

**THE EFFECT OF PREPUTIAL WASHING AND
ANTIBIOTIC TREATMENT ON BACTERIAL LOAD AND
PRESERVABILITY OF BOVINE SEMEN**

**A
DISSERTATION
SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENT
FOR THE DEGREE OF
MASTER OF SCIENCE
IN
DAIRYING
(LIVESTOCK PRODUCTION AND MANAGEMENT)**

**BY
CHAMPAK BHAKAT
(B.V.Sc. & A.H.)**

**DIVISION OF DAIRY CATTLE BREEDING
NATIONAL DAIRY RESEARCH INSTITUTE
(I.C.A.R.)
KARNAL - 132 001 (HARYANA), INDIA**

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DEDICATED

TO

MY FATHER

**FOR THE UNCOMPROMISING PRINCIPLES THAT
GUIDED HIS LIFE**

MY MOTHER

**FOR LEADING HER CHILDREN INTO
INTELLECTUAL PERSUIT**

**THE EFFECT OF PREPUTIAL WASHING AND ANTIBIOTIC TREATMENT ON
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By

CHAMPAK BHAKAT

A thesis submitted to the National Dairy Research Institute
(Deemed University), Karnal in partial fulfilment of the
requirement for the degree of

**MASTER OF SCIENCE
IN
DAIRYING
(LIVESTOCK PRODUCTION AND MANAGEMENT)**

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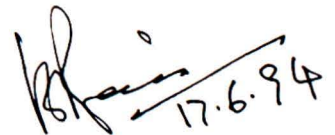
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CERTIFICATE

This is to certify that the dissertation entitled "THE EFFECT OF PREPUTIAL WASHING AND ANTIBIOTIC TREATMENT ON BACTERIAL LOAD AND PRESERVABILITY OF BOVINE SEMEN" submitted by CHAMPAK BHAKAT in partial fulfilment of the requirement for the award of the degree of MASTER OF SCIENCE in DAIRYING (LIVESTOCK PRODUCTION AND MANAGEMENT) of the National Dairy Research Institute (Deemed University), Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.



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LIST OF ABBREVIATIONS

ADF	:	After deep freezing
AI	:	Artificial insemination
AM	:	Morning
ANOVA	:	Analysis of variance
AV	:	Artificial vagina
b	:	billion
DDF	:	Before deep freezing
CD	:	Critical difference
cfu	:	Colony forming unit
cm	:	Centimeter
CR	:	Conception rate
DF	:	Deep freezing
DW	:	Distilled water
EYC	:	Egg yolk citrate
g	:	Gram
h	:	Hour
ISI	:	Indian Standards Institute
IU	:	International Unit
lb	:	Pound
LN2	:	Liquid nitrogen
mg	:	Milligramme
Min	:	Minute
ml	:	Millilitre
MSS	:	Mean sum of square
NR	:	Non-return rate
NRC	:	National Research Council
NS	:	Neat semen
P1	:	At 0 hour
P2	:	After 24 hr
P3	:	After 48 hr
PBS	:	Phosphate buffer saline
PVA	:	Polyvenyl alcohol
PW	:	Preputial wash
Sec	:	Second
SP	:	Streptomycin and penicillin G. sodium combination
SPamp	:	Streptomycin, penicillin G. sodium and ampicillin combination
SPC	:	Standard plate count
SPG	:	Streptomycin, penicillin G. sodium and gentamicin combination
TBC	:	Total bacterial count
TDA	:	Tryptone dextrose agar
ug	:	Microgramme
um	:	Micrometer
UV	:	Ultraviolet
WPW	:	Without preputial wash
W/V	:	Weight/volume
\pm	:	Standard error
%	:	Per cent
°C	:	Degree celsius

CHAPTER 1

INTRODUCTION

1. INTRODUCTION AND IMPORTANCE OF WORK

India is predominantly an agriculture country. The care and keeping of livestock has traditionally been a part of Indian life.

It has been now realised that for the rapid improvement in the milk production, artificial insemination (AI) is an indispensable tool. Proliferation of superior male germ plasm is possible as one normal ejaculate of bull semen could be used for about 200 inseminations. If the bull is not properly screened for pathological problem or the handling of the semen during its processing is unhygienic, the method of AI would result into producing artificial infection. Further, the extender used for extension of semen is rich in nutrients most suitable for the growth of microorganisms.

Microbial contamination of semen may affect spermatozoa directly or may compete for substrate in semen extenders and can infect the inseminated females. This could lead to vulvitis, vaginitis, endometritis, pyometra, lowered conception rates and increased embryonic mortality, abortion and other complications in female.

Preputial cavity is said to be main source of contamination in semen. Therefore, washing of preputial cavity prior to

collection of semen to prevent microbial contamination has been advocated by many investigators. Unhygienic surroundings, order of ejaculate, repeated entry of penis into AV, age of bulls, improper handling of semen and inflammatory condition of reproductive system were reported to influence adversely the bacterial load of semen. Even under best conditions, some microbial contamination can occur.

The generally accepted norms for microbial population in semen are 500 non-pathogenic microorganism per dose of semen (ISI). This was also proposed by Ostaszko (1976) to the International Organisation of Biological Standards to fix the maximum permissible limit for 500 non-pathogenic organisms per insemination dose for satisfactory conception. The proposal, however, was not accepted. No rigid standards with regard to bacterial numbers exist in U.S.A., U.K., Switzerland, Sweden and Australia. The accent in these countries is to produce semen from specific pathogen free sires and to observe maximum hygienic precautions in semen processing. Bartlett *et al.* (1976) stated that under practical conditions, elimination of microbial contamination completely from frozen semen was not practicable and some contamination with non-pathogenic microorganisms was unavoidable and its importance should not be over-estimated. Under these circumstances, there is need for reconsideration by ISI regarding its stipulation limiting 500 non-pathogenic organism as the maximum permissible limit per insemination ~~dose~~ as long as the semen is free from specific pathogens.

However, this does not mean advocacy of any severity on the hygienic measures to be adopted in semen processing. Also on occasional checking of the frozen semen samples for their total bacterial content will go a long way in monitoring the level of contamination in semen and to incorporate corrective measures in semen processing.

With the present trend of shipping the diluted semen over greater distances and inseminating animals after the diluted semen has undergone longer periods of storage, the microbial count is bound to rise. Other factors, in addition to the original quality may become increasingly important in maintaining high levels of fertility. One such factor is bacteriological control.

During the past decade, knowledge on *in vitro* preservation of semen has burgeoned to a greater diversity and revealed useful know-how in semen processing. The problem associated with the presence of bacteria in bull semen used for artificial breeding has received increased attention. Special emphasis has been placed on the use of various antibacterial agents including Penicillin, Streptomycin and various sulfonamides for controlling the bacteria which are always found in the semen of bulls with increasing application of AI in the field of dairying and animal husbandry.

Practically, all workers in the rapidly expanding field of AI stress the necessity of bacteriological control. It,

therefore, seems desirable to know definitely what measure of bacteriological control is necessary or desirable in the collection, handling of storage of semen.

To combat the microorganisms growth in semen, different antibiotics are routinely used. In most of the frozen semen production centres in India and world over, it is a routine practice to add penicillin and streptomycin to extender to control bacterial load in semen.

Penicillin and streptomycin have long been used as semen additives in order to control the microbial growth. There are some findings which suggest that these antimicrobial agents are not fully effective or unaffactive after addition of extenders.

Most of the available information on the bacteria of the reproductive tract or semen of bulls deals with the incidence and spread of disease or with possible causes of sterility of bulls with poor breeding records. Little attention has been given to the subject in relation to the preservation of semen.

Therefore, in the present study, an endeavour has been made to study the effect of preputial washing coupled with antibiotics fortification on preservability of semen. For the purpose, following specific objectives were earmarked for the study:

1. Effect of preputial washing on bacterial count of semen.
2. Effect of antibiotics on bacterial count of semen.
3. Effect of different antibiotics on preservability of semen.

CHAPTER 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

With a view to funnel the existing information available on the topic, the review of literature is being presented under relevant segments as follows:

2.1 PREPUTIAL WASHING AND BACTERIAL LOAD

Preputial cavity is said to be one of the main source of contamination in semen (Reddy *et al.*, 1971; Chandra and Singh, 1961). Ball (1990) observed in bacteriological studies of 586 saline preputial washings of bulls that samples content *Ureaplasma* sp., *M. bovisgenitatum*, *M. canadense*, *Acholeplasma laidlawii*.

Foot *et al.* (1962) cleaned the sheath and underline of the bull and placed a small apron under the chest of the bull. A polyurethane device was fixed at the entrance to the A.V. These measures effectively reduced contamination in the semen at the time of semen collection.

Sokolovaskaja (1962) found a significant reduction in bacterial count in ram semen on intrapreputial wash. He again reported that when insufficient care was taken in handling of samples of ram semen, the microbe content was in the range of 2,80,000 to 120 million/ml. In 24 test ejaculate, it averaged

1.179 million/ml. Washing of prepuce resulted into 20 per cent reduction in microbial load. When a sterile covering was used to prevent contact between the wool of male penis and female, reduction was to the level of 73 per cent. When a sterile covering was fitted round the penis, microbial count was lowered by 83 per cent. All the 3 measures, when practiced, resulted in reducing microbial load to as low as 98 per cent as compared to control. Zamjanis (1962) flushed the preputial cavity of bull just before semen collection to reduce the bacterial count in bull semen.

Hashimoto *et al.* (1963) flushed the intrapreputial region with antibiotic or with antiseptic solution just before semen collection and found that it significantly ($P < 0.05$) reduced the bacterial count in semen. Hashimoto (1966) further conducted an experiment using preputial douche prior to collection which resulted in marked decrease in bacterial contamination of semen.

Prasad *et al.* (1985) washed the preputial cavity with one of the four solutions of antibiotics (benzyl penicillin and/or streptomycin, oxytetracycline) just before collection of semen which resulted into reduction in the number of bacteria in the semen by 61 to 77 per cent.

Moroz *et al.* (1986) suggested that excision of the preputial diverticulum for reducing the risk of *Pseudomonas* contamination. Alternatively, the diverticulum could be disinfected regularly with 3 per cent chloramine solution.

Dagnall (1987) found that the predominant bacteria in the preputial diverticulum of pig were *Proteus* sp., *E. coli*, *Streptococci*, *C. suis*, *C. pyogenes* and *Actinomyces* sp., *Actinobacillus* sp. Penicillin and streptomycin were recommended by him for controlling these contaminations.

Jones (1984) reported that cleaning the preputial region of a stallion with water alone or with soap and water (applied gently with a gloved hand) was indicated before mating or semen collection to reduce the bacterial contamination of the area. It did not, of course, have any effect on bacteria in the semen or in the urethra.

2.2 ANTIBIOTICS AND BACTERIAL LOAD IN SEMEN

Bacteria which are present in semen has deleterious effect on fertility of spermatozoa (Sakale *et al.*, 1961; Roberts, 1971). Place and Kasde (1961) concluded that reduced fertility of bull semen was as a result of contamination with *C. pyogenus*. Meding (1964) studied the semen microflora and found negative correlation between the quality of semen and fertility adversely.

2.2.1 STREPTOMYCIN AND PENICILLIN COMBINATION

Semkov (1975) took 48 ejaculates obtained from 6 bulls, the experimental samples treated with streptomycin and penicillin, 30,000 IU each. Microbial count was of two types of experimental

samples and was highly significantly lower than that of the control samples. However, Wierzbowski *et al.* (1984) did not find any relationship between the concentration of Penicillin (0.5 to 0.3733 IU/ml) and streptomycin (150 to 1,000 ug/ml) and degree of bacterial contamination.

Gangadhar *et al.* (1986) showed that the direct addition of streptomycin in neat semen lead to reduced bacterial load of frozen semen. He again reported that combination of chloramphenicol (250 ug/ml), kanamycin (250 ug/ml), oxytetracycline (100 ug/ml) and the combination of chloramphenicol (250 ug) and either oxytetracycline (100 ug) or kanamycin (250 ug) significantly reduced the number of bacteria but maximum reduction without compromising motility was with chloramphenicol and kanamycin.

Hardjoutomo (1990) concluded that the dose rate of the penicillin-streptomycin added to the diluent routinely used by the Lembang Artificial Insemination Centre was effective against semen bacterial contaminants.

Albertsen (1957) showed that the marked effectiveness of streptomycin against *B. abortus* and *C. pyogenes* was greatly diminished by egg yolk. He recommended incubating the semen with antibiotic for 15 min at 37°C or for 60 min at 30°C before adding egg yolk. On the other hand, Morgan *et al.* (1959) reported that streptomycin treatment of semen extended with egg yolk was ineffective in controlling the spread of infection by AI whereas in milk it was effective.

2.2.2 GENTAMICIN COMBINATION

Rahman (1983) reported that gentamicin, kanamycin and neomycin were the most effective antibiotics against the *S. aureus*, *S. epidermidis*, *E. coli*, *Prateus*, sp., *Klebsiella aerogenes*, *Streptococcus* sp., *P. aeruginosa*, *Bacillus* sp., while penicillin and streptomycin were the least effective.

Stoyanov (1989) reported that the antibiotic most effective against bacteria was gentamicin, followed by penicillin + streptomycin and kanamycin. The bacteria in frozen goat semen were *Proteus mirabilis*, *S. aureus*, *E. coli*, unspecified bacilli and streptococci as found by Sharma et al. (1986). Most of these bacteria were sensitive to gentamicin at 10 ug/ml. Moroz et al. (1986) decontaminated boar semen contaminated with *Pseudomonas aeruginosa* by adding gentamicin at 0.3 per cent.

Saikia et al. (1987) worked out that the organisms which were found in bull semen were highly sensitive to gentamicin (100%) followed by kanamycin (94.59%), neomycin (89.18%) and ampicillin (83.78%). Least effective drugs were bacitracin (0%), polymyxin B (27%), penicillin (10.81%) and streptomycin (45.94%).

Stoyanov (1987) considered that the most effective antibiotic for adding to semen was gentamicin and he reported that the better efficacy of gentamicin over streptopenicillin in controlling bacteria in frozen semen of bull. The same worker

(1989) concluded that the antibiotic most effective against *Compylobacter fetus* and *P. aeruginosa* was gentamicin followed by penicillin, streptomycin and kanamycin.

Gentamicin can control the bacterial growth in the semen sample completely without affecting the semen quality after equilibration at different intervals of preservation and cryopreservation (Ahmed *et al.*, 1989). Nimai Singh *et al.* (1990) found that 94 per cent of the Gram-positive isolates and 96 per cent of Gram-negative isolates were sensitive to chloramphenicol and gentamicin, respectively. He further reported that semen samples to which chloramphenicol and gentamicin were added had significantly lower bacterial counts than those to which benzylpenicillin and streptomycin or no antibiotics were added. Wayda (1991) suggested that gentamicin in combination with penicillin should be used for preparation of diluent for cryopreservation.

2.2.3 AMPICILLIN COMBINATION

There are not many reports available on use of ampicillin as an antibacterial agent in semen.

Golikov *et al.* (1985) reported that 22 strains of *Compylobacter fetus* subsp. *fetus* and 12 strains of subsp. *intestinalis* were most sensitive to a combination of ampicillin and dihydrostreptomycin (0.00125 - 0.011 and 0.027-0.225 ug/ml, respectively), gentamicin 100 per cent sensitive to microflora

which were present in semen. This was followed by kanamycin (94.59%), neomycin (89.18%) and ampicillin (83.78%) (Saikia *et al.*, 1987).

2.2.4 OTHER ANTIBIOTICS COMBINATION

Salisbury *et al.* (1947) added sulfonamide in the dilutor which gave a significant increase in fertility. The addition of polymyxin B has been reported by Elliott (1962). He found increased fertility of frozen semen by its addition. Hamdy *et al.* (1971) had combined lincomycin and spectinomycin to provide bacterial control. This combination alongwith clindamycin is specifically effective against mycoplasma. Shin *et al.* (1988) evolved the combination of gentamicin (500 mug/ml), tylosin (100 mug/ml) and lincospectin (300/600 mug/ml) which was more effective to control the bacteria of bull semen than the standard combination of penicillin, dihydrostreptomycin and polymyxin B sulfate. On the other hand, Pfutzner (1989) reported that lincospectin was capable of inactivating the mycoplasma without adversely affecting spermatozoa.

2.3 ANTIBIOTICS AND SPERMATOZOA

2.3.1 PENICILLIN-STREPTOMYCIN COMBINATION AND SPERMATOZOA

Almquist (1948) evolved that penicillin markedly improved the fertility of semen from certain relatively infertile bulls although an earlier report by same worker showed that penicillin

did not increase the fertility of semen from bulls of relatively high breeding efficiency (Almquist, 1946). Sykes et al. (1951) used the levels as high as 4000 units of penicillin and 8,000 ug/ml of streptomycin or its dihydro form and found these to be harmless for spermatozoa.

In England, Melrose et al. (1958) concluded that 3.4 per cent increase ($P < 0.001$) in fertility when semen from *V. fetus* free bulls was treated with 1,000 ug of streptomycin/ml. The use of 10,000 IU tetracycline/100 ml or 30,000 IU penicillin + 30,000 IU streptomycin + 0.12 IU streptocide/100 ml diluent resulted in a CR of 71 per cent in 8,462 cows and 64.8 per cent in 8,645 cows, respectively (Velieko, 1965), but Golubeva (1970) found that a dose of 50,000 IU streptomycin reduced motility and resistance. A dose of 1,00,000 IU was almost lethal and doses of 2,50,000 IU or 5,00,000 IU further tried were lethal for spermatozoa. Codazza et al. (1971) indicated that tetracycline did not adversely affect sperm survival whereas streptomycin, chloramphenicol, rifamycin and idoform reduced survival time.

^{ga}
Gandhar et al. (1986) reported that a combination of chloramphenicol (250 ug/ml), kanamycin (250 ug/ml) and oxytetracycline (100 ug/ml) reduced motility of sperm by 3 per cent before freezing, 15 per cent after freezing and 18 to 20 per cent after storage for 3 months in liquid nitrogen. He concluded that combination of penicillin, streptomycin, chloramphenicol and either oxytetracyclin or kanamycin did not affect the motility. On the other hand, Ahmed et al. (1989) found that there was no

difference between antibiotics treatment like penicillin (800 IU) and streptomycin (1,000 ug/ml) or gentamicin (500 ug/ml) and between bulls in respect of sperm motility.

2.3.2 AMPICILLIN COMBINATION AND SPERMATOZOA

Duijn *et al.* (1974) added ampicillin to egg yolk citrate diluent which increased the longevity of bovine spermatozoa *in vitro* as compared to that of penicillin + streptomycin sulphate and with doxycycline hyclate.

2.3.3 GENTAMICIN COMBINATION AND SPERMATOZOA

Back *et al.* (1975) worked out the levels of up to 2,500 ug/ml of gentamicin sulphate, lincomycin, nalidixic acid, polymyxin B sulphate or Na-P-G had no detrimental effect on sperm motility. From these results it is suspected that most of these antibacterial agents are fairly innocuous to equine spermatozoa and that adequate levels can be added to extended semen to control pathogenic organism. Similarly, Salisbury *et al.* (1978) found that chloramphenicol and gentamicin were non-toxic to the spermatozoa at the dose level 1,000 ug/ml each, but Stoyanov (1987) observed that the conception rate of cows inseminated with semen containing gentamicin was 15 per cent higher than when penicillin + streptomycin was used. Again, Sullivan *et al.* (1988) reported that there was no significant difference for NR rates between 3 antibiotic combinations, e.g. (i) Gentamicin, tylosin, lincospectin; (ii) Penicillin, dihydrostreptomycin,

polymixin B sulphate, lincospectin, and (iii) Penicillin, dihydrostreptomycin, polymixin B sulphate

Danowski *et al.* (1989) used that gentamicin, kanamycin and neomycin at the rate of 1 to 10 ug/ml without having an adverse effect on spermatozoa. Semen treated in this way could be kept for 5 days at 17°C. Panda *et al.* (1990) reported a significant difference among treatments in sperm motility and live spermatozoa percentage at 0, 24 and 48 h of storage. The antibiotics treatment were gentamicin (250 ug/ml), chloramphenicol (500 ug/ml), ampicillin (500 ug/ml) or penicillin (250 IU) + streptomycin (5 ug/ml). Sperm motility characters were higher in extenders containing gentamicin and chloramphenicol than in those containing the other two antibiotics.

2.3.4 OTHER ANTIBIOTICS COMBINATION

Easterbrooks (1951) observed the oxytetracycline (20 ug/ml), chlortetracycline (50 ug/ml) and chloramphenicol (500 ug/ml) in individual tests were ineffective in raising fertility. Similarly, lincospectin upto 4,500 ug/ml which did not affect sperm motility or acrosomal integrity (Waberski *et al.*, 1990).

Kuroda *et al.* (1973) used seven water soluble antibacterial agents and observed sperm viability and motility which were almost the same immediately after their addition as before. He concluded the spiramycin and terramycin had toxic effects on spermatozoa. Addition of non-spermicidal broad spectrum anti-

biotics promoted the sperm survival in a study conducted by Hovorka (1984).

Salisbury *et al.* (1947) used antibacterial agent like sulfanilamide which depressed the rate of sperm metabolism, increased sperm liveability and partially inhibited bacterial growth. Bonaga *et al.* (1971) used the bacteriostatic levels of neomycin (1500 mg/ml) and sulphanylamide (3000 mg/ml) and observed that these were least toxic to spermatozoa. Henry (1944) found that with proper variation of the factors influencing sulfonamide action inhibit the metabolism of cells of nearly every variety and that certain sulfonamides increased the liveability and fertility of bovine spermatozoa.

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

Six disease free bovine bulls of same age (3-5 years), belonging to A.I. section, NDRI, Karnal were used as semen donors. The bulls were maintained under identical system of management and fed as per N.R.C. standards. Semen collection was done in the morning hours after the bulls were thoroughly washed at least 15 minutes before collection.

Preputial washing was given by the antibiotic solution. Procaine penicillin @ 1,000 IU/ml of distilled water was used for this purpose. Hairs around the preputial orifice were clipped when required. Outer skin surface was cleaned with water. 50 ml antibiotic solution was introduced by the help of catheter and orifice was closed with a rubber band. After introducing solution, the preputial cavity region was massaged with hand. After 10 minute interval band was removed to allow the solution to come out from the cavity. Preputial washing was carried out in all the six bulls. A week before preputial washing, ejaculate from the same bull was taken as control.

3.1 PROCESSING OF SEMEN

3.1.1 SEMEN COLLECTION

Semen was collected from bulls by artificial vagina technique (Walton, 1945) between 7.30 AM to 8.30 AM (January - June). Semen was collected from each bull at weekly intervals.

In each and every step, efforts were made to maintain strict sterilised condition, AV was sterilised by AV steriliser (steam sterilisation) for 40 minute. Glass ware sterilised in hot air oven at 110°C for over night. Surface sterlisation was done by exposure to UV rays.

3.1.2 ASSESSMENT OF VOLUME, COLOUR AND MASS ACTIVITY

Immediately after collection, ejaculate was subjected to gross evaluation. Volume, consistency and colour, etc. were noted.

3.1.2.1 Mass activity

A drop of semen was placed on warm (37°C) clean slide and examined under low power microscope. On the basis of swirling current, semen was rated into five categories and given numerical grading (0 to +5) as per Tomar *et al* (1966).

3.1.2.2 Sperm concentration

Sperm concentration was calculated by Klett Summerson colorimeter by recording optical density of semen. For this, 4.9 ml of sodium citrate (2.9%) buffer was taken in Klett tube. 0.1 ml of semen was added and mixed by inverting the tube. Klett reading was noted using blue filter (wave length, 400-430 um). A ready reckoner was prepared on the basis of which sperm concentration was determined.

3.1.3 PREPARATION OF SEMEN EXTENDER

Egg yolk citrate diluent was used according to Salisbury *et al.* (1941). 2.9 per cent sodium citrate buffer was taken and pH adjusted to 6.8. It was autoclaved at 121°C, 15 lb pressure for 20 minutes. Fresh egg from disease free hen were used. Before using the egg, outer shell surface was cleaned with alcohol. The standard formula is:

Sodium citrate	-	2.9% w/v
Fructose	-	1.0% w/v
Double distilled water	-	1000 ml
Buffer solution	-	80%
Egg yolk	-	20%

To maintain the strict sterilized condition of EYC dilutor, some points should be kept in mind:

- i) 2.9 per cent sodium citrate buffer was properly autoclaved (121°C, 15 lb, 20 min) before it's use.
- ii) Egg was taken from healthy and disease free hen.
- iii) Before using the egg, outer shell surface of it should be sponged with alcohol.
- iv) All glasswares, forceps, filter paper, etc. necessary for dilutor preparation should be properly sterilized.
- v) After preparation of dilutor and before extension of semen, the opening of container should be properly closed.

3.1.3.1 Dilution and antibiotics treatment

Dilution rate for bull semen was fixed on the basis of sperm concentration and per cent motile spermatozoa.

Different combination of antibiotics were added to the EYC dilutor and these were used to dilute the semen samples.

Combination of antibiotics -----	Dose -----
1. Streptomycin + Benzyl Penicillin (Na-salt)	Streptomycin @ 1000 ug/ml of extender Benzyl Penicillin @ 1000 IU/ml of extender
2. Streptomycin + Benzyl Penicillin (Na salt) + Gentamicin sulphate	Gentamicin @ 1000 ug/ml of extender
3. Streptomycin + Benzyl Penicillin (Na-salt) + Ampicillin	Ampicillin @ 1000 ug/ml of extender

All these processes were carried out in contamination free air zone by using spirit lamp. Lab was fumigated at weekly interval.

3.2 ASSESSMENT OF DILUTED, ANTIBIOTIC TREATED SEMEN

Microscopic assessment was carried out for different antibiotic treated, diluted, chilled and freeze-thawed semen.

3.2.1 INDIVIDUAL MOTILITY OF SPERMATOZOA

Individual motility of spermatozoa was observed by placing a small drop of diluted semen on clean, dry warm (37°C) slide and a coverslip was placed on it. The percentage of progressively motile spermatozoa was examined under low power (200 X) of phase contrast light microscope. Progressive forward moving spermatozoa were considered for estimating per cent motile spermatozoa.

3.2.2 ENUMERATION OF LIVE AND DEAD SPERMATOZOA (EOSINOPHILIC)

3.2.2.1 Preparation of stain

The method recommended by Blom (1950) and Hancock (1951) was used. The eosin-nigrosin stain was prepared in sodium citrate buffer (2.9%) of 6.8 pH. The composition of stain was as follows:

Eosin	-	100 mg
Nigrosin	-	500 mg
Sodium citrate buffer	-	10 ml

3.2.2.2 Staining technique

One drop of semen sample was mixed with 2 to 3 drops of eosin-nigrosin stain on clean, dry and warm (37°C) slide. A thin smear was prepared. About 200 spermatozoa were counted in different microscopic fields. Spermatozoa which were non-eosinophilic were taken as live sperm.

3.2.3 ENUMERATION OF MORPHOLOGICALLY ABNORMAL SPERMATOZOA

The slide which was prepared for live and dead count was also used for enumerating abnormalities. The type of abnormalities studied were according to the work of Lagerlof (1934) as given below:

1. Head abnormalities
2. Midpiece abnormalities
3. Tail abnormalities
4. Neck abnormalities

3.2.4 ACROSOMAL INTEGRITY

For staining the acrosome, the method of Hancock (1952) was used. A thin smear prepared from the sample was dried in the air and kept in 5 per cent formaldehyde solution for 30 minutes at 37°C. Then the slides were washed in water and air dried. The staining solution was prepared with the following composition:

Giemsa stain	-	1 g
Methanol	-	66 ml
Glycerol	-	60 ml

They were mixed well and kept overnight and filtered. From this the staining solution was prepared by mixing 3 ml of the

stock solution with 2 ml of phosphate buffer saline (pH 7.2) and 45 ml of carbon dioxide free water.

The slides were kept in staining solution for ³8 hours at 37°C. The stained slides were washed with water dried and examined under microscope. The number of intact and abnormal acrosomes were counted for two hundred spermatozoa.

3.3 STANDARD PLATE COUNT (SPC)

Semen samples were brought to the Dairy Microbiology lab in ice bucket for SPC.

3.3.1 PREPARATION OF MEDIA FOR ENUMERATION OF TOTAL BACTERIAL COUNT

3.3.1.1 Tryptone Dextrose Agar media

Trypton Dextrose Agar media was used as the plating medium for determining the total bacterial count (ISI, 1962).

Composition

		(in grams)
Tryptone	-	5.0
Yeast extract	-	2.5
Dextrose	-	1.0
Agar	-	15.0
Distilled water	-	Added to 1 litre
pH 7.0		

The medium was sterilized by autoclaving at 121°C, 15 lb pressure for 20 min.

3.3.2 SERIAL DILUTION OF NEAT SEMEN

Before culturing the bacteria in neat semen, serial dilution was done by using the normal physiological saline (0.9%). Saline solution was autoclaved at 121°C, 15 lb pressure for 20 min.

Before culturing the bacteria in petri plates, glass were properly sterilised and cabinete was sterilised by UV ray for 30 min. Platting was done at different time interval (0 hr, 24 hr and 48 hr interval) for chilled semen and for frozen and thawed semen.

3.3.3 POUR-PLATE TECHNIQUE

The number of living bacteria or groups of bacteria in TDA media was counted by a cultural method such as the pour plate method.

Neat semen was diluted by serial ten-fold dilutions and for antibiotic treated diluted semen sub-samples, direct platting was done.

With a sterile 1 ml delivering pipette, 1.0 ml of neat semen was transferred into first tube of diluent and thoroughly mixed. From the first tube, 1 ml was added into next tube of diluent with a fresh sterile 1 ml pipette. Made the remaining dilutions

in the same way, using a fresh pipette for each (Cruickshank, 1970).

One ml diluted neat semen and antibiotic treated semen was taken into petriplate and in each plate about 15 to 20 ml of clear TDA, melted and cooled to 45 to 50°C was poured. At once mixed by rapidly moving the plate in circular movements in different direction for 10 sec, taking care not to spill any of the contents. Allowed the agar to set and incubated in inverted position for 2 days at 37°C.

The colonies were counted in different plates and multiplied with dilution factors.

3.3.4 MICROSCOPICAL OBSERVATION OF COLONIES

To see the different colonies forming organism sub-culturing was done by taking the colony with a sterile platinum loop and incubate into tryptone dextrose broth and further incubate for 48 hrs.

Composition of tryptone dextrose broth (ISI,1962)

		(in grams)
Tryptone	-	5.0
Yeast extract	-	2.5
Dextrose	-	1.0
Distilled water	-	1 litre
pH		7.0

(Autoclaved at 121°C, 15 lb pressure for 20 min)

Gram staining technique

One drop of suspension was taken on sterile slide, it was spread, air dried and heat fixed. The slide was stained with crystal violate for 1 min and washed with water. Poured lugol's iodine for 1 min and washed with alcohol. Then counter stained with saforine for 40 to 45 sec and washed with water and observed under oil immersion lens.

3.4 PRESERVATION OF LIQUID SEMEN AT REFRIGERATED TEMPERATURE

Each semen sample split into 3 parts for treating with 3 different antibiotic combinations which were mixed with EYC dilutor. The diluted semen sub-samples were preserved in lab in refrigerator at 5°C. The preserved semen sub-samples were subjected to detailed microscopic tests and TBC. All parameters were repeated at different time intervals. At 0 hr (just after dilution) after 24 and 48 hrs.

3.5 PRESERVATION OF SEMEN IN DEEP FREEZING

Semen samples were split and diluted with different combinations of antibiotic treated dilutor in same way as described before. Deep freezing technique was as follows (Raina, 1987).

(i) Glycerolisation

For the freezing purpose, the diluent was divided into two parts. Both parts A and B were kept at 30°C. Part A was mixed with semen and Part B was mixed with glycerol at the rate of 7

per cent of total diluent. All these processes were done in contamination free air zone in front of spirit lamps.

(ii) Both the parts A and B were cooled from 30°C to 5°C by keeping it in cold cabinet. When both parts reached 5°C, they were mixed together. Thus, the process of glycerolisation was completed.

(iii) Filling and sealing of straws:

Clipped French mini straws of 0.25 ml, bubbler-cum-bath, polyvenyl alcohol (PVA) powder and other accessories required for this purpose were sterilised under UV rays. After sterilisation, all were brought down to 5°C temperature. Separate bubbler-cum-baths were used for different sub-samples to avoid contamination. The filling was done by using a suction pump and the straws were held in a comb specially designed for this purpose. The air space was created in the straws using a bubbler device. The straws were sealed with polyvinyl alcohol powder.

(iv) Equilibration time:

The sealed straws were kept in water bath at 5°C for four hours.

(v) After completion of equilibration time, the straws were taken out of the water bath and dried with pre-cooled filter

paper and were placed horizontally in freezing rack. The rack along with straws was kept in the liquid nitrogen vapour, 4 cm above the level of liquid nitrogen for 10 minutes.

(vi) Storage:

The straws were transferred into goblets with the help of pre-cooled forceps and the goblets were stored in separate canisters in the liquid nitrogen (LN₂) in cryovessel. Cryovessel was always kept three-fourth full by replenishing liquid nitrogen from time to time.

(vi) Thawing and examination of frozen semen:

Straws were thawed after 24 hrs. The straws immediately after removing from the liquid nitrogen were placed in water bath at 37°C for 11 sec. The straws were picked up from water bath and making outer surface dry. The content of straws was transferred into sterilized test tubes at 37°C. All these transfers of straw contents were done in contamination free air zone in front of spirit lamps. The different sub-samples were examined for preservability study (semen quality) and SPC.

3.6 ASSESSMENT OF BACTERIAL LOAD IN AV, GLASSWARE AND EYC DILUTOR

In sterilised and unsterilised AV, 25 ml of sterilised phosphate buffer saline (PBS) was poured and AV was shaken

vigorously for 3 to 5 minutes after closing the open end by sterilized lid of petri dish and the washing was collected.

Glassware was washed with 10 ml of sterilized PBS and washing was collected. All the washing solutions and 1 ml of EYC dilutor were used for SPC.

3.7 STATISTICAL ANALYSIS

The analysis was carried out by using standard statistical methods as described by Snedecor and Cochran (1981).

3.7.1 MATHEMATICAL MODELS

Chilled semen

For the analysis of effect of antibiotic treatment on SPC and semen quality and PW + antibiotic treatment on SPC

Model I

$$Y_{ijkl} = u + R_i + A_j + P_k + e_{ijkl}$$

where, u = overall mean,

- R_i = effect of i^{th} replication ($i = 1, 2, 3 \dots \dots 24$),
 A_j = effect of j^{th} antibiotic treatment,
 P_k = effect of k^{th} period ($P = 1, 2, 3$), and
 e_{ijkl} = random error

For the analysis of P.W. on SPCModel II

$$Y_{ijklm} = u + R_i + W_j + A_k + P_l + e_{ijklm}$$

where, u = overall mean,
 R_i = effect of i^{th} replication ($i = 1, 2 \dots 24$),
 W_j = effect of j^{th} washing ($W = 1, 2$),
 A_k = effect of k^{th} antibiotics,
 P_l = Effect of l^{th} period ($P = 1, 2, 3$), and
 e_{ijklm} = random error

FROZEN SEMENFor the analysis of effect of antibiotics on SPC and semen quality and PW + antibiotic treatment on SPCModel III

$$Y_{ijkl} = u + R_i + A_j + F_k + e_{ijkl}$$

where, u = overall mean,
 R_i = effect of i^{th} replication ($i = 1, 2, 3, \dots 18$),
 F_k = effect of k^{th} freezing ($F = 1, 2$),
 A_j = effect of j^{th} antibiotic,
 e_{ijkl} = random error

For the analysis of P.W. on SPCModel IV

$$Y_{ijklm} = u + R_i + W_j + A_k + P_l + e_{ijklm}$$

where, u = overall mean,
 R_i = effect of i^{th} replication ($i = 1, 2, 3, \dots, 18$),
 W_j = effect of j^{th} washing ($J = 1, 2$),
 A_k = effect of k^{th} antibiotic,
 F_l = effect of l^{th} freezing ($l = 1, 2$)
 e_{ijklm} = random error

For the analysis of SPC, the data were subjected to log transformation. The differences between treatment were analysed with the help of critical difference test (Steel and Torrie, 1981). Analysis of variance was done at Computer Centre.

CHAPTER 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 PHYSICAL ATTRIBUTES

The physical attributes of collected semen samples were studied. The volume per ejaculation ranged from 2.5-6.5 ml. The mass activity of samples varied from +2.5 to +4.5. The colour of the samples were milky to creamy. Sperm concentration ranged from 7.5 b to 1.5 b per ml.

The above attributes were in the normal range and suitable for processing and preservation. The figures obtained were in conformity with the normal range, described by other workers (Bhosrekar and Nagarcenkar, 1971; Gopalakrishna and Rao, 1978; Singh *et al.*, 1983; Saxena, 1978).

4.2 PRESERVATION AT 4-7°C

4.2.1 INDIVIDUAL MOTILITY OF SPERMATOZOA

4.2.1.1 Antibiotic treatment (WPW)

The individual motility of spermatozoa immediately after dilution (0·h) in three treatments, namely SP, SPG and SPAMP was 67.92 ± 1.35 , 69.46 ± 1.25 , 68.00 ± 1.36 per cent, respectively (Table 1a). The corresponding values were 45.83 ± 1.99 , $45.79 \pm$

1.92, 46.84 ± 1.15 per cent after 48 h of preservation. The overall average motility (%) decreased from 68.46 ± 1.12 to 46.15 ± 1.15 after 48 h of preservation. The overall averages for SP, SPG and SPamp were 56.99 ± 1.43 , 57.79 ± 1.47 and 57.28 ± 1.42 per cent, respectively.

Analysis of variance (Table 1b) showed no significant difference between antibiotic treatment and sperm motility but there were significant differences ($P < 0.01$) due to preservation time and due to replications. The CD test revealed significant difference between two time intervals.

4.2.1.2 PW + antibiotic treatment

The per cent of motile spermatozoa in three treatments, namely SP, SPG and SPamp were 65.83 ± 1.73 , 66.67 ± 1.81 and 65.50 ± 1.72 , respectively at 0 h (Table 1a). After 48 h of preservation, these values were gradually reduced to 44.13 ± 1.52 , 44.46 ± 1.79 and 44.33 ± 1.75 per cent, respectively.

No significant difference was found between antibiotic treatment as far as motility percentage was concerned. As expected, preservation interval showed significant ($P < 0.01$) drop in motility percentage at various observations made as confirmed by CD test.

Aydin *et al.* (1984) concluded that antibiotic did not affect the motility of spermatozoa in concentration of up to 1 mg/ml of

Table 1a. Individual motility (%) of chilled semen at various intervals

	Antibiotics treatment (WPW)				PW + antibiotics treatment			
	P1	P2	P3	Overall average	P1	P2	P3	Overall average
SP	67.92 +1.35	57.21 +1.59	45.83 +1.99	56.99 +1.43	65.83 +1.73	56.46 +2.01	44.13 +1.52	55.47 +1.46
SPG	69.46 +1.25	57.92 +1.56	45.79 +1.92	57.72 +1.47	66.67 +1.81	56.54 +1.97	44.46 +1.79	55.89 +1.52
SPamp	68.00 +1.36	57.79 +1.46	46.84 +2.01	57.28 +1.42	65.50 +1.72	56.08 +1.96	44.33 +1.75	55.33 +1.46
Overall average	68.46 ^a +1.12	57.64 ^b +0.89	46.15 ^c +1.15		66.03 ^a +1.02	56.36 ^b +1.18	44.31 ^c +1.04	

Mean with similar superscripts (a,b,c) do not differ significantly from each other (P < 0.05)

Table 1b. ANOVA of Table 1a

Source of variation	D.F.	M.S.S.	
		Antibiotics treatment (WPW)	PW + antibiotics treatment
Replication	23	443.95**	648.06**
Antibiotics	2	9.89NS	6.02NS
Period	2	9174.03**	8527.63**
Error	188	19.31	11.50
Total	215		

* Significant at P < 0.05; ** Significant at P < 0.01; NS = Non-significant

dilutor. Ahmed *et al.* (1989) found that there was no difference between penicillin (800 IU) and streptomycin (1000 ug/ml) or gentamicin (500 ug/ml) on motility. According to Panda *et al.* (1990) motility was higher in extenders containing gentamicin (250 ug/ml) and chloramphenicol than in those containing ampicillin (500 ug/ml) or penicillin (250 IU) + streptomycin (5 ug/ml) at 0, 24 and 48 h of preservation. The present findings are in agreement with the above observation. However, no beneficial effect of the various antibiotics used was observed in the present study as reported by Panda *et al.* (1990).

A significant drop in motility of chilled semen stored at refrigerated temperature is consistent with the observation of several other workers (Almquist, 1959; Tomar *et al.*, 1961; Mahajan and Sharma, 1963).

4.2.2 NON-EOSINOPHILIC SPERMATOOZOA PER CENT

4.2.2.1 Antibiotic treatment (WPW)

The percentage of live spermatozoa at 0 h in SP, SPG and SPAMP treatment were 76.92 ± 1.12 , 76.88 ± 1.16 and 76.49 ± 1.20 , respectively (Table 2a). After 48 h of preservation, the live spermatozoa were 64.75 ± 1.29 , 64.92 ± 1.38 and 64.46 ± 1.38 , respectively. The value decreased from an average of 76.75 ± 0.68 to 64.71 ± 0.79 up to 48 h of preservation. The overall averages of live count (%) for SP, SPG and SPAMP treatment was 71.13 ± 0.94 , 70.97 ± 0.97 and 70.58 ± 0.98 , respectively

The difference between antibiotic treatment on live count was non-significant but there was significant difference due to preservation ($P < 0.01$) with gradual decrease to live count as the storage time passed. The significant ($P < 0.01$) difference among replications was observed.

These variations when further subjected to CD test, live spermatozoa significantly decreased between two storage period.

4.2.2.2 PW + antibiotic treatment

The values were 73.79 ± 1.0 , 73.75 ± 0.88 and 73.96 ± 1.15 per cent for SP, SPG and SPAMP treated samples, respectively at zero h (Table 2a). After 48 h of preservation, the live spermatozoa decreased in similar trend as before. The overall averages for SP, SPG and SPAMP treatment were 67.68 ± 1.39 , 67.76 ± 0.89 and 67.42 ± 0.97 , respectively. The overall averages at different periods of preservation were 73.83 ± 0.60 , 67.69 ± 0.90 and 61.33 ± 0.06 for 0, 24 and 48 h, respectively.

Statistical analysis revealed that there was no significant effect of antibiotic treatment on live spermatozoa but there was significant ($P < 0.01$) influence of period of preservation on live spermatozoa.

The CD test depicted that there was significant difference among periods of preservation on live count.

Table 2a. Non-eosinophilic spermatozoa (%) of chilled semen at various intervals

	Antibiotics treatment (WPW)				PW + antibiotics treatment			
	P1	P2	P3	Overall average	P1	P2	P3	Overall average
SP	76.92 +1.12	71.71 +1.41	64.75 +1.29	71.13 +0.94	73.79 +1.00	67.79 +1.39	61.46 +1.39	67.68 +0.95
SPG	76.88 +1.16	71.13 +1.48	64.92 +1.38	70.97 +0.97	73.75 +0.88	67.92 +1.29	61.63 +1.29	67.76 +0.89
SPamp	76.49 +1.20	70.83 +1.49	64.46 +1.38	70.58 +0.98	73.96 +1.15	67.38 +1.47	60.92 +1.22	67.42 +0.97
Overall average	76.75 ^a +0.68	71.22 ^b +0.85	64.71 ^c +0.79		73.83 ^a +0.60	67.69 ^b +0.90	61.33 ^c +1.06	

Mean with similar superscripts (a,b,c) do not differ significantly from each other (P < 0.05)

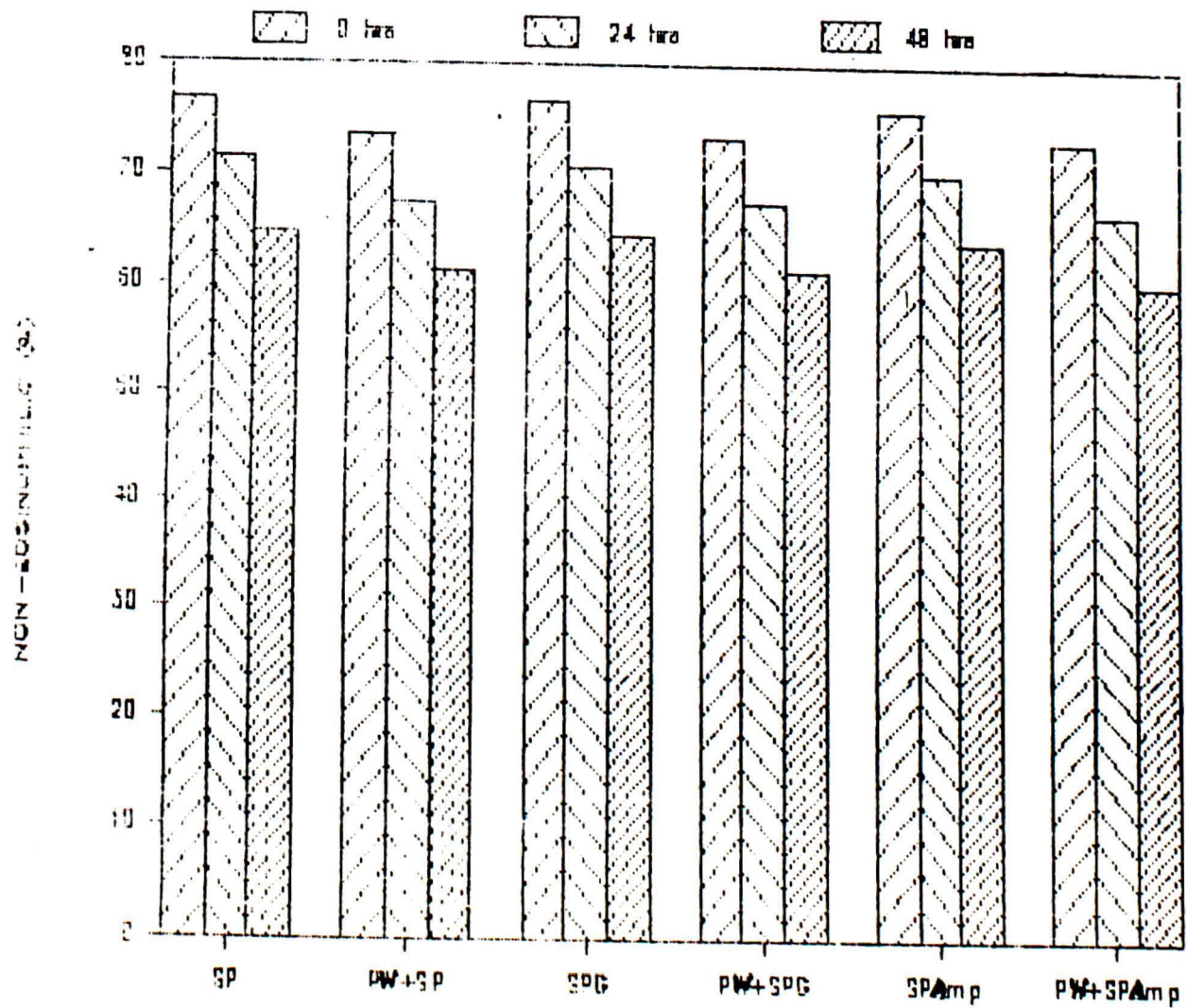
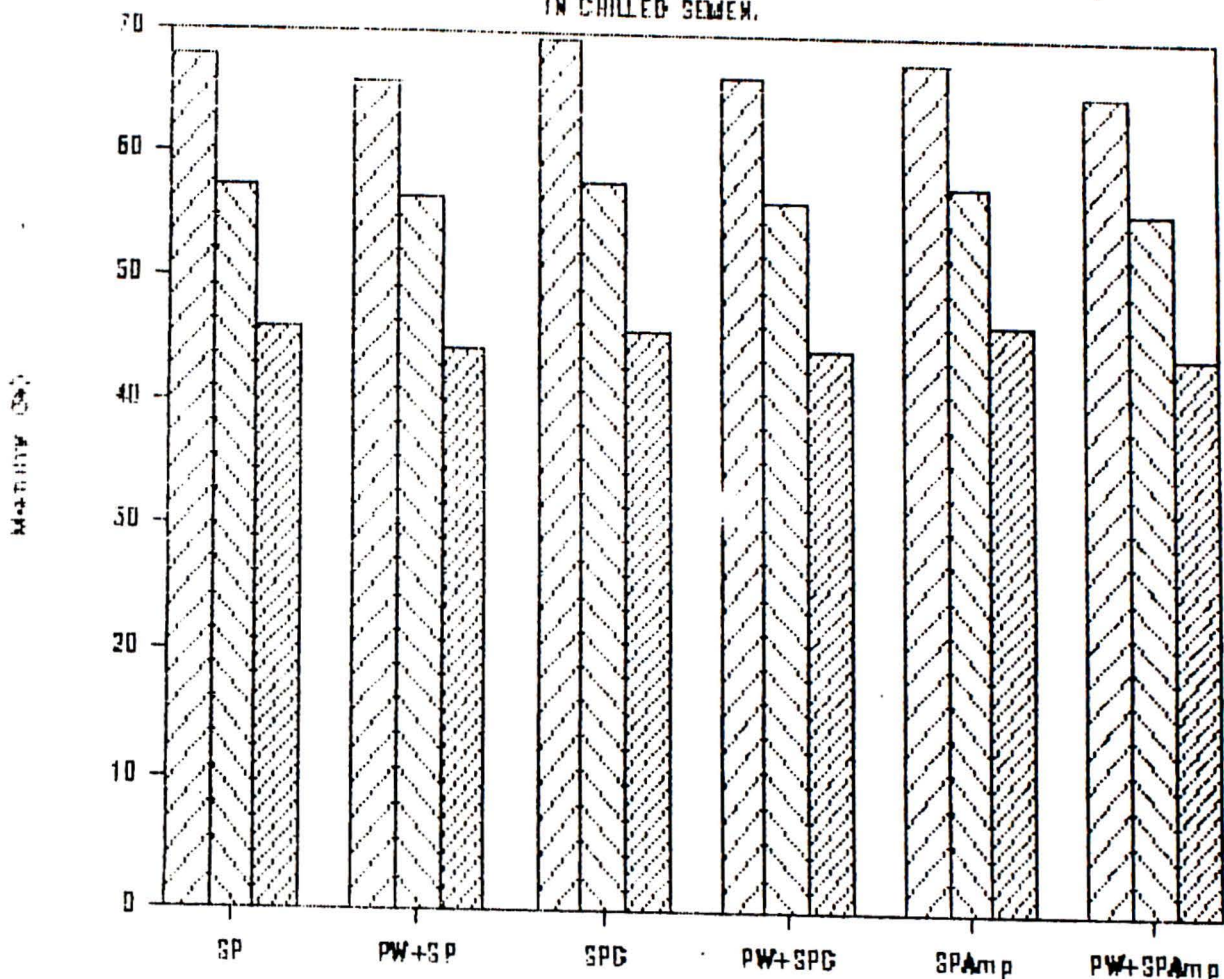
Table 2b. ANOVA of Table 2a

Source of variation	D.F.	M.S.S.	
		Antibiotics treatment (WPW)	PW + antibiotics treatment
Replication	23	351.99**	286.06**
Antibiotics	2	5.62NS	2.37NS
Period	2	2615.87**	2812.80**
Error	188	5.70	7.72
Total	215		

* Significant at P < 0.05; ** Significant at P < 0.01; NS = Non-significant

FIG-1. MOTILITY & NON-EOSINOPHILIC (%)

IN CHILLED SEMEN.



It is clear from the findings that the combination of antibiotics used had no damaging effect on sperm survival. The finding also get substance from the earlier reports of Synes *et al.* (1951). Some workers have reported beneficial effect of certain antibiotics on sperm survival (Duijn *et al.*, 1974; Panda *et al.*, 1990). On the other hand, some lethal effect of different antibiotic treatment on spermatozoa has been reported (Codazza *et al.*, 1971; Bonaga *et al.*, 1971; Golubeva, 1970). However, no such effect was found in the present study for any individual combination of antibiotic. The livability of the spermatozoa has been sustained till 48 h of storage with a decrease in motility by 45.83 ± 1.99 , 45.72 ± 1.92 and 46.84 ± 1.15 for SP, SPG and SPamp treated sub-samples. The figures are appreciably higher than those reported in previous studies (Singh, 1987; Singh, 1990).

4.2.3 SPERM ABNORMALITIES PER CENT

4.2.3.1 Antibiotic treatment (WPW)

The values were 17.33 ± 1.06 , 17.29 ± 1.05 and 17.63 ± 0.90 for SP, SPG and SPamp treated sub-samples, respectively (Table 3a). The corresponding values were 25.08 ± 0.93 , 25.13 ± 0.86 and 25.79 ± 0.87 up to 48 h of preservation. The overall averages gradually increased from 17.42 ± 0.58 to 25.33 ± 0.57 after 48 h of storage. The overall averages of abnormal count

due to SP, SPG and SPamp treatment were 21.17 ± 0.68 , 21.24 ± 0.66 and 21.58 ± 0.65 per cent, respectively.

Statistically, antibiotic treatment had no significant effect on abnormal sperm count. However, abnormal count significantly ($P < 0.01$) increased as preservation time passed.

These variations when further examined by CD test which revealed that abnormalities significantly increased at every interval of storage time.

4.2.3.2 PW + antibiotic treatment

The per cent of abnormal spermatozoa in PW coupled with three antibiotic treatments with SP, SPG and SPamp were 19.79 ± 0.84 , 19.92 ± 0.9 and 19.88 ± 0.84 (Table 3a). An almost similar trend of increment of abnormal count was observed after 48 h of storage as observed in previous case (WPW).

The overall averages for SP, SPG and SPamp treatment were 23.32 ± 0.62 , 23.10 ± 0.65 and 23.14 ± 0.67 per cent, respectively. The overall abnormalities increased from 19.86 ± 0.65 to 26.35 ± 0.59 per cent as preservation time passed.

Statistical analysis showed that antibiotic treatment had no significant effect on abnormal sperm count.

Table 3a. Sperm abnormality (%) of chilled semen at various intervals

	Antibiotics treatment (WPW)				PW + antibiotics treatment			
	P1	P2	P3	Overall average	P1	P2	P3	Overall average
SP	17.33 +1.06	21.08 +0.93	25.08 +0.93	21.17 +0.68	19.79 +0.84	23.46 +0.93	26.71 +0.97	23.32 +0.62
SPG	17.29 +1.05	21.29 +0.91	25.13 +0.86	21.24 +0.66	19.92 +0.90	23.04 +1.00	26.33 +1.10	23.10 +0.65
SPamp	17.63 +0.90	21.33 +0.93	25.79 +0.87	21.58 +0.65	19.88 +0.84	23.54 +1.17	26.00 +1.09	23.14 +0.67
Overall average	17.42 ^a +0.58	21.24 ^b +0.53	25.33 ^c +0.57		19.86 ^a +0.65	23.35 ^b +0.70	26.35 ^c +0.59	

Mean with similar superscripts (a,b,c) do not differ significantly from each other (P < 0.05)

Table 3b. ANOVA of Table 3a

Source of variation	D.F.	M.S.S.	
		Antibiotics treatment (WPW)	PW + antibiotics treatment
Replication	23	171.88**	192.98**
Antibiotics	2	3.59NS	1.00NS
Period	2	1128.59**	758.67**
Error	188	3.28	3.32
Total	215		

* Significant at P < 0.05; ** Significant at P < 0.01; NS = Non-significant

The CD test revealed that period of preservation increased abnormalities significantly at all the intervals.

In the present investigation, abnormalities rose with the passage of time. However, the increment was identical for all antibiotic combinations used in the study. This clearly showed that the sperm morphology was not apparently affected by any of the antibiotic combinations.

The harmless effect of different combinations of antibiotic on sperm morphology, recorded during this investigation, is consistent with the observation of Sykes *et al.* (1951) who also reported that level as high as 4000 units of penicillin and 8000 ug/ml of streptomycin or it's dihydro form were harmless for spermatozoa.

4.2.4 ACROSOMAL INTEGRITY PER CENT

4.2.4.1 Antibiotic treatment (WPW)

The per cent of intact acrosome in SP, SPG and SPamp treated semen samples were 96.17 ± 0.34 , 96.04 ± 0.28 and 95.28 ± 0.28 , respectively at 0 h. Corresponding values were gradually decreased to 91.25 ± 0.47 , 91.21 ± 0.37 and 91.25 ± 0.51 after 48 h of preservation (Table 4a).

The overall averages for SP, SPG and SPamp treated subsamples were 93.60 ± 0.33 , 93.57 ± 0.30 and 93.40 ± 0.32 per

cent, respectively. The overall averages for various time intervals were 96.03 ± 0.25 , 93.31 ± 0.21 and 91.24 ± 0.26 .

Statistical analysis revealed that there was no significant influence of antibiotic treatment on the integrity of acrosome but there was significant ($P < 0.01$) effect of period of preservation on intact acrosome as evidenced from ANOVA table 4b.

From CD test, it was known that integrity of acrosome was gradually reduced in between two preservation time.

4.2.4.2 PW + Antibiotic treatment

The intact acrosome per cent values were 95.54 ± 0.45 , 95.42 ± 0.39 and 95.0 ± 0.42 for the antibiotic treatment, viz. SP, SPG and SPAmP treatment. The overall average corresponding values were 92.92 ± 0.4 , 92.99 ± 0.34 and 92.54 ± 0.37 per cent. The integrity gradually lost during storage of 24 h and 48 h. After 48 h, the values were reached to 90.33 ± 0.67 , 90.5 ± 0.48 and 90.38 ± 0.63 per cent, respectively. The overall average value of intact acrosome decreased from 95.31 ± 0.21 to 90.40 ± 0.25 after 48 h of preservation (Table 4a).

The data when subjected to statistical analysis showed that acrosomal integrity registered a significant ($P < 0.01$) decrease as a result of preservation time. The non-significant effect of various antibiotic combinations on intact acrosome can be evidenced from ANOVA table (Table 4b).

Table 4a. Acrosomal integrity (%) of chilled semen at various intervals

	Antibiotics treatment (WPW)				PW + antibiotics treatment			
	P1	P2	P3	Overall average	P1	P2	P3	Overall average
SP	96.17 +0.34	93.38 +0.38	91.25 +0.47	93.60 +0.33	95.54 +0.45	92.88 +0.44	90.33 +0.67	92.92 +0.40
SPG	96.04 +0.28	93.46 +0.33	91.21 +0.37	93.57 +0.30	95.42 +0.39	93.04 +0.37	90.50 +0.48	92.99 +0.34
SPamp	95.88 +0.28	93.08 +0.37	91.25 +0.51	93.40 +0.32	95.00 +0.42	92.25 +0.45	90.38 +0.63	92.54 +0.37
Overall average	96.03 ^a +0.25	93.31 ^b +0.21	91.24 ^c +0.26		95.31 ^a +0.21	92.72 ^b +0.20	90.40 ^c +0.25	

Mean with similar superscripts (a,b,c) do not differ significantly from each other (P < 0.05)

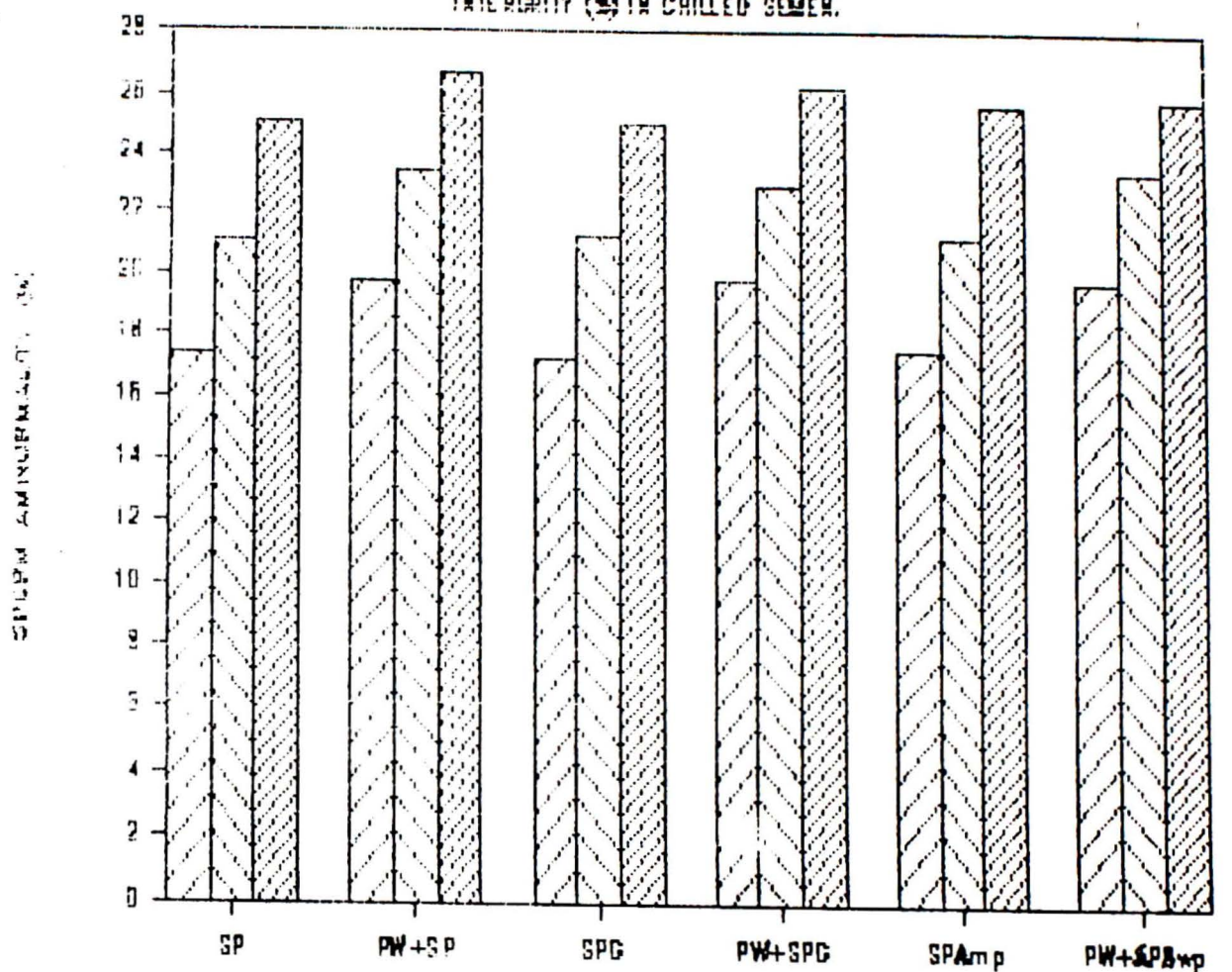
Table 4b. ANOVA table

Source of variation	D.F.	M.S.S.	
		Antibiotics treatment (WPW)	PW + antibiotics treatment
Replication	23	22.60**	33.94**
Antibiotics	2	0.80NS	4.12NS
Period	2	413.84**	435.59**
Error	188	1.20	2.44
Total	215		

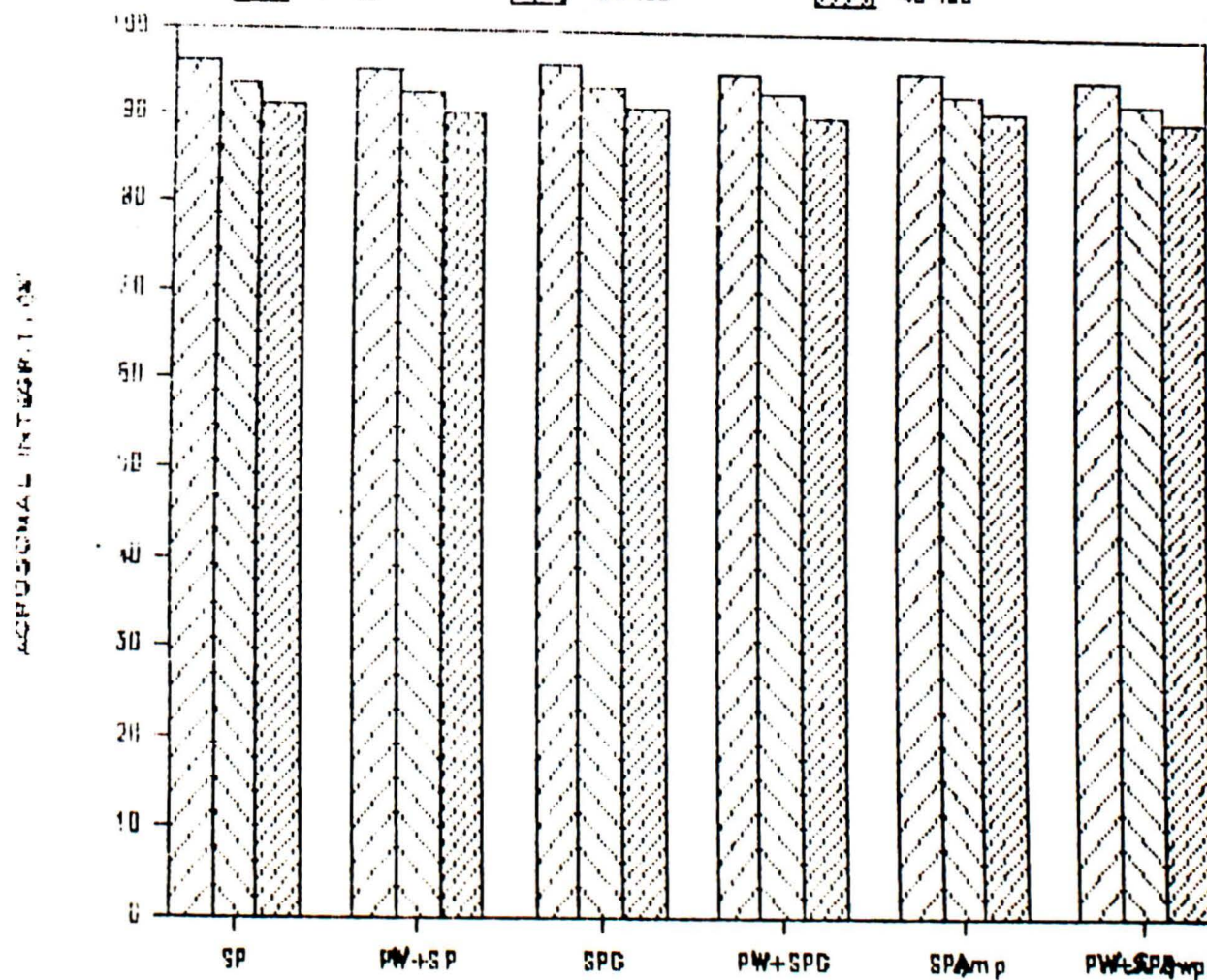
* Significant at P < 0.05; ** Significant at P < 0.01; NS = Non-significant

FIG-2. SPERM ABNORMALITY AND ACROSOMAL

INTEGRITY (%) IN CHILLED SEMEN.



0 hrs 24 hrs 48 hrs



These variations when further examined by CD test could reveal that acrosomal integrity was lost significantly in between each time intervals.

No such study has been reported earlier. However, the acrosomal damage due to preservation at refrigerated temperature observed in the present study is in conformity with various workers (Chinnaiya, 1978) who also reported increased acrosomal damage with the process of semen preservation at 4-7°C temperature.

4.2.5 STANDARD PLATE COUNT

Standard plate counts were determined in antibiotic treated chilled semen subsamples at different time intervals of preservation and the data have been presented in corresponding table.

4.2.5.1 Antibiotic treatment (WPW)

The total bacterial counts at 0 h in four sub-samples, namely NS, SP, SPG and SPAMP were 32,727.08, 1074.58, 523.33 and 798.75, respectively (Table 5a). After 24 h of preservation, the reduction of bacterial count was seen in each sub-samples and after 48 h of preservation further reduction of bacterial count was recorded. The corresponding values of NS, SP, SPG and SPAMP at 48 h of preservation were 1,482.33, 432.08, 229.67 and 239.17.

The data when subjected to statistical analysis showed that bacterial counts registered a significant ($P < 0.01$) decrease after antibiotic treatment. The significant reduction in bacterial counts as a result of preservation time can also be evidenced from the ANOVA table 5b.

These variations when further examined by CD test could reveal that there were significant differences between each treatment combinations and between each combinations of period of preservation as far as bacterial counts were concerned.

In the present study, maximum reduction of TBC was observed in samples treated with SPG followed by SPamp and SP in comparison to NS. Almost a similar trend of reduction was observed in antibiotic treated samples after different period of preservation. Similarly, as a whole maximum reduction was observed at 48 h followed by 24 h as compared with 0 h.

4.2.5.2 PW + antibiotic treatment

The TBC at 0 h in four subsamples, namely NS, SP, SPG and SPamp treatment after preputial washing and antibiotic treatment were 2,812.46, 383.83, 200.63 and 285.88, respectively (Table 5a). However, after 24 h of preservation, a similar trend was observed as was recorded with only antibiotic treatment. Similarly after 48 h of preservation, a further reduction was

seen as evidenced by the lower SPC values, viz. 1,365.00, 178.13, 76.21 and 129.29 in NS, SP, SPG and SPAmP samples.

Antibiotic treatments were highly significant ($P < 0.01$) on TBC and significant difference was also observed due to preservation time and due to replications (Table 5b).

The CD test depicted that there were significant differences between each treatment combinations and between each combinations of period of preservation.

In the present study, maximum reduction of TBC was observed by SPG treatment followed by SPAmP and SP as compared with NS. Same declining trend was seen in each time of preservation and as a whole maximum reduction was observed at 48 h followed by 24 h as compared with 0 h.

The SPC data generated from this study clearly indicated that the antibiotic treatment significantly reduced the TBC in chilled semen sample and the counts reduced with the storage period. The reduction in TBC at 24 h and 48 h could be attributed to injury of some bacterial cells (mesophilic, thermophilic) sensitive to refrigeration temperature (5°C) used for storage of semen samples.

The reduction in bacterial counts in semen samples after antibiotic treatment recorded during this investigation is consistent with the observations of several other workers

(Semkov, 1975; Hardjoutomo, 1990; Nimai Singh *et al.*, 1990) who also obtained very low microbial counts in semen samples after streptomycin and penicillin (30,000 IU) treatment. The present study is generally in agreement with those of Saikia *et al.* (1987) and Rahman (1983) who worked out that the organisms in bulls semen to be highly sensitive to gentamicin (100%) followed by ampicillin (83.78%) and least effective drugs were streptomycin (45.94%) and penicillin (10.81%). Gentamicin has been found to control the bacterial growth in the semen sample completely without affecting the semen quality at different hour of preservation (Ahmed *et al.*, 1989). However, streptomycin treatment of semen extended with egg yolk was ineffective in controlling the spread of infection by AI whereas in milk it was effective. It could be safely concluded from the present study and the earlier studies reported above that gentamicin is the most effective antibiotic followed by ampicillin and SP for checking bacterial load of semen..

4.2.5.3 PW on SPC/antibiotic treatment and pw + antibiotic treatment

Preputial washing significantly reduced the bacterial count in each sub-samples (Table 5a). Before PW, the TBC at 0 h of different subsamples, namely NS, SP, SPG and SPamp were 32727, 1074.58, 523.33 and 798.75, respectively but after PW these counts were reduced to 2812.46, 383.83, 200.63 and 285.88, respectively. After 24 h and 48 h of preservation further lowered TBC in each sub-samples with PW as compared to WPW.

Table 5a. SPC (cfu/ml) of chilled semen at various intervals

	Antibiotics treatment (WPW)				PW + antibiotics treatment			
	P1	P2	P3	Overall average	P1	P2	P3	Overall average
NS	32727.08	19289.58	14833.33	22283.33 ^a	2812.46	1660.42	1365.00	1945.96 ^a
SP	1074.58	780.83	432.08	762.50 ^b	383.83	242.17	178.13	268.04 ^b
SPG	523.33	413.33	229.67	388.78 ^c	200.63	145.54	76.21	148.79 ^c
SPAmP	798.75	472.50	239.17	503.47 ^d	285.88	100.25	129.29	198.47 ^d
Overall average	8780.94 ^a	5239.06 ^b	3933.56 ^c		920.70 ^a	537.10 ^b	437.16 ^c	

Mean with similar superscripts (a,b,c) do not differ significantly from each other ($P < 0.05$)

Table 5b. ANOVA of Table 5a

Source of variation	D.F.	M.S.S.	
		Antibiotics treatment (WPW)	PW + antibiotics treatment
Replication	23	7.96 ^{**}	3.51 ^{**}
Antibiotics	3	258.63 ^{**}	95.56 ^{**}
Period	2	30.81 ^{**}	26.69 ^{**}
Error	259	0.55	0.55
Total	287		

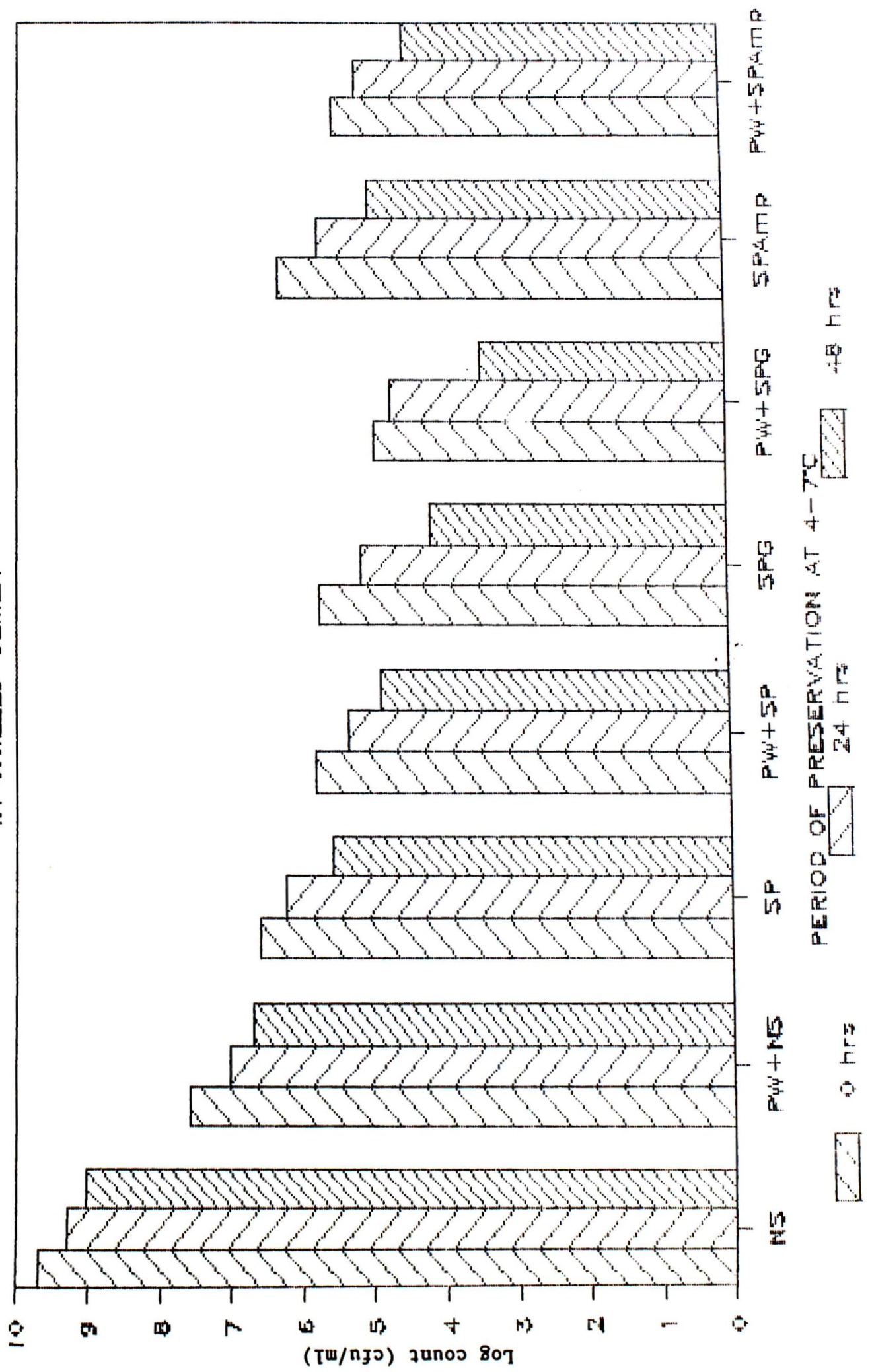
Table 5c. ANOVA of Table 5a.

Antibiotics treatment (WPW) and PW + antibiotics treatment

Replication	23	7.79 ^{**}
Washing	1	167.38 ^{**}
Antibiotics	3	333.43 ^{**}
Period	2	57.41 ^{**}
Error	546	0.79
Total	575	

* Significant at $P < 0.05$; ** Significant at $P < 0.01$; NS = Non-significant

FIG-6. BACTERIAL COUNT (cfu/ml)
IN CHILLED SEMEN



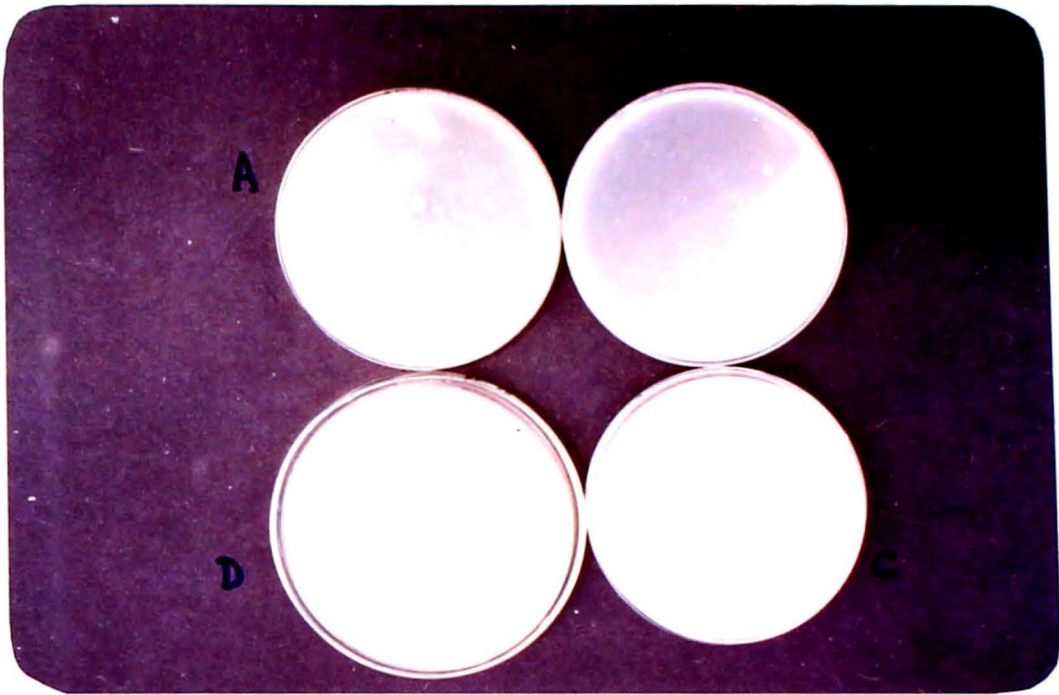


PLATE 1. Bacterial colonies in semen sample (0 hr preservation)
A - NS ($\times 10^3$); B - SPamp treatment ($\times 10$),
C - SP treatment ($\times 10$); D - SPG treatment ($\times 10$).



PLATE 2. Bacterial colonies in frozen semen.
A - SP treatment ($\times 10$)
B - SPG treatment ($\times 10$)



PLATE 3. Bacterial colonies in neat semen.
A - At 0 hr ($\times 10^3$);
B - After preservation (48 hr) ($\times 10^3$)

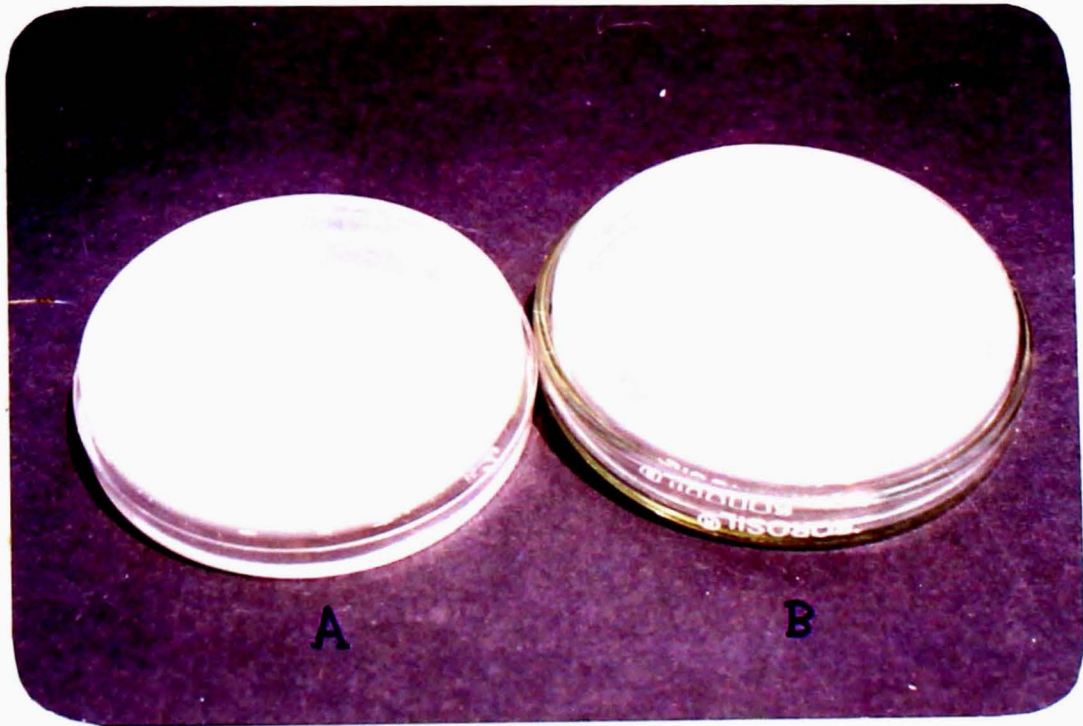


PLATE 4. Bacterial colonies in neat semen (at 0 hr)
A - Without preputial washing ($\times 10^3$)
B - After preputial washing ($\times 10^3$)

In both cases, the overall reduction of TBC were noticed as preservation time passed and maximum reduction was obtained by PW + SPG treatment followed by PW + SPamp, PW + SP and PW + NS as compared to samples without PW.

Since preputial cavity commonly harbours some bacteria which can contaminate the semen, washing with antibiotic solution could reduce the contamination. By flushing out the bacterial contaminants from the cavity as was observed in the present study.

Zamjanis (1962), Hashimoto (1963, 1966) flushed the intrapreputial region with antibiotic solution just before collection which resulted in marked decrease in bacterial contamination of semen. Prasad *et al.* (1985) washed with antibiotic solution and could be able to reduce the bacterial contamination by 61 to 77 per cent.

4.3 PRESERVATION BY DEEP FREEZING

4.3.1 INDIVIDUAL MOTILITY OF SPERMATOOZA

4.3.1.1 Antibiotic treatment (WPW)

The per cent of motile spermatozoa before deep freezing were 67.78 ± 1.93 , 67.78 ± 1.68 and 69.17 ± 1.85 in SP, SPG and SPamp treated semen subsamples. After deep freezing, the post thaw motility of spermatozoa dropped to 41.67 ± 1.84 , 43.06 ± 2.12 and

42.22 \pm 2.05 per cent for SP, SPG and SPamp treatment, respectively (Table 6a). The overall averages for 3 groups of antibiotics were 54.72 \pm 2.55, 55.42 \pm 2.46 and 55.69 \pm 2.64 per cent. The overall average motility dropped from 68.24 \pm 1.05 to 42.31 \pm 1.34 per cent due to freezing.

Statistically antibiotic treatment did not show any significant effect on sperm motility. As expected, significant ($P < 0.01$) drop in motility was observed because of the process of cryopreservation (Table 6b).

4.3.1.2 PW + antibiotic treatment

Before deep freezing, the per cent motile spermatozoa were 69.72 \pm 1.64, 70.00 \pm 1.84 and 69.4 \pm 1.8 for SP, SPG and SPamp treated semen subsamples. The per cent motile spermatozoa, after deep freezing were 46.39 \pm 1.75, 46.94 \pm 1.72 and 46.67 \pm 1.71 per cent in three treatments.

The overall average motility for 3 groups of antibiotics were 58.06 \pm 2.28, 58.47 \pm 2.3 and 58.06 \pm 2.27 per cent. The overall average motility dropped from 69.72 \pm 1.21 to 46.67 \pm 1.51 per cent due to freezing.

The data when subjected to statistical analysis showed that sperm motility registered a significant ($P < 0.01$) decrease after deep freezing. Non-significant effect of various antibiotic on sperm motility can be evidenced from the ANOVA table 6b.

Table 6a. Individual motility (%) of spermatozoa during freezing process

	Antibiotics treatment (WPW)			PW + antibiotics treatment		
	BDF	ADF	Overall average	BDF	ADF	Overall average
SP	67.78 +1.93	41.67 +1.84	54.72 +2.55	69.72 +1.64	46.39 +1.75	58.06 +2.28
SPG	67.78 +1.68	43.06 +2.12	55.42 +2.46	70.00 +1.84	46.94 +1.72	58.47 +2.30
SPamp	69.17 +1.85	42.22 +2.05	55.69 +2.64	69.44 +1.80	46.67 +1.71	58.06 +2.27
Overall average	68.24 +1.05	42.31 +1.34		Overall average 69.72 +1.21	46.67 +1.51	

Table 6b. ANOVA of Table 6a

Source of variation	D.F.	M.S.S.	
		Antibiotics treatment (WPW)	PW + antibiotics treatment
Replication	17	367.16**	315.32**
Antibiotics	2	9.03NS	2.08NS
Freezing	1	18148.15**	14352.08**
Error	87	10.73	6.39
Total	107		

* Significant at P < 0.05; ** Significant at P < 0.01; NS = Non-significant

The non-significant effect of antibiotic on sperm motility as recorded during this investigation is consistent with the observations of Gangadhar *et al.* (1986) who also concluded that the combination of penicillin, streptomycin, chloramphenicol and either oxytetracycline or kanamycin did not affect the post-thaw motility of spermatozoa.

Gangadhar *et al.* (1986) reported that a combination of chloramphenicol (250 ug/ml), kanamycin (250 ug/ml) and oxytetracycline (100 ug/ml) reduced the motility of sperm by 3 per cent before freezing and by 15 per cent after freezing. The present results reveal that the motility was not affected by the combination of antibiotic used in the study. The combinations seem to be appropriate for semen fortification.

4.3.2 NON-EOSINOPHILIC SPERMATOZOA PER CENT

4.3.2.1 Antibiotic treatment (WPW)

The live counts were 74.61 ± 2.55 , 75.11 ± 0.91 and 74.33 ± 1.28 per cent for SP, SPG and SPAMP treated subsamples before deep freezing. The corresponding overall averages were 64.75 ± 1.85 , 64.83 ± 1.92 and 64.44 ± 1.93 per cent. The post thaw live spermatozoa were 54.89 ± 1.40 , 54.56 ± 1.47 and 54.56 ± 1.53 per cent in SP, SPG and SPAMP treated semen samples. The overall averages of live count decreased from 74.63 ± 0.63 to 54.67 ± 0.85 per cent due to freezing (Table 7a).

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Statistical analysis revealed that non-eosinophilic spermatozoa registered a significant ($P < 0.01$) decrease as a result of deep freezing but antibiotic treatment had no significant effect on live and dead spermatozoa count.

4.3.2.2 PW + antibiotic treatment

The number of live spermatozoa before deep freezing were 75.5 ± 0.99 , 75.61 ± 0.95 and 74.22 ± 1.01 per cent in SP, SPG and SPamp treated semen subsamples, respectively but similar decrease trend of live count was observed in post thaw subsamples. After freezing the live count decreased to 54.28 ± 1.21 , 54.39 ± 1.23 and 54.72 ± 1.14 per cent in SP, SPG and SPamp (Table 7a). The corresponding overall average for antibiotic treatment were 64.89 ± 1.93 , 65.0 ± 1.93 and 64.47 ± 1.79 per cent. The overall live count average decreased from 75.11 ± 0.64 to 54.46 ± 0.84 after freezing of semen samples.

Statistical analysis depicted that the difference due to antibiotic treatment on live count being non-significant. However, live count significantly ($P < 0.01$) decreased in frozen semen because of effect of freezing.

From present study, it is clear that the combination of antibiotics used had no damaging effect on sperm survival during the freezing process. Number of live spermatozoa decreased after deep freezing was found in the normal range. In general, the

Table 7a. Non-eosinophilic spermatozoa (%) during freezing process

	Antibiotics treatment (WPW)			PW + antibiotics treatment		
	BDF	ADF	Overall average	BDF	ADF	Overall average
SP	74.61 +0.98	54.89 +1.40	64.75 +1.85	75.50 +0.99	54.28 +1.21	64.89 +1.93
SPG	75.11 +0.91	54.56 +1.47	64.83 +1.92	75.61 +0.95	54.39 +1.23	65.00 +1.93
SPAmp	74.33 +1.28	54.56 +1.53	64.44 +1.93	74.22 +1.01	54.72 +1.14	64.47 +1.79
Overall average	74.69 +0.63	54.67 +0.85		75.11 +0.64	54.46 +0.84	

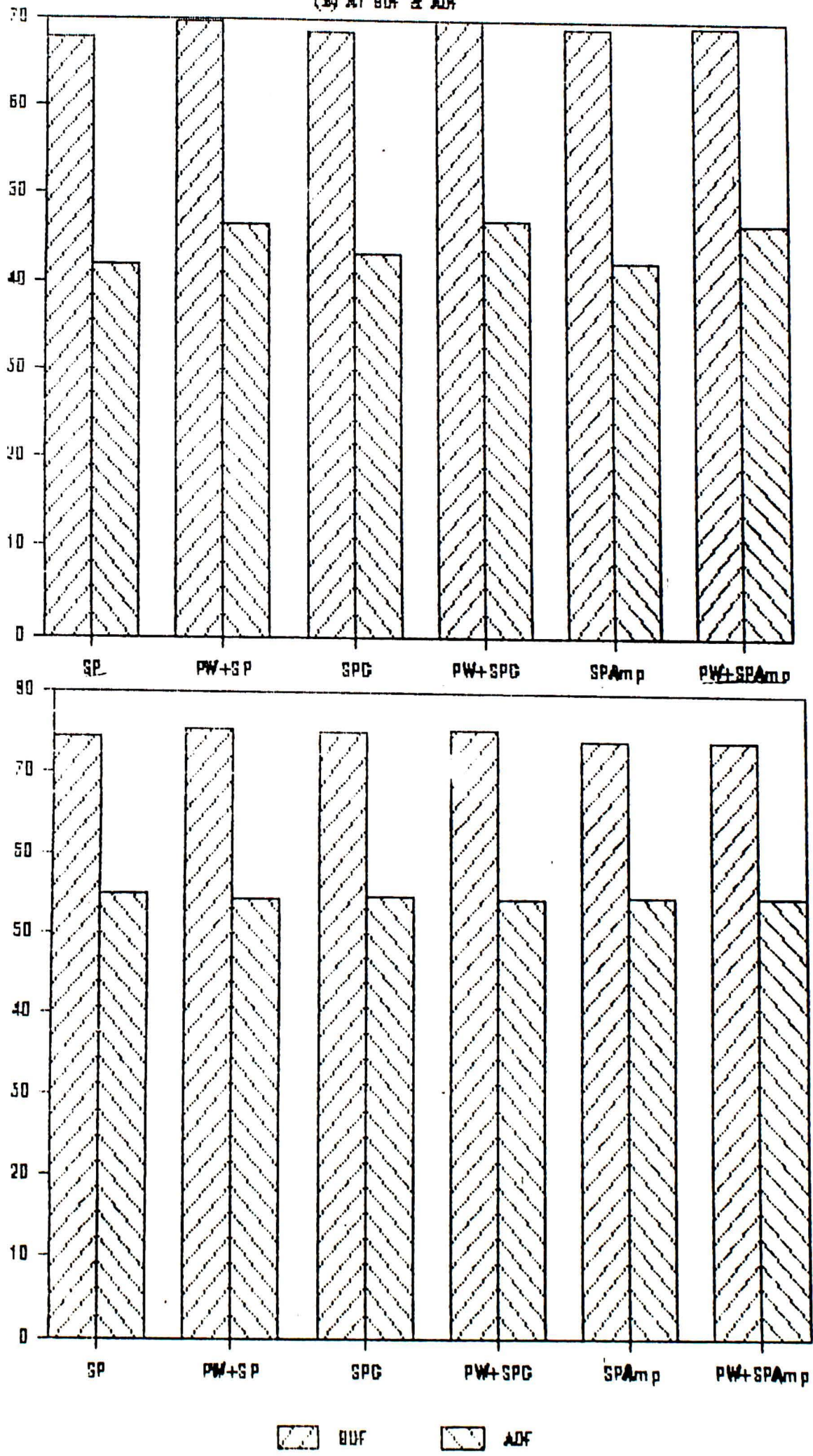
Table 7b. ANOVA of Table 7a

Source of variation	D.F.	M.S.S.	
		Antibiotics treatment (WPW)	PW + antibiotics treatment
Replication	17	112.97**	69.05**
Antibiotics	2	1.51NS	2.79NS
Freezing	1	10820.01**	11511.34**
Error	87	14.67	13.44
Total	107		

* Significant at P < 0.05; ** Significant at P < 0.01; NS = Non-significant

FIG-4. MOTILITY AND NON-EOSINOPHILIC

(%) AT BDF & ADF



post thaw motility obtained from this study was in conformity with the findings of Singh (1987) and Chinnaiya (1978).

4.3.3 SPERM ABNORMALITIES PER CENT

4.3.3.1 Antibiotic treatment (WPW)

Before deep freezing, the number of abnormal spermatozoa were 21.44 ± 0.67 , 20.94 ± 0.85 and 21.72 ± 0.91 per cent in SP, SPG and SPAMP treated subsamples but after deep freezing these counts were increased to 26.67 ± 0.99 , 27.33 ± 1.01 and 26.61 ± 0.82 per cent in same three antibiotic treated subsamples. The overall average of abnormal sperm count increased from 21.37 ± 0.48 to 26.87 ± 0.55 per cent after freezing the antibiotic treated semen subsamples. The overall average of abnormal sperm count in SP, SPG and SPAMP treatment were 24.06 ± 0.74 , 24.14 ± 0.85 and 24.17 ± 0.74 per cent (Table 8a).

Antibiotic treatment did not affect the freezability of semen in terms of increase in abnormal sperm count which was revealed by ANOVA Table 8b. Freezing process, however, significantly ($P < 0.01$) increased the abnormal cell counts in semen samples.

4.3.3.2 PW + antibiotic treatment

The abnormal sperm cells before freezing were 19.39 ± 0.62 , 19.28 ± 0.76 and 19.06 ± 0.75 per cent in SP, SPG and SPAMP

Table 8a. Sperm abnormality (%) during freezing process

	Antibiotics treatment (WPW)				PW + antibiotics treatment		
	BDF	ADF	Overall average		BDF	ADF	Overall average
SP	21.44 +0.67	26.67 +0.99	24.06 +0.74	SP	19.39 +0.62	24.22 +1.01	21.81 +0.72
SPG	20.94 +0.85	27.33 +1.01	24.14 +0.85	SPG	19.28 +0.76	23.44 +1.01	21.36 +0.72
SPAmp	21.72 +0.91	26.61 +0.82	24.17 +0.74	SPAmp	19.06 +0.75	24.67 +1.18	21.86 +0.84
Overall average	21.37 +0.48	26.87 +0.55		Overall average	19.24 +0.46	24.11 +0.45	

Table 8b. ANOVA of Table 8a

Source of variation	D.F.	M.S.S.	
		Antibiotics treatment (WPW)	PW + antibiotics treatment
Replication	17	64.45**	61.36**
Antibiotics	2	0.12NS	2.70NS
Freezing	1	816.75**	640.45**
Error	87	4.95	6.61
Total	107		

* Significant at $P < 0.05$; ** Significant at $P < 0.01$; NS = Non-significant

treated subsamples, respectively. After deep freezing of the same subsamples, abnormalities increased in frozen semen. The values were 24.22 ± 1.01 , 23.44 ± 1.01 and 24.67 ± 1.18 per cent (Table 8b). After freezing, the overall average abnormal count increased from 19.24 ± 0.46 to 24.11 ± 0.45 per cent. The overall averages for SP, SPG and SPAMP treated subsamples were 21.81 ± 0.72 , 21.36 ± 0.72 and 21.86 ± 0.84 per cent, respectively.

The data when subjected to statistical analysis showed that abnormalities registered a significant ($P < 0.01$) increase as a result of deep freezing. The non-significant effect of various antibiotic combination on abnormal sperm count can be evidenced from ANOVA table 8b.

In the present study, abnormalities rose with the freezing process. However, an almost identical increment was found for all treatments of antibiotic combination used in this investigation. From this, it was clear that morphology of spermatozoa was not affected by any of the antibiotic combination.

4.3.4 ACROSOMAL INTEGRITY PER CENT

4.3.4.1 Antibiotic treatment (WPW)

The intact acrosome per cent at before deep freezing were 94.94 ± 0.49 , 93.83 ± 0.39 and 93.83 ± 0.55 in SP, SPG and SPAMP treated semen subsamples, respectively but after freezing

process, these counts decreased to 89.28 ± 0.53 , 88.67 ± 0.55 and 87.67 ± 0.75 per cent for same subsamples (Table 9a). After freezing process and thawing, the integrity of acrosome was lost from 94.28 ± 0.29 to 88.54 ± 0.37 per cent. The overall averages of intact acrosome were 92.11 ± 0.60 , 91.25 ± 0.55 and 90.75 ± 0.69 per cent in SP, SPG and SPAMP treated subsamples, respectively.

Statistical analysis revealed that there was no significant influence of antibiotic treatment on the intact acrosome of spermatozoa but there was a significant ($P < 0.01$) effect of deep freezing on acrosomal integrity as evidenced from ANOVA table 9b.

4.3.4.2 PW + antibiotic treatment

Before deep freezing, the per cent values of intact acrosome were 94.40 ± 0.70 , 94.83 ± 0.47 and 94.78 ± 0.43 in SP, SPG and SPAMP treated subsamples, respectively but after freezing process, all these corresponding values were reduced to 89.22 ± 0.73 , 90.50 ± 0.81 and 88.78 ± 0.60 per cent (Table 9a).

The overall averages for SP, SPG and SPAMP treated subsamples were 91.83 ± 0.67 , 92.67 ± 0.59 and 91.78 ± 0.62 per cent, respectively. The overall average of acrosomal integrity lost from 94.69 ± 0.30 to 89.50 ± 0.39 per cent after freezing and thawing of samples.

Table 9a. Acrosomal integrity (%) of spermatozoa during freezing process

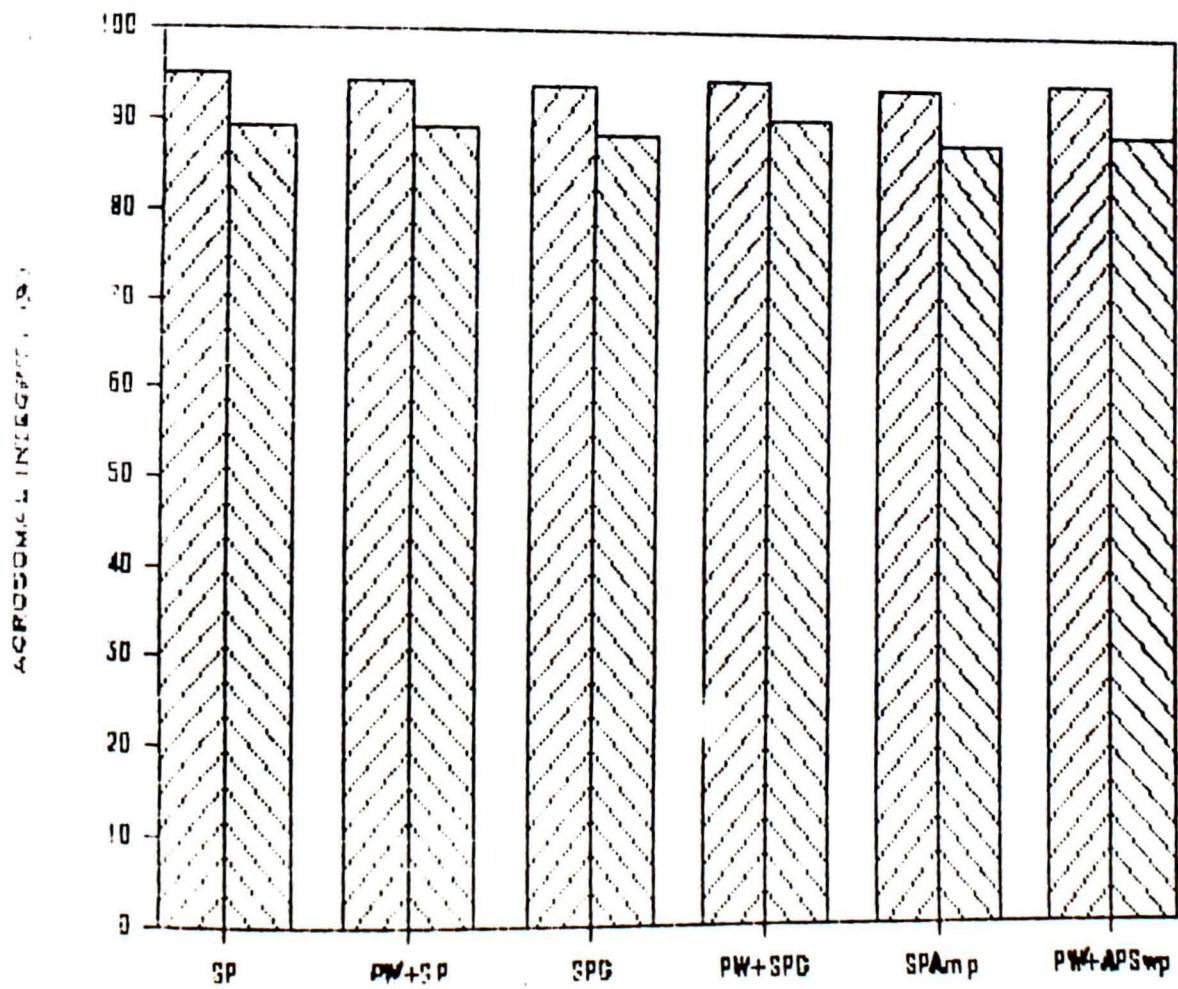
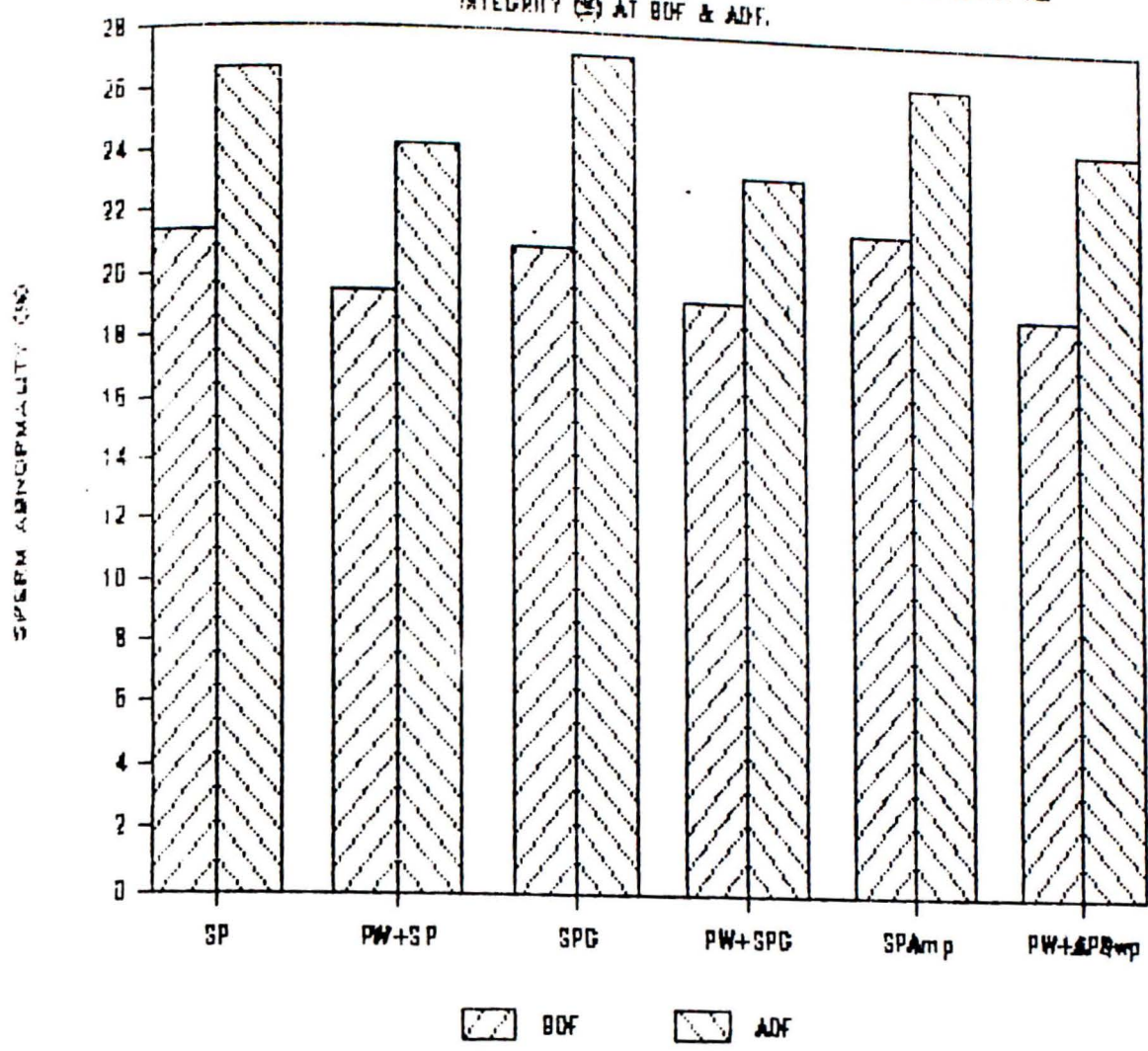
	Antibiotics treatment (WPW)			PW + antibiotics treatment		
	BDF	ADF	Overall average	BDF	ADF	Overall average
SP	94.94 +0.49	89.28 +0.53	92.11 +0.60	94.44 +0.70	89.22 +0.73	91.83 +0.67
SPG	93.83 +0.39	88.67 +0.55	91.25 +0.55	94.83 +0.47	90.50 +0.81	92.67 +0.59
SPamp	93.83 +0.55	87.67 +0.75	90.75 +0.69	94.78 +0.43	88.78 +0.60	91.78 +0.62
Overall average	94.23 +0.29	88.54 +0.37		94.69 +0.30	89.50 +0.39	

Table 9b. ANOVA of Table 9a

Source of variation	D.F.	M.S.S.	
		Antibiotics treatment (WPW)	PW + antibiotics treatment
Replication	17	17.32**	26.04**
Antibiotics	2	7.98NS	8.93NS
Freezing	1	867.00**	725.93**
Error	87	3.58	4.17
Total	107		

* Significant at $P < 0.05$; ** Significant at $P < 0.01$; NS = Non-significant

FIG-5. SPERM ABNORMALITY & ACROSOMAL INTEGRITY (%) AT BDF & ADF.



The data when subjected to statistical analysis showed that acrosomal integrity registered a significant ($P < 0.01$) decrease as a result of deep freezing. The non-significant effect of various antibiotic combination on intact acrosome can be evidenced from ANOVA table 9b.

The acrosomal damage due to cryopreservation observed in the present study is in conformity with various workers (Chinnaiya, 1978; Nehring, 1988) who reported increased acrosomal damage with the process of semen freezing. Terseron *et al.* (1977) reported that spermatozoa were damaged at all the degree of dilution, cooling, freezing and thawing, thereby, resulting in cumulative acrosomal damage of about 40 per cent.

It is clear from the results that the various combinations of antibiotics do not affect the acrosomes and thus revealing no interaction between various combination and freezing process. This further strengthens the previous findings that antibiotic combination have no adverse effect on preservability of bovine semen.

4.3.5 STANDARD PLATE COUNT

Standard plate counts were determined in antibiotic treated semen subsamples before deep freezing (BDF) as well as after deep freezing (ADF) and the data have been presented in corresponding table.

4.3.5.1 Antibiotic treatment (WPW)

The TBC at BDF in three subsamples, namely SP, SPG and SPamp were 334.44, 148.33 and 236.67, respectively (Table 10a). After deep freezing, bacterial count were gradually reduced and the corresponding values were 178.33, 87.22 and 130.56

The data when subjected to statistical analysis, showed that bacterial counts registered a significant ($P < 0.01$) decrease after antibiotic treatment.

The significant reduction in bacterial counts as a result of deep freezing can also be evidenced from the ANOVA table 10b.

The CD test depicted that there were significant difference between different combinations of treatment.

In the present study, reduction of TBC was observed in sample treated with SPG followed by SPamp in comparison to SP treatment. Almost a similar trend of reduction was observed in antibiotic treated semen subsamples after deep freezing.

4.3.5.2 PW + ANTIBIOTIC TREATMENT

The TBC at BDF in three subsamples, namely SP, SPG and SPamp treatment after preputial washing and antibiotic treatments were 221.67, 113.8 and 143.78, respectively (Table 10a). However,

after deep freezing of subsamples a similar trend was observed as was recorded with only antibiotic treatment. The corresponding values were 134.44, 70.66 and 181.67.

Antibiotics treatments were highly significant ($P < 0.01$) on TBC and significant difference was also observed due to deep freezing and due to replications (Table 10b).

From CD test it was known that there was significant difference between each combination of treatment.

In present study, maximum reduction of TBC was observed by SPG treatment followed by SPamp as compared to SP treatment.

The SPC data generated from this study clearly indicate that the antibiotic treatment significantly reduced the TBC in frozen semen samples and the counts reduced by the effect of deep freezing. The reduction in bacterial count after deep freezing could be attributed to injury of some bacterial cells, sensitive to ultra low temperature (-196°C) used for freezing purpose. SPG treatment could reduce the bacterial count effectively, followed by SPamp compared to SP treatment without affecting the spermatozoa and preservability of semen. Though SPG treatment resulted in marked reduction of colonies as compared to SPamp treatment but the count was under permissible level. Ampicillin being cheaper can, therefore, also be recommended for semen fortification for desired results.

The reduction in bacterial counts in frozen semen samples after gentamicin treatment, recorded during this investigation is consistent with the observations of Ahmed *et al.* (1989) who also found that gentamicin can control the bacterial growth in semen sample without affecting the semen quality after equilibration, after different interval of preservation and after cryopreservation.

The suggestion was also given by Wayda (1991) that gentamicin in combination with penicillin should be used for preparation of diluent for cryopreservation.

4.3.5.3 PW on SPC/antibiotic treatment and PW + antibiotic treatment

Preputial washing significantly ($P < 0.01$) reduced the TBC in each antibiotic treated semen subsamples. Before PW, the TBC at BDF of different subsamples, namely SP, SPG and SPamp were 334.44, 148.33 and 236.67, respectively but after PW, these counts were reduced to the level of 221.67, 113.8 and 147.78, respectively (Table 10a).

After deep freezing, lowered TBC were found in each subsamples with PW as compared to without PW. In both cases, the overall reduction of TBC were noticed after deep freezing. Maximum reduction was obtained by PW + SPG treatment followed by PW + SPamp treatment as compared to samples without PW.

Table 10a. SPC (cfu/ml) of semen during freezing process

	Antibiotics treatment (WPW)			PW + antibiotics treatment		
	BDF	ADP	Overall average	BDF	ADP	Overall average
SP	334.44	178.33	256.39 ^a	221.67	134.44	178.06 ^a
SPG	148.33	87.22	117.78 ^b	113.80	70.56	92.22 ^b
SPAmp	236.67	130.56	183.61 ^c	147.78	181.67	124.72 ^c
Overall average	239.81	132.04		161.08	128.89	

Mean with similar superscripts (a,b,c) do not differ significantly from each other ($P < 0.05$)

Table 10b. ANOVA of Table 10a

Source of variation	D.F.	M.S.S.	
		Antibiotics treatment (WPW)	PW + antibiotics treatment
Replication	17	0.52 ^{**}	0.73 ^{**}
Antibiotics	2	5.50 ^{**}	4.01 ^{**}
Freezing	1	10.51 ^{**}	5.47 ^{**}
Error	87	0.14	0.77
Total	107		

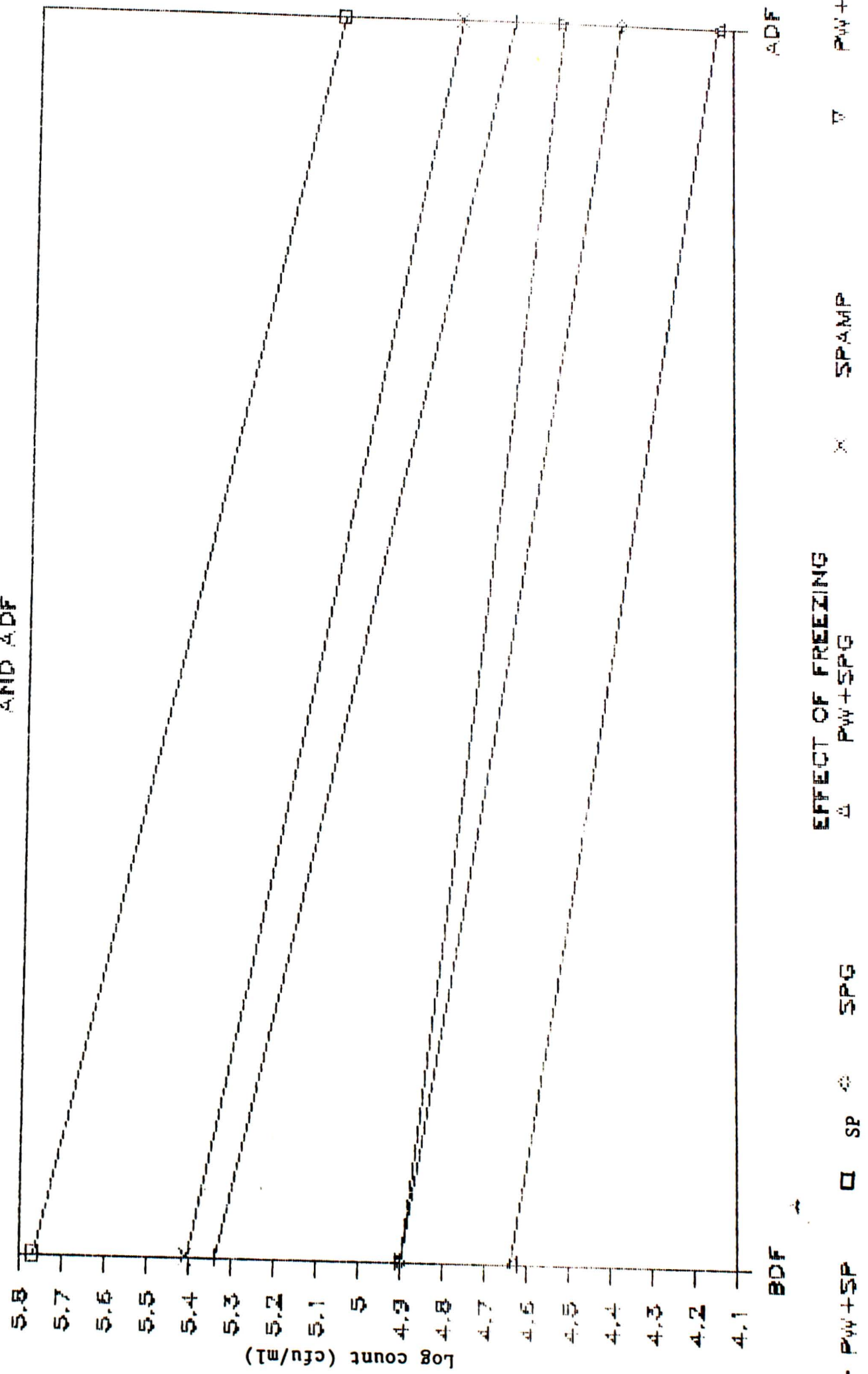
Table 10c. ANOVA of Table 10a

Antibiotics treatment (WPW) and PW + antibiotics treatment

Replication	17	0.94 ^{**}
Washing	1	5.26 ^{**}
Antibiotics	2	9.40 ^{**}
Freezing	1	15.57 ^{**}
Error	194	0.13
Total	215	

* Significant at $P < 0.05$; ** Significant at $P < 0.01$; NS = Non-significant

FIG-3. BACTERIAL COUNT (cfu/ml) AT BDF AND ADF



The reduction in bacterial counts in semen sample after preputial washing recorded during this investigation is consistent with the observations of several other workers (Zamjanis, 1962; Hashimoto, 1963,1966; Prasad *et al.*, 1985) who also obtained marked decrease in microbial counts in semen samples after giving the preputial douche.

In present study, flushing the preputial cavity region with antibiotic solution to remove the bacteria which are commonly harboured in that particular region and resulting in contamination of semen.

4.4 ASSESSMENT OF BACTERIAL LOAD

AV, glassware, semen dilutor, coming in contact with semen during processing are contributing to the bacterial load of the semen. It was felt that the study of bacterial contamination of these contributing factors could help in reducing subsequent bacterial load of the semen.

Keeping above in view, AV, glassware, extender used were subjected to assessment of bacterial load.

A.V.: Different methods of sterilization were followed to get contamination free AV. The average values (cfu) for AV sterilized by steam, flush with alcohol, washing with soap and water and sterilized and just after collection were 616.67, 1312.5, 96,166.67 and 1,95,000 respectively.

Glassware: Glasswares which were subjected to contact with semen during processing and handling were sterilized by hot air oven. The average values (cfu) of sterilized and unsterilized glassware were 130, and 9,40,000 respectively.

EYC dilutor: The average value (cfu/ml) of egg yolk citrate dilutor without any antibiotic treatment was 390 cfu/ml of dilutor.

The TBC data generated from this study clearly indicated that sterilization of AV by 'steam sterilization' was most efficient followed by 'alcohol flush' and 'washing with soap and water'. The AV just after its proper use should not be used repeatedly because it contributed maximum contamination to semen samples. 'Steam sterilization' procedure should be followed to sterilize the AV before its use because it contributed minimum contamination to semen.

Glasswares sterilized by hot air oven (110°C, overnight) contributed a minimum contamination rather than unsterilized glassware. Hence, glasswares should be sterilized by hot air oven before it's actual use during semen processing.

EYC dilutor contributed a minimum contamination to semen sample during it's extension.

4.5 TENTATIVE IDENTIFICATION OF PREDOMINATING BACTERIAL TYPES IN SEMEN SAMPLES

In order to find the nature of predominating bacterial types occurring in the semen samples, an attempt was made to tentatively identify a few randomly picked bacterial colonies from the SPC plates on the basis of their colonies, characteristics and microscopical observation (Table 11).

The illustration of some typical bacterial types recovered from semen samples have been presented in photomicrography plates.

From this observation, it can be concluded that the microbial load in the semen samples appeared to comprise of both Gram-positive cocci as well as Gram-negative coccobacilli. Gram-positive sporeforming rods could also be isolated from some samples. The presence of sporeforming rods in the semen sample could be ascribed to aerial contamination during collection of samples.

Since the exact identity of predominating bacterial types occurring in the semen samples examined in this study could not be established, the occurrence of such contaminants in semen samples does not indicate their true microbiological quality. Hence, more extensive work need to be carried out to draw any meaningful conclusion.

Table 11. Tentative identification of predominating bacterial types in semen samples

Sl. No.	Colony characteristics	Colony No.	Microscopical observation
1.	White mucoid round colony	7	Gram positive, cocci (diplo, mono, bunches)
2.	White submerged colony	11	Gram positive, cocci (bunches)
3.	Mucoid white colony	1	Gram negative rod (single) and Gram positive cocci bunches
4.	Small, smooth white ovoid	8	Gram positive, Coccobacillus (single)
5.	Mucoid wide round	7	Gram negative, small rod
6.	Wide small round	Many	Gram positive rod, cocci (mono, diplo, bunches, short chain)
7.	Submerged wide big round	8	Cocci (mono), Gram positive
8.	Big round, smooth, creamy	10	Gram negative rod, Gram positive cocci
9.	Convex, wide, round, smooth	7	Gram positive, cocci (diplo, mono, bunches)
10.	Pinkish small convex	9	Gram positive, cocci (bunches, mono)
11.	White round convex	11	Gram positive, cocci (mono)
12.	Pinkish round small	4	Gram positive, cocci (diplo, mono, bunches)
13.	Typical, red, small	1	Gram positive rod, Gram negative rod, cocci
14.	White colony small	5	Gram negative rod
15.	Small, white, circular	7	Gram negative sporeforming rod
16.	White convex	11	Gram positive, cocci

contd

contd... table

Sl. No.	Colony characteristics	Colony No.	Microscopical observation
17.	White, smooth, small	12	Gram positive, Coccobacillus (mono)
18.	Smooth, creamy, big	25	Gram positive, cocci, Gram negative rod
19.	Oval small submerged	10	Gram positive cocci (single, diplo, brunch)
20.	Milky, white, big round	11	Gram negative short thick rod
21.	Round white opaque medium size	5	Gram negative rod
22.	Spindle shaped, white	9	Mixed, Gram positive, cocci and rod
23.	Big round white convex	2	Gram negative, typical
24.	Small whitish pinpointed	Many	Gram negative rod, thick

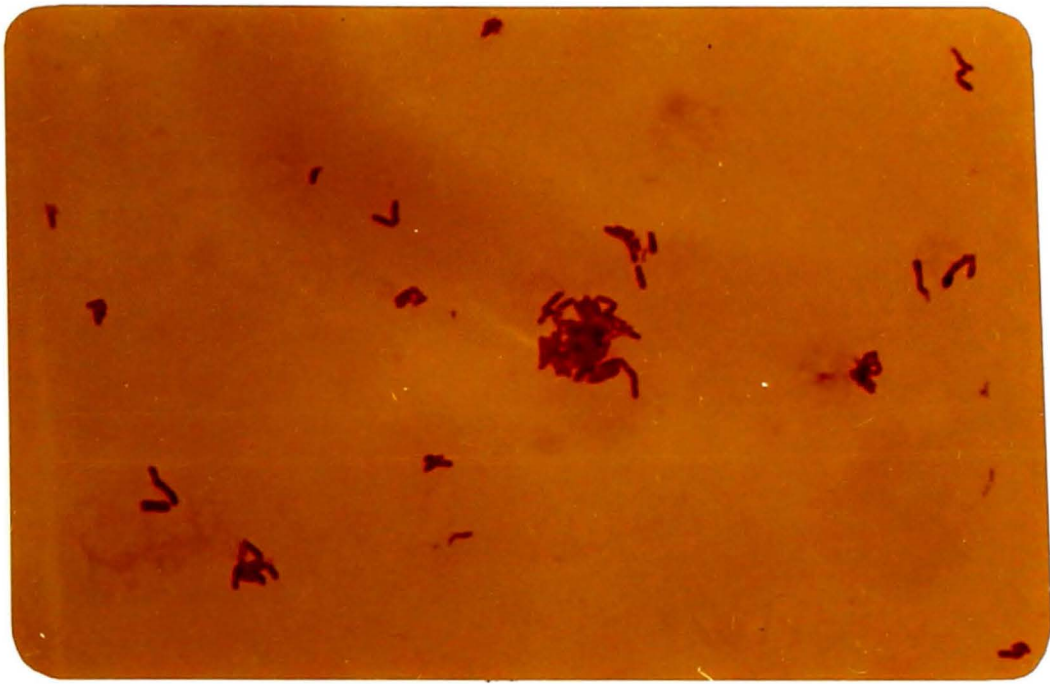


PLATE 5. Gram stained preparation of tentatively identified bacteria 'Gram-negative rod shaped' isolated from neat semen. Colony characteristics - round white, opaque, medium size.

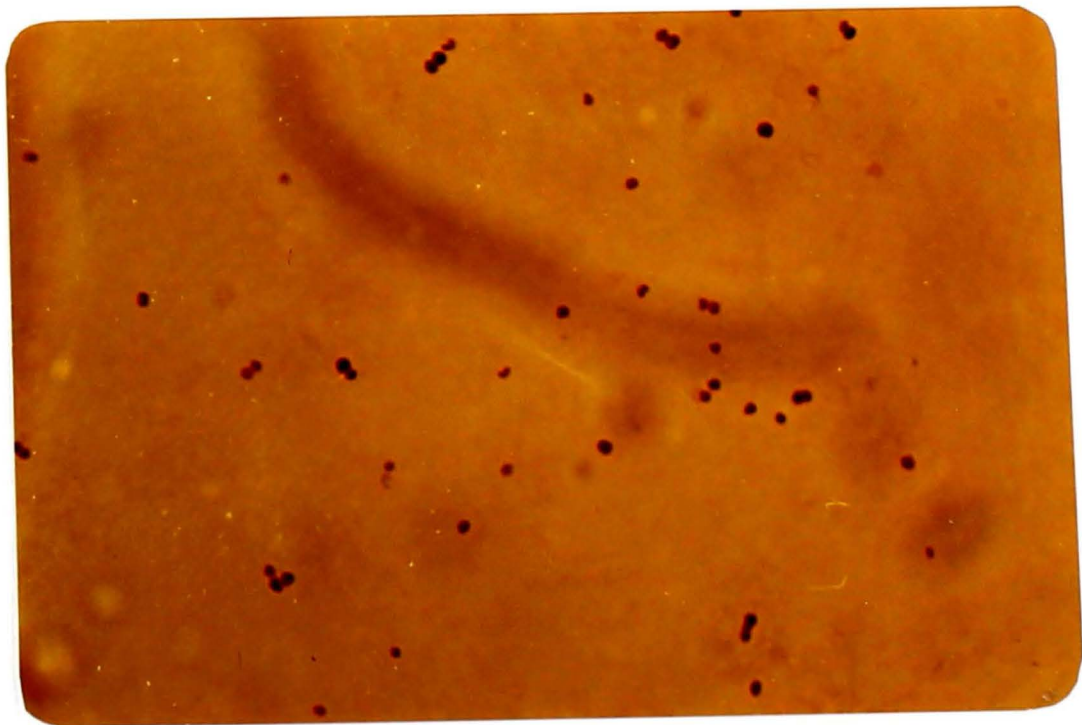


PLATE 6. Gram stained preparation of tentatively identified bacteria, 'Gram positive cocci' isolated from neat semen. Colony characteristics - oval, submerged, white, medium size.



PLATE 7. Gram stained preparation of tentatively identified bacteria, 'Gram negative, short thick rod' isolated from neat semen. Colony characteristics - milky white, round, big size.



PLATE 8. Gram stained preparation of tentatively identified bacteria, 'Gram negative rod', isolated from neat semen. Colony characteristics - big, round, white, convex.



PLATE 9. Eosinophilic and non-eosinophilic spermatozoa in semen sample treated with SPG.

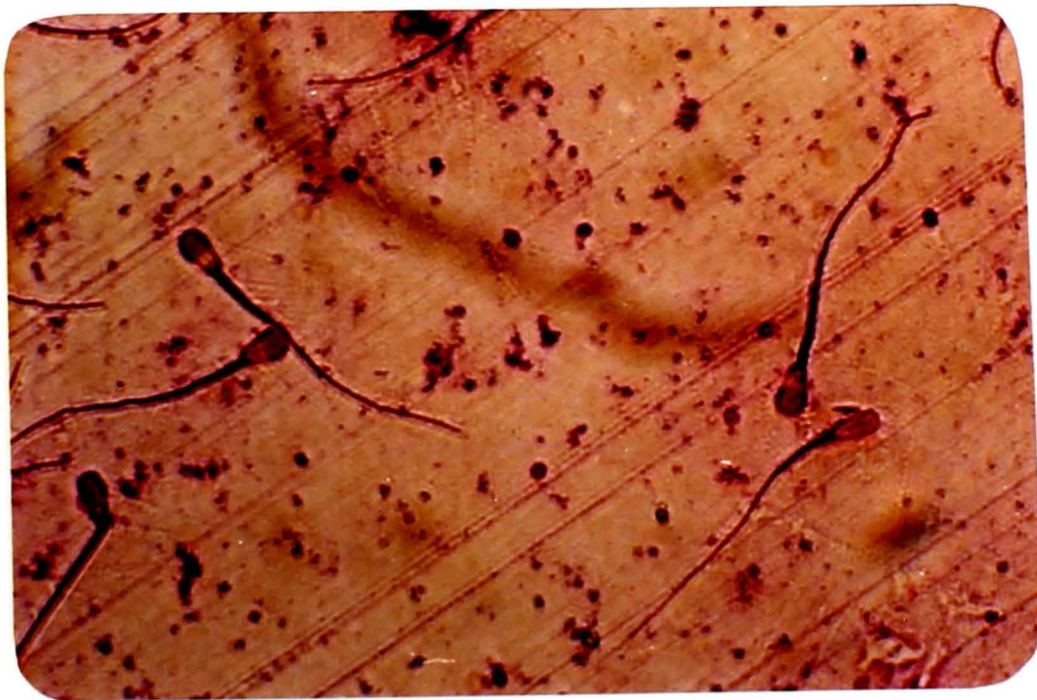


PLATE 10. Spermatozoa having intact acrosome and damaged acrosome in semen sample treated with SPG.

CHAPTER 5

SUMMARY AND CONCLUSION

5. SUMMARY AND CONCLUSION

5.1 SUMMARY

1. The present investigation was an attempt to study the effect of addition of antibiotics and preputial washing on bacterial load as well as preservability of bovine semen.
2. Various combinations of antibiotics used as semen additive were SP, SPG and SPamp. A solution containing procaine penicillin (1,000 IU/ml of DW) was used as preputial wash.
3. Assessment of bacterial load of semen collection & processing equipments and extender was assessed. An attempt was made to tentatively identify a few randomly picked bacterial colonies from the SPC plates on the basis of their ~~colonies~~ characteristics and microscopical observation.
4. Physical attributes of semen, viz., volume, colour, mass activity and sperm concentration studied were in the normal range, suitable for processing and preservation of semen.
5. The individual motility of spermatozoa was not affected in chilled as well as in frozen semen by any of the antibiotic combination.

6. Different combinations of antibiotics had no damaging effect on sperm survival in chilled and frozen semen.
7. Various combinations of antibiotics did not have any effect on sperm abnormalities during preservation of semen at refrigerator as well as ultra low temperature.
8. The acrosomal integrity of spermatozoa was not significantly affected in presence of any of the combinations of antibiotics in chilled as well as frozen semen.
9. The fall in motility, non-eosinophilic count and acrosomal integrity, sperm abnormalities observed at various intervals subsequent to cryopreservation observed was in the normal range.
10. Bacterial load was significantly lowered due to antibiotics treatment. The combination SPG effectively reduced the bacterial load followed by SPamp and SP as compared to NS in case of chilled semen. Preputial washings coupled with antibiotics treatment were proved beneficial in combating the bacterial load.
11. The SPG combination effectively reduced the colony count followed by SPamp as compared to SP at BDF and also at ADF. Deep freezing had a significant effect on lowering down the bacterial load.

12. Preputial washing significantly reduced the bacterial load in chilled semen as well as in the cryopreserved semen. Maximum reduction in TBC was noticed in the treatment of PW + SPG followed by PW + SPamp and PW + SP in chilled and frozen semen.
13. The sterilization of AV by 'steam sterilization' was the most efficient followed by 'alcohol flush' and 'washing with soap and water'. Glassware sterilized by hot air oven (110°C, overnight) contributed a minimum contamination compared to that of unsterilized glassware. EYC dilutor contributed a minimum contamination to semen sample as sodium citrate buffer was autoclaved.
14. A few of the isolate from the semen samples were Gram positive cocci, Gram negative coccobacilli and Gram positive sporeforming rods.

5.2 CONCLUSION

From the present investigation, following conclusion can be safely drawn:

1. Preputial washing (with procaine penicillin @ 1000 IU/ml DW) coupled with SPG treatment (streptomycin @ 1 g/ml of extender, benzyle penicillin @ 1000 IU/ml of extender, gentamicin @ 1000 ug/ml of extender) is the best suited for

chilled and frozen semen to get least bacterial count without affecting semen quality and preservability.

2. Preputial washing coupled with SPAMmp treatment (ampicillin @ 1000 ug/ml of extender) could also be recommended for preservation of semen at refrigerated temperature as well as in frozen state without affecting sperm motility and morphology because Ampicillin costs less compared to that of Gentamicin.
3. The SPG combination was found to be the most efficient in combating bacterial growth even in the absence of preputial washing.

The present study can be better authenticated by conducting further studies on sperm morphology and certain biochemical parameters. Fertility being the acid test, final conclusion can only be drawn after conducting the fertility trials. More extensive work is needed to be carried out on the exact identity of predominating bacterial types occurring in the semen and their sensitivity to specific antibiotics.

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