

**ASSESSMENT OF SPERMATOZOAL
PENETRABILITY THROUGH POLYACRYLAMIDE
GEL COLUMN IN FERTILE AND SUBFERTILE
BUFFALO SEMEN**

Thesis

**Submitted to the Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of**

**MASTER OF VETERINARY SCIENCE
in
VETERINARY GYNAECOLOGY & OBSTETRICS
(Minor Subject : Veterinary Medicine)**

By

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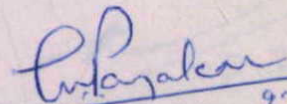
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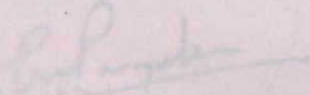
CERTIFICATE I


This is to certify that the thesis entitled, "Assessment of spermatozoal penetrability through polyacrylamide gel column in fertile and subfertile buffalo semen" submitted for the degree of M.V.Sc., in the subject of **Veterinary Gynaecology and Obstetrics** (Minor subject: **Veterinary Medicine**) of the Punjab Agricultural University, Ludhiana is a bonafide research work carried out by **Mr Satwinder Singh** (L-97-V-273-M) under my supervision and that no part of this thesis has been submitted for any other degree.


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


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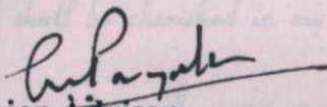

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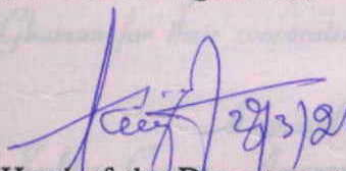

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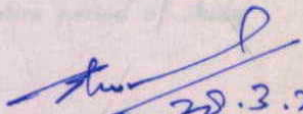

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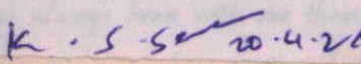
This is to certify that the thesis entitled, "Assessment of spermatozoal penetrability through polyacrylamide gel column in fertile and subfertile buffalo semen" submitted by Mr Satwinder Singh (L-97-V-273-M) to the Punjab Agricultural University, Ludhiana, in partial fulfilment of the requirements for the degree of M.V.Sc., in the subject of Veterinary Gynaecology and Obstetrics (Minor subject : Veterinary Medicine) has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an External Examiner.


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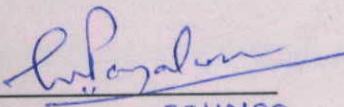
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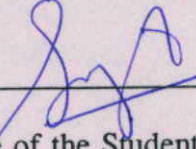
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ABSTRACT

Synthetic migration medium viz. two per cent polyacrylamide gel (PAG) has been used for sperm penetration test (SPT) in the present study for the assessment of spermatozoal penetrability through fertile and subfertile buffalo bull semen. Various batches of two per cent polyacrylamide gel showed non-significant variation with regard to physical characteristics viz. colour and transparency, pH, spinnbarkeit and visible gelification. Seminal characteristics (sperm motility, live sperm count and normal sperm count) exhibited significant ($P < 0.05$) overall positive correlation with spermatozoal penetrability (mm/20 min.). Although spermatozoal penetrability was significantly ($P < 0.05$) decreased in chilled and frozen semen; a good correlation between sperm penetration distance (SPD) values of fresh semen with chilled and frozen semen ($r = 0.823$ and 0.889 ; $P < 0.05$) were established. Sephadex (G-15-120) column filtration recorded significant ($P < 0.05$) improvement in semen quality in terms of seminal characteristics as well as spermatozoal penetrability through PAG column for semen exhibiting poor initial quality as well as poor freezability. Static ejaculates exhibiting significantly ($P < 0.05$) lower sperm motility, live sperm count, normal sperm count and spermatozoal penetrability through PAG column, yet, freezability test appears to be deciding test for the discard of static ejaculates. Cold shocked buffalo bull semen recorded significant ($P < 0.05$) drop in sperm motility, livability, normal sperm count as well as spermatozoal penetrability. In last, sperm penetration test using two per cent polyacrylamide gel was found to be more objective test for evaluation of buffalo bull semen quality.


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INTRODUCTION

Chapter	Topic	Page
I	INTRODUCTION	1 - 4
II	REVIEW OF LITERATURE	5 - 21
III	MATERIALS AND METHODS	22 - 35
IV	RESULTS AND DISCUSSION	36 - 67
V	SUMMARY	68 - 70
	REFERENCES	71 - 83
	VITA	

CHAPTER I

INTRODUCTION

Buffalo is the mainstay of dairy industry in the Indian subcontinent and several South-East Asian countries. The superior ability of buffalo to produce large quantities of high fat milk, ability to sustain and thrive on poor quality roughages and its better ability to resist tropical disease contribute towards the success of this species in sustainable animal production. India owns the largest population (79.5 million) of best quality buffaloes in the world which contribute substantially towards the agricultural economy by producing about 31.9 million tonnes of milk, 1.2 million tonnes of meat and 10 per cent of the total draught power contributed by work animals (Madan 1998). Thus, it is all the more important to deliberate on the problems facing the optimum utilization of the production potential of this great animal.

During the last two decades, extensive efforts have been made to improve and exploit the reproductive potential of the buffalo which is evident by improvement of national stocks through selective breeding programmes and improved husbandry. This is possible only by use of highly fertile bulls with superior genetic potential which ultimately results in improvement of production potential of buffaloes.

Reproductive technologies offer a great opportunity for enhancing the reproductive efficacy of buffaloes. Thereby, apart from normal fertile buffalo bull semen, subfertile semen from bulls of excellence with superior germplasm can also be considered for breeding purposes before being discarded.

Occasionally, freshly collected buffalo semen do not show any mass activity and such non-motile ejaculates are called static or zero ejaculates. The occurrence of static ejaculates are more during extreme climatic conditions and vary from 25-66 per cent (Jindal *et al* 1996). Earlier such non-motile ejaculates were discarded but now it has been observed that about 2/3rd of such ejaculates regain motility when mixed with extender and can be used successfully for breeding purposes (Sahni and Mohan 1990, Kumar and Mohan 1997).

Sometimes, semen samples of bulls carry morphologically normal sperms intermixed with abnormal non-motile sperms and other cellular and non-cellular debris. Such samples will have poor keeping quality and freezability, thereby affecting the fertility. Therefore, fractionation of morphologically normal motile sperms from abnormal sperms and cellular debris is likely to improve the quality of semen samples. A number of techniques for live sperm separation prior to insemination have been reported, claiming varying degrees of success in improving semen quality and thereby the fertility. However, the sephadex column filtration technique has been used by different workers (Heuer *et al* 1983, Graham and Graham 1990, Cisale 1998) with good results.

Artificial breeding of buffaloes on a large scale using semen from bulls of excellence with superior germplasm can solve the problem of low productivity of non-descript buffaloes. On the contrary, in India, adoption of artificial insemination in buffaloes has been poor and still majority of buffaloes are bred either through natural service or using chilled liquid semen. Usually chilled liquid semen is stored and transported in ice-pails having ice for maintaining temperature between 4-6°C, but it is observed that buffalo bull semen is very much

susceptible to cold shock which significantly alter the motility, livability and absolute index of livability of buffalo bull semen (Malik *et al* 1994, Gupta *et al* 1998).

Traditionally employed methods for evaluation of bull fertility such as sperm motility, sperm morphology, sperm concentration etc. are subjective. These parameters provide information about status of spermatogenesis, but due to certain limitations cannot be used as reliable predictor of sperm fertilizing ability (Stewart *et al* 1972, Graham *et al* 1980). Also, the collective traits affecting fertility are difficult to measure in large population of spermatozoa. So, there is a need of some viability test of semen quality that can define or quantify the life of spermatozoa.

The cervical mucus penetration test was originally designed by Kremer (1965) and is known after his name the 'Kremer Test'. The Kremer test, though originally designed for human samples, has also been exploited for assessment of bovine and buffalo bull semen quality (Koefoed-Johnson 1972, Katz *et al* 1981). Thus, there appears considerable potential in the use of cervical mucus as a medium for evaluation of fertilizing ability of spermatozoa *in vitro* (Reichman *et al* 1973, Insler *et al* 1979).

Although the use of cervical mucus has been shown to be reliable, but its quality can be altered by many factors (Kummerfeld *et al* 1981b). However, in andrologic practice, fresh cervical mucus for *in vitro* sperm cervical mucus penetration test (SCMPT) is not always available. Furthermore, cervical mucus has to be taken under standardized conditions, and the quality of mucus

must be carefully controlled to obtain reproducible results and to evaluate the male factor as the main variable (Eggert-Kruse *et al* 1989).

To overcome these problems, an alternative media is needed with sperm migration characteristics similar to those of estrual mucus which could be obtained or prepared freshly with uniform stable characteristic and can also be stored for long periods without deterioration in quality. Lorton *et al* (1981) proposed the use of polyacrylamide gel as a substitute for cervical mucus in human sperm migration studies to overcome problems associated with variability of cervical mucus. A particular advantage of this medium is that it can be produced in large quantities and equal quantity can be stored without problems. But unfortunately, no such media has been tried for buffalo bull semen to judge sperm penetration rate under various conditions. Therefore, purpose of present study is to evaluate buffalo bull semen using polyacrylamide gel as a substitute of cervical mucus on sperm migration test with the following objectives:

1. To study the sperm penetration rate of fertile buffalo semen in polyacrylamide gel column and its correlation with other physical parameters of semen.
2. To study the sperm penetration ability of static ejaculates of buffalo bulls through polyacrylamide gel column.
3. To study the effect of sephadex column filtration in improving semen quality of subfertile semen and its subsequent penetration through polyacrylamide gel column.
4. To study the effect of cold shock on spermatozoa and its subsequent penetration through polyacrylamide gel column.

CHAPTER II

REVIEW OF LITERATURE

2.1 SPERM MIGRATION TEST

Traditionally, semen analysis has been the main method utilized for evaluation of bull fertility. A complete semen analysis includes determination of sperm count, number of motile sperms, number of normal forms, viability, volume, white blood cells, agglutination and viscosity. The semen analysis, though useful in fertility investigations, is imprecise in its measurement of fertility and may not directly simulate any of the events in the conception process (Blasco 1984). Also, these methods of assessing the quality of semen are invariably highly subjective, time consuming and require considerable experience and equipment. On the contrary, sperm mucus penetration test and zona free hamster egg penetration test reveal more authentic information regarding the fertilizing quality of the spermatozoa (Katz *et al* 1980, Murase *et al* 1990). Thus, these tests gain significance as objective tests to assess the quality of the semen.

2.1.1 Sperm Mucus Penetration Test

The sperm mucus penetration test was originally designed by Kremer (1965). This test is based upon measuring the rate of sperm penetration through a column of cervical mucus, either on the surface of glass slide or enclosed in a capillary tube (Moghissi 1972). The migration of spermatozoa could be visualized either under the microscope or by means of scattering light technique based on the Doppler Effect (Jouannet 1979).

Several methods are currently available for the assessment of sperm penetration into cervical mucus viz. slide test, post-coital test, round haematocrit capillary tube test, flat capillary tube test. The post-coital test, although, useful (Blasco 1977) have a long subject of dispute (Grant 1958, Kovacs *et al* 1978). In this test, mucus is collected from the cervix post-coital and number of spermatozoa penetrated are counted. In the slide test (Guard 1960, Moghissi *et al* 1964) a drop of semen is placed in contact with a drop of mucus on a glass slide, covered with coverslip and then slide is examined under high power magnification. Kremer (1965) developed capillary tube technique for human beings. This technique was considered to be more objective which permitted the standardization of the experimental conditions. Mills and Katz (1978) introduced flat capillary tube to enhance the visibility of the penetrated spermatozoa.

Sperm mucus penetration test has been employed in studies of human (Alexander 1981, Pandya *et al* 1986), bovine (Kummerfeld *et al* 1981a, Matousek *et al* 1989) and ovine (Langford *et al* 1984) fertility investigations. Further, in an attempt to standardize these *in vitro* procedures to assess fertilizing ability of bovine spermatozoa the semen of proven bulls has been used (Kummerfeld *et al* 1981a, Okuda *et al* 1988, Murase and Braun 1990).

Matousek *et al* (1989) reported that rate of sperm penetration in same cervical mucus with sperm of different bulls differed significantly. Also, animals which conceived exhibit better rate of sperm migration in oestrial mucus as compared to those which failed to conceive (Sokolovskaya and Skopetes 1986, Dev *et al* 1999).

The sperm penetration provides reliable data for fertility assessment, allows great discrimination of sperm function than semen analysis alone and is a useful tool for diagnosis and management of fertility (Alexander 1981, Dev *et al* 1996a).

2.1.2 Sperm Penetration in Polyacrylamide gel

Cervical mucus has been demonstrated to have potential as a medium to test the quality of semen in terms of sperm function (Insler *et al* 1979, Blasco 1984) and a number of independent studies have indicated that the fertilizing capacity of spermatozoa is correlated with cervical mucus penetration (Alexander 1981, Eggert-Kruse *et al* 1989).

On the contrary, artificial media for sperm migration testing are required because there are various problems in working with cervical mucus. The main problem for most andrologist might be the requirement to collect only periovulatory cervical mucus (human) or estrous mucus (cows), variability of cervical mucus with regard to amount, viscoelastic properties and composition (Moghissi 1972, Wolf *et al* 1977, Panchal 1988). Influencing factors such as microbial colonization (Eggert-Kruse *et al* 1992), antisperm antibodies (Eggert-Kruse *et al* 1991, Kremer and Jager 1992) and pH of the cervical secretions (Eggert-Kruse *et al* 1993b) also have to be regarded. Further disadvantages are the limitation of amount of mucus per collection, the variability because of storage and the impossibility to store cervical mucus over longer time periods without loss of quality (Kummerfeld *et al* 1981b, Devanathan and Pattabiraman 1996, Kaushal 1998).

To overcome these problems a material is needed with sperm migration characteristics similar to those of cervical mucus, which could be obtained or prepared simply with uniform stable characteristics. Various sperm migration media have been tried by various scientists from time to time.

Matousek *et al* (1989) in their experiment compared the SPD (sperm penetration distance) values in body fluids and reported that distance covered by vanguard bull spermatozoa in body fluids such as bovine serum (33 ± 14 mm), ovarian follicular fluid (30 ± 10 mm) and seminal fluid (52 ± 4.2 mm) per 2 minutes was more as compared to cervical mucus (14 ± 11 mm) per 90 minutes.

Similarly, egg white has also been suggested as a cervical mucus substitute (Hanson and Hjort 1979, Eggert-Kruse *et al* 1990) for evaluating penetrability of human spermatozoa, but the pH of egg white can be injurious to sperm and egg white should be buffered. Newwinger and Cooper (1991) reported the use of hyaluronic acid for sperm migration studies.

Bisset (1980) proposed the use of mucus model whose properties can be modified reproducibly to overcome the problems associated with *in vitro* studies. He reported the use of polyacrylamide cross linked with polyethylene glycol bisacrylate (PEG) as a substitute for human cervical mucus. This mucus model was stable for few days at 4°C. Also, freezing of the PEG medium did not alter its rheological properties (viscosity, flow elasticity and spinnbarkeit). The sperm penetrated in fresh medium @ 1.3 mm/minute. He reported identical behaviour in this medium as with Human Cervical Mucus (HCM) in which sperm migrated @ 1.66 mm/minute.

Lorton *et al* (1981) reported the use of synthetic migration medium for capillary sperm migration studies. They observed parallel sperm migration in both polyacrylamide gel (PAG) and bovine cervical mucus (BCM). Cow bull spermatozoa which varied widely in sperm migration distance in bovine cervical mucus, maintained similar relative migration distance in this synthetic medium. They prepared synthetic media containing 1 to 3 per cent (w/v) acrylamide with proportionate N, N'-methylene bis acrylamide (BIS) but 2 per cent medium had gross physical characteristics similar to those of estrous bovine cervical mucus. In addition, this medium could be stored for months together in refrigerator at 4°C without change in sperm penetration characteristics.

Goldstein *et al* (1982) studied the rate of human sperm migration in artificial cervical mucus (ACM) which was synthesized from polyacrylamide. They concluded that ACM can replace BCM in assessing ability of spermatozoa to interact with viscous fluid and use of ACM could provide a more standardized batch of "mucus" for testing purpose in the laboratory. ACM can be stored for longer period (approximately 6 months) and is more uniform than various batches of BCM (Urry *et al* 1986).

Egger-Kruse *et al* (1993a) revealed that sperm ability to penetrate synthetic medium (polyacrylamide gel 1.8%) correlated significantly with penetration of cervical mucus. They also observed significantly higher fertility rate with semen samples that adequately penetrated polyacrylamide gel in sperm migration studies.

Kaushal *et al* (1999) reported the use of polyacrylamide gel as a medium for migration studies of buffalo bull spermatozoa. They concluded that

2 per cent polyacrylamide media provide stable gel for fertility investigations which shows no batch to batch variation and can be stored for months together at 4°C without any change in sperm migration characteristics.

2.1.3 Sperm Penetration in relation to Semen Quality

The fertilizing ability of a spermatozoa can only be ascertained if it successfully fertilize ovum that ultimately results in pregnancy. The various tests used traditionally to assess the possible fertilizing capacity of spermatozoa are sperm motility, sperm count and sperm morphology. Although, these classical semen analysis are basic in male fertility investigations but their significance for fertility evaluation of sperm is limited (Blasco 1984). To have more reliable information regarding fertilizing ability of spermatozoa biologically *in vitro* tests for sperm migration add new dimensions in this field. Ideally, these tests are sperm penetration test and egg penetration test that give more relevant information regarding reproduction quality of sperm (Katz *et al* 1989).

There are some experimental reports dealing with old and new devices and techniques to correlate sperm migration either in estrual mucus or PAG with classical semen analysis parameters. An excellent correlation has been reported between sperm penetrability in mucus and sperm motility in humans (Overstreet and Katz 1977, Mortimer *et al* 1986, Keel and Webster 1988). A significant correlation between sperm penetration and progressive sperm motility in bovines has also been established in the experimental studies (Sidhu *et al* 1986, Okuda *et al* 1988, Murase and Braun 1990).

Reports indicate high correlation between sperm concentration and sperm mucus penetration in human (Mortimer *et al* 1986, Keel and Webster 1988)

and bovines (Sidhu *et al* 1986). On the contrary, David *et al* (1979) and Okuda *et al* (1988) observed non-significant correlation in human and bovine species, respectively.

Perry *et al* (1977) suggested that estrual mucus behaves as a biological filter, preventing abnormal sperms from penetrating the cervical canal. But, there is a poor correlation between normal sperm count and their penetrability both in human (Insler *et al* 1979, Keel and Webster 1988) and bovine (Okuda *et al* 1988).

There exists excellent correlation between sperm viability and penetration in humans (David *et al* 1979) and bovines (Murase and Braun 1990). Also, cryopreservation of semen greatly influence the sperm penetration value (Okuda *et al* 1988, Matousek *et al* 1989).

Goldstein *et al* (1982) studied migration of fresh and cryopreserved human spermatozoa in PAG. They revealed that correlation between sperm motility and sperm migration distance ranged from 0.57 to 0.62. However, correlation coefficient between sperm concentration and migration distance varied from 0.25 to 0.34.

Eggert-Kruse *et al* (1993a) found significant correlation between standard semen parameters and migration testing, however, correlation values decreased with higher concentration of polyacrylamide. They reported correlation coefficient with regard to penetration distance and sperm count (0.367), progressive motility (0.247), morphology (0.350), viability (0.234) in 1.8 per cent PAG.

Dev *et al* (1996b) reported a significant ($P < 0.05$) correlation between sperm penetration distance (SPD) values and sperm motility (0.576), live sperm count (0.758), normal sperm count (0.670) in fresh diluted semen whereas with cryopreserved semen correlation coefficients were 0.574, 0.498 and 0.493, respectively. They further reported a significant ($P < 0.01$) relationship between SPD values of fresh diluted and frozen thawed semen ($r = 0.675$).

Kaushal (1998) studied migration of cryopreserved buffalo bull spermatozoa in 2 per cent PAG and reported significant ($P < 0.05$) correlation coefficient with regard to penetration distance and sperm motility (0.747), live sperm count (0.615) while non-significant ($P < 0.05$) correlation coefficient (0.389) was obtained between penetration distance and normal sperm count.

2.1.4 Clinical Significance of Sperm Penetration in Polyacrylamide Gel

The sperm penetration test is a potential diagnostic tool for evaluation of fertility. *In vitro* SMPT devised by Kremer (1965) have been widely used clinically to evaluate male fertility (Alexander 1981, Takemoto *et al* 1985, Dev *et al* 1996a). The main feature of this test include its physical nature, commercial availability and reproducibility which makes its use easier and popular in domestic species. Commercially available penetration test kits (PenetreeTM) used to evaluate human fertility (Moghissi *et al* 1982) and ovine fertility (Mole and Fitzgerald 1990), proved very useful in clinical investigations.

Kummerfeld *et al* (1981a) and Matousek *et al* (1989) found non-significant correlation between spermatozoal penetrability and bovine fertility. However, Predojovic and Miljkovic (1984) have observed significant ($P < 0.05$) correlation between spermatozoa penetrability and bovine conception rate.

Further, modified sperm penetration and swim up method produced promising results in predicting ram fertility (Suttiyotin *et al* 1992).

The various problems associated with use of bovine cervical mucus as a medium for the sperm penetration studies in human and bovine spermatozoa have resulted in the development of a synthetic media for conducting sperm migration studies.

Lorton *et al* (1981) proposed the use of polyacrylamide gel as synthetic medium for human or bovine sperm migration studies. A particular advantage of this medium is that its ingredients are readily available and gel can be produced in large quantity with uniform quality. The medium can be stored for months in refrigerator at 4°C without change in sperm penetration characteristics. Thus, this medium could prove useful in assessing semen of male infertile patients. This synthetic medium can also serve as a standard in assessing sperm penetrating characteristics of cervical mucus of female partner.

Goldstein *et al* (1982) used PAG as the medium for studying sperm penetration characteristics of fresh and cryopreserved human spermatozoa. They also viewed that this stable gel can be put into application for fertility investigations.

Similarly, Eggert-Kruse *et al* (1993) evaluated the use of PAG as a substitute for human cervical mucus in sperm penetration test and concluded that penetration testing with PAG provide important information in analysing intrinsic motility of spermatozoa which could not be obtained by routine sperm analysis. They reported adequate sperm migration in 1.8 per cent polyacrylamide more frequently in fertile group as compared to infertile.

Lee *et al* (1987) using laser light scattering spectroscopy, reported that rheological and structural proportion of BCM and HCM are similar. Therefore, use of polyacrylamide gel as a substitute of bovine estrual mucus for sperm penetration studies would be more useful. Kaushal *et al* (1999) concluded that both 2 per cent PAG and estrual mucus can be used as adjunct objective test to seminal parameters. However, advantages of polyacrylamide media over mucus make it more useful for assessment of bull semen.

2.2 SEPHADEX COLUMN FILTRATION

Semen samples of buffalo bulls in majority of cases carry morphologically normal sperms intermixed with abnormal non-motile sperms and other cellular and non-cellular debris. Such samples will have poor keeping quality and freezability. Therefore, any technology which could improve the initial quality of poor quality ejaculates of potential sires by fractionation of morphologically normal motile sperms from the abnormal sperms and sperm debris, would be of immense value.

Numerous investigators have tried to separate normal motile sperms from abnormal non-motile sperms by various physical means thereby improving the quality of ejaculated semen of men and animals. The first successful separation of motile from non-motile spermatozoa was performed by passing diluted semen through a layer of small glass beads (Bangham and Hancock 1955). Other methods of sperm separation have been developed using pyrex beads (McGrath *et al* 1977), bovine serum albumin (BSA) gradients (Wall *et al* 1984, Estiennen *et al* 1988), glass wool (Maki-Laurila and Graham 1968, Paulson and Polakoski 1977),

Newtonian gels (Luderer *et al* 1982), sephadex gels (Graham *et al* 1976) and a swim up method (Parrish *et al* 1986).

Sperm separated on BSA gradients increased motility but failed to raise fertility over that of unseparated semen in the pig (Dixon *et al* 1980) and the horse (Goodeaux and Kreider 1978). Newtonian gels also failed to enhance fertility in separated bull semen (Luderer *et al* 1982). Recently, Cisale (1998) compared various methods for improving semen quality in livestock including semen dialysis, the swim up technique, density gradient centrifugation and sephadex filtration. He concluded that sephadex filtration columns with flow controls provide the most useful method for routine preparation of semen doses.

Sephadex is a bead forming gel prepared by cross linking dextran with epichlorohydrin. A partial structure of the sephadex has been shown in Fig.1. Gel is extremely hydrophilic due to presence of a large number of hydroxyl groups and it swells up in water and electrolyte solution. Different grades of sephadex viz. G-15, G-25, G-50, G-75, G-100, G-200 etc. are available in the market and they differ in their degree of swelling and fractionation due to difference in the degree of cross linking. But this degree of swelling is independent of the presence of salts and detergents.

2.2.1 Sephadex Column Filtration vis-a-vis Semen quality

Semen filtration through sephadex column was employed to assess the semen quality in bull semen by Graham *et al* (1976). Later on, same method was used to improve the semen quality by filtering out the dead spermatozoa in cow bull semen (Graham and Graham 1990, Kanakraj and Easwaran 1991, Vyas *et al* 1992a) and in buffalo bull semen (Kumar *et al* 1992, Chauhan *et al* 1993a,

Goyal *et al* 1996, Kumar *et al* 1999). Sephadex ion exchange method was used by Anzar and Graham (1995) to improve semen quality, of course, recovery rate was better than sephadex alone.

Graham *et al* (1976) reported that sephadex (G-15) filtration column retains dead or immotile spermatozoa and filtered semen exhibited higher motility (<90%) and progressive motility (<70%) than unfiltered spermatozoa. Thereafter, numerous investigators evaluated this sephadex filtration technique for improving the semen quality. They observed that sephadex filtration cause increase in initial motility from 45 to 68.05 per cent (Fayemi *et al* 1979); from 71.8 to 78.8 per cent and post thaw motility from 36.6 to 41.7 per cent (Goyal *et al* 1996); from 73 to 88 per cent and upto 63-64 per cent (Kumar *et al* 1996); from 66.5 to 78.8 per cent and 33.8 to 43.3 per cent (Kanakaraj *et al* 1996), respectively.

Vyas *et al* (1992a) compared the different grades of sephadex (G-25 and G-50) with glass wool as filtration media and concluded that sephadex G-25 and glass wool were at par and both were superior to G-50 for improving semen quality of crossbred bulls. They reported increase in initial motility and post-thaw motility from 61.38 to 75.00 per cent and from 25.68 to 39.78 per cent, respectively for sephadex G-25 (Vyas *et al* 1992b).

Chauhan *et al* (1993a) also compared the different grades of sephadex (G-25, G-50, G-75, G-100 and G-200) and reported that sephadex G-75, G-100 and G-200 cause a significant increase in motility but G-75 can be employed for the filtration of buffalo bull semen to get maximum recovery rate and optimum motility. They reported an increase in initial motility from 60 to 77 per cent and

post-thaw motility from 36 to 43 per cent after filtration through sephadex G-75 (Chauhan *et al* 1993b). On the other hand, Kumar *et al* (1999) reported increase in initial motility and post-thaw motility from 71.25 to 80.83 per cent and 36.35 to 38.33 per cent, respectively, while using sephadex G-75 for filtration of buffalo bull semen. Anzar and Graham (1996) used sephadex ion-exchange column where filtered spermatozoa exhibited higher ($P < 0.010$ motility ($> 90\%$) and progressive motility ($> 70\%$) than unfiltered spermatozoa.

Fayemi *et al* (1979) found a decrease in dead spermatozoa from 30 to 12 per cent after sephadex column filtration. Thereafter, various workers reported decrease in dead spermatozoa count from 30.3 to 17.2 per cent (Vyas *et al* 1992a); from 31.7 to 18.1 per cent (Chauhan *et al* 1993a); from 35.6 to 15.8 per cent (Goyal *et al* 1996); from 33 to 22.3 per cent (Kanakaraj *et al* 1996); from 21.7 to 6.5 per cent (Kumar *et al* 1996) and from 20.0 to 12.1 per cent (Kumar *et al* 1999), respectively. Some workers also reported decrease in dead spermatozoa percentage at post-thaw stage viz. from 68.1 to 49.8 per cent (Vyas *et al* 1992a); 54.9 to 46.6 per cent (Goyal *et al* 1996) and from 56.7 to 51.0 per cent (Kumar *et al* 1999).

Sephadex column filtration also decreased the abnormal spermatozoa in the semen from 9 to 4 per cent (Kumar *et al* 1996); from 17.25 to 6.60 per cent (Vyas *et al* 1992a); from 22.83 to 10.3 per cent (Chauhan *et al* 1993a); 10.1 to 8.2 per cent (Goyal *et al* 1996); from 17 to 6.2 per cent (Kanakaraj *et al* 1996) and from 11 to 5 per cent (Kumar *et al* 1999). Some workers also reported decrease in abnormal spermatozoa percentage at post-thaw stage viz. from 23.50 to 9.18 per

cent (Vyas *et al* 1992a); from 27.53 to 15.18 per cent (Chauhan *et al* 1993a) and from 16.4 to 14.3 per cent (Goyal *et al* 1996).

Kumar *et al* (1999) reported that filtered semen at post freeze thaw stage showed significant increase ($P < 0.05$) in SPD as compared to unfiltered semen. This might be due to more number of progressively motile spermatozoa in the filtered semen. Graham and Graham (1990) concluded that this filtration system can be incorporated into a freezing protocol which could effectively remove abnormal sperms and sperm debris from ejaculates of potential sires. Filtration does improve the percentage of motile sperms after freezing and thawing, but the procedure did not improve the fertility of bulls already exhibiting high non-return rates.

2.3 STATIC EJACULATES

Buffalo semen is generally characterized by two types of mass activity viz. ejaculates having wave motion similar to those observed in cattle and ejaculates found to be of cell mass (static) without sperm activity. Such non-motile ejaculates are called either zero ejaculates or static ejaculates. Sahni (1990) reported that the percentage of ejaculates showing normal wave activity in buffalo semen averaged 30-50 as compared to 70-100 in cattle. These static semen samples were previously discarded and non-motile characteristic of ejaculate is attributed to the presence of either spermiostatic factor, motility inhibiting factor or absence of forward motility proteins (Agarwal and Tomer 1998).

The occurrence of static ejaculates is a peculiar phenomenon seen during extremes of cold season. Incidence of static ejaculates vary from 25-66 per

cent as evident from following findings viz. 38 per cent (Adhi *et al* 1968); 43-100 per cent (Sahni and Mohan 1990); 39 per cent (Nainar *et al* 1990); 31 per cent (Kumar *et al* 1993); 33 per cent (Dhami *et al* 1995) and 46 per cent (Jindal *et al* 1996).

It has been observed that about 2/3rd of such ejaculates regain motility when mixed in extender and can be frozen successfully. The mean revival rate of static ejaculates quoted by different workers is 70-80 per cent (Sahni and Mohan 1990); 40 per cent (Nainar *et al* 1990) and 91.67 per cent (Dhami *et al* 1995) and 87.8 per cent (Jindal *et al* 1996).

The average normal semen picture observed both for motile and static ejaculates were: volume 2.56 ± 0.10 and 2.08 ± 0.12 ml, density (0-3 scale), 2.56 ± 0.04 and 2.26 ± 0.07 per cent initially motile sperms (77.82 ± 0.46 and 65.66 ± 1.54), sperm concentration (849.00 ± 22.64 and $710.26 \pm 31.14 \times 10^6/\text{ml}$), number of sperms per ejaculate (1981.26 ± 73.08 and 1432.49 ± 106.26), pH (6.72 ± 0.02 and 6.89 ± 0.04), per cent live sperm (86.57 ± 0.22 and 79.80 ± 0.84), per cent total abnormal sperms (12.51 ± 0.11 and 14.25 ± 0.32), per cent normal acrosomes (90.31 ± 0.16 and 88.74 ± 0.34), per cent normal sperms (87.48 ± 0.17 and 85.71 ± 0.32), total normal sperms per ejaculates (1734.07 ± 64.31 and 1227.62 ± 91.86) and total live sperms per ejaculate (1711.58 ± 62.91 and 1158.00 ± 89.36). The reaction time for motile and static ejaculates were 221.03 ± 14.50 and 254.06 ± 17.06 seconds. All the seminal pictures studied above differed significantly ($P < 0.01$) in motile and static ejaculates and the semen of static ejaculates was of poor quality (Kumar *et al* 1993).

The freezability test as measured by post-thaw motility and intact acrosome percentage indicated significant ($P < 0.01$) reduced freezability for the samples with static spermatozoa. However, the freezability of static semen ejaculates were high enough to pass the standards set for usage of frozen semen (Chinnaiya 1982, Nainar *et al* 1990). These results pointed out that though the static samples may get discarded based on initial motility, the sperms are capable of gaining motility on dilution and freezing successfully though not to the extent of normal semen (Jindal *et al* 1996).

2.4 COLD SHOCK

Artificial breeding of buffaloes on a large scale using semen from bulls of excellence with superior germplasm can solve the problem of low productivity of non-descript buffaloes. However, the use of frozen semen and artificial insemination for improvement of buffalo has not been extensive and still majority of buffaloes are bred either through natural service or using liquid semen. Usually, this liquid semen is stored and transported at temperature between 4 and 6°C. In India, under field conditions, this temperature is maintained in insulated containers by use of ice. But it is well known that spermatozoa of many mammals, including buffalo bulls, undergo irreversible changes on sudden exposure to a low temperature, commonly referred as cold shock (Tomer 1984, Sahni 1986, Tao *et al* 1995). In mammals, a permanent loss of sperm motility and metabolic activity occurs during the process of rapid cooling due to disruption of acrosomal and plasma membranes and leakage of enzymes (Quinn *et al* 1969, Harrison and White 1972, White 1993).

CHAPTER Cold shock significantly alter semen characteristics of cattle and buffalo bulls. A significant ($P < 0.01$) decrease in sperm motility and live sperm counts as well as a corresponding increase in morphological abnormalities were observed in spermatozoa of cattle and buffalo bulls due to cold shock (Salisbury *et al* 1942, Church *et al* 1975, Chinnaiya and Ganguli 1978).

Mohan *et al* (1992) reported that in case of cow and buffalo bulls, however, a significant decrease in sperm motility (44.11 and 33.35%, respectively) following the cold shock treatment and this drop in motility was irreversible. The percentage of dead spermatozoa increased by 43.66 per cent in cattle and 36.66 per cent in buffalo bull semen. In addition, a considerably higher ($P < 0.01$) number of abnormal spermatozoa were observed in two mammalian species after exposure to cold shock.

Similarly, Vaisberg *et al* (1994) studied effect of cold shock on buffalo bull semen and reported that before cold shock, sperm motility and incidence of intact acrosomes was 64.66 and 66.78 per cent, respectively vs 25.68 and 48.51 per cent after cold shock ($P < 0.001$). Also, Malik *et al* (1994) and Gupta *et al* (1998) concluded that cold shock had significant deleterious effect on motility, livability and absolute index of livability of buffalo bull semen.

CHAPTER III

MATERIALS AND METHODS

The study was carried out on seven buffalo bulls (M-1434, M-1437, M-1446, M-1451, M-1506, M-1536 and M-1550) maintained at Punjab Agricultural University Dairy Farm, under the "All India Coordinated Research Project on Buffalo Breeding". In addition, five buffalo bulls (BH-06, BH-14, BH-19, BH-25 and BH-32) maintained at Exotic Cattle Farm, Bhattian, Khanna were also included in this study. All these bulls were provided with identical conditions regarding housing, feeding and exercise. The bulls were kept in half walled concrete sheds in individual pens and were given exercise for half an hour on alternate days by means of manual exerciser. Bulls were given bath and cleaned each time before semen collection. All the bulls were fed green fodder, wheat straw, concentrate mixture throughout the year as per standing feeding schedule followed at respective dairy farms. Water for drinking was provided *ad libitum*.

3.1 EXPERIMENTAL DESIGN

The research work was conducted on the semen of fertile and subfertile buffalo bulls. The semen was collected regularly from the bulls and experiment was designed as under:

3.1.1 Group I: Experiment

In this group, study was carried out on four healthy fertile buffalo bulls (M-1437, M-1451, BH-19 and BH-32). A total of 20 ejaculates, 5 from each

bull were collected and each ejaculate was extended with Tris-egg yolk extender. Then, a 5 ml semen aliquot was further extended with Tris-egg yolk extender to make final volume 10 ml and rest of extended semen was processed for cryopreservation at -196°C . These fresh, extended and cryopreserved semen samples were examined for sperm motility, normal sperm count and live sperm count. Thereafter, SPT was conducted through PAG column. Similarly, Tris-egg yolk extended semen preserved at $4-6^{\circ}\text{C}$ (viz. chilled semen) was evaluated for above said parameters at 24 hrs interval (Flow Diagram-1).

3.1.2 Group II: Experiment

This group was further subdivided into two groups exhibiting initial poor semen quality and poor freezability, respectively.

Group IIa: Experiment

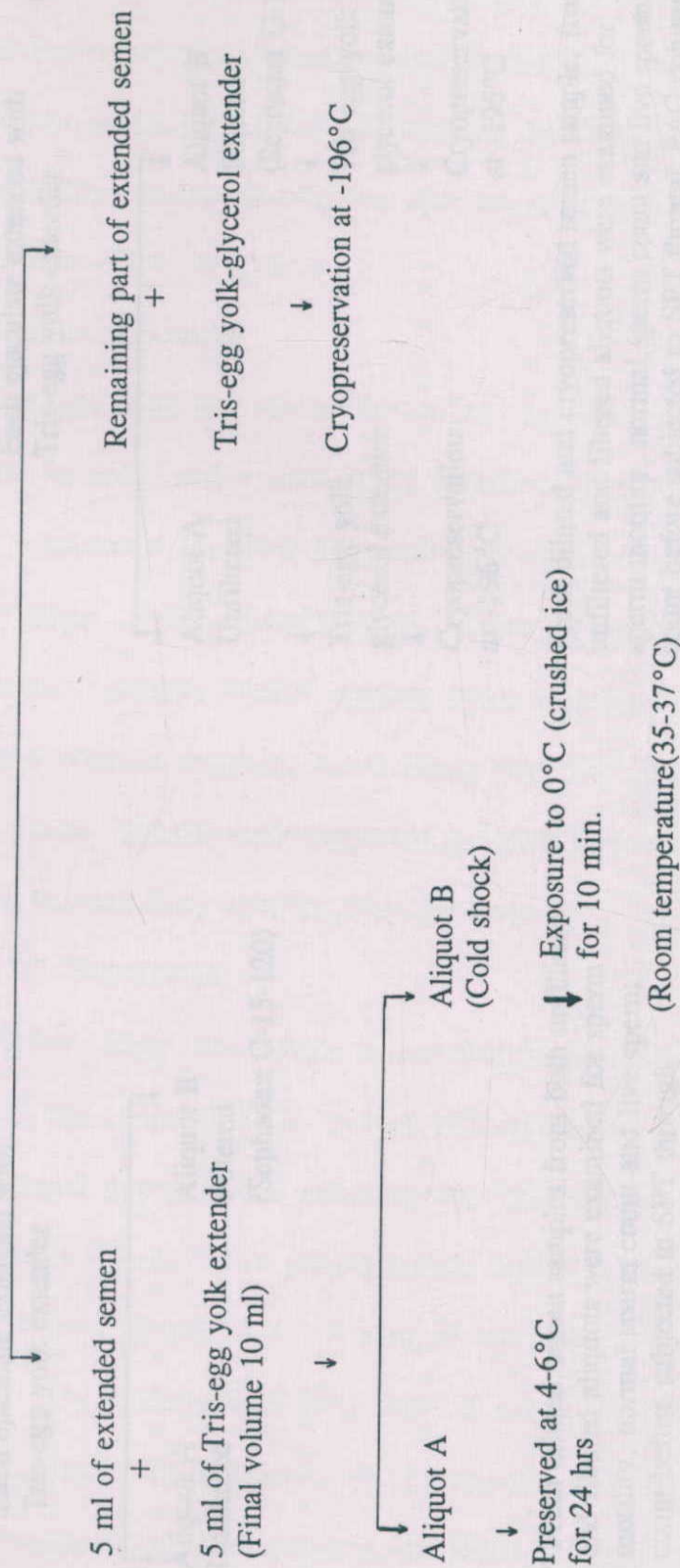
In this group, study was carried on four buffalo bulls (M-1446, M-1506, BH-14 and BH-25) exhibiting initial poor semen quality. A total of 20 ejaculates, 5 from each bull were collected and each ejaculate extended with Tris-egg yolk extender. Here, aliquot A of extended semen was kept unfiltered and aliquot B was filtered using Sephadex (G-15-120) column. These, extended semen samples from both unfiltered and filtered aliquots were examined for sperm motility, normal sperm count, live sperm count along with SPT through PAG column (Flow Diagram-2).

Group IIb: Experiment

In this group, study was carried out on four buffalo bulls (M-1434, M-1536, M-1550 and BH-06) exhibiting poor freezability viz. post thaw motility < 40 per cent. A total of 20 ejaculates, 5 from each bull were collected and each

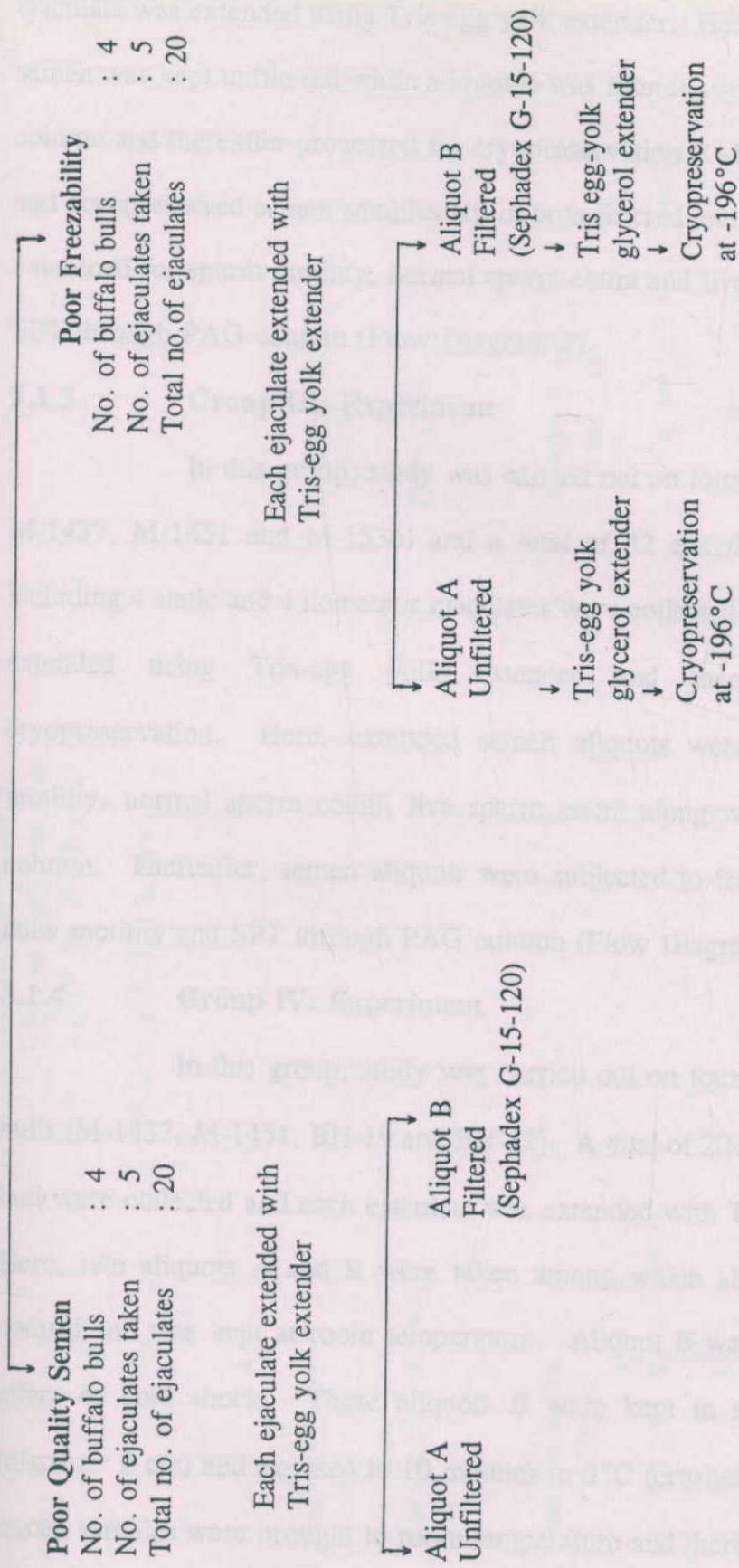
No. of buffalo bulls : 4
No. of ejaculates taken : 5
Total no. of ejaculates : 20

Each ejaculate extended with
Tris-egg yolk extender



- 1) Fresh, extended and cryopreserved semen was examined for sperm motility, normal sperm count and live sperm count alongwith SPT through PAG column.
- 2) Tris-egg yolk extended semen preserved at 4-6°C viz. chilled semen was evaluated at 24 hrs of storage for sperm motility, normal sperm count and live sperm count before subjected to SPT through PAG column.
- 3) Semen aliquot A (control) and semen aliquot B (exposure to 0°C for 10 min.) viz. cold shocked were examined for sperm motility, normal sperm count and live sperm count before subjected to SPT through PAG column.

Flow Diagram 2



* Fresh diluted semen samples from both unfiltered and filtered aliquots were examined for sperm motility, normal sperm count and live sperm count before subjected to SPT through PAG column.

* Fresh diluted and cryopreserved semen samples from both unfiltered and filtered aliquots were examined for sperm motility, normal sperm count and live sperm count before subjected to SPT through PAG column.

ejaculate was extended using Tris-egg yolk extender. Here, aliquot A of extended semen was kept unfiltered while aliquot B was filtered using Sephadex (G-15-120) column and thereafter processed for cryopreservation at -196°C . These, extended and cryopreserved semen samples (from both filtered and unfiltered aliquot) were examined for sperm motility, normal sperm count and live sperm count alongwith SPT through PAG column (Flow Diagram-2).

3.1.3 Group III: Experiment

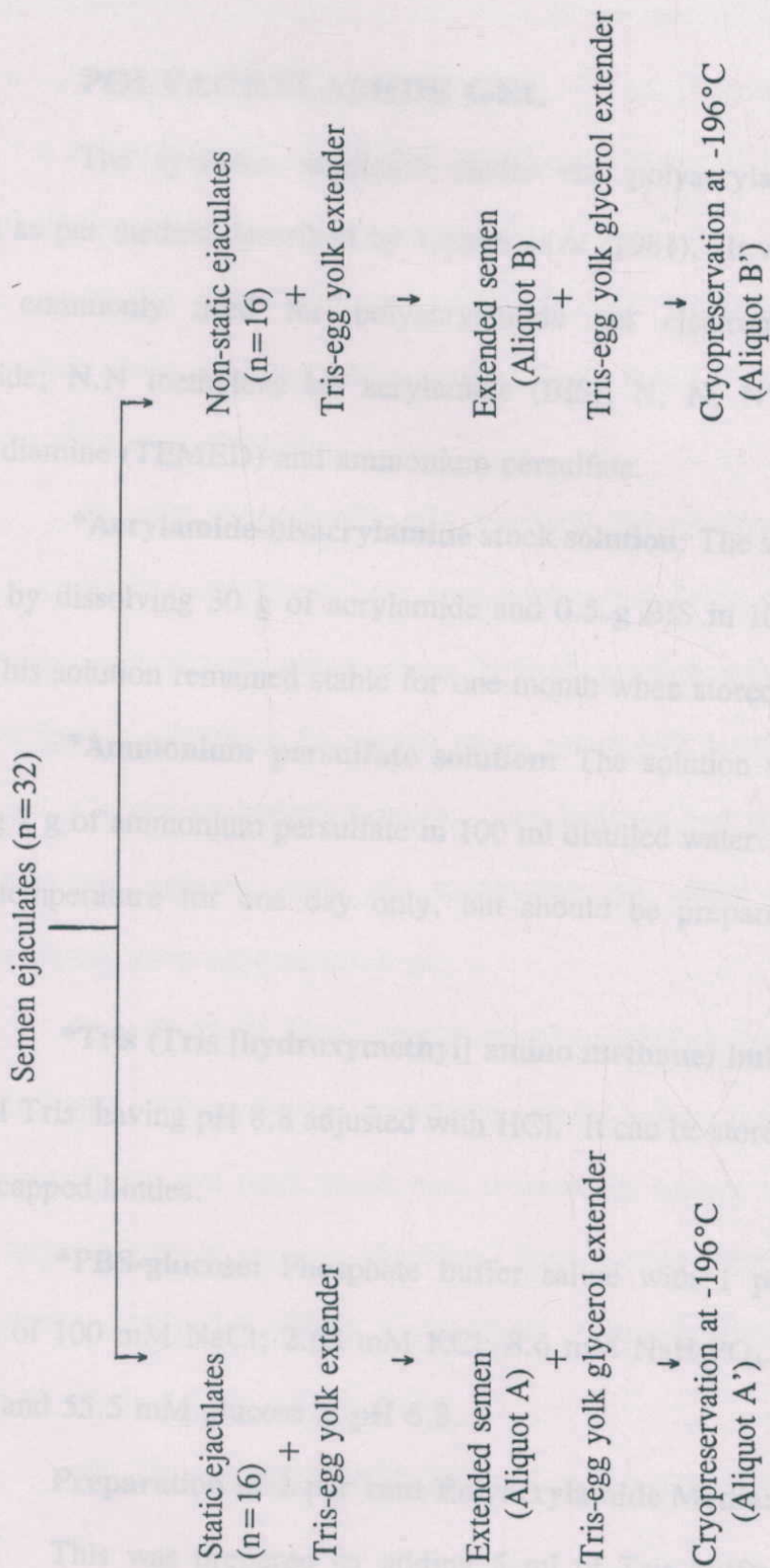
In this group, study was carried out on four buffalo bulls (M-1434, M-1437, M-1451 and M-1536) and a total of 32 ejaculates, 8 from each bull including 4 static and 4 non-static ejaculates were collected. These ejaculates were extended using Tris-egg yolk extender and thereafter processed for cryopreservation. Here, extended semen aliquots were examined for sperm motility, normal sperm count, live sperm count along with SPT through PAG column. Thereafter, semen aliquots were subjected to freezability test viz. post thaw motility and SPT through PAG column (Flow Diagram-3).

3.1.4 Group IV: Experiment

In this group, study was carried out on four healthy fertile buffalo bulls (M-1437, M-1451, BH-19 and BH-32). A total of 20 ejaculates, 5 from each bull were collected and each ejaculate was extended with Tris-egg yolk extender. Here, two aliquots A and B were taken among which aliquot A served as the control and was kept at room temperature. Aliquot B was used to examine the effect of cold shock. These aliquots B were kept in small pre-cooled vials (diameter 2 cm) and exposed to 10 minutes to 0°C (crushed ice). These exposed semen samples were brought to room temperature and thereafter semen aliquots

Flow Diagram-3

No. of buffalo bulls : 4
No. of ejaculates taken : 8
Total no. of ejaculates : 32



- 1) Semen aliquot A and B were evaluated for sperm motility, normal sperm count and live sperm count before subjected to SPT through PAG column.
- 2) Semen aliquot A' and B' were examined for post thaw motility before subjected to SPT through PAG column.

A and B were examined for sperm motility, normal sperm count and live sperm count alongwith SPT through PAG column (Flow Diagram-1).

3.2 POLYACRYLAMIDE GEL

The synthetic migration media viz. polyacrylamide media was prepared as per method described by Lorton *et al* (1981). It was prepared with reagents commonly used for polyacrylamide gel electrophoresis such as Acrylamide; N.N methylene bis acrylamide (BIS); N, N, N', N' tetramethyl ethylene diamine (TEMED) and ammonium persulfate.

***Acrylamide-bisacrylamide stock solution:** The stock solution was prepared by dissolving 30 g of acrylamide and 0.5 g BIS in 100 ml of distilled water. This solution remained stable for one month when stored at -5°C .

***Ammonium persulfate solution:** The solution was prepared by dissolving 2 g of ammonium persulfate in 100 ml distilled water. It can be stored at room temperature for one day only, but should be prepared freshly when required.

***Tris (Tris [hydroxymethyl] amino methane) buffer:** Buffer used was 1.5 M Tris having pH 8.8 adjusted with HCl. It can be stored in refrigerator in tightly capped bottles.

***PBS-glucose:** Phosphate buffer saline with 1 per cent glucose comprised of 100 mM NaCl; 2.68 mM KCl; 8.6 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 12.7 mM Na_2HPO_4 and 55.5 mM glucose at pH 6.8.

3.2.1 Preparation of 2 per cent Polyacrylamide Media:

This was prepared by adding 5 ml of Tris buffer to 1.34 ml of

acrylamide - bisacrylamide stock solution and sufficient water to yield a final volume of 20 ml (Kaushal 1998). Polymerization of media was achieved by incubating overnight at room temperature with the addition of 300 μ l of 2 per cent ammonium persulfate and 10 μ l of TEMED. Polyacrylamide media was then dialyzed for 24 hours at 4°C against three or four changes of at least 10 volumes of PBS-glucose solution.

In the present study, five different batches of 2 per cent polyacrylamide media were prepared freshly every time and subjected to sperm penetration test as per grouping.

3.2.2 Physical Examination of 2 per cent Polyacrylamide Gel

Various batches of 2 per cent polyacrylamide gel were examined for their physical properties viz. colour and transparency; spinnbarkeit; pH and visible gelification before subjecting the gel for sperm penetration studies.

Colour and transparency: This included any abnormal colour or clarity other than normally clear and transparent gel. This was noted for all batches of 2 per cent polyacrylamide gel.

Spinnbarkeit: This property of gel is based on extent of gelification in particular batch of gel. It was measured by taking a drop of particular batch of gel on index finger and then thumb was brought in contact with index finger followed by movement in opposite direction. Spinnbarkeit distance was measured between thumb and index finger at the point where break was observed in continuity of gel.

pH: The pH of particular batch of gel was recorded with the help of pH meter.

Extent of gelification: Based on the extent to which a particular batch of polyacrylamide solution gelled, they were described as absent, slight, moderate and complete gelification.

3.3 SEPHADEX FILTRATION

3.3.1 Preparation of Sephadex Column

A 20 per cent (w/v) slurry of sephadex G-15-120 of particle size 40-120 microns (Pharmacia Fine Chemicals, Uppsala, Sweden) was prepared in 3 per cent (w/v) sodium citrate and allowed to swell at least for 3 hours. A small quantity of glasswool fibres were placed at the bottom of the column to prevent sephadex loss. The fibres were packed with the plunger of the syringe. The sephadex slurry (0.6 ml) was transferred into each syringe which were placed on a rack to allow free drainage of fluid into a collecting vessel. These gel columns were stored at 5°C in the refrigerator until use (Fayemi *et al* 1979, Kumar 1989).

3.3.2 Sephadex Filtration of Semen

Before filtration, the rack containing syringes (with sephadex columns) fixed vertically, was placed in incubator at 37°C. Prior to filtration the slurry fed in each syringe was wet with 3-4 drops of Tris buffer and 2-3 ml of semen was gently placed over the above mentioned columns. The complete filtration process which took about 5-10 minutes was carried out at 37°C in the incubator, protected from bright light and draught.

3.4 COLLECTION AND CRYOPRESERVATION OF SEMEN

3.4.1 Collection of Semen

A total of 74 ejaculates from 12 buffalo bulls were collected twice a week by artificial vagina method. At least 2-3 false mounts were given prior to collection. Immediately after collection, the ejaculates were kept at 37°C in water bath. Each ejaculate was then evaluated for physical characteristics (volume, colour, density) and microscopically for mass activity, per cent motile spermatozoa, sperm concentration and sperm morphology by standard procedures.

3.4.2 Extension of Semen

Semen was extended and processed for deep freezing as per method described by Cassou (1964) and Bindra (1991). Tris buffer for Tris-egg yolk extender was prepared by dissolving 3.786 g of Tris and 2.115 g of citric acid in 100 ml of double glass distilled water. Egg yolk was added to the buffer in the ratio of 1:4. Penicillin-G sodium 1000 IU and streptomycin sulphate 1.00 mg per ml of extender were also added to it.

Tris-egg yolk extender was divided into two equal parts (Part I and Part II) in sterilized tubes. The semen samples and conical flask containing Part I of extender were kept in water bath at 37°C. Semen samples were then extended to have 200 million spermatozoa/ml.

3.4.3 Incorporation of Glycerol and Cooling

Glycerol @ 14 per cent was added in the part II of extender and then part II of extender and extended semen samples were transferred to cold handling cabinet at 5°C for 60-90 minutes. Thereafter, glycerolated extender was mixed

to the extended semen in 2-3 steps at interval of 10-15 minutes, leading final concentration of 100 million spermatozoa per ml. Then, this completely extended semen was kept at 5°C for four hours for equilibration (Tuli *et al* 1981).

3.4.4 Filling and Plugging of Straw

The straws were printed showing bull no., name of extender, date of freezing etc. These straws after drying in hot air oven were exposed to ultraviolet light for one hour. Fifteen straws were fixed in a clamp, keeping the cotton plug on one side. These straws were kept in cold handling cabinet for one hour before filling.

The completely extended semen was filled into the straws with the help of suction pump. Straws were shaken a little to have an air space in the straws to avoid the bursting of straws during freezing. Then, open end of the straw was plugged using polyvinyl alcohol powder. The straws were rubbed with blotting paper so that extra powder was removed.

3.4.5 Freezing and Storage

The rack containing straws were then placed on grill of freezing chamber (wide mouth liquid nitrogen container) in liquid nitrogen vapours for eight to ten minutes. Afterwards, straws were placed vertically in goblets and plunged in liquid nitrogen (-196°C). Suitable thick paper markers were placed for identification purposes.

3.4.6 Thawing of Semen

Straws were individually thawed in water bath at 37°C for 30 seconds and evaluated accordingly.

3.5 SEMEN EXAMINATION

In the present study, semen samples were examined for sperm motility, normal sperm count and live sperm count alongwith SPT through PAG column.

3.5.1 Individual sperm motility

Individual motility of spermatozoa was recorded at 37°C under phase contrast microscope (400X) and scored 0-100, according to per cent of progressively motile spermatozoa.

3.5.2 Normal Sperm Count

Abnormal spermatozoa were studied from the thin smears stained with Rose Bengal stain as per the method of Sharma (1987). Rose Bengal stain was prepared as follows:

Rose Bengal dye	3 g
Commercial formalin	1 ml
Distilled water aa	100 ml

The stained smears were examined under oil immersion lens and a total of 200 spermatozoa from different fields were counted to calculate per cent abnormal sperma-tozoa, thereafter, normal sperm count.

3.5.3 Live Sperm Count

The per cent of live spermatozoa was estimated by differential staining technique using Eosin-Nigrosin stain (Blom 1977). The stain was prepared from stock solution of 5 per cent eosin and 10 per cent nigrosin in the ratio of 1:4 and subsequently filtered. One drop of fresh semen was mixed with 8 drops of eosin-nigrosin stain maintained at 37°C on a glass slide. A thin smear was

prepared from this mixture. A total of 200 spermatozoa were counted in different microscopic fields under oil immersion lens and percentage of live sperm were calculated.

3.5.4 Sperm Penetration Test (SPT)

The SPT was carried out according to method described by Kremer (1965) with little modifications (Dev *et al* 1996a). Polyacrylamide gel (2%) was aspirated into a plain haematocrit round capillary tube (internal diameter of 1.22 ± 0.01 mm and 75 mm length) using either TAPPI-M or scalp vein set (without needle) attached with 2 ml syringe. One end of the capillary tube was sealed with modelling clay and kept at 37°C in incubator. For each semen sample, two capillary tubes were prepared. Each semen sample (fresh or extended or cryopreserved) was taken in specially designed flat bottom glass cup adhered on microscopic glass slide. The capillary tube filled with polyacrylamide gel was then immersed in pool of semen taken in the cup. Thereafter, complete set of glass slide with cup and capillary tubes was incubated under humid conditions at 37°C for 20 minutes. Immediately after incubation, the capillary tubes were removed from glass slide and cooled quickly in crushed ice to give cold shock for preventing further migration. The capillary tube was then placed on microslide and focused for vanguard spermatozoa under phase contrast microscope (X 300). The foremost sperm in capillary tube was located and distance travelled by the sperm was measured with the scale fixed on microscope stage. SPD values were recorded in millimetres per 20 minutes.

3.6 STATISTICAL ANALYSIS

The data was analysed statistically as per methods described by Singh *et al* (1991).

4.1 PHYSICAL EXAMINATION OF POLYACRYLAMIDE GEL

Various batches of 2 per cent polyacrylamide gel were studied for their physical characteristics viz. colour and transparency, spinnbarkeit, pH and visible gelification before subjecting the gel for sperm penetration studies (Table 1).

4.1.1 Colour and Transparency

All the batches of 2 per cent polyacrylamide gel were found to be clear and transparent which is consistent with the findings of Layton *et al* (1981) and Ury *et al* (1986).

4.1.2 pH

The mean pH value for 2 per cent polyacrylamide gel was 6.59 ± 0.00 which fluctuated non-significantly in between various batches. These observations are in accordance with the results of Kaushal (1978).

4.1.3 Spinnbarkeit

The spinnbarkeit of 2 per cent polyacrylamide gel varied non-significantly in between the various batches with mean value of 14.67 ± 0.70 mm. Layton *et al* (1981) reported spinnbarkeit of 15 mm, which is in consistency with the results of present study.

4.1.4 Visible Gelification

Various batches of 2 per cent solution thickened with slight gelification that was visible only at the bottom of container when examined after

CHAPTER IV

RESULTS AND DISCUSSION

4.1 PHYSICAL EXAMINATION OF POLYACRYLAMIDE GEL

Various batches of 2 per cent polyacrylamide gel were studied for their physical characteristics viz. colour and transparency, spinnbarkeit, pH and visible gelification before subjecting the gel for sperm penetration studies (Table 1).

4.1.1 Colour and Transparency

All the batches of 2 per cent polyacrylamide gel were found to be clear and transparent which is consistent with the findings of Lorton *et al* (1981) and Urry *et al* (1986)

4.1.2 pH

The mean pH value for 2 per cent polyacrylamide gel was 8.69 ± 0.00 which fluctuated non-significantly in between various batches. These observations are in accordance with the results of Kaushal (1998).

4.1.3 Spinnbarkeit

The spinnbarkeit of 2 per cent polyacrylamide gel varied non-significantly in between the various batches with mean value of 14.67 ± 0.20 mm. Lorton *et al* (1981) reported spinnbarkeit of 15 mm, which is in consistent with the results of present study.

4.1.4 Visible Gelification

Various batches of 2 per cent solution thickened with slight gelification that was visible only at the bottom of container when examined after

Table 1. Physical characteristics of 2 per cent polyacrylamide gel.

Characteristics	Batch-I	Batch-II	Batch-III	Batch-IV	Batch-V
Colour and Transparency	Clear & Transparent	Clear & Transparent	Clear & Transparent	Clear & Transparent	Clear & Transparent
pH*	8.70	8.69	8.70	8.71	8.69
Spinnbarkeit (mm)*	14.33 ± 0.27	15.00 ± 0.47	14.00 ± 0.47	15.33 ± 0.27	14.66 ± 0.27
Visible Gelification	Slight	Slight	Slight	Slight	Slight

*Non-significant.

overnight incubation during preparation. However, there was no visible solid gelification. This finding was consistent with that of Lorton *et al* (1981) and Kaushal (1998).

However, Duvekot (1987) failed to obtain optimal gel properties particularly spinnbarkeit and viscosity which varied widely in different lots of PAG. But reason for this variation remained undefined.

4.2 SEMEN QUALITY AND ITS RELATION TO SPERM PENETRABILITY

There is an increasing concern that microscopic semen analysis alone may not be sufficient criteria to judge the male fertility potential as it does not simulate any of the events in fertilization process for predicting subsequent fertility. The present study is directed to develop sperm penetration in polyacrylamide gel as a potential diagnostic tool. An attempt has been made to study the correlation between routine semen analysis and SPD values. It could be a noble mean of selecting bulls for commercial purpose and for routine use in AI centres.

4.2.1 Semen Analysis

The mean values of various standard semen parameters and SPD values in fresh, extended, chilled (4-6°C; 24 hrs) and post thawed semen for bulls M-1437, M-1451, BH-19 and BH-32 are presented in Table 2 and Fig.2.

4.2.1.1 Sperm motility

Overall mean spermatozoon motility was recorded as 67.75 ± 1.08 , 67.75 ± 1.08 , 54.75 ± 1.63 and 45.00 ± 0.93 per cent in fresh, extended, chilled

Table 2. Seminal characteristics and spermatozoal penetrability through PAG column for fresh, extended, chilled and post thawed semen.

Parameters	Bull No*				Overall mean
	M-1437	M-1451	BH-19	BH-32	
<i>Fresh Semen</i>					
Sperm motility (%) ^b	66.00 ± 1.67	71.00 ± 1.67	70.00 ± 1.99	64.00 ± 1.67	67.75 ± 1.08
Live sperm count (%) ^a	82.60 ± 2.50	84.00 ± 2.18	86.60 ± 1.88	84.40 ± 2.08	84.40 ± 1.13
Normal sperm count (%) ^a	83.60 ± 0.53	87.40 ± 0.45	88.00 ± 0.48	84.40 ± 0.45	85.85 ± 0.48
SPD value (mm/20min) ^b	21.12 ± 0.32	22.12 ± 0.53	22.76 ± 0.68	21.06 ± 0.23	21.76 ± 0.99
<i>Extended semen</i>					
Sperm motility (%) ^b	66.00 ± 1.67	71.00 ± 1.67	70.00 ± 1.99	64.00 ± 1.67	67.75 ± 1.08
Live sperm count (%) ^a	78.00 ± 2.72	80.00 ± 2.04	83.00 ± 1.12	79.40 ± 1.84	80.20 ± 1.08
Normal sperm count (%) ^a	82.40 ± 0.45	86.00 ± 0.39	86.20 ± 0.59	82.20 ± 0.76	84.20 ± 0.51
SPD value (mm/20min) ^b	21.06 ± 0.20	21.91 ± 0.57	22.82 ± 0.55	20.73 ± 0.29	21.63 ± 0.28
<i>Chilled semen (4-6°C, 24 hrs)</i>					
Sperm motility (%) ^b	53.00 ± 3.34	58.00 ± 1.78	58.00 ± 3.03	50.00 ± 3.16	54.75 ± 1.63
Live sperm count (%) ^a	68.60 ± 2.28	72.60 ± 2.04	75.00 ± 2.15	79.40 ± 0.66	73.90 ± 1.29
Normal sperm count (%) ^a	79.80 ± 0.59	81.80 ± 0.51	82.60 ± 0.72	79.40 ± 0.66	80.90 ± 0.43
SPD value (mm/20min) ^b	20.24 ± 0.15	20.90 ± 0.28	20.84 ± 0.33	20.48 ± 0.35	20.61 ± 0.15
<i>Post thawed semen</i>					
Sperm motility (%) ^b	43.00 ± 1.09	48.00 ± 1.78	46.00 ± 1.67	43.00 ± 1.78	45.00 ± 0.93
Live sperm count (%) ^a	60.20 ± 3.17	57.00 ± 2.52	60.20 ± 1.90	56.80 ± 2.02	58.55 ± 1.28
Normal sperm count (%) ^a	76.00 ± 0.89	80.40 ± 0.35	81.20 ± 0.71	77.60 ± 0.45	78.80 ± 0.56
SPD value (mm/20min) ^b	19.25 ± 0.16	19.51 ± 0.25	19.52 ± 0.28	19.16 ± 0.12	19.36 ± 0.11

* Five ejaculates per bull

a: Mean value for the parameter differ non-significantly between the bulls.

b: Mean value for the parameter differ non-significantly (P < 0.05) between the bulls.

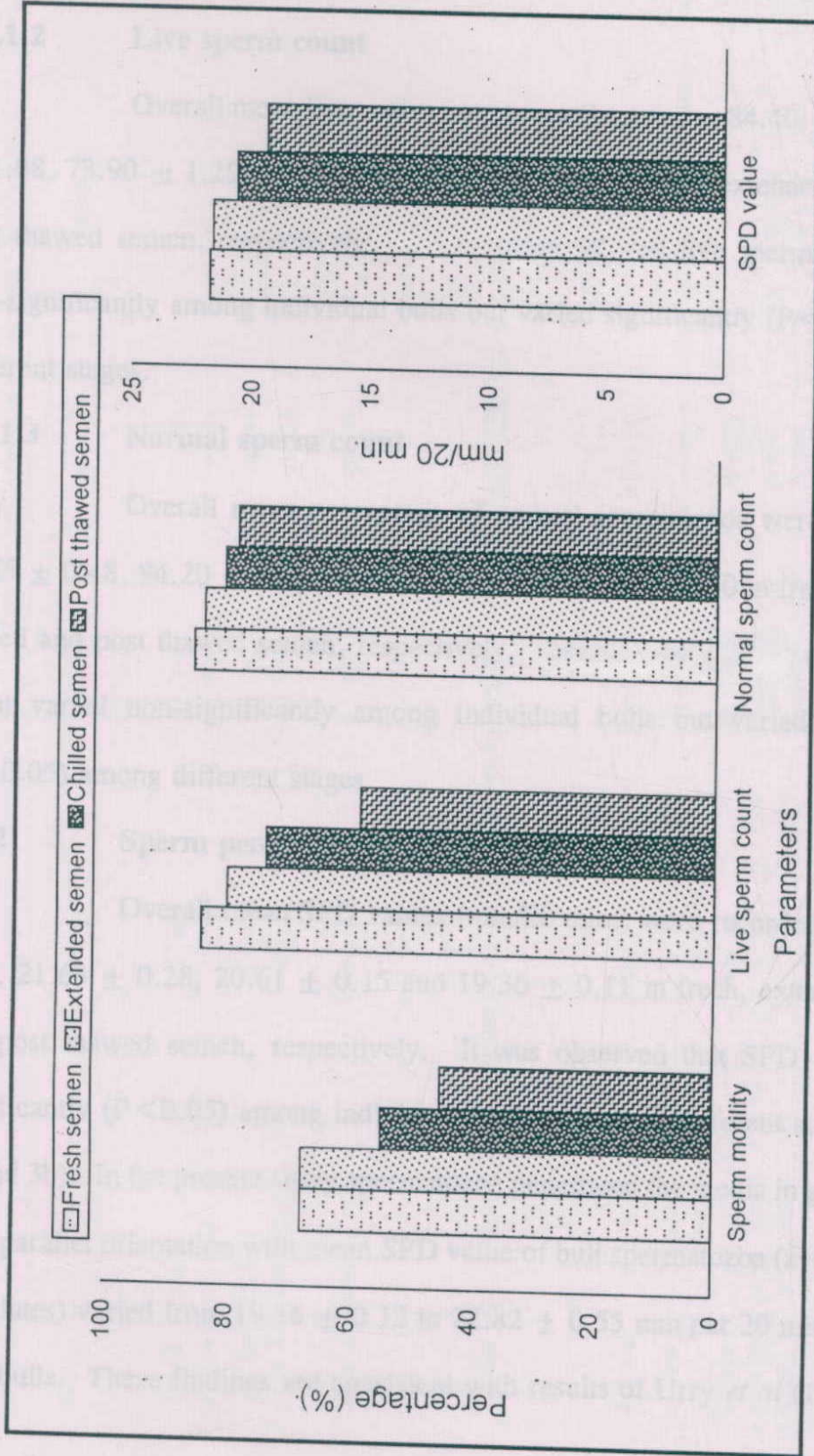


Fig.2. Seminal characteristics and spermatozoal penetrability through PAG column of fresh, extended, chilled and post thawed buffalo bull semen

and post thawed semen, respectively. It was observed that spermatozoon motility varied significantly ($P < 0.05$) among individual bulls as well as among different stages.

4.2.1.2 Live sperm count

Overall mean live sperm count was observed as 84.40 ± 1.13 , 80.20 ± 1.08 , 73.90 ± 1.29 and 58.55 ± 1.28 per cent in fresh, extended, chilled and post thawed semen, respectively. live sperm count varied non-significantly among individual bulls but varied significantly ($P < 0.05$) among different stages.

4.2.1.3 Normal sperm count

Overall mean percentage of normal spermatozoa were recorded as 85.85 ± 0.48 , 84.20 ± 0.51 , 80.90 ± 0.43 and 78.80 ± 0.50 in fresh, extended, chilled and post thawed semen, respectively. normal sperm count varied non-significantly among individual bulls but varied significantly ($P < 0.05$) among different stages.

4.2.2 Sperm penetration distance (SPD) values

Overall mean SPD values (mm/20 min.) were recorded as 21.76 ± 0.99 , 21.63 ± 0.28 , 20.61 ± 0.15 and 19.36 ± 0.11 in fresh, extended, chilled and post thawed semen, respectively. It was observed that SPD value varied significantly ($P < 0.05$) among individual bulls as well as different stages (Tables 3a and 3b). In the present study spermatozoa penetrated the media in good number with parallel orientation with mean SPD value of bull spermatozoa (average of five ejaculates) varied from 19.16 ± 0.12 to 22.82 ± 0.55 mm per 20 minutes among four bulls. These findings are consistent with results of Urry *et al* (1986) and

Table 3a. Mean sperm penetration distance (SPD) values of spermatozoa of different bulls at different stages.

Stage	Bull No*				Overall mean
	M-1437	M-1451	BH-19	BH-32	
Fresh semen	21.12	22.12	22.76	21.06	21.76
Extended semen	21.06	21.91	22.82	20.73	21.63
Chilled semen	20.24	20.90	20.84	20.48	20.61
Post thawed semen	19.25	19.51	19.52	19.16	19.36
Overall mean	20.42	21.11	21.48	20.35	

Table 3b. Analysis of variance in SPD value among different bulls and different stages.

Source	d.f.	Mean of squares	F.ratio	CD (5%)	C.V.
Reps.	4	9.140	1.786		
Between bulls	3	17.635	3.44*	1.432	
Between stages	3	15.899	3.10*	1.432	
Interaction	9	6.245	1.22	NS	
Error	60	5.133			11.00

*(P < 0.05)

Kaushal (1998). But the mean values are comparatively lower than those reported by Lorton *et al* (1981) in cow bulls. This is not surprising in view of the fact that cow bulls provide a better sperm quality than buffalo bulls especially the sperm motility and freezability which affect sperm penetrability.

4.2.3 Correlation of sperm penetration distance (SPD) values in relation to seminal characteristics

The relationship between semen quality and fertility is not clearly established but the availability of several simple quantitative tests increases the opportunity to correctly identify semen of high quality. In the present study, correlation between routine semen analysis and SPD values was also studied (Table 4).

In the present investigation, a significant ($P < 0.05$) positive correlation of 0.838, 0.808, 0.821 and 0.826 has been recorded between per cent motility and SPD values in fresh, extended, chilled and post thawed semen, respectively. Almost similar findings were reported by Kaushal (1998) for post thawed semen. However, Goldstein *et al* (1982) observed lower correlation coefficient with fresh, extended and cryopreserved human semen.

Although a good correlation existed between sperm motility and SPD values, even then a few of semen samples with low sperm motility exhibited good SPD values (Table 2). This shows that the kinetics and quality of progression of spermatozoa is more important than merely the percentage of motile spermatozoa (Keel and Webster 1988).

A significant ($P < 0.05$) positive correlation coefficient of 0.707, 0.628, 0.763 and 0.682 has been observed between live sperm count and SPD

Table 4. Correlation coefficient between seminal characteristics and SPD values in fresh, extended, chilled and post thawed semen.

Seminal characteristics	Correlation coefficient				
	Fresh semen (n=20)	Extended semen (n=20)	Chilled semen (n=20)	Post thawed semen (n=20)	Overall
Sperm motility	0.838*	0.808*	0.821*	0.826*	0.859*
Live sperm count	0.707*	0.628*	0.763*	0.682*	0.820*
Normal sperm count	0.586*	0.583*	0.468*	0.385*	0.744*

* (P < 0.05)

values in fresh, extended, chilled and post thawed semen, respectively. Almost similar observations were recorded by Dev *et al* (1996b) while Kaushal (1998) observed lower correlation coefficients. This clearly indicate that only live spermatozoa are able to exhibit spermatozoal penetrability.

Physiological normal spermatozoa play a crucial role in determining sperm penetration. In the present study, a significant ($P < 0.05$) positive correlation coefficient of 0.586, 0.583, 0.468 and 0.385 (NS) has been observed between normal sperm count and SPD values in fresh, extended, chilled and post thawed semen. These findings are in agreement with the results of Eggert-Kruse *et al* (1993). This correlation further gains support from studies of Fredricsson and Bjork (1977) who reported that the number of normal spermatozoa were higher in cervical mucus collected from internal os than that of external os. This showed that cervical mucus acts as a sperm selector differentiating between morphologically normal and abnormal sperm.

Further, it has been observed that preserved semen either chilled or frozen showed a decreasing trend on sperm motility, live sperm count and normal sperm count. The values of seminal characteristics were significantly ($P < 0.05$) decreased in chilled and post thawed semen (Table 2). Similar observations were recorded by Matharoo and Singh (1980), Pandit (1984) and Chauhan *et al* (1993b). The process of preservation has also influenced the sperm penetrability and later was significantly ($P < 0.05$) reduced following preservation either at chilling temperature or freezing temperature (Tables 3a and 3b). Similar trends were observed by Okuda *et al* (1988). This fall in SPD value might be due

to relative decrease in sperm motility which is an important factor in determining sperm penetration.

Though, there is decline in sperm penetrability following preservation of semen, but significant overall correlation ($r = 0.972, 0.823$ and $0.889; P < 0.05$) were observed between fresh semen SPD value vs extended, chilled and post thawed semen SPD value, respectively (Table 5). However, Dev *et al* (1996a) observed significant correlation ($r = 0.675; P < 0.05$) between fresh diluted vs frozen semen SPD values. The results suggest that SPT can also be performed using extended, chilled or frozen semen instead of fresh semen.

Further, it has been observed that sperm motility in extended ($r = 0.808; P < 0.05$), chilled ($r = 0.821; P < 0.05$) and frozen ($r = 0.826; P < 0.05$) semen are significantly correlated to respective SPD value (Table 4). These results suggest that SPT using PAG column can provide more objective, although indirect evaluation of sperm motility than methods currently used in most of the AI laboratories for grading sperm motility.

4.3 SEPHADEX COLUMN FILTRATION

The comparative poor quality of buffalo bull semen is a serious constraint on the success of artificial insemination in this species. Broadly, poor quality semen ejaculates can be classified as ejaculates with initial poor semen quality ^{and} ejaculates having poor freezability. The present study is directed to check efficacy of sephadex column (G-15-120) in terms of spermatozoal penetrability through PAG column.

Table 5. Correlation coefficients between fresh semen SPD value vs extended, chilled and post thawed semen SPD value.

Stage	Correlation coefficient			
	Bull No*			
	M-1437	M-1451	BH-19	BH-32
Extended semen	0.963*	0.967*	0.981*	0.884*
Chilled semen (4-6°C, 24 hours)	0.414	0.975*	0.956*	0.896*
Post thawed semen	0.800	0.949*	0.984*	0.645
				Overall
				0.972*
				0.823*
				0.889*

* (P < 0.05)

4.3.1 Sephadex column filtration of initial poor semen quality ejaculates

The mean values of various seminal characteristics and SPD values for bulls M-1446, M-1506, BH-14 and BH-25 before and after sephadex filtration are presented in Table 6 and Fig.3.

4.3.1.1 Sperm motility

The overall mean sperm motility percentage of extended unfiltered and sephadex column filtered semen was recorded as 33.00 ± 1.24 and 44.00 ± 1.20 , respectively (Table 6). The percentage of motile spermatozoa increased significantly ($P < 0.05$) after filtration of semen through sephadex column, as compared to unfiltered semen (Table 7). Similar results have been reported by Landa *et al* (1980). This increase in the number of motile spermatozoa is due to retention of dead and immotile sperms in the sephadex column since the dead and immotile cells agglomerate with sephadex particles (Graham *et al* 1976). The other hypothesis is that there is leakage of different macromolecules from the dead immotile or damaged spermatozoa, which might bind to the sephadex particles leading to the retention of dead and immotile spermatozoa (Lodhi and Carbo 1984). Moreover, sephadex column represents a pack of beads which provides a zig-zag path to the moving spermatozoa and the immotile spermatozoa cannot traverse the zig-zag path leading to their retention.

4.3.1.2 Live sperm count

The overall mean live sperm percentage in the extended, unfiltered and sephadex column filtered semen was recorded as 45.80 ± 1.19 and 55.20 ± 1.10 (Table 6). After filtration of semen through sephadex column live sperm



Table 6. Effect of sephadex column filtration of tris yolk extended poor initial quality ejaculates on various seminal characteristics and spermatozoal penetrability through PAG column.

Parameters	Bull No*				Overall mean
	M-1446	M-1506	BH-14	BH-25	
<i>Unfiltered semen</i>					
Sperm motility (%) ^b	34.00 ± 2.60	29.00 ± 0.89	30.00 ± 1.41	39.00 ± 1.67	33.00 ± 1.24
Live sperm count (%) ^b	50.20 ± 3.13	44.40 ± 1.34	41.20 ± 1.27	47.40 ± 0.82	45.80 ± 1.19
Normal sperm count (%) ^b	72.40 ± 1.37	66.80 ± 1.03	68.20 ± 1.03	72.20 ± 0.76	69.90 ± 0.76
SPD value (mm/20min) ^b	17.21 ± 0.31	17.00 ± 0.11	17.51 ± 0.13	18.06 ± 0.04	17.44 ± 0.12
<i>Sephadex column filtered semen</i>					
Sperm motility (%) ^b	45.00 ± 2.44	40.00 ± 1.38	41.00 ± 0.89	50.00 ± 1.41	44.00 ± 1.20
Live sperm count (%) ^b	56.80 ± 2.45	52.20 ± 1.77	53.00 ± 1.93	58.80 ± 0.76	55.20 ± 1.10
Normal sperm count (%) ^b	84.60 ± 0.96	84.00 ± 0.39	83.40 ± 0.35	84.80 ± 0.51	84.20 ± 0.32
SPD value (mm/20min) ^b	18.63 ± 0.19	18.29 ± 0.11	18.54 ± 0.06	19.06 ± 0.12	18.63 ± 0.09

* Five ejaculates per bull.

^b: Mean value for the parameter differ significantly ($P < 0.05$) between the bulls.

Table 7. Mean value of the standard semen parameters and SPD value in Tris-yolk extended poor initial quality ejaculates for unfiltered and sephadex column filtered semen.

Parameters	Unfiltered semen (n=20)	Sephadex column filtered semen (n=20)	t-value
Sperm motility (%)	33.00 ± 1.24	44.00 ± 1.20	8.91*
Live sperm count (%)	45.80 ± 1.19	55.20 ± 1.10	6.26*
Normal sperm count (%)	69.90 ± 0.76	84.20 ± 0.32	19.92*
SPD value (mm/20 min)	17.44 ± 0.12	18.63 ± 0.09	9.26*

* (P < 0.05)

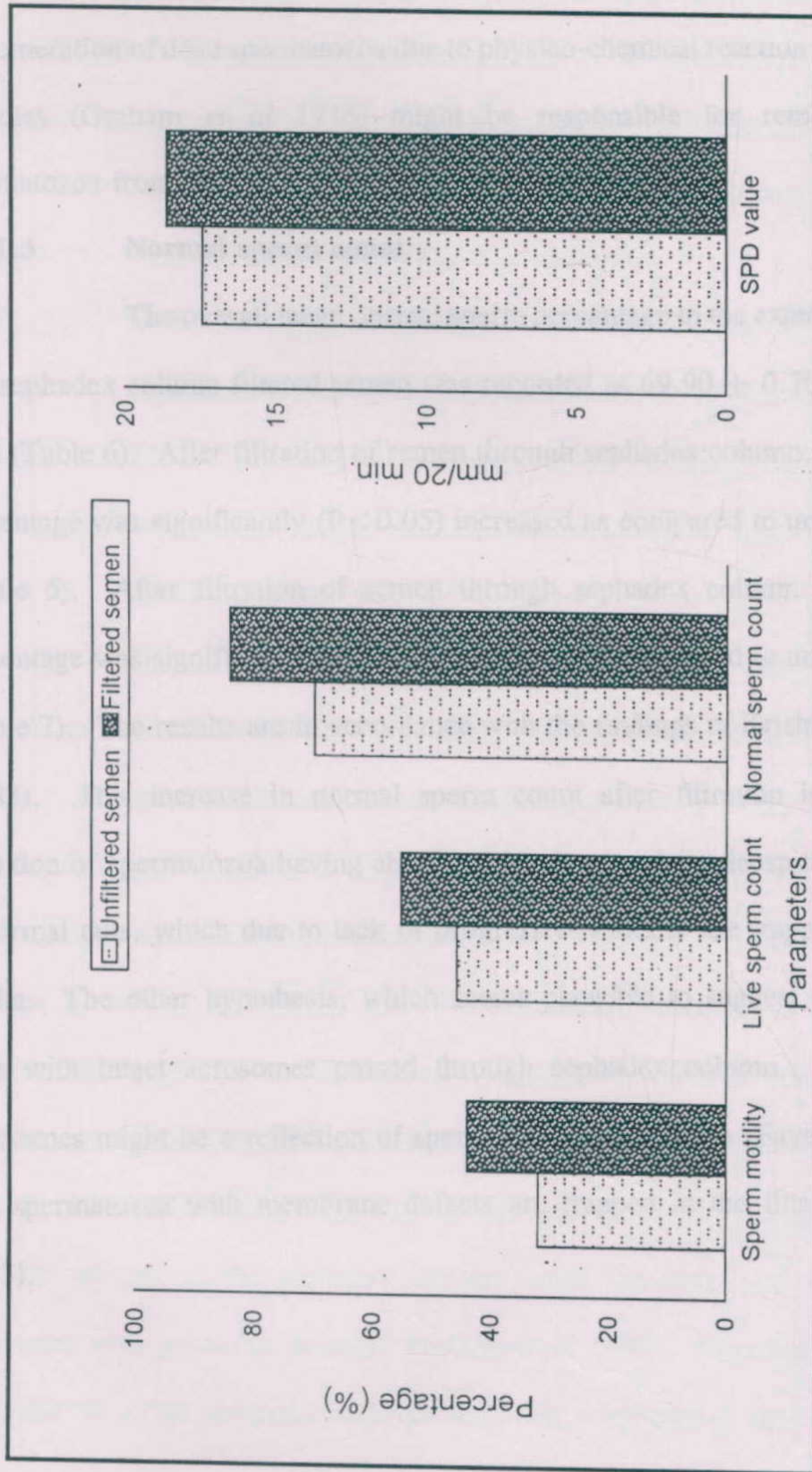


Fig. 3. Effect of sephadex column filtration of Tris-yolk extended poor initial quality ejaculates on various seminal characteristics and spermatozoal penetrability through PAG column

percentage was significantly ($P < 0.05$) increased as compared to unfiltered semen (Table 7). The results are in accordance with the findings of Landa *et al* (1980). Increased stickiness of sperms after their death (Baker and Degan 1972) or agglomeration of dead spermatozoa due to physico-chemical reaction with sephadex particles (Graham *et al* 1976) might be responsible for removal of dead spermatozoa from semen.

4.3.1.3 Normal sperm count

The overall mean normal sperm percentage in the extended unfiltered and sephadex column filtered semen was recorded as 69.90 ± 0.76 and 84.20 ± 0.32 (Table 6). After filtration of semen through sephadex column, normal sperm percentage was significantly ($P < 0.05$) increased as compared to unfiltered semen (Table 6). After filtration of semen through sephadex column normal sperm percentage was significantly ($P < 0.05$) increased as compared to unfiltered semen (Table 7). The results are in accordance with the findings of Krishnamurthy *et al* (1983). This increase in normal sperm count after filtration is attributed to retention of spermatozoa having abnormal heads especially decapitated heads and abnormal tails, which due to lack of progressive motility are trapped by filtering media. The other hypothesis, which seems plausible to suggest that the motile cells with intact acrosomes passed through sephadex column. The damaged acrosomes might be a reflection of sperm surface alterations (Fayemi *et al* 1979) and spermatozoa with membrane defects are trapped in the filter (Heuer *et al* 1983).

4.3.1.4 Sperm penetration distance (SPD) value

The overall average distance (mm per 20 min.) travelled in PAG filled capillary tubes, by the spermatozoa of Tris yolk extended unfiltered and sephadex column filtered semen was recorded as 17.44 ± 0.12 and 18.63 ± 0.12 (Table 6). The filtered semen showed significant ($P < 0.05$) increase in SPD value as compared to unfiltered semen (Table 7). This might be due to more number of progressively motile spermatozoa in the filtered semen.

4.3.2 Sephadex column filtration of semen exhibiting poor freezability

The mean values of various seminal characteristics and SPD values for bulls M-1434, M-1536, M-1550 and BH-06 in extended and post thawed semen before and after sephadex column filtration are presented in Table 8 and Fig.4.

4.3.2.1 Sperm motility

The overall mean sperm motility percentage of extended unfiltered, extended sephadex column filtered, post thawed unfiltered and post thawed sephadex column filtered semen was recorded as 63.75 ± 0.99 , 71.75 ± 0.81 , 29.50 ± 0.85 and 42.75 ± 1.02 per cent, respectively (Table 8). A significant ($P < 0.05$) increase in motile spermatozoon percentage has been observed in extended as well as post thawed semen after filtration of semen through sephadex column, as compared to unfiltered semen (Table 9). Similar results have been reported by Goyal *et al* (1996), Kanakraraj *et al* (1996) and Kumar *et al* (1996). This increase in the number of motile spermatozoa is due to retention of dead and immotile sperms in the sephadex column since the dead and immotile cells agglomerate with sephadex particles (Graham *et al* 1976). Other hypothesis is that separation is on the basis of complex interacting properties of spermatozoal

Table 8. Effect of sephadex column filtration of tris yolk extended poor freezable ejaculates on various seminal characteristics and spermatozoal penetrability through PAG column for extended and post thawed semen.

Parameters	Bull No*				Overall mean
	M-1434	M-1536	M-1550	BH-06	
UNFILTERED					
<i>Extended semen</i>					
Sperm motility (%) ^a	62.00 ± 1.09	67.00 ± 1.09	64.00 ± 2.96	62.00 ± 1.09	63.75 ± 0.99
Live sperm count (%) ^a	75.80 ± 1.48	75.40 ± 1.21	75.20 ± 1.03	74.20 ± 1.36	75.15 ± 0.65
Normal sperm count (%) ^b	79.40 ± 0.53	84.60 ± 0.60	79.00 ± 0.89	83.80 ± 0.33	81.70 ± 0.64
SPD value (mm/20min) ^a	20.45 ± 0.07	20.83 ± 0.26	20.03 ± 0.95	20.01 ± 0.20	20.35 ± 0.26
<i>Post thawed semen</i>					
Sperm motility (%) ^a	28.00 ± 1.09	34.00 ± 0.89	27.00 ± 1.09	29.00 ± 1.67	29.50 ± 0.85
Live sperm count (%) ^a	42.80 ± 1.58	45.60 ± 0.96	40.00 ± 1.12	38.00 ± 1.16	41.60 ± 0.88
Normal sperm count (%) ^b	68.20 ± 0.86	80.40 ± 0.77	71.00 ± 1.22	77.80 ± 0.33	74.35 ± 1.18
SPD value (mm/20min) ^a	17.59 ± 0.09	18.06 ± 0.15	17.59 ± 0.08	17.60 ± 0.10	17.71 ± 0.07
SEPHADEX COLUMN FILTERED					
<i>Extended semen</i>					
Sperm motility (%) ^b	72.00 ± 1.09	73.00 ± 1.78	71.00 ± 2.18	71.00 ± 0.89	71.75 ± 0.81
Live sperm count (%) ^b	83.20 ± 1.50	83.40 ± 1.53	83.40 ± 1.39	82.00 ± 1.01	83.00 ± 0.70
Normal sperm count (%) ^b	89.20 ± 0.33	91.80 ± 0.33	88.40 ± 0.21	90.40 ± 0.21	89.95 ± 0.31
SPD value (mm/20min) ^b	23.09 ± 0.28	22.57 ± 0.68	22.25 ± 0.92	21.82 ± 0.39	22.43 ± 0.32
<i>Post thawed semen</i>					
Sperm motility (%) ^b	44.00 ± 1.67	44.00 ± 2.18	45.00 ± 1.41	38.00 ± 1.09	42.75 ± 1.02
Live sperm count (%) ^b	58.00 ± 1.09	56.20 ± 2.06	51.20 ± 1.90	46.20 ± 1.03	52.90 ± 1.30
Normal sperm count (%) ^b	83.40 ± 0.53	84.60 ± 0.66	83.80 ± 0.71	87.80 ± 0.43	84.90 ± 0.48
SPD value (mm/20min) ^b	19.52 ± 0.22	19.64 ± 0.26	20.04 ± 0.13	18.47 ± 0.11	19.41 ± 0.16

* Five ejaculates per bull.

a: Mean value for the parameter did not differ significantly between the bulls.

b: Mean value for the parameter differ significantly ($P < 0.05$) between the bulls.

Table 9. Mean value of the standard semen parameters and spermatozoal penetrability for Tris-yolk extended poor freezable ejaculates at extended and post thawed stage in unfiltered and sephadex column filtered semen.

Parameters	Unfiltered semen (n=20)	Sephadex column filtered semen (n=20)	t-value
Extended semen			
Sperm motility (%)	63.75 ± 0.99	71.75 ± 0.81	6.91*
Live sperm count (%)	75.15 ± 0.65	83.00 ± 0.70	8.01*
Normal sperm count (%)	81.70 ± 0.64	89.95 ± 0.31	20.33*
SPD value (mm/20 min)	20.35 ± 0.26	22.43 ± 0.32	5.44*
Post thawed semen			
Sperm motility (%)	29.50 ± 0.85	42.75 ± 1.02	11.46*
Live sperm count (%)	41.60 ± 0.88	52.90 ± 1.30	9.74*
Normal sperm count (%)	74.35 ± 1.18	84.90 ± 0.48	19.73*
SPD value (mm/20 min)	17.71 ± 0.07	19.41 ± 0.16	13.81*

* P < 0.05.

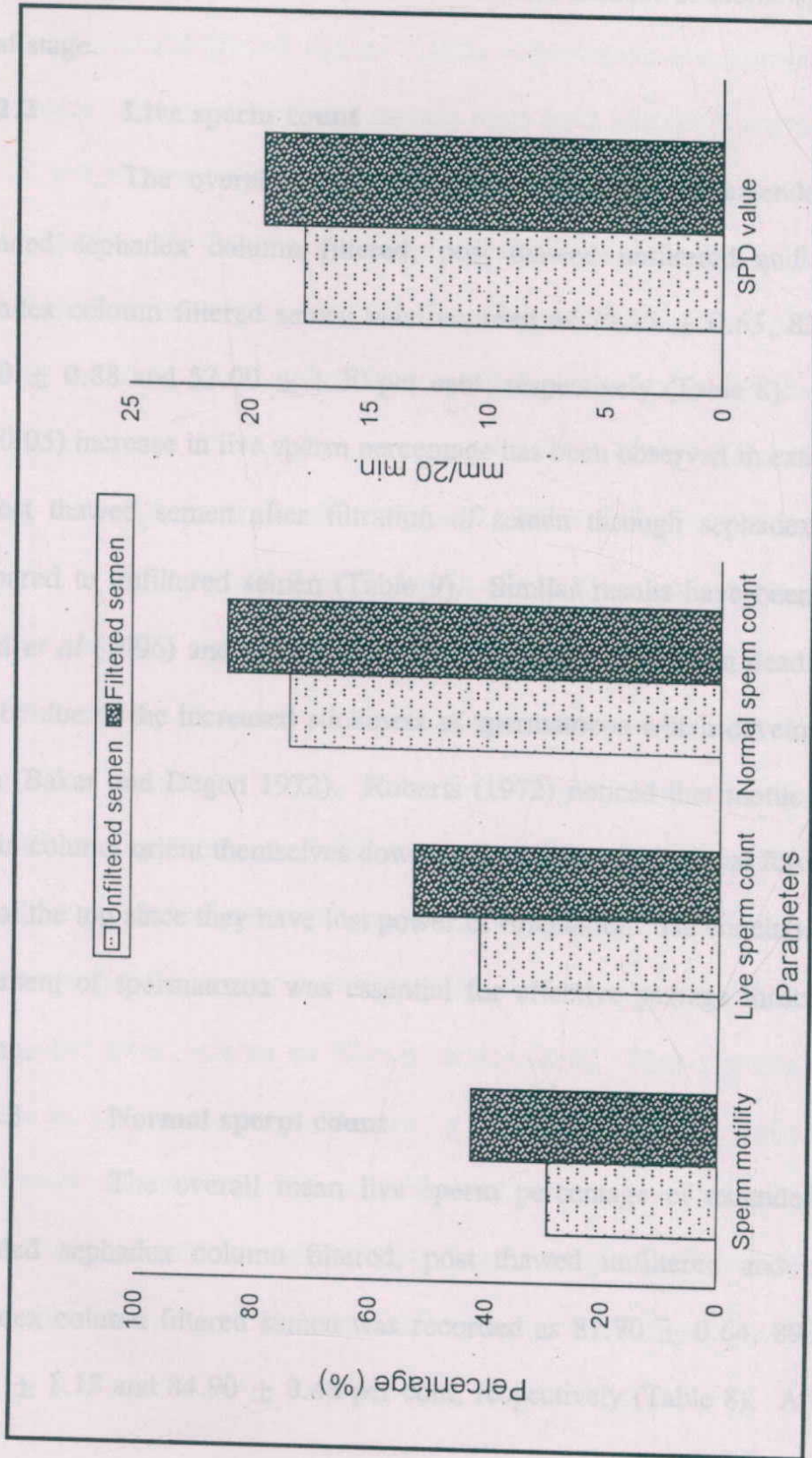


Fig. 4. Effect of sephadex column filtration of Tris-yolk extended poor freezable ejaculates on various seminal characteristics and spermatozoal penetrability through PAG column at post thaw stage

membrane, medium suspending the sperm and sephadex gel (Landa *et al* 1980). A significant ($P < 0.05$) increase in post thawed motility of sephadex column filtered semen might be due to presence of more number of motile spermatozoa at initial stage.

4.3.2.2 Live sperm count

The overall mean live sperm percentage of extended unfiltered, extended sephadex column filtered, post thawed unfiltered and post thawed sephadex column filtered semen was recorded as 75.15 ± 0.65 , 83.00 ± 0.70 , 41.60 ± 0.88 and 52.00 ± 1.30 per cent, respectively (Table 8). A significant ($P < 0.05$) increase in live sperm percentage has been observed in extended as well as post thawed semen after filtration of semen through sephadex column, as compared to unfiltered semen (Table 9). Similar results have been reported by Goyal *et al* (1996) and Kumar *et al* (1996). The reduction in dead spermatozoa may be due to the increased stickiness of spermatozoa which develops after their death (Baker and Degen 1972). Roberts (1972) noticed that motile spermatozoa kept in column orient themselves downwards due to gravitational force while dead float at the top since they have lost power of orientation. He concluded that active movement of spermatozoa was essential for effective passage through sephadex column.

4.3.2.3 Normal sperm count

The overall mean live sperm percentage of extended unfiltered, extended sephadex column filtered, post thawed unfiltered and post thawed sephadex column filtered semen was recorded as 81.70 ± 0.64 , 89.95 ± 0.31 , 74.35 ± 1.18 and 84.90 ± 0.48 per cent, respectively (Table 8). After filtration

of semen through sephadex column, a significant ($P < 0.05$) increase in normal sperm count has been observed in extended as well as post thawed semen as compared to unfiltered semen (Table 9). Similar results have been reported by Kanakaraj *et al* (1976) and Kumar (1989). This increase in normal sperm count is attributed to retention of spermatozoa with head and tail abnormalities, due to lack of progressive motility. Therefore, it seems plausible to suggest that the motile sperms with intact acrosomes passed through sephadex column. The damaged acrosome might be a reflection of sperm surface alterations (Fayemi *et al* 1979) and spermatozoa with membrane defects were trapped in the filter (Heuer *et al* 1983).

4.3.2.4 Sperm penetration distance (SPD) values

The overall average sperm penetration distance (mm per 20 min.) value of extended unfiltered, extended sephadex column filtered, post thawed unfiltered and post thawed sephadex column filtered semen was recorded as 20.35 ± 0.26 , 22.43 ± 0.32 , 17.71 ± 0.07 and 19.41 ± 0.10 mm/20 min., respectively (Table 8). A significant ($P < 0.05$) increase in SPD value (mm/20 min.) has been observed in extended as well as post thawed semen after filtration of semen through sephadex column, as compared to unfiltered semen (Table 9). Similar results have been reported by Kumar *et al* (1996). This significant ($P < 0.05$) increase in SPD value might be due to more number of progressively motile spermatozoa in the filtered semen. Further, a significant correlation has been observed between sperm motility and sperm penetration in polyacrylamide gel column (Goldstein *et al* 1982, Eggert-Kruse *et al* 1993, Kaushal 1998).

4.4 STATIC EJACULATES

Freshly collected buffalo semen sometimes do not show any mass activity and such non-motile ejaculates are called static or zero ejaculates. Non-motile characteristics of ejaculate is attributed to the presence of either spermiostatic factor, motility inhibiting factor or absence of forward motility proteins. The present study is directed towards comparative evaluation of static and non-static ejaculation in terms of seminal characteristics and spermatozoal penetrability through PAG column.

4.4.1 Seminal characteristics

The mean value of various seminal characteristics viz. sperm motility, live sperm count and normal sperm count, for bulls M-1434, M-1437, M-1451 and M-1536 in static and non-static ejaculates are presented in Table 10 and Fig.5.

4.4.1.1 Sperm motility

The overall mean spermatozoon motility was recorded as 68.12 ± 1.07 and 60.93 ± 1.18 for non-static and static tris-yolk extended ejaculates, respectively (Table 10). This spermatozoon motility exhibited significant ($P < 0.05$) differences among static and non-static ejaculates (Table 11). Various workers viz. Nainar *et al* (1990), Kumar *et al* (1993) and Dharni *et al* (1995) quoted higher values for spermatozoon motility which differ significantly between static and non-static ejaculates. This variation in sperm motility from present observations might be due to breed differences, individual variations, seasonal variations (Pangaonkar *et al* 1978), frequency of semen collection and handling of semen.

Bull No

Parameters	Bull No				Overall mean
	M-1434	M-1437	M-1451	M-1536	
<i>Motile (Non-static) ejaculates*</i>					
Sperm motility (%) ^b	63.75 ± 2.07	67.50 ± 1.25	72.50 ± 1.25	68.75 ± 1.08	68.12 ± 1.07
Live sperm count (%) ^a	78.50 ± 1.82	80.50 ± 2.19	82.50 ± 1.03	77.50 ± 1.67	79.75 ± 0.99
Normal sperm count (%) ^b	79.75 ± 0.54	82.75 ± 0.41	85.75 ± 0.41	84.50 ± 1.03	83.18 ± 0.65
SPD value (mm/20min) ^a	20.90 ± 0.29	21.25 ± 0.15	22.41 ± 0.57	21.42 ± 0.26	21.49 ± 0.22
<i>Static ejaculates*</i>					
Sperm motility (%) ^b	58.75 ± 2.72	58.75 ± 2.72	62.50 ± 1.25	63.75 ± 1.08	60.93 ± 1.18
Live sperm count (%) ^a	73.50 ± 2.07	76.00 ± 1.90	76.00 ± 0.93	74.50 ± 1.43	75.00 ± 0.86
Normal sperm count (%) ^b	76.75 ± 1.47	79.00 ± 1.06	81.00 ± 0.61	80.75 ± 1.43	72.37 ± 0.73
SPD value (mm/20min) ^a	19.52 ± 0.58	19.72 ± 0.82	20.26 ± 0.25	20.35 ± 0.13	19.96 ± 0.27

* Four ejaculates per bull.

a: Mean value for the parameter differ non-significantly between the bulls.

b: Mean value for the parameter differ significantly ($P < 0.05$) between the bulls.

Table 11. Mean value of the standard semen parameters and spermatozoal penetrability for Tris-yolk extended motile (non-static) and static ejaculates of buffalo bulls.

Parameters	Non-static ejaculates (n=16)	Static ejaculates (n=16)	t-value
Sperm motility (%)	68.12±1.07	60.93±1.18	4.78*
Live sperm count (%)	79.75±1.18	75.00±0.86	3.30*
Normal sperm count (%)	83.18±0.65	79.37±0.73	5.01*
SPD value (mm/20 min)	21.49±0.22	19.96±0.27	4.27*

*P<0.05.

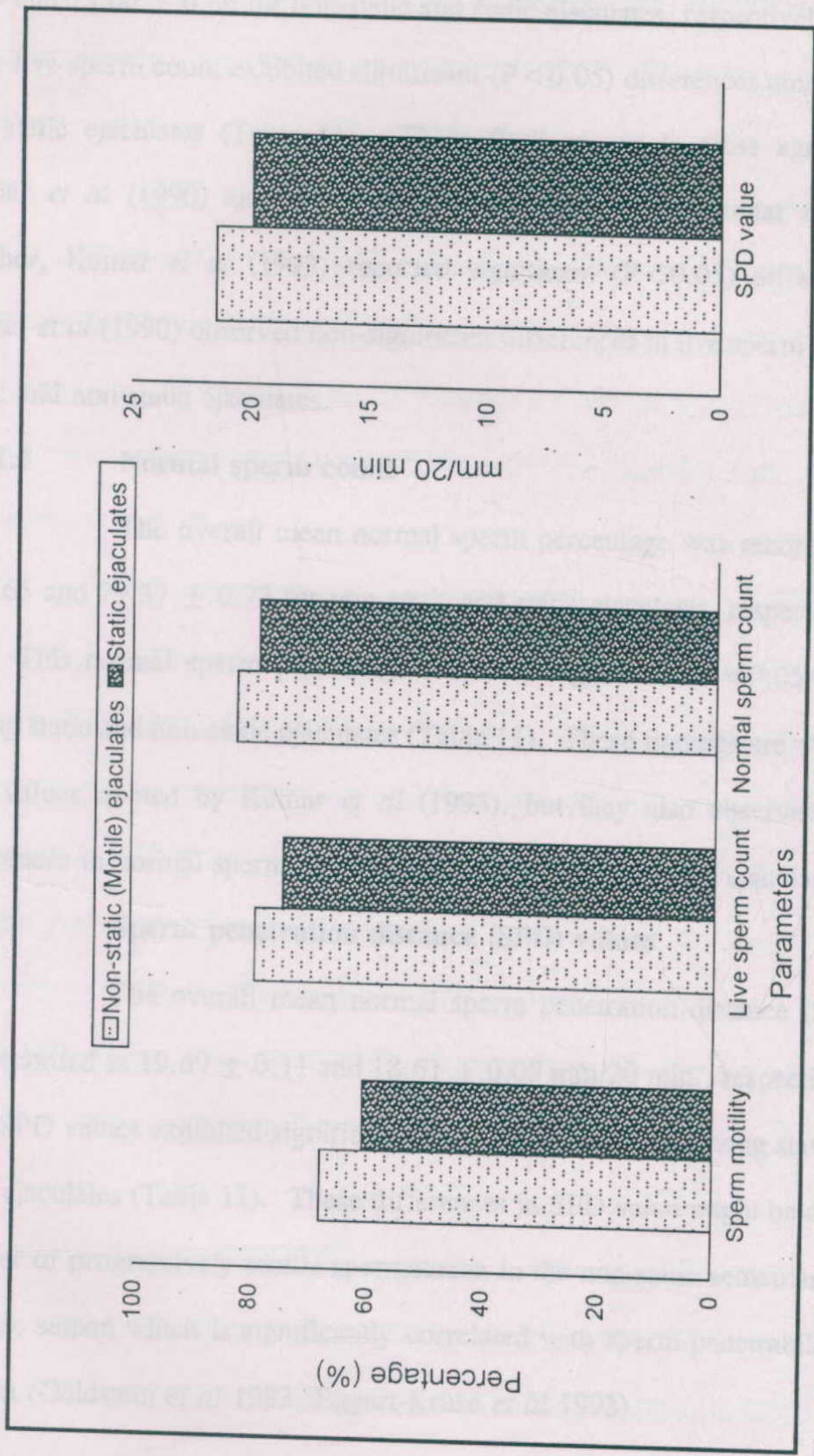


Fig.5. Seminal characteristics and spermatozoal penetrability through PAG column of Tris-yolk extended motile (non-static) and static ejaculates of buffalo bulls

4.4.1.2 Live sperm count

The overall mean live sperm percentage was recorded as 79.75 ± 0.99 and 75.00 ± 0.86 for non-static and static ejaculates, respectively (Table 10). This live sperm count exhibited significant ($P < 0.05$) differences among static and non-static ejaculates (Table 11). These findings are in close agreement with Nainar *et al* (1990) and lower than values reported by Kumar *et al* (1993). Further, Kumar *et al* (1993) reported significant ($P < 0.05$) differences while Nainar *et al* (1990) observed non-significant differences in live sperm count among static and non-static ejaculates.

4.4.1.3 Normal sperm count

The overall mean normal sperm percentage was recorded as 83.18 ± 0.65 and 79.37 ± 0.73 for non-static and static ejaculates, respectively (Table 10). This normal sperm percentage exhibited significant ($P < 0.05$) differences among static and non-static ejaculates (Table 11). These findings are slightly lower than values quoted by Kumar *et al* (1993), but they also observed significant differences in normal sperm count among static and non-static ejaculates.

4.4.2 Sperm penetration distance (SPD) values

The overall mean normal sperm penetration distance (SPD) value was recorded as 19.69 ± 0.11 and 18.61 ± 0.08 mm/20 min., respectively (Table 10). SPD values exhibited significant ($P < 0.05$) differences among static and non-static ejaculates (Table 11). These differences in SPD value might be due to more number of progressively motile spermatozoa in the non-static semen as compared to static semen which is significantly correlated with sperm penetrability in PAG column (Goldstein *et al* 1982, Eggert-Kruse *et al* 1993).

4.4.3 Freezability test

The overall post thaw motility was recorded as 45.63 ± 0.97 and 39.06 ± 0.65 per cent for non-static and static ejaculates, respectively (Table 12). This post-thaw motility exhibited significant ($P < 0.05$) differences among static and non-static ejaculates (Table 13). These findings are in close agreement with Nainar *et al* (1990) and Jindal *et al* (1996) and they also reported significant ($P < 0.01$) differences in post thaw motility among static and non-static ejaculates. Further, overall mean SPD value for post thawed semen was recorded as 19.09 ± 0.11 and 18.61 ± 0.08 mm per 20 minutes for non-static and static ejaculates, respectively, which also varied significantly ($P < 0.05$) among static and non-static ejaculates (Tables 12 and 13).

The freezability test as measured by post-thaw motility and SPD value for post-thaw semen indicated significantly reduced freezability for the samples with static spermatozoa. However, the freezability of static semen ejaculates was high enough to pass the standards set for the usage of frozen semen (Nainar *et al* 1990). Chinnaiya (1982) also reported that 25 per cent of static semen samples may get discarded based on initial motility, the sperms are capable of gaining motility on dilution and freezing successfully though not to the extent of normal semen. Thus freezability test appears to be the deciding test for the discard of semen samples with static sperms.

4.5 COLD SHOCK

It is well known that the spermatozoa of many animals; including buffalo bulls undergo irreversible changes on sudden exposure to low temperature;

Table 12. Post thaw motility and spermatozoal penetrability through PAG column for Tris-yolk glycerol extended motile (non-static) and static ejaculates of buffalo bulls.

Parameters	Bull No.			Overall mean
	M-1434	M-1437	M-1451	
<i>Motile (Non-static) ejaculates*</i>				
Sperm motility (%) ^a	45.00 ± 1.77	43.75 ± 1.08	48.75 ± 1.08	45.00 ± 2.50
SPD value (mm/20 min) ^a	19.68 ± 0.23	19.41 ± 0.10	19.85 ± 0.14	19.82 ± 0.27
<i>Static ejaculates*</i>				
Sperm motility (%) ^a	38.75 ± 1.08	37.50 ± 1.25	38.75 ± 1.08	41.25 ± 1.08
SPD value (mm/20 min) ^a	18.41 ± 0.11	18.73 ± 0.09	18.93 ± 0.11	18.36 ± 0.11

* Four ejaculates per bull.

a: Mean value of the parameters differ non-significantly between the bulls.

Table 13. Mean value of post thawed motility and spermatozoal penetrability for Tris-yolk glycerol extended motile (non-static) and static ejaculates of buffalo bulls.

Parameters	Non-static ejaculates (n=16)	Static ejaculates (n=16)	t-value
Sperm motility (%)	45.63±0.97	39.06±0.65	5.27*
SPD value (mm/20 min)	19.69±0.11	18.61±0.08	7.74*

* P<0.05.

commonly referred as cold shock. The present study is directed towards the comparative evaluation of Tris-yolk extended buffalo bull semen before and after cold shock, for routine seminal characteristics and spermatozoal penetrability through PAG column.

4.5.1 Semen analysis

The mean values of various seminal characteristics in Tris-yolk extended semen of bulls M-1437, M-1451, BH-32 and BH-19 before and after cold shock are presented in Tables 14-15 and Fig.6.

4.5.1.1 Sperm motility

The overall mean value of spermatozoon motility before and after cold shock was recorded as 67.75 ± 1.08 and 32.00 ± 0.54 per cent, respectively which vary non-significantly between individual bulls but vary significantly ($P < 0.05$) before and after cold shock. These results are in accordance with observations of Salisbury *et al* (1942), Church *et al* (1975), Chinnaiya and Ganguli (1978), Mohan *et al* (1992) and Vaisberg *et al* (1994). Further, Mohan *et al* (1992) and Vaisberg *et al* (1994) observed significant ($P < 0.01$) drop in sperm motility after cold shock treatment.

4.5.1.2 Live sperm count

The overall mean value of live sperm percentage before and after cold shock were recorded as 80.20 ± 1.09 and 44.00 ± 0.73 per cent, respectively which vary non-significantly between individual bulls but vary significantly ($P < 0.05$) before and after cold shock. The results are in accordance with observations of Salisbury *et al* (1942), Church *et al* (1975), Chinnaiya and

Table 14. Seminal characteristics and spermatozoal penetrability through PAG column for Tris-yolk extended semen before and after cold shock.

Parameters	Bull No.*			Overall mean
	M-1434	M-1451	BH-19	
<i>Before cold shock</i>				
Sperm motility (%) ^a	66.00 ± 1.67	71.00 ± 1.67	64.00 ± 1.67	70.00 ± 1.99
Live sperm count (%) ^a	78.00 ± 2.72	80.40 ± 2.04	79.40 ± 1.84	83.00 ± 1.12
Normal sperm count (%) ^b	82.40 ± 0.45	86.00 ± 0.39	82.20 ± 0.77	86.20 ± 0.59
SPD value (mm/20 min) ^a	21.06 ± 0.20	21.98 ± 0.59	20.80 ± 0.28	22.62 ± 0.68
<i>After cold shock</i>				
Sperm motility (%) ^a	36.00 ± 1.09	34.00 ± 0.89	36.00 ± 1.09	38.00 ± 1.09
Live sperm count (%) ^a	42.80 ± 1.63	44.20 ± 1.24	43.80 ± 1.03	45.20 ± 1.65
Normal sperm count (%) ^b	71.60 ± 1.09	69.80 ± 1.17	68.20 ± 0.86	71.60 ± 0.77
SPD value (mm/20 min) ^a	16.36 ± 0.21	16.21 ± 0.17	16.64 ± 0.25	16.59 ± 0.20

* Five ejaculates per bull.

a: Mean value of the parameters differ non-significantly between the bulls.

b: Mean value for the parameter differ significantly ($P < 0.05$) between the bulls.

Table 15. Mean value of standard semen parameters and spermatozoal penetrability for Tris-yolk extended semen before and after cold shock.

Parameters	Before cold shock (n=20)	After cold shock (n=20)	t-value
Sperm motility (%)	67.75 ± 1.08	36.00 ± 0.64	29.95*
Live sperm count (%)	80.20 ± 1.09	44.00 ± 0.73	27.78*
Normal sperm count (%)	84.20 ± 0.51	70.30 ± 0.57	21.77*
SPD value (mm/20 min)	21.65 ± 0.29	16.45 ± 0.11	16.94*

*P < 0.05

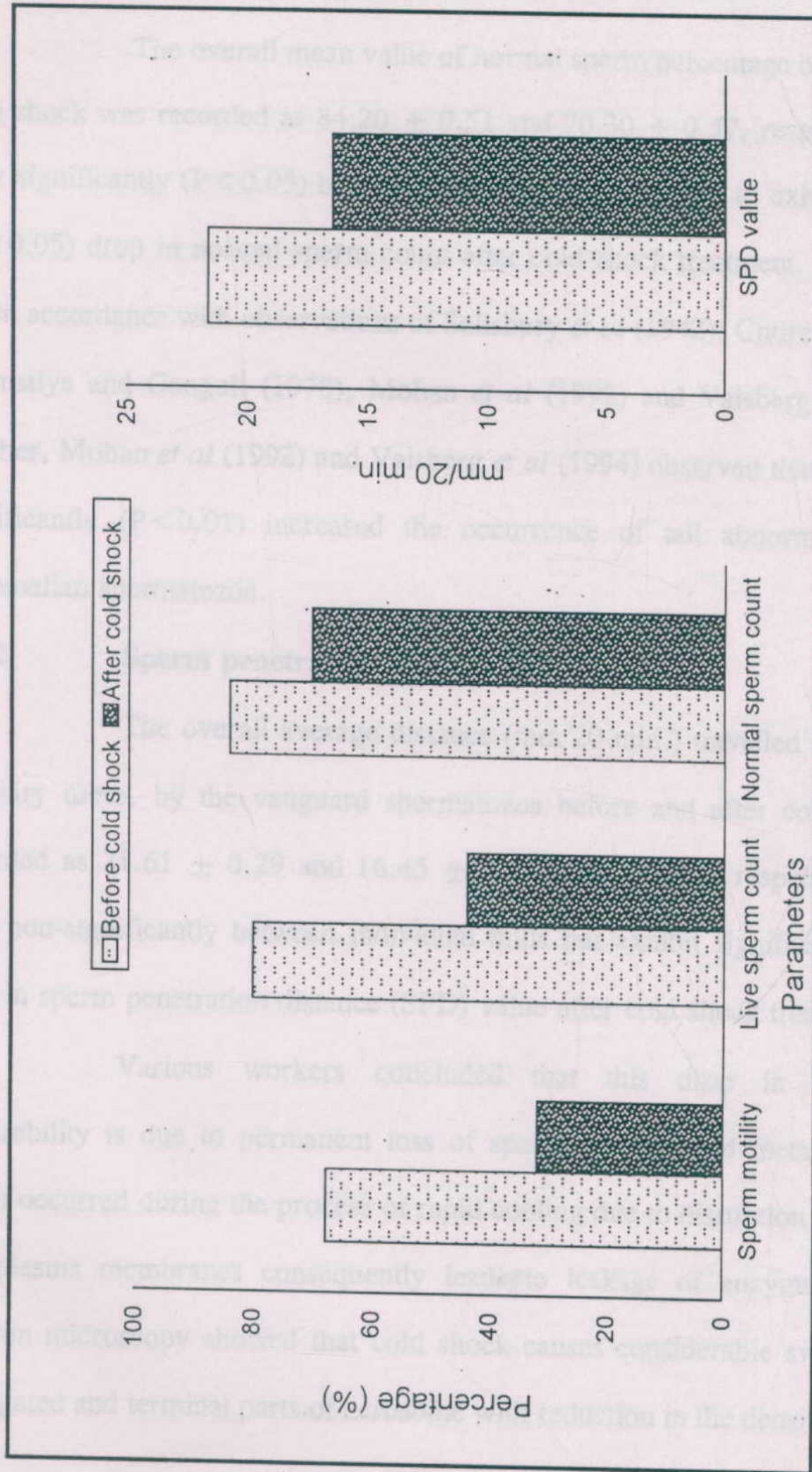


Fig.6. Seminal characteristics and spermatozoal penetrability through PAG column of Tris-yolk extended buffalo bull semen before and after cold shock

Ganguli (1978) and Mohan *et al* (1992). Further, Mohan *et al* (1992) observed significant ($P < 0.01$) drop in live sperm count after cold shock treatment.

4.5.1.3 Normal sperm count

The overall mean value of normal sperm percentage before and after cold shock was recorded as 84.20 ± 0.51 and 70.30 ± 0.57 , respectively which vary significantly ($P < 0.05$) between individual bulls as well as exhibit significant ($P < 0.05$) drop in normal sperm count after cold shock treatment. These results are in accordance with observations of Salisbury *et al* (1942), Church *et al* (1975), Chinnaiya and Ganguli (1978), Mohan *et al* (1992) and Vaisberg *et al* (1994). Further, Mohan *et al* (1992) and Vaisberg *et al* (1994) observed that rapid cooling significantly ($P < 0.01$) increased the occurrence of tail abnormalities in the mammalian spermatozoa.

4.5.2 Sperm penetration distance (SPD) value

The overall average distance (mm/20 min.) travelled in PAG filled capillary tubes, by the vanguard spermatozoa before and after cold shock was recorded as 21.61 ± 0.29 and 16.45 ± 0.11 mm/20 min., respectively, which vary non-significantly between individual bulls but exhibit significant ($P < 0.05$) drop in sperm penetration distance (SPD) value after cold shock treatment.

Various workers concluded that this drop in spermatozoal penetrability is due to permanent loss of sperm motility and metabolic activity which occurred during the process of rapid cooling due to disruption of acrosomal and plasma membranes consequently leading to leakage of enzymes. Further, electron microscopy showed that cold shock causes considerable swelling in the corrugated and terminal parts of acrosome with reduction in the density of material

contained within the acrosomal membranes. This leads to substantial loss of non-dialysable ornicol and biuret reactive substances along with acid soluble phosphorus and phospholipids after cold shocking. It is difficult to see how a change in the acrosome of this nature could cause such profound changes in distant motor apparatus and it is not even clear whether this loss of material could be a cause of functional changes induced in the spermatozoon by cold shock (Quinn *et al* 1969, Harrison and White 1972, Mohan *et al* 1992, White 1993).

with bovine cervical sperm from the test of late year of animals; the main factor is main variable. Therefore, for these and other reasons, a synthetic artificial medium (polyacrylamide gel) with stable and uniform characteristics having similar migration characteristics was undertaken in the present investigation to study spermatozoal penetrability through female and artificial barrier wall.

The study was carried out on semen collected from buffalo bull maintained at Punjab Agricultural University Dairy Farm, Ludhiana and District Cattle Farm, Bhaini Sahib. Semen specimens were checked for various physical characteristics i.e. sperm motility (5%), live sperm count (1%), and normal sperm count (2%) and spermatozoal penetrability (mean 20 min.) through polyacrylamide gel column.

In the present study, various batches of 1% polyacrylamide gel were synthesized in the laboratory under standardized conditions and checked for their physical characteristics (colour and transparency, pH, substructure and extent of gelification) which varied non-significantly among various batches.

Mean penetrability (20 min.) values were significantly correlated with various seminal characteristics i.e. sperm motility ($r = 0.553$), live sperm percentage ($r = 0.220$) and normal sperm count ($r = 0.744$). Although significant

SUMMARY

The *in vitro* penetration tests can be an effective diagnostic test in bull evaluation. Although measurement of sperm penetration in cervical mucus could be of prospective value for subsequent bull fertility, but problems associated with bovine cervical mucus limit the use of this test to evaluate the male factor as main variable. Therefore, for these and other reasons, a synthetic artificial medium (polyacrylamide gel) with stable and uniform characteristics having similar migration characteristics was undertaken in the present investigations to study spermatozoal penetrability through fertile and subfertile buffalo bull semen.

The study was carried out on semen collected from buffalo bulls maintained at Punjab Agricultural University Dairy Farm, Ludhiana and Exotic Cattle Farm, Bhattian, Khanna. Semen ejaculates were studied for various seminal characteristics viz. sperm motility (%), live sperm count (%) and normal sperm count (%) and spermatozoal penetrability (mm/20 min.) through polyacrylamide gel column.

In the present study, various batches of 2 per cent polyacrylamide gel were synthesized in the laboratory under standardized conditions and assessed for their physical characteristics (colour and transparency, pH, spinnbarkeit and extent of gelification) which varied non-significantly among various batches.

Sperm penetration distance (SPD) values were significantly correlated with routine seminal characteristics i.e. sperm motility ($r = 0.859$), live sperm percentage ($r = 0.820$) and normal sperm count ($r = 0.744$). Although significant

decrease in seminal characteristics were observed in both chilled and frozen semen, but there was parallel fall in SPD values in the chilled and frozen semen. A significant correlation between SPD values of fresh semen with chilled and frozen semen ($r = 0.823$ and 0.889 ; $P < 0.05$) were established.

Sephadex column filtration of poor initial quality semen cause significant ($P < 0.05$) increase in sperm motility (33.00 ± 1.24 to 44.00 ± 1.20), live sperm count (45.80 ± 1.10 to 55.20 ± 1.10), normal sperm count (69.90 ± 0.76 to 84.20 ± 0.32) and spermatozoal penetrability (17.44 ± 0.12 to 18.63 ± 0.09). Similarly, filtration of poor freezable semen through sephadex column has proved to be significantly ($P < 0.05$) better in terms of post thaw motility (20.50 ± 0.85 to 42.75 ± 1.02), livability (41.60 ± 0.88 to 52.90 ± 1.30), normal sperm count (74.35 ± 1.18 to 84.90 ± 0.48) and spermatozoal penetrability (17.71 ± 0.07 to 19.41 ± 0.16).

Static ejaculates extended in Tris-egg yolk recorded significantly ($P < 0.05$) lower values of sperm motility (68.12 ± 1.07 vs 60.93 ± 1.18), live sperm count (79.75 ± 0.90 vs 75.00 ± 0.86), normal sperm count (83.18 ± 0.65 vs 79.37 ± 0.73) and spermatozoal penetrability (21.49 ± 0.22 vs 19.96 ± 0.27) as compared to motile (non-static) ejaculates. These recorded significant reduced freezability in terms of post thaw motility (45.63 ± 0.97 vs 39.06 ± 0.65) and spermatozoal penetrability (19.69 ± 0.11 vs 18.61 ± 0.08). However, the freezability of static semen ejaculates was high enough to pass the standards set for the usage of frozen semen.

Cold shocked buffalo bull semen recorded significant ($P < 0.05$) drop in sperm motility (67.75 ± 1.08 to 36.00 ± 0.64), livability (80.20 ± 1.09 vs

44.00 \pm 0.73), normal sperm count (84.20 \pm 0.51 vs 70.30 \pm 0.57) as well as spermatozoal penetrability (21.61 \pm 0.29 vs 16.45 \pm 0.11).

CONCLUSIONS

1. Polyacrylamide gel (2%) can be prepared from readily available ingredients in any amount and exhibiting no batch to batch variation with regard to viscoelastic properties as well as the composition.
2. As there exhibit significant correlation of spermatozoal penetrability in fresh, extended, chilled and frozen semen; sperm penetration test using polyacrylamide gel can be employed either in fresh, extended, chilled or frozen semen samples for predicting fertility.
3. The per cent spermatozoon motility, live sperm count, normal sperm count and spermatozoal penetrability through PAG column increased significantly after sephadex filtration of poor quality buffalo bull semen.
4. Sephadex filtration improves the freezability of buffalo bull semen by increasing its post thaw motility, livability, normal sperm count and spermatozoa penetrability through PAG column.
5. Although static ejaculates exhibit significantly lower sperm motility, live sperm count, normal sperm count and spermatozoal penetrability through PAG column, yet, freezability test appears to be the deciding test for the discard of static ejaculates.
6. Cold shocked semen exhibit significant drop in routine seminal characteristics and spermatozoal penetrability through PAG column.

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