ASSESSMENT OF BACTERIAL LOAD IN CHILLED AND FROZEN BUCK SEMEN

By
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THESIS

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DECLARATION

I hereby declare that this thesis entitled **"ASSESSMENT OF BACTERIAL LOAD IN CHILLED AND FROZEN BUCK SEMEN"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"ASSESSMENT OF BACTERIAL LOAD IN CHILLED AND FROZEN BUCK SEMEN"** is a record of research work done independently by Smt. Liz Simon, 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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Introduction

1. INTRODUCTION

Goat rearing is one of the important agricultural enterprises of rural Kerala by providing considerable source of income and occupation for the rural farmers. As a result, there is steady increase of goat population in Kerala which registered an increase of 17.71 per cent between 1986 and 1996, and reached 18,60,501 in 1996 (Livestock Census, 1996).

Since maintenance cost of breeding bucks are high and does are spread over a scattered population, difficulty is being experienced in breeding does. It is in this context that Artificial Insemination of goats has gained momentum in recent years. However, one of the important drawbacks of Artificial Insemination is that if semen is not free from microbial agents, it can act as a source of infection to a large number of female population.

Semen can be contaminated from within the body by microorganisms from the testis, accessory organs, tissue fluids, urethra, preputial cavity and preputial orifice. Once collected, there can be contamination from the atmosphere, the teaser, the extender and the equipment. The rich nutritional status and the presence of cryoprotectants in extenders help in the perfect preservation of the microbial viability. The bacterial cell, because of its diploid nature, seems to be more resistant to freezing than haploid spermatozoa. The conventional antibiotics used in semen extenders are becoming resistant and found less effective in controlling the microbial growth. Moreover by artificial insemination the natural

defence mechanism of the vaginal and cervical mucus against invading micro organisms are normally by-passed. Thus the likelihood of infecting the female is increased.

In view of all these it has become necessary to fix norms for bacterial load for the processed buck semen by way of fixing standards. Though, extensive work has been done on bacterial load of bovine semen, perusal of literature shows paucity of major works on bacterial load of buck semen and the factors that regulate them. In fact no work has been taken up in Kerala in this regard. Since Kerala is now planning for popularisation of Artificial Insemination programme in goats there is an urgent need for the study of bacterial load of buck semen and factors controlling it. With the above objective, the present study is aimed:

1. To assess the effect of processing and preservation on the bacterial load of buck semen.

2. To identify the steps that increase the bacterial contamination during processing of buck semen.

3. To assess the effect of bacterial load with the semen quality.

Review of Literature

2. REVIEW OF LITERATURE

Semen has a great potential for the spread of infectious diseases as evidenced by the presence of pathogenic microorganisms found in semen. Apart from pathogenic organisms, semen gets contaminated by non-specific microorganisms during collection, processing and preservation (Hare, 1985).

Informations on the bacterial load in fresh and frozen buck semen appears to be scanty.

2.1 Microbial load in semen

2.1.1 Standards of microbial load

The Indian standard Institute (1976) recommended that the frozen semen should be free from pathogenic bacteria and the number of saprophyptic bacteria per dose of bull semen should be below 500.

Willis (1978) also recommended that the maximum number of bacterial organisms present in frozen bull semen should be limited to 500 per dose.

Though the limit of 500 organisms per dose of frozen semen was accepted by the International Organisation of Biological Standards, the number of organisms per millilitre has not been accepted (Raghavan *et al.*, 1982).

2.1.2 Bacterial load in fresh semen

The average bacterial load of thirty three samples of neat semen from three breeds of bucks were 4477 organisms per millitre for Jamnapari, 5505 for Barbari and 6988 for Black Bengal goats (Sinha *et al.*, 1987).

Rosza (1950) found that the bacterial count of bull semen ranged from 250 to 400000 per millilitre, While Marinov *et al.* (1966) reported the variations to be two to 6,00,00,000 per millilitre. Out of forty two bulls used for artificial insemination in New Zealand, the total number of viable microorganisms were between 20 and 5,34,000 per millilitre (Brown *et al.*, 1974). Kher and Dholakia (1984) found that the bacterial count per millilitre of neat bull semen in three artificial insemination centres were 520-17000, 1280-9700 and 3600-21500. The bacterial load per millilitre of neat bull semen was found to be 15,485 ± 3861 (Mohanty *et al.*, 1988). Out of three hundred and fourteen samples from twenty seven Holstein Friesian bulls the bacterial count in neat semen ranged from 7280 to 20900 organisms per millilitre (Rathnamma *et al.*, 1997).

The average bacterial load in pre-sperm and sperm rich portions of semen from five Large White Yorkshire boars were $0.832 \pm 0.054 \times 10^6$ and $0.29 \pm 0.017 \times 10^5$ organisms per millilitre respectively (Pandey and Singh., 1997).

2.1.2.1 Level of contamination

Bacteriological studies on 1293 ejaculates from 146 bulls showed that 208 ejaculates from 104 bulls were free of bacteria (Balashov *et al.*, 1977). Stoyanov (1985) reported that out of 7636 samples of bull semen, 42 percentage had no contamination, 40 had slight contamination and 18 had severe contamination. Aleem *et al.*, (1990) found that twenty seven out of sixty bulls produced contaminated semen which had a single species of bacteria, while ten had mixed flora. Gupta and Maurya (1993) found that of 88 bull semen samples 59 had more than one type of bacteria.

2.1.3 Bacterial load in chilled semen

Brown *et al.*, (1974) found that bacterial population of extended bull semen at 15° centigrade declined to a low level within three days of preservation.

The average bacterial load per millilitre of diluted semen of Jamnapari, Barbari and Black Bengal goats were 26554, 15832 and 37851 organisms respectively (Sinha *et al.*, 1987).

Ahmed *et al.*,(1987) found the overall mean bacterial load in thirty two samples of preserved bull semen with egg yolk citrate extender and supplemented with penicillin and streptomycin was 3286.11 ±1004.11 organisms per millilitre. The average bacterial load of extenders in three artificial insemination centres were 3133, 1260 and 3587 organisms per millilitre and the corresponding

bacterial load in diluted preserved bull semen were 9817, 1965 and 7480 organisms per millilitre respectively (Kher and Dholakia, 1987).

Bindra *et al.* (1994) got the bacterial count of chilled buffalo semen as 14399.16 ± 1003.48, 10541.00 ± 1428.83, 8141.67 ± 819.89 and 5998.33 ± 723.76 organisms per millilitre for four treatments namely no preputial washing, one two and three preputial washings respectively.

2.1.4 Bacterial load in frozen semen

Sharma and Deka (1986) found that twenty out of thirtyfive samples of frozen buck semen were contaminated and the bacterial count ranged from three to sixty organisms per dose in the positive samples. Sinha *et al.* (1987) found that the average bacterial load per millilitre of frozen buck semen were 11075 for Jamnapari, 6293 for Barbari and 6981 for Black Bengal goats.

According to Mazurova *et al.* (1975) the number of bacteria in 0.2 millilitre of samples of fresh, diluted, after equilibration, after freezing on dry ice and after three months of storage in liquid Nitrogen averaged 11962, 19016, 8014, 4932 and 1710 organisms respectively. Nowakowski and Wierzbowski (1980) observed that the average bacterial load of frozen bull semen was 60000 organisms per millilitre. Svelan *et al.* (1982) reported that 8.2 per cent of samples were contaminated in frozen bull semen pellets. The bacterial count in frozen bull semen was lower than that in diluted semen, the count indicating the effect of freezing on the total bacterial load (Kher and Dholakia, 1984). The mean bacterial load of frozen bull

semen from three different production centres were 110.61 ± 25.01, 2639.84 ± 764.89 and 30.54 ± 6.98 organisms per dose (Gangadhar *et al.*, 1986). Nimai Singh *et al.* (1990) found that the mean bacterial load in semen without antibiotics reduced from 11.56 ± 1.67 x 10^{10} per millilitre before freezing to 5.84 ± 0.19 x 10^{3} per millilitre ninety days post freezing. The mean bacterial load of frozen bull semen from three centres of Kerala were 2260 ± 880.62, 825,91 ± 186.06 and 80.33 ± 18.47 organisms per dose (Mathew, 1996). Rathnamma *et al.* (1997) reported that the bacterial load in 314 semen samples from twenty seven Holstein Friesian bulls ranged from 5050 to 171400 organisms per millilitre.

Bindra *et al.* (1994) worked on the bacterial load of frozen buffalo semen and recorded it as 3301.67 ± 323.18, 1318.67 ± 250.92, 907.33 ± 204.75 and 735.67 ± 208.22 respectively for no preputial washing, one, two and three preputial washings respectively.

2.2 Factors effecting bacterial load in semen

2.2.1 Preputial condition

Subramanyam *et al.*, (1991) observed that semen was mainly contaminated due to infected prepuce. The mean number of colonies in buck semen collected prior to washing of prepuce were found to be 80.9, 34.5, 13.2 organisms per millilitre for $1/10^{2}$, $1/10^{3}$, $1/10^{4}$ dilutions respectively. Subsequent to preputial washing it reduced to 36.5, 19.1 and 12.8 organisms per millilitre at the respective dilutions. Reddy *at al* (1971) found that bulls with tucked up prepuce had lower

bacterial count in their preputial washings and semen than bulls with pendulous sheath.

Bindra *et al.* (1994) reported that the mean bacterial load in fresh buffalo semen was 38522.22 ± 4431.55, 27398.00 ± 3371.21 19128.33 ± 2610.90 and 15116.66 ± 1537.6 bacteria per millilitre respectively in no preputial washing, one, two and three preputial washings. They further opined that washing of prepuce at weekly intervals would reduce the bacterial load in semen.

2.2.2 Ejaculate number

Ajitkumar *et al.* (1996) found that there was an increase in the bacterial load of the second ejaculate. They found the bacterial load in the first ejaculate to be 1100 organisms per millilitre as against 4860 organisms per millilitre in the second ejaculate and opined that the use of the same artificial vagina repeatedly could be the reason for increased bacterial load in the second ejaculate.

2.2.3 Age

Young bulls had a lower bacterial count in their preputial washings and semen than that of older bulls (Reddy *et al.*, 1971). Kher and Dholakia (1984) found the average bacterial load per millilitre of neat semen for bulls below six years was 3235 and that above six years was 6474. They further found that bulls above six years showed nearly two times the bacterial load (69322 per millilitre) in preputial washing than that of younger bulls (38093 per millilitre). The mean

bacterial load per dose of semen from bulls below four years was significantly higher than those of bulls between four to six years of age (Mathew, 1996).

2.2.4 Environment

Microorganisms commonly found in the environment have been isolated from semen and is reported to cause a wide variety of reproductive disorders (Rahman *et al.*, 1983). Rahman *et al.* (1983); Kher and Dholakia (1984) and Gangadhar *et al.* (1986) further remarked that the bacterial load of preserved semen depended upon the extent of hygienic measures taken during processing of semen.

2.2.5 Season

Kher and Dholakia (1984) found that the bacterial contamination was higher in summer followed by monsoon and winter season. They have attributed the high bacterial load on equipment in summer as the reason for more bacterial load of semen.

2.2.6 Artificial vagina

According to Petelikova (1974) the microbial count per millilitre of semen averaged 9029 in traditional artificial vagina (50 cm long with a reasonable cup collector), 1503 in the "pulawy type" (33 cm long with a rubber funnel and a double walled glass collection tube) and 2229 in "Russian type" (30 cm long with a disposable polythene collector in an insulating muff). Serdyuk *et al.* (1974)

compared artificial vagina of 23 and 36 centimetre length, without a diaphragm, with a perforated external diaphragm and one fitted eight centimeter inside the opening and found that semen with the least microbial count was that obtained with short type artificial vagina with an external diaphragm. The bacterial count in one millilitre of thawed frozen bull semen averaged 983560 and 238070 in two stations with the standard artificial vagina and 54230 in ejaculate collected by modified artificial vagina which prevented bacterial contamination (Boryczko, 1978).

Ajitkumar *et al.* (1996) observed an increase in bacterial load of the second ejaculate of bull semen when first and second collections were taken with the same artificial vagina and attributed it to contamination of artificial vagina with preputial discharge.

2.2.7 Semen processing equipment

Kher and Dholakia (1987) found that the average bacterial load of artificial insemination equipment were 523, 336 and 2568 organisms per millilitre in three artificial insemination centres and attributed the high variation due to handling of equipment in semen processing centres.

2.2.8 Diluents

The bacterial count in processed semen is in proportion to neat semen except in cases where additional contamination has taken place from diluent. (Kher and Dholakia, 1984).

Sampathkumaran (1960) suggested that egg yolk used in diluents could be easily contaminated. Mazurova (1975); Kher and Dholakia (1984) and Sinha *et al.* (1987) found that the mean bacterial load in diluted semen was higher than in neat semen. Sinha *et al.* (1987) suggested the higher count to be due to egg yolk, fructose and buffer being conducive for growth of microorganisms or to resistance of some bacteria to Penicillin and Streptomycin.

2.2.8.1 Antimicrobial agents

Although Penicillin and Streptomycin were the oldest and most widely used antibiotics, they had little effect on flora of bacterial origin (Rahman *et al.*, 1983). Gentamicin was found to be the most sensitive of antibiotics against bacteria in semen (Rahman *et al.*, 1983, Sharma and Deka, 1986 and Saikia *et al.*, 1987). Chloramphenicol and gentamicin were found to be the most effective antibiotics (Nimai Singh *et al.*, 1990 and Bindra *et al.*, 1994). Mathew (1996) recommended the addition of new antibiotics other than the conventionally used antibiotics to semen extenders.

2.2.9 Liquid nitrogen

Liquid nitrogen used to store semen contained many species of bacteria (Pospelov *et al.*, 1973, Roberts, 1986). Carbollo (1981) reported that semen could be contaminated from liquid nitrogen or its container especially when semen is frozen in pellets.

2.2.10 Semen packing systems

Darii and Deku (1975) found that contamination was lesser in straws than in pellets. Korudzhiski (1979) found that bacterial contamination was least in plastic straws followed by granules and glass ampoules.

2.2.11 Artificial insemination technicians

The percentage of frozen bull semen straws contaminated before despatch from semen production centres and after distribution to artificial insemination technicians were 11.7 and 38.8 (Fejes, 1975). Mazurova and Krpatova (1990) found the percentage of semen with no fungal contamination from semen production centres, regional semen banks and artificial insemination technicians to be 41.5, 12 and 0.5 respectively.

2.3 Microbial types in semen

2.3.1 Specific bacteria

Specific bacteria transmitted via semen are diphtheroids, *Corynebacterium renale*, *Leptospira*, *Brucella abortus*, *Brucella ovis*, *Brucella melitensis*, *Mycobacterium tuberculosis*, *Mycobacterium paratuberculosis*, *Campylobacter fetus* and *Trichomonas fetus* (Roberts, 1986; Cupps, 1991 and Arthur *et al.*, 1996).

2.3.2 Non specific bacteria in semen

The organisms isolated from frozen buck semen were *Proteus mirabilis*, *Staphylococcus aureus*, *E. coli*, *Citrobacter*, *Bacillus* and *Streptococcus* (Sharma and Deka, 1986). The organisms isolated from neat, diluted and frozen samples from three breeds of bucks were *E. coli* which had the highest frequency followed by *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Aerobacter*, *Alcaligenes*, *Paracolobacterium*, *Citrobacter*, *Klebsiella aerogenes*, *Aeromonas*, *Staphylococcus*, *Micrococcus*, *Streptococcus*, *Enterococci*, *Bacillus* and *Corynebacterium* (Sinha *et al.*, 1987). Subramanyam *et al.* (1991) found that the most common contaminants of buck semen were *Bacillus*, *E. coli*, *Hafnia* and *Corynebacterium*.

The saprophytic bacteria contaminating bull semen were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus*, *Pseudomonas aeruginosa*, *E. coli*, *Bacillus*, *Corynebacterium* (Sorel, 1961; Rahman *et al.*, 1983; Stoyanov,

1985; Saikia *et al* , 1987; Aleem *et al*., 1990; Gupta and Maurya, 1993 and Singh *et al*., 1995).

Bacillus had the highest percentage among the isolated bacteria from bull semen followed by *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Streptococci* and other organisms (Kher and Dholakia, 1984).

Hussain *et al*. (1990) reported the prevalence of different types of bacteria in percentage as *E. coli* (21.1), *Bacillus subtilis* (17.9), *Proteus vulgaris* (16.3), *Micrococcus* (11.9), *Pseudomonas aeruginosa* (9.2.), *Corynebacterium pyogenes* (5.5), *Streptococcus haemolytica* (4.3), *Staphylococcus aureus* (3.8) and unidentified gram negative bacilli (2.1).

Borah (1995) isolated *Staphylococci*, *Bacillus*, *E. coli*, *Citrobacter*, *Micrococcus* and *Enterobacter* as the common bacteria from frozen bovine semen straws. The bacterial isolates from four crossbred bulls of Artificial Insemination Centre, Mannuthy were *Corynebacterium*, *Alcaligenes*, *Kurthia*, *Streptococcus*, *Micrococcus*, *Enterobacteriaceae* and *Pseudomonas* (Ajitkumar *et al*., 1996).

2.4 Effect of bacteria on semen quality

2.4.1 Sperm motility

Ram spermatozoa were completely motionless after exposure for three hours to *Vibrio fetus* lipopolysaccharide, lipopolysaccharide of *Pastuerella multocida* and *Serratia marcescens* (Dennis, 1962). The percentage of sperm

motility of bull semen was found to be 80 and 30 for 12756, 27100 microorganisms per millilitre respectively (Runceanu *et al.* 1973). Panangala (1981) found that four out of twenty bulls were positive for Mycoplasma and had 20 per cent or less sperm motility.There was a negative influence on the motility of spermatozoa after six hours incubation with *E. coli* but not with *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (Boryczko *et al.*, 1985). Gangadhar *et al.* (1986) found that of three artificial insemination centres, there was no significant correlation between post thaw motility and total bacterial count in two centres but the correlation coefficient just touched the significant level in one centre.

2.4.2 Live sperm

Scwerdtner (1961) reported that the quality of bull semen contaminated with *Pseudomonas pyocyanaea* was not inferior but viability of sperms was lower after storage for five days or more. *E. coli* and *Bacterium prodiogiosum* were the most spermicidal due to glucose dehydrase activity (Marinova, 1967). Gangadhar *et al.*, (1986) found that of 221 frozen bull semen straws, the mean coliform load was below 300 million per millilitre which is the count spermicidal for bull spermatozoa.They further stated that a level of 5000 pseudomonads per millilitre is detrimental to semen quality. Borah *et al.* (1995) reported that most of the species isolated from frozen bull semen were of no pathogenic significance but increasing load might reduce viability of sperms.

2.4.3 Sperm abnormality

Lein and Nielson (1975) reported development of sperm abnormalities following inoculation of *Mycoplasma bovigenitalium* or *Mycoplasma agalactiae* subspecies *bovis* into the seminal vesicle of bulls. Sobech *et al.* (1991) reported that in young bulls reacting serologically positive to *Chlamydia psittaci* antigen, there was a decrease in volume and concentration of spermatozoa, and an increase in sperm abnormalities.

2.4.4 Acrosomal abnormalities

Meyer *et al.* (1980) found that extracellular proteins of *Staphylococcus aureus* damaged spermatozoa, as evidenced by loss of motility and loss of acrosomal cap.

2.4.5 Fertility status

Bush *et al.* (1950) observed that conception rate decreased as the number of bacteria increased, but the number of organisms or type of bacteria did not affect the length of oestrus cycle after unsuccessful artificial insemination. Romanuik (1965) demonstrated that the conception rates were 81 per cent and 73.8 per cent respectively for semen containing bacteria upto or over 300000 per millilitre. In a study by Marinov (1967) it was found that *E. coli* and *Bacterium prodiogiosum* caused embryonic mortality and a decrease in the conception rate.

2.5 Semen quality during processing and preservation by freezing

2.5.1 Sperm motility

The motility in frozen semen was high in Tris egg yolk citric acid fructose glycerol diluent (Deka and Rao, 1985, Choudhury *et al.*, 1987 and Sinha *et al.*, 1992)

Monji (1987) found that the percentage of sperm motility in fresh semen, was 86.45 ±0.82; for semen cooled to 5°C at 2.5h, was 80.95 ± 0.83; after glycerolisation and equilibration, 79.45 ± 0.84 and after thawing at 37°C it was 69.55 ± 0.67. Post thaw motility was recorded to be higher in samples frozen after four hours and six hours of equilibration, the percentage being 57.65 and 57.60 respectively (Sinha *et al.*, 1987).

Washing of buck semen before freezing in Tris based diluent with egg yolk and glycerol significantly increased post thaw motility (Westhusyen Van der, 1978). Perez (1985) reported that ejaculates from goats washed twice had higher sperm motility and higher percentage acrosome defects than non washed semen after freezing. The percentage of progressively motile sperms upto 12 months of storage at -196°C did not differ between washed and unwashed buck semen (Deka and Rao 1987).

Haunhorst (1990) suggested that centrifugation of samples did not affect sperm motility. Tuli and Holtz (1994) reported that the removal of seminal plasma had an unfavourable effect on post thaw motility of buck semen.

Das and Rajkonwar (1995) found that the mean percentage of motility of fresh semen was 87.09 ± 0.22 after three hours of equilibration and after freezing it was 47.79 ± 0.98. The initial sperm motility of 82.00 ± 0.61 was found to drop to 44.00 ± 0.79 after freezing and thawing of buck semen (Ranjini, 1998).

2.5.2 Live sperm

The percentage of live sperms in frozen goat semen at different storage periods of zero day, three months, six months, nine months, and twelve months was 69.60 ± 1.96, 68.06 ± 1.78, 66.67 ± 2.12, 68.13 ± 2.01, 68.91 + 1.74 respectively with seminal plasma and 71.18 ± 2.15, 70.67 ± 1.26, 70.07 ± 1.25, 62.31 ± 1.34, 70.91 ± 0.25 respectively without seminal plasma (Deka and Rao, 1986).

Deshpande and Mehta (1991) reported the percentage of live sperms in buck semen immediately after dilution, five hours after dilution, immediately after freezing and 24 hours after freezing to be 82.00 ± 2.14, 76.83 ± 2.23, 45.33 ± 4.52, 44.50 ± 2.98 at six per cent glycerol level. The mean live percentage of fresh semen was 90.03 ± 0.80. The corresponding figures after initial extension, glycerolisation equilibration and freezing were 86.75 ± 0.79, 83.00 ± 0.72 74.32 ± 0.84 and 54.25 ± 0.59 respectively (Ranjini, 1998).

2.5.3 Sperm abnormality

Deka and Rao (1984) reported that the mean percentage of abnormalities of head, mid piece and tail at different stages of processing and freezing did not differ significantly from that of fresh semen in any extender. The percentage in Tris diluent were 0.20 ± 0.07, 0.85 ± 0.15, 1.23 ± 0.19 and 1.26 ± 0.17 respectively. The average percentage of abnormal sperms in fresh buck semen was observed as 3.05 ± 0.25. The percentage of abnormal sperms on processing were 3.75 ± 0.57, 4.7 ± 0.30, 5.50 ± 0.43 and 7.13 ± 0.71 respectively at the end of initial extension, glycerolisation, equilibration and freezing and thawing (Ranjini, 1998).

2.5.4 Acrosomal abnormality

Watson (1975) opined that there was progressive disruption of acrosome during chilling and deep freezing of sperms.

The proportion of acrosomal deformities (2.9 per cent) in fresh semen increased after glycerolisation to 20.7 per cent but there was no relationship between the proportion of deformed spermatozoa and motility (Gocken and Asti, 1980). Sokolovskaya *et al.* (1981) classified acrosomal abnormalities as swollen, exfoliated and lost and suggested that thawed semen is suitable for insemination if thirty per cent of spermatozoa have normal acrosome. The per cent of normal acrosome was better in semen of rams centrifuged at 5°centigrade than at 12° centigrade. Deka and Rao (1985) estimated the percentage of total damaged

acrosome in buck semen to be 1.5 ± 0.3, 2.5 ± 0.23, 5.07 ± 0.52, 12.37 + 1.84 respectively in fresh semen, after cooling, dilution, equilibration and after freezing in Tris egg yolk citric acid fructose diluent. The incidence of swollen acrosome and total damaged acrosome in buck semen did not differ significantly between egg yolk levels before freezing but differed after freezing and thawing. The acrosome damage was significantly lower with ten and twenty per cent egg yolk when compared with seven per cent egg yolk (Deka and Rao 1986a). They further reported (1986b) that the percentage of damaged acrosome was lower with four per cent glycerol and one hour equilibration while freezing buck semen. The percentage of intact acrosome after equilibration and after freezing and thawing varied significantly between equilibration periods and glycerol level but not due to interaction. A combination of four hour equilibration and six per cent glycerol level in Tris extender resulted in a better quality of frozen buck semen (Sinha *et al.* 1992).

Das and Rajkonwar (1994) reported that a higher percentage of damaged acrosome could be recorded with five per cent level of glycerol than seven per cent. The lowest percentage of swollen, separating and entirely lost acrosome was recorded at three hour equilibration period with seven per cent glycerol. Singh and Purbey (1996) reported that in eight per cent glycerol, intact acrosome in buck spermatozoa diluted in Tris extender and frozen in liquid nitrogen declined from 88.76 ± 0.81 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. Ranjini (1998) reported a significant increase in the acrosome abnormalities during glycerolisation, equlibration, freezing and thawing.

2.6 Semen quality during processing and preservation by chilling

2.6.1 Sperm motility

Jelam and Nambiar (1968) observed the percentage of motility and live sperms in egg yolk citrate and goat milk dilutent as 59.3, 51.4 at forty eight hours and 54.4 and 43.6 at 72 hours. The percentage of live sperms were 61.27 and 50.09 after 72 hours of chilling. Balakrishnan (1979) reported an average motility percentage of 75.39, 62.97, 49.90, 37.42 and 19.30 after 24, 48, 72, 96 and 120 hours of storage at 5° centigrade.

Singh *et al.*, (1982) found the percentage of motility to be 76.60 ± 3.48, 53.60 ± 2.81, 41.90 ± 0.96, 29.50 ± 1.01, 13.30 ± 0.78 in Tris yolk glycerol diluent at 0, 24, 48, 72 and 96 hours of preservation. The motility of buck semen preserved under refrigeration temperature was found to be 79.63 per cent, 73.75 per cent and 65.50 per cent respectively at 0, 24, 72 hours of preservation with Tris yolk diluent (Sarmah 1983). Singh *et al.* (1985) found the percentage of motility to be 87.78 ± 2.5 and 66.60 ± 3.69 for Barbari and Jamnapari bucks respectively at 24 hours of preservation.

Puranik *et al.* (1994) suggested that Tris-fructose-citric acid was a good diluent with 52 per cent sperm motility at 72 hours of preservation.

2.6.1 Live sperm

Jelam and Nambiar (1968) observed the live sperm percentage in egg yolk citrate and goat milk diluents as 61.27 and 50.09 after 72 hours of preservation. The percentage of live sperms at 0, 24, 48, 72 and 96 hours of preservation in Tris yolk glycerol was found to be 86.00 ± 2.38, 63.10 ± 2.01, 49.30 ± 2.67, 36.60 ± 1.81, 21.00 ± 1.69 respectively. (Sarmah, 1983), Singh *et al.* (1985) found the percentage of live sperm to be 75.30 ± 3.33 and 78.37 ± 1.76 for Jamnapari and Babari bucks at 24 hours of chilling.

2.6.3 Sperm abnormality

Rao and Rao (1979) found a significant increase in tail abnormalities on increase in storage periods. The total sperm abnormalities were found to be 5.42, 6.57 and 6.76 per cent at 0, 24 and 72 hours of preservation of buck semen in Tris diluent. (Sarmah 1983).

2.6.4 Acrosomal abnormalities

There is progressive disruption of acrosomes during chilling (Watson 1975). Sarmah (1983) estimated the acrosome defects of buck semen at refrigeration temperature to be 0.87, 2.76 and 3. 62 per cent at 0, 24 and 72 hours of preservation in Tris diluent.

Materials and Methods

3. MATERIALS AND METHODS

Seventy two semen ejaculates from six cross bred Malabari bucks aged between 1.5-2 years maintained at the Artificial Insemination centre, under the Department of Animal Reproduction, College of Veterinary and Animal Sciences, Mannuthy, Thrissur were utilised for the study. The bucks selected were normal, healthy and maintained under standard feeding and management conditions.

3.1 Semen collection

Semen samples were collected using Danish type artificial vagina remodelled by reducing the length of rubber cylinder to 15 centimetre to suit the buck. Special care was taken in the preparation of the artificial vagina to minimise the chance of contamination. Separate artificial vagina was used for collecting each ejaculate. Two false mounts were given for stewing the bucks just before taking each collection. Immediately after collection, the labelled samples were transferred to a water bath maintained at 37°centigrade and further subjected to various evaluation procedures.

3.2 Semen evaluation

The physical attributes of semen like volume, colour density and mass activity were assessed by standard procedures (Sane, *et al.* 1991).

3.3 Semen quality

The parameters examined under sperm morphology were sperm motility, live sperms, sperm abnormalities and acrosomal abnormalities.

3.3.1 Live sperm

Live sperm percentage of the semen samples was assessed by Eosin-Nigrosin staining technique. One drop of two per cent eosin and three drops of ten per cent Nigrosin were mixed gently with a small drop of semen and smears were prepared immediately.

A total of hundred sperms from each sample were counted from different microscopic fields under oil immersion objective of the microscope, keeping a note of dead and live sperms from the staining characters and the percentage worked out (Sane *et al.*, 1991).

3.3.2 Sperm abnormality

Percentage of abnormal sperms was estimated in Nigrosin- eosin smears under oil immersion objective of the microscope. A total of hundred sperms were counted from different microscopic fields keeping a note of specific abnormalities and expressed in percentage (Sane *et al.*, 1991).

3.3.3 Acrosomal abnormality

Acrosomal abnormality of spermatozoa was assessed by Giemsa staining technique (Sarma, 1996).

Giemsa staining

Semen smears were prepared on grease free glass slides, air dried and fixed with methanol for 15 minutes. These slides were then air dried and immersed in Giemsa working solution for three hours. Giemsa working solution was prepared by mixing Giemsa stock solution six millilitre, 0.2 mol phosphate buffer four millilitre and deionised water 90 millilitre. Giemsa stock solution was prepared by mixing one gram of Giemsa powder, 66 millilitre of methanol and 60 millilitre of glycerol. The stained slides were then washed in tap water, air dried and mounted in DPX and observed under oil immersion. Based on the morphological appearance the acrosome of sperms were scored as normal, swollen, separating and lost (Das and Rajkonwar, 1994).

3.3.4 Microbial count

Microbial count of the semen samples were estimated by Pour plate technique (Cruickshank *et al.*, 1975).

3.3.4.1 Pour plate technique

Media for total bacterial count

Tryptone soya agar (Himedia) (Culture media)

Ingredients	grams/litre
Casein enzymic hydrolysate	15.0
Papaic digest of soyabean meal	5.0
Sodium chloride	5.0
Agar	15.0

Final pH (at 25° centigrade) 7.3 ± 0.2

Autoclaved for 15 minutes at 121° centigrade and 15 lbs pressure.

Peptone water (Himedia) (Dilution media)

Ingredients	grams/litre
Peptic digest of animal tissue	10
Sodium chloride	5

Final pH (at 25° centigrade) 7.2 ± 0.02

Autoclaved for 15 minutes at 121° centigrade and 15 lbs pressure.

Procedure

Ten fold seriel dilutions of samples were made before preparing petri plates for viable count of bacteria. Hundred microlitres of a sample was mixed with 0.9 millilitre of peptone water taken in a sterile test tube. From this, hundred

microlitre was transferred into a second test tube containing 0.9 millilitre of peptone water in order to passage the sample serially.

The number of dilutions for each sample were regulated in such a way that the number of colonies were between 30 and 300. Usually single, double or triple dilutions were enough to get the desired range. Hundred microlitre of this serially diluted sample were transferred using micropipette fitted with sterile disposable plastic tips into 50 millilitre of Tryptone Soya Agar medium taken in a sterile beaker. The medium was previously sterilised and held at 50° centigrade in a water bath. The sample mixed with medium was then poured into duplicate petri plates and held horizontally to solidify. The plates were then incubated at 37° centigrade for 24-48 hours.

At the end of incubation the number of colonies were counted by an illuminated Quebec colony counter. The mean number of colonies counted for the highest dilution of each sample was taken as the mean colony count. The total bacterial load was determined by multiplying the mean colony count with dilution factor.

3.4 Semen preservation

For the purpose of this study semen samples were preserved by two different processing techniques, chilling, and freezing in liquid Nitrogen.

3.4.1 Selection of samples

Based on preliminary evaluation only those semen samples having +++ mass activity, DDDD density and a motility of seventy per cent or more were used for the study.

3.4.2 Washing of spermatozoa

The pooled ejaculates were extended ten fold in phosphate buffered saline (PBS) and centrifuged at 3000 rpm for five minutes. The supernatant fluid was pipetted out. The pellet was again extended ten fold in PBS and centrifuged at 3000 rpm for five minutes and the clear supernatant was removed.

The pellet was reconstituted again with PBS to the original volume. The sample was assessed for sperm motility, dead and live count, sperm abnormality, acrosome abnormality and total microbial count.

The sample was then split into two portions, one for chilling and the other for freezing.

3.4.3 Semen extenders

In both the types of preservation techniques Tris extender was used as the basic component.

3.4.3.1 Extender for chilling

Ingredients	g/50 ml
Tris hydroxy methyl amino methane (SRL)	1.36
Citric acid (Merck)	0.75
Fructose (Merck)	0.56
Benzyl penicillin	1000 IU/ml
Streptomycin sulphate	1000 µg/ml

Twenty five millilitre of deionised water was added to a hundred millilitre flask into which the ingredients were added and stirred well. Five millilitre of egg yolk collected aseptically from fresh, hen egg was also added to the same flask and mixed into a homogenous fluid using a magnetic stirrer. The final volume was made upto fifty millilitre by adding deionised water and kept in a water bath at 37°centigrade.

3.4.3.2 Extender for freezing

Nonglycerolated fraction of extender

Ingredients	g/50 ml
Tris hydroxy methyl amino methane (SRL)	1.21
Citric acid (Merck)	0.67
Fructose (Merck)	0.5
Benzyl penicillin	1000 IU/ml
Streptomycin sulphate	1000 µg/ml

Glycerolated fraction of extender

Ingredients	g/50 ml
Tris hydroxy methyl amino methane (SRL)	1.21
Citric acid (Merck)	0.67
Fructose (Merck)	0.5
Glycerol (Merck)	6 ml
Benzyl penicillin	1000 IU/ml
Streptomycin sulphate	1000 µg/ml

Twenty five millilitre of deionised water was added into two, hundred millilitre flasks into which the ingredients were taken and stirred well. Egg yolk was collected aseptically from fresh, hen egg and five millilitre each was added to both the flasks. The final volume of both the fractions of the extender was made upto fifty millilitre with deionised water and kept in a water bath at 37° centigrade.

The microbial load of the extenders for chilling and freezing (nonglycerolated and glycerolated fraction) was assessed immediately before dilution with the semen samples.

3.4.4 Procedure for freezing

The major steps involved in freezing of semen were initial extension, cooling, glycerolisation, equilibration, filling, sealing, freezing and storage.

3.4.4.1 Initial extension

The sample was extended five times the volume with non-glycerolated fraction of the extender. This was then taken in a sterile 15 millilitre glass vial and closed with aluminium foil. Equal volume of glycerolated fraction of extender was also taken similarly in another sterile glass vial. After initial extension the sperm motility, dead and live count, sperm abnormalities, acrosomal abnormalities and bacterial load were assessed.

3.4.4.2 Cooling

The vials containing half diluted semen and glycerolated fraction of extender were kept in a five hundred millilitre water bath at 37°centigrade and transferred to a cold handling unit maintained at 5° centigrade. Straw clips, straw filling combs, sterile cotton, sterile towel, poly vinyl alcohol powder, air bubblers, air bubbler dish, rack, ramp, 0.5 millilitre straws of different colours and a tray containing water with two drops of disinfectant were also placed in the cold handling unit for cooling. The cooling rate of half diluted semen and the glycerolated fraction were regulated so as to bring the temperature to 5°centigrade within a period of two hours.

3.4.4.3 Glycerolisation

After cooling to 5° centigrade the glycerolated fraction of the extender was added to half extended semen samples in three steps at fifteen minutes interval to make a final glycerol concentration of six per cent. The sample was assessed for sperm motility, dead and live count, sperm abnormalities, acrosome abnormalities and total bacterial count.

3.4.4.4 Equilibration

The fully extended semen sample was equilibrated for a total duration of four hours before final freezing. On completion of equilibration period, the semen samples were evaluated for sperm motility, dead and live count, sperm abnormalities, acrosomal abnormalities and total bacterial count.

3.4.4.5 Filling and sealing

Filling of straws was carried out after one hour of equilibration. Straws were arranged in straw clips. Straw in the clips were attached to a filling comb by the factory seal end. The comb was connected to a vacuum pump. After mixing gently the semen was transferred to the bubbler dish and the open end of the straws attached to the comb were dipped in it and semen was filled using negative pressure. Air spaces were created in the straws by applying the open end of the straws in the bubbler comb. Then the open end of the straws were sealed with polyvinyl alcohol powder. The excess powder sticking to the straws were removed using a cotton pad. After releasing from the clips and comb the straws were

immersed in water kept at 5° centigrade. After forty five minutes filled straws were taken out and wiped with a dry towel and arranged on freezing racks.

3.4.4.6 Freezing and storage

Wide mouthed liquid nitrogen refrigerator was used for freezing. The liquid nitrogen was poured into the refrigerator upto the level touching the freezing grill. Few goblets were placed over the grill for cooling. It was then closed with a wooden lid and kept for a few minutes so that the vapour temperature in the refrigerator got stabilised. After stablising the vapour temperature, freezing racks with straws were transferred quickly from the cold handling unit to the freezing cabinet. The straws in the racks were four centimetre above the liquid nitrogen level. The refrigerator was closed. After eight minutes the lid was opened, the frozen straws were collected and loaded into the precooled goblet and plunged into liquid nitrogen. After this the goblets along with straws were transferred into storage containers. A paper label showing the freezing date, buck number and straw colour was tied on the handle of the canister for identification of semen straws.

The straw for thawing was picked up from the storage can within few seconds of opening of the lid using a precooled forceps. By holding one end, the straw was shaken to remove drops of liquid nitrogen if any sticking to it. Then placed it in a water bath containing drops of disinfectant at 30°centigrade for 30 seconds. After thawing, the straw was dried with a sterile cotton pad and semen was transferred to a sterile glass vial for evaluation.

Sperm motility, dead and live sperm count, sperm abnormalities, acrosomal abnormalities and total bacterial load were assessed on zero day, fifteenth day and thirthieth day of freezing.

3.4.5 Procedure of chilling

The sample was extended ten times the volume with the extender for chilling and the motility, dead and live count, sperm abnormalities, acrosomal abnormalities and total bacterial load were assessed.

It was then kept in a water bath at 37°centigrade and transferred to a refrigerator for cooling to 5°centigrade. The temperature in the refrigerator was counter checked and was found to range between 5-8° centigrade.

The sperm motility, dead and live count, sperm abnormalities, acrosomal abnormalities and total bacterial load were assessed at twenty four hours and forty eight hours of preservation.

Results

4. RESULTS

A study was carried out to assess the bacterial load of buck semen after collection, during processing and preservation by chilling and freezing. Semen samples from six Malabari crossbred bucks of the Artificial Insemination Centre, Department of Animal Reproduction, College of Veterinary And Animal Sciences Mannuthy, Thrissur were collected and used for the purpose.

A total of six collections comprising of 12 ejaculates from each buck were used for this study.

4.1 Semen Volume

The mean volume of semen from two ejaculates on pooling was 1.23 ± 0.03 millilitre.

4.2 Semen quality and Bacterial load of fresh sample

Samples with creamy colour, ++++ mass activity and DDDD density were only used for further processing. The percentage of live sperms, sperm abnormalities and acrosome abnormalities were 94.69 ± 0.67, 1.31 ± 0.67 and 0.70 ± 0.15 respectively and the total bacterial load was 1166.67 ± 348.64 organisms per millilitre (Table 1).

4.3 Semen quality and bacterial load during processing and preservation by freezing

4.3.1 Washing and reconstitution

The sperm motility, live sperms, sperm abnormalities and acrosome abnormalities were 75.14 ± 1.42, 89.69 ± 0.46, 3.76 ± 0.46 and 7.14 ± 0.65 per cent respectively. The mean bacterial load was 3493.05 ± 734.90 organisms per millilitre (Table 2). There was no significant correlation between the bacterial load and the semen quality at this stage (Table 5).

4.3.2 Initial Extension

The percentage of sperm motility, live sperms, sperm abnormalities and acrosome abnormalities were 70.00 ± 0.68, 82.44 ± 1.42, 3.94 ± 0.46 and 8.27 + 0.41 and the bacterial load was 27272.22 ± 4012.70 organisms per millilitre (Table 3a). There was significant difference in the bacterial load between washing and reconstitution and initial extension (Table 8a). However there was no significant correlation between the bacterial load and semen quality (Table 5).

4.3.3 Glycerolisation

The sperm motility and live sperm percentage reduced to 61.80 ± 0.95 and 78.79 ± 1.43 respectively after glycerolisation. The percentage of sperm abnormalities, acrosomal abnormalities and the mean bacterial load were 5.32 ± 0.39, 9.62 ± 0.75 and 24466.67 ± 3682.40 organisms per millilitre (Table 3b).

There was no significant correlation between the bacterial load at initial extension and after glycerolisation (Table 8a).

4.3.4 Equilibration

There was a significant decrease in the bacterial load on equilibration, the mean bacterial load being 2691.11 ± 664.81 organisms per millilitre (Table 8a). The percentage of sperm motility, live sperms sperm abnormalities and acrosomal abnormalities were 55.83 ± 1.12, 70.82 ± 2.01, 5.44 ± 0.42 and 10.81 ± 0.79 respectively (Table 3c). The bacterial load and the semen quality was found to have no significant correlation (Table 5).

4.3.5 Zero day of freezing

The percentage of sperm motility, live sperms, sperm abnormalities and acrosomal abnormalities were 35.42 ± 0.71, 59.70 ± 1.03, 6.79 ± 0.41 and 14.50 ± 0.95 respectively. There was a significant decrease in the bacterial load to 221.81 ± 129.77 organisms per millilitre (Table 3d and Table 8a). The correlation between the semen quality and the bacterial load was non significant (Table 5).

4.3.6 Fifteen days of freezing

The sperm motility after fifteen days of freeze preservation was 34.45 ± 0.74 per cent. The live sperms were 57.69 ± 1.30 per cent, the sperm abnormalities 6.72 ± 0.55 per cent, acrosome abnormalities 14.76 ± 0.96 and the bacterial load was 161.00 ± 19.94 organisms per millilitre (Table 3e). The difference in the

bacterial load at zero day and fifteen days of freezing was non-significant (Table 8b). The bacterial load and semen quality were also found to have no significant correlation (Table 5).

4.3.7 Thirty days of freezing

The percentage of sperm motility, live sperms, sperm abnormalities and acrosome abnormalities were 33.17 ± 1.14, 57.83 ± 0.90, 7.42 ± 0.45, 14.76 ± 0.77 respectively. The bacterial load of frozen semen was 162.78 ± 29.03 organisms per millilitre (Table 3f). There was no significant correlation between the bacterial load and semen quality (Table 5). So also the difference between the bacterial load of frozen semen at zero and thirty days or between fifteen and thirty days were non-significant (Table 8b).

4.4 Semen quality and bacterial load during preservation by chilling

4.4.1 At zero hour of preservation

The percentage of sperm motility, live sperms, sperm abnormalities and acrosome abnormalities were 73.47 ± 4.53, 88.24 ± 0.56, 2.97 ± 0.37 and 7.2 ± 0.58. The bacterial load per millilitre of diluted and chilled semen was 41563.89 ± 5359.58 organisms (Table 4a). The bacterial load and semen quality were not correlated significantly (Table 6).

4.4.2 At 24 hours of preservation

The bacterial load after 24 hours of preservation was 56611.11 ± 8236.55 organisms per millilitre. The percentage of sperm motility, live sperms, sperm abnormalities and acrosome abnormalities were 70.55 ± 0.17, 80.82 ± 0.53, 3.68 ± 0.51 and 8.58 ± 0.60 (Table 4b). There was no significant correlation between the bacterial load and semen quality (Table 6). Similarly there was no significant difference in the bacterial load of zero and 24 hours of preservation (Table 8c).

4.4.3 At 48 hours of preservation

The percentage of sperm motility, live sperms, sperm abnormalities, acrosome abnormalities and bacterial load after 48 hours of preservation were 62.50 ± 1.27, 72.72 ± 1.70, 4.74 ± 0.48, 9.31 ± 0.66 and 86458.33 ± 35433.74 organisms per millilitre respectively (Table 4c). There was significant increase in the bacterial load between zero and forty eight hours of preservation. However, the difference in bacterial load between 24 and 48 hours was non-significant (Table 8c). Similarly, the bacterial load and semen quality were non-significantly correlated (Table 6).

4.5 Bacterial load of extenders

4.5.1 Extender for freezing

The mean values of the bacterial load of the non-glycerolated and glycerolated fractions of the extender for freezing were 44069.20 ± 23404.11 and 54402.79 ± 2273.79 organisms per millilitre. There was no significant correlation between the bacterial load of the non-glycerolated fraction and initial bacterial load on semen extension and between the glycerolated fraction and semen after complete glycerolisation. (Table 7a, 7b)

4.5.2 Extender for chilling

The mean value of the bacterial load of extender for preservation under refrigeration temperature was 35866.67 ± 9263.63 organisms per millilitre. The correlation of the bacterial load of extender and zero hour of preservation was non significant (Table 7c).

Table 1. Bacterial load and semen quality of fresh semen

Buck	Bacterial load (organisms per ml)	Live sperms (%)	Sperm abnormalities (%)	Acrosomal abnormalities (%)
1.	466.67± 118.09	96.27±0.52	1.19±0.20	0.82±0.09
2.	1016.67 ± 461.28	95.24±1.39	1.44±0.29	0.17±0.15
3.	675.00± 266.07	95.09±0.62	1.42±0.26	0.41±0.18
4.	533.33± 145.87	92.40±1.21	1.44±0.17	0.17±0.21
5.	1633.33±1052.43	95.83±1.01	1.33±0.19	0.87±0.18
6.	2675.00±1203.87	93.30±1.05	1.03±0.33	1.23±0.16
Mean	1166.67± 348.64	94.69±0.67	1.31±0.67	0.70±0.15

Table 2. Bacterial load and semen quality of reconstituted sample

Buck	Bacterial load (organisms per ml)	Live sperms (%)	Sperm abnormalities (%)	Acrosomal abnormalities (%)	Sperm motility (%)
1.	6250.00±2307.68	90.20±1.12	2.45±0.29	7.50±0.30	76.67±1.67
2.	2108.33±1046.30	90.67±2.01	3.70±0.76	7.45±0.35	68.33±4.77
3.	2308.33±1133.90	88.12±0.56	3.89±0.83	8.70±0.66	75.00±3.42
4.	4908.33±3628.21	88.48±0.83	5.80±0.54	7.82±0.36	77.50±1.71
5.	1691.67± 576.11	90.68±1.61	3.50±0.27	7.32±0.48	75.83±2.01
6.	3691.67±1088.46	89.98±0.76	3.22±0.32	4.03±0.56	77.50±1.71
Mean	3493.05± 734.90	89.69±0.46	3.76±0.46	7.14±0.65	75.14±1.42

Table 3 Bacterial load and semen quality during freezing
Table 3a. Initial extension

Buck	Bacterial load (organisms per ml)	Live sperms (%)	Sperm abnormalities (%)	Acrosomal abnormalities (%)	Sperm motility (%)
1.	30550.00±10592.41	86.83±0.90	2.61±0.40	9.06±0.39	70.83±0.83
2.	20916.67±11763.23	85.43±2.94	3.31±0.42	9.02±0.47	68.33±4.94
3.	32416.67±10539.15	81.45±1.08	4.66±0.92	8.70±1.07	67.50±4.43
4.	18666.67± 4013.87	81.58±2.24	5.77±0.56	8.84±0.26	70.83±0.83
5.	18166.67± 4952.45	76.95±1.09	3.88±0.43	7.02±0.37	71.67±3.07
6.	42916.67±22472.36	82.37±1.91	3.41±0.55	6.97±0.42	70.83±2.17
Mean	27272.22±4012.70	82.44±1.42	3.94±0.46	8.27±0.41	70.00±0.68

Table 3b. Glycerolisation

Buck	Bacterial load (organisms per ml)	Live sperms (%)	Sperm abnormalities (%)	Acrosomal abnormalities (%)	Sperm motility (%)
1.	22250.00± 6944.72	79.78±1.37	4.54±0.30	12.35±0.33	62.50±1.71
2.	19466.67± 6640.11	83.50±1.64	4.37±0.45	8.72±0.45	58.33±4.77
3.	31083.33±12264.05	78.70±0.80	5.44±0.30	10.15±0.94	60.00±3.65
4.	13083.33± 8517.06	80.19±1.38	6.95±0.30	10.87±0.66	61.67±3.07
5.	19916.67± 4461.68	72.90±0.47	5.86±0.79	8.01±0.40	64.17±5.23
6.	38000.00±16360.52	77.65±1.77	4.76±0.58	7.63±0.37	64.17±5.23
Mean	24466.67± 3682.40	78.79±1.43	5.32±0.39	9.62±0.75	61.80±0.95

Table 3c. Equilibration

Buck	Bacterial load (organisms per ml)	Live sperms (%)	Sperm abnormalities (%)	Acrosomal abnormalities (%)	Sperm motility (%)
1.	2766.67±1147.73	69.17±2.02	4.65±0.23	12.81±0.35	56.67±4.77
2.	1833.33± 581.19	67.12±2.02	4.41±0.23	11.36±0.35	51.67±4.77
3.	5833.33±4928.92	78.60±1.06	5.68±0.18	12.19±0.70	55.00±4.28
4.	1216.67± 415.87	65.77±2.16	7.28±0.48	11.20±0.62	56.67±4.22
5.	2091.67±1099.05	69.35±1.19	5.61±0.56	9.05±0.32	60.00±5.16
6.	2400.00± 761.14	74.92±1.23	5.03±0.23	8.23±0.42	55.00±4.28
Mean	2691.11±664.81	70.82±2.01	5.44±0.42	10.81±0.79	55.83±1.12

Table 3d. Frozen zero day

Buck	Bacterial load (organisms per ml)	Live sperms (%)	Sperm abnormalities (%)	Acrosomal abnormalities (%)	Sperm motility (%)
1.	410.83±113.80	63.08±2.29	5.61±0.43	17.50±0.35	35.83±4.16
2.	30.00± 8.17	56.18±1.87	5.82±0.36	14.35±0.97	33.33±3.33
3.	221.67± 31.67	58.81±3.30	8.18±0.93	13.22±0.43	33.33±4.94
4.	310.83± 38.95	61.00±2.27	7.68±0.86	16.16±0.79	36.67±3.33
5.	183.33± 96.98	57.84±4.36	6.59±0.58	14.90±0.83	37.50±2.50
6.	174.17± 62.22	61.22±0.81	6.83±0.42	10.88±0.56	35.83±2.00
Mean	221.81±129.77	59.70±1.03	6.79±0.41	14.50±0.95	35.42±0.71

Table 3e. Frozen 15 days

Buck	Bacterial load (organisms per ml)	Live sperms (%)	Sperm abnormalities (%)	Acrosomal abnormalities (%)	Sperm motility (%)
1.	187.50±28.69	53.70±1.86	5.71±0.52	14.90±0.83	36.67±2.11
2.	79.17±23.18	58.25±1.25	6.05±0.39	10.69±0.56	35.00±2.24
3.	157.67±78.83	62.42±2.32	5.73±0.26	17.88±0.43	34.67±4.17
4.	200.00±67.28	55.57±2.60	6.12±0.37	14.99±0.85	31.67±3.07
5.	133.33±39.62	59.62±3.05	9.02±0.59	14.30±0.79	33.33±4.84
6.	208.33±69.01	56.03±2.16	7.71±0.60	15.83±0.83	35.83±2.71
Mean	161.00±19.94	57.69±1.30	6.72±0.55	14.76±0.96	34.45±0.74

Table 3f. Frozen 30 days

Buck	Bacterial load (organisms per ml)	Live sperms (%)	Sperm abnormalities (%)	Acrosomal abnormalities (%)	Sperm motility (%)
1.	187.50±68.43	57.45±2.36	6.37±0.30	17.75±0.39	33.33±4.01
2.	30.00± 9.31	59.62±1.89	6.98±0.47	15.33±1.26	30.00±4.47
3.	153.33±74.42	60.08±2.84	8.98±0.63	14.31±0.57	30.00±3.65
4.	241.67±95.02	59.10±1.64	8.54±0.61	15.68±0.64	32.50±1.71
5.	181.67±59.24	54.40±1.24	6.33±0.50	13.36±0.81	37.50±2.50
6.	182.50±69.01	56.25±2.33	7.34±0.44	12.15±0.81	35.00±2.24
Mean	162.78±29.03	57.83±0.90	7.42±0.45	14.76±0.77	33.17±1.14

Table 4. Bacterial load and semen quality during chilling

Table 4a. At zero hour of preservation

Buck	Bacterial load (organisms per ml)	Live sperms (%)	Sperm abnormalities (%)	Acrosomal abnormalities (%)	Sperm motility (%)
1.	40666.67±17764.04	89.33±0.98	1.53±0.24	7.25±0.29	72.50±1.71
2.	45500.00±19104.54	89.15±0.93	2.63±0.29	7.24±0.46	66.67±4.22
3.	33216.67± 8511.19	88.02±0.90	2.59±0.22	8.77±0.86	73.33±3.33
4.	32500.00±20078.18	89.17±1.52	3.43±0.39	6.41±0.95	76.67±2.11
5.	31666.67±16969.91	88.04±2.02	4.08±0.78	6.33±0.37	75.00±2.24
6.	65833.33±17342.47	85.73±0.87	3.55±0.39	6.45±0.47	76.67±2.11
Mean	41563.89±5359.58	88.24±0.56	2.97±0.37	7.20±0.58	73.47±4.53

Table 4b. At 24 hours of preservation

Buck	Bacterial load (organisms per ml)	Live sperms (%)	Sperm abnormalities (%)	Acrosomal abnormalities (%)	Sperm motility (%)
1.	85333.33±21377.04	87.25±0.88	1.88±0.48	8.92±0.25	68.33±2.11
2.	34833.33± 9744.79	79.70±1.71	2.59±0.40	8.31±0.33	66.67±4.22
3.	41666.67±14727.90	82.12±1.79	3.95±0.28	9.98±0.60	70.00±2.58
4.	61166.67±29343.28	80.73±0.70	4.12±0.35	9.65±0.53	70.00±3.65
5.	43000.00±11.38	76.09±2.14	5.34±0.37	8.80±0.67	73.33±2.11
6.	73666.67±19093.92	79.10±0.82	4.20±0.38	5.83±0.38	74.17±2.01
Mean	56611.11±8236.55	80.82±0.53	3.68±0.51	8.58±0.60	70.55±0.17

Table 4c. At 48 hours of preservation

Buck	Bacterial load (organisms per ml)	Live sperms (%)	Sperm abnormalities (%)	Acrosomal abnormalities (%)	Sperm motility (%)
1.	131416.67±23516.10	77.18±2.40	3.01±0.41	9.08±0.16	61.67±3.07
2.	91000.00±40333.61	66.32±2.71	3.62±0.37	8.99±0.60	58.33±5.43
3.	137500.00±75684.76	76.28±1.62	5.83±0.71	11.79±0.75	63.33±4.94
4.	60000.00±20312.56	76.28±1.62	5.83±0.71	11.79±0.75	60.00±0.15
5.	49000.00±13296.62	69.92±1.53	4.87±0.52	8.19±0.33	65.00±5.77
6.	49833.33±15862.78	74.45±1.21	5.32±0.38	7.32±0.46	66.67±4.22
Mean	86458.33±35433.74	72.72±1.70	4.74±0.48	9.31±0.66	62.50±1.27

Table 5. Coefficient of correlation of bacterial load and semen quality during processing and preservation by freezing

Semen parameters	Fresh	Reconstitute	Initial extension	Glyceroli-sation	Equili-bration	Frozen 0 day	Frozen 15 day	Frozen 30 day
Live sperms	-0.31	0.18	-0.05	0.03	0.27	0.23	-0.01	0.18
Sperm abnorma-lities	0.03	-0.23	-0.25	-0.19	-0.07	0.11	-0.14	0.05
Acrosome abnormalities	0.32	0.16	-0.04	-0.27	-0.06	0.26	0.12	-0.08
Sperm motility	-	0.14	0.23	0.19	0.24	0.11	-0.17	-0.01

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

Table 6. Coefficient of correlation of bacterial load and semen quality during preservation by chilling

Semen parameters	0 hour	24 hour	48 hour
Live sperms	0.04	0.28	0.11
Sperm abnormalities	-0.13	-0.07	-0.04
Acrosomal abnormalities	-0.17	-0.17	0.08
Sperm motility	0.18	0.11	-0.13

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

Table 7. Bacterial load of extenders

Table 7a. Non glycerolated fraction of extender for freezing

Sample No	Organisms/millilitre
1.	38666.67 ± 11485.26
2.	145083.33 ± 92298.47
3.	29333.00 ± 17390.93
4.	15750.00 ± 6732.69
5.	18333.33 ± 6194.98
6.	17250.00 ± 6318.95
Mean	44069.20 ± 23404.11

Coefficient of correlation between nonglycerated extender and after initial extension = 0.34

Table 7b. Glycerolated fraction of extender for freezing

Sample No	Organisms/millilitre
1	55116.67 ± 22397.52
2.	65883.33 ± 23623.86
3.	46166.67 ± 14234.74
4.	47166.67 ± 23338.33
5.	44416.67 ± 23523.89
6.	67666.67 ± 29243.42
Mean	54402.79 ± 2273.79

Coefficient of correlation between glycerolated extender and after glycerolisation = 0.05

Table 7c. Extender for chilling

Sample No.	Organisms/millilitre
1	41916.67 ± 12649.85
2.	45083.33 ± 92298.47
3.	27500.00 ± 7973.92
4.	33833.33 ± 15702.26
5.	26000.00 ± 7416.19
6.	30866.67 ± 9541.02
Mean	35866.67 ± 9263.63

Coefficient of correlation between chilled extender and 0 hour of preservation = 0.06

Table 8a. Students t test on the mean bacterial load during processing by freezing

Process	t value
Fresh versus reconstituted	2.55
Reconstituted versus initial extension	6.09**
Initial extension versus glycerolisation	0.37
Glycerolisation versus equilibration	6.99**
Equilibration and zero day of freezing	3.73**

Table 8b. Students' t test on the mean bacterial load during different days of freeze preservation

Day	t value
Zero day versus 15 days	1.49
15 days versus 30 days	0.12
Zero day versus 30 days	1.65

Table 8c. Student's t test on the mean bacterial load during preservation by chilling

Hours	t value
Zero hour versus 24 hours	1.93
Zero hour versus 48 hours	2.41**
24 hours versus 48 hours	1.66

* Significant at 1 per cent level
** Significant at 5 per cent level

Fig.1 BACTERIAL LOAD DURING PROCESSING AND PRESERVATION BY FREEZING

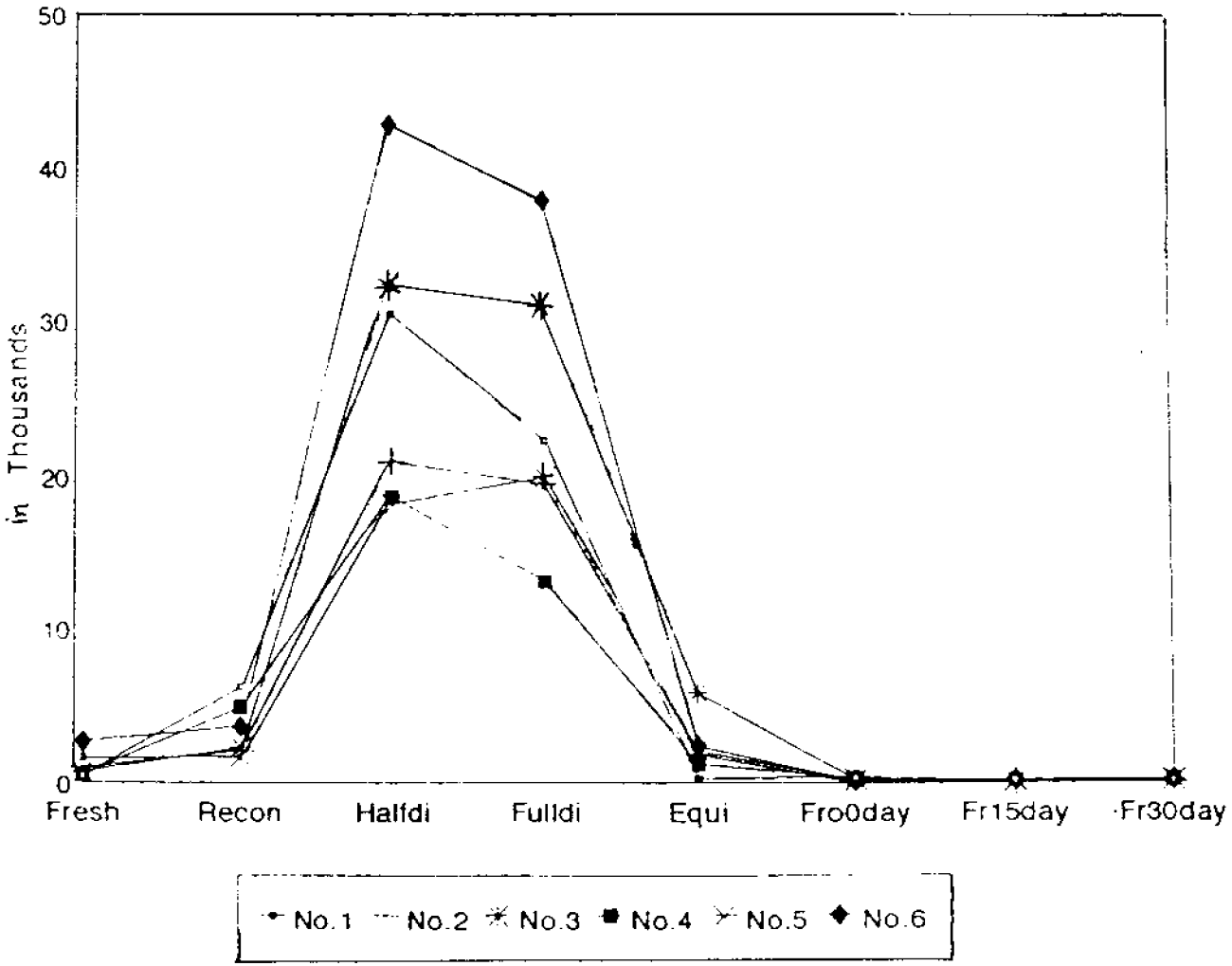

Fig.2 . SEMEN QUALITY DURING PROCESSING AND PRESERVATION BY FREEZING

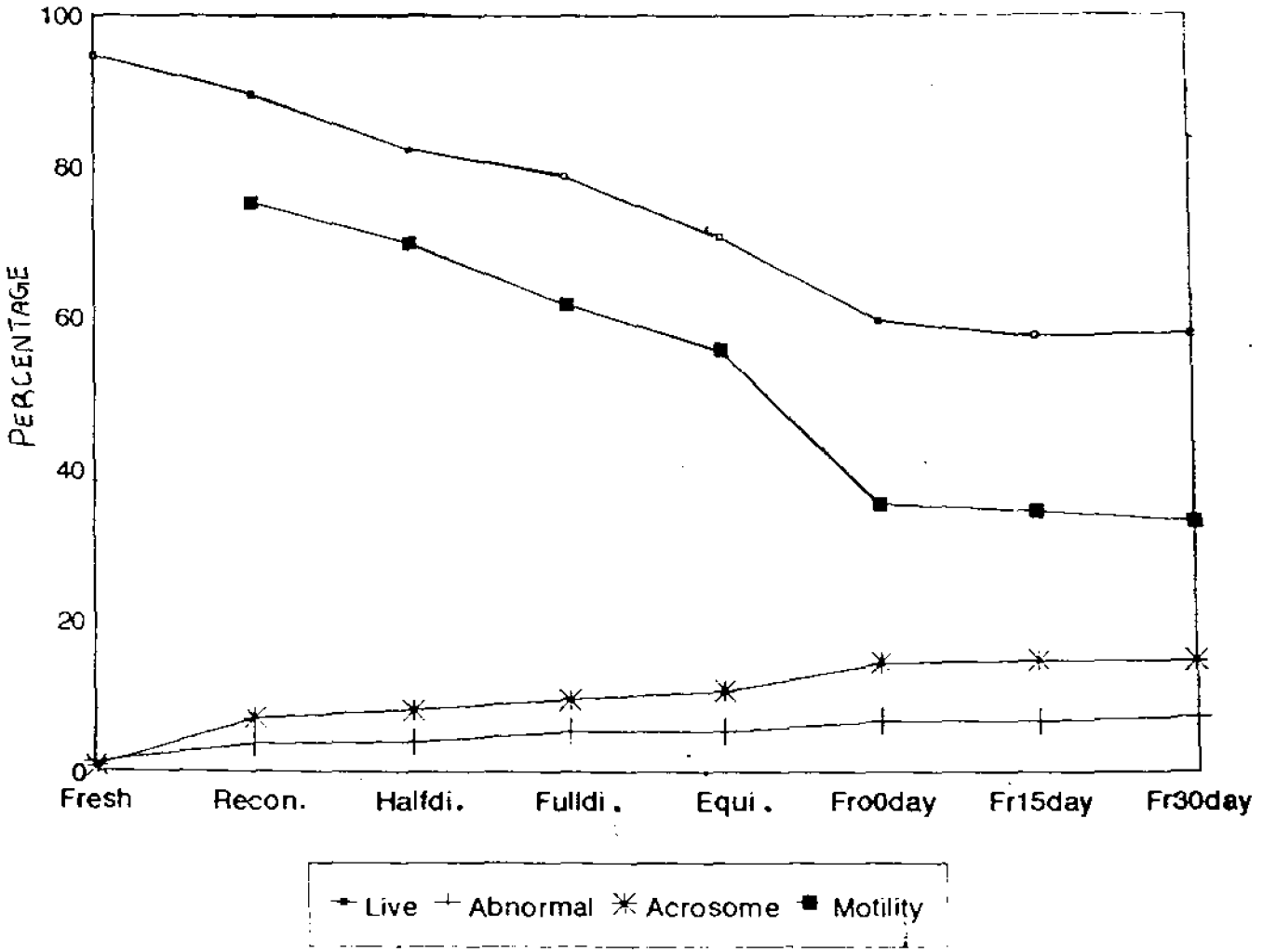

Fig.3 BACTERIAL LOAD DURING PRESERVATION AT REFRIGERATION TEMPERATURE

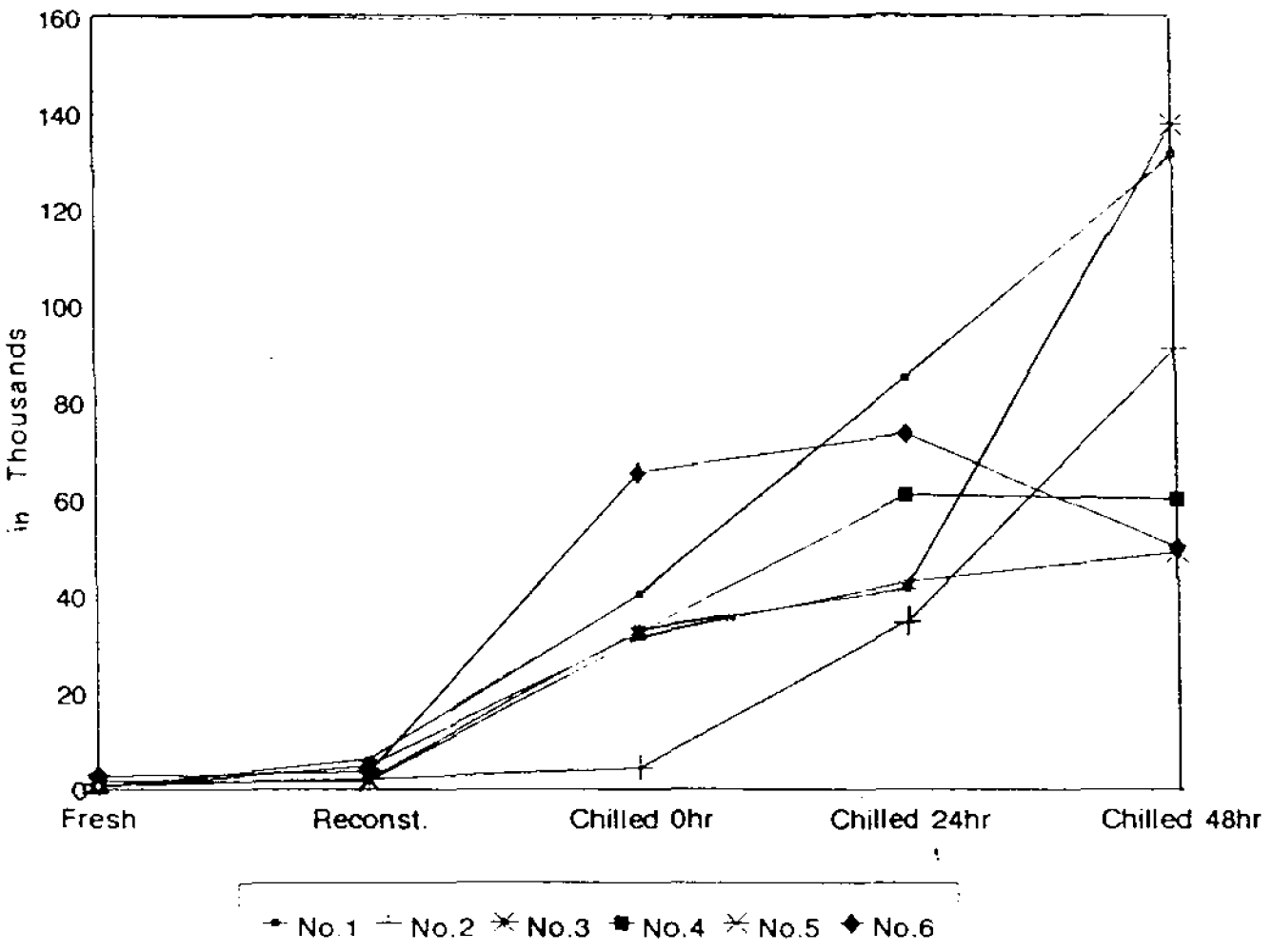

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Fig. 4 SEMEN QUALITY DURING PRESERVATION AT REFRIGERATION TEMPERATURE

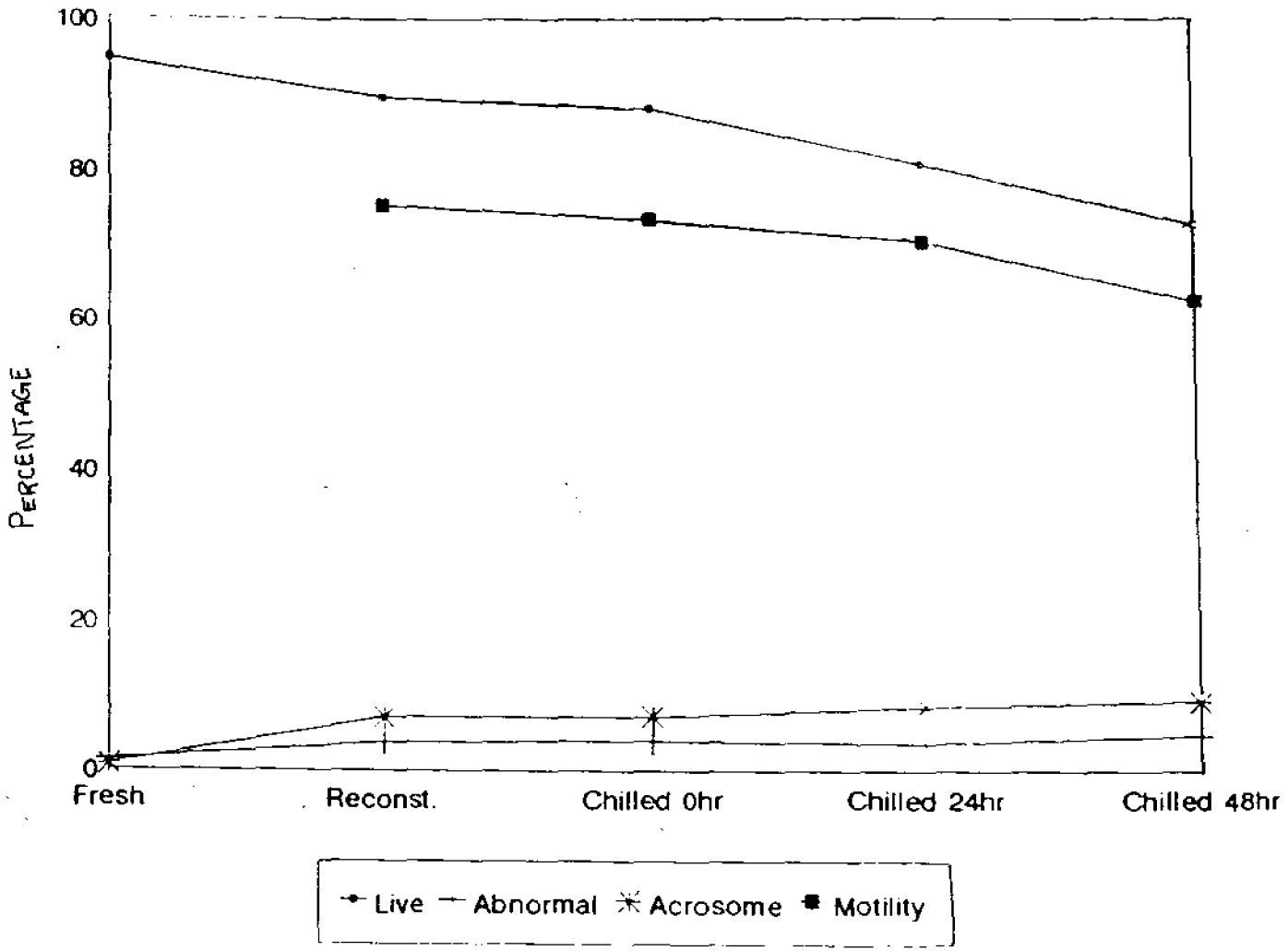

Plate 1. Bacterial colonies - Growth in Tryptone soya Agar

Plate 2. Swollen, separating and lost acrosomes in frozen semen smear - Giemsa staining

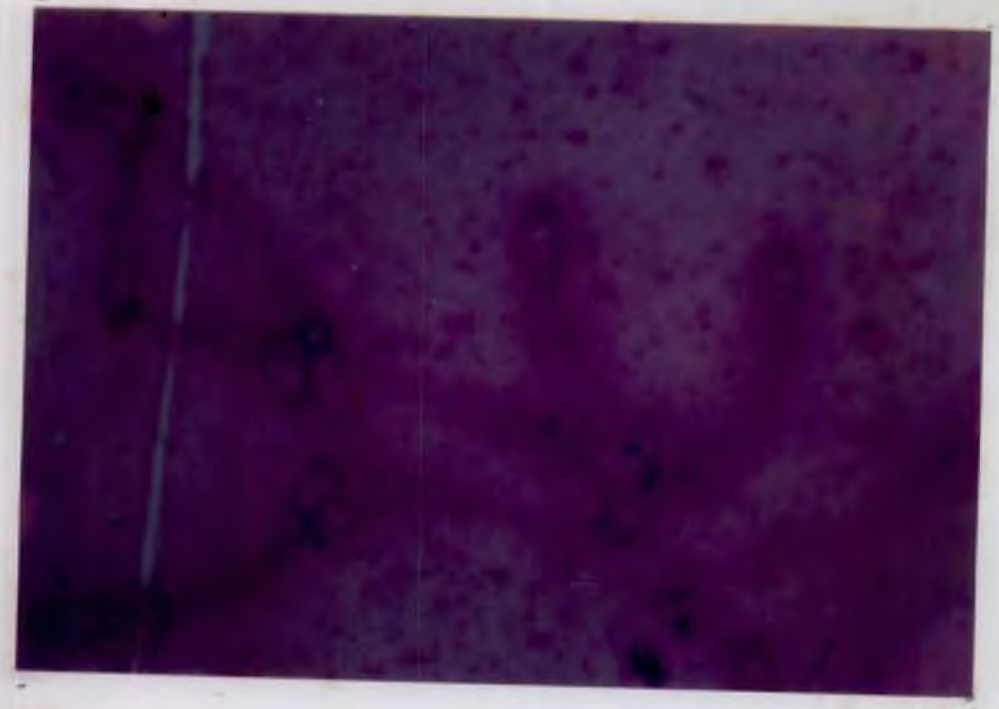

Plate 3. Detached heads (after glycerolisation) Nigrosin-eosin staining

Plate 4. Coiled tail (preservation by chilling) Nigrosin-eosin staining

Discussion

5. DISCUSSION

Semen samples from six Malabari crossbred bucks were subjected to screening for assessing the bacterial load after collection, chilling and freezing. A total of six collections (12 ejaculates) from each buck were utilized for the study.

5.1 Semen volume

The mean volume of semen from two ejaculates was found to be 1.23 ± 0.03 millilitre which is in full agreement with earlier reports (Knoblauch, 1962; Cetinkaya, 1980; Pattnaik *et al.*, 1991 and Ranjini, 1998). However, Singh *et al.* (1982) reported lower values.

5.2 Semen quality and bacterial load of fresh sample

5.2.1 Live sperm

The percentage of live sperms was 94.69 ± 0.67 for fresh semen (Table 1) which is within the range reported by Sarmah (1983); Singh *et al.* (1985); Prasanth (1995) and Ranjini (1998) and slightly higher than that reported by Mann (1980) and Pandey *et al.* (1985).

5.2.2 Sperm abnormality

The percentage of sperm abnormalities was observed to be 1.31 ± 0.67 which is slightly lower than the values reported by Ranjini (1998) and is in agreement with that of Singh *et al.* (1985).

5.2.3 Acrosomal abnormality

The percentage of sperms with abnormal acrosomes was 0.7 ± 0.15. This is lower than the values reported by Ranjini (1998).

5.2.4 Bacterial load

The bacterial load per millilitre of neat semen was found to be 1166.67 ± 348.64 organisms per millilitre which is lower than that reported by Sinha *et al.* (1987). Variation in the bacterial load of semen samples at different centres depends on the extent of variation in the hygienic practises and sanitary environment provided during collection, processing and preservation (Rahman *et al.*, 1983). Wiezerbowski (1981) also reported that management practises followed at different centres reflected on the bacterial load of semen produced from these centres. The better hygienic practises and sanitary environment provided might have helped to reduce the microbial load in neat semen in this study. However, no significant correlation was observed between the semen quality and bacterial load.

5.3 Semen quality and bacterial load during processing and preservation by freezing

5.3.1 Washing and reconstitution

5.3.1.1 Sperm motility

The percentage of sperm motility of buck semen after washing and reconstitution further reduced to 75.14 ± 1.42. Westhuysen Van der (1978), Perez (1985) and Memon *et al.* (1985) reported that the percentage of sperm motility was higher in samples devoid off seminal plasma because of the removal of an egg coagulating factor present in the seminal plasma. However, Tuli and Holtz (1994) reported that glutamic oxaloacetic transaminase (GOT) level in the extracellular fluid was lower in washed spermatozoa before freezing, but after thawing more GOT was released thus leading to an adverse effect on the post thaw motility.

5.3.1.2 Live sperm

Deka and Rao (1987) opined that the percentage of live sperms upto 12 months of storage at -196° centigrade did not differ between buck semen frozen with and without seminal plasma. The percentage of live sperms after washing and reconstitution reduced to 89.69 ± 0.46 which is higher than the values (74.78 ± 1.69) reported by Ranjini (1998).

5.3.1.3 Sperm abnormality

The percentage of sperm abnormalities increased to 3.76 ± 0.46 due to washing and reconstitution which may be due to mechanical damage caused in the centrifuge.

5.3.1.4 Acrosomal abnormality

There was an increase in the number of sperms with acrosomal abnormalities (7.14 ± 0.65). Perez (1985) reported a higher percentage of acrosome defects in washed than unwashed buck semen. The values obtained in this study is in agreement with that of Perez (1985) and Deka and Rao (1987).

5.3.1.5 Bacterial load

The average bacterial load increased to 3493.05 ± 734.90 organisms per millilitre and was not significantly correlated to the semen quality. However no earlier reports could be traced out on the bacterial load in washed semen. The increase in the bacterial load of washed semen may be attributed to bacterial multiplication at ambient temperature during processing.

5.3.2 Initial extension

5.3.2.1 Sperm motility

The percentage of sperm motility after initial extension was 70.00 ± 0.68, which is not differnt from after washing and reconstitution and is in agreement with the values observed by Prasanth (1995).

5.3.2.2 Live sperm

The percentage of live sperms was 82.44 ± 1.42 which is slightly lower than that obtained by Ranjini (1998).

5.3.2.3 Sperm abnormality

The percentage of sperm abnormalities (3.94 ± 0.46) was almost similar to the value obtained after washing and reconstitution which is slightly lower than that obtained by Ranjini (1998).

5.3.2.4 Acrosomal abnormality

The percentage of acrosomal abnormalities was 8.27 ±0.41 which is not different from the percentage value obtained after washing and reconstitution.

5.3.2.5 Bacterial load

There was a significant increase in the bacterial load to 27272.22 ± 4012.70 organisms per millilitre. This increase in the bacterial load is in full agreement with Mazurova *et al.* (1975) and Sinha *et al.* (1987). Kher and Dholakia (1984) suggested that the bacterial count might be higher in diluted semen due to contaminated diluents or resistance of organisms to routinely used antibiotics. Sinha *et al.* (1987) opined that the higher bacterial load in diluted semen might be due to the fact that egg yolk, fructose and buffer are quite conducive for the growth of microorganisms. However there was no significant correlation between semen quality and bacterial load.

5.3.3 Glycerolisation

5.3.3.1 Sperm motility

The percentage of motile sperm was 61.80 ± 0.95 which was below the values observed by Ranjini (1998) and almost within the range observed by Prasanth (1995).

5.3.3.2 Live sperm

The percentage of live sperms (78.79 ± 1.43) is slightly lower than that reported by Ranjini (1998) and almost similar to the values obtained by Prasanth (1995).

5.3.3.3 Sperm abnormality

The percentage of total sperm abnormalities progressively increased to 5.32 ± 0.39 which is similar to the values obtained by Ranjini (1998). The increase in sperm abnormalities might be due to cold shock as the samples were kept at 5° centigrade.

5.3.3.4 Acrosomal abnormality

The percentage of acrosomal abnormalities was 9.62 ± 0.75 which is lower than that observed by Gocken and Asti (1980) and within the range observed by Ranjini (1998). Das and Rajkonwar (1994) reported slightly lower values for damaged acrosomes after glycerolisation.

5.3.3.5 Bacterial load

The bacterial load was 24466.67 ± 3682.40 organisms per millilitre which is not significantly different from the bacterial load after initial extension. However there was a slight reduction in the bacterial load after glycerolisation. The reduction in the temperature of the sample to 5° centigrade coupled with the bactericidal effect of antibiotics might have acted detrimentaly on the microbes, thereby reducing their number. There was no significant correlation between semen quality and bacterial load at this stage.

5.3.4 Equilibration

5.3.4.1 Sperm motility

There was reduction is sperm motility to 55.83 ± 1.12. However, Das and Rajkonwar (1995) reported slightly higher values.

5.3.4.2 Live sperm

There was a further reduction in the live sperm percentage to 70.82 ± 2.01 on equilibration. Desphande and Mehta (1991) reported that the percentage of live sperm after equilibration was 76.83 ± 2.23.

5.3.4.3 Sperm abnormality

The percentage of sperm abnormalities (5.44 ± 0.42) were almost similar to the values observed after glycerolisation and was similar to that reported by

Ranjini (1998). Deka and Rao (1984) reported very low values for sperm abnormalities after equilibration.

5.3.4.4 Acrosomal abnormality

The percentage of sperms with total damaged acrosomes were 10.81 ± 0.79. The values are not different from those observed after glycerolisation. Das and Rajkonwar (1994) and Ranjini (1998) reported slightly higher values.

5.3.4.5 Bacterial load

The bacterial load significantly reduced to 2691.11 ± 664.81 organisms per millilitre. This reduction in the bacterial load might be due to the fact that most of saprophytic bacteria cannot survive at lower temperatures. The bactericidal effect of antibiotics also might have helped to reduce the bacterial load. The present observation is in agreement with that reported by Mazurova *et al.* (1975). However there was no significant correlation of the bacterial load and semen quality.

5.3.5 Frozen zero day

5.3.5.1 Sperm motility

There was a reduction in sperm motility to 35.42 ± 0.71 after freezing. This abrupt reduction in motility might be due to formation of intracellular ice crystals or increased solute concentration in the media or due to their interaction (Arthur 1996). These values are slightly lower than those obtained by Ranjini

(1998) and Das and Rajkonwar (1995) but is almost in the agreement range with Prasanth (1995).

5.3.5.2 Live sperm

The percentage of live sperms after freezing reduced to 59.70 ± 1.03 and is higher than the values observed by Deshpande and Mehta (1991) and similar to the values observed by Ranjini (1998). Deka and Rao (1987) reported that the percentage of live sperms were not affected by removal of seminal plasma. They obtained a percentage of 69.60 ± 1.96 and 71.68 ± 2.15 with and without seminal plasma.

5.3.5.3 Sperm abnormality

The percentage of sperm abnormalities was 6.79 ± 0.41 which is higher than the value observed by Deka and Rao (1984) but is in full agreement with that of Singh *et al.* (1994) and Ranjini (1998).

5.3.5.4 Acrosomal abnormality

The percentage of acrosome abnormalities was 14.50 ± 0.95. Singh and Purbey (1995) reported a very high percentage of abnormal acrosomes after freezing. According to Jones and Stewart (1979) the exposure of spermatozoa to ultra low temperature causes considerable ultrastructural changes of acrosome leading to the disruption of plasma and the outer acrosome membrane resulting in

the loss of acrosomal content. Das and Rajkonwar (1994) and Ranjini (1998) also reported similar values in their studies.

5.3.5.5 Bacterial load

There was a significant reduction in the bacterial load after freezing, the average bacterial load being 221.81 ± 129.77 organisms per millilitre. Mazurova (1975); Kher and Dholakia (1984); Sinha *et al.* (1987) and Rathnamma (1997) also made similar observations. The lower microbial count might be due to destruction of microorganisms on freezing of semen due to dehydration or higher solute concentration (Bindra *et al.*, 1994).

5.3.6 Preservation by freezing upto 30 days

5.3.6.1 Sperm motility

The percentage of sperm motility was not significantly different on different days of preservation. The percentage of sperm motility was 34.45 ± 0.74 and 33.17 ± 1.14 at 15 and 30 days of preservation. There was no significant effect of storage at-196° centigrade on motility upto 12 months (Deka and Rao, 1987).

5.3.6.2 Live sperm

The percentage of live sperms after 15 and 30 days of preservation was 57.69 ± 1.30 and 57.83 ± 0.90. Deka and Rao (1987) observed that the percentage

of live sperms were not significantly affected by duration of storage at -196° centigrade upto 12 months.

5.3.6.3 Sperm abnormality

The percentage of sperm abnormalities were 6.72 ±0.55 and 7.42 ± 0.45 respectively on 15 and 30 days of preservation. There was not much difference in the sperm abnormalities after storage at -196° centigrade.

5.3.6.4 Acrosomal abnormality

There was no significant difference in the percentage of spermatozoa with abnormal acrosomes, the percentage being 14.76 ± 0.96 and 14.76 ± 0.77 at 15 and 30 days respectively. Deka and Rao (1987) reported that the mean percentage of swollen acrosomes increased significantly during storage. The percentage of total damaged acrosomes increased gradually with increase in storage period.

5.3.6.5 Bacterial load

There was no significant difference in the bacterial load during different days of preservation. The bacterial load at 15 and 30 days of preservation was respectively 161.00 ± 19.94 and 162.78 ± 29.03 organisms per millilitre. Nimai Singh *et al.* (1990) found that the mean bacterial load decreased regularly after storage for 30 to 90 days.

5.4 Preservation by chilling upto 48 hours

5.4.1 Sperm motility

The sperm motility at zero, 24 and 48 hours of preservation was 73.47 ± 4.53, 70.55 ± 0.17 and 62.50 ± 1.27 respectively. Singh *et al.* (1982), Sarmah (1983) and Singh **et al.** (1985) reported decreasing percentages of sperm motility at different hours of preservation.

5.4.2 Live sperm

The live sperm percentage of semen samples preserved under refrigeration temperature was 88.24 ± 0.56, 80.82 ± 0.53 and 72.72 ± 1.70 respectively at zero, 24 and 48 hours of preservation. There was a progressive decrease in the percentage of live sperms during different hours of preservation.

5.4.3 Sperm abnormality

The percentage of sperm abnormalities after zero, 24 and 48 hours were 2.97 ± 0.37, 3.68 ± 0.51 and 4.74 ± 0.48 respectively. The increase in the percentage was due to tail abnormalities. Similar observations were made by Balakrishnan (1979).

5.4.4 Acrosomal abnormality

The percentage of acrosomal abnormalities at zero, 24 and 48 hours of preservation were 7.20 ± 0.58, 8.58 ± 0.60 and 9.31 ± 0.66 respectively. Watson

(1975) opined that the in crease in acrosomal abnormalities might be due to progressive disruption of acrosomal membrane during preservation by chilling.

5.4.5 Bacterial load

The bacterial load after preservation by chilling at zero, 24 and 48 hours were 41563.89 ± 5359.58, 56611.11 ± 8236.55 and 86458.33 ± 35433.74 organisms per millilitre respectively. The increase in the bacterial load might be due to the presence of some of the Psychrotrophic organisms like *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, *Micrococcus*, *lactobacillus*, *Enterobacter* and *Arthrobacter* which multiply at lower temperature (James, 1978). These bacterial types were isolated from semen samples by Sinha *et al.* (1987); Borah *et al.* (1995) and Ajit *et al.* (1996). The increase in the bacterial load during chilling might also be due to variation in temperature in the refrigerator.

5.5 Semen extenders

The bacterial load of nonglycerolated fraction of extender was 44069.20 ± 23404.11 organisms per millilitre and the load immediately after dilution was 27272.22 ± 4012.70 organisms per millilitre and that of glycerolated fraction of extender was 54402.79 ± 2273.79 as against 24466.67 ± 3682.40 organisms per millilitre of glycerolated semen. There was no significant correlation between the bacterial loads indicating that contamination was from extenders. The bacterial load of chilled extender was 35866.67 ± 9263.63 and that of diluted semen was 41563.89 ± 5359.58 organisms per millilitre, and there was no significant

correlation between the bacterial load. Mazurova *et al.* (1975) and Sinha *et al.* (1987) opined that the bacterial load in diluted semen was higher than in neat semen due to contamination from extenders.

It can be concluded that the bacterial load of semen samples was at its maximum during the stages of dilution in both the preservation methods However, the process of freezing helped to reduce the bacterial load significantly. With respect to chilling, the bacterial load was found to increase progressively throughout the period of study. However, no correlation could be worked out between semen quality and the bacterial load at any stage of processing or method of preservation.

Summary

6. SUMMARY

Semen samples from six Malabari crossbred bucks were processed and preserved by freezing and chilling to assess the bacterial contamination after collection, during processing and preservation.

After prelimimary evaluation, seventy two ejaculates having creamy colour, ++++ mass activity and DDDD density were selected for further processing. The semen samples were diluted ten fold with phosphate buffered saline (PBS) centrifuged twice and the sperm pellets were reconstituted to the original volume with PBS. It was then split into two portions, one for chilling and the other for freezing. The samples for chilling were diluted ten fold with Tris-citric acid-fructose-egg yolk diluent and preserved under refrigeration temperature for 48 hours. The samples for freezing were extended five fold with non-glycerolated fraction of Tris-citric acid-fructose - Egg yolk-glycerol diluent. After chilling to 5° centigrade it was further diluted five fold with glycerolated fraction of extender, equilibrated for four hours, frozen in liquid nitrogen and preserved upto 30 days. The bacterial load and semen quality were evaluated at each stage of processing in both the methods of preservation. The results were compiled to evaluate the effect of processing and preservation on the bacterial load of semen. The semen sample had an overall mean volume of 1.23 ± 0.03 millilitre.

The initial live sperm percentage dropped to 57.83 ± 0.90 after freezing and preservation upto 30 days. The initial sperm motility, after washing and reconstitution, 75.14 ± 1.42, was found to drop to 33.17 ± 1.14 percentage on freezing and preservation upto 30 days. This significant drop in live sperm percentage and sperm motility signifies the adverse effect of processing and freezing on the viability of sperms.

The percentage of abnormal sperms, increased from 1.31 ± 0.67 to 7.42 ± 0.45, after freeze preservation. The percentage of acrosomal abnormalities increased from 0.7 ± 0.15, to 14.76 ± 0.77 at the end of freeze preservation. The percentage of acrosomal abnormalities were found to increase progressively during freezing.

The bacterial load of semen samples increased from 1166.67 ± 348.64 organisms per millilitre to 3493.05 ± 734.90 after washing. Then there was a significant increase in bacterial load to 27272.22 ± 4012.70 organisms per millilitre after initial dilution. However, after final dilution the bacterial load reduced to 24466.67 ± 3682.40 organisms per millilitre. A significant decrease in bacterial load to 2691.11 ± 664.81 after equilibration and to 221.81 ± 129.77 after final freezing was very characteristic. However, the bacterial load was not significantly different between zero, 15 or 30 days of storage.

The percentage of live sperms at zero, 24 and 48 hours of preservation at refrigeration temperature was 88.24 ± 0.56, 80.82 ± 0.53 and 72.72 ± 1.70

respectively. The sperm motility reduced from 73.47 ± 4.53 at zero hour to 70.55 ± 0.17 at 24 hours and 62.50 ± 1.27 at 48 hours. The percentage of sperm abnormalities increased from 2.97 ± 0.37 at zero hour to 3.68 ± 0.51 at 24 hours and 4.74 ± 0.48 at 48 hours of preservation. The percentage of acrosomal abnormalities was 7.20 ± 0.58, 8.58 ± 0.60 and 9.31 ± 0.66 respectively at zero hour, 24 and 48 hours of preservation.

The bacterial load of samples after preservation at refrigeration temperature was found to increase from 41563.89 ± 5359.58 at zero hour, to 56611.11 ± 8236.55 at 24 hours and 86458.33 ± 35433.74 organisms per millilitre at 48 hours of preservation. The increase in the bacterial load was not significant between zero and 24 hours or between 24 and 48 hours. However, the increase was significant between 0 and 48 hours of preservation.

It can be concluded that the contamination of semen samples was at its maximum during the stages of dilution in both the preservation methods. However, the process of freezing helped to reduce the bacterial load significantly. With respect to chilling, the bacterial load was found to increase progressively throughout the period of preservation. No correlation could be worked out between the semen quality and bacterial load in any stage of processing or method of preservation.

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ASSESSMENT OF BACTERIAL LOAD IN CHILLED AND FROZEN BUCK SEMEN

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ABSTRACT OF THE THESIS

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ABSTRACT

With the object of assessing the bacterial load of buck semen during processing and preservation by freezing and chilling, a study was carried out at Artificial Insemination Centre, College of Veterinary And Animal Science, Mannuthy, Thrissur, using 72 ejaculates from six Malabari cross bred bucks.

The average volume of semen from two pooled ejaculates was 1.23 ± 0.03 millilitre. Semen samples with creamy colour, ++++ mass activity and DDDD density were only used for processing and preservation.

The samples were diluted 10 fold in phosphate buffered saline before centrifuging twice and the pellet was reconstituted to the original volume with PBS. These were then split into two portions, one for chilling and other for freezing. The sample for chilling was diluted ten fold with Tris-citric acid-fructose egg yolk diluent and preserved under refrigerated conditions for 48 hours. The sample for freezing was diluted five fold in nonglycerolated fraction of Tris-citric acid-fructose-egg yolk-glycerol diluent, cooled to 5° centigrade, glycerolated, equilibrated for 4 hours, frozen in liquid nitrogen and preserved upto 30 days.

The initial live sperm percentage was 94.69 ± 0.67 which dropped to 57.83 ± 0.90 after freezing and storage for 30 days. Similarly, the initial sperm motility of 75.14 ± 1.42 after washing and reconstitution dropped significantly to 33.17 ±

1.14 during the same period. There was an increase in the percentage of sperm abnormalities from 1.31 ± 0.67 to 7.42 ± 0.45 and that of acrosomal abnormalities from 0.70 ± 0.15 to 14.76 ± 0.77 during the same period.

The bacterial load of neat semen was 1166.67 ± 348.64 organisms per millilitre which increased on washing and reconstitution to 3493.05 ± 734.90 organisms per millilitre. Further there was a significant increase on initial extension to 27272.22 ± 4012.70 organisms per millilitre. The declining trend started after glycerolisation with a reduction of bacterial load to 24466.67 ± 3682.40 organisms per millilitre. But on equilibration, reduction in the bacterial load was much more faster and significant and reduced to 2691.11 ± 664.81 organisms per millilitre. This further reduced significantly to 221.81 ± 129.77, 161.00 ± 19.94 and 162.78 ± 29.03 organisms per millilitre on storage at zero, 15 and 30 days of freeze preservation.

With respect to preservation by chilling the live sperm percentage at zero, 24 and 48 hours were 88.24 ± 0.56, 80.82 ± 0.53 and 72.72 ± 1.70 respectively. The sperm motility also reduced from 73.47 ± 4.53 to 70.55 ± 0.17 and 62.50 ± 1.27 during the same period. There was a slight increase in the percentage of sperm abnormalities from 2.97 ± 0.37 at zero hour to 3.68 ± 0.51 and 4.74 ± 0.48 respectively at 24 and 48 hours of preservation. The percentage of acrosomal abnormalities were 7.20 ± 0.58, 8.58 ± 0.60 and 9.31 ± 0.66 respectively at zero, 24 and 48 hours of preservation.

The bacterial load per millilitre of samples on preservation at refrigeration temperature was found to increase from 41563.89 ± 5359.58 at zero hour to 56611.11 ± 8236.55 at 24 hours and 86458.33 ± 35433.74 at 48 hours of preservation. This increase in the bacterial load was not significant between zero and 24 hours and 24 and 48 hours, but was significant between 0 and 48 hours of preservation.

However no significant correlation was found between semen quality and bacterial load at any stage of processing and preservation either by freezing or by chilling.