STUDIES ON DEEP FREEZING OF BULL AND BUFFALO SEMEN FOR FREEZABILITY, ENZYME LEAKAGE AND FERTILITY.

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A Thesis
Submitted to the
Gujarat Agricultural University
in Partial Fulfilment of the Requirements
for the Award of the Degree
of

Master of Veterinary Science
(Obstetrics & Gynaecology)

By
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B. V. Sc. & A. H

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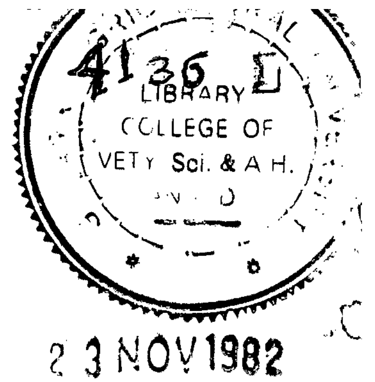
1982.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To My Parents...

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CERTIFICATE

This is to certify that the thesis entitled "STUDIES ON DEEP FREEZING OF BULL AND BUFFALO SEMEN FOR FREEZABILITY; ENZYME LEAKAGE AND FERTILITY" submitted by Shri VINOD R. JANI in partial fulfilment of the requirements for the degree of MASTER OF VETERINARY SCIENCE (Obstetrics and Gynaecology) of the Gujarat Agricultural University is a record of bonafide research work carried out by him under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

(S.B. KODAGALI)
Major Advisor

Approved by the advisory committee:

Chairman: 26/10/82

Members :

1.

2.

3.

External member of the examination committee

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N.S.	:	Non Significant
O.D.	:	Optical Density
r.p.m.	:	revolutions per minute
S.E.	:	Standard error
Sec.	:	Seconds
Soln.	:	Solution
temp.	:	temperature
TFYG	:	Tris fructose yolk glycerol
u	:	units
WMG	:	Whole milk glycerol.

A C K N O W L E D G E M E N T

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Anand,

Date : 3-9-82

(V.R. JANI)

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A B S T R A C T

STUDIES ON DEEP FREEZING OF BULL AND BUFFALO SEMEN FOR FREEZABILITY, ENZYME LEAKAGE AND FERTILITY

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The present studies on deep freezing of bull and buffalo semen for freezability; enzyme leakage and fertility were carried out at the Department of Obstetrics and Gynaecology, Gujarat Veterinary College, Anand.

An experiment was undertaken to study the freezability of spermatozoa for 46 ejaculates (2 ejaculates/bull) from 23 bulls (20 Surti + 3 H.F.). Ten bulls (7 buffalo bulls and 3 H.F. bulls 43.5%) provided the semen ejaculates having very good freezability. The semen ejaculates from nine (39.2%) buffalo bulls were moderately suitable for freezing. Four buffalo bulls (17.3%) provided the semen ejaculates; which could not stand to freezing satisfactorily.

Twenty five ejaculates from five Surti buffalo bulls were split diluted in Tris fructose yolk glycerol (TFYG) and lactose fructose yolk glycerol (LFYG) diluents. On the basis of post-thaw spermatozoal recovery; TFYG was proved to be the better

diluent for buffalo semen freezing as compared to LFYG. Fifteen semen ejaculates from 3 H.F. bulls were split diluted in five diluents. TFYG and egg yolk citrate glycerol (EYCG) ranked high, followed by Laiciphos-478 (L-478), whole milk glycerol (WMG) and AMUL spray glycerol (ASG) for freezing of bull semen, on the basis of post-thaw spermatozoal recovery.

For all the ejaculates of bull and buffalo semen, per cent dead and morphologically abnormal spermatozoa significantly ($P < 0.01$) increased subsequent to freezing. The spermatozoal head breadth in bull and buffalo semen decreased significantly ($P < 0.05$) after freezing, whereas the spermatozoal head length measurements showed decrease in freeze-thawed samples, but the difference was non-significant.

Seventeen semen ejaculates from five buffalo bulls were processed to estimate the pre-freeze and post-thaw levels of transaminases (GOT and GPT) in seminal plasma and hyaluronidase activity in sperm extract (25×10^6 spermatozoa). The seminal plasma levels of both the transaminases (GOT and GPT) were increased significantly ($P < 0.01$) in freeze-thawed samples. TFYG diluent protected the spermatozoa more efficiently, as the pre and post-freeze release of GOT and GPT enzymes in seminal plasma was lower in TFYG as compared to LFYG in diluted semen samples. The levels of GOT enzyme were relatively higher

in 'flat' semen samples before and after freezing, as compared to those in semen samples having initial spermatozoal motility. The hyaluronidase enzyme activity in sperm extract of buffalo semen, significantly ($P < 0.05$) decreased after freezing and thawing. The difference in hyaluronidase activity between the dilutors was non-significant.

The overall fertility following 426 inseminations in Surti buffaloes was 57.24%, requiring on an average 2.69 inseminations per conception. In Kankrej cows the overall fertility percentage following 285 inseminations using Friesian frozen semen was 46.21%, requiring on an average 2.46 inseminations per conception. In repeating Kankrej cows and heifers in a farm, 3.5 inseminations were required per conception. The fertility percentages between eight bulls under study; varied apparently.

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INTRODUCTION

I. INTRODUCTION

Artificial Insemination has become the focal point of overall improvement of livestock. The contribution of AI to improve performance; increase productivity and to promote betterment of the quality of end products has long been a recognized reality in the dairy-animal industry. AI technology has been developing since its starting with the introduction of Artificial vagina in nineteen thirties, use of dilutors in forties and following discovery of glycerol as cryoprotective agent for freezing of semen, in fifties. The frozen semen technology is gaining momentum of its popularity in most of the advanced and developing countries and its increased use has been accepted in improving animal breeding and augmenting production at faster rates. During the year 1977-78; the use of frozen semen has been reported in 38 countries of the world. In 27 of these countries 99% or more of the cows inseminated were with frozen semen; 32 countries used frozen semen on more than 90% and only 3 countries used frozen semen on less than 60% of the AI cows (Iritani, 1980).

The burst of research on semen collection, processing, evaluation, extenders, buffers, insemination techniques etc., has contributed extensively to the sweeping changes and rapid establishment of AI industry. However, the buffalo semen has received relatively little attention (Bhattacharya, 1974). Buffaloes are mainly found in

recently developing countries of Asia; where AI is not widely applied. India has the best germ plasm of this species, which has an exceptional production potentiality. Involvement of this important domestic animal in the specially selected breeding programmes and the quicker dissimination of the superior germ plasm can be of much help.

Number of reports exist relating to deep freezing of cow bull semen. Freezing technique for cow bull semen is fully established, but it could not be satisfactorily applied in total, to freeze buffalo semen, which is said to be having lower freezability, regardless of t[illegible]lutor or the method of freezing used (Sengupta, 1981). [illegible] much is known apropos the lower freezability of buffalo semen. More controlled and exhaustive field trials are required, to establish the real merit of this new technique for improving the dairy buffalo.

An effective diluent is an essential link in the frozen semen technology. Several diluents have been tried for freezing bull semen to achieve higher conception rates. Relatively few reports deal with the use of suitable diluents for buffalo semen freezing, based on actual fertility trials.

An evaluation of the quality of frozen-thawed semen is of paramount importance to predict the fertility of

bulls. Laboratory tests of seminal quality are used extensively in research on seminal physiology, semen biochemistry and quality control. No single test or combination of tests have been proved to be totally reliable for accurate prediction of semen quality in relation to fertility. However, enzymatic tests can be applied for the better evaluation of bovine semen pre and post-freezing (Graham et al. 1973). Studies on enzymatic systems of the spermatozoa are essential to understand their life processes. The detailed elucidations of some of these fundamental processes can be of great value for the development of the technique of sto[illegible] [illegible]nd use of bovine semen over a long period of time. So[illegible] [illegible]ch work has been reported for bull semen; however; very little is known in this regards for buffalo semen.

Mensuration characters of spermatozoa are reported to be related with fertility (Bishop et al., 1974). The knowledge regarding the changes in mensuration characteristics of spermatozoa due to the effect of freezing is scarce, which should be given due importance in relation to the attempts towards [illegible] better fertility.

In AI programme, the genetic improvement must come from greater selection pressure on the bull side followed by more extensive use of the superior [illegible]lasm. Fertility of the male is the greatest influencing factor,

As levamisole has been shown in both experimental animal and man to be immunostimulant (Renoux and Renoux, 1978) and has been used as an adjunct for therapy of certain malignancies (Chirigas, 1978), immunostimulation seems to be an attractive way to treat patients in whom some component of immune system is suppressed. Therefore, immunomodulatory effect of levamisole might be of vital significance in such suppressed immunological status. Since viral infections including attenuated viral vaccines, can also suppress immunological functions (Brody *et al*.,1964; Reed *et al*.,1972; Mangi *et al*.,1974 and Pike *et al*.,1977), it appears that simultaneous administration of levamisole and viral vaccine would be a very useful method of increasing immunity during vaccination.

A limited work has been carried out to study the immunomodulatory effect of levamisole in India. Haemorrhagic Septicaemia in cattle and buffaloes and Ranikhet Disease in poultry are very important diseases in our country and requires special attention, particularly regarding vaccinal immunity to avoid heavy losses through immunity breakdown.

In view of the above facts, the study on immunomodulatory effect of levamisole is taken up with the following objectives.

(1) To study the immunomodulatory effect of levamisole in chicks vaccinated against Ranikhet Disease.

(2) To study the effect of dose of levamisole in immunopotentiation amongst vaccinated chicks.

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REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

The present project involves the deep freezing of cow and buffalo bull semen using a simple freezing technique in various diluents; studies on freezability of semen from different bulls; the effect of freezing on enzyme levels and mensuration characters of spermatozoa and the fertility of frozen bovine semen. The relevant literature is reviewed as under.

(A) FREEZING OF SEMEN:

(a) Dilutors:

Dilutors have some significant effect on the freezability of semen sample as far as the post-thaw motility is concerned (Mohanty, 1973). Many dilutors have been tried for freezing the bovine semen. This is still an active field of research, as far as buffalo semen is concerned.

(i) Cow bull semen:

According to Saroof and Mixner (1955) dilutors containing 20% egg yolk and 2.9% sodium citrate solution with level of 7% glycerol were ideal for sperm survival and motility after freezing the bovine semen.

For preservation of bovine semen in deep frozen state, Bruce (1956) found milk based diluent with 10% skim milk and 10% glycerol, giving better spermatozoal revivability as compared to standard egg yolk citrate glycerol diluent. O'Dell and Almquist (1957) also reported that the spermatozoa frozen in milk based diluents maintained higher post-thaw

motility than those frozen in egg yolk citrate glycerol extender.

Amann and Almquist (1957) compared the heated reconstituted milk and heated fresh skim milk for freezing bovine semen. They observed 42% post-thaw motility in heated fresh skim milk diluent.

Davis *et al.* (1963) and Stainbach and Foote (1964) in their separate experiments on freezing bovine semen found Tris buffered fructose yolk glycerol extender superior as compared to citrate yolk glycerol extender.

In their studies regardi[illegible] motility of bovine spermatozoa, [illegible]. (1970) observed that pre and post-freezing mot[illegible] in egg yolk tris (EYT), intermediate in egg yolk citrate (EYC) and lowest in Minnesota GO (Minn GO). Post freezing mean motility was 32%, 26% and 19% for EYT, EYC and Minn GO, respectively. However, Muller (1972) did not find the significant difference in post-thaw motility for the bovine semen diluted and frozen using three glycerol containing media viz., sodium citrate yolk, Tris yolk lactose and laiciphos.

Simmet (1975) used 'Triladyl' (a concentrated form of Tris) for diluting bovine semen at room temperature and directly filling, sealing and freezing after equilibration at 5°C. Better results were achieved with this method as compared to Cassou's method of freezing.

Stoyanov and Kostadnov (1978) compared a raffinose yolk diluent (without glycerol) with a lactose yolk glycerol diluent for freezing bull semen. For the two diluents respectively, the sperm motility was 58.5 and 60.9% in freshly diluted semen, which reduced to 34.2% and 32.7% post freezing.

Ala-ud-Din et al. (1979) studied the effect of four different dilutors: whole milk yolk glycerol, laiciphos-271, lactose yolk glycerol and glucose yolk citrate glycerol on freezability of bull semen. They did not observe the significant difference in post-thaw spermatozoal motility and longevity at 37.5°C. On the basis of absolute index of longevity of spermatozoa lactose yolk glycerol diluent seemed to be the best.

Bonia et al. (1980) in their studies on the freezability and revivability of bovine spermatozoa used three different extenders; viz., egg yolk lactose (EYL), egg yolk citrate (EYC) and egg yolk tris citric-glucose (EYTCG). On thawing, EYL gave consistently better recovery (53.20 ± 0.01%) than EYC and EYTCG (42.50 ± 0.01%).

Sattar et al. (1980) studied the freezability of Bovine semen using two dilutors, viz., egg yolk citrate and skim milk extender. The post-thaw spermatozoal motility was 59.10 ± 0.93% and 63.45 ± 0.56% for these two dilutors, respectively. However, Sahni and Roy (1972) reported very

poor post-thaw spermatozoal recovery (0 -30%) of frozen buffalo semen; extended in egg yolk citrate and milk diluent.

El Kafrawi and Barrada (1974) in two trials, tested four different extenders for freezing bovine semen. In first trial, ejaculates from 8 buffalo bulls and 10 Friesian bulls were diluted and frozen in either egg yolk lactose glycerol (EYL) or egg yolk fructose (EYF). Post-thaw spermatozoal motility in EYL and EYF for Friesian semen was 41.7 and 25.4% respectively. Motility of buffalo semen in EYL and EYF was 37% and 20.8%. In a second trial, the ejaculates were diluted and frozen using either egg yolk skim milk glycerol (EYS) or egg yolk citrate glycerol (EYC). After 8 months storage in liquid nitrogen, the post-thaw recovery of spermatozoa was 52.3 and 48.9% respectively for Friesian semen in EYS and EYC. The corresponding recovery rates for buffalo semen were 46.2% and 38.1%.

(ii) Buffalo semen:

In an attempt to find out the suitable dilutor for buffalo semen, Flukinger et al. (1976) tried six different dilutors - viz., EYC (without pH adjustment), EYC (with pH adjustment) CAW, Laiciphos, Tris (without pH adjustment) and Tris (with pH adjustment). They observed the highest post-thaw spermatozoal motility in semen diluted with Tris, with or without pH adjustments, followed by EYC, Laiciphos and CAW.

In a study with buffalo semen, which was diluted in three different extenders, frozen and stored in liquid

nitrogen, gave the highest post thaw spermatozoal motility for semen diluted in Tris (34.50 ± 1.45%) followed by citric acid whey (14.75 ± 1.82%) and egg yolk glucose bicarbonate (11.52 ± 1.63%) (Takkar et al., 1980).

Chinnaiya (1979) tested three dilutors for freezing Murrah buffalo semen. For semen diluted in egg yolk citrate, citric acid whey and Tris egg yolk glycerol, sperm motility prior to freezing averaged 60%, 60.75% and 60.85%, which was reduced post thawing to 29.5%, 29.35% and 31.10% respectively.

Five different diluents comprising of lactose egg yolk glycerol, fructose egg yolk glycerol and three different combinations of lactose and fructose with equal proportion of egg yolk and glycerol were tried for buffalo semen freezing. Based on post-thawing recovery, livability at 37°C and absolute index of livability, the diluent comprising lactose (11%), fructose (6%), yolk (20%) and glycerol (5%) ranked high among the diluents tested (Ahmad and Chaudhry, 1980).

Gunzel et al. (1979) diluted 55 ejaculates from a water buffalo with Tris diluent and frozen in straws. The percentage of motile spermatozoa on thawing averaged 62.

In a comparison of 6 diluents for freezing buffalo semen, Crabo et al. (1980) found the highest post-thaw spermatozoal motility in Tris citrate egg yolk (56%), followed by TEST milk egg yolk (48%), lactose egg yolk (33%), citrate egg yolk (29%) and milk citrate (25%).

Shetti *et al*(1981) successfully carried out buffalo bull semen freezing using concentrated form of Tris (Triladyl). At 20°C, 5°C and -196°C, the mean progressive motility percentages were 79.45, 74.48 and 35.36% respectively.

Meharsing *et al.* (1980) reported the post thaw motility of frozen buffalo semen diluted in Tris yolk glycerol, to be 54 and 48% for 15 mt. and 1 month stored semen samples.

In a trial to evaluate the suitability of citric acid whey (CAW) and Tris buffered diluent for the preservation of buffalo semen at -196°C, Dharmasena and Rajamahendran (1980) processed 47 ejaculates from 8 buffalo bulls. They split diluted all the ejaculates with the dilutors under study. They reported that the post thaw motility with Tris at 0, 24 and 96 hr after freezing was significantly better than with CAW.

Patil *et al.* (1981) frozen the buffalo semen by Landshut ministraw (0.3 ml) method in various dilutors, viz., Triladyl, Tris and citric acid whey (CAW). The post thaw revival rates of spermatozoa for these three dilutors were 51.55%, 30.59% and 22.41% respectively. They further found the addition of PMSF (Progressive Motility Sustaining Factor) to semen was beneficial irrespective of dilutor.

(b) Extension rate:

The optimal number of live spermatozoa in frozen semen dose is an important factor to achieve the desired conception rate. The requirement of number of live spermatozoa in a frozen semen dose has however, not remained fixed in the studies reported earlier.

Hapen (1961a) studied the different dilution rates for freezing and storage of bovine semen. He reported that for optimum results, a concentration of atleast 10 million spermatozoa per ml of semen dose is a must. However, Foote (1970a) used 8 million and 4 million motile sperms per ml in a frozen semen dose without any significant effect on fertility. In this contrast, Pavithran et al. (1972) reported that diluted buffalo semen should contain about 100 million spermatozoa per ml before freezing. They further stated that, the rate of dilution of the buffalo semen had profound effect on the survival rates after freezing.

Darii and Nauk (1979) noted that sperm survival time of bovine frozen thawed semen was higher at dilution of 1:10 than at 1:4, 1:20 and 1:30. Chinnaiya and Ganguli (1980) studied the freezability of buffalo semen diluted with egg yolk citrate, citric acid whey and Tris diluents at 1:10 and 1:20 dilution rates; without affecting the fertility results; due to different dilution rates.

(c) Packaging of semen:

Since the freezing technique for semen was established; experiments have successfully been conducted to freeze the extended semen in pellets, in glass ampoules, in plastic pipettes or in polyvinyle straws. But it is world wide practice to package frozen semen in single dose container.

Plastic straws for packaging the bovine semen appear to offer some advantages over glass ampoules. Adler (1961) replaced the use of glass ampoules with plastic straws for packaging and freezing the semen in liquid nitrogen. Cassou (1964) gave real impetus for using straw technique for freezing semen, who first introduced a polyvinyle straw with the capacity of 1.2 ml (large) semen. Further, it was modified to 0.5 ml (medium) and 0.25 ml (mini) capacities.

Minitub, a plastic straw with globe seal was developed in West Germany by Simmet (1972). In 1975; he replaced plug in French medium straw by steel and glass balls, which sealed straws perfectly. He further reduced the size of minitub to 0.3 ml semen capacity. This method of packaging and freezing of semen is known as "Landshut method".

Pavithran et al. (1972), Roy and Bhatt (1973) and Bandopadhyaya et al. (1974) used glass ampoules for freezing of semen, successfully. Roy (1974) used "Tupol" polythene tube for successful freezing of buffalo semen.

Shetti et al. (1981) and Patil et al. (1981) used mini straws (0.3 ml) for buffalo semen freezing with good results.

Method of bovine semen freezing in straws and in ampoules have been compared by many workers. Linares (1969) concluded that the straw method is better than ampoule method of freezing as far as economy and efficiency are concerned.

Ganguli (1974) reported that semen frozen in ampoules gave 43.2% conception rate as compared to 50% with samples frozen in polyvinyle straws. According to Palson (1977), the conception rate of buffalo semen frozen in the French medium straws exceeded by 10% over the results with ampoule frozen semen.

Monji et al. (1980) compared the ampoule and straw freezing of buffalo semen. Spermatozoa frozen in straws showed 25 to 30% more post-thaw recovery than in ampoules.

Higher post thaw motility ($P < 0.005$) was observed when spermatozoa were frozen in 0.5 ml french straws than in 0.25 ml continental straws (Zarazua et al., 1977).

(d) Glycerolization:

Since Polge, Smith and Parkes (1949) first reported the action of glycerol in low temperature preservation of bovine spermatozoa, glycerol has been widely used as the primary cryoprotective agent in attempts to freeze the semen.

(i) Cow bull semen:

Graham et al. (1958) suggested that glycerol can be added to bull semen at 35°C or 5°C with no significant effect on fertility. Clegg et al. (1965) found that glycerol addition at higher temperature was detrimental to spermatozoa, when egg yolk citrate extender was used. However, if a Tris yolk extender was used, glycerol could be added to bovine spermatozoa prior to cooling (Foote, 1970b).

Foulkes et al. (1977) reported that lactose based diluents for freezing bovine semen did not require the separate addition of glycerol containing fraction. However, addition of glycerol containing fraction of the diluent in more than one steps at 5°C was found to be better for milk diluent (Almquist, 1959).

Foote (1970b) could observe that the fertility rate in citrate glycerol yolk extender was the same when glycerol was added in different fractions or in single fraction at 5°C.

Mathew et al. (1975) investigated the effect of addition of the glycerol containing portion of bovine semen extender in different fractions or in a single lot. They could not observe any appreciable difference on post-thaw recovery or fertility among frozen samples extended with glycerol fraction of the diluent.

Miller and Van Demark (1953) reported optimum survival of bull spermatozoa following freezing in yolk citrate extender with 6 and 8% glycerol; while 4, 10, 12 and 20% were comparatively less satisfactory. Whereas, Mapen (1961b) while freezing the bull semen at four different levels of glycerol observed that glycerol levels of 7 and 10% maintained maximum motility compared to 0 and 15%.

Almquist and Wickersham (1962) tried 5, 10 and 20% glycerol levels with skim milk dilutor. There was a highly significant increase in mean livability with a decrease in glycerol percentage.

Rodriguez *et al.* (1975) tested 5, 7, 9 and 11% glycerol levels for freezing of bovine semen. They found that 7 to 11% glycerol levels provided optimal spermatozoal survival. They also pointed out the relationship between glycerol level and freezing rate.

Robbins *et al.* (1976) examined different glycerol levels in their freezing trial of bull semen. Among the glycerol levels tested (1, 4, 7, 10 and 13%) 8.5% showed optimum post-thaw spermatozoal recovery. They established the interaction between the glycerol levels and thawing rate.

(ii) Buffalo bull semen:

Ahmad and Chaudhry (1980) suggested 5[illegible] to be optimum for freezing buffalo semen in LFYG dilutor.

Dass and Jainudeen (1980) tried two glycerol levels. viz., 3 and 5% with TFYG dilutor for freezing buffalo semen. They found 5% glycerol level to be better on basis of post-thaw recovery. Crabo et al. (1980) tried five glycerol levels (5 to 9%) for freezing buffalo semen in LFYG diluent. They found 5 to 7% glycerol levels to be beneficial. Among three glycerol levels tried: 5, 7 and 9% for buffalo semen freezing in LFYG diluent, Heuer (1982) found 7% to be optimum.

Chinnaiya and Ganguli (1980) in [illegible] buffalo semen freezing, tried 6, 7 [illegible] volume with three diluents viz: ci[illegible], Tris and egg yolk citrate. They could not find any significant difference in post-thaw recovery of spermatozoa due to variation in glycerol levels.

Bhandari and Chauhan (1980) could freeze buffalo semen with good recovery using 6.4% glycerol by volume in Tris extender. Shetti et al. (1981) and Patil et al. (1981) used 6% glycerol in "Triladyl" diluent for satisfactory freezing of buffalo semen.

(e) Equilibration:

The spermatozoa require time to adjust themselves with glycerol and other constituents of the extender before they are subjected to freezing. An abrupt freezing may give freezing shock to spermatozoa and therefore

it is necessary to equilibrate their metabolic activities at 5°C for some time.

Wiggin and Almquist (1975) equilibrated the milk diluted bovine semen at 5°C for 30 mts or 120 mts before freezing in liquid nitrogen. The difference in sperm motility and per cent intact acrosome between two equilibration time was not significant.

Ennen et al. (1976) found no significant effect on post-thaw spermatozoal motility, when they equilibrated the bull semen at 5°C for 2, 4, 6, 10 or 18 hrs, eventhough they recommended 4 to 10 hrs equilibration period to be optimum.

Gilbert and Almquist (1978) studied the effect of 0, 3 or 9 hrs equilibration time on 10 bovine ejaculates extended in Tris yolk glycerol extender and packaged in 0.3 ml continental straws. On the basis of post-thaw acrosomal retention and spermatozoal motility, they reported that 3 or 9 hrs of equilibration time was superior to 0 hr of equilibration.

In recent studies, the buffalo semen diluted in Tris and equilibrated for periods ranging from 0 to 6 hrs at 5°C gave maximum post-thaw recovery when the equilibration time was 4 -5 hrs (Bhandari and Chauhan, 1980).

Working with buffalo semen extended in LFYG extender and frozen in liquid nitrogen, Crabo et al. (1980) noted

that an equilibration time of 7 hrs gave better results than of 5 hrs in terms of post-thaw motility and percentage of spermatozoa passing through sephadex G^{15} filters.

(f) Freezing technique:

Vapour freezing using liquid nitrogen as a refrigerent is now well accepted technique every where. Most of the laboratories use liquid nitrogen refrigerator for this purpose.

Shetti et al. (1981) and Patil et al. (1981) adopted the "thermocole box freezing unit". They adjusted two-wire meshes at four and 15 cms distance from the level of liquid nitrogen in a 5 litre capacity thermocole box. At both the levels 5 mts exposure of liquid nitrogen vapour was given to the straws on a wire mesh. The freezing was effected by immersing the straws in liquid nitrogen containing goblets 10 mts after total vapour exposure.

(g) Thawing:

The process of liquification of frozen semen seems to be simple, but is one of the most important factors affecting post-thaw spermatozoal viability.

Robbins et al. (1972) compared different thaw rates by plunging straws into water either at 5°C/4 mts, 26°C/1 mt, 35°C/30 sec, 75°C/6 sec, or 75°C/12 sec. They found that thaw rates achieved by using 35°C and 75°C water resulted in significantly greater retention of the

acrosome and maintenance of post-thaw motility than did the lower temperatures.

Considering mean per cent acrosomes and per cent motility, Senger *et al.* (1976) stated that the 35°C thaw for 1 or 3 mts resulted in better recovery as compared to 5°C thaw for bull semen diluted in Tris yolk glycerol and frozen in liquid nitrogen.

Rapid thawing rates (50°C/15 sec or 65°C/7.5 sec.) for semen packaged in plastic straws have shown to significantly increase the post-thaw acrosomal maintenance and per cent motile spermatozoa (Robbins *et al.*, 1976). They further reported that, when rapid thaw rates were used, the level of glycerol had to be increased to achieve optimum post-thaw recovery of spermatozoa.

Kim and Kim (1978) studied the effect of rapid and slow thawing on motility and fertility in bovine frozen semen. They observed that sperm motility was higher in semen thawed in water at 30°C for 1 mt than in iced water for 12 hrs.

For the bull semen extended in three different extenders and frozen in straws, Chandler *et al.* (1979) deduced the common thaw rates to be 40°C for 30 sec for optimum post-thaw motility and per cent intact acrosomes.

In an experiment involving large number of inseminations, Almquist *et al.* (1979) using milk diluted

bovine semen frozen in 0.3 ml straws, compared thawing times of 12 sec. and 30 sec. in a warm water bath at 32° to 35°C. They observed significantly higher fertility when semen was thawed for 30 sec. than for 12 sec.

Hube and Uribe (1980) froze the bovine semen in mini straws and thawed in water at 5°, 17° or 40°C or in air at 21°C. Samples thawed at 40°C showed significantly higher post-thaw motility and higher percentage of normal acrosomes than those thawed at lower temperatures.

Pace *et al.* (1981) reported that 90 days non-return rate for spermatozoa frozen in 0.5 ml straws and thawed in 37°C. water was higher than that for spermatozoa thawed in either ambient temperature water or ice-water.

(B) FREEZABILITY:

All the bulls do not give the ejaculates suitable for freezing. An ability of spermatozoa to undergo the freezing at ultralow temperature varies from bull to bull. Age (Il'Inskaya and Osipova, 1972), season (Boehnke *et al.*, 1976), breed (Biborski *et al.*, 1971) and species are some of the established factors, which affect the freezability of bull semen. As compared to bull semen, buffalo semen has been reported having low freezability. Patil (1980) reported that out of 14 frozen semen stations in operation at present in India, only 3 or 4 are involved in freezing of buffalo semen.

Vlachos et al. (1965) found considerable difference in freezability of semen between bulls. Only 40% of the total bulls used, ejaculated semen suitable for deep freezing in liquid nitrogen.

Of 108 bulls at an AI centre, 60.18% provided semen suitable for deep freezing as reported by Molinari and Valpreda (1968).

Biborski et al. (1971) investigated the sexual development and freezability in 46 Polish Black and White Lowland (BWL) and 48 Polis Red & White Lowland(RWL) bulls aged 12 months. 71.7% of BWL bulls were sexually mature at 12 months [illegible] 31.1% produced semen suitable for freezing versus 22.9% and 12.5% respectively for the RWL bulls.

Roberts(1971) reported that about 30% of the bulls reared as potential sires in AI units don[illegible]men that does not freeze satisfactorily with conventional extender.

Haave (1972) evaluated 270 bulls (3 ejaculates/bull) for freezability. He found 88.1% of samples showed satisfactory freezability (atleast 25% forward motility post-thawing).

In their study on freezability of bull semen Otel et al. (1972) evaluated 51 bulls and 9[illegible]se bulls were found suitable for freezing. They did not find the significant differences between adult bulls and young bulls aged 18 - 24 months in relation to freezability.

Szilagyi and Muller (1972) carried out deep freezing experiments with 2022 semen ejaculates from 75 bulls. They reported that the ejaculates of 61 bulls showed good freezability, 12 bulls were not good and the ejaculates from 2 bulls were totally unsuitable for freezing.

Sahni and Roy (1972) carried out the trial on deep freezing of semen samples collected from 10 buffalo bulls. They reported that the semen of only one bull could stand to freezing. The results of post-thaw motility of remaining nine bulls were very poor.

Pavithran et al. (1972) froze the semen from five buffalo bulls. They found significant variation between the freezability of semen from different bulls.

Verses et al. (1972) in their study on freezability of bovine semen observed the ejaculates from 18 bulls with suitable, reasonably suitable and unsuitable for freezing under electron microscope. The ejaculates of 2 bulls, which were not freezable, showed consistently damaged acrosomes (swelling, detachment or rupture) in fresh semen and to a greater extent in freeze-thawed semen.

Boehnke et al. (1976) examined 29000 semen ejaculates from bulls for freezability. Ejaculates with less than 6.0×10^9 spermatozoa exhibited below-average freezability. They further stated that freezability varied with season.

(C) LIVE-DEAD AND MORPHOLOGICALLY ABNORMAL COUNTS OF SPERMATOZOA:

Morphology of spermatozoa is one of the criteria employed for the evaluation of bull semen. Cytomorphological characteristics of spermatozoa are affected by genetic factors like strain, breed and species difference (Mukharjee and Singh, 1965) and by non-genetic factors like age of the male, season, temperature, shock, dilutors and preservation (Tomar et al., 1964).

Lagerlof (1934) stated that the presence of 18 - 20% of abnormal spermatozoa was comparable with good fertility. Herman and Swanson (1941) reported this level to be upto 30%.

Storage of semen at 4°C for 48 hrs had no effect on percentage of live sperm (Hafez and Dar-Wish, 1956). They observed 81.1% live spermatozoa in the buffalo semen.

Kumaran (1965) carried out the comparative studies on morphology of fresh and frozen bovine semen. He found that percentage of abnormal spermatozoa varied from 7 to 20 in fresh semen and from 23.7 to 32.0 in frozen semen.

Rob and Jiranek (1971) studied the spermiological criteria of deep frozen bovine semen. They reported the morphologically abnormal sperm percentage to be 16.9% post-thawing versus 12.5% in fresh semen.

Kodagali et al. (1973b) in their studies on semen characteristics of Surti buffalo bulls reported the abnormal percentage to be 9.79 ± 1.35% in fresh ejaculates.

Shetti *et al.* (1981) studied percentage of live and morphologically abnormal spermatozoa in buffalo semen diluted with 'Triladyl' diluent. They observed live spermatozoa percentages at 20°, 5° and -196°C, which averaged 80.98 ± 0.88%, 77.9 ± 1.19% and [illegible].22 ± 7.35% respectively. At the same three temperature levels, the abnormal spermatozoa were 7.50 ± 0.29%, 8.33 ± 0.31% and 9.10 ± 0.15% respectively.

Patil *et al.* (1981) in their trial on buffalo semen freezing, used three different dilutors. They reported, that on freezing the overall live percentage of spermatozoa in various dilutors significantly decreased from 83.64 ± 0.94 to 52.74 ± 3.21 and the overall percentage of morphologically abnormal spermatozoa increased from 12.59 ± 1.09 to 14.08 ± 1.30% at 5° and -196°C respectively.

(D) MENSURATION CHARACTERS OF SPERMATOZOA:

Qualitative and quantitative changes in sperm morphology affect the preservation of semen in vitro (Roberts, 1971). Mann (1964) showed that spermatozoan head is mainly concerned with the fertility of mammalian spermatozoa. Mukherjee and Kumar (1971) reported that the fertility of bulls is positively correlated with spermatozoan head length. Bishop *et al.* (1954) and Mukherjee and Rajwar (1971) also found the mensuration characteristics of spermatozoa to be related to fertility.

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Rajendrakumar et al. (1977) in their studies on biometrics of buffalo-bull spermatozoa in relation to fertility reported the overall mean for the spermatozoal head length, head breadth and head shape as 7.83 ± 0.0327 μ, 4.79 ± 0.0305 μ and 1.63 ± 0.09 μ respectively.

Rajwar and Mukherjee (1970) studied the effect of temperature shock on cytomorphology of bull and buffalo spermatozoa. They observed that the head breadth of bull spermatozoa and head area of buffalo spermatozoa were greater after a temperature shock of 5°C than at 30°C.

Kodagali et al. (1973[a]) studied the biometrical norms of the Surti buffalo spermatozoa. The average values for head length, head breadth, midpiece and tail length and total sperm length were 7.618 ± 0.22 μ, 4.591 ± 0.51 μ, 56.715 ± 0.93 μ and 64.333 ± 2.17 μ respectively.

Rao and Sidhu (1975) studied the biometrics of spermatozoal dimensions in bulls. The Hariana and Jersey bulls' spermatozoa had the head length of 9.7 μ and 9.16 μ and the head breadth of 5.10 μ and 4.72 μ respectively.

Sharma and Gupta (1978) studied the mensuration of spermatozoa of buffalo bulls. The head length averaged 8.43 μ, total length 60.9 μ and tail main piece length 47.8 μ.

Jain (1979) studied the effect of deep freezing on morphology of the spermatozoa in buffalo semen. He noted

the decrease in average head length from 7.5376 ± 0.0585 to 7.1699 ± 0.0624 microns, which was highly significant in two bulls, but non-significant in other two. The head breadth decreased from 4.5966 ± 0.0258 to 4.5128 ± 0.0252 microns, which was nonsignificant for individual bulls.

(E) STUDIES ON ENZYME LEAKAGE:

With all the current research and sophisticated tecaniques, the damage in frozen-thawed spermatozoa can not be prevented, which lead the release of certain important enzymes. The enzymatic tests for the evaluation of semen quality have been of much help in bovines to predict the fertility of bulls. Stewart *et al.* (1972) conducted six conventional subjective laboratory tests on fresh and frozen semen samples relating to motility, live/dead ratio, morphology and survival after incubation. They could not find a significant correlation between any of the subjective tests and fertility. The post-thaw motility of frozen semen is being widely accepted in a routine evaluation of frozen semen quality. But Kelly and Hurst (1963) in their study regarding the relationship between certain laboratory criteria and fertility of bovine semen, observed that motility immediately on thawing and survival time of spermatozoa were not correlated with fertility.

The assay of the released acrosomal enzymes into the seminal plasma is an early and sensitive indication of

damage sustained by spermatozoa during the processing of semen (Allison and Hartee, 1970).

(a) Transaminases:

Enzymes which transfer an amino group of aminoacid to a keto acid are known as transaminases. Transaminase enzyme activity has been found in semen. Graham et al. (1974) reported the use of aspartate aminotransferase (GOT) level in semen as an indicator of semen quality. The release of Glutamic Oxaloacetic Transaminase (GOT) has been used, by many workers as a measure of the sperm membrane permeability. An increased membrane permeability reflected by elevated extracellular GOT concentration in the seminal plasma. Pace and Graham (1970) showed that the release of GOT was inversely correlated with fertility in bulls.

Pace and Graham (1970) stated that GOT found in semen is mainly associated with the sperm cell and that the amount found in seminal plasma mainly arose from leakage from the sperm cells. They further reported that the amount of GOT released was significantly correlated (0.80) with the sperm concentration.

Chauhan and Srivastava (1973) in their studies on enzyme composition of buffalo seminal plasma reported that GOT:GPT ratio was much lower in buffalo than in bull seminal plasma (i.e. 5:1 V 42:1).

Varshney et al. (1978) determined transaminases in seminal plasma of buffalo semen. The Glutamate Pyruvate Transaminase (GPT) value averaged 20.4 ± 0.47 units/ml and the GOT was 83.5 ± 3.0 units/ml, the later being less than half of the similar value reported in case of bull semen by Flipse (1960), who assayed 63 samples of bovine semen for GOT and GPT values. It gave an average of 623 ± 48 units of GOT and 15 ± 1 units of GPT per ml of seminal plasma. He further stated that the concentration of both of which in seminal plasma was significantly correlated with number of the spermatozoa per ml of semen.

Crabo et al. (1971) suggested the extracellular GOT as a measure of membrane injury in spermatozoa. The semen diluted with citrate phosphate, Tricin or TES showed the increased levels of GOT with time and cooling.

Breeuwsma (1972) observed the significant increase in seminal plasma level of GOT after freezing.

In their studies on the transaminase activity of bull semen Semkov et al. (1973) did not observe the significant correlation between the spermatozoal motility and GOT activity. The GOT activity averaged 210 units for seminal plasma and 58 units for spermatozoa.

Coulter and Foote (1973) diluted the bovine semen in low lipid diluent and frozen in liquid nitrogen vapour.

They observed the higher GOT concentration extracellularly in freeze thawed semen samples.

Zahariev *et al.* (1974) in their studies on transaminase activity of bull's semen, found the GPT activity in spermatozoa and seminal plasma to be correlated with the percentage of motile spermatozoa in freshly collected semen. The enzyme activity was 17.2 units in spermatozoa and 13.0 units in seminal plasma before freezing and 12.6 and 11.6 units respectively after freezing.

Roychaudhury *et al.* (1974) studied the effect of cold shock on GOT and GPT release. They observed that cold shock caused significant release of GOT and GPT from bull spermatozoa, if diluted with sodium citrate buffer. The enzymatic release was not significantly high, when the semen was extended with Tris-citric-acid fructose extender.

In their study on biochemical changes in frozen bull semen Buruiana *et al.* (1975) found the sperm GOT activity in decreasing trend in fresh, frozen and frozen stored semen. It was between 52.5 to 80.2 u in fresh semen, 36 to 48.4 u in one month old semen and from 10.9 to 11.6 U in 13 month old semen.

Chinnaiya *et al.* (1979) studied the extracellular release of transaminases from buffalo spermatozoa on freezing. They reported that the citric acid whey diluent

showed significantly lower release of GOT and thereby better protectotive action on spermatozoa as compared to egg yolk citrate or Tris diluents.

Jain (1979) studied the effect of deep freezing on release of GOT from buffalo spermatozoa. He found the significant difference in GOT value pre-freeze and post-freeze which decreased from 351.28 ± 69.01 to 201.74 ± 20.74 units/mg protein.

(b) Hyaluronidase:

There are very few reports on hyaluronidase activity in buffaloes. The hyaluronidase is one of the important enzymes concerned with fertilization. (Austin, 1960). The enzyme hyaluronidase is believed to permit spermatozoa to disperse and penetrate the cumulus oophorus, thereby facilitate the fertilization of ovum. Loss of acrosomal integrity is a sign of sperm damage. It allows the release of hyaluronidase found to be located in acrosomic system.

Swyer (1947b) in his studies found the close correlation between hyaluronidase activity and sperm density. He reported, 100 million sperm pack of 1 ml bull semen contained 7 units hyaluronidase.

Kooner and Ludwick (1971) found the significant correlation (-0.96) between hyaluronidase activity and spermatozoal motility. Dhanda et al. (1981) in their study with Murrah buffalo semen observed that the sperm motility was inversely related to the hyaluronidase activity in semen.

Holmaan *et al.* (1978) observed the significant difference between hyaluronidase activity between undiluted, diluted and frozen thawed semen, the activity being 12.0, 22.6 and 37.7% respectively. They also studied the relationship between sperm characters and hyaluronidase activity and found non-significant correlation between hyaluronidase activity and percentage of sperm abnormality.

Chinnaiya et al (1979), using different diluents found the significant rate of release of hyaluronidase on dilution. Tris showed low rate, followed by citric acid whey and sodium citrate.

Ganguli (1979) estimated the hyaluronidase activity in spermatozoa diluted in Tris and citric acid whey. He found on freezing and thawing the extent of damage to spermatozoa and the hyaluronidase release were almost similar for both the dilutors.

Raizada *et al.* (1980) observed the positive correlation between hyaluronidase activity of sperm extract and sperm count in HRS (Bos indicus) and Murrah bulls. They also reported the significant correlation between hyaluronidase activity in sperm extract and fertility ($R = 0.98$, $P < 0.05$).

Patil *et al.* (1981) undertook a study to estimate acrosomal damage and assay of hyaluronidase leakage in buffalo spermatozoa in fresh and freeze-thawed semen in

various dilutors. The hyaluronidase activity markedly declined from 636.25 ug in fresh semen to 406.00 ± 0.70 ug/25 x 10^6 of sperm extract post-freezing in various dilutors.

(F) FERTILITY TRIALS

The real and practical success of the attempts made on extending, freezing or preserving spermatozoa for longer duration can be best judged by their ability to fertilize the ova, when artificially inseminated. Large scale field trials in U.S.A. and Europe have clearly shown that properly frozen semen, when inseminated gave conception rates comparable to conventionally preserved semen (Rice *et al.*, 1957).

At Reading in England, Stewart (1951) reported for the first time, the birth of a calf from inseminations with deep frozen semen. Kumaran (1965) reported for the first time fertility of frozen bull semen in India, though the results were not satisfactory. During the same period Basirao (1964) and Settergren (1966) reported the fertility of 40 -77% following the first ever inseminations with frozen water buffalo semen in India.

(i) Cow bull semen:

Jainudeen (1968) reported the conception rate of 44.4% to first inseminations by using imported frozen Friesian semen.

Davidovic *et al.* (1971) obtained over 71% of conception rate by using deep frozen semen for inseminating over 550 cows.

For 558 cows, the first service inseminations as reported by Jondet (1964) gave 68% conception rate.

Picket *et al.* (1959) artificially inseminated 329 cows with frozen semen and reported 72.9% fertility on 60 -90 day non return rate basis.

A study was made on the conception rate by inseminations with deep frozen semen performed in 87,224 cows over the period of 3 years by Nair (1975). He reported that the overall conception rate varied from 38.88 to 41.48% (Av. 40.18%).

On the basis of 66 days non return rates, involving over 30,000 first service inseminations with milk diluted Friesian frozen semen. Almquist *et al.* (1979) obtained over 70% conception rate.

Foote (1970b) reported 74.1%, 60 to 90 days non returns for Tris extended semen involving over 50,000 inseminations in field fertility trials in cows.

Maulic *et al.* (1975) found the conception rate of 42.2% with Friesian frozen semen in Haryana cows.

Guha (1972) reported the first service conception rate of 46% using frozen semen for Friesian half bred of Haryana.

Roy (1974) reported 85.9% and 80.6% conception rates on 60 days non return basis by semen frozen with "Tupol" freezing technique in Murrah buffaloes and Friesian cows respectively.

For 110 and 107 females inseminated with frozen thawed semen, extended in conventional egg yolk citrate and in a chemically defined medium Tris, Olar and Pickett (1980) obtained the conception rate to first inseminations 72.7% and 71.0% respectively.

Austin *et al.* (1978) reported the conception rates of 44.6% and 43.6% respectively for 1425 confirmed first inseminations carried out on randomly selected cows with Jersey frozen and liquid semen.

In a field study on 319 cows, the conception rate of females inseminated with semen frozen in dry ice plus alcohol was 52.6% against 58.0% for frozen semen in liquid nitrogen (Ishii *et al.*, 1978).

Chinnaiya *et al.* (1974) compared the conception rates obtained by using imported frozen Friesian semen and the one frozen in laboratory and reported the conception rate ranging from 30.8 to 52.1% and 42.6 to 60. [illegible] ctively.

Tomar (1981) reported the overall conception rate of 61.34% involving 194 inseminations in [illegible]ana cows with deep frozen semen of Holstein Friesian bulls.

Singhal et al. (1981) found higher conception rates for Friesian frozen semen than those for Brown Swiss and Jersey bulls on farm level.

(ii) Buffalo bull semen:

On 60 days non return basis Pavitharan et al. (1972) found the conception rate to be 80% for about 200 frozen semen inseminations in buffaloes.

By using Murrah buffalo frozen semen in 145 buffaloes, most of them had previously failed to conceive to natural mating, Elimwadi et al. (1974) obtained the conception rate of 25.5% in a field level fertility trial.

Takkar et al. (1980) reported the conception rate of 36.12% using frozen buffalo semen extended with Tris.

Roychaudhury (1978) obtained 50% conception rate in Surti buffaloes with frozen semen extended in egg yolk citrate glycerol extender.

Sharma et al. (1979) in field trials obtained the conception rates of 28.4% and 24.5% using buffalo semen frozen in egg yolk citrate glycerol and citric acid whey dilutors respectively.

Chinnaiya et al. (1979) carried out fertility trials using frozen Murrah buffalo semen. The semen was diluted with egg yolk citrate glycerol, citric acid whey (CAW) and Tris yolk glycerol. The corresponding fertility rates

were 57.14, 58.44 and 46.22% based on 119, 77 and 119 inseminations, respectively.

Shafi (1979) reported, 50% conception rate for frozen buffalo semen.

Vasanth (1979) has successfully frozen the semen of Surti and Murrah buffalo bulls using Tris yolk glycerol diluent. Under field conditions, using frozen semen in 13659 buffaloes, he reported the conception rate of 45%.

Jainudeen (1980) obtained the average pregnancy rate of 30% by using the frozen semen in Asian Swamp buffaloes.

Chinnaiya and Ganguli (1980) reported first service and overall conception rates of 45% and 45%, [illegible] and 44% and 31% and 40% using buffalo semen frozen in three different dilutors viz., citric acid whey, egg yolk citrate and Tris respectively.

Toelihere (1980) obtained the conception rate of 30% with frozen buffalo semen, which was lower than, 58% obtained using fresh liquid semen.

Meharsing *et al.* (1980) reported conception rate of 45.8% to first service 72 inseminations with frozen buffalo semen extended with Tris dilutor.

The first service inseminations in 993 and 221 buffalo cows with semen frozen in pellets and straws gave the conception rates of 53.9% and 57.2% respectively on farm level fertility trials (Zhou, 1981).

Austin et al. (1981) carried out 450 first inseminations on randomly selected buffaloes with chilled, and frozen semen of Murrah buffalo bulls and obtained the mean conception rates 28.55% and 42.60% respectively.

Patil et al. (1981) undertaken a study on deep freezing of buffalo semen in three dilutors with and without addition of epididymal constituent. Based on 34 inseminations, the overall fertility rate of freeze thawed semen was 47.06%, for Triladyl, Triladyl + MSF, Tris and Tris + MSF dilutors it was 42.86, 50.00, 55.55 and 50.00% respectively.

Heuer (1982) reported the fertility percentages of 53.8%, 54.3%, 56.7% and 53.8% for buffalo semen diluted and frozen in LFYG, Tris, Tris milk and skim milk diluents respectively following 1154 total inseminations.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

The present research project was carried out at the department of Veterinary Obstetrics and Gynaecology; Gujarat Veterinary College, Anand; for a period of 12 months commencing from 1st June '81 to 31st May '82.

Experimental breeding bulls:

In all, 23 bulls were available for study. Twenty of them were Surti buffalo bulls ranging between 4 to 7 years of age; and three were cow-bulls of Holstein Friesian breed aging between 4½ to 5½ years. The Holstein Friesian bulls used for this study were owned by Livestock Research Station, Veterinary College, Anand but, were stationed at the Gynaecology department of the college; where the two other buffalo bulls were also maintained in the identical managemental condition. The remaining 18 buffalo bulls were stationed at a well organized farm and a Semen Bank situated at the distance of about 80 and 40 kms respectively.

All the bulls selected for this study were in good health; under constant veterinary care and supervision and maintained in nearly identical managemental conditions. All these bulls had normal libido and service behaviour.

Sterilization:

All the glass articles used during the study were sterilized in hot air oven at 160°C for 30 mts.

Buffer solutions, lubricant, rubber articles and artificial vagina etc., were autoclaved at 10 lb. pressure (115.6°C) for 20 mts.

Collection of semen:

The schedule of semen collection was twice in a week for all the bulls. The semen ejaculates were obtained in sterilized graduated collection tubes; attached through a rubber cone to artificial vagina for bulls, with smooth rubber liner; at a inner temperature of 42-44°C.
A separate artificial vagina was used for each bull. Usually the semen collections were made between 6.30 to 7.30 A.M. For semen collection, the bulls of the same species were used, as teasers.

After the collection, the semen containing tubes were placed in a beaker; half filled with water at 32° -35°C, till processing.

Evaluation of semen:

(a) In a graduated collection tube volume, colour and consistency of semen were observed and noted immediately after collection.

(b) [illegible]ivity of fresh semen was estimated putting a drop of semen on a clean glass slide placed on a biotherm stage at 37°C, and viewing through 10 X objective of a phase contrast [illegible]cope.

This activity was expressed in 0 to +++++ grade as per the standard technique of Herman and Madden (1953). The sperm concentration was estimated by using a portable photoelectric colourimeter employing 4.9 ml of 2.9 [illegible]ium citrate solution and 0.1 ml of neat semen; at 540 m μ.

(c) Percentage of Live and Abnormal spermatozoa:

Differential staining technique was used for counting live-dead and abnormal spermatozoa. The eosinnigrosin stain was used as per Hancock (1951) dissolving eosin 1.67 gms and nigrosin 10 gms in 100 ml of double glass distilled water. For staining to be done, the proportion of stain and semen was kept as 4 drop: 1 drop respectively. The uniform thin smears were prepared. One hundred spermatozoa were counted for each of the two [illegible]tions from each stained slide under oil immersion objective of phase contrast microscope.

(d) The individual progressive motility and per cent motile spermatozoa were estimated by putting a diluted semen drop on a glass slide under a cover-[illegible], using biotherm at 37°C, on 25 X objective of a [illegible] contrast microscope.

Dilution:

Following these initial evaluations, the samples showing required optimum quality (Minimum +++ Mass activity and 70% motility) were processed further, diluting them in the selected dilutors. In present studies, the different dilutors used for bull and buffalo bull semen were as follows:

For bull semen:

(i) Egg yolk citrate glycerol (EYCG),
(ii) Tris-fructose-yolk glycerol (TFYG),
(iii) Laiciphos-478,
(iv) AMUL spray-glycerol (ASG),
(v) Whole-milk glycerol (WMG),

For buffalo bull semen:

(i) Lactose fructose yolk glycerol (LFYG),
(ii) Tris fructose yolk glycerol (TFYG).

Using standard procedures, the required amount of dilutors were prepared just before use. The EYCG was prepared as per Salisbury *et al.* (1941). The TFYG was prepared as per the recommendation of [illegible] (1979). For, LFYG dilutor, the ingredients were used in a proportion suggested by Ahmad and Chaudhry (1980). The Laiciphos was imported and used as per the instructions given by the manufacturer*. The preparation of A[illegible] glycerol

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*IMV -France.

dilutor was based on the formula of El Alamy (1979). The whole milk glycerol was prepared as per the standard procedures, according to Thacker & Almquist (1953).

Antibiotics - Penicillin @ 1000 i.u./ml and Streptomycin @ 1000 mg/ml were added in each dilutor. (For composition of dilutors pl. see Appendix II).

While diluting each semen sample, the concentration of spermatozoa in a sample was taken into consideration. The dilution rate was adjusted; keeping about 50 to 60 million spermatozoa per ml of semen before freezing; and thereby allowing at least 12.5 to 15.0 million live spermatozoa available in each 0.5 ml frozen semen dose, after thawing.

<u>Freezing of semen</u>:

(a) <u>Glycerolization</u>

Dilutors LFYG and TFYG were incorporated with 5% and 6% glycerol respectively. When these two dilutors were used, glycerolization was carried out at room temperature; by diluting in single step the semen with glycerol containing dilutor. The diluted semen was maintained at room temperature till filling of straws.

The other dilutors used during the study contained 7% glycerol by volume. [illegible] were EYCG; Laiciphos-478, ASG, and WMG; where the basic dilutor (without glycerol) was divided into two fractions - A and B. The 'A' portion

of the dilutor consisted of the basic dilutor, without glycerol, which made 50% of the total requirement of the dilutor. Fraction 'B', the other half portion, consisted of the basic dilutor with twice the desired final concentration of glycerol, by volume. Immediately after collection and initial evaluation, each ejaculate was practically half diluted at room temperature, using fraction 'A'. The partially diluted semen was then placed in a water bath containing water at 32°C, and cooled gradually to 5°C. Simultaneously, the fraction 'B' was also cooled to 5°C. Glycerolization was carried out at 5°C by addition of equal volume of fraction 'B' to the fraction 'A', in four parts at 15 minutes interval. The diluted semen, was then drawn into the 0.5 ml plastic straws.

(b) Filling of the straws:

The straws were fixed in clamps, which hold 15 medium straws. The closed ends of the straws were fitted into the nozzles of the filling comb, which was connected at the other end, to a vacuum suction pump, through a plastic tube. Assuring all the connections to be perfect, the open ends of the straws were dipped into a beaker containing diluted semen and the switch was put on. The filling of the straws was obtained due to aspiration.

The uniform 1 to 1½ cm air space was created in each straw by fitting the open ends of the clamped straws into the teeth of the plastic bubbler.

The sealing powder - polyvinyl alcohol powder - was spreaded evenly in about 4 - 5 mm thickness on a glass plate. The open ends of the straws held in the clamp were then dipped into the powder; when the powder had penetrated about 5 mm into the straws, a satisfactory plug was made.

After sealing, the straws were placed in the water bath at the temperature, similar to that of diluted semen i.e. at 32°C when one step dilution was practiced and at 5°C in case of two - step dilution.

(c) Equilibration:

Sealed straws were gradually cooled to 5°C temperature by putting them into a plastic box. This was transferred into a thermocole box containing ice; where the straws were further maintained at 5°C for 5 hrs equilibration time.

(d) Freezing technique:

A "thermocole freezing unit" was used for freezing of the semen. Vapour freezing method using liquid nitrogen was adopted. A rectangular thermocole box of 10 litre capacity was used. Inside of which, on the upper part, two flat wooden strips were fixed transversely at the distance of about 2". A line was marked 4 cms below the level of wooden strips in the thermocole box. About 6 - 7 litres of liquid nitrogen was poured into

the thermocole box, upto the level of marked line, while freezing.

Following the equilibration, the straws were taken out of the water and dried rolling gently in pre-cooled turkish towel.

The dried straws were immediately placed on the wooden strips, horizontally and the thermocole box lid was closed. The straws were exposed to the liquid nitrogen vapour exactly for 10 mts. The lid of the thermocole box was then opened and the straws were transferred into the goblet containing liquid nitrogen (-196°C), assuring that the full length of the straws were dipped into the liquid nitrogen. Immediately after deep freezing, one straw was thawed and the semen was examined for spermatozoal motility. The samples showing more than 40% post-thaw motility were transferred through the canisters in liquid nitrogen storage containers. The substandard samples were discarded.

The different colours of the straws were used, bull-wise and dilutor-wise for easy identification.

(e) Thawing:

Thawing of frozen semen was effected by immersing the straws in water kept at 102° - 104°F in a thermos for 15 to 20 seconds.

Mobile freezing:

With a view to facilitate the working a "mobile freezing unit" was established for freezing the semen of buffalo bulls, stationed at the farm & Semen Bank, away from the laboratory. The dates for freezing of the semen were pre-determined with the respective authorities. All the necessary equipments and buffers etc., kept at 5°C in a thermocole box containing ice cubes, were carried to the place of work, in a jeep, on the previous night of the day of work. On the next day morning, observing the strict hygienic measures the semen was collected; from the selected bulls, evaluated and diluted as per the requirements.

The diluted semen was filled and sealed in the French medium straws and maintained separately bull wise and dilutor-wise in a thermocole box at 5°C for equilibration. During the equilibration period, the filled and sealed straws were transported smoothly in the jeep to the pre-cooled laboratory, where the actual freezing was carried out.

The present research project was divided in five separate experiments, for easy working and better interpretation. A test freezing was done previous to each experiment.

I. A STUDY ON FREEZABILITY OF SEMEN:

The ability of spermatozoa to undergo the freezing at ultra low temperature varies from bull to bull. To study the freezability of semen of various bulls, an experiment was carried out, involving all the 23 bulls - (20 Surti and 3 H.F.). Two ejaculates from each bull were collected one at a time at weekly interval.

The semen was evaluated for volume, colour consistency, sperm concentration, mass and individual motility and live and abnormal counts. The samples fulfilling the required standards were diluted depending upon the concentration of spermatozoa and frozen in liquid nitrogen vapour. For all the ejaculates of cow bull and buffalo bulls, TFYG dilutor (FAO: 1979) was used.

The post-thaw recovery was estimated twice, immediately after freezing and 24 hrs after storage in liquid nitrogen, using a biotherm at 37°C on 25 X objective of a phase contrast microscope looking for progressive motile sperms.

II. A STUDY ON REVIVABILITY OF SPERMATOZOA IN DIFFERENT EXTENDERS:

An attempt was made to study the efficiency of various extenders for freezing of bull and buffalo semen, using split ejaculate technique.

Three H.F. Bulls and five Surti buffalo bulls were involved in this study. For freezing of H.F. bull semen, five extenders were tried viz., EYCG, TFYG, ASG, [illegible] and Laiciphos-478. Two extenders, LFYG and TFYG were [illegible] for buffalo semen freezing. Five ejaculates from [illegible] bull were processed separately for this experiment.

After collection and initial evaluation, the semen samples with optimum quality were split into five aliquots in case of H.F. bull ejaculates and in two aliquots in case of buffalo semen. These samples were then diluted using dilutors under trial at the rate of 1:20; packaged in French medium straws and frozen in liquid nitrogen as described earlier.

The post-thaw recovery of spermatozoa was estimated immediately after freezing and 48 hrs after storage in liquid nitrogen. The live and abnormal counts of the spermatozoa were assessed, using the same eosin-nigrosin stained slides for each ejaculate.

III. EFFECT OF DEEP FREEZING ON RELEASE OF CERTAIN ENZYMES FROM BUFFALO SPERMATOZOA:

The enzymatic tests for the evaluation of semen quality have been of much help particularly with bovine species. Effect of deep freezing on release of trans-aminases and hyaluronidase enzymes was studied on semen samples of five Surti buffalo bulls.

Seventeen ejaculates from the five buffalo bulls were used for pre-freeze and post-freeze study of release of enzymes.

(a) Hyaluronidase assay:

After collection and evaluation, the semen samples were split up in two parts, each of 0.5 ml. They were then diluted separately with TFYG and LFYG dilutors, in such a way that the diluted sample contained approximately 25 million spermatozoa per ml. The 25 million sperm bead was prepared by centrifuging one ml of diluted sample at 1500 r.p.m. for 15 mts. The estimation of enzyme Hyaluronidase was carried out in fresh diluted samples within shortest possible time after collection of semen and preparation of sperm beads (usually within 6 hrs). The samples were kept at 5°C till the estimation.

The remaining quantity of the diluted semen in both dilutors, was frozen and stored. The post-freeze estimation of the enzyme activity in sperm extract was carried out after 24 hrs of storage in liquid nitrogen.

Based on liberation of N-acetylglucosamine from enzyme the estimation of Hyaluronidase activity in sperm extract was carried out as per the procedure detailed by Bollet et al. (1953). The procedure involved the following steps:

1. In a centrifuge tube containing approximately 25 million sperm pack :

   0.1 ml sodium acetate buffer (0.05 M, pH 3.8),

   + 0.2 ml Hyaluronic acid (6 mg/ml),

   + 0.1 ml Sodium chloride soln. (2 M soln.),

   + 0.01 ml Sodium hydroxide soln. (4 N soln.),

   + 0.1 ml Potassium tetraborate soln. (0.8 M, pH 9.1) were added.

2. Incubated for 1 hr at 37°C.

3. Incubation was followed by heating the tubes in boiling water bath for exactly 3 mts.

4. The solution in the tube, then cooled to room temp.

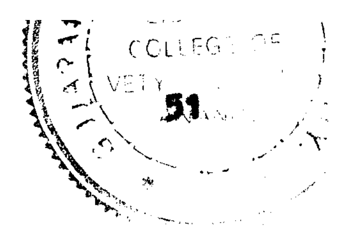
5. Then 3.6 ml of P-Dimethylamine benzaldehyde (P-DMAB) (10 gms/100 ml acetic acid to be diluted 10 times in acetic acid) was added to the solution.

6. Tubes were again placed in a water bath at 37°C for 20 mts.

7. Then the tubes were centrifuged for 10 mts at 2000 r.p.m.

8. The intensity of colour developed in a supernatant was measured in Spectrophotocolourimeter at 580° μ.

The amount of hyaluronidase enzyme released from the sperm extract pre and post freezing was expressed as O.D. (i.e. Optical Density) readings, and the difference in enzyme activity was noted.



(b) <u>Estimation of GOT and GPT</u>:

The same bulls and the very ejaculates were utilized for estimation of the transaminases in the semen before and after freezing. From the diluted samples, 1 ml of semen (containing approx. 50 million spermatozoa) was centrifuged at 1500 r.p.m. for 15 mts.

Till the estimation of the enzymes, which was carried out in most cases six hrs after the collection of semen, the separated seminal plasma was maintained at 5°C in sterilized vials.

The activity of transaminases (GOT and GPT) was estimated in the sperm-free seminal plasma separated from the frozen-thawed samples, after one week storage in the liquid nitrogen. Two straws were thawed for each sample and 1 ml of seminal plasma was processed for the enzymatic estimations immediately after thawing. The transaminases (GOT and GPT) were determined by the colorimetric method according to Reitman and Frankel (1957). GOT reacts with substrate (α-ketoglutaric acid and aspartic acid) to produce oxaloacetic acid which then reacts with 2,4-dinitrophenylhydrazine to produce colour in alkaline medium. GPT reacts with substrate (α-ketoglutaric acid and alanine) to produce pyruvic acid which then reacts with 2,4-dinitrophenylhydrazine to produce colour in alkaline medium. The enzymes were measured in international units (IU) of enzyme/lit.

IV. CHANGES IN CYTOMORPHOLOGY OF SPERMATOZOA DUE TO FREEZING:

The ejaculates from three H.F. bulls and five Surti buffalo bulls were available for this study. Two ejaculates from each bull were utilized. Two semen smears were prepared from each ejaculate prefreeze and post-freeze by mixing a drop of semen with three to four drops of nigrosin-eosin stain. Fifty straight live sperms were randomly selected for each ejaculate and their head length, head breadth (maximum), and total length were measured on projection microscope (Lensmeter) having 40 X objective lense and overall magnification of 900 X on a circular graduated ground glass.

V. A STUDY ON FERTILITY TRIALS OF FROZEN BULL AND BUFFALO SEMEN:

The ability of spermatozoa to fertilize the female has been the most reliable test for the quality of semen. As a part of the present study, fertility trials were conducted at field and farm levels for the bulls selected on the basis of the results of 1st and IInd experiments of this project. Bulls with good freezability and dilutor with good suitability were selected for their use in fertility trials. In all, for fertility study eight bulls - 3 H.F. and 5 Surti - were selected. The required numbers of semen ejaculates were collected and evaluated. Ejaculates showing optimum quality were diluted and frozen in a way that at least 12 to 15 millions live spermatozoa

were available per dose of frozen semen, packaged in a 0.5 ml capacity medium straws.

TFYG extender was used for diluting both, bull and buffalo semen. The frozen semen was stored in liquid nitrogen at least 10 days before it was used. All the inseminations were performed between October '81 to March '82, during the period of six months.

(a) Fertility trial at field level:

The frozen Holstein bulls' semen doses were distributed for insemination in Kankrej cows and heifers at field level in two advanced districts of Gujarat state. The trained inseminators at village AI centres were involved with the inseminations and pregnancy diagnosis work. The pregnancy diagnosis was performed per rectally, 90 days after inseminations. Totally 251 cows and heifers were inseminated using 285 frozen semen doses.

The frozen buffalo bull semen was utilized for inseminating the Surti buffaloes and heifers coming for inseminations at the College A.I. Centre, where qualified and well experienced veterinarians performed the inseminations and the pregnancies were diagnosed by per rectal palpations 60 to 90 days after inseminations. Totally 342 buffalo and buffalo heifers were inseminated using 426 frozen semen doses.

(b) Fertility trial at farm level, using frozen semen in repeat breeder cows:

About 98 frozen semen doses of two Holstein bulls were utilized to study the efficacy of frozen semen to settle the repeating Kankrej cows and heifers of Livestock Research Station, Gujarat Agricultural University, Anand. A farm veterinarian performed the inseminations. The pregnancy diagnosis was based on rectal palpations 75 - 90 days after inseminations. All the inseminations were performed randomly on repeat breeding Kankrej cows and heifers.

Statistical analysis:

The percentage values of progressively motile, live and abnormal spermatozoa were transformed to arcsin $\sqrt{\%}$ before statistical analysis. The analysis of variance and other standard statistical procedures were carried out as described by Snedecor and Cochran (1971).

IV. RESULTS

The last couple of decades have witnessed a spate of research in processing technology of semen and the use of frozen semen for genetic improvement of dairy animals. However, comparatively poor head way has been made in AI programmes involving buffaloes. The research leading to progress in deep freezing of buffalo bull semen has been engaging the attention of many workers in India and abroad, but the results lack consensus. During the present research project, certain important aspects on deep freezing of buffalo semen were elaborated, and the results achieved were as follows.

A. SEMINAL ATTRIBUTES OF BUFFALO AND COW BULL SEMEN:

Using standard procedures, 50 ejaculates from 20 Surti buffalo bulls and 25 ejaculates from 3 H.F. bulls were examined for normal seminal attributes. The perusal of Table-1 shows that for the buffalo, the volume of semen averaged 3.08 ± 0.19 ml. The mass activity and individual motility averaged 2.11 ± 0.17 and 77.4 ± 1.63% respectively. The average sperm concentration was 1022.34 ± 22.22 million/ml and the per cent live and morphologically abnormal spermatozoa averaged 85.36 ± 0.46 and 9.72 ± 0.35 respectively.

For cow bull semen the average semen volume was 5.65 ± 0.095 ml. The mass activity and individual motility

averaged 3.44 ± 0.078 and 79.8 ± 0.68% respectively. The sperm concentration averaged 1514.8 ± 2.50 million/ml. The averages for per cent live and morphologically abnormal spermatozoa were 87.08 ± 0.49 and 9.2 ± 0.42, respectively.

The colour and consistency of semen for all the ejaculates varied from milky white to thick creamy depending upon the concentration of spermatozoa in particular ejaculate.

B. FREEZABILITY OF SPERMATOZOA:

Two semen ejaculates from each of 23 bulls - 20 Surti and 3 H.F. were subjected to freezing at ultra low temperature (-196°C). The ejaculates were studied for the freezability of spermatozoa on the basis of post-thaw motility. The difference amongst the bulls for freezability of spermatozoa was significant (Table-3, Fig. 1). Out of 20 buffalo bulls, seven provided semen having very good freezability (≥ 50% post-thaw spermatozoal motility). [illegible] semen ejaculates from other nine bulls were mode[illegible] [illegible]uitable for freezing (post-thaw spermatozoal motility > 30% but < 50%) and the remaining four bulls showed poor freezability. (< [illegible] [illegible]t-thaw spermatozoal motility). Three H.F. bu[illegible] provided the semen ejaculates with very good freezability (Table-2).

From the analysis of variance, it was revealed that, the post-thaw motility at 0 hr varied significantly between the bulls, but the difference in freezability between the

ejaculates of the same bull was found to be non-significant (Table-3). After 24 hrs storage of semen in liquid nitrogen, it was observed that the post-thaw motility differed significantly between bulls as well as between the ejaculates.

It was observed that, the correlation between mass activity and freezability of spermatozoa was non-significant ($r = 0.23 \pm 0.15$).

C. EFFICACY OF DILUTORS:

Five ejaculates from each of the five Surti buffalo bulls and three H.F. bulls were utilized to study the efficacy of different dilutors. It was observed that the individual motility of spermatozoa in buffalo semen averaged 76.20% and 73.40% for semen diluted in TFYG and LFYG diluents before freezing; it declined to 57.80% and 54.2 [illegible] [illegible]ediately after freezing and 55.20% and 49.40% afte[illegible] [illegible] storage in liquid nitrogen, respectively (Table-4, Fig. 2).

The post-thaw motility immediately after freezing and after 48 hrs storage in liquid nitrogen did not differ significantly between the buffalo bulls, while the difference in post-thaw motility of spermatozoa was significant between the dilutors at 0 hr and 48 hrs post-freezing (Table-5). The difference due to dilutors on the spermatozoal motility before and after freezing was found to be non-significant. However, the difference in

spermatozoal motility between temperatures and the interaction between dilutors and temperature was highly significant (Table-6).

The comparison of progressive motile percentage of spermatozoa in freeze-thawed buffalo semen in different dilutors was done by critical difference test. It was observed that TFYG dilutor was having significantly ($P < 0.01$) higher percentage of progressive motile spermatozoa compared to LFYG dilutor at 0 hr, as well as 48 hrs post freezing (Table-17).

For H.F. bull semen, the individual progressive motility of spermatozoa before freezing in ASG; L-478, WMG, TFYG and EYCG diluents averaged 76.33%, 75.00%, 75.33%, 82.00% and 79.66%, which reduced to 34.33%, 35.66%, 38.00%, 61.66% and 56.66% immediately after freezing and further declined to 29.33%, 33.00%, 33.00%, 56.66% and 54.66% after 48 hrs storage in liquid nitrogen; respectively (Table-7, Fig. 3).

As shown in Table 8, when the post-thaw motility of bull spermatozoa was observed at 0 hr, the difference between the bulls was found to be non-significant, but the same varied significantly on the basis of post-thaw motility itself, when examined after 48 hrs of storage in liquid nitrogen. The five dilutors used for freezing the bull semen were found to be differing significantly from

each other on the basis of post-thaw motility of spermatozoa at 0 hr and after 48 hrs post-freezing.

Critical difference test was employed to compare the progressive motile spermatozoa of bull semen in various dilutors (Table-16). It was observed that, TFYG diluted semen showed significantly ($P < 0.01$) higher percentage of spermatozoa as compared to EYCG, WMG, L-478 and ASG diluted semen at 0 hr as well as 48 hrs post-freezing. There was no significant difference between the dilutors WMG and L-478. At 0 hr and 48 hrs post-freezing, WMG and EYCG dilutors differed significantly from ASG dilutor, while the differences between L-478 and WMG were non-significant. ASG dilutor differed significantly ($P < 0.01$) from WMG and L-478, 48 hrs post-freezing (Table-16).

The comparison between bulls showed the significant differences in post-thaw motility, ($P < 0.01$). MF-316 gave a significantly higher post-thaw spermatozoal motility followed by MF-317 and MF-307 (Fig. 3).

The analysis of variance for spermatozoal motility of cow bull semen revealed that the differences due to temperature and interaction (dilutor x temperature) were significant ($P < 0.01$) (Table-9).

D. PER CENT LIVE SPERMATOZOA:

The per cent live spermatozoa was calculated in neat (pre-freeze) and freeze-thawed semen samples diluted in

various dilutors for bull and buffalo semen. The live percentage of the spermatozoa in neat buffalo semen averaged 86.24%, which declined to 65.32% and 65.24% in freeze-thawed samples diluted in TFYG and LFYG diluents, respectively (Table-10).

The statistical analysis revealed that the per cent live spermatozoa did not show significant difference between buffalo bulls as well as between dilutors (Table-11). However, the difference in per cent live spermatozoa between temperatures was highly significant (Table-12).

The average live percentage of spermatozoa in HF bulls' neat semen was 88.26%, which in fre[illegible] samples diluted in ASG, L-478, WMG, TFYG and EYCG, diluents declined to 6[illegible], [illegible], [illegible], 65.43% and 62.93% respectively (Table-13).

The comparison of per cent live spermatozoa in bull semen diluted in various dilutors was done by critical difference test. It was observed that [illegible]YG diluted semen had significantly ($P < 0.01$) higher percentage of live spermatozoa as compared to EYCG, L-478, WMG and ASG diluted semen. The differences between ASG, L-478 and [illegible] were non-significant. EYCG and L-478 dilutors did not differ significantly for per cent live spermatozoal count (Table-16).

The analysis of variance for per cent live spermatozoa of bull semen revealed that the differences between various bulls were non-significant whereas they were found to be significant between dilutors as well as between temperatures (Table-14, 15).

E. MORPHOLOGICALLY ABNORMAL SPERMATOZOA:

The percentage of morphologically abnormal spermatozoa was estimated in neat and freeze-thawed semen samples of cow and buffalo bulls diluted in various diluents.

In buffalo neat semen, the morphologically abnormal spermatozoa averaged to 7.96% which increased to 15.52% and 15.68% in freeze-thawed samples diluted in TFYG and LFYG diluents, respectively (Table-10).

Analysis of variance for percentage of morphologically abnormal spermatozoa in buffalo semen showed that the difference between temperature was highly significant (Table-12); whereas it was non-significant between bulls and between dilutors (Table-11, 17).

The percentage of morphologically abnormal spermatozoa in H.F. bulls' neat semen aver[illegible] [illegible].93%, which increased to 15.46%, 16.00%, 15.26%, 16.66% and 16.06% in freeze thawed semen samples, diluted in ABG, L-47[illegible], [illegible]G, TFYG and EYCG diluents, respectively (Table-13).

On statistical analysis, it was revealed that the difference in percentage of morphologically abnormal

spermatozoa was significant between dilutors as well as between temperatures, but it was found to be non-significant between the bulls (Table-14, 15).

For post-thaw comparison of per cent morphologically abnormal spermatozoa in various dilutors, critical difference test was done. The observations revealed that, dilutors TFYG and EYCG did not differ significantly for this character. Dilutors ASG, L-478, and WMG also gave non-significant differences in per cent abnormal spermatozoal count. Dilutor WMG was significantly different from TFYG and EYCG dilutors (Table-16).

F. ENZYME LEAKAGE:

(a) Transaminases:

The seminal plasma levels of Glutamic oxaloacetic transaminase (GOT) and Glutamic pyruvic transaminase (GPT) were estimated pre and post freeze. The levels of GOT in seminal plasma before freezing averaged 8.02 and 9.42 i.u. for TFYG and LFYG diluted semen samples, which increased to 12.82 and 16.23 i.u. respectively in freeze-thawed samples (Table-18, Fig. 4).

The levels of GPT in seminal plasma averaged 2.67 i.u. and 3.05 i.u. before freezing, which increased to 3.65 and 4.03 i.u. respectively after freezing in TFYG and LFYG diluents (Table-18, Fig. 5).

Analysis of variance revealed that the difference in GOT levels was found to be significant between temperatures as well as between dilutors, whereas the difference in levels of GPT was found to be significant between temperatures, but it was non-significant between the dilutors (Table-19).

(b) Hyaluronidase activity:

The activity of hyaluronidase enzyme in spermatozoa pre and post-freezing was estimated for 17 semen ejaculates from five Surti buffalo bulls. The hyaluronidase activity in spermatozoa before freezing averaged 0.064 and 0.065 (O.D.) for TFYG and LEYG diluted semen samples; which decreased to 0.039 and 0.030 (O.D.) for the two dilutors respectively in freeze thawed samples (Table-20, Fig. 7).

Analysis of variance for hyaluronidase activity in spermatozoa was carried out. The difference in the enzyme activity between dilutors was found to be non significant. The paired 't' test revealed that the difference in hyaluronidase activity was significant ($P < 0.01$) between the temperatures (pre and post-freeze).

6. MENSURATION CHARACTERS OF SPERMATOZOA:

The mensuration characters were studied for cow bulls' and buffalo bulls' spermatozoa before and after freezing.

The head breadth and the head length of the buffalo spermatozoa averaged 4.6175 and 7.8282 microns in neat

semen, which decreased to 4.4384 and 7.7113 microns respectively, in freeze-thawed samples (Table-21).

The average head breadth and head length of the H.F. bull spermatozoa in neat semen were 5.2273 and 9.4052 microns which decreased to 4.8585 and 9.1665 microns respectively, after freezing (Table-22).

The total length of the spermatozoa averaged 58.7890 and 55.620 microns in neat semen and 59.0440 and 55.7187 microns after freezing for buffalo bull and cow bull semen, respectively (Table-21, 22).

The paired 't' test was carried out to study the differences in mensuration characters before and after freezing. It was observed that the difference in head breadth were significant for both cow bull and buffalo bull spermatozoa between temperatures (pre and post freeze), whereas the head length measurements did not show significant difference for both the species before and after freezing.

H. FERTILITY TRIALS:

(a) In buffaloes and heifers:

During the present study totally 426 inseminations were performed in 342 Surti buffaloes, using Tris diluted frozen semen from five Surti buffalo bulls.

The overall fertility percentage achieved was 57.24%. On an average 2.69 inseminations were required per conception.

The fertility amongst the buffalo bulls under study varied. It was 62.02%, 52.17%, 61.43%, 53.33% and 50.00% respectively for five buffalo bulls viz., SB_{AS}, SB_{AJ}, SB_{RM}, SB_{PR} and SB_{AT} (Table-23, Fig. 8).

Number of inseminations required per conception were 2.57, 2.83, 2.27, 3.37 and 3.20 for buffalo bulls SB_{AS}, SB_{AJ}, SB_{RM}, SB_{PR} and SB_{AT}, respectively (Table-24, Fig. 8).

(b) In cows and heifers:

In all 285 inseminations were performed in 251 Kankrej cows and heifers, using Tris diluted frozen semen from three H.F. bulls. The overall fertility achieved was 46.21%. On an average 2.46 inseminations were required per conception.

For the three bulls, H.F. 316, H.F. 317 and H.F. 307, the fertility percentages were 52.87, 48.75 and 36.90 respectively (Table-25, Fig. 8).

The number of inseminations required per conception were 2.06, 2.93 and 2.50 for the three bulls, H.F. 316, H.F. 317 and H.F. 307 respectively (Table-26, Fig. 8).

There was difference in fertility results of these bulls semen at various AI centres. The centre-wise fertility results are presented in Table-28.

(c) Use of frozen semen in repeating Kankrej cows and heifers:

Total 98 inseminations were carried out using frozen semen from H.F. 316 and H.F. 317 bulls in 37 repeat breeding Kankrej cows and heifers, which had repeated 7 to 18 times. It was possible to settle 28 repeat breeders using frozen semen. The semen of H.F. 316 bull required 3.35 inseminations per conception and H.F. 317 bull required 3.87 inseminations per conception. On an average 3.5 inseminations were required per conception in these repeating animals (Table-27).

V. DISCUSSION

To achieve rapid and sure success in dairy cattle and buffalo breeding programmes, the use of frozen semen technology has no substitute so far. The preservation and usage of semen has become one of the important branches of research in the field of Animal Reproduction. The superior germ-plasm has a great role to play in dairy cattle and buffalo breeding programmes. The present research project involves studies on freezability, post-thaw revivability, enzyme leakage (pre and post freezing) and fertility of bull and buffalo semen. The results obtained have been discussed.

A. SEMINAL ATTRIBUTES:

The important seminal attributes observed in the present study as volume, colour, consistency, individual motility, sperm concentration, per cent live spermatozoa and morphologically abnormal spermatozoal percentage were within the optimal range for both the species - buffalo bull and cow bull, in accordance with their breed characteristics (Table-1). The mass activity of bull semen was in the optimal range, with the average 3.44 ± 0.07. But the average mass activity of buffalo semen, in present study was 2.11 ± 0.17, which was lower than the normal; due to the fact that, some of the bulls used in this experiment sometimes provided the semen ejaculates without the initial motility. Such static samples gained motility

on dilution and could be frozen with good post-thaw spermatozoal recovery. These findings are comparable with the observations of Chinnaiya (1982), who removed the inhibitory effect of seminal plasma from visible immotile semen samples, washed and treated the spermatozoa with three semen extenders to regain the motility in 30% of the samples, which could be frozen, and the post-thaw motility was observed to be above 40%. Abhi *et al*. (1968) in their study on initially non-motile sperms in buffalo bulls, observed the motility in varying degrees, following dilution in more than 85% 'flat' semen ejaculates. These findings also support to the observations made in the present experiment relating to initially non-motile semen samples.

B. FREEZING OF SEMEN:

(a) Extension Rate:

As, many sperms are killed ever under the best freezing conditions, the total sperm concentration per dose of frozen semen is kept always higher than that in chilled semen. It varies from 12 to 60 million per dose as suggested by Sulivan (1970).

The concentration of spermatozoa preferred in the present study was approximately 12 to 15 million per frozen semen dose, which was higher than recommended by Branton *et al*. (1954) who found 10 million sperms per insemination as optimum and Foote (1970a) who could observe even 4 million spermatozoa per insemination dose, without

affecting the fertility. However, the dilution effected in present study is well in agreement with the levels suggested by Eapen (1961[a]), Willett (1950), Willett and Larson (1952) who preferred minimum 12 million of sperms needed for insemination without loss of fertility.

Pavithran et al. (1972) reported that the rate of dilution of the buffalo semen had a profound effect on the post-freezing spermatozoal survival rates. He suggested to keep at least 100 million spermatozoa per ml before freezing. At the same time, it should not be forgotten that the decreased seminal dilution rates result in decreased dilution of microorganisms present in semen and so provide a greater challenge to the action of antibiotics in the diluted semen (Pickett et al., 1976). Mann (1964) also mentioned that a good concentration of motile spermatozoa in a semen sample is occasionally misleading. These findings support the dilution rate adopted in the present study.

(b) Packaging of Semen:

Packaging of bovine semen in plastic straws has been advocated by a growing segment of the A.I. industry. Automated systems for packing bovine semen in plastic straws, reports of higher conception rates; greater spermatozoal utilization and greater storage efficiency have all made processing of semen in straws increasingly popular.

The French medium straws of 0.5 ml capacity were used for packaging of semen in the present study. The increasing experimental evidences suggest that, the reduced volume of dose delivery in AI is better tolerated by the uterus. This indicates the use of 0.25 ml straws rather than 0.5 ml. However, Zarazua et al. (1977) observed significantly higher post-thaw motility, when spermatozoa were frozen in 0.5 ml French medium straw than in 0.25 ml straws. This observation supports the use of 0.5 ml straws, which was employed in the present study.

(c) Glycerol levels and Glycerolyzation:

Glycerol is being used universally as a primary cryoprotective agent for freezing of semen. The amounts of glycerol and methods of glycerolyzation vary depending upon the extenders, freezing method, species and the recipes developed by individual laboratory.

In the present study, 5% and 6% glycerol was used, respectively, in LFYG and TFYG dilutors. In ASG, L-478 and MMG dilutors 7% glycerol was preferred.

The amount of glycerol used (5%) with LFYG dilutor, was in accordance with the level suggested by Ahmad and Chaudhry (1980) and Crabo et al. (1980). However, it was lower as compared to 7%, a optimal level in this dilutor, suggested by Heuer (1982), recently. The necessity of such higher level of glycerol in presence of two sugars in a

dilutor is questionable according to the findings of Krishnamurthy (1981), who compensated the glycerol reduction from 6 to 3% by using in an extender, the combination of fructose and lactose, with good post-thaw spermatozoal recovery. He supported the proposition that certain beneficial effects could be derived by reducing the level of glycerol in frozen semen extender. Stoyanov and Kostadnov (1978) agreed with these findings, who could successfully freeze the bull semen using raffinose yolk diluent, without glycerol. Though glycerol is considered to be the best cryoprotectant in frozen semen extenders, its incorporation is increasingly being doubted. The extent to which the use of combination of sugars to replace or reduce the glycerol levels in frozen semen extender, yet remains to be determined.

The satisfactory results in terms of post-thaw spermatozoal motility and fertility achieved by incorporating 6% glycerol in TFYG diluent in the present study agree with the results obtained by Bhandari and Chauhan (1980) who used 6.4 [illegible] [illegible]cerol in Tris extender with satisfactory post- [illegible] [illegible]rmatozoal recovery. The present findings can, also, be compared with those of Shetti *et al.* (1981) and Patil *et al.* (1981) who used 6% glycerol in Triladyl and Tris extenders for successful freez[illegible] [illegible] buffalo semen. Dasand Jainudeen (1980) observed 5% glycerol to be beneficial in Tris diluent for freezing buffalo semen, which was lower than that used in present study.

However, Chinnaiya and Ganguli (1980) could not find any significant difference in post-thaw recovery of buffalo spermatozoa when they used three levels of glycerol 6, 7 and 8% each in three dilutors.

In the present study for EYCG diluent, 7% glycerol was used, which can be compared with the study of Miller and Van Demark (1953) who reported 6 to 8% glycerol in EYCG to be beneficial. The observations made by Kapen (1961b), Roussel et al. (1964) and Rodriguez et al. (1975) also support the choice of 7% glycerol levels in present study. However, Robbins (1976) recommended 8.5% glycerol level, to be better, which was higher, than what was used in present experiment.

In milk based diluents, Almquist and Wickersham (1962) observed 5% glycerol level to be better, but as it is a general practice to use 10% glycerol in milk based diluents, 7% glycerol was preferred for present study in ASG, L-478 and WMG dilutors. The post-thaw recovery of spermatozoa was not as good as reported by other workers.

(d) Equilibration:

There exists a difference in opinion regarding the time during which semen should be equilibrated with glycerol before freezing.

In the present study, 5 hrs equilibration time was used with all the dilutors, which is in agreement with

the study of Bhandari and Chauhan (1980). The 7 hrs equilibration period suggested by Crabo et al. (1980) was longer than the one used in the present study. However, Ennen et al. (1976) recommended 4 to 10 hrs equilibration period to be optimum which supports the present study. The glycerol equilibration time used in present study was quite longer as compared to the findings of Jondet (1972) and Wiggin and Almquist (1975) who suggested 1 mt and 30 mts equilibration time, respectively without any adverse effect on post-thaw spermatozoal motility and intact acrosomes. However, the period of equilibration preferred in present study falls in between 3 hrs and 9 hrs, the two periods suggested to be optimum by Gilbert and Almquist (1978).

(e) Thawing:

Although much work has been reported on the relative merits of different thawing methods, there is no general agreement on whether any particular technique offers significantly better results. Rate of thawing is one of the most important factors affecting post-thaw spermatozoal viability (Robbins et al., 1972; 1976; Rodriguez, et al., 1975).

In the present study, the thawing rate used was 102 - 104°F for 15 - 20 seconds, with satisfactory results. This can be compared with the results of Robbins et al. (1972), Kim and Kim (1978), Chander et al. (1979),

Almquist et al. (1979), Hube and Uribe (1980) and Pace et al. (1981) who all recommended the use of thawing rates for straw frozen semen ranging between 35° to 40°C for 15 - 30 seconds. However, Robbins et al. (1976) advocated the rapid thawing rates (50°C/15 sec or 65°C/7.5 sec.) which were higher than the one preferred in the present study.

C. FREEZABILITY OF SPERMATOZOA:

In the present experiment, it was observed that, there were significant differences amongst the bulls in respect of the ability of their ejaculates to withstand the ultra low temperature during freezing (Table-3). The results of this experiment revealed that out of 23 cow bulls and buffalo bulls, only 10 (3 bulls + 7 buffalo bulls - 43.5%) bulls provided the ejaculates with very good suitability for freezing. In the other nine buffalo bulls (39.2%) ejaculates were moderately suitable for freezing, while the semen of remaining four buffalo bulls (17.3%) was totally unsuitable for freezing. These findings are in accordance with the observations of Sahni and Roy (1972) and Pavithran et al. (1972) who observed significant differences between the freezability of semen from different buffalo bulls. The overall observations of the present experiment are also supported by the findings of Roberts (1971), Vlachos et al. (1965), Molinari and Valpreda (1968), Biborski et al. (1971), Haave (1972), Szilagyi and Muller (1972), Otel et al. (1972) and Verses et al. (1972), who

observed the significant differences between the bulls in respect of freezability.

The lower freezability of some of the buffalo bulls' semen in the present experiment, might be explained in relation to the findings of Sengupta (1981), who stated that, regardless of the dilutor or the method of freezing used, post-thaw motility in general was poorer in buffalo semen than bull semen. He concluded that the differences in the biochemical profile of semen of the two species were mainly responsible for the differences in their ability to maintain the viability of spermatozoa during in-vitro preservation.

The buffalo semen exhibits much seasonal variation in the quality of semen (Kodagali et al., 1973[b]; Raja, 1982). Hence, variation in freezability of bull semen as reported by Boehnke et al. (1976) may be true for buffalo semen too.

The freezability of sperms seems to be related with their morphology. The fresh bovine semen ejaculates which were not freezable, when viewed under electron microscope, exhibited large number of swollen, detached or ruptured acrosomes, as reported by Verses et al. (1972), who could also observe the damage to the sperm membrane in ejaculates with good motility, which did not stand to freezing. The age factor also is responsible for the freezability of semen, as observed by Molinari and

Valpreda (1968), who reported that the semen ejaculates from younger bulls showed lower freezability as compared to those from adult bulls.

The probable reasons for no freezability or lower freezability of the ejaculates from some of the bulls in the present experiment, may be due to some alterations in acrosomal morphology or break in sperm membrane integrity, which could not have been identified under simple microscope. Individual variations in some biochemical profiles of the semen or inherent inability of spermatozoa to withstand the ultra low temperature as probable reasons also cannot be rejected. The factors responsible for low freezability such as age and season, can easily be ruled out, as the bulls studied in the present experiment were in same age group and the ejaculates were processed for freezing during the most favourable period of the year (September to February). This is also supported by the observations of Singh (1982), who found the percentage of unsuitable buffalo ejaculates lowest during January to March and October to December. Haranath *et al.* (1982) observed in buffalo bulls, the average post-thaw motility to be better in winter season, however he could not find the differences between ages of bulls on freezability of spermatozoa.

D. EFFICACY OF DILUTORS:

By using proper dilutor and correct procedure, the fertilizing ability of semen can be maintained for years, by deep freezing. It is a common practice to select a dilutor on the basis of its ability to maintain the motility of spermatozoa after thawing. Eventhough it is well known fact that, motility and fertility do not reside in the same parts of the sperm cell and may suffer different degree of damage from an identical treatment. Using non-motile sperm, successful AI has been reported (Tyler, 1973), but the finding that, non motile sperm is not able to facilitate it's passage through cervical mucus (Hoyes et al., 1958) is suggestive of the importance of progressively motile spermatozoa in a semen dose for AI.

Number of dilutors have been tried for freezing of bull semen by using various buffering media and varying levels of cryoprotective agents as glycerol, sugars or egg yolk etc. The introduction of Zwitter ion buffers for buffering egg yolk as extenders for bull semen has led to the application of Tris (hydroxymethyle amino-methane) as a common buffering agent.

Buffalo semen:

Two widely used buffalo semen extenders, TFYG and LFYG were tried to test their efficacy. On the basis of post-thaw motility of spermatozoa, TFYG was found to be the better extender for buffalo semen as compared to LFYG.

In the present experiment, the post-thaw motility of the spermatozoa diluted in TFYG dilutor, averaged 57.80% and 55.20% respectively at 0 hr and after 48 hrs post-freezing (Table-4). The present findings are in accordance with the results of Crabo et al. (1980) who observed the progressively motile spermatozoal percentage to be 56% in freeze-thawed buffalo semen diluted in Tris extender. The post-thaw spermatozoal motility observed in TFYG diluted buffalo semen in present study was higher compared to the findings of Chinnaiya (1979) as 31.10%, Vasanth (1979) as 45%, Shetti et al. (1981) as 35.36%, Patil et al. (1981) as 50.59%, Meharsing et al. (1980) as 54% and Bhandari et al. (1982) as 38.60%. However, the value of post-thaw revivability of spermatozoa obtained in present study was lower as compared to Gunzel et al. (1979) who reported the post-thaw spermatozoal motility to be 62% in Tris diluted buffalo semen.

The post-thaw motility obtained in the present study in TFYG diluted semen was comparatively higher (Table-17). This may be due to the inclusion of fructose in the diluent. Hafez (1962) observed the beneficial effects on post-thaw spermatozoal motility following inclusion of fructose in the diluent. The similar observations of Marczewski (1967), supported the findings of present experiment. Though the level of fructose suggested by him was not in accordance with the level used in the present study. The presence of fructose in the diluent said to be providing the source

of metabolisable energy. But, as it is known that the metabolism of spermatozoa in vitrified state is extremely low, the role of fructose solely as a metabolisable source of energy is doubtful. Eventhough it is well established that spermatozoa preferentially utilize glucose or mannose rather than fructose (Mann, 1964), the inclusion of fructose in frozen semen diluents is more beneficial than other monosaccharides.

With LFYG dilutor, the post-thaw motility of buffalo semen averaged 54.20 and 49.40% respectively at 0 hr and 48 hrs post-freezing (Table-4), which was higher as compared to Ahmad and Chaudhry (1980) who reported it to be 40.71%. In this relation Stoyanov and Kostadnov (1978) observed lower spermatozoal motility as 32.7% post-freezing, in bovine semen extended with lactose based diluent. The present findings are in well accordance with the observations of Bonia *et al.* (1980) who found spermatozoal motility to be 53.20% in freeze thawed bovine semen diluted in lactose based diluent.

It was expected to observe better post-thaw spermatozoal recovery in the semen diluted with LFYG dilutor. Though it could not exceed the recovery rates obtained in TFYG dilutor, the revival rate was quite satisfactory.

Nagase (1964) and Krishnamurthy (1981) studied in detail the cryoprotective action provided by sugars. Martin and Emmens (1961) suggested that addition of fructose to lactose in an extender brought about an increase in overall survival rate of spermatozoa. Ahmad and Chaudhry (1980) agreed with these findings, who justified the inclusion of two sugars in the diluent stating that after the depletion of fructose present in seminal plasma, fructose present in the diluent comes into play. By the time this source is exhausted, breakdown products of lactose in the form of glucose and galactose start serving as the energy inputs. Also, these sugars in diluent allowed the least variation in osmotic equilibration surrounding the cell.

Bull semen:

In the split diluted H.F. bull semen the post-thaw spermatozoal motility averaged 61.66%, 56.66%, 38.00%, 35.66% and 34.00% in TFYG, EYCG, WMG, L-478 and ASG diluents, respectively (Table-7). On perusal; these observations showed that on the basis of post-thaw spermatozoal motility, TFYG and EYCG diluents ranked high for freezing cow bull semen, as compared to WMG, L-478 or ASG dilutors (Table-16).

The results achieved in terms of post-thaw spermatozoal recovery in the present experiment with TFYG dilutor

are in accordance with the findings of Davis et al. (1963) and Simmet (1975). The percentage of progressively motile spermatozoa was higher in present experiment, compared to the results obtained by Beatty et al. (1970) and Bonia et al. (1980) as 32% and 42.50% respectively.

By using EYCG dilutor, the post-thaw spermatozoal recovery obtained in present experiment is well in agreement with the observations of Saroof and Mixner (1955), and Beatty et al. (1970), who found satisfactory recovery of freeze-thawed spermatozoa extended and frozen in EYCG diluent.

In the present experiment, it was observed that the post-thaw spermatozoal recovery of bull spermatozoa was relatively lower when L-478, ASG or WMG dilutors were used. These results are not in accordance with the findings of Ala-ud-Din et al. (1979) who did not find the significant differences in post-thaw motility of spermatozoa in bovine semen diluted in Laiciphos, WMG, lactose yolk glycerol or glucose yolk citrate glycerol. Amann and Almquist (1957) observed 42% post-thaw motility in heated fresh milk diluent, which was higher than what was achieved in the present study.

The results obtained in present study using milk based diluents did not agree with the findings of Bruce (1956) and O'Dell and Almquist (1957) who reported that the spermatozoa frozen in milk based diluents maintained higher post-thaw motility than those frozen in EYCG extender.

The observations made by Muller (1972) suggested that, the post-thaw spermatozoal recovery did not differ significantly in three dilutors viz., Tris yolk lactose, EYCG and Laiciphos. These findings are not in agreement with the observations of the present experiment, in which Laiciphos dilutor was ranked third on basis of post-thaw spermatozoal motility.

The poor recovery of spermatozoa in these dilutors may be due to the fact that, the relationships between glycerol level and freezing rate (Rodriguez et al., 1975) and, also, between glycerol level and thawing rate (Robbins et al., 1976) were not delt in the present study. This might have contributed, at least in part, to the poor recovery rates in milk based diluents in the present study. Moreover, the probable reason for not getting better post-thaw spermatozoal recovery in ASG diluent, may be attributed to reduction of equilibration time, as ElAlamy (1979) with "infant milk formula" (SMAG-I), diluent had recommended 15 hrs of equilibration time to be optimum.

E. LIVE SPERM COUNT:

Though the concentration of live spermatozoa may be predicted by observing the initial motility of semen (Lasley, 1951), yet the actual evaluation of the live percentage is desirable. Stone et al. (1950) and Lasley (1951) stated that semen having more than 30%

initial dead spermatozoa may not be good for preservation. Tomar (1970) cited Lasley and Bogart (1943) stating that the fertility is questionable from semen samples with less than 50% live spermatozoa. As 50% destruction of spermatozoa is a common finding for frozen semen (Pavithran et al., 1972); this itself explains the probable reason for the lower fertility obtained by using frozen semen in earlier fertility trials.

In the present study, the post-thaw live percentages of buffalo spermatozoa averaged 65.32% and 65.24% in TFYG and LFYG dilutors respectively (Table-10). The difference in the live sperm percentage between dilutors was non-significant, whereas the decrease in live percentage at temperature 37°C to -196°C was significant ($P < 0.01$). These observations suggest the higher values of post-thaw live sperm percentage as compared to those reported by Shetti et al. (1981) as 38.22% and Patil et al. (1981) as 52.74%.

As observed in the present study, the average live percentage of spermatozoa in H.F. fresh semen was 88.28%, which in freeze-thawed samples diluted in ASG, L-478, MMG, [illegible] and EYCG declined to 60.46%, 61.66%, [illegible], [illegible] and 62.93% respectively (Table-13). These findings are well in accordance with the observation of Jondet and Rabadeux (1976) who reported 68.34% live spermatozoa in

freeze-thawed bovine semen. Similar observation was, also, made, by Balasov (1969) who found 69% live sperm count in freeze-thawed samples of bull semen.

In the present study, the per cent live count of spermatozoa was expected to show variations in accordance with per cent post-thaw motility, but it was not observed so. It needs to be pointed out here that the probable interference due to presence of glycerol in frozen semen diluents, which did not allow the preparation of semen smears to be uniform and ideal owing to the greesyness of the medium. Moreover, the egg yolk present in diluents is, also, reported to be interfering in live-dead staining of the spermatozoa (Chaube and Sengupta, 1972). These factors at least, in part, might have affected in arriving at the actual live spermatozoal count in present study. It has been found necessary to seek for a new or modified staining procedure for live-dead count in freeze-thawed semen sample.

F. ABNORMAL SPERM PERCENTAGE:

All specimens of semen contain a proportion of abnormal cells (Laing, 1979). Many workers regard the extent to which abnormal forms of spermatozoa are present coupled with types of abnormality as the best indicator of fertility. Conventionally, semen with more than 20% total abnormal cells is discarded (Laing, 1979).

The percentage of morphologically abnormal spermatozoa observed in the present study on buffalo semen average 7.96% in neat semen, which increased to 15.52% and 15.68% in freeze-thawed samples diluted in TFYG and LFYG diluents, respectively (Table-10). The values in neat semen found, were in agreement with the same reported by Kodagali *et al.* (1973b) as 9.79%. Shetti *et al.* (1981) reported 9.10% abnormal spermatozoa in freeze-thawed buffalo semen samples, which was lower than the values observed in present experiment. However, morphologically abnormal spermatozoal percentage observed by Patil *et al.* (1981) as 14.08%, was comparable with the present findings.

As revealed from the results of the present experiment, the per cent morphologically abnormal spermatozoa in H.F. neat semen averaged 8.93%, which increased to 15.46%, 16.00%, 15.26%, 16.66% and 16.06% in freeze-thawed semen samples diluted in ASG, L-478, WMG, TFYG and LYCG, respectively (Table-13), were quite low as compared to the findings of Kumaran (1965), who reported 23.7 to 32.0% abnormal spermatozoa in frozen bovine semen. However, the present findings are well in accordance with the study of Rob and Jiranek (1971) who found post-thaw abnormal spermatozoal percentage to be 16.9% in bull semen.

G. ENZYME LEAKAGE:

Evaluation of the quality of frozen semen has remained a subject of research in many of the frozen semen

laboratories during the recent years. For successful fertilization to occur many of the properties of spermatozoa are responsible and most evaluation techniques, evaluate only one or two of them. Though fertility of animals inseminated alone can reflect exactly the quality of semen, fertility trials are often time consuming and may involve the risks by inadvertent usage of semen having low fertility. It would certainly be easier if laboratory techniques could substitute for fertility trials. The motility estimation under microscope is the most widely used seminal character for assessing the quality of semen, may be because it is quicker method. Pickett et al. (1961) observed that the correlation of motility estimates with the fertility varies from 0.21 to 0.79.

It has been observed with the use of frozen semen that the spermatozoal recovery rates, though present after thawing, have resulted in very low fertility, in some cases. This may be due to damage of spermatozoa during the process of freezing. There is no satisfactory method, which can tell about the extent of damage that occurred to spermatozoa during the process of freezing. One can depend upon the biochemical tests by estimating the presence of enzymes, which are limited only to sperm cells, in seminal plasma after freezing and thawing. Although most of the enzymes found in seminal plasma have their origin in accessory glands, at least some might be the result of

leakage from spermatozoa (Anand, 1981). In this context, acrosome should be given a special attention, as its apical ridge in the bull spermatozoa deteriorates with aging or injury to the cell and the important acrosomal enzymes may be released out (Saacke, 1972).

(a) Transaminases:

Graham et al. (1974) have stressed the importance of the use of Glutamic oxaloacetic transaminase (GOT) enzyme level in the semen as an indicator of quality of frozen semen. GOT enzyme is reported to be purely cellular (Pace and Graham, 1976). However, Weitze (1976) found much of it to be derived from disintegrated protoplasmic droplets and only small part from spermatozoa. If these findings are believed to be of value, the levels of GOT in seminal plasma as an indicator of sperm cell damage occurring during freezing, needs re-evaluation. Anand (1981) considered the epididymis as one of the sources of GOT and GPT (Glutamic Pyruvic Transaminase) in bull seminal plasma, but he did not reject the possible leakage of these enzymes from the damaged spermatozoa.

The results of the present experiment revealed significantly more values of GOT as well as GPT enzymes in freeze-thawed samples as compared to pre-freeze levels (Fig. 4 & 5). These findings can be compared with the observations of Roychoudhury et al. (1974), who found the significant release o[illegible] [illegible]d GPT from bull spermatozoa

diluted with sodium citrate buffer on cold shock. The significantly higher GOT concentration extracellularly in freeze-thawed semen samples was observed by Coulter and Foote (1973), Breeuwsma (1972) and Chinnaiya et al. (1979), which is well in accordance with the results of the present experiment.

The loss of GOT enzyme activity from spermatozoa and the release of the same extracellularly in seminal plasma is suggestive of sperm membrane injury, as reported by Crabo et al. (1971). These findings explain the negative correlationship between spermatozoal and seminal plasma concentration of transaminases. So the decrease in sperm level of GOT concentration after freezing as observed by Buruiana et al. (1975) and Jain (1979) and also the decreased levels of GPT in spermatozoa post-freezing, as observed by Zahariev et al. (1974) are also very well comparable with the results of the present experiment. However, the decreased seminal plasma levels of GPT in freeze-thawed samples from bull (from 13.0 units before freezing to 11.60 u after freezing) as observed by Zahariev et al. (1974) did not agree with the findings of the present experiment.

The seminal plasma levels of GOT and GPT in fresh buffalo semen determined by Varshney et al. (1978) as 83.5 ± 3.0 units/ml and 20.4 ± 0.47 units/ml respectively, were not in accordance with the levels found in the

present experiment. This might be due to the positive correlationship between spermatozoal concentration and GOT levels in semen as observed by Singhal *et al.* (1976); Varshney *et al.* (1978) and Pace and Graham (1980). It may be recalled here that the enzyme values obtained in the present experiment were in diluted semen containing about 50 million spermatozoa per ml.

The levels of GOT reported in bull seminal plasma by various workers viz., Flipse (1960) as 623 ± 48 units, Semkov *et al.* (1973) as 210 i.u. and Roychaudhury *et al.* (1974) as 205.56 mu. are more as compared to the values obtained of the enzyme in present experiment. This can be explained by citing the findings of Varshney *et al.* (1978) who observed that GOT value in buffalo semen was less than half of similar value reported in case of bull, whereas GPT value was comparable to that in bull semen.

The results of the pre[illegible] experi[illegible] that the extracellular release of GOT in TFYG dilute[illegible] samples was significantly lower as compared to LFYG diluted [illegible]on, i.e. TFYG dilutor provided better protection to spermatozoa, as compared to LFYG. These findings can very well be compared with those of Roychaudhury *et al.* (1974), who did not find the GOT release significantly high in Tris diluted bull semen on cold shock. However, Chinnaiya *et al.* (1979) reported that extracellular release of transaminases from buffalo spermatozoa on

freezing was significantly lower in CAW dilutor as compared to EYCG and Tris.

It is interesting to note overhere that in the present experiment, out of 17 samples processed for estimating activity of transaminases in seminal plasma, five semen samples were static i.e. without initial spermatozoal motility. The GOT values obtained pre freeze in such static samples were relatively more than the similar values obtained in initially motile samples. The former showed still more increase in freeze-thawed samples (Fig. 6).

These findings are well in accordance with the observations made by Crabo et al. (1971) who found the negative correlation between motility and aspartate amino-transferase (GOT) release. Similar observation was also made by Sadykov and Puzii (1971), who found significant correlation between GOT activity and sperm survival. However, the present findings did not agree with the study of Semkov et al. (1973) who did not observe the significant correlation between the spermatozoal motility and GOT activity. Zahariev et al. (1974) found GPT activity in spermatozoa and seminal plasma to be correlated with the percentage of motile spermatozoa in freshly collected bull semen, which did not agree with the results of present study with buffalo semen.

From the limited observations made on static semen samples and their pre and post freezing seminal plasma GOT levels, it appears that the level of GOT is indicative of the possibility of cell damage in initially flat semen samples. The mean level of GOT pre freeze and post freeze in motile semen samples and initially static samples were 7.65 i.u., 12.63 i.u. and 12.1 i.u. and 19.1 i.u. respectively.

Currently, the problem of obtaining initially non motile semen samples in buffaloes at various buffalo semen banks is increasing, which warrant more intensive studies on this aspect. However, the results obtained in the present study on GOT enzyme level pre and post-freezing in initially motile and static semen samples in buffaloes point out to one of the probable aetiologies for initially static semen samples.

(b) Hyaluronidase:

The hyaluronidase enzyme which is solely of testicular origin has been considered to be the best "marker enzyme" for the acrosomal integrity. Hyaluronidase activity in sperm extract found to be significantly related with fertility (Raizada et al., 1980). Acrosome has been found to contain an enzyme hyaluronidase and the physiological significance of acrosome is connected with the vital function for fertilization by releasing lytic enzymes.

The role of seminal hyaluronidase in fertilization process consists in denuding the egg of its surrounding follicular cells by acting on hyaluronic acid, so as to enable penetration and fertilization.

Many workers reported the increased percentage of damaged acrosomes in bull spermatozoa following freezing and thawing treatments (Chaves, 1979; Ganguli, 1979; Gilbert and Almquist, 1978; Patil et al., 1981), and the protection of acrosome is necessary for preserving the normal hyaluronidase activity of spermatozoa (Swyer, 1947[b]).

In the present experiment, the significant difference in hyaluronidase activity in spermatozoa was found between diluted buffalo semen samples before freezing and in freeze-thawed samples. The enzyme activity obtained was expressed as difference in optical density readings read from Spectrophotocolourimeter. It averaged 0.064 and 0.065 (O.D.) for TFYG and LFYG diluted samples before freezing, which decreased to 0.039 and 0.030 for the two dilutors respectively in freeze thawed samples (Table-20). These findings are in agreement with the observations of Holzmann et al. (1978), Ganguli (1979) and Patil et al. (1981). No significant difference was found in hyaluronidase enzyme activity in spermatozoa between TFYG and LFYG diluted buffalo semen pre and post freeze. Similar results were obtained by Ganguli (1979) who did not observe the significant difference in release of hyaluronidase enzyme

from spermatozoa between two dilutors Tris and CAW. However Chinnaiya (1979) found significant difference in rate of release of hyaluronidase on dilution. The lowest rate of enzyme release was from Tris diluted semen followed by CAW and citrate diluted semen samples.

Standard graph of known q[illegible] acetyle glucosamine against Optical Den[illegible] could not be plotted in present study, as N-acetyle-glucosamine was not readily available so the actual values of enzyme in relation to known quantity of N-acetyle glucosamine could not be obtained, and so the values had to be expressed in optical density readings. However, on availability of N-acetyle glucosamine the actual values of enzyme would be ascertained.

H. MENSURATION CHARACTERS:

Mensuration characters of spermatozoa are of much value, as they are reported to be related with fertility (Mukherjee and Kumar, 1971; Bishop *et al.*, 1954; Mukherjee and Rajwar, 1971).

(a) Buffalo spermatozoa:

In the present experiment the head breadth and head length of the buffalo spermatozoa averaged 4.6175 and 7.8282 microns in neat semen (Table-21) which were lower than the similar values of 5.3 μ and 8 μ respectively, as reported by Fischer and Gunzel (1978), for

Egyptian water buffaloes. However, the present values are in close agreement with the study of Rajendrakumar et al. (1977) who reported the overall mean for the spermatozoal head length and head breadth as 7.83 and 4.79 µ respectively. In buffalo semen, Kodagali et al. (1973a) found the average values of head length and head breadth of Surti buffalo spermatozoa as 7.618 and 4.591 microns, which also agree with the present findings. The head length measurement in Murrah buffalo spermatozoa reported by Sharma and Gupta (1978) as 8.43 microns was higher than the values observed in present experiment.

As observed in the present study, the head length and head breadth of buffalo spermatozoa in freeze thawed samples averaged 7.7113 microns and 4.4384 microns respectively (Table-21). These findings agree with the observations of Jain (1979), who noted that due to the effect of freezing there was a decrease in average head length from 7.5376 to 7.1699 microns and the head breadth from 4.5966 to 4.5128 microns. However, unlike his observations the decrease in head length was non-significant in present experiment, instead, the pre freeze and post-thaw difference in head breadth, was highly significant. Rajwar and Mukherjee (1970) observed the increase in head area of buffalo spermatozoa after a temperature shock of 5°C, which do not agree with the findings of the present experiment.

(b) Bull spermatozoa:

The H.F. bulls under study showed the average spermatozoal head length and head breadth in neat semen as 9.4050 microns and 5.2273 microns respectively, which on freezing decreased to 9.1665 and 4.8585 microns respectively (Table-22).

These values are more than those reported by Fischer and Gunzel (1978), who found in Friesian semen, the spermatozoal head length and head breadth to be 8 μ and 4.25 μ respectively. However the present values in neat semen can be compared to those reported for Haryana bulls' spermatozoa as 9.7 μ and 5.1 μ respectively, by Rao and Sidhu (1975). The same workers also reported the values for Jersey bull spermatozoa as 9.161 microns and 4.72 microns respectively, which were lower than the findings of present experiment with Friesian spermatozoa. There was a significant difference in values of head breadth measurement between pre freeze and freeze-thawed spermatozoa, however, such temperature effect in head length measurements was non significant.

The values obtained for total length of spermatozoa pre-freeze and post-freeze do not appear to be of much significance.

I. FERTILITY TRIALS:

Obviously, the only valid measure of semen quality is to assess fertility by examining for pregnancy the

inseminated females. The research on semen extension and preservation cannot be considered to be complete without fertility trials.

(a) Buffalo semen:

In the present experiment on fertility trials in buffaloes, the overall fertility percentage achieved with TFYG diluted semen was 57.24% (Table-23), which is comparatively higher than fertility rates obtained by Takkar et al. (1980) as 36.12%, Vasanth (1979) as 45%, Chinnaiya et al. (1979) as 46.22%, Patil et al. (1981) as 55.55% and Reddy et al. (1982) as 47%, in buffaloes using Tris diluted frozen semen. On first service insemination basis, Meharsing et al. (1980) and Chinnaiya and Ganguli (1980) obtained the conception rates of 45.8% and 40.0% respectively, using Tris diluted freeze-thawed semen, these results were also lower as compared to the fertility rate obtained in present study. But this may be due to the fact that, the former values are based on first service results. Similar results were observed by Austin et al. (1981) as 42.60% and Zhou (1981) who reported the first service conception rates of 53.9% and 57.2%, on farm level fertility trials in buffaloes using semen frozen in pellets and straws, respectively.

The overall fertility percentages obtained by Heuer (1982) as 53.8%, 54.3%, 56.7% and 53.8% for buffalo semen

diluted and frozen in LFYG, Tris , Tris milk and skim milk diluents respectively. These are well in accordance with the results of present experiment. Roychaudhury (1978) and Shafi (1979) obtained 50% conception rates using frozen semen from buffaloes which were slightly lower than the present findings. However, t[illegible]lity results obtained by Pavitharan *et al.* (1972) as [illegible] quite higher, might be due to the fact that, it was on 60 days non-return basis. On field level fertility trials. Elimwadi *et al.* (1974) obtained 25.5%, Sharma *et al.* (1979) obtained 24 to 28%, Toelihere (1980) and Jainudeen (1980) obtained 30% conception rates using frozen semen in buffaloes. These results are quite lower as compared to the results found in present fertility trials.

Ahmad *et al.* (1982) reported in buffaloes the average requirement of number of inseminations per conception to be 1.77 to 2.75, which is very well in agreement with the findings of present study, in which average 2.69 inseminations were required per conception (Table-24).

The reasons for comparatively higher fertility rates achieved in the present experiment may be mainly due to the following factors:

(i) Well awareness amongst the buffalo owners in the area regarding the oestrous signs and time of insemination.

(ii) Involvement of well experienced and technically competent veterinarians in inseminations and pregnancy diagnosis work.

(iii) Post-insemination antibiotic treatment as and when required.

__Bull semen:__

The overall mean fertility results of H.F. bull frozen semen obtained in the present study was 46.21% (Table-25). The fertility results obtained are very well comparable with those obtained by Guha (1972) as 46.00%, Chinnaiya __et al.__ (1974) as 30 to 60% and Maulic __et al.__ (1975) as 42.2%, who used frozen semen from Friesian sires in indigenous cows. However, the present fertility results are quite low as compared to the results reported by Davidovic (1971) as 71%, Jondet (1964) as 68%, Ishii __et al.__ (1979) as 52 to 58%, Tomar (1981) as 61.34% and Olar and Pickett (1980), who reported over 70% conception rates by using Friesian frozen semen. The present fertility results agree with the first service conception rates of 44.8% and 44.6% reported by Austin __et al.__ (1978) and Jainudeen (1968), respectively. However, Almquist __et al.__ (1979), Foote (1970), Pickett __et al.__ (1959) and Roy (1974) reported over 70% conception rates, on 60 to 90 days non-return basis, were naturally higher than the fertility results obtained in the present study.

The average number of inseminations per conception required in the present study was 2.45 (Table-26), which is very well comparable with the observations of Nair (1975) who reported that, Brown Swiss frozen semen required 2.49 inseminations per conception. However, the fertility trials undertaken by Qureshi (1979) and Tomar (1981) showed the requirements of 2.76 and 3 to 4 inseminations per conception, respectively. These were higher than the present finding. The requirement of the number of inseminations per conception in Kankrej cows in the present fertility trials was quite less as compared to the results obtained by Kumaran (1965), who reported the number of services required per conception as 8.0, 6.12 and 8.3 in Red Sindhi, Sahiwals and Tharparkar cows, respectively.

It was observed from the present fertility trials that there were significant differences in fertility rates amongst the bulls and buffalo bulls studied for the purpose. This may be due to the bull - difference in post-thaw motility of the semen (Singh et al., 1980; Reddy et al., 1982). The fertility results obtained by using Friesian semen, in present study are comparatively low. Such low conception results, using frozen semen may be attributed to the probable causes as suggested by Kumaran (1965) and Bhosrekar (1973), which include :

Lower post-thaw spermatozoal motility,

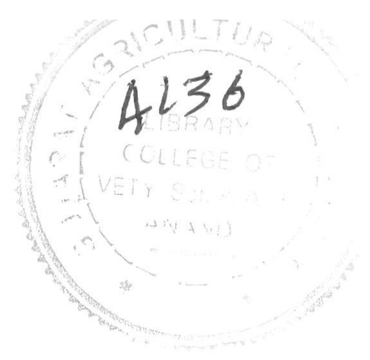
faulty insemination technique,

exposure of semen straw to warm temperature before thawing,

improper condition of thawing of frozen semen and delayed insemination after thawing. The lower conception rates might have also been due to poor nutritional status and poor fertility of cows (Nair, 1975).

The perusal of the fertility results of bull semen in present study, showed much variation in fertility results obtained at various AI centres (Table-27). This may be attributed to the efficiency of inseminators; as they play an important role in artificial insemination services (King, 1973).

In the present study, the use of frozen semen in repeating Kankrej cows and heifers required on an average 3.5 inseminations per conception (Table-28), which is quite lower to settle a repeat breeder. These findings are much in contrast to the study of Bhosrekar (1973), who observed more cases of repeat breeders due to the inseminations with using frozen semen from Brown Swiss sires. However, the exact role of frozen semen in settling the repeating cows with liquid semen, is not clear.



SUMMARY AND CONCLUSIONS

VI. SUMMARY AND CONCLUSION

The present research project was designed to attempt deep freezing of the bull and buffalo semen at the Department of Veterinary Obstetrics and Gynaecology, Gujarat College of Veterinary Science and Animal Husbandry, Anand. The project was aimed to study the freezability of semen from different bulls, to test the efficacy of various dilutors for freezing; to estimate extracellular release of certain enzymes (pre and post freezing) and to know the changes in cytomorphology as well as mensuration characters of spermatozoa subsequent to freezing. It also involved the fertility trials in the field and at farm levels using the freeze-thawed semen in Surti buffaloes and Kankrej cows.

The freezability of spermatozoa in 46 ejaculates from 23 bulls (20 Surti and 3 H.F.) was studied in Tris fructose yolk glycerol (TFYG) diluent. The significant difference ($P < 0.01$) was observed amongst the bulls under study in their ability to provide the semen ejaculates suitable for freezing. Out of 23 bulls, 10 bulls (43.5%) provided the semen ejaculates having very good freezability ($\geq$ 50% post-thaw motility of spermatozoa). The semen ejaculates from nine buffalo bulls (39.2%) were moderately suitable for freezing (post-thaw spermatozoal motility between 30 and 50%). The remaining four buffalo bulls (17.3%) ejaculated the semen which did not stand to freezing

satisfactorily (less than 30% post-thaw spermatozoal motility). The initial motility and freezability of spermatozoa were not found to be related positively ($r = 0.23$) to some extent, under the present study.

For buffalo semen, two diluents and for bull semen, five diluents were tried to study their effic[illegible] as frozen semen extenders. For freezing buffalo semen, TFYG proved to be better as compared to lactose fructose yolk glycerol diluent (LFYG). The post-thaw motility averaged 57.80%, 54.20% and 55.20%, 49.40% at 0 hr and after 48 hrs post freezing in the TFYG and LFYG diluents, respectively. Out of five diluents tested for freezing bull semen, TFYG and egg yolk citrate glycerol (EYCG) ranked high, followed by whole milk glycerol (WMG), Laiciphos-478 (L-478) and AMUL spray glycerol (ASG).. At 0 hr and 48 hrs post-freezing, the post-thaw spermatozoal motility percentages averaged 34.33, 35.66, 38.00, 61.00, 56.66 and 29.33, 33.00, 33.00, 56.60, 54.60 in ASG, L-478, WMG, TFYG and EYCG diluents, respectively.

Live sperm per cent in buffalo neat semen averaged 86.24%, which later declined significantly ($P <$ [illegible]) [illegible] 65.32[illegible] [illegible]d 65.24% in freeze-thawed semen diluted in TFYG and LFYG diluents respectively. The H.F. bull semen showed the presence of 88.26% live sperms before freezing, which significantly ($P < 0.01$) decreased to 60.46%, 61.66%, 60.20%, 65.43% and 62.9[illegible] [illegible]eezing and thawing in

ASG, L-478, WMG, TFYG and LYCG diluents, respectively. In respects of post-thaw live sperm percentage bulls did not vary significantly, however there were significant differences between dilutors.

The morphologically abnormal spermatozoal percentage in buffalo neat semen averaged 7.96, which in freeze-thawed semen samples increased significantly to 15.52% and 15.68%. The post-thaw abnormal sperm per cent did not show significant differences between bulls or between dilutors. In H.F. bulls' neat semen the morphologically abnormal sperm percentages averaged 8.93%, which significantly increased to 15.46%, 16.00%, 15.26%, 16.66% and 16.06% in ASG, L-478, WMG, TFYG and LYCG diluents after freezing and thawing, respectively. The differences in per cent post-[illegible] abnormal sperm counts were non-significant betw[illegible] bulls but significant differences were observed between the dilutors.

The levels of transaminases in buffalo seminal plasma were determined pre and post freezing. [illegible]els of Glutamic oxaloacetic transaminase (GOT) before freezing averaged [illegible] and 9.42 i.u. in TFYG and LFYG dil[illegible] samples, which significantly ($P < 0.01$) increased to 12.[illegible]2 and 16.23 i.u. in freeze-thawed semen samples, respectively. The difference in GOT release was significant ($P < 0.01$) between dilutors, being lesser in TFYG diluent,

thereby protecting spermatozoa more efficiently during freezing, as compared to LFYG diluent. Glutamic pyruvic transaminase (GPT) levels averaged 2.67 and 3.05 in buffalo seminal plasma, before freezing, which significantly ($P < 0.01$) increased to 3.65 and 4.03 i.u. in TFYG and LFYG diluents after freezing, respectively. The difference in release of GPT was non-significant between dilutors. The levels of GOT enzyme appeared to be related negatively with initial motility of spermatozoa.

The activity of hyaluronidase enzyme in sperm pack (25×10^6 spermatozoa) averaged 0.064 and 0.065 (O.D.) before freezing, which significantly ($P < 0.01$) declined in freeze-thawed samples to 0.039 and 0.030 (O.D.) in TFYG and LFYG diluents, respectively. The difference in hyaluronidase activity between the dilutors was found to be non-significant.

The changes in mensuration characters of spermatozoa in bull and buffalo semen were studied pre and post-freezing. The head breadth and head length of buffalo spermatozoa averaged 4.6175 and 7.8282 in neat semen, which decreased to 4.4384 and 7.7113 microns respectively in freeze thawed semen samples. For H.F. bull, the spermatozoal head breadth and head length averaged 5.2273 and 9.4052 microns before freezing, which declined to 4.8585 and 9.1665 after freezing. The difference in head breadth was statistically significant ($P < 0.05$), unlike the head length.

The overall fertility following frozen semen inseminations in Surti buffaloes and buffalo heifers was 57.24%. On an average 2.69 inseminations were required per conception. The fertility percentages obtained for five buffalo bulls were 62.02, 52.17, 61.43, 53.33 and 50.00 for SB_{AS}, SB_{AJ}, SB_{RM}, SB_{PR} and SB_{AT} respectively. The frozen semen inseminations in Kankrej cows and heifers resulted in overall fertility of 46.21%. On an average 2.46 inseminations were required per conception. For the three H.F. bulls, the fertility percentage were 52.87, 48.75 and 36.90 for H.F. 316, H.F. 317 and H.F. 307 respectively. On an average 3.5 inseminations were required per conception on farm level fertility trial in repeating Kankrej heifers and cows.

Conclusion:

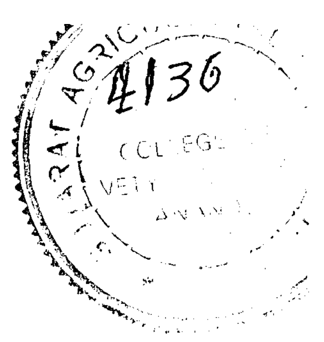
1. Bulls showed significant variations in respect of their ability to provide the semen ejaculates suitable for freezing.
2. The correlation between mass activity and freezability of spermatozoa was found to be rather non-significant.
3. Tris fructose yolk glycerol (TFYG) is a better dilutor for freezing both, bull and buffalo semen.
4. The estimation of extracellular release of transaminases in freeze-thawed semen samples can reliably be used for selecting the suitable dilutor and to know the quality of frozen semen.

5. The GOT enzyme levels were comparatively more, pre and post freezing in the semen samples with initially non-motile spermatozoa which can be suggestive of sperm cell injury in static semen samples. More intensive studies are necessary in this regards.

6. Hyaluronidase was found to be liberated from damaged spermatozoa. The hyaluronidase assay in frozen semen can be helpful to predict the fertility.

7. Mensuration characters are affected due to freezing. This can be considered as one of the methods to evaluate the quality of frozen semen. Fertility studies are required to be carried out in relation to the mensuration characters of freeze-thawed spermatozoa.

8. Frozen semen usage in a proper manner, through experienced technicians in healthy females offer encouraging fertility results.



APPENDIX - I

TABLES & FIGURES

Table - 1. Observations on normal seminal attributes of bull and buffalo semen.

Sr. No.	Seminal attributes	Buffalo semen			Bull semen		
		Mean	S.E.	C.V.%	Mean	S.E.	C.V.%
1	Volume (ml)	3.08 * (50)	0.19	31.09	5.65 (25)	0.095	8.40
2	Colour and consistency	Thin to thick milky DD(D)			Thin to thick creamy DDD(D)		
3	Mass activity	++(+) (2.11)(50)	0.17	56.87	+++(+) (3.44)(25)	0.078	11.37%
4	Individual motility	77.4% (50)	1.63	14.87	79.8% (25)	0.68	4.24
5	Sperm concentration (million/ml)	1022.34 (50)	22.22	15.37	1514.80 (25)	2.50	10.28
6	Per cent live sperms	85.36% (50)	0.46	3.78	87.08 (25)	0.49	2.81
7	Per cent abnormal sperms	9.72% (50)	0.35	25.20%	9.20 (25)	0.42	22.93

* The figures in parenthesis indicate the number of samples.

Table -2. Freezability of semen from different bulls on the basis of post-thaw spermatozoal motility at 0 hr and after 24 hrs post freezing.

Bulls	Mass activity			Indi.motility (%)			Post-thaw motility (%)					
							At 0 hr			After 24 hrs		
	1	2	Av.	1	2	Av.	1	2	Av.	1	2	Av.
HF 316	4.0	3.5	3.75	85	90	87.5	60	50	55.0	60	45	52.5
HF 317	3.5	3.0	3.25	80	80	80.0	65	55	60.0	60	55	59.0
HF 307	3.0	3.5	3.25	85	80	82.5	60	60	60.0	60	55	57.2
SB_{AS}	3.0	3.5	3.25	80	85	82.5	50	50	50.0	45	55	50.0
SB_{AJ}	4.0	3.0	3.50	80	90	85.0	60	50	55.0	55	50	52.5
SB_{AT}	2.5	3.0	2.75	85	80	82.5	40	45	42.5	40	50	45.0
SB_{BJ}	2.5	2.0	2.25	80	85	82.5	55	40	47.5	50	40	45.0
SB_{RM}	3.0	3.0	3.00	90	85	87.5	60	60	60.0	50	55	52.5
SB_{BH}	4.0	3.0	3.50	80	90	85.0	60	55	57.5	60	55	57.5
SB_{PR}	2.0	2.5	2.25	80	70	75.0	50	55	52.5	45	60	52.5
SB_{SH}	3.0	2.5	2.75	75	80	77.5	40	50	45.0	35	40	37.5
SB_{BL}	2.0	0.5	1.25	80	80	80.0	20	30	25.0	00	20	10.0
DF_{LD}	1.5	2.0	1.75	80	70	75.0	55	50	52.5	50	50	50.0
DF_{KV}	0.0	0.0	0.00	80	75	77.5	45	35	40.0	40	30	35.0
DF_{RV}	3.5	3.0	3.25	75	80	77.5	20	30	25.0	00	10	5.0
DF_{CK}	2.0	1.0	1.50	85	70	77.5	40	50	45.0	35	30	32.5
DF_{KN}	3.0	1.5	2.25	70	70	70.0	50	40	45.0	30	30	30.0
DF_{TJ}	0.0	0.0	0.00	80	60	70.0	40	55	47.5	30	40	35.0
DF_{HR}	3.0	2.5	2.75	80	75	77.5	30	40	35.0	30	20	25.0
DF_{JN}	0.0	1.0	0.50	70	80	75.0	40	50	45.0	35	45	40.0
DF_{BG}	0.0	0.0	0.00	80	80	80.0	50	40	45.0	30	35	32.5
CB_1	0.0	1.0	0.50	70	75	72.5	60	50	55.0	35	50	52.5
CB_2	1.0	0.0	0.50	80	70	75.0	50	50	50.0	40	50	45.0

Table - 3. Analysis of variance for post-thaw spermatozoal motility between bulls and ejaculates at 0 hr and after 24 hrs post freezing.

Source of Vatiation	D.F.	At 0 hr		After 24 hrs	
		M.S.	'F' value	M.S.	'F' value
Between bulls	22	66.54	4.56**	214.82*	7.20**
Between ejaculates	1	0.34	N.S.	155.38	5.21**
Error	22	14.58		29.85	

$P < 0.01$

N.S. = Non Significant.

Table -4. Buffalo spermatozoal motility (pre freeze) and post-thaw at 0 hr and after 48 hrs post freezing in different diluents.

Bulls	Individual motility(%) (pre freeze)		Post-thaw motility (%)			
			At 0 hr		After 48 hrs	
	TFYG	LFYG	TFYG	LFYG	TFYG	LFYG
SB_{RM}	70	75	60	55	60	50
	75	70	55	65	50	60
	80	75	60	60	55	50
	80	70	70	50	60	50
	70	60	65	60	65	50
Av.	75.0	70.0	62.0	58.0	58.0	52.0
SB_{AS}	80	80	55	60	50	60
	80	75	50	55	50	50
	85	75	60	50	60	40
	80	80	55	60	50	50
	70	75	60	50	55	50
Av.	79.0	75.0	56.0	55.0	53.0	50.0
SB_{AJ}	80	80	60	55	55	50
	85	75	65	50	60	50
	70	80	60	50	55	40
	70	74	50	55	50	40
	70	80	45	45	45	45
Av.	75.0	77.0	56.0	51.0	55.0	45.0
SB_{PR}	80	75	65	60	60	60
	70	60	55	55	50	55
	70	75	60	50	60	45
	80	70	50	50	50	40
	75	70	60	60	55	55
Av.	75.0	70.0	58.0	55.0	55.0	51.0
SB_{SH}	85	80	50	50	50	45
	70	75	60	50	55	50
	80	75	60	45	60	40
	75	75	60	55	60	50
	75	80	55	60	50	60
Av.	77.0	75.0	57.0	52.0	55.0	49.0
Overall Av.	76.20	73.40	57.80	54.20	55.20	49.40

Table -5. Analysis of variance for post-thaw buffalo spermatozoal motility between bulls in various dilutors at 0 hr and after 48 hrs post freezing.

Source of variation	D.F.	At 0 hr		After 48 hrs	
		M.S.	F value	M.S.	F value
Between bulls	4	18.82	1.83 N.S.	4.472	1 NS
Between dilutors	1	82.22	7.99**	119.74	10.89**
Error	44	10.29		10.99	

** $P < 0.01$

N.S. = Non Significant.

Table-6. Analysis of variance for buffalo spermatozoal motility between various dilutors and temperatures pre and post-freezing.

Source of variation	D.F.	M.S.	F value
Between dilutors	1	70.75	1.09 NS
Between temperatures	1	3507.85	53.94**
Dilutor X temperature	1	261.47	4.02**
Error	96	65.03	

** $P < 0.01$

N.S. = Non Significant

Table -7. Pre freeze and post-thaw spermatozoal motility in bull semen at 0 hr and after 48 hrs post freezing in different diluents.

Bulls	Individual motility(%) (Pre freeze)					Post-thaw motility (%) At 0 hr					Post-thaw motility (%) After 48 hrs				
	ASG	L-478	WMG	TFYG	EYCG	ASG	L-478	WMG	TFYG	EYCG	ASG	L-478	WMG	TFYG	EYCG
H.F. 316	80	70	75	85	80	30	30	45	60	50	30	40	40	60	50
	70	75	75	90	75	35	35	40	65	55	20	35	35	60	50
	75	70	80	80	80	35	35	40	60	55	30	30	40	60	55
	85	80	70	80	85	35	40	35	60	60	35	40	35	55	55
	75	70	80	80	75	40	35	30	65	60	35	35	30	55	60
Av.	77	73	76	83	79	35	37	38	62	56	30	36	36	58	54
H.F. 317	80	80	80	90	75	40	40	50	70	60	40	40	40	60	60
	75	75	70	80	80	30	45	40	60	55	20	40	30	50	55
	70	75	80	80	85	30	40	30	55	60	30	30	30	55	55
	80	70	70	80	85	35	35	30	60	55	30	35	30	60	50
	70	80	70	80	80	30	35	40	60	60	30	30	35	55	60
Av.	75	76	74	82	81	33	39	38	61	58	30	35	33	56	56
H.F. 307	70	70	75	80	85	30	40	45	60	60	20	40	40	60	55
	75	75	75	85	80	35	30	40	65	60	35	30	30	50	60
	80	85	80	80	85	40	35	40	60	50	20	30	20	55	50
	80	80	70	80	70	30	20	30	60	55	30	20	30	60	55
	80	70	80	80	75	40	30	35	65	55	35	20	30	55	60
Av.	77	76	76	81	79	35	31	38	62	56	28	28	30	56	54
Over-all Av.	76.33	75.00	75.33	82.00	79.66	34.33	35.66	38.00	61.66	56.66	29.33	33.00	33.00	56.66	54.66

Table -8. Analysis of variance for post-thaw spermatozoal motility in various dilutors for different bulls at 0 hr and after 48 hrs post freezing.

Source of variation	D.F.	At 0 hr		After 48 hrs	
		M.S.	F value	M. S.	F value
Between Bulls	2	5.465	1 N.S.	33.06	28.74**
Between Dilutors	4	711.25	43.26**	894.96	778.22**
Error	68	16.44		1.154	
Total	74				

** $P < 0.01$, N.S. = Non Significant.

Table -9. Analysis of variance for bull spermatozoal motility at various temperatures in different dilutors.

Source of variation	D.F.	M.S.	F value
Between Dilutors	4	675.59	78.65**
Between temperatures	1	14576.95	1696.97**
Dilutor X temperature	4	265.86	30.95**
Error	140	8.59	
Total	149		

** $P < 0.01$

Table - 10. Pre-freeze and post-thaw percentages of live and abnormal spermatozoa in buffalo semen.

Bulls	Pre freeze		Post freeze			
	Live %	Abnormal %	Live %		Abnormal %	
			TFYG	LFYG	TFYG	LFYG
SB_{RM}	88	9	61	66	13	15
	87	8	63	65	17	16
	85	8	67	64	18	18
	83	7	62	60	15	19
	89	6	69	62	18	20
Av.	86.4	7.6	64.4	63.4	16.2	17.6
SB_{AS}	85	7	61	66	17	15
	82	9	66	71	12	13
	88	9	69	62	14	16
	91	6	71	66	11	18
	87	8	63	65	16	14
Av.	86.6	7.8	66.0	66.0	14.0	15.2
SB_{AJ}	86	8	63	66	17	16
	90	11	71	69	18	18
	87	8	62	66	13	12
	82	7	68	62	10	14
	83	6	64	65	16	15
Av.	85.6	8.0	65.6	65.2	14.8	15.0
SB_{PR}	90	8	68	60	19	12
	85	6	66	68	14	13
	88	8	61	72	13	16
	88	11	65	65	16	19
	83	9	64	65	15	14
Av.	86.8	8.4	64.8	66.0	15.4	14.8
SB_{SH}	82	10	65	65	16	14
	88	8	68	62	18	20
	85	8	70	68	16	14
	88	8	62	66	17	15
	86	6	64	67	19	16
Av.	85.8	8	65.2	65.6	17.2	15.8
Overall	86.24	7.96	65.32	65.24	15.52	15.68

Table - 11. Analysis of variance for buffalo semen for different bulls in various dilutors for post-thaw per cent live and abnormal spermatozoa.

Source of variation	D.F.	% Live spermatozoa		% Abnormal spermatozoa	
		M. S.	F value	M. S.	F value
Between Bulls	4	2.4975	$1^{N.S.}$	5.115	$1^{N.S.}$
Between Dilutors	1	0	N.S.	3.61	$1^{N.S.}$
Error	44	3.0059		13.925	
Total	49				

N.S. = Non Significant

Table - 12. Analysis of variance for per cent live and abnormal spermatozoa in buffalo semen at different temperatures.

Source of variation	D.F.	Live count		Abnormal count	
		M.S.	F value	M.S.	F value
Between temperatures	1	2574.6	1176.6^{**}	594.502	272.27^{**}
Error	48	2.188		2.1835	
Total	49				

** $P < 0.01$

Table - 13. Pre and post freezing, per cent live and abnormal spermatozoa in bull semen.

Bulls	Pre freeze		Post freeze									
	Live %	Abnormal %	Live %					Abnormal %				
			ASG	L-478	MMG	TFYG	LYCG	ASG	L-478	MMG	TFYG	LYCG
HF 316	91	8	52	61	59	66	68	12	16	13	14	15
	89	11	68	66	60	68	62	15	16	19	13	16
	86	7	60	52	63	64	66	18	12	13	16	15
	89	8	63	66	60	68	64	19	13	21	20	12
	87	7	65	61	64	69	59	17	19	19	13	16
Av.	88.4	8.2	61.6	61.2	61.2	67.0	63.8	16.2	15.2	17.0	15.2	14.8
HF 317	88	12	62	62	58	64	65	14	19	16	17	20
	90	9	58	68	66	68	62	18	17	16	18	14
	86	8	59	59	62	61	63	16	17	15	18	17
	89	10	60	66	64	66	61	13	18	14	19	18
	90	7	61	62	61	67	60	12	16	16	18	12
Av.	88.6	9.2	60.0	63.4	62.2	65.2	62.5	14.6	17.4	15.4	18.0	16.2
HF 307	87	10	52	59	60	66	63	19	17	12	14	16
	85	8	59	60	56	62	67	16	12	16	18	20
	90	12	60	58	61	61	63	13	12	14	20	18
	88	9	61	59	60	63	61	12	17	12	19	17
	89	8	67	66	52	68	60	18	19	18	13	15
Av.	87.8	9.4	59.8	60.4	57.2	64.1	62.8	15.6	15.4	13.4	16.8	17.2
Over-all Av.	88.26	8.93	60.46	61.66	60.20	65.33	62.93	15.46	16.00	15.26	16.66	16.06

Table - 14. Analysis of variance for post-thaw live and abnormal spermatozoal percentages for different bulls in various dilutors.

Source of variation	D.F.	Live count		Abnormal count	
		M. S.	F value	M. S.	F value
Between Bulls	2	15.19	3.67 N.S.	7.48	1.56 N.S.
Between Dilutors	4	22.73	5.49**	213.46	44.66**
Error	68	4.14		4.78	
Total	74				

** $P < 0.01$, N.S. = Non Significant.

Table - 15. Analysis of variance for per cent live and abnormal spermatozoa in bull semen at different temperatures.

Source of variation	D.F.	% Live Spermatozoa		% Abnormal spermatozoa	
		M. S.	F value	M. S.	F value
Between temperatures	1	2356.02	1463.36**	272.02	157.23**
Error	28	1.61		1.73	
Total	29				

** $P < 0.01$

Table - 16. Comparison of means of post-thaw spermatozoal motility at 0 hr and after 48 hrs, per cent live and abnormal spermatozoa in bull semen in various dilutors.

Dilutors	Post-thaw motility (%)		Live sperm count (%)	Abnormal sperm count (%)
	At 0 hr	After 48 hrs		
TFYG	*61.66^{a}	56.66^{a}	65.43^{a}	16.66^{a}
EYCG	56.66^{b}	54.66^{b}	62.93^{b}	16.06^{ab}
WMG	38.00^{c}	33.00^{c}	60.20^{c}	15.26^{c}
L-478	35.66^{cd}	33.00^{c}	61.66^{bc}	16.00^{abc}
ASG	34.33^{d}	29.33^{d}	60.46^{c}	15.46^{bc}

* Averages bearing different superscripts differ significantly.

Table - 17. Comparison of means of post-thaw spermatozoal motility at 0 hr and after 48 hrs and per cent live and abnormal spermatozoa of buffalo semen in various diluents.

Dilutors	Post-thaw motility (%)		Live sperm count (%)	Abnormal sperm count (%)
	At 0 hr	After 48 hrs		
TFYG	*57.80^{a}	55.20^{a}	65.32^{a}	15.52^{a}
LFYG	54.20^{b}	49.40^{b}	65.24^{a}	15.68^{a}

* Averages bearing different superscripts differ significantly.

Table - 18. Levels of transaminases (GOT and GPT) before and after freezing in different dilutors for buffalo seminal plasma.

Buffalo Bull	Mass activity	GOT i.u./Litre (in 50 x 10^6 spermatozoa)				GPT i.u./Litre (in 50 x 10^6 spermatozoa)			
		Pre freeze		Post freeze		Pre freeze		Post freeze	
		TFYG	LFYG	TFYG	LFYG	TFYG	LFYG	TFYG	LFYG
SB_{AS}	+++	10.0	8.0	13.0	18.0	4.0	4.0	4.5	4.0
	++	9.5	8.0	14.0	21.0	3.0	3.5	4.0	4.0
	++(+)	6.5	8.5	9.0	10.0	2.0	3.0	2.0	3.5
Av.	2.41	8.66	8.16	12.0	16.33	3.0	3.5	3.5	3.83
SB_{SH}	++	6.0	9.0	10.0	11.0	2.0	2.0	4.0	4.5
	++(+)	7.5	9.5	11.0	15.0	4.0	5.0	4.0	5.0
	++(+)	6.0	7.0	15.0	16.0	3.0	4.0	4.0	5.0
Av.	2.33	6.5	8.5	12.0	14.0	3.0	3.66	4.0	4.83
SB_{AJ}	+++	5.0	7.0	9.0	13.0	4.0	2.0	4.5	2.0
	+++	6.0	7.5	9.0	12.0	3.0	4.0	3.0	4.5
	++(+)	6.5	8.0	10.0	15.0	2.0	2.5	3.0	4.0
Av.	2.83	5.83	7.33	9.33	13.33	3.0	2.83	3.5	3.5
CB_1	+(+)	6.0	10.0	9.0	16.0	2.0	2.0	3.0	4.0
	-	9.0	10.0	16.0	18.0	2.0	2.0	3.0	4.0
	-	12.0	15.0	19.0	22.0	2.0	3.0	5.0	6.0
	+	6.0	13.0	14.0	20.0	2.0	3.0	5.0	4.0
Av.	0.5	8.25	12.0	14.5	19.0	2.0	2.5	4.0	4.5
CB_2	-	13.00	12.0	19.0	21.0	3.0	2.0	4.0	2.0
	-	11.0	10.0	18.0	19.0	2.5	3.0	4.0	4.0
	+	6.5	6.5	10.0	13.0	2.0	4.0	2.0	4.0
	-	13.0	16.0	18.0	21.0	2.0	2.0	3.0	4.0
Av.	0.12	10.87	11.12	16.25	18.5	2.37	2.75	3.25	3.5
Overall Av.		8.02	9.42	12.82	16.23	2.67	3.048	3.65	4.032

Table - 19. Analysis of variance of pre freeze and post-thaw seminal plasma levels of GOT and GPT enzymes in different dilutors and at various temperatures.

Source of variation	D.F.	G O T		G P T	
		M. S.	F value	M. S.	F value
Between temperatures	1	168.31	31.28**	4.81	21.86**
Between dilutors	1	29.00	5.39**	0.72	3.27 N.S.
Error	17	5.38		0.22	
Total	19				

** $P < 0.01$

N.S. = Non Significant.

Table -20. Hyaluronidase activity (HA) in sperm pack (25×10^6 spermatozoa) before and after freezing in different dilutors.

Buffalo bulls	Hyaluronidase activity in 25×10^6 million sperm extract			
	Pre freeze (OD)		Post freeze (OD)	
	TFYG	LFYG	TFYG	LFYG
SB_{AS}	0.07 0.04 0.05	0.06 0.05 0.05	0.03 0.02 0.03	0.03 0.025 0.02
Av.	0.053	0.053	0.026	0.025
SB_{AJ}	0.06 0.05 0.07	0.05 0.05 0.06	0.04 0.04 0.05	0.025 0.025 0.02
Av.	0.06	0.053	0.043	0.023
SB_{SH}	0.07 0.06 0.08	0.10 0.05 0.06	0.05 0.04 0.05	0.05 0.03 0.025
Av.	0.07	0.07	0.046	0.035
CB_1	0.06 0.07 0.09 0.071	0.05 0.08 0.08 0.07	0.04 0.045 0.05 0.045	0.02 0.03 0.04 0.03
Av.	0.072	0.07	0.045	0.03
CB_2	0.06 0.08 0.06 0.06	0.07 0.09 0.08 0.08	0.04 0.035 0.03 0.035	0.035 0.04 0.04 0.038
Av.	0.065	0.08	0.035	0.038
Overall Av.	0.064	0.0652	0.039	0.030

Table -21. Pre and post freezing mensuration characters of spermatozoa in buffalo semen.

Buffalo bulls	Head length (microns)		Head breadth (microns)		Total length (microns)	
	Pre freeze	Post freeze	Pre freeze	Post freeze	Pre freeze	Post freeze
SB_{AS}	7.788	7.832	4.488	4.368	59.90	59.56
	7.701	7.268	4.516	4.389	61.20	60.44
Av.	7.7445	7.550	4.502	4.3785	60.55	60.00
SB_{SH}	8.090	7.992	4.928	4.334	56.48	57.53
	7.892	7.732	4.638	4.598	59.32	61.02
Av.	7.991	7.862	4.783	4.466	57.90	59.28
SB_{AJ}	7.964	7.917	4.356	4.291	59.94	60.23
	7.826	7.633	4.598	4.512	58.69	59.66
Av.	7.895	7.775	4.477	4.402	59.32	59.95
CB_1	7.852	7.870	4.342	4.288	59.84	59.00
	7.724	7.783	4.519	4.497	57.02	58.23
Av.	7.788	7.827	4.431	4.393	58.43	58.62
CB_2	7.832	7.348	4.884	4.444	57.64	56.32
	7.612	7.744	4.906	4.664	57.86	58.45
Av.	7.722	7.546	4.895	4.554	57.75	57.39
Overall Av.	7.8282	7.7113	4.6175	4.4385	58.787	59.044

Table -22. Pre and post freezing mensuration characteristics of spermatozoa in bull semen.

Bulls	Head length (microns)		Head breadth (microns)		Total length (microns)	
	Pre freeze	Post freeze	Pre freeze	Post freeze	Pre freeze	Post freeze
HF 316	9.412 9.548	8.994 9.590	5.654 5.614	5.400 4.906	52.89 54.06	54.34 55.66
Av.	9.480	9.292	5.634	5.153	53.46	55.00
HF 317	9.020 9.636	9.188 8.844	4.752 5.056	4.430 4.422	53.02 58.52	51.192 58.89
Av.	9.328	9.016	4.902	4.426	55.77	55.02
HF 307	9.429 9.386	9.242 9.141	5.269 5.019	5.010 4.983	56.28 58.95	57.39 56.84
Av.	9.408	9.192	5.144	4.997	57.62	57.82
Overall Av.	9.4052	9.1665	5.2273	4.8585	55.62	55.7187

Table -23. Fertility of frozen semen for different buffalo bulls.

Bulls	No. of animals inseminated	No. of animals followed	No. of animals pregnant	Fertility (%)
SB_{AS}	99	79	49	62.02
SB_{AJ}	85	69	36	52.17
SB_{RM}	78	70	43	61.43
SB_{PR}	45	30	16	53.33
SB_{AT}	35	28	14	50.00
Total	342	276	158	
Overall mean fertility				57.24%

Table -24. Number of inseminations required per conception for different buffalo bulls under study.

Bulls	No. of inseminations	No. of animals conceived	No. of insem./concep.
SB_{AS}	126	49	2.57
SB_{AJ}	102	36	2.83
SB_{RM}	98	43	2.27
SB_{PR}	54	16	3.37
SB_{AJ}	46	14	3.20
Total	426	158	
Av.			2.69

Table -25. Fertility of frozen semen for different bulls.

Bulls	No. of animals inseminated	No. of animals followed	No. of animals pregnant	Fertility (%)
HF 316	87	87	46	52.87
HF 317	80	80	39	48.75
HF 307	84	84	31	36.9
Total	251	251	116	
Overall fertility				46.21

Table - 26. Number of inseminations required per conception for different bulls.

Bulls	No. of inseminations	No. of animals conceived	No. of insem./concep.
HF 316	96	46	2.06
HF 317	98	39	2.93
HF 307	91	31	2.50
Total	285	116	
Av.			2.45

Table - 27. Fertility in repeat breeding Kankrej cows and heifers following inseminations with frozen semen.

Bulls	No. of inseminations	No. of animals conceived		No. of insem./concep.
		Cows	Heifers	
HF 316	67	16	4	3.35
HF 317	31	6	2	3.87
Total	98	22	6	
Av.				3.50

Table -28. Fertility results for frozen semen from different bulls at various centres.

Bulls	Centre A			Centre B			Centre C			Centre D			Total		
	No. insem	No. preg	Ferti (%)	No. insem	No. preg	Ferti (%)	No. insem	No. preg	Ferti (%)	No. insem	No. preg	Ferti (%)	No. insem	No. preg	Ferti (%)
HF 316	14	5	35.71	20	9	45.00	24	19	79.16	29	13	44.83	87	46	52.87
HF 317	31	19	61.29	8	4	50.00	32	11	34.36	9	5	55.55	80	39	48.75
HF 307	28	14	50.00	6	4	66.66	42	8	19.08	8	5	62.50	84	31	36.90
Total	73	38	52.05	34	17	50.00	98	38	38.77	46	23	50.00	251	116	46.21

FIG-1

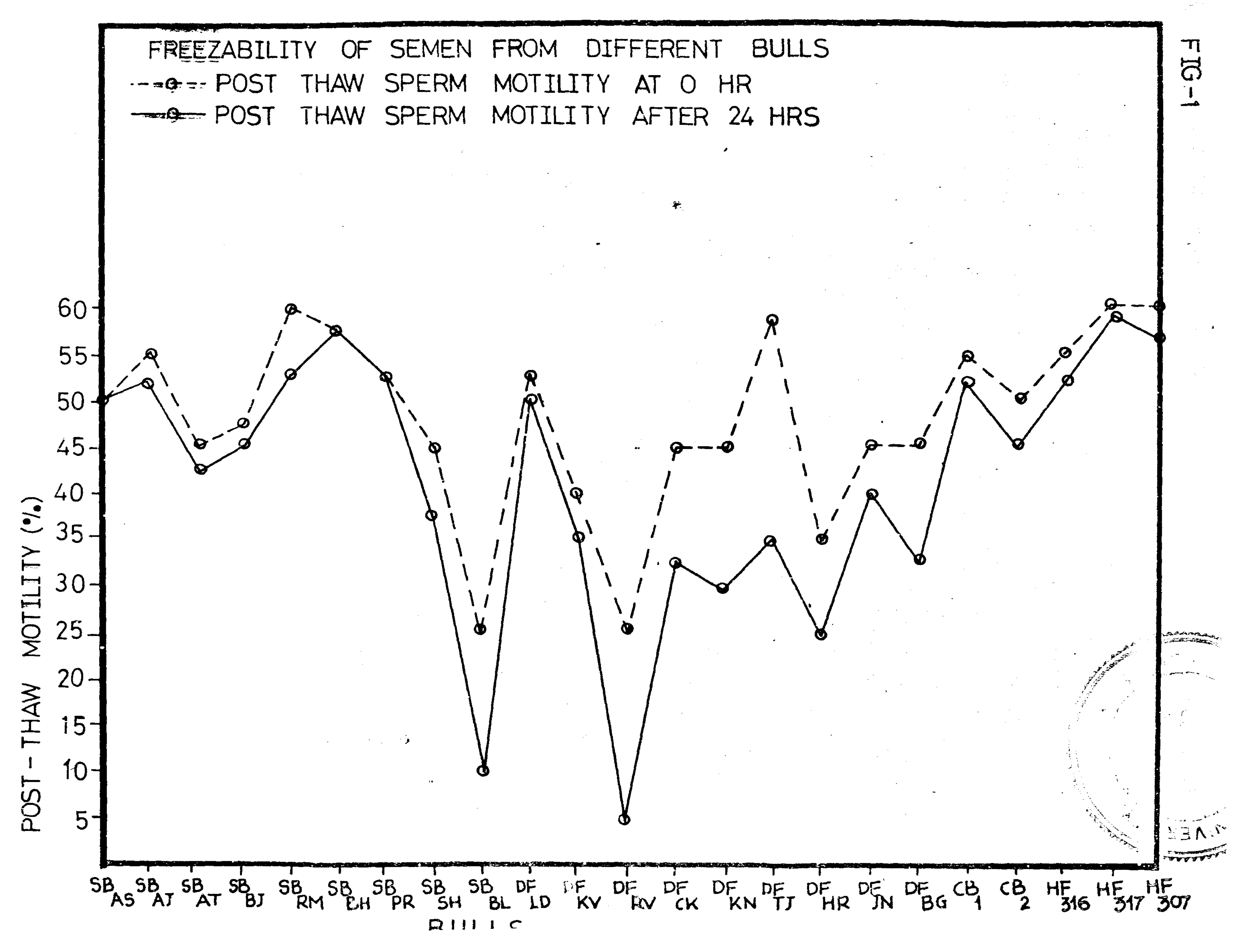


FIG - 2

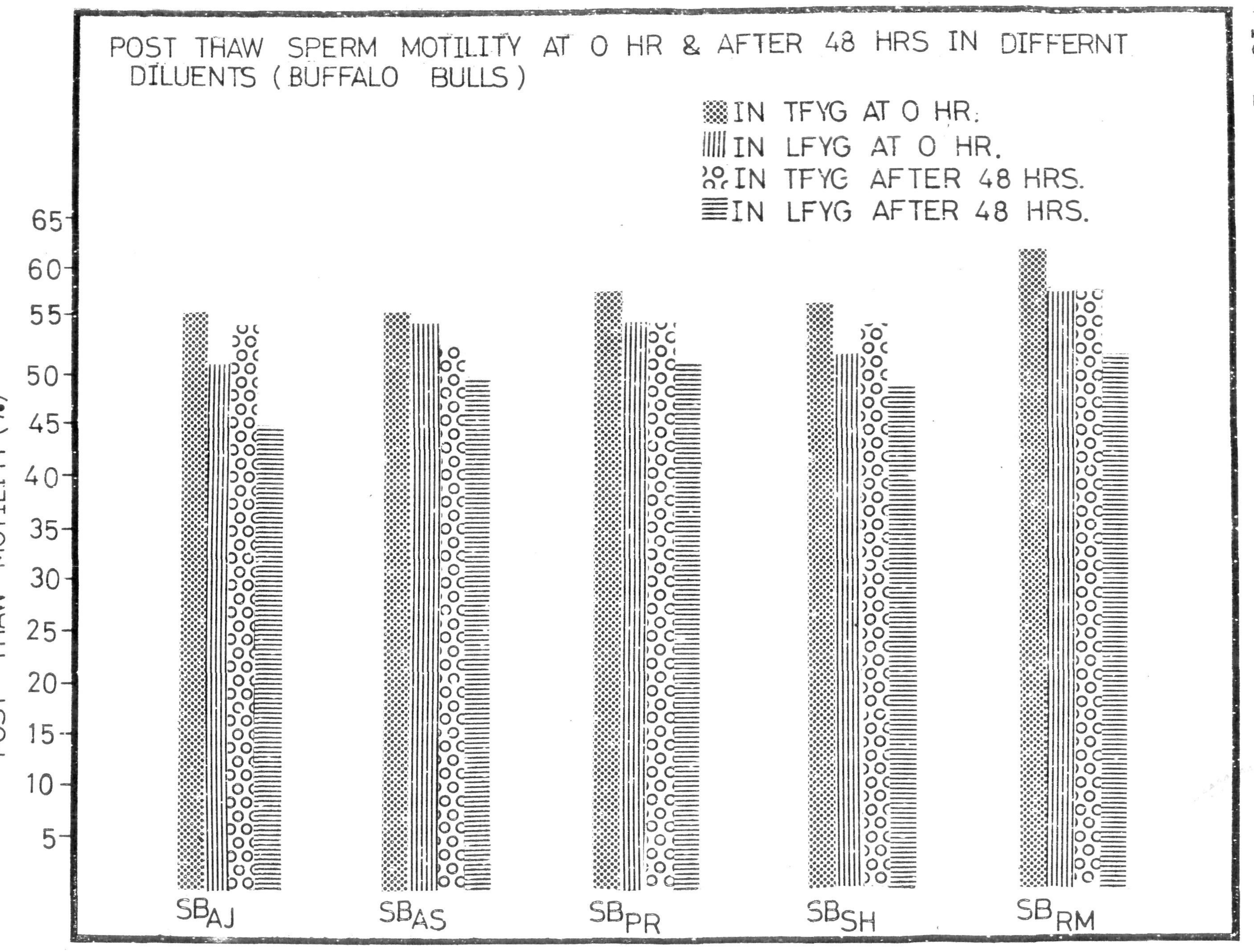


FIG.- 3

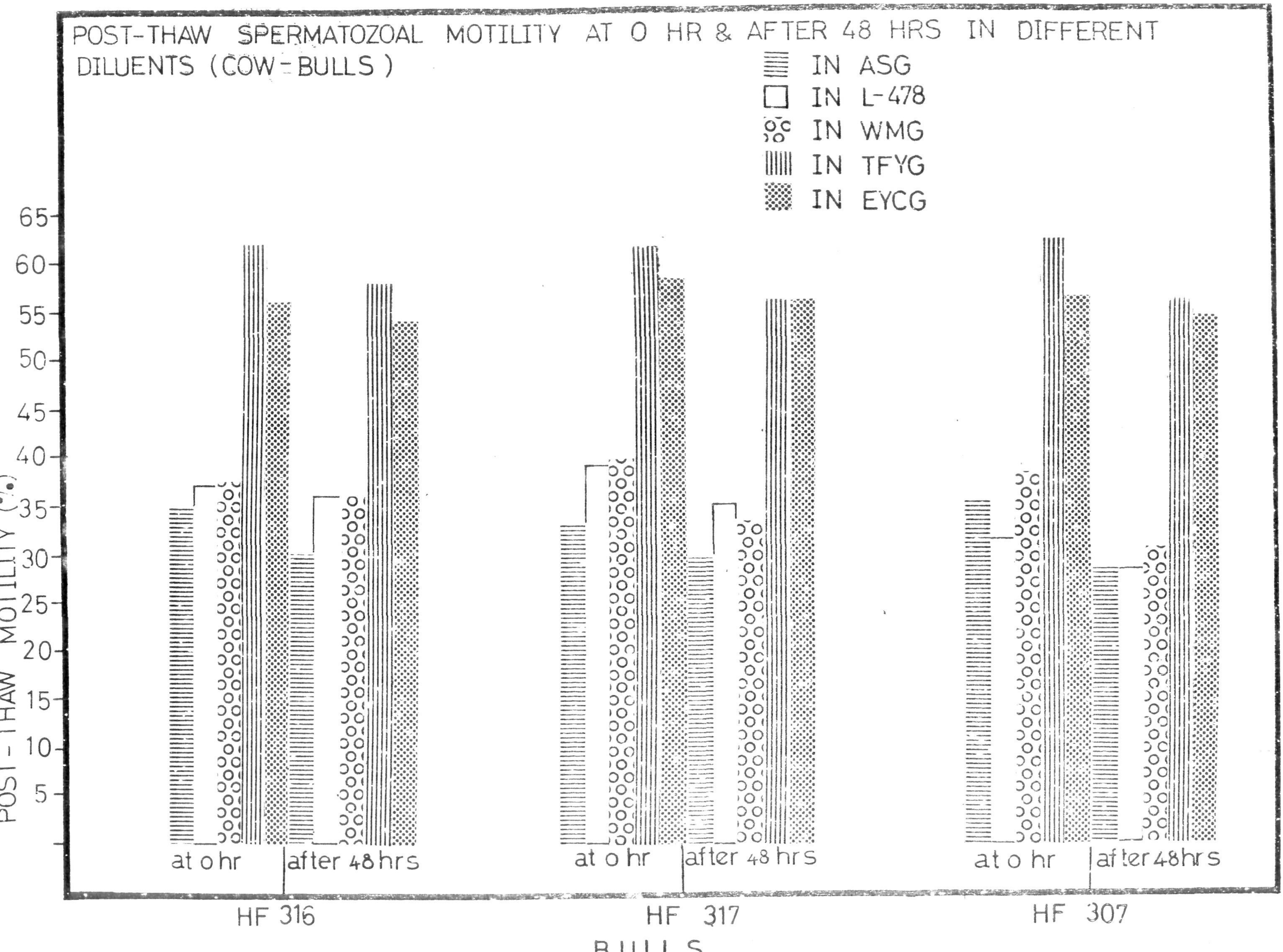


FIG.-4

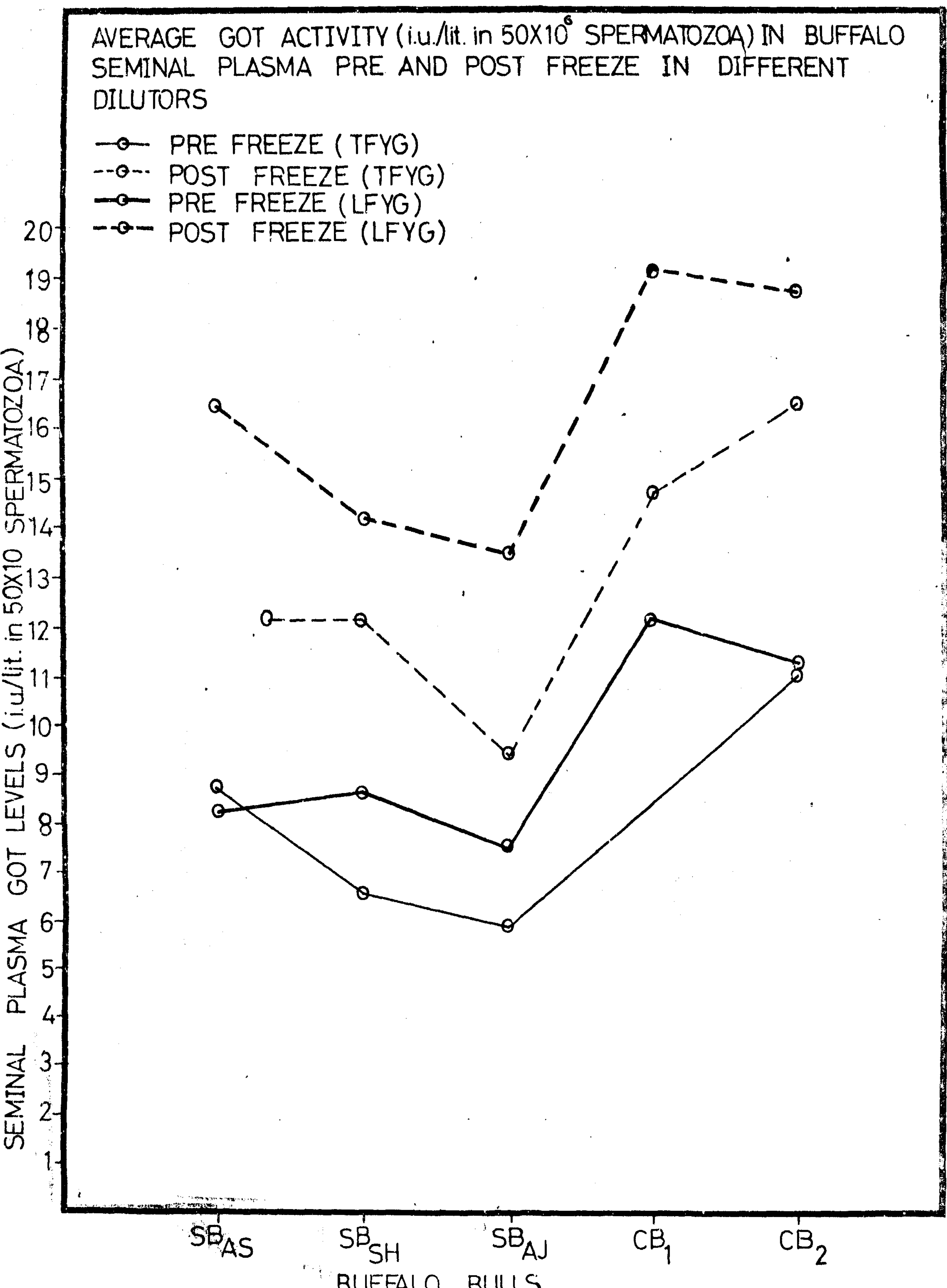


FIG - 5

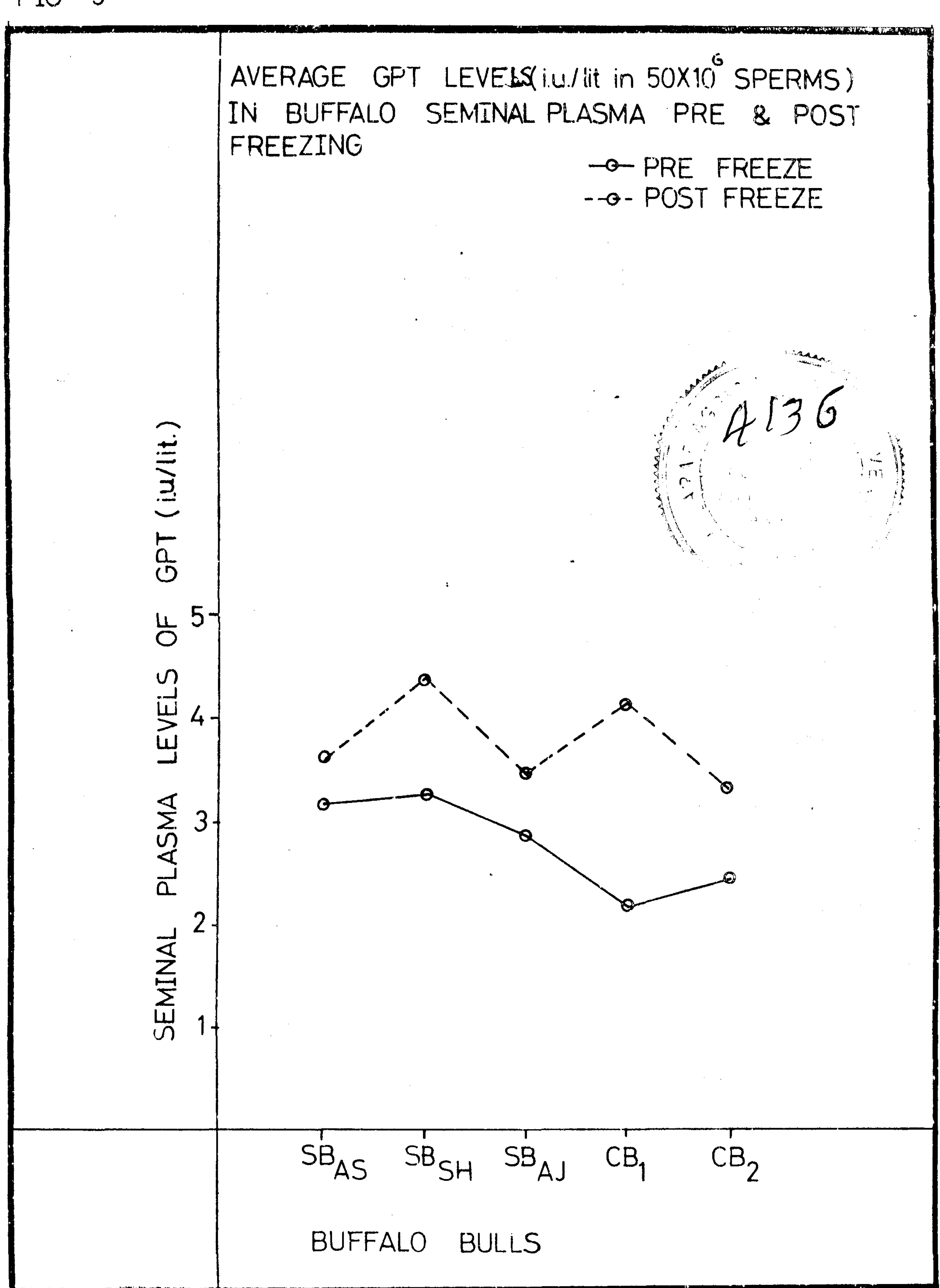


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FIG-6

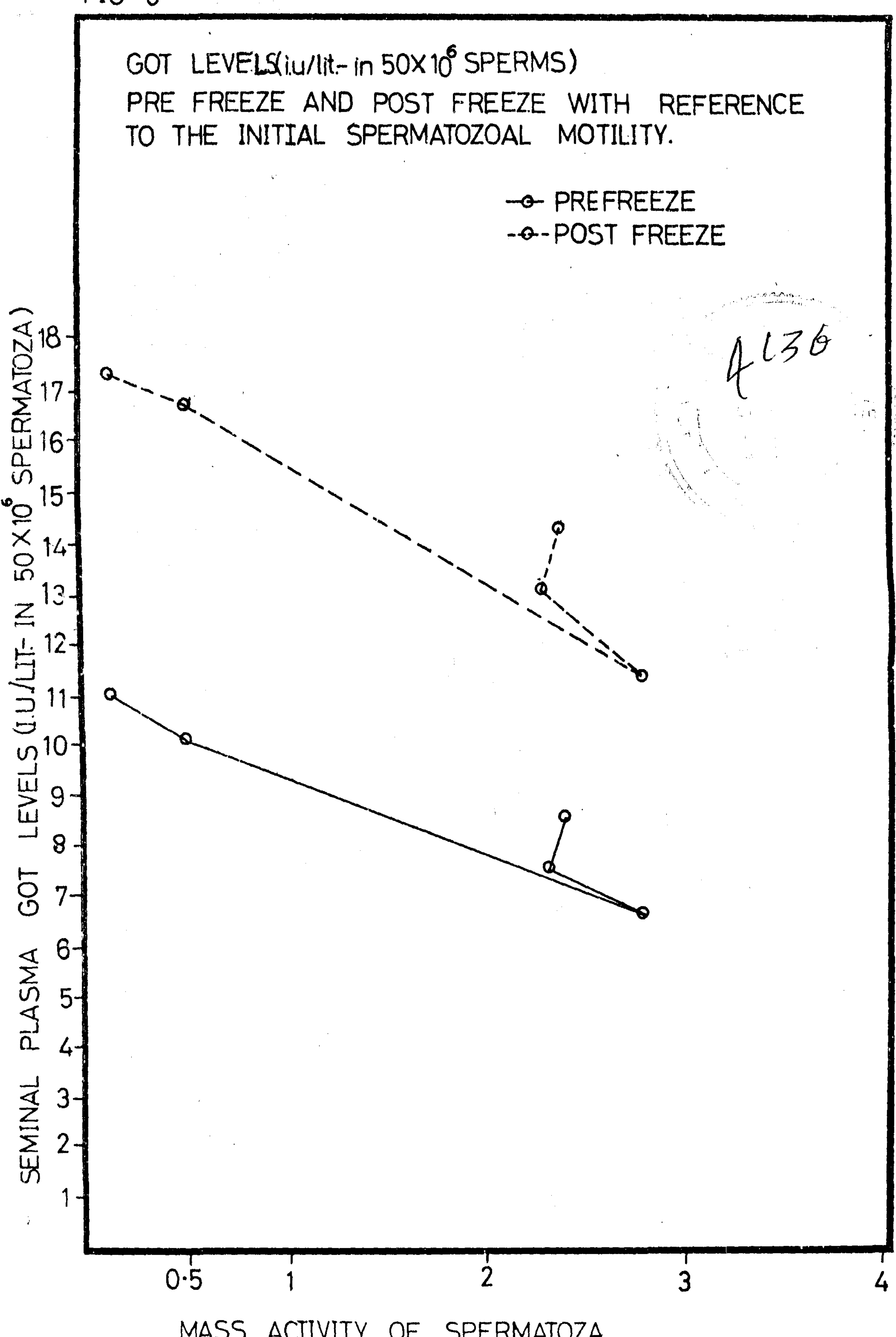
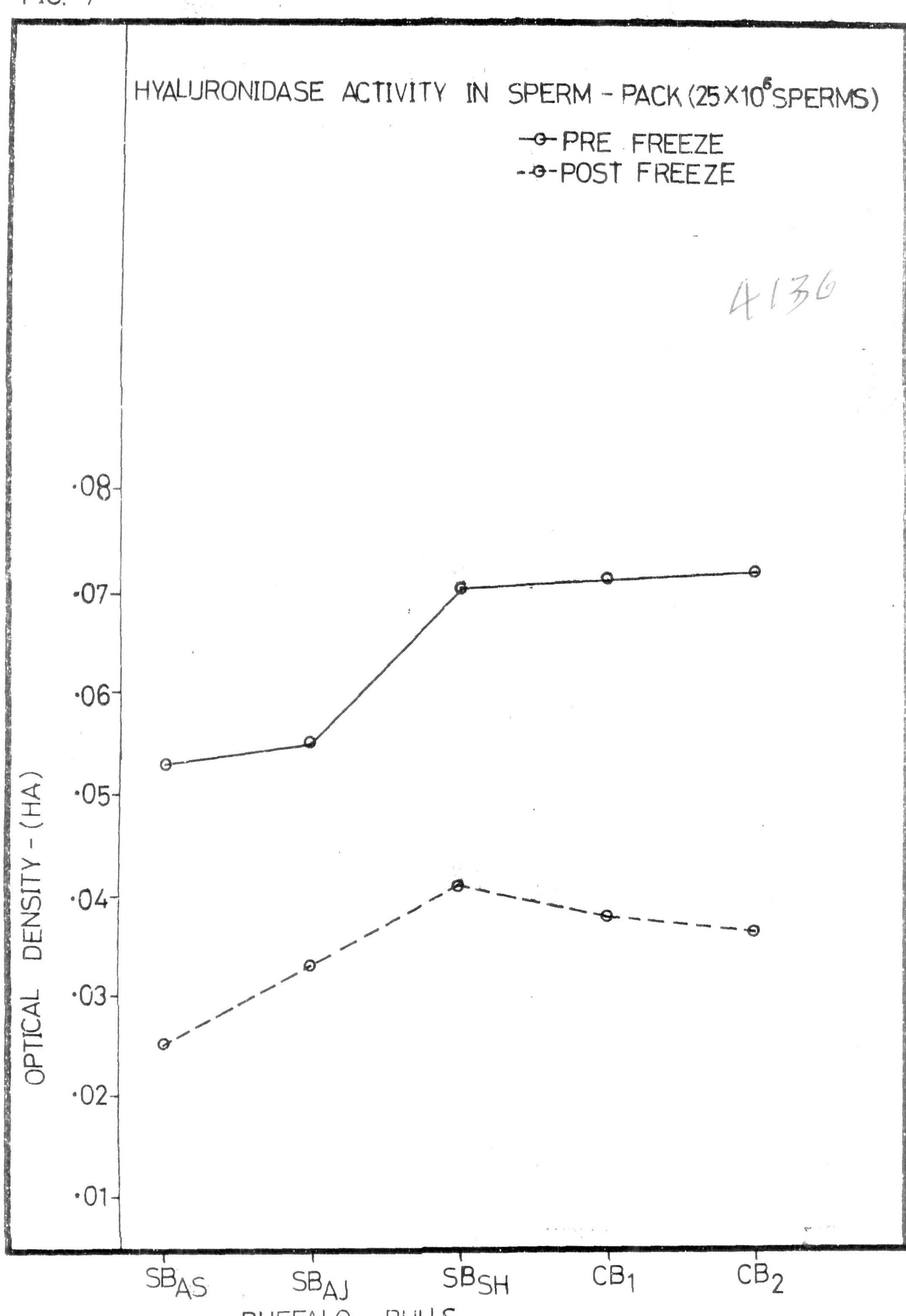


FIG.-7



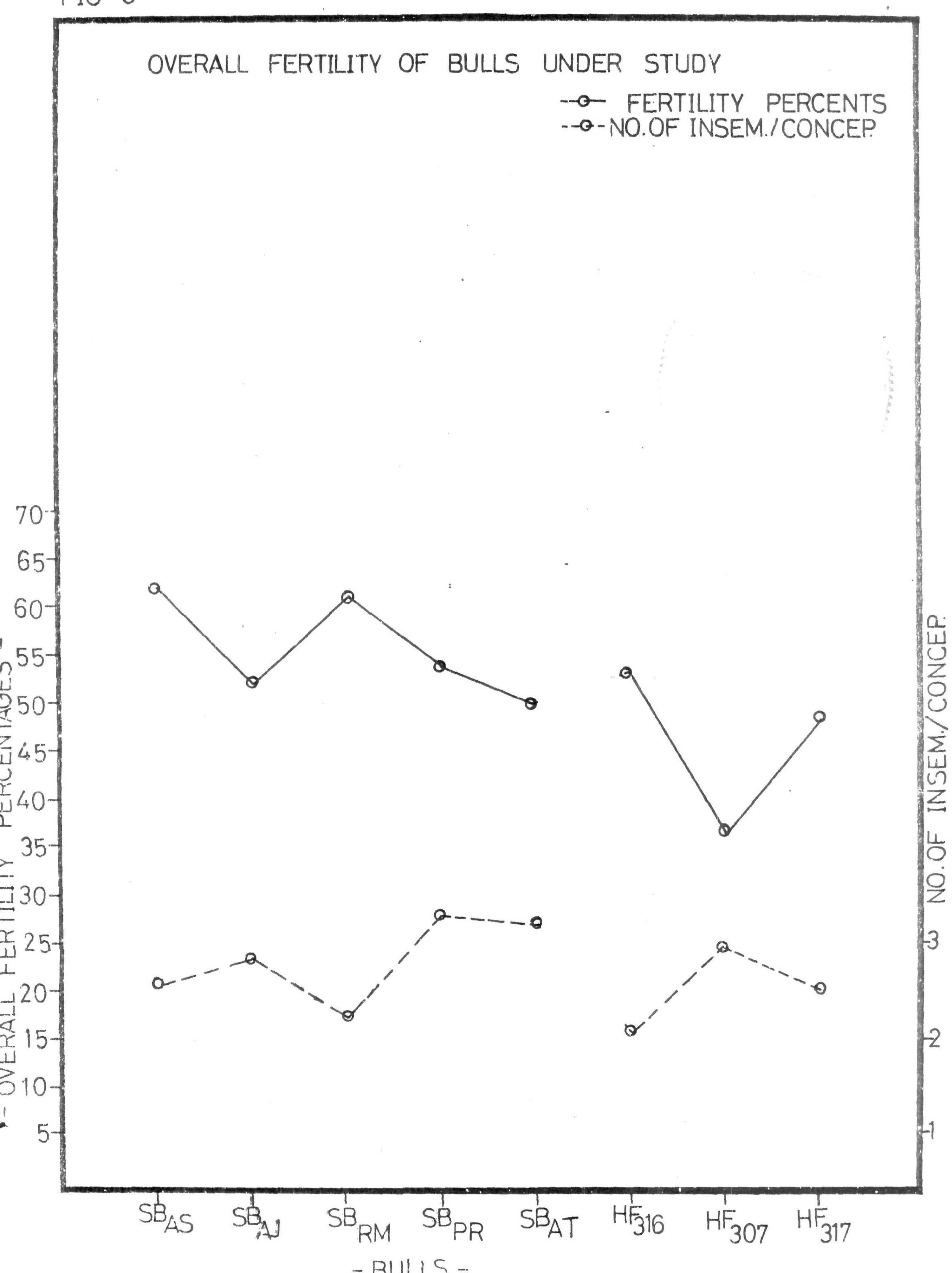


FIG- 8
OVERALL FERTILITY OF BULLS UNDER STUDY
FERTILITY PERCENTS
NO.OF INSEM./CONCEP.
- OVERALL FERTILITY PERCENTAGES -
70
65
60
55
50
45
40
35
30
25
20
15
10
5
NO.OF INSEM./CONCEP.
3
2
1
SB AS
SB AJ
SB RM
SB PR
SB AT
HF 316
HF 307
HF 317
- BULLS -

APPENDIX - II

PREPARATION OF DILUTORS

1. Tris fructose yolk glycerol (TFYG):

Tris buffer:

Tris	:	30.4812 gms
Citric acid	:	17.0000 gms
Fructose	:	12.5000 gms
Add Dist. water	:	850.0000 ml.

Tris buffer	:	740 ml
Egg yolk	:	200 ml
Glycerol	:	60 ml.

2. Lactose fructose yolk glycerol (LFYG):

11%	Lactose	:	56.25 ml
6%	Fructose	:	18.75 ml
	Egg yolk	:	20.00 ml
	Glycerol	:	5.00 ml.

3. AMUL spray glycerol (ASG):

- 16 gms AMUL spray powder (Infant Milk Formula) was suspended in 96 ml of double glass distilled water, heated first to boiling and then cooled to room temperature and filtered.
- 4 ml sodium citrate (2.9%) solution was then added to neutralize the acidity to pH around 6.8.
- The basic suspension as prepared above was divided into two equal fractions A and B.
- To fraction B, 14% glycerol was added. (86 ml basic suspension + 14 ml glycerol).
- The fresh semen was initially diluted with fraction A at room temp.

- Both the fractions gradually cooled to 5°C.
- At 5°C, fraction B was added to fraction A in three-four parts, at 10 mts interval.

4. Whole milk glycerol (WMG):
   - Buffalo milk containing 5% fat was heated at 92°C for 10 mts in a water bath.
   - Heated milk was cooled to room temperature filtered and divided into two equal parts, A and B.
   - To fraction B added 14% glycerol (14 ml glycerol and 86 ml milk).
   - Freshly collected semen was diluted with fraction A at room temperature.
   - Fraction A and B were then cooled gradually to 5°C.
   - Fraction B was added to fraction A in three-four parts at 10 mts interval, at 5°C.

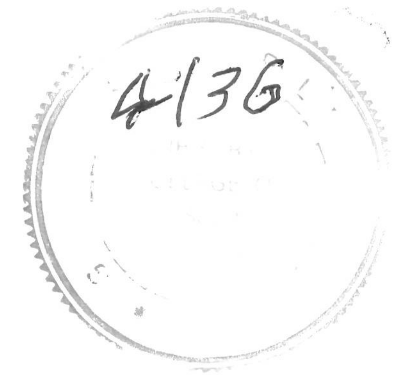
5. Laiciphos-478 (L-478):
   - 50 gms Laiciphos-478 into 400 ml bidistilled water at 40°C.
   - 50 ml fresh egg yolk in 100 ml bidistilled water at 50°C.
   - Egg yolk was mixed into Laiciphos preparation and then divided into two equal parts A and B.
   - Initial dilution was carried out in fraction A at room temperature.
   - To fraction B 14% glycerol was added (86 ml aqueous soln. + 14 ml glycerol).
   - Fraction A and B were cooled to 5°C and then B fraction of diluent was added to A fraction in three-four parts at 10 mts interval.

6. Egg yolk citrate glycerol diluent (EYCG):

2.9% sodium citrate soln - 80 ml
Egg yolk - 20 ml.

- The egg yolk citrate soln. was divided into two equal parts A and B.
- The initial dilution was effected in fraction A at room temperature.
- To fraction B, 14% glycerol was added (14 ml glycerol in 86 ml egg yolk citrate soln.).
- Both the fractions of diluent were cooled to 5°C gradually.
- Fraction B was added then to fraction A in three-four equal parts at 10 mts interval.

To all the diluents 1000 i.u. of Penicillin and 1000 ug of dihydro Streptomycin were added per ml of diluent.



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