immediately after freezing post thaw (P3) and 24 hours after freezing post thaw (P4) to be 82.00 ± 2.14, 76.83 ± 2.23, 45.33 ± 4.52 and 44.50 ± 2.98 at six per cent glycerol level.

2.2.8 Sperm abnormalities after processing and freezing

Deka and Rao (1984 b) reported that the mean percentage of abnormalities of head, mid piece and tail in different stages of processing and freezing did not differ significantly from that of fresh semen in any extender. In Egg yolk citrate the mean abnormality in fresh semen, after initial dilution and cooling, after equilibration and after freezing and thawing were 0.20 ± 0.07, 0.78 ± 0.14, 1.05 ± 0.20, and 1.50 ± 0.19 respectively, 0.20 ± 0.07, 0.85 ± 0.15, 1.23 ± 0.19 and 1.26 ± 0.17 respectively in Tris diluent, 0.20 ± 0.07, 0.85 ± 0.15, 1.05 ± 0.17 and 1.18 ± 0.16 respectively in Skim milk and 0.20 ± 0.07, 1.28 ± 0.24, 1.53 ± 0.30 and 1.54 ± 0.27 respectively in Raffinose.

2.2.9 Acrosome abnormalities after processing and freezing

Jones and Martin (1973) reported that incubation of diluted ram semen at 35^0 C for one to two hours did not cause significant changes in acrosomes, but a significant portion of spermatozoa showed condensation of mitochondria or loss of material. Acrosomes and mid pieces were affected by cooling to 5^0 C and storage at this temperature for up to 72 hours. Visser (1974) opined that higher thawing temperature and thawing in a solution favoured the maintenance of acrosomal integrity following the freeze thaw incubation procedures.

The proportion of acrosome deformities to the extent of 2.9 per cent in fresh ram semen, increased to 20.7 per cent after glycerolisation, but there was no relationship between the proportion of deformed spermatozoa and motility. (Gokcen and Asti, 1980)

Sokolovskaya *et al.* (1981) classified a scheme of abnormal spermatozoa as swollen, exfoliated, lost and complete loss of head and suggested that thawed semen is suitable for insemination if 30 per cent of spermatozoa have normal acrosome. The percentage of normal acrosomes was better in semen of rams centrifuged at 5^0 C. Centrifugation at 12^0 C increased the incidence of abnormal spermatozoa.

Papa (1982) reported that thawing at 70^0 C increased the per cent intact acrosomes in bull semen. Thawing at 25^0 C and 40^0 C increased the incidence of loose and detached acrosomes in bull semen (Bhosrekar *et al.*, 1984).

Aalseth and Saacke (1985) observed the swelling of apical ridge and anterior acrosome of motile bovine spermatozoa using differential interference contrast optics. They concluded that storing spermatozoa in seminal plasma at 4^0 C for one day was most conducive to the swelling of apical ridge. Replacing seminal plasma with Egg yolk citrate inhibited swelling.

The percentage of total damaged acrosomes was reported to be 1.50 $\pm$ 0.3, 2.10 $\pm$ 0.40, 5.73 $\pm$ 0.91, and 18.60 $\pm$ 2.74 respectively in fresh semen, in cooled diluted semen at 5^0 C, after equilibration and after freezing and thawing in Egg yolk citrate fructose glycerol diluent and 2.50 $\pm$ 0.23, 5.07 $\pm$ 0.52, 12.37 $\pm$ 1.84 respectively in cooled diluted semen, after equilibration and after freezing and thawing in Tris egg yolk citric acid fructose diluent. (Deka and Rao, 1985).

The incidence of swollen acrosomes and total damaged acrosomes in buck semen did not differ significantly between egg yolk levels before freezing but after freezing and thawing. The acrosome damage was significantly lower with 10 and 20 per cent egg yolk when compared with seven per cent egg yolk (Deka and Rao, 1986 a). They also reported (Deka and Rao, 1986 b)that

the percentage of damaged acrosomes was lower with four per cent glycerol and one hour equilibration period while freezing buck semen.

Deka and Rao (1986 c) confirmed that while freezing buck semen the different forms of damaged acrosomes did not differ between thawing methods. The percentage of total damaged acrosomes was 15.5 $\pm$ 0.81 for rapid thawing and 17.05 $\pm$ 1.10 for slow thawing. The incidence of swollen acrosomes increased significantly during storage which contributed to the progressive increase in acrosome damage on storage (Deka and Rao, 1987a).

Rao *et al.* (1989) studied the acrosomal alteration in deep frozen buffalo spermatozoa and reported that the incidence of damaged acrosomes increased from 16.3 per cent in fresh semen, 25.5 per cent after equilibration 44.5 per cent after thawing and 47.5 per cent after three hours of incubation at 37^0 C. Pontbriand *et al.* (1989) suggested that the damage of loosening of acrosomal cap is markedly influenced by the cryo protective properties of the diluent . Ahmed *et al.* (1990) reported the mean percentage of acrosomal abnormalities as 6.12 $\pm$ 0.35 and 53.35 $\pm$ 2.65 in fresh and frozen semen respectively. They also found that freezing did not affect the dimension of sperm head significantly but caused significant damage to acrosomal integrity in buffalo spermatozoa. Misra *et al.* (1993) reported the total acrosomal

changes in unwashed and washed semen to be 26.85 ± 0.59 and 17.29 ± 0.47 respectively.

The lowest percentage of swollen, separating and entirely lost acrosome after freezing of buck semen in Fructose egg yolk glycerol extender with seven per cent glycerol and three hours equilibration time was recorded to be 11.97 ± 0.82 and 0.45 ± 0.18 and 0.29 ± 0.20 respectively (Das and Rajkonwar, 1994).

Das and Rajkonwar (1994a) reported the percentage of swollen, separating and detached acrosomes after freezing and thawing of buck spermatozoa to be 9.44, 3.37 and 0.16 respectively in Skimmed milk egg yolk glycerol extender. Equilibrating for one hour in seven per cent glycerol resulted in least sperm damage before freezing but after freezing damage was least among spermatozoa that had been equilibrated for three hours .

Singh and Purbey (1996) reported that the per cent intact acrosome in buck spermatozoa diluted in Tris extender and frozen in liquid nitrogen declined from 88.76 ± 0.43 in pre freeze to 68.90 ± 0.82 in post thaw semen. The percentage of swollen, ruffled, fractured, separating and entirely lost acrosomes were estimated as 14.68 ± 0.21, 7.94 ± 0.35, 13.12 ± 0.06. and 3.98 ± 0.20 in tris extender.

Materials and Methods

MATERIALS AND METHODS

3.1 Semen Collection

Normal semen ejaculates from five Malabari crossbred (Alpine X Malabari) bucks aged one to two years, maintained at the Artificial Insemination Centre, under the Department of Animal Reproduction, College of Veterinary and Animal sciences, Mannuthy, Thrissur were utilized for this study. The buck's selected were normal, healthy and maintained at standard feeding and management practices. Two ejaculates each were collected from five bucks once in two weeks during the period from November 1996 to December 1997 by artificial vagina method.

Semen was collected using Danish type artificial vagina remodelled with a rubber hose length of 15 cm to suit the bucks. A 15 ml graduated collection vial was attached to the free end of the rubber cone for easy reading of the volume soon after collection. The artificial vagina was prepared so that the inside temperature of 45 to 50^0 C and adequate pressure was provided by blowing in air. The mouth of the artificial vagina was lubricated and the collection vial was insulated with a polystyrene cube.

Adult male bucks were used as teasers. Two false mounts were given for stewing the bucks before taking collection and six collections were made from each buck. Immediately after collection the semen samples were transferred to a water bath maintained under 37^0 C before centrifugation.

3.2 Semen evaluation

The physical attributes of semen as volume, colour, density, mass activity and pH were observed for.

3.2.1 Volume

The volume of semen was recorded from the graduations on the collection vial.

3.2.2 Colour

The colour of semen was recorded by visual observation and graded on the intensity of cream colour.

3.2.3 Density

The density of semen was graded and expressed as D to DDDD based on visual examination of opacity of a drop of semen taken on a glass slide.

3.2.4 Mass activity

A small drop of semen was taken on a glass slide and examined under low magnification of microscope for waves, swirls and intensity and was expressed from + to ++++.

3.2.5 Hydrogen ion concentration

A small piece of pH indicator paper was immersed in a drop of semen placed on a glass slide. The change in colour obtained on the pH paper was matched with that of the standard. The value thus obtained was recorded as pH of the sample.

3.2.6 Sperm concentration

The sperm concentration was determined using haemocytometer. Number of sperms in eighty small squares was multiplied by 10^4 to obtain the number of sperms per cubic mm.

3.2.7 Sperm morphology

a) Sperm vitality

Nigrosin-Eosin smears were prepared (one drop of one per cent aqueous Eosin and three drops of 10 per cent Nigrosin were taken on a glass slide and a small drop of semen was mixed with it and smears were

prepared after 10 to 15 seconds). Under oil immersion objective of the microscope a total of 100 sperms were counted from different microscopic fields keeping a note of dead and live sperms from the staining characters and percentage of each was worked out.

b) Sperm abnormalities

Percentage of abnormal sperms was estimated in a Nigrosin-Eosin smear under oil immersion objective of the microscope. From different microscopic fields on the slides a total of 100 sperms were counted keeping a note of specific abnormalities as head, mid piece and tail abnormalities and the proportion of each were worked out and expressed in percentage.

c) Acrosome abnormalities

This was assessed by two different staining procedures.

1) Geimsa staining

Dried semen smears were fixed in four per cent buffered formaldehyde or four per cent formalin in distilled water for 15 minutes. The smears were washed in running tap water for 15 to 20 minutes. The smears were dried and immersed in buffered Geimsa solution for 90 minutes. They were then rinsed in distilled water, dried and mounted in DPX and observed under

immersion objective of the microscope and scored as intact, ruffled swollen and lost acrosomes according to the intensity of staining of the cells and the contrast with the background.

2) Nigrosin -Eosin-Geimsa staining

The Nigrosin-Eosin stained smears used for vitality estimation were fixed in formaldehyde for 10 minutes and then rinsed in tap water for 10 minutes. These were then immersed in Geimsa stain for 10 minutes. The smears were rinsed first in tap water, then in distilled water, dried and mounted in DPX.

3.3 Processing and Freezing of semen

3.3.1 Semen extenders

Tris hydroxy methyl amino methane (2.42 g), citric acid (1.34 g) and fructose (1.0 g) were weighed accurately into five sterile 100 ml volumetric flasks numbered I to V. Then 25 ml double glass distilled water was added in all the flasks and stirred well. Fresh egg yolk was collected aseptically and added 10 ml each to flasks numbered III, IV and V and mixed well using sterile glass rod. Twelve ml and fourteen ml each of glycerol were added into the flasks numbered IV and V respectively. Benzyl penicillin and streptomycin sulphate were added at the rate of 50,000 iu and 50,000 micro g respectively in all flasks and stirred well. Then the volume of

extenders were made up to 100 ml by adding double glass distilled water in all the five flasks. Extenders were placed in a water- bath maintained at 37^0 C before dilution with semen. The solution in the flasks I and II were used for washing of fresh semen, that in flask II was the non glycerolated portion of the extender for initial dilution and those in IV and V, the glycerolated fraction of the extender used in protocols I and II of freezing of semen.

3.3.2 Selection of samples

Based on the preliminary evaluation semen samples with more than 70 per cent initial motility were used for freezing trials. After preliminary evaluation they were split into two parts for freezing by two different protocols.

3.3.3 Washing of spermatozoa

In protocol I semen sample was extended 10 times with Tris buffer and centrifuged at 3000 rpm for five minutes. The supernatant fluid was pipetted out. The pellet was extended again by 10 fold in Tris buffer and repeated the centrifugation for five more minutes at 3000 rpm and the clear supernatant fluid was removed. In protocol II only single washing in the ratio of extension of 1:15 for 10 minutes was done.

3.3.4 Initial extension

The pellet formed after washing was extended five times the original volume (1:5) with the non glycerolated Tris extender. The initial motility of the sample was assessed by placing a drop of semen on a glass slide and keeping a cover slip on it. Motility was assessed under high magnification of the microscope and expressed as percentage. The semen with non glycerolated Tris extender was taken in the vials, glycerolated extenders were also taken in vials and closed with aluminium foils. After initial extension all the samples were assessed for motility, live-dead count, sperm abnormalities and acrosome abnormalities.

3.3.5 Cooling of semen

The vials containing semen with non glycerolated extenders and vials containing glycerolated extenders were kept in a 500 ml beaker containing water at 37^0 C. This beaker was kept in the cold handling chamber maintained at 5^0 C. Straw clips, straw filling combs, sterile cotton, sterile towel, polyvinyl alcohol powder, air bubblers, air bubbler dish, 0.5 ml straws of different colours and a tray containing water were placed inside the cold handling chamber. Diluted semen and extenders were kept in the cold handling chamber for one and a half to two hours or till the temperature of the water bath was lowered to 5^0 C. After cooling one drop each of the mixture from all the samples were taken for assessing the

motility, live-dead count, sperm abnormalities and acrosome abnormalities.

3.3.6 Glycerolisation

Equal quantities of the glycerolated fraction of the extender was mixed and added to the semen with non glycerolated extender in three parts at 15 minutes intervals so that the final concentration of glycerol is six per cent in protocol I and seven per cent in protocol II.

3.3.7 Filling and sealing of straws

Different coloured straws (orange, yellow, green, blue, red) of 0.5 ml capacity were used for filling semen under both protocols. Straws were arranged in straw clips. Polyvinyl alcohol powder was spread about five mm thickness evenly in a glass dish nine cm diameter. Straws in clip were attached to a filling comb by the factory seal end. The comb was connected to the vacuum pump by a small rubber tubing. After mixing gently the semen was transferred to the bubbler dish in the cold handling chamber and the open end of the straws attached to the comb was dipped in it and filled using a vacuum pump. Air spaces were created in the straws by applying the open end of straws into the bubbler comb. Then the open end of the bubbler comb was dipped into polyvinyl alcohol powder to prepare the laboratory seal. The excess powder sticking to the

straws were removed using a cotton pad. The straws were immersed in water at 5^0 C after releasing from the clips and comb. After 45 minutes filled straws were taken out and wiped with dry towel and arranged on freezing racks.

3.3.8 Equilibration

The straws were given four hours equilibration in protocol I and three hours in protocol II.

3.3.9 Freezing and storage

Wide mouthed liquid nitrogen refrigerator was used for freezing. The liquid nitrogen was poured into the refrigerator up to the level touching the freezing grill 30 cm below the brim of the container. This was closed and kept for a few minutes so that the refrigerator is cooled. After attaining the vapour pressure, freezing racks with straws were transferred quickly from the cold handling chamber to the freezing cabinet. The straws in the rack were four cm above the liquid nitrogen level. Goblets were also placed over the grill. Closed the lid of the refrigerator and allowed the straws to remain there for eight minutes in protocol I and 10 minutes in protocol II. Then the straws were collected by cooled gloved hand and placed into the pre cooled goblet and plunged into liquid nitrogen. After this the goblets along with the straws frozen under both the protocols were transferred

into different goblets of the storage container cryocan BA-42. A thick paper label showing the protocol number, date of collection and number of straws was tied on the handle of the canister for identification of semen straws. The post thaw motility was assessed immediately after freezing of each group of straws.

3.3.10 Thawing of semen

After removing the neck tube plug of liquid nitrogen refrigerator, the tip of the forceps was cooled by holding it in the vapour through the neck for 30 seconds. Then lifted the canister up to the bottom level of the neck. With the pre cooled forceps, straws were picked up within five seconds. By holding one end of the straw shook it twice to remove the drops of liquid nitrogen if any. Then placed the straws in water at 25^0 C for 30 seconds in protocol I and warm water at 60^0 C for 10 seconds in protocol II.

Sperm motility, percentage of dead sperms and acrosome abnormalities of spermatozoa were estimated at the end of initial extension, cooling, glycerolisation, equilibration and deep freezing.

Results

RESULTS

A study was carried out to find out the effect of processing and freezing procedures on the acrosome morphology of buck spermatozoa using semen samples from five Malabari crossbred bucks maintained at the Artificial Insemination centre under the Department of Animal Reproduction, College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

4.1 Semen evaluation

The results of preliminary semen evaluation are presented in table.4.1. The overall average volume of buck semen was 1.328 $\pm$ 0.067 ml. The colour of all the ejaculates was found to be creamy, density was DDDD and the mass activity was ++++.The overall average pH was 7.275 $\pm$ 0.040. The average sperm concentration was 2972 $\pm$ 293 millions per ml. The mean volume, pH and sperm concentration were found to have no significant variation between bucks.

4.2 Sperm motility

The percentage of sperm motility was evaluated after washing of semen and initial extension (stage I), after cooling to 5^0 C (stage II), after glycerolisation and equilibration (stage III) and after freezing and thawing (stage IV) during processing and freezing spermatozoa by two different protocols. The average percentage of sperm motility was 82.000 $\pm$ 0.606 in protocol I as against 81.375 $\pm$ 1.089 in protocol II at the end of stage I . At the end of stage II, the average percentage of

progressively motile sperms were 75.875 ± 1.305 and 74.625 ± 0.644 in the protocols I and II respectively. The average percentage of progressive sperm motility at the end of stage III were 70.500 ± 0.960 in protocol I and 69.125 ± 0.579 in protocol II. The average post thaw motility of the frozen semen were 44.000 ± 0.790 in protocol I and 44.750 ± 1.075 in protocol II. There was no significant difference in the motility between corresponding stages of the two protocols (table 4.2, fig 4.1). However, there was significant difference in the motility of sperms between the four stages of processing and freezing by the two different protocols ($p<0.05$) .

4.3 Live sperms

The mean live sperm percentage of fresh semen in the Nigrosin-Eosin smears (NE) and Nigrosin-Eosin-Geimsa (NEG) smears were 90.025 ± 0.801 and 80.850 ± 1.494 respectively. The corresponding figures after the end of stage I, II,III and IV were 86.750 ± 0.788, 83.000 ± 0.724, 74.325 ± 0.842 and 54.250 ± 0.593 respectively in NE smear as against 74.775 ± 1.693, 74.350 ± 1.992, 64.475 ± 0.518 and 54.875 ± 0.677 respectively in the NEG smear in protocol I. In protocol II, the mean percentage of live sperms at the end of stages I, II, III and IV were 86.500 ± 0.369, 80.625 ± 0.506, 74.225 ± 0.257 and 53.125 ± 0.793 respectively in NE smear and 76.700 ± 0.829, 70.875 ± 0.956, 65.800 ± 0.942 and 53.400 ± 0.730 respectively in the NEG smear.(table 4.3.1 and table4.3.2 ; fig 4.2 and 4.3). There was no significant difference in the live sperm percentage between the corresponding stages of protocol I and II with both the staining techniques. On the other hand,

significant difference in the live sperm percentage between stages within protocol I and II by NE and NEG staining were noticed. ($p<0.05$) It was also observed that there was significant difference in the mean percentage of live sperms by the two different staining methods at the end of all the stages in both the protocols. ($p<0.05$)

4.4 Sperm abnormalities

The average percentage of abnormal sperms in the fresh semen as observed in the NE smear was 3.050 ± 0.245. The percentage of abnormal sperms were 3.750 ± 0.570, 4.700 ± 0.303, 5.500 ± 0.428 and 7.125 ± 0.706 respectively at the end of stages I, II, III and IV of processing and freezing by protocol I and 4.025 ± 0.235, 4.700 ± 0.208, 5.275 ± 0.601 and 6.300 ± 0.369 respectively in protocol II (table.4.4 ; fig 4.4).

There was no significant difference in the percentage of sperm abnormalities between stages I, II and III. However stage IV differed significantly from the first two stages in both the protocols (table.4.6).

4.5 Acrosome abnormalities

The average percentage of intact acrosomes in the fresh semen stained with Geimsa and NEG smears were 91.175 ± 0.636 and 91.275 ± 0.710 respectively. The corresponding figures after the end of stages I, II, III and IV were 86.050 ± 1.247, 81.825 ± 2.180, 79.575 ± 1.703 and 76.425 ± 2.044 respectively with Geimsa smear as against 88.925 ± 1.489, 86.025 ± 1.168, 83.600 ± 0.843 and 80.850 ±

0.713 respectively with NEG smear in protocol I. In protocol II, the percentage of intact acrosomes at the end of stages I, II, III and IV were 86.700 ± 0.843, 80.425 ± 2.105, 78.175 ± 2.400 and 79.825 ± 1.327 respectively with Geimsa staining and 87.500 ± 0.489, 85.400 ± 1.005, 82.575 ± 1.036 and 81.300 ± 0.676 respectively with NEG staining in protocol II. (table 4.5.1and 4.5.2 ; fig 4.5 and4.6)

Analysis of data has revealed that there was no significant difference in the mean percentage of intact acrosomes between corresponding stages of the two protocols with Geimsa and NEG staining . However, there was significant difference between stage I and III and I and IV in protocol I with Geimsa staining as against the observance of significant difference between stage I and II, I and III and I and IV in protocol II with the same staining method ($P<0.05$). On the other hand, with NEG staining significant difference in the intact acrosome was observed between stages I and III, I and IV, II and IV and III and IV in protocol I and between I and III, I and IV and II and IV in protocol II. It was further observed that there was no significant difference in the percentage of intact acrosomes by the two staining methods at the end of each stage in both the protocols.

The percentage of swollen, ruffled and entirely lost acrosome in fresh semen were 3.925, 3.300 and 1.600 with a total of 8.825 . The corresponding figures were 6.200, 4.575, and 3.000 at the end of stage I, 6.350, 5.650 and 5.875 at the end of stage II, 7.150, 6.750 and 6.725 at the end of stage III and 9.400, 7.800 and 6.175 at the end of stage IV in protocol I. Similarly in protocol II, the mean percentages of swollen,

ruffled and entirely lost acrosome were 5.025, 5.700 and 5.550 at the end of stage I, 7.425, 6.825 and 5.000 at the end of stage II, 7.825, 6.925 and 6.200 at the end of stage III and 8.200, 5.975 and 5.625 at the end of stage IV.(table 4.7, fig 4.7) The percentage of total damaged acrosome after the end of stage I, II, III and IV were 13.775, 17.875, 20.625 and 23.375 respectively in protocol I as against 13.275, 19.250, 20.625 and 19.825 respectively in protocol II. There was significant difference between stages I and III and I and IV in protocol I as against the observance of significant difference between stage I and II , I and III and I and IV in protocol II with the same staining method ($p<0.05$) . There was no significant difference in the percentage of damaged acrosome between the corresponding stages of the two protocols. There was also no significant difference in the proportion of different acrosomal abnormalities between stages within protocols I and II.

Table 4.1 **Evaluation of Buck semen - Physical attributes (Average of six)**

Buck no	volume (ml)	colour	density	mass activity	pH	spermconcentration millions/ml
1	1.750	creamy	DDDD	++++	7.180	3060
2	1.400	creamy	DDDD	++++	7.250	3872
3	1.150	creamy	DDDD	++++	7.250	3542
4	1.210	creamy	DDDD	++++	7.500	1854
5	1.120	creamy	DDDD	++++	7.250	2532
Mean	1.328	creamy	DDDD	++++	7.275	2972
SE	0.067				0.040	293

Table 4.2 Percentage of progressively motile sperms at the end of different stages of processing and freezing of buck spermatozoa

Buck no	Motility at the end of stage							
	I		II		III		IV	
	PI	PII	PI	PII	PI	PII	PI	PII
1	83.125	83.125	75.000	75.000	70.625	66.875	46.250	46.250
2	83.125	83.750	80.000	76.875	70.000	69.375	43.750	43.750
3	81.250	81.250	77.500	74.375	66.875	69.375	42.500	41.250
4	80.000	77.500	72.500	73.125	65.625	70.000	42.500	45.000
5	82.500	81.250	74.375	73.750	69.375	70.000	45.000	47.500
Mean	82.000	81.375	75.875	74.625	70.500	69.125	44.000	44.750
SE	0.606	1.089	1.305	0.644	0.960	0.579	0.729	1.075
tvalue	0.5013		0.8591		_0.5573		_0.5774	

Table 4.3.1 Percentage of live sperms at the end of different stages of processing and freezing of buck spermatozoa Nigrosin-eosin smear

Buck no	Live sperms at the end of stages								
	fresh	I		II		III		IV	
		PI	PII	PI	PII	PI	PII	PI	PII
1	91.375	85.125	85.750	83.625	80.000	75.375	74.500	53.250	50.375
2	91.750	88.500	87.500	83.250	80.875	73.625	73.500	54.250	54.500
3	89.125	84.750	86.500	82.625	81.750	71.375	75.000	56.250	54.375
4	87.375	87.000	85.625	83.375	79.000	75.125	74.250	52.875	52.375
5	90.500	88.375	87.125	82.125	81.500	76.125	73.875	54.625	54.125
Mean	90.025	86.750	86.500	83.000	80.625	74.325	74.225	54.250	53.125
SE	0.801	0.788	0.369	0.274	0.506	0.842	0.257	0.593	0.793
t value		0.2875		4.1265		0.1136		1.1109	

Table 4.3.2 Percentage live sperms at the end of different stages of processing and freezing of buck spermatozoa Nigrosin-Eosin-Geimsa smear

Buck no	Live sperms at the end of stages								
	fresh	I		II		III		IV	
		PI	PII	PI	PII	PI	PII	PI	PII
1	82.250	78.375	76.250	82.000	71.875	67.875	68.375	53.000	54.000
2	77.375	76.500	75.000	70.625	69.250	59.250	62.750	55.875	55.625
3	82.375	69.875	79.875	72.625	73.875	63.125	66.875	56.500	53.750
4	77.375	71.625	76.000	72.375	68.500	65.625	66.000	53.500	51.500
5	84.875	77.500	76.375	74.125	70.875	66.500	65.000	55.250	52.125
Mean	80.850	74.775	76.700	74.35	70.875	64.475	65.8	54.875	53.400
SE	1.494	1.693	0.829	1.992	0.956	0.518	0.942	0.677	0.730
t value		_1.0212		_1.5730		_0.7414		1.4306	

Table 4.4 Percentage abnormalities at the end of different stages of processing and freezing procedures of buck spermatozoa

Buck no	Abnormalities at the end of stages								
	fresh	I		II		III		IV	
		PI	PII	PI	PII	PI	PII	PI	PII
1	2.250	3.375	4.375	3.875	4.625	4.875	5.375	7.875	6.000
2	3.000	3.375	3.625	4.250	4.500	5.500	3.500	5.250	5.375
3	2.875	2.250	3.500	5.625	4.875	5.625	4.875	6.875	6.125
4	3.625	5.750	4.750	5.000	1.125	4.500	5.375	6.250	6.375
5	3.500	4.000	3.875	4.750	5.375	7.000	7.250	9.375	7.625
Mean	3.050	3.750	4.025	4.700	4.700	5.500	5.275	7.125	6.300
SE	0.245	0.574	0.235	0.303	0.208	0.428	0.601	0.706	0.369
t value		_0.4432		0.0000		0.3050		1.0350	

Table 4.5.1 Percentage of intact acrosomes at the end of different stages of processing and freezing of buck spermatozoa Geimsa smear

Buck no	Intact acrosomes at the end of stages								
	fresh	I		II		III		IV	
		PI	PII	PI	PII	PI	PII	PI	PII
1	90.375	83.375	85.250	75.125	75.125	76.250	71.000	70.000	75.250
2	90.750	85.375	85.625	80.250	79.625	75.125	80.625	76.375	83.125
3	89.500	84.875	85.250	82.125	77.000	81.625	74.125	74.250	81.625
4	92.050	90.750	89.375	83.000	84.000	80.625	81.375	81.000	79.500
5	93.000	85.875	88.000	88.625	86.375	84.250	83.750	80.500	79.625
Mean	91.175	86.050	86.700	81.825	80.425	79.575	78.175	76.425	79.825
SE	0.636	1.247	0.843	2.180	2.105	1.703	2.400	2.044	1.327
t value		_0.4318		0.4019		0.4757		_1.3953	

Table 4.5.2 Percentage intact acrosomes at the end of different stages of processing and freezing of buck spermatozoa Nigrosin-Eosin-Geimsa smear

Buck no	Intact acrosomes at the end of stages								
	fresh	I		II		III		IV	
		PI	PII	PI	PII	PI	PII	PI	PII
1	92.125	92.250	88.375	86.375	88.125	81.375	84.125	80.625	83.250
2	91.000	87.875	86.750	86.375	85.375	85.750	84.750	83.000	82.500
3	93.375	92.625	88.750	88.750	87.125	85.375	78.875	78.625	80.000
4	90.750	86.500	87.500	86.500	83.125	82.875	83.125	81.500	79.875
5	89.125	85.375	86.125	81.625	83.250	82.625	82.000	80.500	80.875
Mean	91.275	88.925	87.500	86.025	85.400	83.600	82.575	80.850	81.300
SE	0.710	1.489	0.489	1.168	1.005	0.843	1.036	0.713	0.676
t value		0.9094		0.4032		0.7677		_0.4579	

Table 4.6 Comparison of seminal attributes between various stages (t value)

	I&II		I&III		I&IV		II&III		II&IV		III&IV	
	PI	PII	PI	PII	PI	PII	PI	PII	PI	PII	PI	PII
% Motility	4.26*	6.33*	11.89*	9.92*	40.09*	23.92*	4.55*	6.35*	21.32*	23.84*	20.32*	19.95*
% Livesperm (NE)	4.50*	9.38*	10.78*	27.30*	32.97*	38.14*	9.80*	11.27*	44.01*	29.20*	19.50*	25.28*
% Live sperm (NEG)	0.16	4.60*	4.53*	8.68*	10.94*	21.08*	3.94*	3.78*	9.28*	14.58*	5.80*	10.40*
% Abnorm-alities	1.46	2.15	2.44*	1.94	3.71*	5.18	1.52	0.90	1.15*	3.77*	1.97	1.45
% Intact acrosomes (Geimsa)	1.68	2.76*	3.06*	3.35*	4.01*	4.37*	0.81	0.71	1.80	0.24	1.18	0.60
% Intact acrosome (NEG)	1.53	1.88	3.11*	4.30*	4.90*	7.43*	1.67	1.96	3.75*	3.38*	2.49*	1.03

Table 4.7 Percentage of acrosome abnormalities at the end of different stages of processing and freezing of buck spermatozoa

Buck no	fresh			SI								SII								SIII								SIV							
				PI				PII				PI				PII				PI				PII				PI				PII			
	S	R	L	T	S	R	L	T	S	R	L	T	S	R	L	T	S	R	L	T	S	R	L	T	S	R	L	T	S	R	L	T	S	R	L	T
1	2.87	3.75	2.00	8.82	6.25	6.12	5.37	17.75	4.12	7.25	3.87	15.25	7.37	7.12	8.75	23.75	7.75	9.12	6.50	23.37	7.00	8.50	9.00	24.50	8.75	8.25	9.62	26.62	10.00	12.00	7.00	29.00	9.12	6.37	9.50	23.00
2.	4.37	2.75	2.25	9.37	6.25	5.12	3.87	15.25	5.37	6.75	2.75	14.87	5.87	8.12	5.87	19.87	6.12	9.12	4.75	20.00	6.62	7.25	9.50	23.37	7.00	6.12	6.62	19.75	9.75	6.37	7.75	23.87	7.50	3.60	4.00	15.10
3.	5.12	4.62	0.62	10.37	6.50	5.00	3.50	15.00	6.50	5.62	2.12	14.25	7.00	4.00	6.87	17.87	9.75	6.50	6.75	23.00	7.62	6.62	5.25	19.50	8.62	8.12	6.75	23.50	9.25	8.00	8.25	25.50	7.62	7.25	3.60	18.00
4.	3.75	2.75	1.25	7.75	8.62	2.75	0.87	9.25	4.00	4.00	2.00	10.00	6.75	4.62	5.62	17.00	7.87	4.75	3.62	16.25	7.12	5.87	6.37	19.37	7.50	7.75	3.37	18.62	8.32	5.80	4.75	19.00	8.37	6.62	5.12	20.00
5	3.50	2.62	0.87	7.00	6.37	3.87	1.37	11.62	5.12	4.87	2.00	12.00	4.00	4.37	2.25	11.37	5.62	4.62	3.37	13.62	7.12	5.87	6.37	19.37	7.50	7.75	3.37	18.62	8.32	5.80	4.75	19.00	8.37	6.62	5.12	20.00
Av	3.92	2.62	0.87	7.00	6.20	4.57	3.00	13.77	5.02	5.70	2.56	13.27	6.35	5.66	5.37	17.87	7.42	6.82	5.00	19.25	7.15	6.75	6.72	20.62	7.82	6.92	6.20	20.96	9.40	7.80	6.17	23.37	8.20	5.97	5.65	19.00

S-Swollen acrosome R-Ruffled acrosome L-Lost acrosome T-Total damaged acrosome

Fig. 4.1 Percentage of progressively motile sperms at the end of different stages of processing and freezing of buck spermatozoa

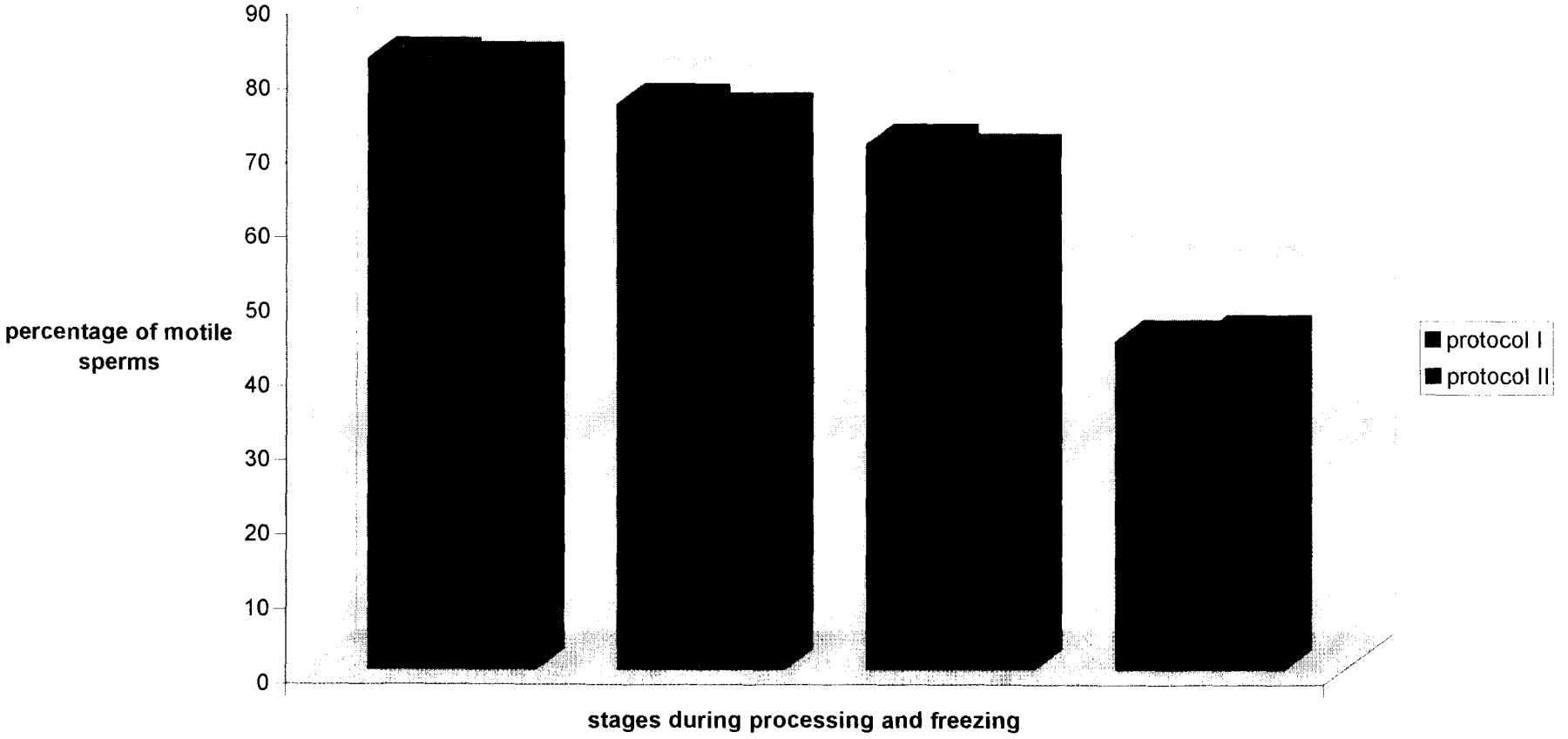


Fig 4.2 Percentage of live sperms at the end of different stages of processing and freezing of buck spermatozoa Nigrosin-Eosin staining

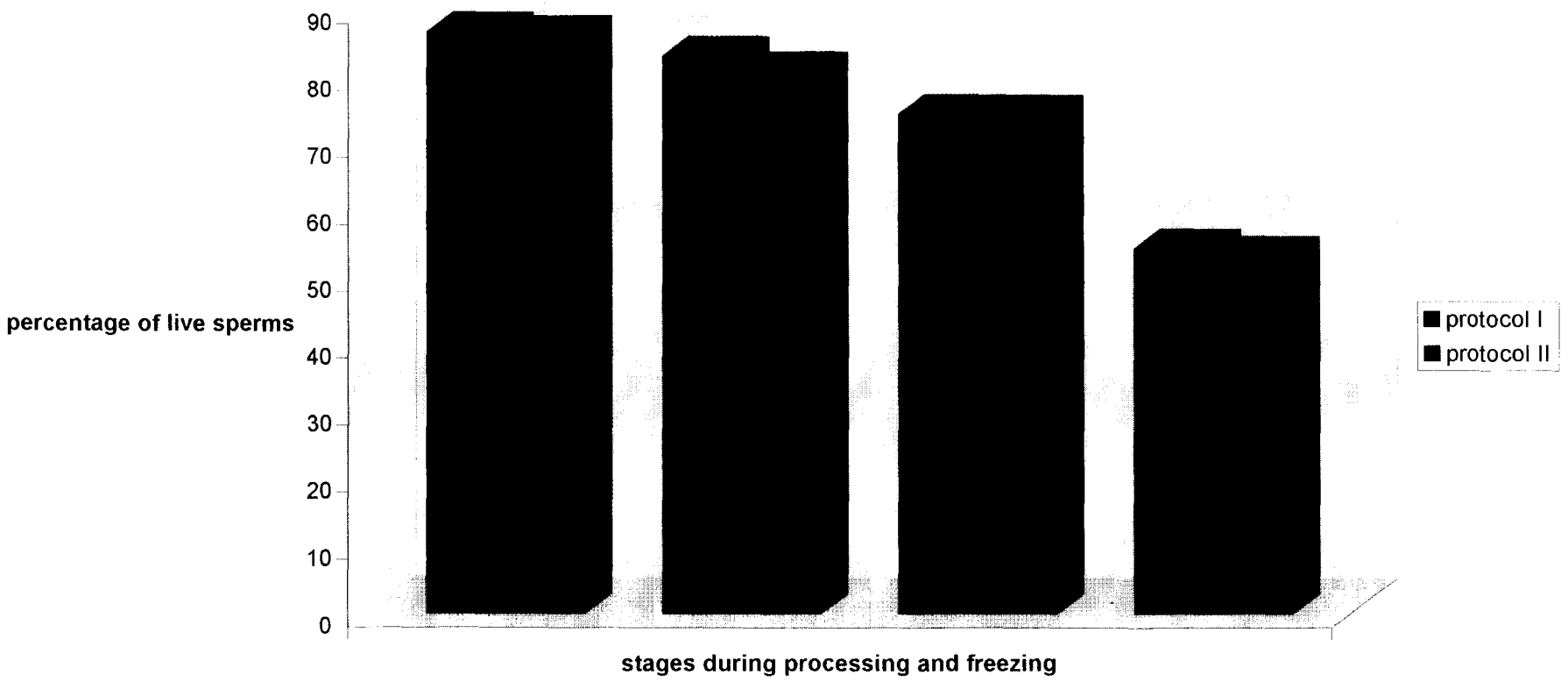


Fig 4.3 Percentage of live sperms at the end of different stages of processing and freezing of buck spermatozoa Nigrosin-Eosin-Geimsa staining

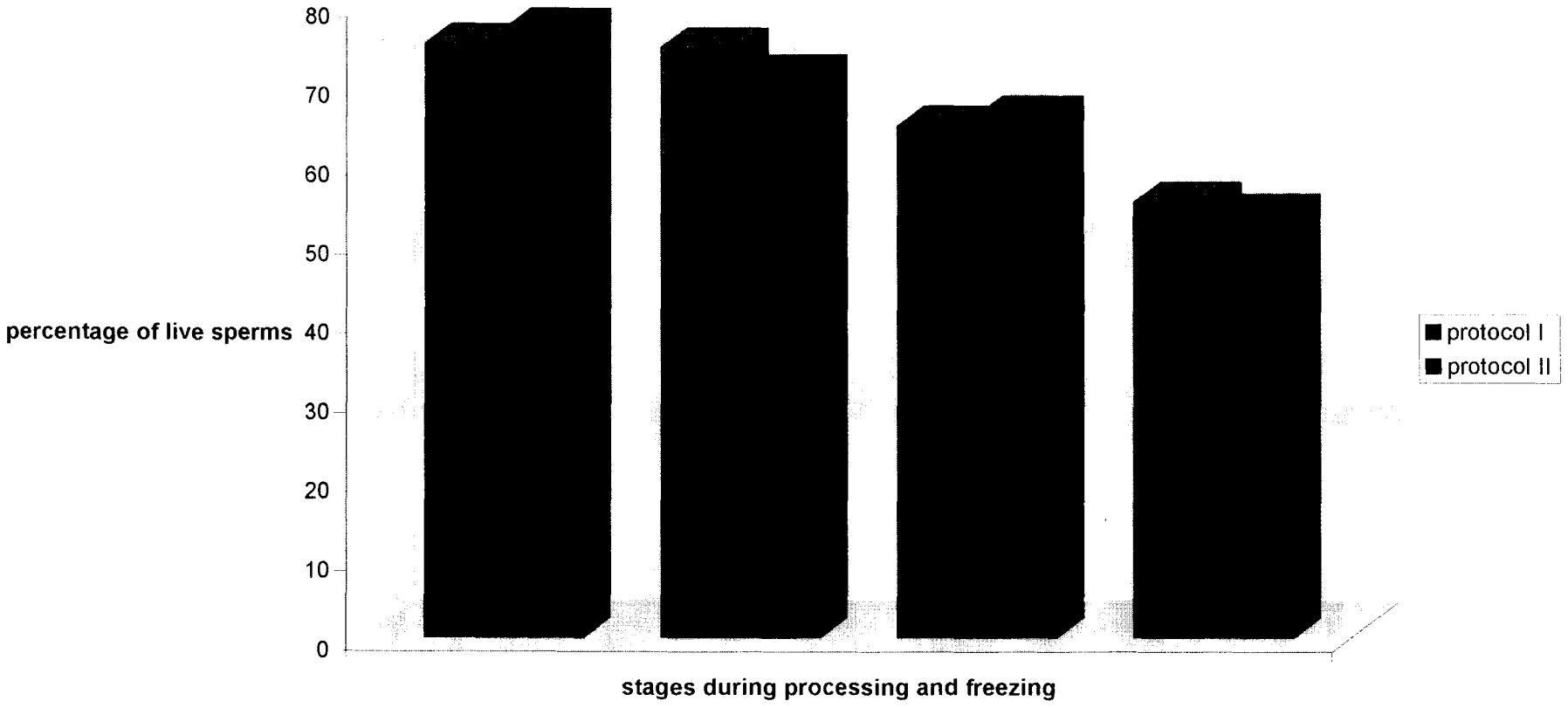


Fig 4.4 Percentage of sperm abnormalities at the end of different stages of processing and freezing of buck spermatozoa Nigrosin-Eosin staining

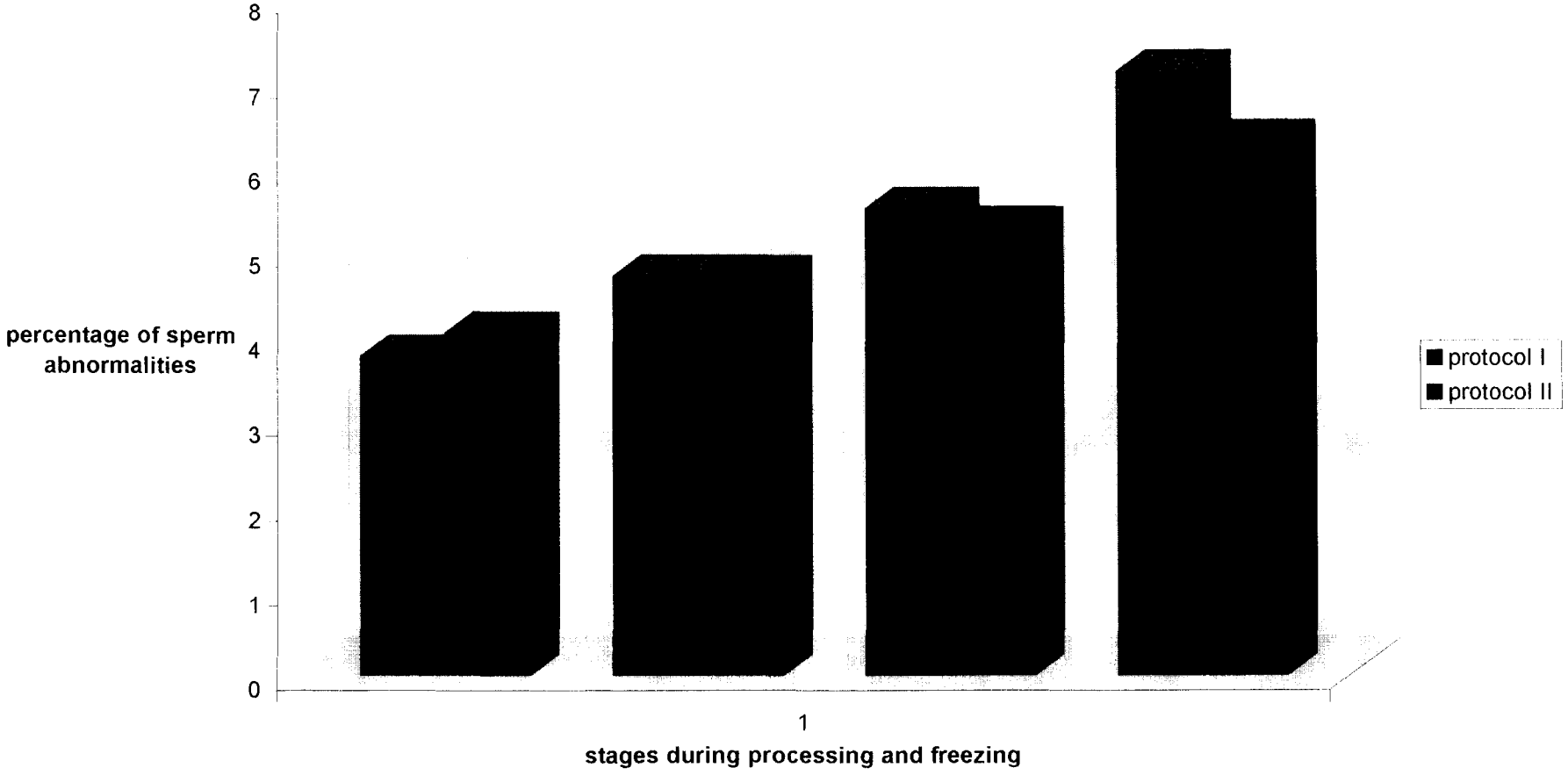


Fig 4.5 Percentage of intact acrosomes at the end of different stages of processing and freezing of buck spermatozoa Geimsa staining

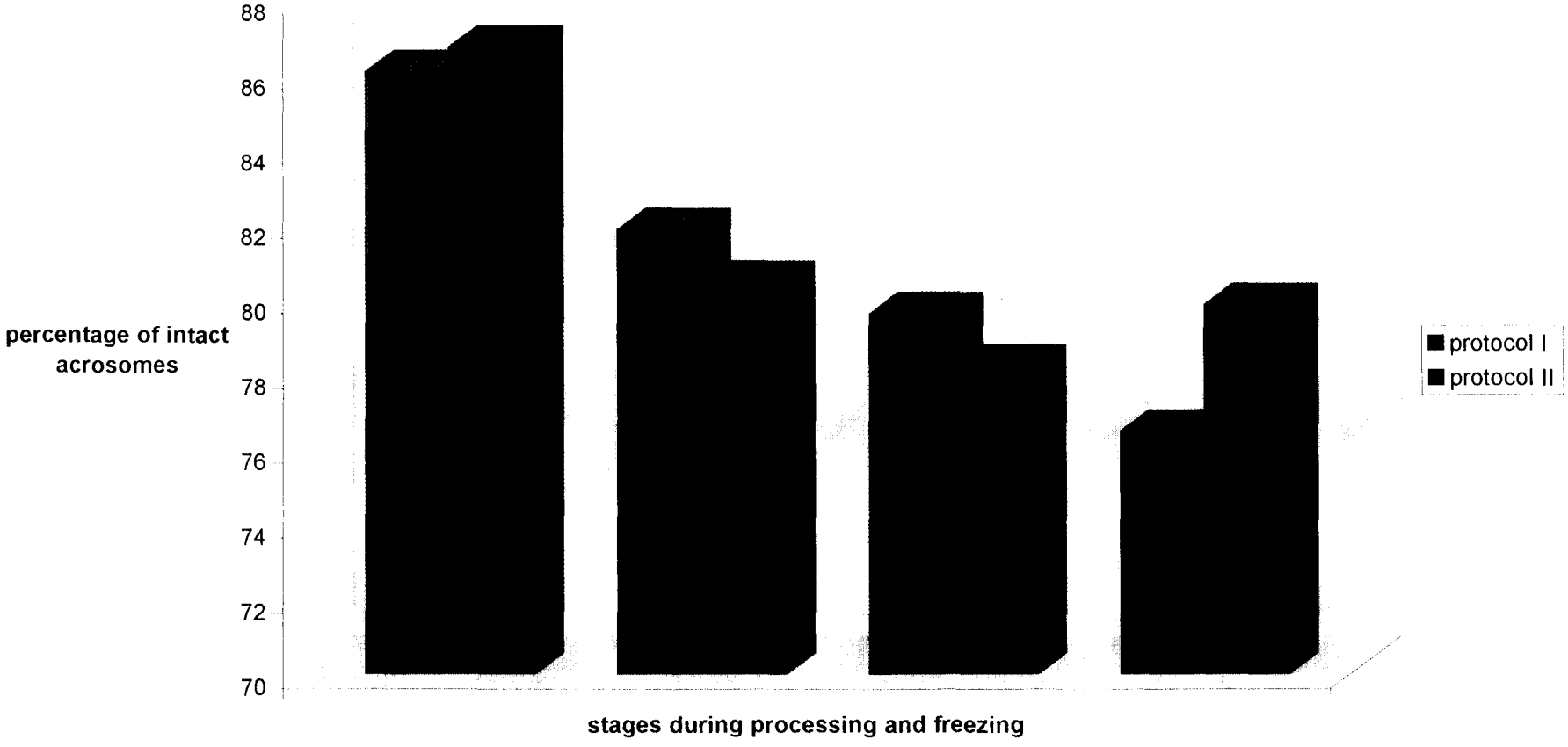


Fig 4.6 Percentage of intact acrosomes at the end of different stages of processing and freezing of buck spermatozoa Nigrosin-Eosin-Geimsa staining

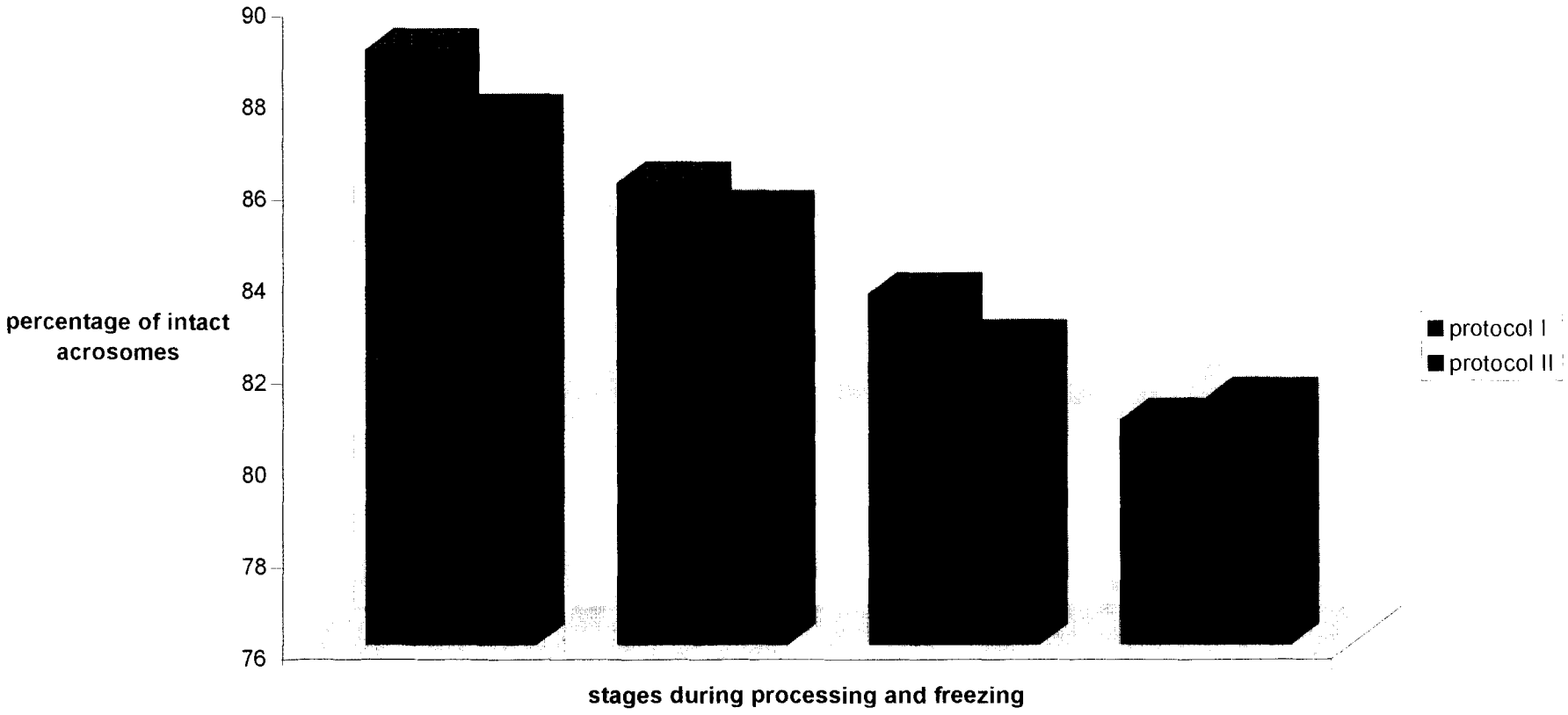


Fig 4.7 Percentage of different acrosomal abnormalities at the end of different stages of processing and freezing of buck spermatozoa Geimsa staining

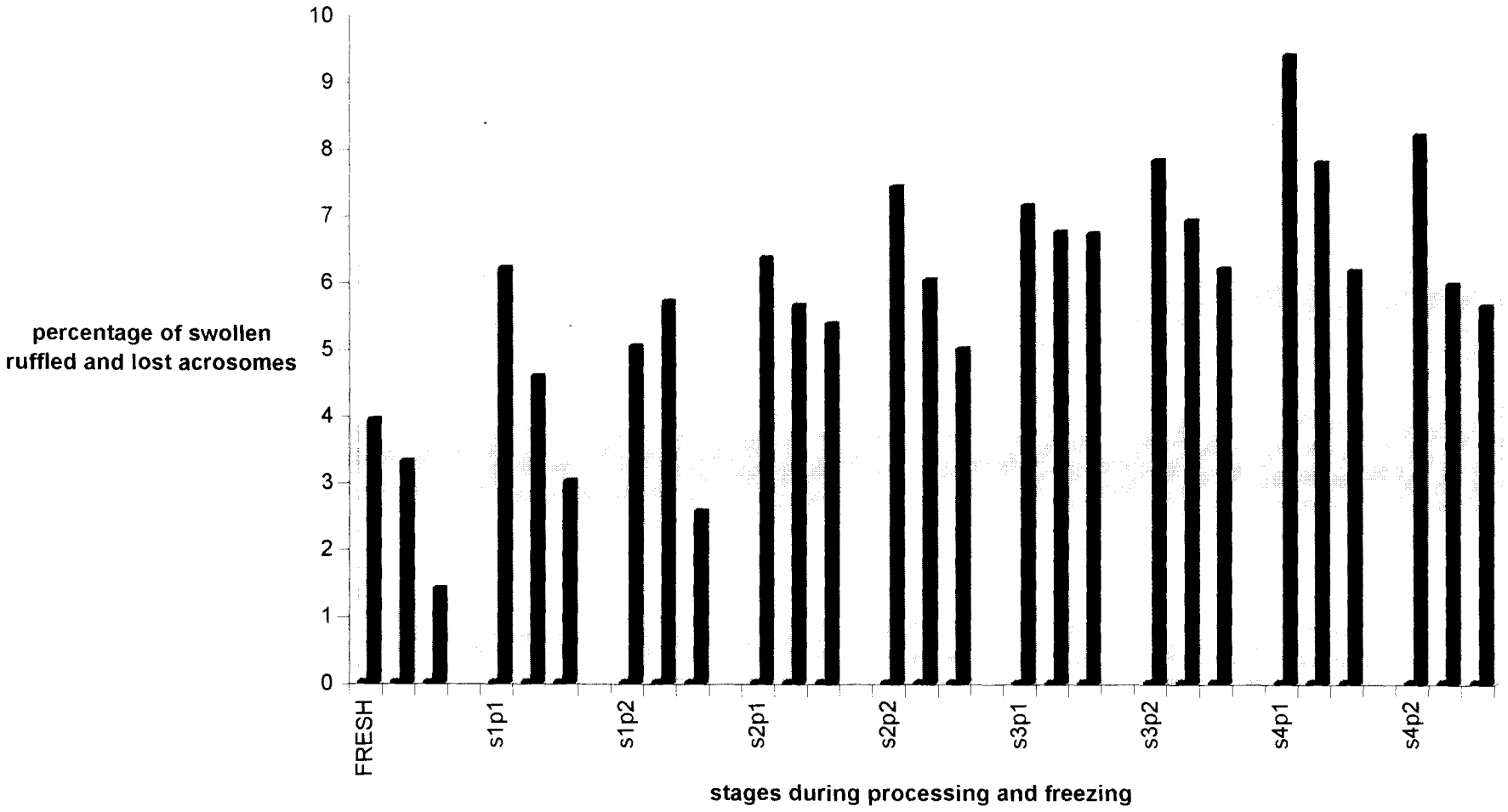
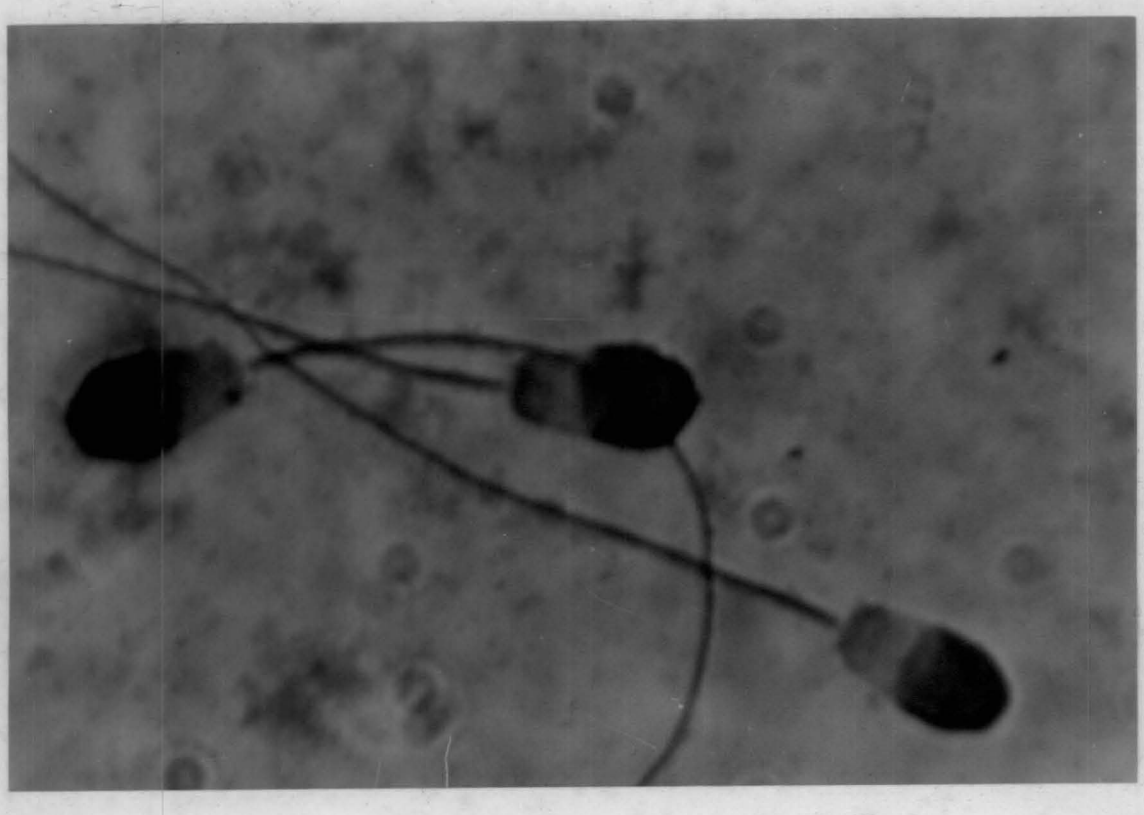


FIG 4.8 Buck spermatozoa showing a normal acrosome - Geimsa staining

FIG 4.9 Lost acrosome Geimsa staining - taken at the end of processing and freezing



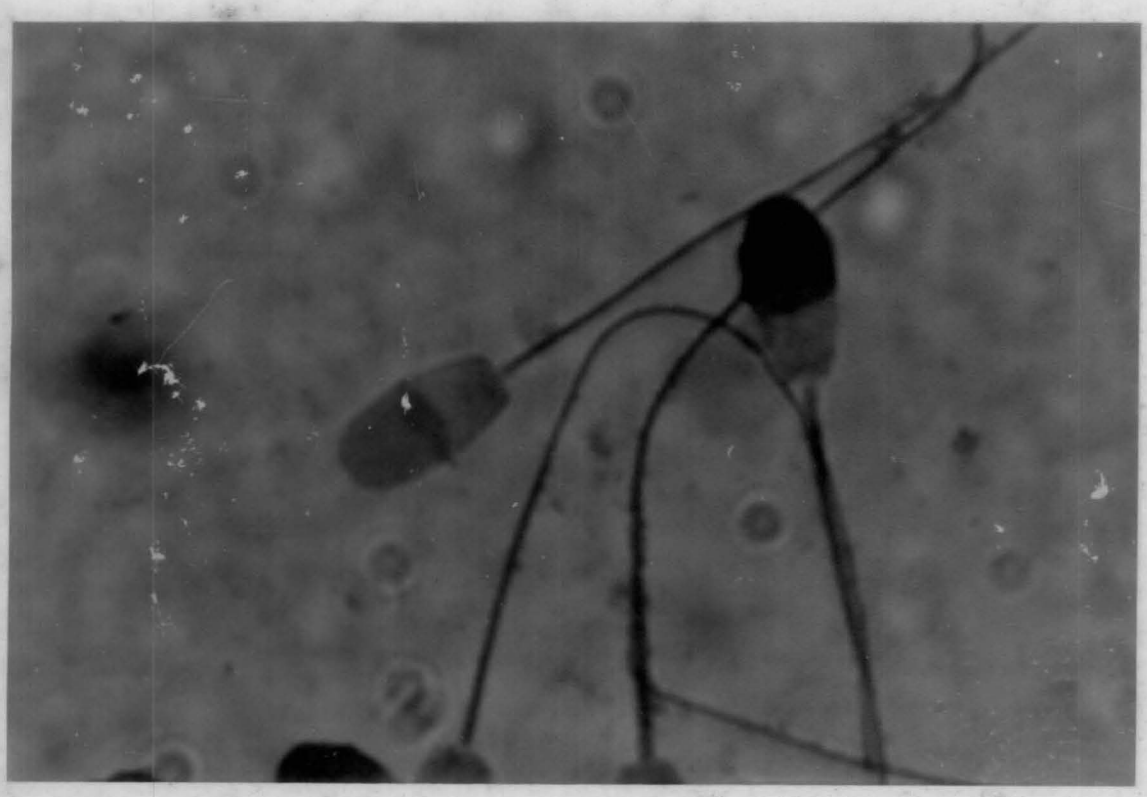
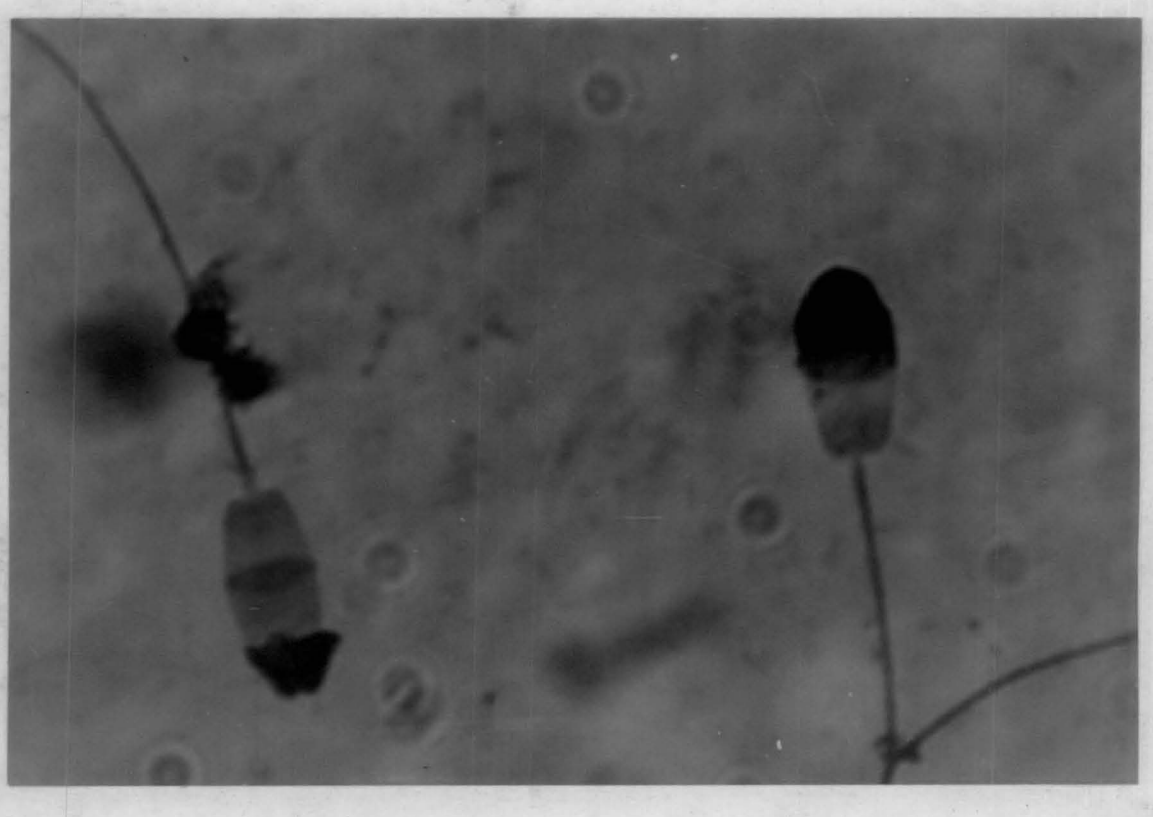


FIG. 4.10 Separating acrosomal membrane -fresh semen smear—Geimsa staining

FIG 4.11 A Swollen acrosome- frozen semen smear—Geimsa staining



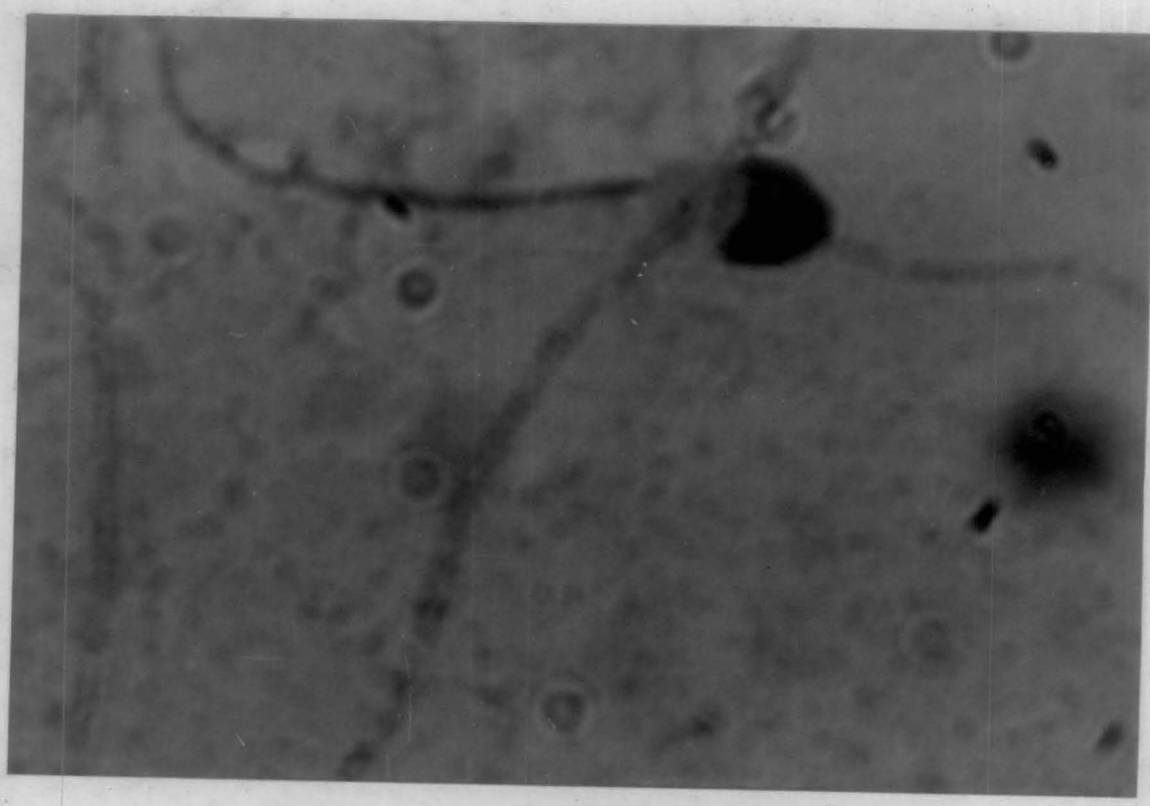
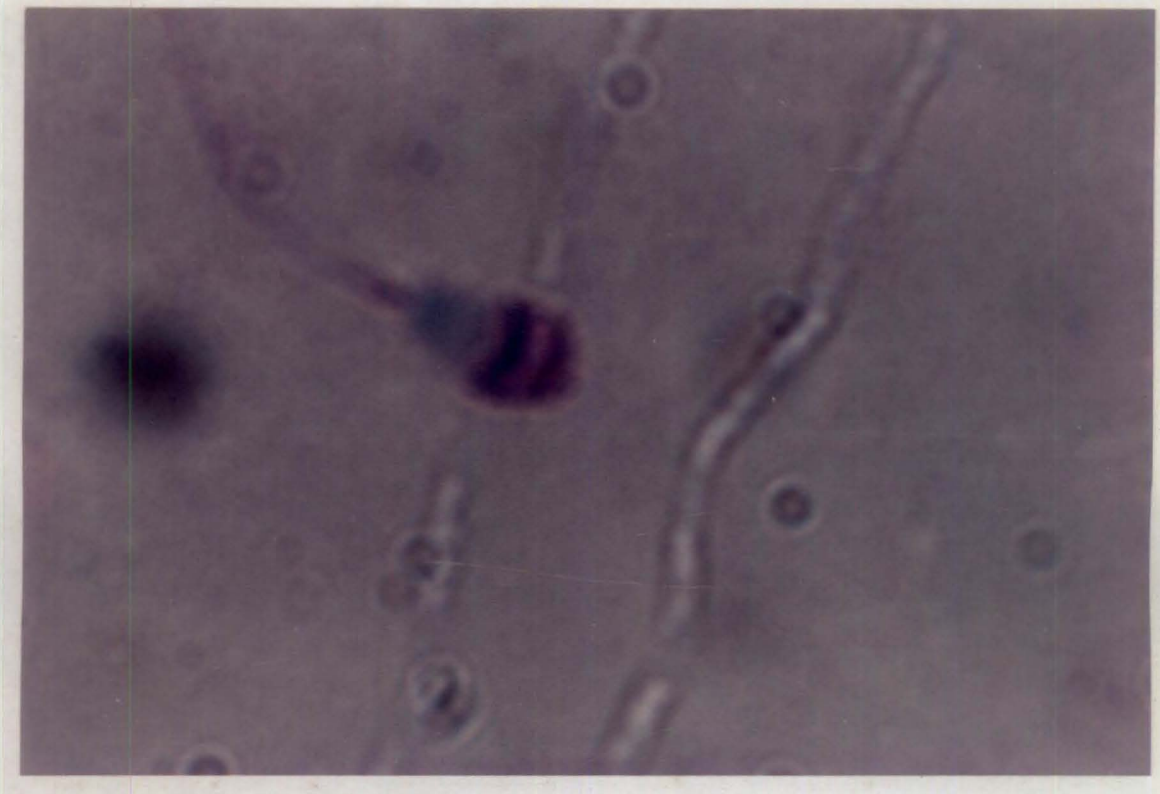


FIG 4.12 Ruffled acrosome at the end of stage III (after equilibration)—Geimsa staining

FIG 4.13 Live intact acrosome in fresh semen smear NEG staining



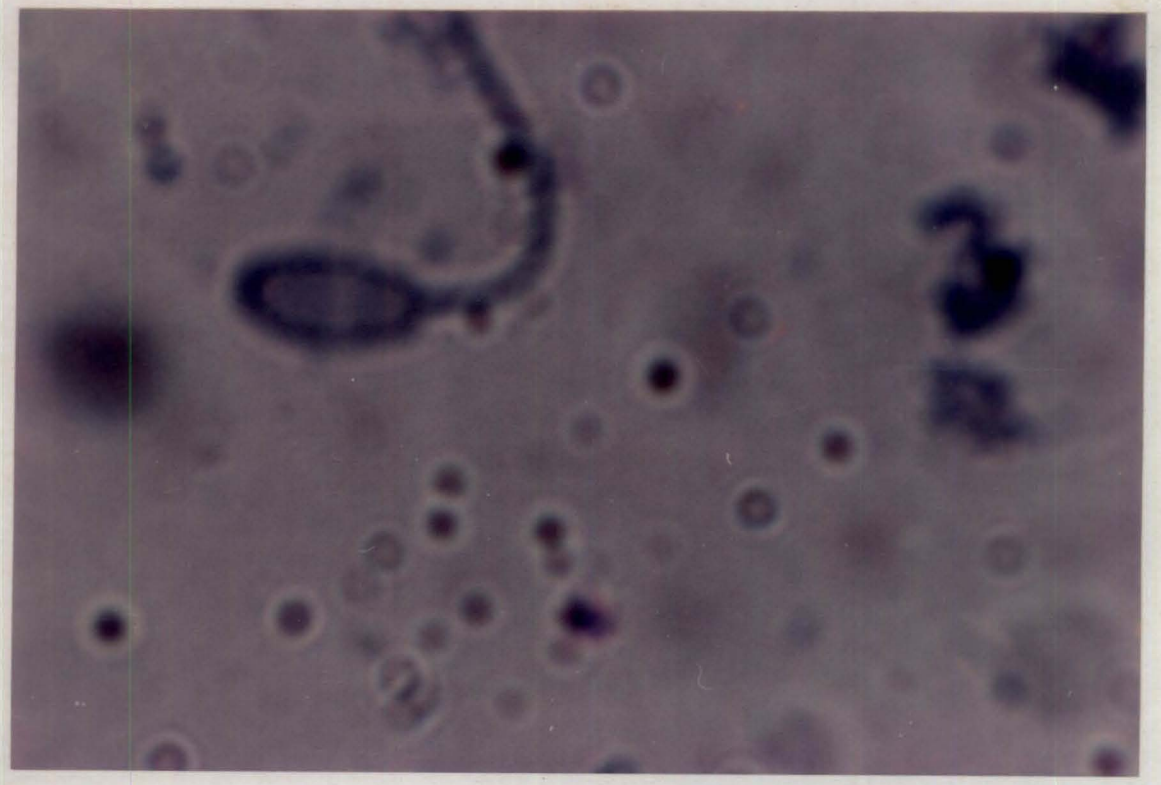
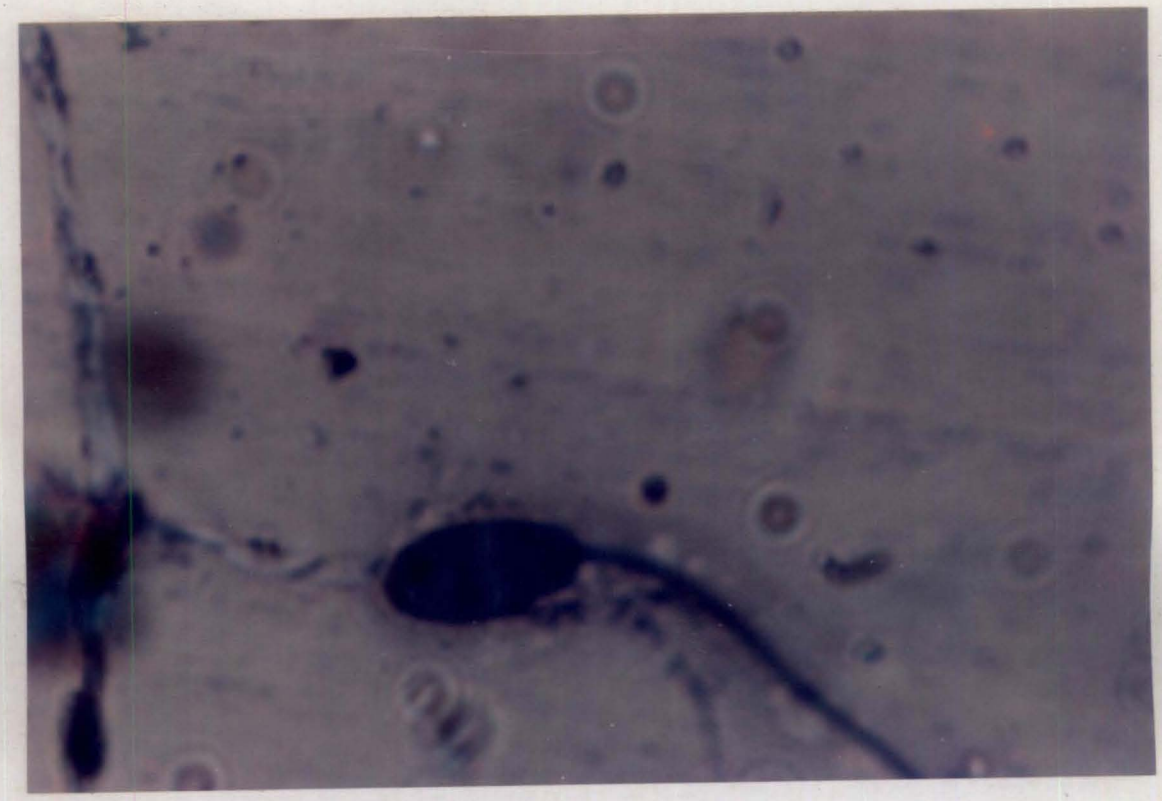
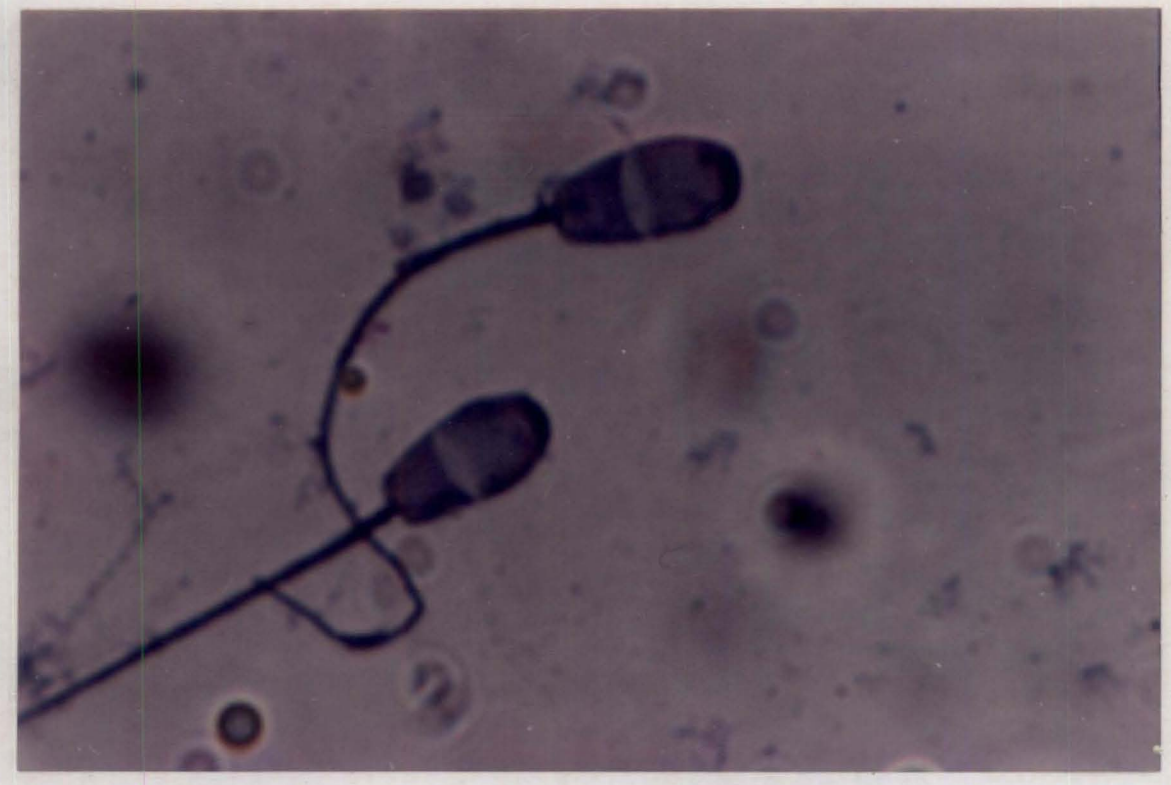


FIG 4.14 Dead intact acrosome - NEG staining

FIG 4.15 Dead damaged acrosome – NEG staining





Discussion

DISCUSSION

Six pooled semen samples (two ejaculates) of good quality from five Malabari cross bred bucks were frozen in two different protocols in order to evaluate the effect of processing and freezing procedures on the acrosome morphology of buck spermatozoa.

5.1 Semen evaluation

The mean semen volume was found to be 1.328 ± 0.067 ml which is in full agreement with the earlier reported values. (Knoblauch, 1962 ; Kurian and Raja, 1965 ; Igboeli, 1974 ; Cetinkaya *et al.*, 1980 ; Singh *et al.*, 1982 ; Sevinc *et al.*, 1985 ; Pandey *et al.*, 1985 ; Ali and Mustafa, 1986 ; Bakshi *et al.*, 1987 ; Mahmood *et al.*, 1988 ; Pattnaik *et al.*, 1991). The colour of all semen samples was found to be creamy as was reported earlier. (Knoblauch, 1962 ; Patil, 1970 ; Igboeli, 1974 ; Mann, 1980 ; Pandey *et al.*, 1985 ; Ali and Mustafa, 1986 and Prasanth, 1995) The overall density of buck semen was DDDD which is in conformity with the earlier observations. (Knoblauch, 1962 ; Sarmah, 1983 ; Joseph, 1983 and Prasanth, 1995) The mean mass activity of buck semen sample was ++++, which is in full agreement with the earlier reports. (Patil, 1970 ; Sarmah, 1983 ; Mahmood *et al.*, 1988 ; and Prasanth, 1995) The average pH was found to be 7.275 ± 0.040 which is comparable to the values

reported (Mikus and Pilko, 1975 ; Mann, 1980 ; Mohan *et al.*, 1980 and Prasanth, 1995). The mean sperm concentration of buck semen was 2972 ± 293 millions per ml which is comparable with the earlier reported values.(Kurian and Raja, 1965 ; Patil, 1970 ; Mann, 1980 ; Mohan *et al.*, 1980 ; Mahmood *et al.*, 1988 and Prasanth, 1995). The mean volume, pH and sperm concentration were found to have no significant variation between bucks.

5.2 Sperm motility

The progressive sperm motility of the samples was evaluated after washing and initial extension in Tris diluent and thereafter at the end of four stages of processing and freezing. The initial sperm motility of 82.000 ± 0.606 observed at the end of washing and initial extension in protocol I was found to drop to 44.000 ± 0.790 per cent post thaw motility at the end of freezing and thawing. There was significant drop in the motility of sperms between the four stages of processing and freezing under protocol I. Similarly the percentage of progressively motile sperms registered a fall from 81.375 ± 1.089 to 44.750 ± 1.075 at the end of stage IV in protocol II indicating a significant drop in motility during various stages of processing and freezing. However, there was no significant difference between corresponding stages of the two protocols. It may be concluded that both the protocols are comparable as far as the post thaw motility is concerned. The maximum drop in the motility was registered during the freezing of

semen due to freezing injury. The differences in the procedure for washing (single or double washing), percentage of glycerol (six and seven per cent), equilibration time (three and four hours), freezing time (eight minutes and 10 minutes) and thawing temperature (25^0 C for 30 seconds and 60^0 C for 10 seconds) did not significantly influence the post thaw motility. Since comparable post thaw motility was recorded with single washing and centrifugation with 50 per cent more of Tris buffer it is felt that this can be adopted as a routine measure.

5.3 Live sperms

The average live sperm percentage of 90.025 $\pm$ 0.801 of fresh semen by Nigrosin - Eosin (NE) staining was found to decrease to 54.250 $\pm$ 0.593 at the end of freezing and thawing in protocol I. The decrease in the percentage of live sperms between the four stages of processing and freezing was significant. Similarly in protocol II the mean percentage of live sperms showed a fall from 90.025 $\pm$ 0.801 in fresh semen to 53.125 $\pm$ 0.793 at the end of stage IV with the same staining method. It was also observed that there was no significant difference between corresponding stages of the two protocols. By the NEG staining the mean live sperm percentage registered a significant reduction from initial live sperm percentage of 80.850 $\pm$ 1.494 to 54.875 $\pm$ 0.677 in protocol I as against 53.400 $\pm$ 0.730 in protocol II. In this staining method also there was no significant difference between corresponding stages of the two protocols.

On the other hand, there was significant difference in the mean sperm percentage of live sperm by the two different staining methods at the end of all the stages in both the protocols. ($p<0.05$).

Though it has been reported that the live and dead sperms can be evaluated by NE staining and also by NEG staining methods, the former staining is considered to be more reliable and accurate based on the observations given under. In the NE staining, eosin, a vital stain penetrates the plasma membrane of dead sperms and stains the nucleus red, while the live sperms stay white. On the other hand, in the NEG staining the dead sperms is reported to take blue colour and live sperms colourless. However, the live sperms seems to take light blue colour which makes differentiation of dead and live sperms difficult. This might have been responsible for a proportionally higher percentage of dead sperms recorded in all the four stages in both the protocols by NEG staining. The percentage of live sperm obtained by NE staining is comparable to the percentage of motile sperms recorded in the different stages of processing and freezing. It is therefore concluded that NE staining technique is more reliable than NEG staining for evaluation of live and dead sperms.

5.4 Sperm abnormalities

The mean percentage of abnormal sperms in the fresh semen by the Nigrosin-Eosin staining was 3.050 ± 0.245. The percentage of abnormal

sperms of 3.750 ± 0.570 at the end of washing and initial extension was found to increase progressively to 7.125 ± 0.706 at the end of freezing and thawing by protocol I. Similarly, in protocol II the abnormal sperms was found to increase from 4.025 ± 0.230 to 6.300 ± 0.369. There was no significant difference in the percentage of sperm abnormalities between stages I, II and III. However, stage IV differed significantly from the first two stages in both the protocols.

It can be concluded that there is no significant increase in the percentage of abnormal sperms during washing and initial dilution, cooling, glycerolisation and equilibration. However, there was significant increase in the abnormal sperm percentage during freezing.

5.5 Acrosome abnormalities

The mean percentage of damaged acrosomes was evaluated at the end of different stages of processing and freezing by two protocols with the Geimsa staining. The percentage of swollen ruffled and entirely lost acrosomes registered an increase from 8.825 in fresh semen to 23.375 after the end of stage IV in protocol I and 19.825 in protocol II with the Geimsa smear. There was significant difference between stage I and III and I and IV in protocol I as against the observance of significant difference between stage I and II, I and III and I and IV in protocol II with the same staining method. However, there was no significant difference in the mean percentage of damaged acrosomes between corresponding

stages of the two protocols. It was also observed that there was no significant difference in the percentage of damaged acrosomes by the two staining methods at the end of each stage in both the protocols. There was also no significant difference in the proportion of different acrosomal abnormalities between stages within protocols I and II. The results obtained with NEG staining was comparable with that of Geimsa staining. All the three acrosomal abnormalities were found to register an increase during the processing and freezing, more so during glycerolisation and equilibration and freezing in both the protocols.

Summary

SUMMARY

Six pooled semen samples (two ejaculates) of good quality from five Malabari crossbred bucks were processed and frozen in two different protocols in order to evaluate the acrosome morphology of buck spermatozoa.

After preliminary evaluation, the semen samples confirming to good quality were processed under two protocols. In protocol I, the samples were diluted 10 fold before centrifuging twice and the final pellet was re-suspended in the non glycerolated fraction of tris yolk diluent. The sample was then chilled, glycerolated (six per cent), equilibrated (four hours), frozen in liquid nitrogen vapour (eight minutes), and thawed (25^0 C for 30 seconds). In protocol II, the semen samples were diluted 15 fold and centrifuged once and the pellet re-suspended in Tris yolk diluent, cooled to 5^0 C, glycerolated (seven per cent), equilibrated (three hours), frozen (10 minutes) and thawed (60^0 C for 10 seconds). The semen characters such as motility, live sperm, sperm abnormalities and acrosome abnormalities were evaluated at the end of washing and initial extension (stage I), cooling to 5^0 C (stage II), glycerolisation and equilibration (stage III) and freezing and thawing (stage IV). The results were compiled to

evaluate the effect of different processing and freezing procedures on the semen characters in general and acrosome morphology in particular.

The semen sample used in the study had a mean volume of 1.328 ± 0.067 ml, creamy in colour, DDDD density, ++++ mass activity, pH of 7.275 ± 0.040 and a concentration of 2972 ± 293 millions per ml. No significant difference in the above semen characters was found between bucks.

The initial sperm motility of 82.000 ± 0.606 was found to drop to 44.000 ± 0.790 at the end of stage I, II, III and IV respectively in protocol I. Similarly in protocol II the initial motility dropped from 81.375 ± 1.089 to 44.750 ± 1.075 at the end of stage IV. There was significant drop in motility between stages in both the protocols signifying the adverse effect of processing and freezing on sperm motility. However, there was no significant difference between corresponding stages of the two protocols indicating that both the protocols are equally good in obtaining good post thaw motility of semen. However, the second protocol is preferred over first as it eliminates the second washing and centrifugation.

The mean live sperm percentage of fresh semen in NE and NEG smears were 90.050 ± 0.801 and 80.850 ± 1.494 respectively. The corresponding figures at the end of stage I, II, III and IV were 86.750 ± 0.788, 83.000 ± 0.724, 74.325 ± 0.842 and 54.250 ± 0.593 respectively in the NE smear as against 74.775 ± 1.693, 74.350 ± 1.992, 64.475 ± 0.518 and 54.875 ±

0.677 respectively in NEG smear in protocol I . Similarly in protocol II, the mean percentage of live sperms at the end of stages I, II, III and IV were 86.500 ± 0.369, 80.625 ± 0.506, 74.225 ± 0.257 and 53.125 ± 0.793 respectively in the NE smear and 76.700 ± 0.829, 70.875 ± 0.956, 65.800 ± 0.942 and 53.400 ± 0.730 respectively in the NEG smear. There was no significant difference in the live sperm percentage between the corresponding stages of protocol I and II with both the staining techniques. On the other hand, significant difference in the live sperm percentage between stages within protocol I and II and also between two different staining methods at the end of all the stages in both the protocols. The percentage of live sperms obtained by NE staining was comparable to the percentage of motile sperms recorded in all four stages of both the protocols. In contrast a proportionately higher percentage of dead sperm was obtained with NEG staining. This could be attributed to the fact that even live sperms took a light bluish hue with this staining which made differentiation of live and dead sperms difficult and unreliable. The mean percentage of abnormal sperms in the fresh semen was 3.050 ± 0.245. The percentage of abnormal sperms were 3.750 ± 0.57, 4.700 ± 0.303, 5.500 ± 0.428 and 7.125 ± 0.706 respectively at the end of stages I, II, III and IV of processing and freezing by protocol I and 4.025 ± 0.235, 4.700 ± 0.208, 5.275 ± 0.601 and 6.300 ± 0.369 respectively in protocol II. There was no significant difference in the percentage of sperm

abnormalities between stages I, II and III. However, stage IV differed significantly from the first two stages in both the protocols. It can be concluded that significant increase in the abnormal sperm percentage occurred only during the freezing process.

The average percentage of intact acrosomes in fresh semen stained with Geimsa and NEG smears were 91.175 ± 0.636 and 91.275 ± 0.710 respectively. The corresponding figures at the end of stages I, II, III and IV were 86.050 ± 1.247, 81.825 ± 2.180, 79.575 ± 1.703 and 76.450 ± 2.044 respectively in Geimsa smear and 88.925 ± 1.489, 86.025 ± 1.168, 83.600 ± 0.843 and 80.850 ± 0.713 respectively in NEG smear in protocol I. Similarly in protocol II, the percentage of intact acrosomes at the end of stage I, II, III and IV were 86.700 ± 0.843, 80.425 ± 2.105, 78.175 ± 2.400 and 79.825 ± 1.327 respectively with Geimsa staining as against 87.500 ± 0.489, 85.400 ± 1.005, 82.575 ± 1.036 and 81.300 ± 0.676 with NEG staining in protocol II. There was no significant difference in the percent intact acrosomes between corresponding stages of the two protocols with Geimsa and NEG staining as well as between the two staining methods at the end of each stage in both the protocols. On the other hand, there was significant difference between stage I and III and I and IV in protocol I and stage I and II, I and III and I and IV in protocol II with the same staining method. However, with NEG staining significant difference in the intact acrosome was observed between stages I and III, I and IV and III

and IV in protocol I and between I and III, I and IV and II and IV in protocol II.

The percentage of swollen, ruffled and entirely lost acrosome in fresh semen were 3.925, 3.300 and 1.600 with a total of 8.825. The corresponding figures were 6.200, 4.575 and 3.000 at the end of stage I, 6.350, 5.650 and 5.875 at the end of stage II, 7.150, 6.750 and 6.725 at the end of stage III and 9.400, 7.800 and 6.175 at the end of stage IV in protocol I. Similarly in protocol II, the mean percentages of swollen, ruffled and entirely lost acrosome were 5.025, 5.700 and 5.550 at the end of stage I, 7.425, 6.825 and 5.000 at the end of stage II, 7.825, 6.925 and 6.200 at the end of stage III and 8.200, 5.975 and 5.625 at the end of stage IV. The percentage of total damaged acrosome after the end of stage i, II, III and IV were 13.775, 17.875, 20.625 and 23.375 respectively in protocol I as against 13.775, 19.250, 20.625 and 19.825 respectively in protocol II. Significant difference was observed between stages I and III and I and IV in protocol I as against the observance of significant difference between stage I and II, I and III and I and IV in protocol II with the same staining method. There was no significant difference in the percentage of damaged acrosome between corresponding stages of the two protocols as well as the proportion of different acrosomal abnormalities between stages within protocols I and II. Comparable result was obtained with NEG staining with that of Geimsa. The acrosomal

abnormalities of swollen, ruffled and entirely lost acrosomes were found to increase during processing and freezing, more so during glycerolisation, equilibration and freezing in both the protocols.

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EFFECT OF PROCESSING AND FREEZING PROCEDURES ON THE ACROSOME MORPHOLOGY OF BUCK SPERMATOZOA

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ABSTRACT

Six pooled semen samples (two ejaculates) of good quality from five Malabari crossbred bucks were processed and frozen in two different protocols to evaluate the effect of processing and freezing procedures on the acrosome morphology of buck spermatozoa.

In protocol I, the samples were diluted 10 fold in Tris buffer before centrifuging twice and the final pellet was re-suspended in the non glycerolated fraction of Tris yolk diluent. The sample was glycerolated (six per cent), equilibrated (four hours), frozen (eight minutes), and thawed (25^0 C for 30 seconds). In protocol II, centrifugation was done only once, after 15 fold dilution in Tris buffer. The re suspended pellet was glycerolated (seven per cent), equilibrated (three hours), frozen (10 minutes) and thawed (60^0 C for 10 seconds). The semen characters such as motility, live sperm, sperm abnormalities and acrosome abnormalities were evaluated at the end of washing and initial extension (stage I), cooling to 5^0 C (stage II), glycerolisation and equilibration (stage III) and freezing and thawing (stage IV). The results were compiled to evaluate the effect of different processing and freezing procedures on the semen characters in general and acrosome morphology in particular.

The semen sample used for split sample dilution had a mean volume of 1.328 ± 0.067 ml, creamy in colour, DDDD density, ++++ mass activity, pH of 7.275 ± 0.040 and a concentration of 2972 ± 293 millions per ml. No significant difference in the above semen characters were found between bucks.

The initial sperm motility of 82.000 ± 0.606 was found to drop significantly during processing and freezing and the final post thaw motility obtained was 44.000 ± 0.790 in protocol I. Similarly in protocol II the initial motility dropped from 81.375 ± 1.089 to 44.750 ± 1.075 at the end of stage IV. Even though there was significant drop in motility between stages in both the protocols, there was no significant difference in the corresponding stages of the two protocols. It could be inferred that good post thaw motility was obtained in both the protocols. The fact that a single washing and centrifugation was only adopted in protocol II makes it a more acceptable procedure for buck semen freezing.

The mean live sperm percentage of fresh semen was evaluated using both NE and NEG staining technique. The percentage of live sperms of 90.050 ± 0.801 was found to decrease to 54.250 ± 0.593 after freezing and thawing in protocol by NE staining. Similarly in protocol II, the mean percentage of live sperms was found to reduce to 53.125 ± 0.793 with the same staining. Even though there was significant difference in the live sperm percentage between stages within protocol I and II no significant

difference in the live sperm percentage between the corresponding stages of protocol I and II . With NEG staining the initial live sperm percentage of 80.850 ± 1.494 was found to drop to 54.875 ± 0.677 in protocol I as against 53.400 ± 0.730 in protocol II. While there was significant difference in the live sperm percentage between stages within protocol I and II there was no variation between corresponding stages of the two protocols. A significantly lower percentage of live sperms was recorded with NEG staining when compared with NE staining probably on account of the fact that the differentiation of live and dead sperm was difficult in the former staining method as live sperms were stained light blue instead of colourless.

The mean percentage of abnormal sperms of 3.050 ± 0.245 in fresh semen did not register any significant increase during processing. However, there was significant increase in the percentage of sperm abnormalities during freezing and thawing with the final abnormality percentage of 7.125 ± 0.706 in protocol I and 6.300 ± 0.36 in protocol II.

The initial acrosomal abnormality of 8.825 in the fresh semen steadily rose to 23.375 in protocol I as against 19.825 in protocol II at the end of stage IV. There was no significant difference in the percentage of various acrosomal abnormalities between corresponding stages of the two protocols. However, there was significant increase in the acrosomal abnormalities during glycerolisation, equilibration, freezing and thawing

under both the protocols. It was concluded that the processing and freezing under two different protocols did not significantly alter the post thaw motility, percentage abnormal and dead sperms and acrosomal abnormalities. A good post thaw motility and low acrosomal abnormality was obtained with a single washing of buck semen with 15 fold Tris buffer which was comparable with double washing with 10 fold Tris buffer.