CHAPTER III

MATERIALS AND METHODS
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III.1. General

The buffaloes used in the study were from the herd maintained at the Institute for artificial insemination purposes. These bulls were regularly used for collection of semen for the use of different centres distributed in this state as well as in neighbouring states. Semen collections for the experiments were made from 5 Murrah buffaloes. The bulls were trained to mount females which were not in heat.

The usual sterile precautions necessary for all the materials handled were observed. Glassware was washed thoroughly, rinsed with distilled water and sterilised in hot air oven. Rubber goods were boiled in distilled water after cleaning.

Collection of semen was carried out by the use of artificial vagina. The bulls were allowed one or two false mounts before they were allowed to serve in to the artificial vagina. Usually two collections were taken one after another in separate artificial vaginas and each collection was evaluated separately.

The collected semen were subjected to usual laboratory examinations as colour, volume density, pH, mass activity, etc. Only samples which were normal and good mass activity were used for experimental work. Usually first ejaculate was used for further processing if it was agreeable. Otherwise second ejaculate was used.
As the usual technique of cattle semen freezing was not satisfactory for freezing buffalo semen an attempt was made to evolve a new procedure for the freezing of buffalo semen. The various reported diluents with different additives and freezing with different techniques were tried. Finally a skim milk diluent with added sodium citrate and lactose was evolved by which buffalo semen could be successfully frozen. The method is named "Dairysearch method" after the telegraphic code word of the Institute.

II.2. Dairysearch method of freezing of buffalo semen

Diluter:- Fresh milk of cows after separation of cream was used. The skim milk was heated in a water bath to 90-93°C for 10 minutes and cooled slowly before use. The pH of milk was adjusted to 7 by weak sodium hydroxide solution. The diluter was prepared by adding the necessary constituents in the following proportion:

<table>
<thead>
<tr>
<th></th>
<th>First portion</th>
<th>Second portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>50 %</td>
<td>50 %</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>20 %</td>
<td>20 %</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0.33 %</td>
<td>0.33 %</td>
</tr>
<tr>
<td>Lactose</td>
<td>nil</td>
<td>4 %</td>
</tr>
<tr>
<td>Glycerol</td>
<td>nil</td>
<td>14 %</td>
</tr>
<tr>
<td>Water</td>
<td>30 %</td>
<td>16 %</td>
</tr>
</tbody>
</table>

Sodium citrate was added as 6.6% solution, 0.5 ml. for 10 ml. of diluter. Lactose (Analar) was added as powder.
The diluter was prepared fresh every time. For freezing as pellets over solid carbon dioxide, diluter was prepared as a combination of the first and second portions of the above composition giving the same final concentrations of components.

Dilution: The diluter after preparation was kept in warm water till the collection of semen was ready. A thick sample of semen was diluted to get a final dilution rate of 1:10 and a thin sample 1:5. The collected semen was diluted to half the final volume by the first portion of the diluter and then examined for motility. The tubes containing diluted semen were placed in a beaker of water at the same temperature. Care was taken to keep the level of water above the level of diluted semen. For effecting better cooling only small quantities (5 ml.) were kept in long and narrow test tubes of about 15 ml. capacity. The beaker of water with semen tubes was kept in the refrigerator and cooled over a period of one hour to 4°C, by adding pieces of ice at intervals. Care was taken to avoid sudden fluctuations of temperature.

Equilibration: After one hour of cooling the diluted semen, equal quantity of the second portion of the diluter which was precooled to the same temperature was added to each tube. The content of the tube was mixed by gentle rotation and by pouring back and forth between the tubes slowly. After mixing, it was kept for 5 hours at 4°C for equilibration. Diluted semen which are to be frozen as pellets were
equilibrated for 5 hours.

The equilibrated semen was examined for motility at the end of the period when it was transferred to small glass ampoules, which were precooled, either directly from the test tubes or by means of a cooled automatic delivery syringe which can deliver pre-set quantities of fluid. About 1 ml was poured to each ampoule. The ampoules were tightly closed by rubber corks. Variation in temperature was avoided as far as possible during the process of pouring and corking. The ampoules were previously marked by suitable ink, when they are for storage purposes. Otherwise, the corks of the ampoules were marked by permanent ink when they are for routine trials only.

III.2.a. Freezing in alcohol bath

As the container for the bath, an ordinary wide mouthed thermosflask was used when there are only few ampoules to be frozen. Otherwise a wide mouthed aluminium container having thermocole insulation was used as the container. Sufficient alcohol was poured in the container to keep the ampoules submerged at least one inch below the alcohol level, after they were transferred. A thermometer which could read up to or below -80°C was placed in the bath and the temperature of alcohol was brought down to +5°C by adding small pieces of dry ice. The ampoules containing the equilibrated semen was transferred to this bath and the temperature was again brought down by adding pieces of dry ice. The temperature fall was adjusted by adding small
pieces of dry ice at a time and noting the temperature and time by the thermometer and a stop watch respectively. The following schedule of temperature let down was adopted:

- From +5°C to -6°C, at the rate of 1°C drop per minute,
- From -6°C to -12°C, at the rate of 3°C drop per minute,
- From -12°C to -32°C, at the rate of 4°C drop per minute,
- From -32°C as fast as possible.

After the temperature had reached near -72°C and remained at that level for some time, few ampoules were taken out and thawed and examined for motility. Ampoules which were to be stored were transferred directly to Linde flasks containing liquid nitrogen.

For studying the effect of storage on motility, ejaculates collected from bulls whose semen have got good freezability, was frozen by this method and stored in liquid nitrogen. Samples were examined occasionally after thawing during a period of 30-90 days storage. The motility ratings immediately after freezing were compared with those after storage for 30-90 days by analysing the data by paired t-test (Snedecor, 1956).

III.2.b. Freezing in liquid nitrogen vapour

For this purpose a wire mesh rack with a long vertical handle was used. An ampoule half filled with alcohol was fixed on to the handle vertically, keeping the bottom of the ampoule at the same level with the rack. A thermometer which could record temperatures below -100°C was placed in the ampoule,
keeping the bulb immersed in the alcohol and the thermometer was fixed to the handle.

Liquid nitrogen was poured in to a wide mouthed insulated jar. The rack was suspended above the liquid nitrogen jar by fixing the handle of the rack to a stand having arrangement for lowering and lifting the rack by adjusting screws. The rack with the thermometer was cooled to -5°C by the liquid nitrogen vapour and the corked ampoules containing equilibrated semen was placed horizontally over the rack.

Temperature fall of the ampoules as recorded in the thermometer was controlled by adjusting the height of the rack from the level of liquid nitrogen and noting the time from the stop watch. Trials were conducted by adjusting the temperature fall at 5-7°C as well as 10-12°C per minute. After the temperature decreased below -50°C the thermometer was removed and the whole rack with the ampoules were dipped into the liquid nitrogen. After allowing 5-10 minutes, few ampoules were thawed and the semen was examined for motility. Ampoules which were to be stored were transferred directly to the Linde container having liquid nitrogen.

Samples of equilibrated semen from the same lot were frozen simultaneously in alcohol bath and were used as controls for comparison with vapour freezing. The survival rates in the two methods of freezing were compared by paired t-test (Snedecor, 1956).
Ampoules containing equilibrated semen were placed on powdered dry ice, contained in a wide mouthed flask, in a single layer and covered with a layer of powdered dry ice. More than 15 minutes were allowed for the ampoules to freeze inside sufficiently thick layer of powdered dry ice. Then few ampoules were thawed and examined. Frozen ampoules which were to be stored were transferred to liquid nitrogen container.

Samples of the same lot of equilibrated semen were frozen in alcohol bath to be used as control and the survival rates in both techniques of freezing were compared by paired t-test (Snedecor, 1956).

III.2.d. Freezing as pellets

Semen after collection and preliminary examination was split into two and one sample was diluted and processed for freezing in alcohol bath as described previously. This was used as control. The other sample of semen was diluted in a diluent containing glycerol and having the same final concentration of components as that of the diluter previously described. The diluted semen was cooled to 5°C slowly by keeping it in a refrigerator for 5 hours.

After the equilibration period was over the diluted semen was dropped on the surface of solid carbon dioxide. Small depressions were made on the surface to hold the drops. Each drop was about 0.15 ml. After allowing a time of about 10 minutes for the drops to freeze, they were transferred to
ampoules containing small quantities of the diluter. The ampoules were closed and thawed by keeping them in warm water for 2-3 minutes.

The survival rates in the two treatments were compared by analysing the data by paired t-test (Snedecor, 1956).

III.2.e. Thawing

Thawing of frozen ampoules were done by immersing in warm water of about 38°C for 3-4 minutes with occasional mixing up of the contents of the ampoules. Drops of warm semen were examined under cover slip on a warm stage of a microscope.

For each treatment at least two ampoules and from each ampoule at least two samples were examined. All the samples were examined by two or more personnel working in the laboratory and when there was a discrepancy in the motility ratings by different individuals, the average of the ratings only were recorded against that treatment.

The quality of semen after thawing was recorded as motility percentage by visually assessing the percentage of progressively motile spermatozoa in the samples. From this motility ratings "survival percentages" were calculated as the percent spermatozoa having progressive motility compared to the initial motility, that at the time of collection: i.e.,

\[
\text{Percent survival} = \frac{\text{percent motility after freezing}}{\text{percent motility at collection}} \times 100
\]
III.2.f. Differential staining

The procedure of staining by nigrosin-eosin recommended by Campbell et al. (1966) was used in the study for the differential staining of live and dead spermatozoa. The stain consisted of 10 grams nigrosin and 1.67 grams eosin-Y dissolved in 100 ml. of distilled water. Equal quantities of stain and semen sample were mixed and smears prepared from that after allowing about two minutes of time.

III.3. Fertility studies with frozen semen

Inseminations of the female buffaloes were carried out at the Key village centre attached to the Institute. Inseminations were conducted with frozen semen stored for different periods in liquid nitrogen. The thawed samples of semen were examined on the spot for motility and only samples which had motility ratings of more than 30% were used for inseminations. Inseminations were done for female buffaloes without selection.

Inseminations were carried out during the period of November, 1968 to March, 1969. It was not done on all days but only when it was convenient for the work. Thus during the period of November to March, both liquid semen as well as frozen semen were used for inseminations at the Centre and as such the inseminations by the liquid semen could be used as control for the experimental data.

Sixty-ninety day nonreturn rates were calculated for both frozen semen and liquid semen inseminations. The frozen semen used was that from two bulls (SVR and 638) whose semen
had good freezability. Non return rates were calculated for the period for those two bulls for both frozen and chilled semen, as well as for all the bulls put together in the case of liquid semen inseminations.

### III.4. Effect of lactose, citrate and glycerol

After trials with different levels of sodium citrate \((2\text{H}_2\text{O})\) and lactose added to skim milk, the best combinations were selected. A comparison of these with milk alone as well as a combination of both lactose and citrate were tried, thus making four types of diluters. Each of these were tried with three levels of glycerol \((5, 7\) and \(9\%\)). All the combinations were tried by freezing as pellets with five hour equilibration and in alcohol bath with 5 and 18 hour equilibrations. The composition of the diluents were:

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Component</th>
<th>1st portion (%)</th>
<th>2nd portion (%)</th>
<th>Pelletting (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk (M)</td>
<td>Milk</td>
<td>80</td>
<td>62</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Yolk</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>(\ldots)</td>
<td>10,14 &amp; 18</td>
<td>5,7 &amp; 9</td>
</tr>
<tr>
<td>Milk-Citrate (MC)</td>
<td>Milk</td>
<td>65</td>
<td>47</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Yolk</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Citrate</td>
<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>(\ldots)</td>
<td>10,14 &amp; 18</td>
<td>5,7 &amp; 9</td>
</tr>
<tr>
<td>Milk-Lactose (ML)</td>
<td>Milk</td>
<td>80</td>
<td>62</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Yolk</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Lactose</td>
<td>(\ldots)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>(\ldots)</td>
<td>10,14 &amp; 18</td>
<td>5,7 &amp; 9</td>
</tr>
<tr>
<td>Milk-Lactose-Citrate (MLC)</td>
<td>Milk</td>
<td>65</td>
<td>47</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Yolk</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Lactose</td>
<td>(\ldots)</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Citrate</td>
<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Glycerol</td>
<td>(\ldots)</td>
<td>10,14 &amp; 18</td>
<td>5,7 &amp; 9</td>
</tr>
</tbody>
</table>
The volume in each case was made up to 100 with glass distilled water wherever it was necessary.

Semen collections were made from all bulls without any selection, according to the routine allotment of bulls in the artificial insemination centre. Six freezing were done for each treatment and as far as possible split samples were used in each group of treatments. The rate of dilution was at varying levels.

The motility percentages after thawing under different treatments were compared by analysing the data in a 4x3x3 factorial design.

III.5. Level of citrate and glycerol in yolk citrate diluent

In a 3x4x3 factorial experiment 3 levels of sodium citrate(2H₂O) and 4 levels of glycerol were tried in a yolk-citrate diluent. Each combination of the diluter was frozen in alcohol bath with 5 and 13 hours equilibration and as pellets with 5 hours of equilibration. Each treatment was repeated thrice and average motility after freezing in each case was recorded. Split samples of ejaculates were used in each group of treatments and no selection was done regarding the bulls.

Levels of sodium citrate used were 2.5, 2.0 and 1.6 % and final glycerol concentrations were 5, 7, 9 and 11 %. 30 % yolk was used for each treatment. Other details were as described earlier. Dilution rates of semen were not fixed and in many cases were about 1:20.
III.6. Comparison of three freezing techniques

To compare the efficiency of pellet freezing and alcohol bath freezing with 5 as well as 18 hours of equilibration periods, the data on survival after freezing in items III.4 and III.5 were tabulated accordingly. The data in item III.4 gives survival rates in four different milk diluents with three levels each of glycerol. The data in item III.5 gives survival rates in three levels of citrate with four levels of glycerol combinations. In both cases no selection regarding bulls from which semen was collected was made.

III.7. Variation in freezability between semen of different bulls

To find the amount of variation in freezability of semen of different bulls, the motility ratings after freezing in different treatments in item III.4 were tabulated for each of the five bulls. The average motility after freezing in case of each bull for different diluters and techniques of freezing were calculated.

III.8. Levels of milk solids and glycerol

A 3x3 factorial experiment was designed to study the effect of different levels of milk solids, levels of glycerol and the possible interaction between them. For adjusting the solids, reconstituted milk prepared from buffalo skim milk powder was used. Reconstituted milk was prepared with milk solids levels of 7, 10 and 13%. In each level 7, 10 and 13% glycerol levels were combined. The composition of the diluent for each milk solid level was as follows:-
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<table>
<thead>
<tr>
<th></th>
<th>First portion (%)</th>
<th>Second portion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Glycerol 7% 10% 13%</td>
</tr>
<tr>
<td>Milk</td>
<td>75</td>
<td>61 55 49</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>0.33</td>
<td>0.33 0.33 0.33</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>20</td>
<td>20 20 20</td>
</tr>
<tr>
<td>Glycerol</td>
<td>..</td>
<td>14 20 26</td>
</tr>
<tr>
<td>Water to 100</td>
<td>..</td>
<td>.. .. ..</td>
</tr>
</tbody>
</table>

Split samples of ejaculates were used for all the treatments and four repetitions were carried out from different bulls. Freezing was done in alcohol bath with five hours of equilibration time. The dilution rate of semen used was 1:10.

Fresh skim milk of cows were used along with as a control as in the case of Dairysearch method. The motility rating after freezing from each treatment was recorded and percent survival in each case was calculated.

**III.9. Levels of milk and yolk**

a) The effect of fresh skim milk at three different levels were tried in order to find out the optimum level. Keeping the levels of yolk(20%), sodium citrate (0.33%), lactose (2%) and glycerol (7%) constant, the level of milk was varied to 60, 50 and 40%. Freezing was carried out in alcohol bath similar to Dairysearch method.

Five repetitions of the treatment were carried out with split samples of ejaculates from bulls whose semen has got good freezability and the percentage survival in each case after freezing were recorded.
The data were analysed by Analysis of Variance technique (Snedecor, 1956).

b) A 2x2 factorial experiment was carried out to explore the possible interaction of levels of milk with levels of yolk. Two levels of milk with two levels of yolk in the diluter were tried. Composition of the diluter and other procedures were same as in (a) above.

The data were analysed by the Analysis of variance technique in a 2x2 factorial experiment.

III.10. Effect of pH of milk on freezability

The pH of milk to be used for the preparation of diluter was altered and its effect on freezability of spermatozoa was studied. The pH of the heated milk was altered by the addition of dilute sodium hydroxide or citric acid solution. The pH was read by bromothymol blue indicator by comparing against standard colours (supplied by B.D.H.). Colour standards with 0.2 pH difference were available.

The pH of the milk was adjusted to 6.6, 7.0 and 7.4 and the diluters were prepared from them. Other procedures were same as that of Dairysearch method. The fresh heated milk usually recorded a pH around 6.6. Milk without any adjustment of pH was used as control. Freezing was done in alcohol bath.

Split samples of ejaculates were used for the different treatments and five repetitions were carried out with ejaculates collected from bulls whose semen has got good freezability. Percentage survival in each case was recorded.
III.11. Comparison of cow and buffalo milk as diluter

Skim milk of cows and buffaloes were used separately for the preparation of diluters as per Dairysearch method. Split samples of ejaculates were used for the two types of diluters. Semen was collected from bulls whose ejaculates were having good freezability and frozen in alcohol bath as per Dairysearch method. Five repetitions were carried out for both treatments and the percentage survival in each case were averaged.

III.12. Comparison of Dairysearch, citrate-yolk and lactose-yolk diluters

III.12.a. Freezability

Citrate-yolk-glycerol and lactose-yolk-glycerol diluters were compared in freezability with Dairysearch diluter. For the citrate-yolk-glycerol diluter 2.9% sodium citrate solution, 25% egg yolk and 7% glycerol were used. For lactose-yolk-glycerol diluter 11% lactose solution, 25% egg yolk and 7% glycerol were used. Except for the change in diluters other procedures for freezing were same as that of Dairysearch method.

Split samples of ejaculates were used for different treatments and no selection was done as for the bulls. Four replications were done with semen of four different bulls and the motility of semen after dilution, after equilibration and after freezing were recorded and averaged.
III.12.b. Abnormalities

The effect of diluters, processing and freezing technique on the morphology of the spermatozoa and the increase in the abnormalities due to these treatments were studied from the trials described in section III.12.a. above. The abnormalities looked into were detached head, bent tail and coiled tail. For observing the abnormalities, one drop of semen was mixed with one drop of nigrosine-eosine stain (Campbell et al., 1956) over a warm slide and smears were prepared from it after allowing about two minutes time. These films were examined under oil immersion lens of a microscope and a minimum 300 sperms from different portions of the smear were examined. The percentage of each type of abnormality was worked out.

The abnormalities were recorded from fresh semen, after equilibration and from frozen semen. The increase in the abnormalities from the initial were calculated for different treatments.

III.13. Effect of concentration of sperms on freezability

Using the diluter and freezing procedure of Dairysearch method the effect of varying concentrations of spermatozoa on freezability was evaluated. The semen immediately after collection and examination was tested for concentration of spermatozoa by counting, using a haemocytometer. After determining the concentration of sperms, it was diluted to contain final concentrations of 10, 25, 50, 100 and 150 million sperms per ml. of diluted semen. The semen was
equilibrated and frozen in alcohol bath as in Dairysearch method. The survival rates were expressed in terms of percent survival.

Split samples of ejaculates were used for each set of trials and five repetitions were made from selected bulls having good freezability of semen. The data were analysed by the Analysis of Variance technique (Snedecor, 1956).

III.14. Effect of seminal plasma on freezability

The role of seminal plasma with reference to freezability of semen was examined by centrifuging out the seminal plasma and comparing its freezability with whole semen. Collected semen after preliminary examination was subjected to 15 minutes centrifugation. The supernatent plasma was removed and sperms were mixed with necessary quantity of diluter. The control sample of semen was also subjected to the same amount of centrifugation but it was diluted without the removal of seminal plasma.

Composition of the diluter, processing etc., were same as in the case of Dairysearch method. Semen was collected from different bulls without selection and five repetitions of the trials were conducted with split samples of ejaculates. The survival percentages in the two types of treatments after freezing were recorded. The effect of two treatments were compared by 'paired t-test' (Snedecor, 1956).

III.15. Effect of addition of antibiotics on freezability

The possible beneficial effect of addition of antibiotics to the diluter on the freezability of semen was evaluated.
Penicillin G sodium and Dihydrostreptomycin sulphate were added to the diluent at the rate of 5,000 I.U. and 5000 microgram per ml. respectively. No antibiotics were added to the control.

Composition of the diluter and other processing methods were same as in Dairysearch method.

Semen from different bulls were used without selection and split samples of ejaculates were used for both treatments. Five replications were tried and survival percentages after freezing in both treatments were tabulated.

**III.16. Effect of EDTA treated milk on freezability**

Cows' skim milk was treated with 0.1, 0.2, 0.3 and 0.4 ml. of a solution of 0.2 M ethylene diamine tetraacetic acid (EDTA) per 10 ml. of milk. The final concentration of EDTA in milk was at the rate of 0.2, 0.4, 0.6 and 0.8 mM EDTA per ml. The milks thus treated were used for the preparation of diluter as per Dairysearch method. The control consisted of fresh skim milk used as in Dairysearch method.

Four repetitions of the trial were conducted from semen collected from different bulls without selection and the percentage survival in each treatment were recorded. The results were compared by the analysis of variance technique.

**III.17. Effect of dimethyl sulphoxide as cryoprotectant**

Dimethyl sulphoxide was tried instead of glycerol in the preparation of diluters at final concentrations of 3, 6, 9 and 12 % levels. The diluters were prepared as in Dairysearch method. The diluted and equilibrated semen was
frozen over liquid nitrogen vapour at different equilibration periods of 15 minutes, 45 minutes, 1 hour and 15 minutes, 2 hours, 3 hours and 5 hours. Three replications were carried out for each treatment combinations with split ejaculates.

Control semen from split samples from each of the replications were frozen as in Dairysearch method with 5 hours of equilibration period over liquid nitrogen. The rate of cooling in all the cases were at 5-7°C per minute. Only ejaculates from bulls whose semen has got good freezability were used.

III.18. Method of addition of glycerol

The time interval between dilution and glycerelation and the temperature at the time of glycerol addition and their effect on freezability were studied. Four methods of glycerol additions were tried:

a) incorporating glycerol in the diluent as for pelleting,
b) dilution of semen to half its final volume with the nonglycerol portion followed in five minutes with full dilution with glycerol containing portion and the diluted semen cooled to 5°C in one hour and equilibrated for 5 hours,
c) dilution was done as in (b) above but cooling was done slowly over a period of 4-5 hours,
d) control, as in the case of Dairysearch method.

Split samples of ejaculates were used for the four treatments and all the samples were frozen together in alcohol bath and the survival percent in each case was recorded.
Five repetitions of trial were conducted with semen from selected bulls. Other details were as in Dairysearch method.

The effect of the four treatments were compared by analysing the data by the method of Analysis of variance technique (Snedecor, 1956).

III.19. Effect of different equilibration periods

In order to assess the effect of different equilibration periods on the freezability, semen was frozen with 3, 5 and 7 hours of equilibration periods. Freezing was done over liquid nitrogen vapour with a cooling rate of 10-12°C per minute. The experimental samples were compared with control samples frozen in alcohol bath with 5 hour equilibration. The percentage survival in each case after freezing were recorded.

Semen collected from bulls whose ejaculates had good freezability only were used for the trials. Split samples of ejaculates were used for different treatments and five replications were carried out. Other details of the procedure were those of Dairysearch method. The survival rates in the different treatments were compared by Analysis of Variance technique (Snedecor, 1956).

III.20. Effect of different rates of freezing

To find out whether the rate of freezing has got any effect on freezability, two rates of freezings were tried:

1) at the rate of 5-7°C drop per minute,
2) at the rate of 10-12°C drop per minute. The two rates were tried by freezing over liquid nitrogen. This was
compared with control which was frozen in alcohol bath with dry ice. The diluters and other details of processing were same as described for Dairysearch method. The survival percentages after freezing for different treatments were recorded.

Split samples of semen collected from selected bulls were used for the study and five replications were carried out. The survival percentages in the two rates of freezing were compared by t-test (Snedecor, 1956).

III.21. Starting temperature of alcohol bath

In the case of alcohol bath freezing, the temperature at which the bath could be set to start with was tried at varying temperature levels. The levels tried were +5°C, -10°C, -20°C, -30°C and -70°C. The equilibrated semen according to Dairysearch method was used. The freezing was started with bath set at +5°C having ampoules of equilibrated semen to be used as controls and as the freezing proceeded, further sets of ampoules were placed in the bath when the temperature reached the above mentioned levels. The survival percent in each treatment was recorded from the frozen-thawed semen.

Semen from selected bulls only were used with split samples of ejaculates for the different treatments. Five replications were carried out.

The data were analysed by Analysis of Variance technique (Snedecor, 1956).

III.22. Storage method of frozen semen

Two methods of storage of ampoules which were frozen in alcohol bath were tried. In one case the ampoules after
freezing were kept in the bath itself with sufficient quantity of dry ice for overnight storage. In the other case the frozen samples were transferred to liquid nitrogen containers for storage. Both the samples were examined for motility together on the next day and these rates were compared with that of the motility just after freezing.

Five replications were carried out with semen collected from selected bulls. The survival percentages in the two methods of storage were compared by paired t-test (Snedecor, 1956).

III.23. Motility of frozen semen kept at 38°C

To determine the viability of frozen thawed semen at 38°C, samples of semen frozen and stored for different periods were thawed and examined at intervals while keeping them in a rack in water bath at 38°C. The samples were examined for motility just after warming (0 time) and after 5, 15, 30, 45 and 60 minutes. Ten samples of semen of different periods of storage were examined and the motility recordings were averaged. The averages were plotted on a graph.

III.24. Motility of frozen-thawed semen kept in ice water

Buffalo semen which were frozen by Dairysearch method and stored in liquid nitrogen for periods of 3 to 5 months were thawed in warm water and examined for motility. The ampoules of semen after examination were closed tightly with rubber corks and kept vertically in racks and kept partially immersed in tap water. The temperature of water was brought down to 5°C within 10-15 minutes by the addition of ice.
Samples from each ampoules were examined after warming at half hour intervals, upto four hours. A total of ten frozen samples were examined and the averages plotted on a graph.

III.25. Methylene blue reduction time of frozen semen

The procedure given by Beck and Salisbury (1943) for the methylene blue reduction time of semen was followed with slight modifications. Methylene blue solution was prepared by dissolving 50 mg. methylene blue in 100 ml. sodium citrate solution (dihydrate, 2.96%). A quantity of 2 ml. of diluted semen (in milk diluent or yolk-citrate as the case may be) was placed in test tubes of approximately 1 cm. outer diameter and 0.1 ml. of methylene blue solution was added and mixed. The contents of the tube was sealed by the addition of half inch layer of liquid paraffin at the top and kept in water bath at 45°C. The time taken to decolourise the methylene blue was noted.

The dilution rate of the semen in all the treatments were 1:10. The following treatments were tried:

i) frozen semen: frozen by Dairysearch method in alcohol bath and stored in liquid nitrogen overnight,

ii) Sample of diluted and equilibrated semen from the previous treatment which was stored overnight in refrigerator,

iii) Semen diluted in yolk-citrate diluter and stored in refrigerator overnight,

iv) Sample of diluter prepared for treatment (i) and (ii) without addition of semen.
All the four treatments were kept in water bath at the same time. Five replications of the trial were carried out with semen from selected bulls and the data were analysed by Analysis of Variance technique (Snedecor, 1956).