Preservation of Buffalo-Bull Semen in Various Diluents containing Skimmed Milk And its Effect on Spermatozoal Livability & Metabolism

A Thesis
Submitted to Magadh University in Partial Fulfilment
of the Requirements for the Degree of
Master of Science (Veterinary)
in
Gynaecology, Obstetrics
and
Artificial Insemination

BY
Mohd. Kouser, B.V.Sc.
Bihar Veterinary College, Patna
November, 1965

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Post Graduate Dept. of Gynaecology
Bihar Vety. College.
Patna the 20th Nov. 1965.

This is to certify that this thesis entitled "Preservation of Buffalo-Bull Semen in Various Diluents Containing Skimmed Milk and its Effect on spermatozoal Livability and Metabolism" submitted for the degree of Master of Science (Vet.) in Gynaecology, Obstetrics and Artificial Insemination to Magadh University by Shri Mohd. Kousar embodies the results of research carried out by him independently under my supervision and guidance.

20.XI.65

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

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C O N T E N T S.

PART ONE.

LIVABILITY :

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C O N T E N T S.

P A R T T W O

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P A R T - O N E

L I V A BI L I T Y

C H A P T E R - I

I N T R D U C T I O N

INTRODUCTION

Indian economy is very much dependent upon the live-stock and its products. Bullocks alone provide employment to three crores of people and many more earn their livelihood from the cow. Besides cattle, buffaloes also play an important role in the development of agrarian economy of the country. Although cattle and buffalo of India constitute 23% of the world bovine population (175.0 million cattle and 51.0 million buffaloes, as per 1961 cattle census). The contribution of livestock industry to the national income is very meagre. (7% of the national income). The average milk production of a cow is 369 lbs. and that of buffalo 1077 lbs. per year as compared to 4126 lbs. in U.S.A., 5576 lbs. in U.K. and 7005 lbs. in Denmark per year. Our per capita consumption of milk is as low as 4.6 ounces a day against 10 ounces as recommended by nutritionist. (Parnerkar 1965)

ROLE OF BUFFALO :-

From economy point of view buffaloes are also of not less importance. They produce 59.9% of the total milk produced, though they constitute only thirty percent of the total milch animals in the country. Besides milk, bone, hoves, horns and hide also add to the national income. Therefore attempts for the improvement of our native buffalo is the immense need of the hour. A great deal of genetic variability exists in buffaloes and there is wide scope for the improvement in productive potentiality of these animals by modern breeding techniques.

The artificial insemination is a modern tool in the hands of animal breeders for raising healthy and economic livestock. Presently it has served as the only key to chronic problem of paucity of proven sires in many of the developing agricultural countries of the world, and means to improve, in shortest possible time. Thus it is very fine zootechnique for exploiting the animal resources for the welfare of humanity and as well as for improvement of livestock; but success of A.I. is very much dependent on the ability of dilutors in preserving the "in vitro" vitality and fertility of spermatozoa, as A.I. is simply deposition of the spermatozoa into the female reproductive passage by mechanical means than by male himself. A proven sire can be utilised to the maximum through A.I. and thus hundreds of calves can be produced within the breeding life of a bull. A.I. made possible to use semen of a bull fifty to hundred times more than the natural service. Even after the death of sire the semen can be utilised, preserved by freeze dried method through A.I.

A. I. is now practised throughout the world and is accepted as the most practicable zootechnique for improvement of livestock.

HISTORY OF ARTIFICIAL INSEMINATION :-

The idea of artificial insemination is not new. The Arab horse-breeders were practising it as far back as 1322 A.D. as reported by de-Bonne. This has been cited by Heape(1897) and Loesse (1934); however on sound scientific lines it was first employed in 1779 by Abbott Spallanzani, an eminent Italian physiologist, in his experiment on dogs.

Russians were the first to employ A.I. in practical field on large scales and first A.I. centre was established by E.I. Ivanoff in the year 1907. In India sporadic attempts were made to take up

the work of A.I. have been made since 1939, but comprehensive studies into the problems of A.I. with a special reference to Indian conditions have been taken up at Indian Vety. Research Institute since 1942 under a scheme sponsored by Indian Council of Agricultural Research, New Delhi. Published works on various aspects of A.I. in buffaloes are scanty. It has been also observed that buffalo bull semen cannot be preserved as effectively as bull semen in U.P. & M.P. Further it has also been observed that the quality of buffalo bull semen detoriates earlier during winter season than bull semen, therefore, factors influencing quality of semen and advancement of some of dilutors for preserving buffalo-bull semen still remained an interesting field of ~~text~~ investigation, because short survival of buffalo-bull spermatozoa during winter season and the seasonal behaviour exhibited by the buffaloes during the same season, makes it very difficult to carry out the breeding programme effectively and efficiently. It was reported that 92% of the total number of buffaloes bred annually, are bred during autumn and winter season alone (Dharmapal 1956).

Donne used milk as semen diluent as early as 1837, although he could not get better results. The toxicity of raw milk for spermatozoa was also not known in early days.

OBJECT OF THE STUDY :- The present investigation aims at :-

To find out a better and more effective diluent for preservation of buffalo-bull semen which could fulfil the basic need of the day.

To evaluate the comparative efficiency of certain diluents for preservation of buffalo-bull semen,

And to determine whether any recognisable deviations occur in the livability and metabolism of spermsdue to preservation .

C H A P T E R - II

REVIEW OF LITERATURE

REVIEW OF THE LITERATURE

About one and half century ago, an Italian physiologist Abbott Spallanzani (1780) found out the zootechnique of Artificial Insemination and practised in dogs successfully. Hunter produced a pregnancy in the human by this method in the year 1799. The field of A.I. remained untapped for about one century except a few sporadic attempts to exploit this method for regular breeding programme. In 1899 Ivanov, a Russian Academician commenced research in this field, and established first A.I. centre in 1907. Later in early tens of 20th century many attempts were made by different workers to find ways and means for preserving semen in different media, and many diluents were recommended, like, solution containing glucose, chick-brain substance, evaporated milk, boiled muscle extract, buffered egg-yolk and gelatine etc.

Milk and other biological fluids were also tried, but no encouraging results could be obtained by Donne (1937), Kolleiker (1956), and Hoffman (1905).

The protective action of egg-yolk was first demonstrated by Lardy and Phillips (1939). They pointed out that the action of yolk protecting sperms against cold shock was due to presence of an active substance lecithin or similar substance phospholipoid present either in separate form or in combination with protein.

Donne was first to use milk as semen diluent in the year 1837, and further Phillips and Lardy (1940) reported that bull spermatozoa could survive in an evaporated milk diluent for 48 hours, but were non-motile after 72 hours.

Underbjerg et al. (1942) concluded that autoclaved milk had no beneficial effects on fertility, but Mihailove (1949)

reported encouraging results with milk as diluent for stallion semen. Mihailove (1950) also reported the use of milk as semen.

Later Jaequet (1951) recommended that use of canned skimmed milk for diluting semen, and also suggested that it was superior to milk-yolk and having many advantages over E.Yolk citrate. Sobek (1951) reported that the toxic effect of milk on the spermatozoa can be easily eleminated by heating. He obtained satisfactory results with milk yolk and milk yolk citrate. 5% high fertility was obtained with the use of milk yolk, than E.Y.C.

Thacker and Almquist (1951) in their experiment observed that a satisfactory spermatozoal survival rate could be got in either hemogenised milk or pasteurised skim milk only after they had been boiled for at least 10 minutes in covered vessel, and allowed to cool prior to the addition of semen.

In Algeria semen used in 1951 was diluted in E.Y.C. average conception rate was 60. In 1952 the semen was diluted with milk, 64% conception rate was obtained. A total of 997 cows was inseminated in 1952.

Vlachos (1952) observed the conception rate as 76-83%, when boiled cows milk was used as semen diluent in 82 cows, and 72.55% when Egg yolk plus N/15 sodium citrate was used in 510 cows.

Jacquet and Cassau (1952) recommended the use of powdered skim milk dissolved in glass dist. water, 10 times to the volume of powder milk and 10% egg yolk. Semen diluted in this diluent could be used for 6 days, and sperms can be preserved for 15-20 days. They discarded the canned milk, as it is much variable in reliability.

Ayyar (1952) recommended the use of heated milk with citrate buffer for diluting bull and buffalo semen and found that semen can be preserved as effectively as E.Y.C. Weiss et al.(1952) obtained 58.5% conception rate when 2 C.C. egg yolk + 100 C.C. skim milk + 10 drops of dihydrostreptomycin was used as semen diluent and when 5000 cows were inseminated with E.Y.C. 59.3% conception rate was noted by him.

Sanfile (1952) observed the average conception rate in 1210 cows inseminated with semen diluted with skimmed milk (previously heated to 95-97°C) as 4.2% higher than the conception rate in 1130 cows for which E.Y.C. was used as semen diluent.

In the year 1953 Thacker and Almquist reported that there was no appreciable difference in the percentage of surviving spermatozoa in boiled skim milk or yolk citrate diluent; it was slightly higher in boiled homogenised milk during first 10 days than in yolk citrate, but there after motility was retained longer in yolk citrate. Various methods of heating milk for various periods proved that the length of time of heating was not as important to livability as heating to a temp. of at least 92°C.

Plerchinger et al. (1953) found in his experiment that semen preserved in milk for 3 days gave more satisfactory results of conception than E.Y.C. They also compared boiled homogenised milk without antibiotics with egg yolk citrate containing penicillin and streptomycin as bull semen diluent (1:1), and obtained conception rate as 72.7% for boiled homogenised milk diluent, and 71.4% for E.Y.C. on the basis of 60-90 day non-return rate. Collins (1953) in his experiment observed that the fertility results were similar with milk and

E.Y.C. when used as semen diluents.

Dreher and Webb (1953) obtained a 69.8% conception rate on 60-90 day non return rate, when homogenised milk was used as semen diluent in 6009 cows for insemination, and 59.2 %on 60-90 day non return rate when 6060 cows were first inseminated with E.Y.P. They also observed that semen of those bulls, which gave poor response on preserving in E.Y.P. gave very good results with homogenised milk. Peters (1953) described the preparation and advantages of E. yolk diluent, however he could not find any appreciable differences in the motility and fertility rates between samples preserved either in milk yolk or in yolk-spermasol diluent. In the year 1953 Sobek observed that when milk diluent was used the conception rate was not appreciably different from that when E.Y.C. diluent was used, but the calves have greater birth wt. the percentage of male calves was greater and there were more twins. Before use, boiled milk was added to the yolk and Y.C. diluents in the ratio of 4:1.

Almquist et al. (1954) reported that there was no difference among fertility of bull semen diluent with heated fresh homogenised milk, heated fresh pasteurised skim milk and E.Y.C. Almquist (1954) reported that when semen from high and low fertility bulls was diluted in fresh heated homogenised milk and E.Y.C., and used for insemination, heated milk with commonly used antibiotics i.e. streptomycin and penicillin, was of special value as diluent for semen from bulls of relative low fertility and that it improved the fertility rate.

Perkins et al.(1954) made a comparative study of the fertility of bull semen diluted in E.Y.C. and heated homogenised

milk and found no significance difference between these two in 60-90 day non-return, although they found a highly significant difference in non-return percent, between 2 day and 3 day old semen with these two dilutors. Milk dilutor was best with 2day old semen. They also found improvement in non-return percentage with bulls of lower fertility when milk dilutor was used.

Bolton et al.(1954) made comparison of 3049 of 3049 cows inseminated with either yolk citrate diluted semen, or semen diluted in skimmed milk, which was heated to 96-100°C for 10-15 minutes. He found 4.28% more conception in E.Y.C. at 60-90 day non-return rate. It was suggested that poor results in milk diluents may be due to high population of bacteria.

Bonnadonna (1954) found conception rate in 2091 cows inseminated during autumn with semen diluted with milk + 10% egg yolk, was 50.4% Vs. 47.9% in 5341 cows inseminated during summer with semen from the same bulls, diluted with egg-yolk-citrate. Seasonal effect might have accounted for the difference in conception rates. Almquist, Flipse and Thacker (1954) found that differences were not statistically significant in non-return rates for 4689 first insemination with 105 semen samples diluted in milk, milk corn starch or yolk-citrate, or for 8399 first inseminations with 72 samples diluted in homogenised milk + corn starch, skimmed milk + corn starch, or yolk citrate. Results were not affected by age of semen upto 48 hrs. The addition of corn starch to increase the viscosity of the milk diluent, was therefore found to be unnecessary.

Veiga et al.(1954) found, following 84 insemination of Friesian cattle (in Brazil) in which a skimmed milk diluent was used, conception rate was 60.7% Vs. 46.0% in case of 1094

insemination using an egg yolk citrate.

Simunic (1955). studied skimmed milk heated to 95°C for 10 minutes to replace citrate in a yolk diluent. Semen characteristics were also studied with and without milk, when 6304 cows were inseminated with semen in milk diluent, the 90 day non-return rate to 1st service was 51.4%. He recommended the use of milk as semen diluent. Kerruish (1956) compared heat treated skimmed milk and heat treated homogenised milk with E.Y.C. and obtained the following results.

T A B L E -1.

Sl. No.	Name of dilutor	No. of Insemination	Conception rate	Remarks.
1.	Heated treated homogenised milk	4637	68.8%	
2.	Yolk-citrate	5051	64.9%	
3.	Heat treated Skim milk	6850	70.7%	
4.	Yolk citrate	7978	69.1%	

The low cost of skimmed milk, together with the results obtained justify its wider use as a dilutor.

Srivastava and Prabhu (1955) tried six dilutors for diluting buffalo-bull semen namely:-

1. Active Principle.
2. Egg-yolk phosphate.
3. Egg-yolk citrate.
4. Autoclaved milk.
5. Spermasol.
6. Glucose, Sod. bicarb. Sulphamezathin and egg-yolk (G-Dilutor).

G-Dilutor had given best results followed in order of decreasing

suitability and efficiency by spermasol, E.Y.C. Autoclaved milk, Active Principle. Further the fertility results were favourable with G as E.Y.P./ E.Y.C. Jaskowaski (1956) made a comparative investigation on the use of four diluents namely :-

1. E.Y.C. (after Salibury).
2. E.Y.G.C.(after Milavanov).

Skimmed boiled milk and skimmed boiled milk + 10% egg yolk upto a dilution of 1:40, he did not find any significant difference in the livability of spermatozoa among the dilutors used. At 1:80 dilution he found a statistically significant and higher sperm livability in heated skimmed milk with 10% egg yolk.

Melrose (1956) compared conception rates using 9% skim milk powder diluent with citrate diluent, both diluents containing 500 units of streptomycin, which are represented in table 2.

T A B L E 2.

Table showing conception rates in skimmed milk and Egg-yolk citrate.

Sl. No.	Name of the dilutor	No. of Inseminations	Conception Rate	Remarks.
1.	Skim milk diluent	5076	68.4	
2.	Sodium citrate diluent	5113	63.5	

No increase in conception rate could be obtained on increasing the quantity of streptomycin from 500 Mg. to 1000 Mg. per C.C. Better fertility rates were obtained with semen of poor fertile bulls when diluted in milk-diluent. Schmidt (1956) observed that the use of boiled milk as a diluent had no adverse effect on fertility upto a dilution rate of 1:15, at 1:31 there was slight decline.

Mihailov (1957) reported that when cows milk was used to dilute bull semen and 956 cows were inseminated at 2 stations. The conception rates were 91.7 and 94.0% respectively. Milk Marketing Board of England and Walse (1957) reported the results of use of heat treated homogenised milk and skim milk as semen diluent. The conception rates obtained were 69.6% with standard. E.Y.C. The board also reported that there was practically no difference between heat treated liquid skim milk powder and reconstituted milk powder in the conception rate. Hendrikse and Joling (1957) found that skim milk diluent containing 5% egg yolk maintained better motility than those containing 10-15% of egg yolk. They got 2.2% better results with skim milk than E.Y.C. on inseminating 8000 cows with E.Y.C. and 600 with skimmed milk and Egg yolk.

Gokhale (1958)recommended the use of glucose, soda-bicarb. and sulphamezathine dilutor for diluting buffalo-bull semen, and found it very effective with higher conception rate as compared to the citrate or phosphate buffers. He obtained the following conception rates with different dilutors,glucose dilutor 52.29%, E.Y.C. dilutor 40.8%, E.Y.P. dilutor 41.0%,on the rate of 90-120 day non-return.

Reidel (1958) used fresh skimmed milk with 10% E.Y.C. and antibiotics for dilution of bull semen and got encouraging results.Albright, Ehlers and Ebb (1958) made a comparative study of E.Y.C., E.Y. glycine, skim milk and whole milk, with or without addition of glycerol and their various combinations. Four semen samples from each of three bulls were divided into a glycerolated and non-glycerolated group, and extended 1:24

In 10 different diluents. It was found that E.Y.C., W.M., S.M. and E.Y. glycine were on an average lower in motility, after 5-7 days of storage than their combinations. Both combinations whole milk yolk glycine and skim milk yolk glycine, showed higher average motility on day 7 than did whole milk yolk citrate, skim milk yolk citrate, whole milk with skim milk or yolk citrate yolk glycine. ylk citrate yolk glycine was superior to either yolk citrate or yolk glycine alone. Whole milk, skim milk and milk yolk citrate gave higher motility after extension then the same extenders containing glycerol. No other combinations involving glycerol showed statistically significant difference.

O' Conner and Smith (1959) in their experiment obtained the following results over a period of six weeks one collection was made per week from each of 16 Friesian bulls at 16 different A.I. centres one half of each sample was diluted with heat treated skimmed milk and antibioties to the normally in use at Milk Marketing Boards A.I. centres; the other half differed in containing 10% glycerol. Each half was then divided into four parts for use on 1st, 2nd, 3rd and 4th day of age respectively. For a total of 14,319 inseminations the difference between the two diluents was significant except on the fourth day when the percentage of non-returns was 50.0 for the milk diluent and 60.6 for the milk glycerol diluent, but on the 1st and 2nd days of storage the percentage non-return were more for skim milk with antibiotics in case of skim milk with antibiotics, and 2.9% per day with skim milk and glycerol with antiboities.

Almquist (1959) found out that the use of milk

glycerol as semen diluent was cheaper and more efficient than heated skimmed milk containing antibiotics when heated skim milk without glycerol was used in a large A.I. practice 95% of diluted semen was used before 25th hour after collection, and a conception rate of 71% based on 60-90 day non-return was obtained. After the introduction of 10% glycerol he obtained an average non-return value of 73% when 44% of the semen was used after 25hours, and 12% on 3rd and 4th days.

Comparison of conception rates in skimmed milk and egg yolk diluents are represented in table no. 3

TABLE NO. 3.

Comparison of conception rates in skimmed milk and egg yolk diluents.

Reference	Diluent and treatment	No. of insemination.	Non return conception rate%	REMARKS
Almquist et al(1954)	Skim milk heated yolk citrate	2873 2674	67.8% 66.8%	Average high fertility bulls used.
Kerruish(1956)	Skim milk heated Yolk citrate	6850 7978	70.7 69.1	90-120 day Non-return conception rate.
Bolton and Durrell(1954)	Skim milk heated Yolk citrate	1494 1555	66.4 70.7	Suggested reduced conception rate in milk due to bacterial contamination.
Adler and Rasbech (1956 b)	Skim milk heated Cream-homogenised and sterilised Yolk-citrate	2263 2931 2370	69.3 69.4 63.6	
Melrose et al.(1958)	9% Skim milk powdered heated Yolk citrate	5076 5113	68.4 63.5	Conception rate increase confined to 6 of 17 bulls, 112 day non return conception rate.
Melrose et al(1958)	9% S.M.powdered heated Fresh skim milk htd.	2025 1449	67.3 68.9	112 days non-return conception rate.

Table quoted from the book " The semen of animals and A.I. by Maule.

Pickett et al.(1960) in their experiment to determine whether motility and fertility of bull semen would be improved by the addition of egg yolk to heated skimmed milk it was found that better results could be obtained by adding the yolk in 4 parts at 15 minute interval than by adding all at once or over different periods e.g. 10 minutes 30 minutes and constant drips (30 minutes). There was no statistically significant difference between the use of skim milk + yolk + glycerol and E.Y.C. glycerol from the point view of over all conception rates. There was no significant difference in the 60-90 day non-return percentage between extenders on the 1st & 2nd days, but there was a possible significance in favour of E.Y.C. on the 3rd day, spermatozoa stored in skim milk-yolk-glycerol at 5°C showed slightly better motility during a 10-day period. Albright, Erb, and Ehlers(1960) on the basis of their experiments had shown that extenders consisting soley of milk constituents i.e. whole milk or skim milk gave favourable results at 5°C and poor results with prolong storage at 37°C.

Joshi (1961) used four diluents for diluting semen of buffalo-bull, namely whole-milk, milk-yolk reconstituted powdered milk and G. dilutor.(Glucose-Sod.Bicarb. egg yolk diluent). He found that milk yolk gave better results than either whole milk or G dilutor. Reconstituted powder milk was not found to be as statisfactory as G. dilutor or other two diluents.

Tomar and Desai (1961) tried three dilutors for diluting buffalo bull, namely, Skimmed milk, skimmed milk egg

yolk glycine and skimmed milk + Egg-yolk + glycine + fructose. The buffalo-bull semen was diluted (1:1) and stored at 5-7°C. Motility was evaluated after varying storage periods, and +3 motility was preserved for an average of 2.62, 8.1 and 7.65 days by the three dilutors respectively. The difference between skimmed milk and the two egg yolk diluents were highly significant.

Roslanowski (1961) made a study of the influence of glycerol and of antibiotics on bull semen stored in a milk yolk diluent at + 5°C. The methods of adding glycerol were investigated. The most effective method under lab. conditions was found to be its addition in portions after cooling the diluted semen to +5°C. The diluent that gave the longest survival of spermatozoa consisted of milk 80 C.C., Glycerol 10 C.C., streptomycin 0.1 micro-gramme.

Pickett and Cowan (1961) in their experiments found out that in an unheated skim milk-yolk-glycerol diluent spermatozoal motility was maintained as effectively by the addition of 0.5 mg. cystein hydrochloride per ml. as by 1.0 or 1.5 mg. , with 0.5 mg. there were 48% motile spermatozoa vs. 44% in the control heated skim milk yolk diluent. In motility studies 10% egg yolk was nearly as effective in inhibiting the spermicidal factor in unheated skim milk as egg yolk + heat treatment. In field trials using semen diluted in unheated skim milk-yolk glycerol and in heated whole homogenised milk glycerol there were 70.6% non-returns to 391 first inseminations with the former and 72.3% to 394 with the later.

Sharma and Mahajan (1961) tried five dilutors for preserving buffalo semen, namely E.Y.C., E.Y.P., E.Y. Glycine, E.Y. Milk and E.Y. Glucose sod.-bi-carb. The semen was diluted in 1:10 proportion and 1:50. The motility was best preserved in E.Y. Glucose soda-bi-carb. at the 1:10 level of dilution.

Hutchinson and Cooper (1961) carried out an extensive study with diluent composed of 10% egg-yolk, 3% glycerol and 87% treated skim milk to determine its keeping quality when used over a 2 day period. Routine use of this diluent showed a 3 months conception rate of 75.3% for 106745 cows. The number of repeat services has been considerably reduced.

Oskoca and Osan (1961) in their experiment diluted semen samples with E.Y.C. and milk glycerol and stored in referigesater. Initial motility was 75.66 and 68.133% for E.Y.C. and M.G. diluted samples and motility after 10 days was 10.5 and 12.72% respectively. Conception rate to 1st insemination was 57.4% and 65.8% respectively for M.G. and E.Y.C. diluted semen.

Ehters & Mikota (1962) carried out analysis of variance on data, collected over 2 non-concurrent periods of two years, on % non-return to 1st insemination in four breeds of cattle. Semen was diluted with milk in one period and milk and 10% glycerol in the other. With the milk diluent there was a decline in average monthly percentage non-return of 4.8% for semen used on the 3rd day after collection compared with that used on the 2nd day. For the milk plus glycerol diluent the figure was 2.6%.

Mahajan and Sharma (1963) reported that G dilutor

(Glucose soda. bicarb.-egg yolk diluent) was superior significantly to other diluents, E.Y.C., E.Y.P., E.Y. Glycine and E.Y. boiled milk for preserving buffalow-bull semen. The average/of no. days for which the sperm survival in this diluent were 16 and 12 at dilution rate 1:10 to 1:50.

Kale (1963) reported that buffalo semen can be preserved for 3 days with desirable motility of spermatozoa(upto 40%) in diluents containing milk which were previously heated upto 92°C to 95°C.

HEAT TREATMENT OF MILK.

In early days the toxicity of raw milk as spermatozoal livability was not known.

Sebek (1951) was first to report that the toxic effect of milk on spermatozoa can be easily eleminated by heating.

Thacker and Almquist (1953) studied various methods of heating for various periods and concluded that the length of time of heating was not as important to livability as heating to temp. of at least 92°C. During the same year they observed in the second experiment that gentle boiling at 95°C for 1-10 minutes gave better spermatozoal survival than did vigorous boiling. They also observed that boiling the milk in open vessel was harmful to livability of spermatozoa whereas boiling in covered vessel was not harmful even for an hour.

Plipse et al. (1954) reported that the factors present in unheated milk, which were toxic to bovine spermatozoa were associated with albumin containing fraction. Lactinin had been found highly toxic to a spermatozoa. Lacto-per-oxidase when added to heated skim milk and used as semen diluent in storage trial, exhibited no toxicity for bovine spermatozoa at the concentration used.

Thacker, Plipse and Almquist (1954) in their experiment observed that bovine spermatozoa were sensitive to products developed in milk upon excessive heating, as well as to chemical and physical factors present in unheated milk. In attempts to identity a factor in unheated skimmed milk which was toxic to spermatozoa caesein and protein free milk serum were prepared and found devoid of toxic factor. They found that toxicity was associated only with albumin containing fraction. They also observed that heating those fraction at 92°C for 10 minutes eleminated the toxicity. It was also noted by them that heating of euglobulin and preudoglobulin fractions resulted in decrease in sperm livability when compared to similar factor in unheated milk.

Sancke et al. (1956) studied in detail heat treatment of skim milk which was used in place of egg yolk citrate. Firstly the skimmed milk was heated rapidly to temperature ranging from 73°C to 105°C for 3-9-27 minutes. There was no significant difference in the livability of spermatozoa when the milk was heated from 73°C to 97°C for 27 minutes or from 81°C to 105°C for 3 or 9 minutes, but significant decrease in livability was obtained when it was heated at 37°C for 3 and 9 minutes or from 81 to 105°C for 27 minutes indicating that the heating time was important only at 73°C and 105°C. Secondly the skimmed milk was heated to 77°C to 97°C for 1-10 minutes, optimum livability of sperm was obtained when heating was for one minute at 87-97°C. There was a significant decrease in livability when the milk was heated for 1 minute at 77°C and 82°C.

POWER OF HYDROGEN (pH).

Concentration of hydrogen and hydroxyl ions are

conveniently expressed as pH.

The effects of pH on motility of spermatozoa were studied as far back as 1934 (Dubinick, 1934). He reported that at pH 4.2 the spermatozoa of man, dog and stallion became immotile, but the motility was resumed again with shift towards alkalinity. At a pH of 3.5 - 3.4 an irreversible immotility ensued. Rashlebnove (1937) pointed that the resistance of spermotozoa to pH changes was influenced by the type of diluent employed. Lardy and Phillips (1939) and latter Phillips and Lardy (1940) recommended that bovine semen be stored in a narrow range of pH 6.7 - 6.8 in a diluent developed by them.

Guerreiro (1940) reported that when ram semen was acidified with lactic acid, all motility was retained at pH 5.35. At pH 4.65 only 5% oscillatory motility was observed followed by death of spermatozoa. At pH 4.45 motility was irreversibly destroyed. Addition of $NaHCO_3$ stimulated motility at pH 7.6; it ceased at pH 8.6, but was reversible. The addition of Na-oH did not affect motility at pH 8.6, but caused it to cease at pH 9.2, and at pH 9.2, and at pH 9.8 the motility could not be reversed.

Moore et al. (1940) noticed that the degree of acidity directly affected the motility of ram spermatozoa at pH values below 6.0. At pH 5.37 motility ceased completely.

Sergin et al. (1941) reported that optimum pH for storage of ram and bull spermatozoa was to be in the range of neutrality and survival was reduced below pH 6.0. Easley et al. (1942) Willelt and Saalisbury (1942) stressed the importance of proper pH during storage.

Emmense (1947) observed that rabbit spermatozoa were

more rapidly killed in acid than in alkaline media. Sperms remained immotile for one to two hours at pH 5.8 before actual death. Anderson (1947) believed that a well buffered medium which tended to be more acidic showed least pH changes during storage and maintained sperm motility best.

Blackshaw and Emmens (1951) found pH 7.0 to be optimum for motility of bull sperms and indicated wide range of tolerance between pH 5.5 and 8.5 in which the sperms were not greatly affected.

Fiser (1952) ascertained the pH of 130 samples of ram and bull semen. After storage for 15 and 30 minutes respectively pH was again ascertained, in both cases the pH of good quality semen was 0.4 lower, and that poor quality semen had decreased less or not at all.

Jean Blain (1953) observed that the higher the sperm concentration the more rapid was the lowering of pH in bull semen. He also concluded that when the semen reached a pH of 6 or less, spermatozoal viability was irremedially affected, even the addition of sodium citrate failed to delay sperm mortality much longer, and so long as pH was maintained between 6.5-7.0 by the addition of egg yolk citrate or egg-yolk phosphate diluents and semen stored at 37°C. there was good vitality after 210 minutes.

Peresy Perez (1953) reported that when diluents with different pH values ranging from 5.0 - 8.25 were added to samples of ass semen in which the pH arranged 7.6. Temporary anamolies in spermatozoal motility were observed in those samples in which the difference in pH between semen and diluents were greater than 0.4. Schwarz (1955) reported that when the pH of the semen was acid, the sex-ratio tended to be in favour of ♀♀

Rivas Doday (1956) recorded the changes in pH in diluted semen samples of stallion bull and ram semen incubated both aerobically and anaerobically at 36.5°C for 45 minutes. The sum of change under aerobic and anaerobic conditions was used as an expression of spermvitality. The values for the stallion, bull and the ram respectively were 0.34 0.49 and 0.58.

Rickard (1957) observed better livability at pH 7.1 - 7.4 when bovine semen was incubated at 37 - 39°C for 12 hours. The trend was reversed during storage at 4 - 6°C when better livability was observed at pH 6.5 - 6.8 over a ten day period.

Kulesova (1958) reported that the spermatozoal viability was greatest when the pH was neutral (7.07) of buck semen. He also observed that when the pH of the semen used to inseminate Angora does was neutral, conception rate was highest and the viability of young greatest.

Willett and Ohms (1958) reported that activation of spermatozoa was caused by an increase in pH in their experiment. They concluded that in most instances motility was greater after the addition of sodium hydroxide than after that of sodium bicarbonate, but the differences were not significant.

Schindler et al. (1958) studied initial and post storage pH, buffering capacity fructose content, as well as initial and post storage level of total reducing substances and lactic acid in cock and bull semen. They reported that the pH of cock semen changed slightly during storage where as that of bull semen decrease considerably. Whole cock semen and semen plasma had a small buffering capacity than whole semen and semen plasmaóf bull.

C H A P T E R - III

MATERIAL AND METHODS

MATERIALS AND METHODS

In the present study seven adult murrah buffalo-bulls were used maintained at Semen Bank Patna, having 5-10 years of age. The bulls were daily given exercise in the morning, and given water baths. The animals were fed groundnut cake, wheat bran and crushed maize, two pounds each, and to it were added mineral mixture and salt one ounce each. Hay was given with the rate of 15 lbs. per animal per day. The concentrates were given in morning and evening divided into two equal parts. The animals were allowed to drink water twice in a day.

The experiment was continued for three months from April to June.

An ancestrous buffalo was used as dummy for collection. Prior to proceeding with experimental observations, the technique to be followed for examination of various semen characteristics were standardised. Thirty five semen collections were studied, five from each animal using an artificial vagina. Collections were taken twice in a week in forenoon hours. For each bull separate A.V. was used, with temp. varying from 40-45°C. Each A.V. was provided with an insulating bag covering the rubber cone and semen collection tube to protect the spermatozoa from external atmospheric influence during the process of collection.

The semen was taken in the laboratory immediately after collection where it was kept at 32°C. in a water bath and examined for different characteristics and diluted for preservation. At the time of collection thrust and sex libido of the animals were recorded. The following seminal attributes were studied:-

1. Volume.
2. Colour.
3. Consistency.
4. Mass activity.
5. Motility.
6. Power of Hydrogen (pH).
7. Sperm concentration.
8. Percentage of dead sperms.

1. VOLUME :- Volume of the semen was recorded directly from the graduated collection tube.

2. COLOUR :- It was observed by naked eye and recorded as creamy Milky or Watery.

3. CONSISTENCY :- Consistency was recorded as thick, medium or thin, and observed by naked eye.

4. MASS ACTIVITY :- Immediately after collection the semen was examined under low power (10 x 5) magnification of the microscope, putting one droplet of semen on the slide without cover slip at 30°C. The mass activity was graded in the following five scales.

Individual movement of sperm. no wave.	+
Slow Waves.	+ +
Fairly rapid waves.	+ + +
Very rapid waves.	+ + + +
Waves formation like lightening in the clouds	+ + + + +

5. MOTILITY :- Motility was considered as an extensive index in the evaluation of semen. The movement of individual sperm was taken into consideration while grading the motility. The semen was diluted with corresponding dilutor (1:100). A droplet of

diluted semen was taken on a clean glass slide, gently warmed up-to body temperature, covered with a coverslip and examined under high power (40 x 10) magnification. Spermatozoa which were progressive motile, only were taken into consideration. Other type of motile spermatozoa were neglected.

The motility grading was done by the following method advocated by Herman and Swanson(1941) with slight modifications.

T A B L E NO. 4

Table showing different gradings of semen.

Grade	Percentage of live sperm.	Movements.
0	No motility	No movement.
0.5 to 1.0	10 to 20% motile spermatozoa.	No waves, movement mostly weak and oscillatory.
1.0 to 1.5	20 to 30% motile spermatozoa	Movements mainly vigorous and rapid but no waves and eddies are formed.
1.5 to 2.0	30 to 40% motile spermatozoa	-do-
2.0 to 2.5	40 to 50% motile spermatozoa	Wave formation with slight whorls which move slowly across the field.
2.5 to 3.0	50 to 60% motile spermatozoa	-do-
3.0 to 3.5	60 to 70% motile spermatozoa with swirling motion.	Rapid and vigorous movements, waves and whorls or eddies fxx form and change with great rapidity.
3.5 to 4.0	70 to 80% motile spermatozoa.	-do-
4.0 to 4.5	80 to 90% motile spermatozoa.	The movements and churning of the swirls and eddies are extremely rapid and can be compared with a tide in the sea.
4.5 to 5.0	90 to 100% motile spermatozoa.	-do-

6. POWER OF HYDROGEN (pH) :- Capillary tubes of similar bore were used. They were marked with greese pensil at equal distance. Little quantity of semen was taken in a watch glass. It was sucked with capillary tube upto the mark and transfer to next clean watch glass. Bromothymol Blue was sucked in next capillary tube upto the mark and transferred to the watch glass containing semen and mixed well. The mixture was sucked in the same capillary tube, and compared with colour in the capillirator (B.D.H.) and the value of the pH was noted.

pH of neat semen just after collection was recorded. pH of diluted semen was recorded at every 24 hour interval upto 96 hours.

7. SPERM CONCENTRATION :- The concentration of spermatozoa was estimated by direct method with the help of haemocytometer. The procedure employed is as follows:-

1. The tube containing semen after collection was kept in a water bath at 40°C.

2. The semen tube was rotated between the palms with a quick motion for uniform distribution of the spermatozoa.

3. The semen was drawn into the micropipette (0.1 ml. capacity) carefully upto the mark.

4. The exterior of the pipette was wiped off carefully with a piece of a sterilised filter paper, and then transferred the semen to the next tube containing 9.9 ml. of 0.05% eosin in physiological saline.

5. The test tube containing diluted semen (1 in 100) was rotated with a quick motion for uniform distribution of spermatozoa in the diluting fluids.

6. 1.0 ml. of diluted semen was drawn in a fresh 1.0 ml. capacity graduated pipette, the exterior of the pipette was wipped off carefully, and transferred to an another test tube, containing 9 mls. of 0.05% eosin in physiological saline. (Final dilution 1:1000).

7. Two samples of an ejaculate for counting were taken. They were diluted in the fashion described above.

8. Two samples of one ml. of diluted semen(1:100) were taken from each and followed step 6.

9. The tubes containing diluted semen were kept in the freezing chamber of the referigerator for half an hour, for killing the spermatozoa.

10 Each tube was taken by turn and rotated with a quick motion for uniform distribution of spermatozoa in the diluent.

11. Uniformly distributed supermatozoa from (10) were taken in the micropipett, for charging the hoemocytometer.

12. The coverslip of hoemocytometer was fixed perfectly, as such that the Newton's ring was formed.

13. Then the hoemocytometer was charged.

14. Charged hoemocytometer was kept for 10-15 minutes to allow spermatozoa to settle down.

15. Two hoemocytometers were used for each sample.

16. Four large squares were counted at random from each of two sides of the two hoemocytometers to give an over all total for 16 large squares. A zero added to the figure gives the estimate of concentration of sperms in million/ ml.

8. PERCENTAGE OF DEAD SPERMATOZOA :- The Nigrosin & Eosin method

advocated by Hancock (1951) for counting the no. of dead and live spermatozoa was used in this experiment.

The Nigrosin - Eosin stain was prepared as follows:-

Eosin (Water & Spirit Soluble)	5 gms.
Nigrosin (BDH).	30 gms.
Dist. Water	300 c.c.

After preparing the stain it was kept in hot water bath for some hours and then filtered.

PREPARATION OF SLIDES :- On clean grease free glass slides, a drop of diluted or neat semen was taken, to it four drops of Nigrosin-Eosin were added and allowed to stand for one to two minutes. Exposure of sperms to stain more than 3 minutes gave wrong results. Exposure time varying from 30 seconds to 3 minutes showed no significant difference. One minute exposure time was taken as normal as suggested by Nyudu et al. (1961). Thin smear were drawn on clean slides, and marked with grease pensil with bull no., date of collection, neat or diluted semen. The smears were then air dried, and then transferred to a hot air oven at 40°C. and kept there for 1/2 hour. Then the slides were mounted with a coverslip and neutral Canada Balsam. The prepared slides were again put back in the oven at 45°C. till the Canada Balsam got thoroughly dried. The slides were made in duplicates.

Live sperms remained unstained where as dead sperms took pink colour, some spermatozoa took colour only anteriorly or posteriorly. As the opinion about the partially stained spermatozoa was not uniform but the majority of workers were of the view that the partially stained spermatozoa were on the way of death, hence they might be considered as dead sperms.

PREPARATION OF DILUENTS :- In the present experiment six dilutors were tried, all of them were containing heated buffalo skim milk, obtained from Milk Union, Patna. Different constituents of diluents were represented in table no. 5.

T A B L E 5.

TABLE SHOWING PROPORTIONS OF VARIOUS DILUENTS.

Sl. No.	Name of the dilutor	Skim milk.	Egg yolk.	Sodium citrate 2.94%	Glucose 5%	Sod. bi-carb. 1.3 %	Glycerol.	Abbrevation.
1.	Skim milk yolk	90	10	-	-	-	-	MY
2.	-do- citrate	60	30	10	-	-	-	MYC
3.	-do- Glucose	60	30	-	10	-	-	MYD
4.	-do- Soda-bi-carbonate	60	30	-	-	10	-	MYS
5.	-do- Glycerol	60	30	-	-	-	10	MYG
6.	Skim Milk Glycerol	90	-	-	-	-	10	MG.

SKIM MILK :- It was obtained from Govt. dairy from Patna containing 0.1% fat. Buffalo milk was used throughout the experiment. It was procured one day before its use, and heated in water bath upto 92-95°C. for ten minutes. Over heating and underheating was avoided. The heated milk was allowed to cool down to room temperature, and then kept over night in the referigerator. Next day morning the milk was filtered through a thin cotton layer, and then used for preparing dilutors.

EGG-YOLK:- Unfertilised fresh hens eggs were used through out the experiment. The external shell of the egg was cleaned and sterilised with a swab of alcohol, and then opened with the help of a thumb forcep. The white of the egg was drained off, and the yolk was placed on a sterilised filter paper. The

vitelline membrane was punctured with a sterilised rod, and the yolk was poured out into a graduated cylinder.

PREPARATION OF BUFFERS :- All the chemicals used in the experiment were of analar reagent grade to ensure maximum purity. The buffer solutions were prepared as given in Appendix A.

DILUTION :- Split sample technique was employed. The dilutors were prepared as details given in table No. 5 in 15 c.c. capacity test tubes, and penicillin G sodium and streptomycin were added at the rate of 500-1000 I/U & 500-1000 micrograms per ml. respectively, and then labelled with bull no., date of collection, and name of dilutor. The test tubes containing dilutors were then kept in water bath at 32°C.

After collection the semen was kept in the water bath at 32°C. and examined for Mass activity, volume, colour, consistency, pH, and Dead and live percentage of spermatozoa.

To each of the dilutor 1 c.c. of neat semen was transferred, care was exercised that at the time of dilution, the temperature of dilutor and semen was the same. It was maintained at 30-32°C. After dilution the tubes were rotated gently between the palms, for uniform distribution of sperms in the dilutor. Motility, pH and dead percentage of spermatozoa were examined immediately after dilution of the semen.

24 Clean and sterilised test tubes of 3 c.c. capacity were taken. They were divided into four lots each containing 6 tubes for six dilutors, to be examined at 24,48,72,96 hours. They were labelled with bull no., date of collection and name of dilutor, and date of examination after expiry of 24, 48, 72 and 96 hours. Each tube received 2 c.c. of diluted semen of the corresponding dilutor, and tightly corked. Separate lots were

kept for examination at the end of different interval of preservation, as otherwise if the diluted semen was kept in bulk in one tube for examination at different intervals, the handling of the tube at the end of 24 hours of preservation for examination of semen characteristics could conceivably affect the observations that were to be made at the end of 48, 72 and 96 hours interval of preservation. The four lots of tubes each containing 6 6 tubes were kept in four beakers having 400 c.c. of water at 25 - 30°C. and kept in the referigerator for gradual cooling. The beakers were also labelled as 24, 48, 72, and 96 hours for easily identification. At the end of 2-3 hours when the water of the beakers attained 4°C., it was removed and the tubes were kept in the same empty beakers at 4 ± 1°C. for preservation.

The semen samples were examined on every 24 hrs. interva for motility, pH, and dead % of spermatozoa as follows :-

On every 24 hours interval corresponding beaker was taken out from the referigerator. The semen tubes were thawed well one by one between the palms of the hands sufficiently long to warm them up to the body temperature, and also made up and down twice or thrice gently to ensure uniform distribution of spermatozoa in the dilutor. The tubes were then transferred to constant temperature water bath at 30 32°C. Then the samples were examined as described for different characteristics of semen.

METHOD OF ANALYSIS :

The date were statistically analysed according Snedecor (1946). Percentage of dead sperms were transformed to angles before subject to analysis of variance. The average was calculated with the formula - $\Sigma n/n$ (n = number of observations). Standard error (S.E.) was calculated after calculating standard deviation (S) with the formula $\sqrt{\frac{\Sigma x^2 - \left(\frac{\Sigma x^2}{n}\right)}{n-1}}$

Standard error = $\frac{S}{\sqrt{n}}$

Coefficient of variance was calculated with the formula,

$= \frac{S \times 100}{\bar{X}}$, ($\bar{X}$ = average).

Analysis of variance was run separately for motility, pH and dead percentage of spermatozoa for semen diluted in all the diluents, and preserved for 96 hours. Separate analysis of variance was run for every 24 hours interval upto 96 hours. When "p" test was found significant, a further test of all comparisons among means was taken up, as "p" test gives no clue as to how many differences there are. In a group of "a" means there are in all a (a - 1)/2 potential differences. The test was performed by computing "p" critical difference value(C.D.) at 1% and 5% level, and with this, the all means individually were compared. If the value of mean found higher than C.D. value the difference was taken as significant.

C H A P T E R - IV

RESULTS

R E S U L T S

The following characters of neat semen samples were examined immediately after collection.

1. Volume.
2. Colour.
3. Consistency.
4. Mass activity.
5. Power of Hydrogen (pH).
6. Sperm concentration.
7. Dead percentage.

1. V O L U M E :-

Volume of the semen was recorded directly from the graduated collection tube. The average colume was 4.5 ± .02 ml. with coefficient of variation 31.11%. The range was 2.0 - 8.0mls.

2. C O L O U R :-

Colour of the semen was observed creamy in 50% samples, and rest 50% samples were milky in colour.

3. C O N S I S T E N C Y :-

13% semen samples were recorded as having thick consistency and 75 were observed of medium consistency. The rest 12% were of thin consistency.

4. M A S S A C T I V I T Y :- The average mass activity of spermatozoa was observed to be +4 ± 0.5 with 85% coefficient of variation. The range was +3 ± 4.5.

5. P O W E R O F H Y D R O G E N (pH):- The pH was recorded as 6.7 ± .002 with coefficient of variation 2.98% and the range 6.3 - 7.0.

6. SPERM CONCENTRATION :-

The semen samples were having 1254 ± 6 millions of spermatozoa per ml. with the range from 670 - 1960 millions per ml.

7. DEAD PERCENTAGE :-

Dead percentage of spermatozoa was 10.8 ± .06% with coefficient of variation of 34.25%. The range was 4-19%.

Mean and range of different characters of semen, are represented in table 6.

TABLE 6.

Treatment mean and range of different characters of semen along with standard error and coefficient of variation.

Sl. No.	Hem.	No. of observation.	Average	Range Min.	Range Max.	Standard error and	Coefficient of variation
1.	Volume	35	4.5	2	8	.02	31.11
2.	Mass activity	35	4.0	3	4.5	.05	85.00
3.	pH	35	6.7	6.3	7.0	.002	2.98
4.	Sperm conc.	35	1254.8	670	1960	6.0	28.43
5.	Dead %	35	10.8	4	19	.06	34.25

Neat semen was diluted in diluted diluents containing milk Motility, pH and dead percentage of spermatozoa were recorded at 0 - 24 - 48 - 72 and 96 hours intervals. The mean, range along with standard error and coefficient of variation of motility spermatozoa diluted in various diluents containing milk, and preserved over different hours of interval are given in table 7 and figure No.1.

TABLE - 7.

Mean, range, with standard error coefficient of variation of motility of spermatozoa diluted in different diluents, and preserved over different periods of interval.

(No. of observations 35)

Interval	MILK YOLK			MILK YOLK CITRATE			MILK YOLK GLUCOSE		
	Av.	S.E. ±	C.V.	Av.	S.E. ±	C. V.	Av.	S.E. ±	C.V.
0 hr.	3.50	.06	16.01	3.48	.01	18.26	3.50	.20	12.12
24 hrs.	3.28	.07	14.02	3.44	.07	13.08	3.48	.10	18.67
48 hrs.	3.17	.13	24.40	2.94	.28	54.82	3.27	.10	18.65
72 hrs.	2.84	.03	8.09	3.21	.07	13.70	2.72	.22	49.63
96 hrs.	2.25	.25	68.44	1.81	.28	92.26	2.17	.21	58.06

TABLE - 7 (Continued)

Interval	MILK YOLK SOD.BI-CARB.			MILK YOLK GLYCEROL			MILK GLYCEROL		
	Av.	S.E. ±	C. V.	Av.	S.E. ±	C. V.	Av.	S.E. ±	C.V.
0 hr.	3.81	.01	12.20	3.20	.09	25.19	2.50	.11	24.14
24 hrs.	3.62	.08	13.81	2.61	.14	32.56	1.52	.17	68.42
48 hrs.	3.34	.06	11.37	2.44	.17	40.98	1.17	.16	84.61
72 hrs.	2.85	.16	34.38	2.00	.33	99.50	0.82	.03	26.82
96 hrs.	2.23	.20	52.32	1.48	.30	75.67	0.71	.04	33.80

Motility of +3 was maintained for 48 hrs. in MY, MYC, MYD and MYS, and then it was progressively declined to +2 and above in 96 hours. In milk yolk of glycerol and milk glycerol motility lowered down to +3.20 to +2.61 and +2.50 to 1.50 with 24 hours of preservation respectively. In milk-glycerol

spermatozoa showed +1.52 ± .17 motility upto 24 hours. In 96 hours period motility was lowered down to 0.71 ± .04 with coefficient of variation 33.80%.

pH :- The mean, standard error and coefficient of variation of pH of diluted semen in various diluents. preserved for 96 hours are represented in table 8 and figure 2.

T A B L E No. 8.

Mean, standard error and coefficient of variation of pH of diluted semen in various diluents.

(No. of observations 35)

	MILK YOLK			MILK YOLK CITRATE			MILK YOLK GLUCOSE		
Interval	Av.	S.E.	C.V.	Av.	S.E.	C.V.	Av.	S.E.	C.V.
0 hour.	6.12	.02	5.03	5.81	.03	5.51	5.88	.03	4.21
24 hours	6.02	.04	4.31	5.86	.03	3.41	5.75	.05	5.20
48 hours	5.85	.05	5.12	5.70	.04	4.21	5.62	.04	4.98
72 hours	5.68	.04	4.92	5.55	.04	4.68	5.48	.05	5.47
96 hours	5.46	.05	6.04	4.84	.01	2.06	5.35	.05	5.60

T A B L E-8 (Contd.)

	MILK YOLK SOD. BI-CARB.			MILK YOLK GLYCEROL			MILK GLYCEROL		
Interval	Av.	S.E.	C.V.	Av.	S.E.	C.V.	Av.	S.E.	C.V.
0 hour	6.17	.01	5.12	6.72	.03	9.12	6.60	.01	3.21
24 hours	6.06	.04	4.62	6.68	.07	8.27	6.50	.02	2.15
48 hours	5.88	.05	5.10	5.61	.04	4.63	6.36	.03	3.14
72 hours	5.75	.01	1.73	5.48	.04	4.37	6.20	.04	4.19
96 hours	5.62	.05	6.04	5.35	.04	5.23	6.06	.06	6.27

The pH of semen in all the diluents decreased progressively during preservation for 96 hours at 4 ± 1°C. The pH was maintained highest in MG followed by MYG, MYS, MY, MYD and MYC on storage for 96 hours.

DEAD PERCENTAGE OF SPERMATOZOA :-

The mean, standard error with coefficient of variation of percentage of dead spermatozoa of diluted semen in various diluents, preserved for 96 hours, are given in table No. 9 and figure 3.

T A B L E NO. -9.

The mean, standard error with coefficient of variation of dead percentage of spermatozoa in diluent semen, preserved for 96 hours (The values are retransformed from angles)

(No. of observations 35)

	MILK YOLK			MILK YOLK CITRATE			MILK YOLK GLUCOSE		
Interval	Av.	S.E. ±	C.V.	Av.	S.E. ±	C.V.	Av.	S.E. ±	C.V.
0 hour	26	0.3	49	17	0.1	30	25	0.1	12
24 hours	30	0.2	58	28	6.2	49	29	0.1	20
48 hours	37	0.1	23	35	0.1	27	34	0.1	13
72 hours	41	0.2	37	47	0.1	17	46	0.2	29
96 hours	52	0.4	53	56	0.2	26	56	0.2	32

T A B L E - No. 9(Contd.)

	MILKYOLK-TOR-BI-CARB.			MILK YOLK GLYCEROL			MILK GLYCEROL		
Interval	Av.	S.E.	C.V.	Av.	S.E.	C.V.	Av.	S.E.	C.V.
0 hour	18	0.1	5	25	0.1	12	33	0.1	6
24 hours	27	0.1	12	45	0.1	21	78	0.1	8
48 hours	35	0.2	37	54	0.2	32	77	0.3	29
72 hours	42	0.2	41	62	0.2	25	88	0.1	9
96 hours	56	0.2	33	70	0.3	27	89	0.1	8

The percentage of dead spermatozoa increased progressively in all the diluents during preservation for 96 hours as it is evident from table 9. The highest no. of dead % of spermatozoa was observed in EG followed by EYG, EYD, EY, .EY and EYS over 96 hours of preservation.

ANALYSIS OF VARIANCE :-

I - After 24 hours preservation :- Analysis of variance and treatment means with S.E. and ~~mx~~ critical difference values at 5% and 1% level of motility, pH and dead % of spermatozoa preserved in various diluents over a period of 24 hours are presented in table No. 10 and 11.

T A B L E No. 10

Analysis of variance of motility, pH and dead % of spermatozoa after 24 hours preservation.

Source of variance	D. F.	Motility M.S.	Motility F.Ratio	pH M.S.	pH F.Ratio	Dead % + M.S.	Dead % + F.Ratio
Diluents	5	22.55	59.5**	3.04	33.7**	5144.45	31.8**
Bulls	6	2.98	7.5**	0.27	3.0**	366.40	2.2*
D x B	30	0.60	1.34	0.52	5.7	119.56	0.7
Error	168	0.38	-	.09	-	161.51	-

N. B :- + The values are transformed to angles.

** Significant at 1% level of probability

* Significant at 5% level of probability.

The analysis of variance given in table No. 10 shows that there was significant contribution of diluents and bulls in the variation of motility, pH and dead percentage of spermatozoa preserved for 24 hours after collection.

T A B L E NO.11

Means of motility, pH, and percentage of dead spermatozoa after 24 hours of preservation.

(Average of 5 x 7 observations)

Bull No.	Motility						pH						Dead % of Spermatozoa +					
	EY	EYC	EYD	EYS	EYG	EG	EY	EYC	EYD	EYS	EYG	EG	EY	EYC	EYD	EYS	EYG	EG
11	3.8	3.6	3.5	3.7	3.4	1.6	6.02	5.82	5.90	5.76	5.84	6.44	24	28	31	27	53	74
0/8	3.0	3.6	2.8	4.6	2.4	1.8	6.10	5.98	5.90	6.16	5.58	6.60	38	29	43	23	50	73
38	2.9	3.3	3.4	3.2	2.8	2.1	5.82	5.62	5.42	6.00	5.52	6.42	13	32	29	39	39	78
0/4	3.8	4.0	3.9	3.9	3.3	1.7	6.12	6.00	5.90	6.18	5.84	6.58	28	20	21	23	35	85
M	3.7	3.3	3.7	3.7	2.0	0.9	5.84	5.86	5.78	6.10	5.62	6.52	25	22	25	24	47	85
4	3.5	3.8	3.8	3.6	3.2	1.7	6.14	5.86	5.68	6.04	5.74	6.48	30	25	24	29	37	67
137	2.3	2.5	3.3	2.5	2.0	0.9	6.10	5.90	5.74	6.18	5.64	6.50	58	46	34	29	55	86
Average	3.28	3.44	3.48	3.62	2.61	1.58	6.02	5.86	5.76	6.06	5.68	6.50	31	29	29	28	45	79

S. E. of Mean =		0.14	0.22	3.03
Critical difference value (C.D.).	At 5% level	.27	0.43	5.94
	At 1% level	.36	0.57	7.78

N. B.:- + The values are retransformed from angles.

Table 11 shows that motility of spermatozoa ~~is~~ remained highest in MYS and lowest in MG. The difference was found statistically significant between MYS, and MYG and MG,(1% level of probability), but there was no significant difference between MYS and MYC, MYD. Significant difference (or significant at 5% level) observed between MYS and MY. motility was found lowest in MG. No difference was recorded in motility in MY, MYC, MYD.

The mean of pH was found lowest in MYG and highest in MG. The difference between mean of MG and MYC, MYD, MYG was found highly significant. The difference was observed non significant between MY, MYC, MYD, MYG, MYS and between MG and MYS.

The mean of percentage of dead spermatozoa was recorded highest in M G and lowest in MYS. The difference between MG and MYS, MYD, MYC and MY was observed as highly significant, where as the difference with MYG was significant (or significant at 5% level). The difference between all other diluent (MY, MYC, MYD, MYS and MYG) failed to be significant.

II. <u>After 48 hours of preservation</u> :-

Analysis of variance and treatment means with S.E. and critical difference values at 5% and 1% level of motility, pH and dead % of spermatozoa preserved in various diluents over a period of 48 hours are presented in table 12 and 13.

TABLE NO.12

Analysis of variance of motility, pH, and dead percentage of spermatozoa after 48 hours of preservation.

Source of variance	D. F.	Motility		pH		Dead % +	
		M. S.	F.Ratio	M. S.	F.Ratio	M. S.	F.Ratio
Diluent	5	23.94	27.00**	2.71	38.7**	3675.45	19.6**
Bulls	6	4.54	5.00**	0.22	3.1**	499.99	2.6*
D x B	30	1.11	1.29	0.47	6.7**	155.12	0.8
Error	168	.86	-	.07	-	187.02	-

N. B.:- + The values are transformed to angles before subject to analysis of variance.

** Significant at 1% level.

* Significant at 5% level.

Table 12 indicates that there was significant contribution of diluents and bulls in the variation of motility, pH and dead percentage of spermatozoa preserved for 48 hours interval at 48 ± 1°C. The mean motility in MYS on 48 hours preservation was found highest than all the diluents used. MG stood lowest in keeping motility of semen. Highly significant difference was observed between MYS and MYG, MG (or significant at 1% level) in keeping motility. There was no difference in motility between MYS and other two diluents namely MY, and MYD. No difference was also observed among the three diluents (MY, MYC and MYD).

MG maintained highest pH and lowest was observed in MYG. Highly significant difference prevailed between MG and other five diluents viz MY, MYC, MYD, MYS. Highly significant

T A B L E - NO. 13

Treatment means of motility, pH and dead % of spermatozoa after 48 hours of preservation.(Average of 5 x 7 observations)

Bull No.	MOTILITY						pH						DEAD % OF SPERMATOZOA +					
	MY	MYC	MYD	MYS	MYG	MG	MY	MYC	MYD	MYS	MYG	MG	MY	MYC	MYS	MYD	MYG	MG
11	3.4	3.6	3.2	3.7	2.4	1.2	6.06	5.66	5.70	5.56	5.64	6.30	31	27	24	39	54	83
0/8	3.5	3.5	3.5	3.9	2.8	1.6	5.92	5.74	5.70	5.92	5.64	6.46	28	29	23	28	40	74
38	2.1	1.6	2.3	2.3	2.0	1.2	5.62	5.58	5.34	5.80	5.48	6.28	52	50	59	54	61	58
0/4	3.5	2.6	2.5	3.3	2.8	0.7	5.94	5.72	5.64	6.00	5.78	6.46	35	32	37	29	56	67
M	3.5	3.3	3.7	3.3	1.8	1.1	5.72	5.68	5.56	5.94	5.52	6.34	40	44	30	24	68	88
4	3.3	3.1	3.4	3.4	2.9	1.5	6.00	5.78	5.78	6.02	5.66	6.36	43	22	29	33	44	77
137	2.9	3.9	3.3	3.5	2.4	0.9	5.86	5.78	5.64	5.98	5.60	6.32	41	39	50	33	53	88
Av.	3.17	2.94	3.27	3.34	2.24	1.17	5.85	5.70	5.62	5.88	5.61	6.36	47	35	35	34	55	77

S. E. of semen =		0.2	0.06	3.2
Critical difference value(C.D.)	At 5% level	0.39	0.12	6.27
	At 1% level	0.51	0.15	8.22

N.B. - + The values are retransformed from angles.

difference was also observed between MYS and MYC, MYD and MYC. No difference was recorded between MYS and MY.

The mean of percentage of dead spermatozoa was found highest in MG and lowest in MYD. Highly significant difference was noticed between MG and other five diluents in maintaining dead % of spermatozoa, however the difference failed to be significant between MY, MYC, MYD and MYS. The difference remained highly significant between MYG and the other diluents i.e. MY, MYC, MYD and MYS.

III - After 72 hours of preservation :

Analysis of variance and treatment means with S.E. and critical difference value at 5% and 1% level, of motility, pH and dead % of spermatozoa, preserved in various diluents over a period of 72 hours are given in table 14 - 15.

T A B L E -NO. 14

Analysis of variance of motility pH and dead % of spermatozoa after 72 hours of preservation.

Source of variation	Motility		pH		Dead % +	
	M. S.	F.Ratio	M. S.	F. Ratio	M. S.	F. Ratio
Diluents	23.90	17.7**	2.61	32.6**	4534.32	25.9**
Bulls	8.24	6.0**	0.26	3.2**	1137.25	6.5**
Dil. x Bulls	.24	0.1	0.36	4.5	138.57	0.7
Error	1.35	-	.08		174.49	-

\+ The values are transferred to angles before subject to analysis.

** Significant at 1% level.

* Significant at 5% level.

Table No. 14 shows that Diluents and bulls significantly contributed in the variation of motility, pH and dead percentage of spermatozoa diluted in various diluents and preserved for 72 hours at 5 ± 1°C.

The motility was maintained highest in MYS and lowest in MG. It is also evident from table No. 10 that the difference was highly significant between motility in MYS and MG, MYG and MYC (significant at 1% level). The difference failed to be significant between MYS, MYD and MY. Highly significant difference was also observed in motility between MG, and other five diluents viz. MY, MYC, MYD, MYS and MYG. The difference was obtained as highly significant between MYG and other five diluents i.e. MG, MY, MYC, MYD, MYS. The difference was observed as non significant between MYD, MYS and MY.

MG showed highest pH on preserving semen over a period of 72 hours and the lowest was observed in MYG and MYD. Highly significant difference was recorded between the pH of MG and other five diluents. The difference failed to be significant between MYG and MYD. Highly significant difference (or difference at 1% level) was observed between MY, MYC, MYD, MYS and MYG in maintaining pH value.

Highest percentage of dead spermatozoa was observed in MG, and lowest in MY. Highly significant difference was recorded in having dead % of spermatozoa between MG, and other five diluents. No difference recorded between MYC, MYD and MYS. Significant difference was observed between MY and MYC.

TABLE NO. 15

Treatment means of motility, pH and dead percentage of spermatozoa after 72 hours of preservation.

(Average of 5 x 7 ejaculates)

Bull No.	Motility						pH						Dead % of spermatoa +					
	MY	MYC	MYD	MYS	MYG	MG	MY	MYC	MYD	MYS	MYG	MG	MY	MYC	MYD	MYS	MYG	MG
11	2.8	3.1	3.0	3.1	1.7	0.9	4.6	5.5	5.6	5.4	5.5	6.1	44	44	42	38	71	85
0/8	3.1	2.6	3.0	2.4	2.3	0.8	5.7	5.6	5.5	5.8	5.5	6.2	36	48	34	30	50	85
38	1.9	2.2	1.6	2.2	1.5	0.6	5.5	5.4	5.2	5.6	5.4	6.8	48	63	82	80	76	91
0/4	3.0	3.2	2.0	2.4	2.5	0.5	5.7	5.5	5.5	5.9	5.6	6.3	41	43	37	30	51	96
M	3.3	3.5	2.8	2.5	2.6	0.9	5.6	5.5	5.4	5.8	5.4	6.1	35	47	46	47	53	87
4	3.1	3.4	3.4	3.3	2.4	1.2	5.8	5.6	5.6	5.8	5.5	6.3	37	32	32	30	49	75
137	2.9	2.5	3.3	3.1	1.0	0.6	5.6	5.5	5.4	5.7	5.4	6.2	46	52	51	42	80	90
Av.	2.84	2.29	2.72	2.85	2.00	0.82	5.68	5.55	5.48	5.75	5.48	6.20	40	47	46	43	62	88

S.E. of mean =		.077	.004	3.15
Critical difference Value (C.D.)	At 5% level	.151	.008	6.17
	At 1% level	.198	.010	8.09

N. B. - + The values are retransformed from angles.

IV. After 96 hours of preservation :

Analysis of variance and treatment means with standard error and critical difference values at 5% and 1% level, of motility, pH and dead % of spermatozoa preserved in various diluents over a period of 96 hours are presented in table 16 - 17.

TABLE NO. 16

Analyses of variance of motility, pH, and dead % of spermatozoa after 96 hours preservation.

Source of variance.	Motility		pH		Dead % of sperm +	
	M. S.	F.	M. S.	F.	M. S.	F.
1. Diluents	13.65	9.03**	2.60	26.0**	3100.94	10.9**
2. Bulls	4.70	3.11**	0.32	3.2**	993.95	3.5*
3. Dil. x Bull	2.16	1.43	0.53	5.3	150.97	0.5
4. Error	1.51	-	0.10	-	283.03	-

N. B.:

\+ The values are transformed to angles before subject to analysis.

** Significant at 1% level.

* Significant at 5% level.

Table No. 16 shows that there was significant contribution of bulls and different diluents used, in variation in motility, pH and Dead percentage of spermatozoa on preservation for 96 hours at $4 \pm 1^{\circ}C$.

Table 17 represents that the highest motility was maintained in MYS and lowest in MG on preserving for 96 hours. Highly significant difference was found in maintaining motility between MYS and MY, MYD,

TABLE NO.17

Treatment means of motility, pH and dead percentage of spermatozoa after 96 hours of preservation.

(Average of 5 x 7 ejaculates)

Bull No.	Motility						pH						Dead % of sperm +					
	MY	MYC	MYD	MYS	MYG	MG	MY	MYC	MYD	MYS	MYG	MG	MY	MYC	MYD	MYS	MYG	MG
11	2.5	0.8	3.6	2.7	1.5	0.9	5.6	5.4	5.5	5.3	5.4	6.0	50	66	51	46	74	87
0/8	2.7	1.5	2.3	3.1	1.1	0.6	5.5	5.3	5.4	5.7	5.3	6.1	51	69	48	34	65	92
38	0.8	1.9	1.0	1.9	0.8	0.5	5.9	5.3	5.1	5.4	5.2	5.9	56	63	88	68	88	91
0/4	2.6	2.2	2.0	1.2	1.4	0.2	5.6	5.5	5.3	5.9	5.4	6.0	51	59	63	84	64	93
N	3.1	1.1	2.1	2.7	2.7	1.2	5.4	5.3	5.2	5.6	5.1	6.0	40	37	37	44	49	83
4	2.1	3.2	3.1	2.7	1.9	1.2	5.7	5.5	5.5	5.8	5.4	6.2	45	30	37	57	63	84
137	2.0	2.0	2.1	2.3	1.0	0.4	5.4	5.4	5.1	5.5	5.4	6.1	57	63	60	55	82	93
Av.	2.25	1.81	2.17	2.37	1.48	0.71	5.4	4.8	5.3	5.6	5.3	6.0	52	56	56	56	70	89
S.E. of mean =						0.29				.075						4.02		
Critical difference				At 5% level		0.57				.147						7.88		
value (C.D.)				At 1% level		0.75				.192						10.33		

N. B. - + The values are retransformed from angles.

MYC. Highly significant difference was also recorded between MG and other four diluents viz. MY, MYC, MYD and MYS. MY MY and MYD maintained no significant difference.

The mean of pH value was noted highest in MG and lowest in MYC. Highly significant difference was observed between pH of MG and other five diluents. Difference remained nil between pH values in MYC and MYD. Difference was observed as significant (or significant at 5% level) between MYS and MY. Difference was also observed as highly significant between MY and MYC.

The mean percentage of dead spermatozoa was found highest in M.G. and lowest in MY. Highly significant difference was recorded between MG and other five diluents viz. MY, MYC, MYD, MYS and MYG. Difference failed to be significant between MY, MYC, MYD and MYS. Difference remained nil between MYC, MYD and MYS. Highly significant diff. was also found between MYG and other five diluents MY, MYC, MYD, MYS and MG.

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C H A P T E R - V

D I S C U S S I O N

D I S C U S S I O N

In the present investigation an attempt was made to find out a better and suitable diluent for preservation of buffalo-bull semen. In view of this object six diluents containing skimmed milk along with different chemical substrates were tried. Semen samples were tested for three characters namely motility, pH and dead percentage of spermatozoa on every 24 hours interval for a period of 96 hours, and the behaviour of different diluents in keeping quality of semen was recorded.

MOTILITY :-

Motility was expressed in terms of +1 - +2 and so on up to +5, (Herman and Swanson 1941) where +1 motility was considered as equal to 20 progressive motile spermatozoa. Motility was examined immediately after dilution and it was found that highest motility was maintained in MYS and lowest in MG. In all the six diluents motility declined progressively over a period of 96 hours which is represented in figure no. 1. In table 18 the mean percentage of progressive motile spermatozoa in different diluents and preserved for zero hour and 96 hours is represented.

T A B L E No. 18

Mean of percentage of progressive motile sperms at zero and 96 hours preservation.

Sl. No.	Interval	MY	MYC	MYD	MYS	MYG	MG
1.	0 hour	70	68	70	76	64	50
2.	96 hours	45	36	42	46	28	14

Table No. 18 shows that highest no. of progressive motile spermatozoa were maintained in MYS on zero hr. preservation followed by MY, MYD, MYC, MYG and MG. On 96 hours of preservation sperm life was preserved in the same order. In table No. 19 and figure No.7 the mean percentage of decline in progressive motile spermatozoa in various diluents on preservation for 96 hour is presented.

TABLE NO.19

Average percentage of decline in progressive motile spermatozoa preserved in various diluents for 96 hrs.

Interval	MY	MYC	MYD	MYS	MYG	MG
0 hour	10.0	104	10.0	3.8	16.0	30.0
24 hours	4.4	0.8	2.0	3.8	11.8	19.6
48 hours	2.2	10.0	4.2	5.6	3.4	7.0
72 hours	6.6	-5.4	11.0	9.8	8.8	7.0
96 hours	12.0	28.0	11.0	9.4	10.8	2.2

In MG highest decrease in progressive motile sperm was recorded on preservation for 96 hours, and the lowest in MYS (Table No. 19). It was observed that the decrease in progressive motile spermatozoa was highest in all the diluents except MG and MYC during last 24 hours. This finding does not tally with the work of Herman and Swanson 1941, who found the greatest loss of progressive motile spermatozoa during first 24 hours of preservation.

It is evident from table 7 and 19 that Glycerol containing diluents MG and MYG proved inferior to other diluents. It was thus established that yolk played an important role in protecting the spermatozoa from temperature shock. (Salisbury 1942); Kampschmidt et al.(1953); Blockshaw(1957); Mayer (1957). It was also confirmed that spermatozoa, besides yolk for protecting them from shock, also required some chemical substrate for acting as buffer against lactic acid and other end metabolities, and as well as to metabolise as a source of energy (Lardy and Phillips 1942; Vandemark 1945; Kampschmidt et al.1951; Smith et al, 1954). MYG even though contained egg yolk could not preserve semen better than MY. This finding points out that glycerol containing diluents which were useful for preserving bull semen (Almquist 1959, Ozkoca and Ozan 1961) were proved unsuitable for preserving buffalo-bull semen. This may be due to species difference or due to some other factors which could not be accounted for.

In the present investigation it was also observed that diluents containing sodium-bi-carbonate was proved superior to other diluents in maintaining optimum motility during storage. This finding confirmed the finding of Kampschmidt et al. (1953) and Smith et al. (1954). Kampschmidt et al. (1951) included bicarbonate in their diluent and found it satisfactory for long term storage. Citrate was reported to be superior to carborate and phosphate containing yolk diluents, but carbonate gave more protection against cold shock (Bogart and Mayer 1950).

Minimum percentage of decline of progressive motile spermatozoa was recorded in MYS immediately after dilution and as well as on 96 hours preservation indicated that bicarbonate protected spermatozoa from shock, better than sodium citrate, Motility of spermatozoa is an expression of certain at present little understood biochemical activities within the protoplasm of the cell. On preservation the metabolic activities of the spermatozoa was reduced thus decrease in motility was observed.

POWER OF HYDROGEN (pH) :-

Spermatozoa are very delicate cells and react to change of environment. In preserving maximum number of spermatozoa motile, optimum control of pH is very essential. The change in pH of semen is caused by the metabolic activity of the spermatozoa, and it is generally observed that increased acidity of the semen during storage is due to accumulated metabolic products. In the process of glycolysis and fructolysis there is accumulation of lactic acid which results in acid pH. Change in pH was used as an expression of sperm vitality. (Rivas Doday 1956) Dilutors containing buffers act against pH changes, and protect sperm life, however resistance and livability depend on the type of diluent used. (Bashlebnove 1937). The lesser the changes in pH the more efficient is the buffering capacity of the diluent. Means of decline in pH in various diluents on preservation for 96 hours are represented in table no. 20 and figure no.7.

TABLE NO. 20

Mean of decline in pH in various diluents on preservation for 96 hours.

Sl. No.	Interval	MY	MYC	MYO	MYS	MYG	MG
1.	24 hours	.10	.03	.12	.11	.04	.10
2.	48 hours	.17	.16	.14	.18	.03	.14
3.	72 hours	.17	.27	.14	.13	.13	.16
4.	96 hours	.22	.22	.13	.13	.13	.4

Table No. 20 indicates that highest decline in pH occured in MG followed by MYC, MY, MYD, MYG and MYD on preservation for 96 hours. Difference in decline in pH remains non-significant between MY, MYD, MYS and MYG. The resistance of spermatozoa to pH was very much influenced by the type of diluent used. The pH was not observed directly proportional to motility. In MG pH was recorded nearest to optimum 605 (Rickard 1957) the motility was found the lowest. In all the diluents pH was maintained above 5.5 on storage for 72 hours.

The decrease in progressive motile spermatozoa was recorded highest in all diluents except MG. during last 24 hours (72 hours to 96 hours) when pH tended to fall below 5.5; this finding tallies with the work of Blackshaw and Emmens 1951, who reported that bovine spermatozoa are having wide range of tolerance between 8.5 - 5.5, in which they were not affected much. In the present study it was observed that buffalo spermatozoa were having fairly

resistance power to wide range in pH variation in their environment, but the wide range of tolerance perhaps a fortunate feature, and does not rule out the possibility of there being a narrow range of maximum efficiency. In fact the effects of pH which seemingly are tolerated, may have been enough to trip the sensitive enzyme system.(Sharma 1962).

DEAD PERCENTAGE OF SPERMATOZOA :-

Table No.9 indicates that no. of dead percentage of spermatozoa increased progressive in all the diluents during storage for 96 hours, and it was observed, that the decrease in progressive motile spermatozoa concurrently caused increase in dead percentage of sperms. The no. of percentage of dead spermatozoa was noted highest in MG and lowest in MYS. The increase in percentage of dead sperm on preservation for 96 hours is represented in table No. 21 and figure No. 3.

TABLE NO. 21

Mean of increase in percentage of dead spermatozoa on preservation for 96 hours.

Sl. No.	Interval	MY	MYC	MYD	MYS	MYG	MG.
1.	24 hours	4	11	4	9	20	45
2.	48 hours	7	7	5	8	9	-1
3.	72 hours	4	12	12	7	8	11
4.	96 hours	11	9	10	14	8	1
	Total	26	39	31	38	45	57

The percentage of dead spermatozoa was recorded highest in glycerol containing diluents (MG and MYG). This finding indicated that these diluents have less capacity to protect sperm life against cold shock. Between MG and MYG, MG was observed as having higher percentage of dead spermatozoa. It indicated that yolk factor in MYG was responsible for protecting sperm life. Spermatozoal life was maximumly protected in MY diluent followed by MYD, MYS, MYC, MYG and MG (table no. 21) whereas highest motility was recorded in MYS. (Table No. 7, figure no. 3). The possible explanation for this finding may be that on storage certain spermatozoa though failed to show motility, but remained alive and thus did not take eosin stain. This fact justifies the less no. of stained spermatozoa in MY diluent.

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C H A P T E R - VI

CONCLUSION

and

SUMMARY

C O N C L U S I O N

The aim of present investigation was to find out a better and more suitable diluent containing skimmed milk for preserving buffalo-bull semen, to meet the pressing need of the day. In view of this object six diluents containing skimmed milk were tried. From the results of this experiment with "in vitro" tests the following conclusions were drawn.

1. Skim milk and yolk along with other chemical substrates can very well be employed for preservation of buffalo-bull semen for 72 hours.
2. Egg-yolk was confirmed as an essential constituent of diluents to protect sperms against cold shock.
3. MYS diluent proved superior to all other diluents tried.
4. Very less difference was observed in maintaining sperm motility between MY, MYC, MYD and MYS.
5. Glycerol containing diluents were proved inferior to non-glycerol containing diluents.
6. Linear correlation was observed between decrease in motility and increase in percentage of dead spermatozoa on preservation for 96 hours.

7. Fall in motility was recorded highest during last 24 hours suggesting that semen should be used before 72 hours.

8. No direct relation between motility and pH was recorded.

However only on " in vitro " tests it would not be advisable to advocate use of HYS, for routine A.I. work as fertility rates should be obtained before recommending wider use of this diluents under field conditions.

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S U M M A R Y

In the present investigation semen was collected from seven healthy murrah buffalo-bulls and was diluted in six diluents containing skim milk, and preserved at 4 ± 1°C. for 96 hours. Volume, colour consistency, mass activity, dead percentage of spermatozoa, and sperm concentration of the neat semen were examined. Thirty five semen samples were studied, five from each buffalo-bull. Each sample was diluted in six diluents namely MY, MYC, MYD, MYS, MYG and MG, preserved at 4 ± 1°C., and tested on every 24 hours interval for motility, pH and dead percentage of spermatozoa for 96 hours.

It was observed that motility was best maintained in MYS followed by MYD, MY, MYC, MYG and MG, during preservation for 96 hours.

Glycerol containing diluents MYG and MG proved inferior to non-glycerol diluents in maintaining better motility, indicated that yolk and other chemical substrates are essential for preservation of sperm life for longer time. Very less difference of motility was observed between MY, MYC, MYD and MYS.

pH decreased progressively in all the diluents on preservation for 96 hours. It was maintained highest in MG, followed by MYG, MYS, MY, MYD and MYC. It was

observed that spermatozoa were having fairly resistance power to wide range in pH variation in their environment.

Percentage of dead spermatozoa were declined progressively in all the diluents on preservation for 96 hours. Highest percentage of dead spermatozoa were recorded in MG, followed by MYG, MYC, MYS, MYD and MY. Very negligeable difference was recorded between MY, MYC, MYD and MYS in containing no. of dead % of sperms. Decrease in motility concurrently caused increase in dead percentage of spermatozoa.

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P A R T - T W O

M E T A B O L I S M

C H A P T E R - I

INTRODUCTION.

I N T R O D U C T I O N

The metabolism of spermatozoa is very complicated process, and there is much yet to be learnt about this interesting aspect of sperm physiology, specially from the stand point of the effects on metabolic activity of the handling and treatment necessary in A.I. practice.

The mature spermatozoa is an atypical cell, unique both morphologically and physiologically. It possesses a remarkable degree of permeability, and exchange of gas and minerals between sperm and surrounding medium take place at a rapid rate (Mann 1949). Sperm metabolism must be studied under the condition permitting accurate measurements of small quantitative changes in the amounts of metabolic fuels in the surrounding fluids, and extremely small gaseous exchange.

The effects of varicus inorganic and organic substances added to the diluting media on normal physiological behaviour of the sperm also demand serious consideration in metabolic studies. In the presence of utilisable carbohydrates spermatozoa show a preference for glycolytic metabolism as a source of energy both aerobically and anaerobically (Mann 1954). Under anaerobic conditions spermatozoa tend to utilise preferentially glucose as compared to fructose (Mann 1949; Salisbury and Vandemark 1945). Utilisation of glucose, fructose and

mannose cause formation of latic acid and these sugars benefit motility (Rendenz 1933). Formation of lactic acid and other end metabolites are having deterimental effect on sperm life.

The present study aims at to find out the rate of utilisation of fructose and non-fructose xxxds reducing substances in fresh and preserved, diluted semen samples and to determine whether any recognisable deviations occur on preservation in the normal metabolic capacity of the spermatozoa.

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C H A P T E R - II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

As early as 1933 Redeun reported that bull spermatozoa utilised glucose, fructose and mannose and produce lactic acid. The presence of these sugars, but not of sucrose, lactose and glycogen is beneficial for sperm motility. It was also observed by him that in absence of oxygen spermatozoa rely on carbohydrate metabolism as the chief source of energy.

Mann in year 1946 demonstrated that fructose and not glucose as was erroneously believed, is the glycolysable sugar present in semen, had opened a new chapter in the field of sperm physiology.

Later it was reported that spermatozoa utilise fructose and produces lactic acid (Mann 1945 ; Mann and Lutwak Mann 1948). One very significant advantage had emerged out of these studies, was a new and simple method for assessing the quality of semen; namely fructolysis Index. (Milligrammes of fructose utilised by 10 sperm in one hour at 37^{0}C).

In the presence of utilisable carbohydrate substrates, spermatozoa show a preference for glycolytic metabolism as a source of energy both aerobically and unaerobically (Mann 1954).

Salisbury (1946) studied the effects of different rates of dilution levels of glucose on motility and glycolysis of spermatozoa after four and ten days of preservation. He observed that increasing levels of glucose, resulted in better motility after storage, with a maximum response at 464 mgs. per 100 mls. Addition of glucose also stimulated greater lactic acid production during preservation, and the maximum lactic acid gain was at 1:4 dilution rate.

Roy et al. (1950a) reported normal variations in the level of fructose in semen of buffalo-bulls, rams and goats. They found that in rams and goats 85% of total reducing substances was fructose, whereas in buffalo, fructose formed only 70% of total reducing substances. They also observed that the sperm concentration was inversely correlated with initial fructose and directly correlated with fructolysis (Roy et al. (1950b).

Anderson (1951) reported a direct relationship between fructolysis and change in pH on incubation of bull semen, higher values being associated with a higher initial pH, a poorer buffering capacity and a lower specific gravity. He further suggested that the direct estimation of fructolysis appeared to give a better estimate of spermatozoal metabolic activity than the pH change.

Under anaerobic conditions spermatozoa tend to utilise preferentially glucose to fructose.(Mann 1949;

Salisbury and Vandernark 1945). The sparing effect of yolk diluent on fructose utilisation was due to preferential utilisation of glucose by spermatozoa at 37°C. and it was confirmed by Vantien-hoven et al. (1952).

Branton et al. (1952) found an inverse relationship between the concentration of fructose and the density of semen. They correlated the quality of semen in terms of fructose concentration and fertility, with a degree of excitement preceeding ejaculation, and recommended a false mount for routine practice in stud bulls.

Rasbech (1953) reported that the addition of antibiotics added to diluted semen depressed fructolysis during storage. Later on the work of Branton and Panther (1954) confirmed that antibacterial agents inhibit the metabolic activities of spermatozoa. They pointed out that penicillin, streptomycin and sulphanomide, each one of the antibacterial agents depressed utilisation of fructose, and other total reducing substances, and also glycolysis by spermatozoa. A combination of streptomycin and penicillin was as effective as sulphanomide in inhibation of metabolism.

Melrose (1953) attempted to investigate a correlation between " fructolysis index " and fertility of semen samples of A.I. bulls. He obtained significant correlation between conception rate and fructolysis index in three out of eight tested bulls.

Mann (1954) reported that under anaerobic conditions lactic acid cannot be oxidised further, but in the presence of oxygen the rate of fructolysis was smaller,

and the lactic acid undergoes further oxidation thus providing an additional source of metobolic energy.

Flipse and Almquist (1955) investigated the qualitative anaerobic catabolism of labelled glucose and concluded that spermatozoa metobolise glucose to lactic acid but the lower recovery rates of lactic acid were due to further break down of lactic acid into acetate, carbon-di-oxide and other products.

Mayer (1956) found that depression of anaerobic glycolysis by cold shock for ten minutes was slight as compared to the effect on aerobic glycolysis or respiration. At 15°C. glycolysis rate was not affected but as the cold shock temp. was lowered, the rate was depressed to greater extent, the maximum effect occuring at 1°C (62% of control).

Flirchinger et al. (1956) observed high positive correlation between increase of lactic acid and loss of fructose, sperm cont. and motility during aerobic incubation at 37°C. for one hour.

Salisbury and Kinney Jr. (1957) observed a marked effect of pH, on aerobic fructolysis. High the pH, the greater was the lactic acid production in excess of fructose utilisation.

Clark et al. (1957) reported that the heat production in the first hour of incubation at 37°C by live spermatozoa in semen appeared to be associated with the break down of fructose to lactic acid.

Salisbury and Nakabeyashi (1957) reported that under aerobic condition phosphate ions increased fructose utilisation and lactic acid production, where as chloride ions depressed both fructolysis and glycolysis. They further observed that oxygen uptake and motility was increased in presence of chloride ions and decreased in presence of phosphate ions.

Blackshaw et al. (1957) observed that fructolysis decreased more from 37°C. to 21°C. than it did from 20-5°C. Marked differences between 9% Nacl. and phosphate diluents at 37°C. were not observed at either 21 - 5°C.

Freund et al. (1957) determined the influence of sperm concentration and initial level of fructose on fructolysis. It was found that sperm concentration and the initial fructose level of semen influenced the amount of sugar used by spermatozoa.

Blackshaw and Salisbury (1957) reported that rapidly cooling diluted semen from 25°C to near 0°C greatly reduced motility respiratory activity and glycolysis Metobolic damage was retarded by lecithin, and almost completely by egg yolk, which also stimulated glycolysis of unshocked semen.

Blackshaw et al. (1958) reported that glycolysis was greatly depressed by reduction of incubation temperature. Cold shock damaged the metabolism of sperm, and yolk did not give sufficient protection to enable sperm to maintain.

their metobolic activity during a subsequent period of incubation.

Norman et al. (1958) studied the preservation of bull semen at room temperature and found that with progression of time lactic acid accumulated causing a decline in pH. In a range of 5.50 - 5.80, the sperm metabolism was maximumally inhibited. The reversible acid inhibition helped in preservation.

Pickett and Berilan (1959) reported that Glycerol plus fructose maintained a greater degree of motility and livability of bovine spermatozoa at the end of 105 minutes of incubation at 38°C. than did either fructose or glycerol alone. They pointed out that 1% glycerol and 1% fructose was the apparent optimum for maintenance of spermatozoa\motility and livability.

Freund and Murphee (1959) studied three semen characteristics - sperm concentration - initial frutose level, and initial % motility in 262 semen specimen. They observed no significant difference among bulls in fructolysis, on subject to multiple - covariance analysis, indicating that, in this system, differences in fructose utilization may be accounted by differences in sperm concentration, initial fructose level and initial percent motility.

Freund et al. (1959) reported that for every 1°C increase in incubation temperature there was 4.22% increase in fructose utilization. They suggested the

routine addition of fructose to all incubated semen samples to present fructose concentration for becoming rate - limiting factor.

De Vuyst (1959) reported that the addition of a million I.U. of vitamin A to the weekly ration of bulls, the % non-return to A.I. increased from 53.9 to 76.6. Spermatozoal fructolysis was found to be greatest in the first hour after collection. It was conducted that the sperm fructolysis was related to spermatozoal concentration and to motility.

Romstein and Steberl (1959) reported that the preservation of spermatozoa for one week in a synthetic medium did not affect the sugar metabolism of bovine spermatozoa.

Salisbury (1960) reported that there was no inhibition effect of penicillin on utilisation of hexose or lactic acid production, however he observed that streptomycin had a measurable effect on glycolysis, but not as prominant as sulphonamide. When all three were combined the inhibition effect on metabolism was primarily due to sulphonamides.

Srivastava and Rozanasir (1960) reported that the unaerobic metabolism as studied by fructolysis index was lower in EYGB diluent with sulphamezathin as compared to eggyolk citrate and Egg-yolk-glycine.

Mayudu (1961) observed that provision of exogenous glucose resulted in a higher rate of sugar

intake by buffalo spermatozoa. The presence of glucose had an inhibitory effect on the utilisation of fructose by buffalo-spermatozoa and the depression was proportional to the level of added glucose.

Lodge and Salisbury (1963) reported that metabolic carbon di-oxide significantly stimulated the oxygen uptake of the sperm cells in the presence of seminal plasma, but not in its absence. Added fructose depressed O_2 uptake during four hours when seminal plasma was present, and also during the first hour with washed cells. However after 1st hour when the washed cells had utilised all their endogenous substrates, fructose was necessary for continued metabolism.

C H A P T E R - III

MATERIAL AND METHODS

MATERIALS AND METHODS.

For observing the metabolic activities of spermatozoa of semen, diluted in various diluents, and preserved for 72 hours at 4 ± 1°C, known quantities of neat and diluted semen, fresh and preserved were incubated at 37 - 38°C. for three hours, and the contents of fructose and total reducing substances(T.R.S.) in mgs. were estimated by colourimetric procedures. In this experiment Klett - Summerson photoelectric colorimeter was used. Semen was obtained from three buffalo bulls which were used for livability studies. The sperm concentration of semen samples used in this experiment ranged from 880 to 1500 millions per ml. It was estimated by the use of Neubauer's hoemocytometer (Laing 1945).

In the present experiment two important aspects of sperm metabolism were studied.

1. Fructolysis Index.

2. T. R. S. Index.

The metabolic activities of spermatozoa can very will be judged by their capacity to utilise fructose and total reducing substances(T.R.S.) in various diluents.

BUFFER AND REAGENT SOLUTIONS :-

The different buffer solutions for the diluents and other reagent solutions for the experiments were prepared with analargrade chemicals and double glass dist. water, as discribed in Appendix A

METHODS :-

For conducting this experiment the dilutors were

prepared similar to the previous experiment for livability. The dilution rate was maintained (1: 1) and the antibiotics were added in the same concentration as in previous experiment. Semen of good quality having more than 80% progressive motile spermatozoa was only used in this investigation. Immediately after collection, the semen samples were kept in, constant temperature water bath at 37°C for further processing. 0.1 ml. of neat semen was pipetted out and deproteinised, similarly 0.1 ml. of semen with 0.3 ml. of phosphate buffer was transferred to constant temperature water bath at 37°C. for three hours for incubation to determine the utilisation of fructose and T.R.S. in mgs. by the spermatozoa.

DILUTION :-

Six tubes each 3 ml. capacity were arranged each containing 1 ml. of six dilutors. The tubes were labelled with name of the dilutor, no. of the bull and date of collection. Care was exercised that the temperature of dilutor and semen was the same at the time of collection to ensure maximum preservation of sperm life. One ml. of neat semen was transferred to every tube containing diluents. The tubes were rotated gently between the palms of the hand for uniform distribution of spermatozoa.

One ml. from each of diluted semen tubes was kept in referigerater at 4 ± 1°C for preservation for 72 hours.

Second set of six tubes were arranged for estimating fructose and T.R.S. 0.2 c.c. of the diluted semen

from each diluent was transferred to each tube and deproteinised.

INCUBATION :-

In the third set six tubes were arranged each having 0.3 ml. of phosphate buffer (pH = 7.4) and to each of the tube 0.2 ml. of diluted semen from each diluent was added, and then incubated in a constant temperature water bath at 37°C. for three hours and then deprotinised, for determinig the utilisation of fructose and T.R.S.

DEPROTEINISATION :-

To each tube containing 0.2 c.c. of diluted semen were added 2 c.c. of zinc sulphate 2% and 2 c.c. of sodium hydroxide 0.4% and then the volume was made up to 8 c.c. Each tube was kept in hot water bath for one minute for max deproteinisation and then they were centrifuged at 1200 R.P.M. for five to ten minutes.

Twelve tubes were arranged of 10 c.c. capacity, in two separate sets. One set was used for determining fructose content, and other for T.R.S. 2 c.c. of clear supernatant fluid was transferred to each tube of the two set from each diluent. The tubes were labelled with Fructose T.R.S., Bull No., name of the dilutor, and date of collection. Tubes which were deproteinised before incubation were also treated in the above fashion for estimating initial fructose and T.R.S. content.

After 72 hours preservation semen samples were taken

out and rotated between the palms of hand for uniform distribution of spermatozoa.

0.2 ml. of semen from each tube was transferred to other tubes containing 0.3 ml. of phosphate buffer and kept in water bath at 37 - 38°C. for incubation for three hours. After three hours the tubes were taken out and deproteinised as described, for estimating fructose and T. R. S.

Fructose and T. R. S. were also estimated of the preserved samples before incubation.

Estimation of fructose and T.R.S. was carried in three separate experiments without adding phosphate buffer.

ESTIMATION OF FRUCTOSE UTILISATION WORKING STANDARD :-

Two tubes were taken and marked as A and B. 1.8 ml. of dist. water was added to tube A and 1 ml. to tube B. Exactly 0.2 ml. of 0.2% (S) of fructose solution was pipetted and then 1 ml. of solution from tube A was transferred to tube B and again mixed the contents thoroughly. One ml. was pipetted out from tube B and discarded, so that finally tube A and B should contain equal volumes of solution each representing 0.1 mg. and 0.05 mg. of fructose in 1 ml. standard solutions were prepared in duplicates.

To two mls. of deproteinised supernatant were added 6 mls. of 30% Hcl and 2 ml. of alcoholic resorcinol, mixed well and kept in hot water bath at 80 - 80°C for 10 - 20 minutes or till the brick red colour developed. The tubes were taken out and cooled in running water.

Klett's ~~kmcolori~~-meter was adjusted to zero zero. The standard solution was kept in the colourimeter. The reading noted after bringing the deniated indicator to zero zero line. Colorimeteric reading was also recorded of semen samples.

The quantity of fructose in the unknown sample was estimated by the following formula.

$$\frac{\text{Quantity of fructose in standard solution}}{\text{Reading of standard solution}} \times \frac{\text{Reading of unknown sample}}{1} \times \frac{4}{1}$$

This gave the quantity of fructose in mg. in 0.1 ml. of neat or 0.2 ml. of diluted semen with the help of quantity ~~of~~ of fructose utilised by known no. of spermatozoa in three hours. Fructolysis index was calculated.

ESTIMATION OF TOTAL REDUSABLE SUBSTANCE :

T. R. S. (Indian) were estimated by the method of Nelson (1944).

WORKING STANDARDS :

Two standard solutions were prepared from 1.0% glucose stock solution (preserved) with benzoic acid of this stock solution 1.0 ml. was added to 9 ml. of dist. water. This solution contained 1 mg. glucose per ml. In two tubes marked A and B were placed 0.6 ml. and 0.7 ml. of dist. water. To these tubes were added 0.4 ml. and 0.3 ml. respectively of the diluted stock solution containing 1 mg. of glucose per ml. followed by 0.5 ml. of zinc sulphate

and 0.5 ml. of 0.1 NaoH solution. The two mls. of standard solutions A and B contained 0.4 mg., 0.30 mg. of glucose respectively.

2 mls. of deproteinised supernatant fluid was taken in a test tube, to it was added 2 mls. of alkaline copper reagent. The tube was allowed to remain in hot water bath at 80 - 85°C till the colour changed to yellow colour. It was taken out from the bath and cooled in renning tap water. To each of the tube was added 2.0 mls. of molybdate reagent and shaken vigorously. The colour changed to dark blue or light green shades. Then the volume was made up to 8 mls. by adding dist. water.

Readings of working standards were recorded first by keeping tubes containing solution in klett-Summersons bio-colorimeter. In the same readings were noted of semen samples tube. The quantity of T.R.S. was calculated with the following formula.

$$\frac{\text{Quantity of glucose in standard solution}}{\text{Reading of standard}} \times \frac{\text{Reading of semen samples}}{\frac{1}{4}}$$

Thus the amount of T.R.S. in 0.1 ml. of semen was estimated. The same procedure was followed for semen samples, fresh preserved and incubated without adding phosphate buffer.

Control tubes were prepared by keeping the semen samples in freezing chamber of referigerator for killing all the spermatozoa, and then incubated, at 37°C. for three hours, to find out whether utilisation of fructose and T.R.S. was made by any other source besides spermatozoa.

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C H A P T E R - IV

RESULTS

R E S U L T S

UTILISATION OF FRUCTOSE AND TOTAL REDUCING SUBSTANCE BY THE SPERMATOZOA :

In the presentation of the results of metabolic activities of the spermatozoa the following expression are used:

FRUCTOLYSIS INDEX :

The utilisation of fructose in mgs. by one billion (10^9) spermatozoa at $37^{\circ}C$ in one hour.

TOTAL REDUCING SUBSTANCE INDEX (TRS):

The utilisation of TRS in milligrammes by one billion of spermatozoa at $37^{\circ}C$. in one hour.

The original values of utilisation of fructose and TRS in fresh and preserved semen samples with and without phosphate buffer are represented in Appendix C. The mean of fructolysis Indices and TRS Indices of fresh, and diluted semen in various diluents with phosphate buffer, before and after preservation for 72 hours at $4 \pm 1^{\circ}C$., are represented in table 1.

Table No.1 indicates that maximum utilisation of fructose by sperms occurred in neat semen except MYC, followed by MYD, MYS, MY, MG, MYG. In MYD most of the utilised substances consisted of hexose (non-fructose substance); the same trend was also maintained on

T A B L E NO. 1

Means of fructolysis Indices, TRS Indices along with the standard error and coefficient of variation in milligrammes before and after preservation with phosphate buffer.

Sl. No.	Diluents	Fructolysis Indices (Fresh)			Fructolysis Indices (preserved)			TRS Indices (Fresh)			TRS Indices (Preserved)		
		Av.	S.E. ±	C.V.	Av.	S.E. ±	C.V.	Av.	S.E. ±	C.V.	Av.	S.E. ±	C.V.
1.	Neat semen	1.24	.13	10.48	-	-	-	1.34	.17	12.68	-	-	-
2.	MY	1.11	.09	8.10	.21	.05	23.80	1.22	.04	3.28	.23	.11	47.92
3.	MYC	1.33	.15	11.27	.21	.05	23.80	1.16	.05	4.31	.39	.11	28.20
4.	MYD	.78	.05	4.25	.08	.19	50.00	1.44	.07	8.03	.45	.05	26.31
5.	MYS	1.12	.11	9.82	.22	.05	22.72	1.14	.05	4.38	.15	.04	26.60
6.	MYG	1.02	.12	11.76	.31	.11	35.48	1.20	.15	12.50	.39	.21	53.84
7.	MG	1.03	.12	11.65	.35	.23	65.71	1.00	.05	5.00	.24	.05	20.83

preservation. Spermatozoa utilised preferably non-fructose hexose than fructose, and more than four fifth of utilised hexose consisted of non-fructose substances.

The data pertaining to fructolysis Indices, TRS Indices of spermatozoa before and after preservation in six diluents were subjected to analysis of variance which are represented in Table no. 2.

T A B L E NO. 2.

Analysis of variance of fructolysis Indices and TRS Indices of spermatozoa in fresh and diluted semen before & after preservation with phosphate.

Sl. No.	Source of variance.	D. F.	Fructolysis Indices		T.R.S. Indices	
			M. S.	F. Ratio	M. S.	F. Ratio
1.	Fresh Vs. preserved(A)	1	6.55	6.1*	6.86	6.3*
2.	Diluents (B)	5	.018	.01	.03	.02
3.	A x B	5	.03	.02	.02	.009
4.	Error	24	1.07	-	1.08	-
	Total	35	-	-	-	-

N. S. - * Significant at 5% level.

Table No. 2 indicates that the difference between fresh and preserved samples was significant for both fructolysis Indices and TRS Indices values. The difference between the diluents remained non-significant for both the values.

TABLE No. 3

Means of fructolysis Indices, and TBS Indices, along with standard error and coefficient of variance in milligrammes before and after preservation without phosphate buffer.

Sl. No.	Diluents	Fructolysis Indices (Fresh)			Fructolysis Indices (preserved)			TBS Indices (Fresh)			TBS Indices (preserved)		
		Av.	S.E. ±	C. V.	Av.	S. E. ±	C. V.	Av.	S.E.	C.V.	Av.	S.E. ±	C. V.
1.	Neat semen	0.25	.05	20.0	-	-	-	.39	.16	41.02	-	-	-
2.	MY	0.53	.21	33.33	.25	.12	48.0	.23	.05	21.73	.14	.05	35.71
3.	MYC	0.38	.21	55.26	.72	.41	56.94	.28	.04	14.28	.45	.19	42.22
4.	MYD	0.46	.09	98.56	.44	.21	47.72	.28	.11	39.28	.20	.04	20.00
5.	MYS	0.54	.27	50.0	.82	.50	60.9%	.18	.05	37.77	.13	.04	30.76
6.	MYG	0.34	.09	26.47	.39	.09	23.07	.19	.04	21.05	.35	.03	65.71
7.	MG	0.29	.05	17.24	.61	.15	24.59	.24	.04	16.66	0.85	.05	33.33

Table 3 indicates that utilisation of fructose was maximum in MYS diluent in fresh condition followed by MY, MYD, MYC, MYG, Mg, and neat semen in descending order. On preservation spermatozoa had also utilised fructose to maximum extent in MYS only, followed by MYC, MG, MYD, MYG, and MY. In MYD spermatozoa utilised maximum amount of hexose and minimum in MYS in fresh condition; even on preservation utilisation of hexose was minimum in MYS and maximum was utilised by spermatozoa diluted in MYC followed by MYG, MYD, and MY.

Comparing Table no. 1 and 3 it is evident that utilisation of fructose and as well as hexose was much reduced in semen samples incubated with phosphate buffer than without it. These differences were represented in figure no. 1. From the difference between the corresponding values between fresh and preserved semen samples before incubation, the utilisation of fructose and TRS by 10^9 spermatozoa during preservation for 72 hours at 4 ± 1°C were calculated and the mean values are represented in table 5.

TABLE No. 4

Mean of fructose and TRS in milligrammes utilised by 10^9 spermatozoa at 4 ± 1°C during 72 hours.

Sl. No.	Item	MY	MYC	MYD	MYS	MYG	MG
1.	Fructose	.19	.13	.09	.12	.13	.09
2.	TRS	.54	.36	.41	.38	.36	.33

Table 4 indicates that utilisation of fructose by glucose containing diluents is the fraction of TRS utilised, even on preservation for 72 hours at 4 ± 1°C. the trend of utilisation of carbohydrates was maintained as on incubation in fresh and preserved semen. In order to find out the whether besides spermatozoa, fructose and TRS are metabolised by any other factor. Control experiments were run, by killing the spermatozoa in freezing chamber of the referigerator.

Mean of fructose and TRS in fresh and preserved semen sample before and after incubation are given in table No. 5.

T A B L E NO. 5.

Mean of fructose and TRS in control semen samples before and after incubation.

	Fresh		Preserved	
	Before incubation	After incubation	Before incubation	After incubation.
Fructose	.19	.15	.19	.17
TRS	.62	.61	.62	.61

Table No. 5 indicates the values for fructose and total reducing substance of fresh and preserved samples were more or less same, as there was no source of utilisation of carbohydrates except sperms.

C H A P T E R - V

D I S C U S S I O N

DISCUSSION

Redenz (1938) was first to report that under anaerobic conditions glycolysable substrate was necessary for life. Glucose was utilised preferentially to fructose by sperms (Salisbury and Vandemark 1949). In the present investigation it was observed that sperms in glucose containing dilutor (MYD) utilised glucose preferentially to fructose. Seven parts out of ten parts of TRS utilised by sperms were Non-fructose Reducing Substances, which were utilised by the spermatozoa diluted in MYD diluent. Salisbury (1946) reported that utilisation of glucose (TRS) was much influenced by the level of glucose in the diluent. The rate of utilisation of TRS was 1.44 which is about double than the rate of fructose utilisation. This finding suggests that spermatozoa metabolise glucose at a more rapid rate than fructose.

The lower values of fructolysis and TRS Indices in diluted semen in comparison to neat semen may be accounted as due to the antibacterial agents used in this experiment. Rasbech (1953) reported that the addition of antibiotics added to the diluted semen depressed fructolysis during storage. Branton and Prather (1954) confirmed this finding. They showed that penicillin, streptomycin and sulphonamides, each one of the antibacterial agents depressed utilisation of fructose and

Non-fructose Reducing Substances, and also glycolysis by spermatozoa. Utilisation of fructose was observed highest in MYC and lowest in MG in fresh diluted semen (Table No. 1 and figure No. 1).

It was observed that diluent containing citrate was having higher utilisation of fructose than other diluents. It was reported that phosphate and citrate have stimulating effect on fructose utilisation and depressing effect on motility, (Salisbury and Nakabayashi 1957).

The utilisation of fructose and TRS was lower in MYS than MYC before preservation. This can be explained that bicarbonate containing diluents have inhibition effect on fructose utilisation.

Preservation for 72 hours at 4 ± 1°C caused decrease in hexose utilisation. Though a loss of livability could have been responsible for it in the MYG, MG diluents, but in MY, MYC, MYD, this decline may have been due to " aging ", since the loss of livability due to preservation was insignificant in these diluents.

Utilisation of fructose was observed higher in MY, MYC, MYS, MYG, and MG after preservation, since the higher values of utilisation of fructose in these diluents except in MYG and MG were associated with better preservation of livability. In MYG and MG diluents, the higher values of fructose utilisation after preservation, suggest that spermatozoa possibly catabolise glycerol to fructose (White 1957).

Table No. 2 shows that preservation period to a greater extent contributes in variation of fructose and TRS. The values of fructolysis and TRS Indices were significant.

As we know that production of lactic acid inhibits metabolic activities of spermatozoa, therefore, buffers are needed to neuteralise the effect of lactic acid produced by the metabolic action of sperms. It was reported that phosphate ions play profound part in controlling aerobic metabolism of bull spermatozoa (Bishop and Salisbury 1952). The phosphate ions inhibited the oxidation of the lactic acid formed and depressed motility, but it forced the system to greater aerobic glycolysis in order to meet the energy demands.

In the present investigation separate experiments were run with and without phosphate buffer. Table 1 and 3 and figure 1 to 8 indicate that the capacity of utilisation of fructose and TRS had much reduced in diluents incubated without phosphate buffer, than those which were treated with it. The corresponding values of fructose and TRS utilisation varied considerably in diluents incubated with and without phosphate buffer (Table 1 and 3).

The increased values of utilisation of fructose in diluents incubated without phosphate buffer(MYG, MYS, MYG, and MG) can be explained as follows :

In milk yolk containing diluents increased values of fructose utilisation could not be explained. In MYG and MG, Glycerol was possibly catabolised to fructose (White 1957).

Table No. 4 shows that utilisation of fructose was maximum in MYD except in MY diluents, whereas utilisation of TRS in the same diluent was observed lowest except MY on preservation for 72 hours. This finding points out that even on preservation at 4 ± 1°C for 72 hours, spermatozoa tended to utilise glucose preferentially to fructose (Mann 1954).

In control semen samples the values for fructolysis and TRS Indices in fresh and preserved semen samples before and after incubation remained more or less same (Table No. 5). This finding suggests that there were no other factors except spermatozoa which could change the quantities of fructose and TRS in the semen.

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C H A P T E R - VI

CONCLUSION

and

SUMMARY

CONCLUSION

Semen of buffalo-bulls was diluted in six diluents containing skimmed milk, and they were tested for fructose and TRS utilisation capacity of spermatozoa before and after preservation at 4 ± 1°C with and without phosphate buffer. The object of this study was to determine whether any recognisable deviations occur in the metabolic activity of the spermatozoa on preservation in various diluents and to what extent they effect the efficiency of the dilutors for preserving life of spermatozoa. From the results of the present study following conclusionswere drawn.

1. The capacity of spermatozoa to utilise fructose and TRS was reduced to a greater extent in fresh and preserved semen, suggesting that these may be due to " aging " of spermatozoa.

2. The values both for fructose and TRS remained higher in diluents incubated with phosphate buffer than without it, suggesting that phosphate was having stimulating effect on fructolysis (Salisbury and Nakabayashi 1957).

3. Provision of exogenous glucose resulted in a higher rate of sugar intake by the buffalo-bull spermatozoa both at 4 and 37°C.

4. Glucose was utilised preferentially by the sperms to fructose in Glucose containing diluent and it was having inhibition effect on the utilisation of fructose.

5. Increased values of fructose Indices in Glycerol containing diluents on preservation suggest that the spermatozoa possibly catabolise glycerol to fructose as a source of energy (White 1957).

6. Spermatozoan metabolism tended to be "conditioned" or acclimatised on exposure to specific environment.

7. Phosphate ions played a profound part in controlling aerobic metabolism as utilisation of fructose and TRS was much more in diluents incubated with phosphate than without it (Bishop and Salisbury 1955).

8. On the basis of increased values of hexose utilisation in some diluents, egg yolk appeared to be involved as sources of energy for the spermatozoa in diluted semen samples both at 4 and 37°C.

9. Antibacterial agents used in the experiment were having inhibition effect on fructose and TRS utilisation (Rasbech 1958).

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SUMMARY

In the present study semen from buffalo-bulls maintained at Semen Bank Patna was diluted in six diluents containing skimmed milk. Sperm concentration of the semen samples used in this experiment ranged between 880-1500 Million per ml. In order to determine the rate of metabolic activities of spermatozoa two important aspects were studied i.e.

1. Fructolysis Index.
2. TRS Index.

Neat and diluted semen samples were incubated, before and after preservation for 72 hours at 4 ± 1°C, with and without phosphate buffer. After incubation at 37°C for three hours the semen samples were deproteinised and centrifused. To the known quantity of supernatant fluid alcoholic resorcinol with concentrated Hydrochloric acid were added. The tubes were kept in water bath at 80 - 85°C till brick red colour developed, then they were cooled down in running tap water.

For estimation of TRS to the supernatant fluid of the same semen sample 2 c.c. of alkaline copper reagent was added and kept in water bath till the colour changed to yellow. The tubes were also cooled down in running tap water, to the cooled tube 2 c.c. of Arsino-molybdate reagent was added and shaken well. The colour changed to dark blue. Semen samples, before and after

preservation with and without phosphate buffer were treated as above.

STANDARD SOLUTIONS :

Known quantities of fructose and glucose were dissolved in dist. water to prepare standard solutions.

Klett Summerson Photoelectric colorimeter was used in this experiment to determine the quantity of fructose and TRS in unknown semen samples, before and after preservation with and without phosphate on incubation at 37°C.

Rate of utilisation of fructose and TRS was also recorded in control semen samples. The control semen samples were kept in freezing chamber of refrigerator for ½ hour before processing.

Readings of the standard tubes were recorded by the colorimeter. Then the unknown semen samples tubes were kept in the colorimeter and the reading were noted.

With the help of reading of standard solutions the quantity of fructose and TRS utilised by spermatozoa were calculated.

The results of the experiment indicate that sperms preferentially utilised glucose to fructose aerobically.

Phosphate buffer was having profound control over aerobic metabolism of spermatozoa (Bishop & Salisbury 1958).

Antibacterial agents used in the experiment were having inhibition effect on fructose and TRS ~~xxx~~ utilisation (Rasbech 1953).

Preservation caused decrease in capacity of spermatozoa of utilising Fructose TRS.

Provision of exogenous glucose resulted in a higher rate of hexose utilisation by the spermatozoa both at $4^{o}C$ and $37^{o}C$.

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A P P E N D I X

(A - B - C)

APPENDIX - 'A'.

STANDARD SOLUTIONS

1. **2.94% Sodium Citrate Solution.**

Sodium Citrate dihydrate ($Na_3C_6H_5O_7.2H_2O$.)	....	2.94 gms.
Glass dist. water	....	100 mls.

2. **1.3% Sodium bicarbonate solution.**

Sodium bicarbonate ($NaHCO_3$)	....	1.3 gms.
Glass dist. water	....	100 mls.

3. **5% Glucose solution.**

Glucose ($C_6H_{12}O_6$)	....	5 gms.
Glass dist. water	....	100 mls.

(The solutions were autoclaved before use)

4. **0.25 M phosphate buffer (pH-7.4).**

Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)	....	17.9 gms.
Hydrochloric acid (1 N HCl)	....	10 mls.
Glass dist. water add	....	190 mls.

5. **2% Zinc sulphate solution.**

Zinc sulphate ($ZnSO_4.7H_2O$)	....	2 gms.
Dist. water to make up	....	100 mls.

6. **0.1 N sodium hydroxide solution.**

Sodium hydroxide	....	0.4 gms.
Dist. water to make up	....	100 mls.

The carbonate and Rochelle salts were dissolved in 250 mls. dist. water and the bicarbonate was added. The sulphate was dissolved in 500 mls. dist. water and boiled to expel the air. The solutions were added and brought to 800 mls. volume and labelled as no. 1.

Reagent II (Copper reagent)

Copper sulphate	....	4 gms.
Sodium sulphate	....	30 gms.
Dist. water to make up	....	200 mls.

Placed 40 mls. of 10% solution of copper sulphate in a graduated cylinder, dissolved 36 gms. sodium sulphate in 100 mls. water and boiled and added the copper sulphate solution and made up to 200 mls.

Four parts of Reagent I were added to one part of Reagent II at the time of estimation of TRS and used as copper reagent.

12. Arsinomolybdate reagent.

Dissolved 25 gms. Ammonium molybdate in 450 mls. of dist. water. To it was added 21 mls. of con. sulphuric acid and mixed well.

Dissolved 3 gms. of $Na_2 H AsO_4 . 7 H_2O$ (Disodium hydg. Arsinate) in 25 mls. of dist. water, and mixed the two solutions. The mixed solution was placed in a constant temp. water bath at 37°C. for 24 - 48 hours and then stored in cool and dark place.(The solution was kept in brown bottle).

——:::::——

7. 0.2% Fructose solution

Fructose	....	0.2 gms.
Water to make up	....	100 mls.

The solution was preserved with benzoic acid.

8. 0.1% Alcoholic Resorcinol solution.

Resorcinol	....	0.1 gm.
Ethanol (98%) to make up	....	100 ml.

(The solution was stored in dark and cool place)

9. 2.5% copper sulphate

Copper sulphate ($CuSO_4 \cdot 7 H_2O$)	....	2.5 gms.
Dist. water to make up	....	100 mls.

10. 1% glucose solution.

Glucose	....	1 gm.
Dist. water to make up	....	100 mls.

11. Alkaline copper reagent.

Two solutions were prepared.

Reagent I

Anhydrous Sodium carbonate	....	24 gms.
Sodium bicarbonate	....	16 gms.
Pot. sodium tartarate (Rochelle salt).	....	12 gms.
Sodium sulphate	....	144gms.
Dist. water to make up	....	800mls.

APPENDIX - 'B'.

Table No.1

Mass activity of neat semen samples.

Collection	Bull Numbers.						
	11	0/8	38	0/4	M	4	137
1.	++++	++++	++++(+)	++++	++++	++++(+)	++++
2.	++++(+)	++++	++++	++++	++++(+)	++++(+)	+++(+)
3.	++++	++++	+++(+)	++++(+)	++++(+)	++++	++++
4.	++++	++++	++++	++++	++++	++++	+++
5.	++++(+)	+++(+)	++++	++++	++++(+)	+++(+)	++++

Table No.2

Volume of neat semen in c.c.

Collection	Bull Number						
	11	0/8	38	0/4	M	4	137
1.	4.5	3.5	5.5	3.5	5	4.0	8.0
2.	3.5	3.0	3.5	3.5	5.5	5.5	4.5
3.	3.5	3.5	3.5	4.0	2.5	5.0	7.0
4.	2.0	3.5	4.5	8.0	5.0	5.5	4.0
5.	3.5	7.5	7.0	5.0	4.5	4.0	4.0

Table No. 3

Colour of the neat semen samples.

Collection	Bull Numbers						
	11	0/8	38	0/4	M	4	137
1.	C	C	M	M	M	M	C
2.	C	M	M	C	C	C	C
3.	M	C	M	C	C	M	C
4.	M	M	C	C	C	M	M
5.	C	M	M	M	C	M	M

N. B. - M Milky.
C Creamy.

Table No. 4

Consistency of neat semen samples.

Collection	Bull Numbers.						
	11	0/8	38	0/4	M	4	137
1.	Medium	Thick	Medium	Medium	Medium	Medium	Medium
2.	Medium	Medium	Medium	Medium	Medium	Medium	Medium
3.	Medium	Thick	Medium	Medium	Thick	Thin	Medium
4.	Thin	Medium	Medium	Medium	Medium	Thin	Medium
5.	Medium	Thin	Medium	Medium	Thin	Thin	Thick

Table No. 5

Power of Hydrogen (pH) of neat semen samples.

Collection	Bull Numbers						
	11	0/8	38	0/4	M	4	137
1.	6.9	6.8	6.7	6.5	6.7	6.9	6.5
2.	6.9	6.6	6.5	6.3	6.7	6.8	6.5
3.	6.9	6.5	6.9	6.4	6.7	6.5	6.9
4.	6.6	6.4	7.0	7.0	7.0	6.7	6.9
5.	6.9	7.0	6.9	6.6	6.9	6.9	6.9

Table No. 6.

Sperm concentration of neat semen samples.
in million per Ml.

Collection	Bull Numbers.						
	11	0/8	38	0/4	M	4	137
1.	1120	1809	1320	1120	1290	670	1205
2.	1502	892	1510	1202	1502	890	1900
3.	891	1923	1502	920	1510	920	792
4.	792	1202	1809	1130	1120	1360	930
5.	1960	1200	1201	1390	1680	890	830

Table No. 7

Dead percentage of the spermatozoa in neat semen samples.

Collection	Bull Numbers.						
	11	0/8	38	0/4	M	4	137
1.	10.0	12.0	18.0	13.0	19.0	4.0	12.0
2.	12.0	9.0	5.4	9.9	8.5	6.0	11.0
3.	10.0	12.0	9.8	12.0	16.0	8.0	16.0
4.	9.0	11.0	16.0	6.5	9.2	12.0	6.5
5.	19.0	7.5	10.0	7.0	11.0	19.0	12.0

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Table No. 8

Motility of spermatozoa in diluted semen samples after 24 hours preservation at 4 ± 1°

Diluent	Collection	Bull Numbers.						
		11	0/8	38	0/4	M	4	137
Milk yolk.	1.	+4.0	+3.5	+3.5	+4.0	+4.0	+3.5	+0.5
	2.	+4.0	+4.0	+1.5	+4.0	+3.5	+4.5	+3.0
	3.	+3.5	+3.5	+4.0	+4.0	+4.0	+3.5	+0.5
	4.	+3.5	+0.5	+2.5	+3.5	+4.0	+3.0	+3.0
	5.	+4.0	+3.5	+3.0	+4.0	+3.0	+3.0	+3.5
Milk yolk citrate.	1.	+3.5	+3.5	+3.5	+4.0	+3.5	+3.5	+3.0
	2.	+3.5	+4.5	+3.0	+4.0	+4.0	+4.0	+3.5
	3.	+4.0	+3.5	+3.0	+4.0	+3.5	+4.0	+2.0
	4.	+3.5	+3.0	+3.5	+4.0	+3.5	+4.0	+3.0
	5.	+3.5	+3.5	+2.5	+4.0	+2.0	+3.5	+2.0
Milk yolk glucose.	1.	+4.5	+3.0	+4.0	+4.0	+4.0	+3.5	+3.5
	2.	+3.5	+4.0	+3.5	+4.0	+4.0	+4.0	+3.5
	3.	+3.0	+3.3	+3.0	+4.0	+3.5	+3.5	+3.0
	4.	+3.0	+0.5	+3.5	+3.5	+3.5	+4.0	+3.5
	5.	+3.5	+3.0	+3.0	+4.0	+3.5	+4.0	+3.0
Milk yolk Sod.bi-carb.	1.	+3.5	+4.0	+4.0	+3.5	+3.5	+3.5	+3.5
	2.	+3.5	+4.0	+3.0	+4.0	+3.5	+4.5	+3.5
	3.	+4.5	+4.0	+3.5	+4.0	+4.0	+3.0	+3.0
	4.	+4.0	+3.0	+2.0	+4.0	+4.0	+4.0	+4.0
	5.	+3.0	+4.0	+3.5	+4.0	+3.5	+3.0	+3.5

Table No. 8 (Contd.)

Diluent	Collection	Bull Numbers						
		11	0/8	38	0/4	M	4	137
Milk yolk Glycerol.	1.	+4.0	+3.0	+3.5	+3.5	+3.0	+3.5	+2.0
	2.	+2.0	+4.0	+3.0	+3.5	+3.0	+4.0	+3.0
	3.	+2.0	+2.0	+3.0	+3.0	+0.5	+3.0	+2.0
	4.	+2.0	+1.0	+2.5	+3.0	+3.0	+2.5	+2.0
	5.	+2.0	+2.0	+2.0	+3.5	+0.5	+3.0	+2.0
Milk glycerol.	1.	+3.0	+2.5	+4.0	+3.5	+2.0	+4.0	+0.5
	2.	+1.0	+4.5	+1.5	+2.0	+1.0	+1.5	+2.0
	3.	+2.0	+1.0	+0.5	+0.5	+0.5	+2.5	+0.5
	4.	+1.0	+0.5	+0.5	+1.0	+0.5	+0.5	+0.5
	5.	+0.5	+1.0	+0.5	+0.5	+0.5	+0.5	+0.5

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Table No. 9

Motility of spermatozoa in diluted semen samples after 48 hours preservation at 4±1°C.

Diluent	Collection	Bull Number						
		11	0/8	38	0/4	N	4	137
Milk yolk	1.	3.0	4.0	3.0	3.5	3.5	4.0	3.5
	2.	3.0	3.5	0.5	3.0	3.5	4.5	3.0
	3.	3.5	3.5	4.0	3.5	3.5	3.0	2.0
	4.	3.5	3.0	1.0	3.5	3.5	3.0	3.0
	5.	4.0	3.5	2.0	4.0	3.5	2.0	3.0
Milk yolk citrate.	1.	4.0	3.5	4.0	4.0	3.5	3.5	3.5
	2.	3.0	4.0	0.5	3.5	4.0	4.0	3.5
	3.	4.0	4.0	3.5	3.0	3.5	3.5	2.0
	4.	3.5	3.0	2.0	3.5	2.5	4.0	3.5
	5.	3.5	3.0	2.0	3.0	3.5	4.0	2.0
Milk yolk glucose.	1.	3.0	3.0	3.0	3.5	3.5	3.0	3.0
	2.	3.0	4.0	2.0	3.5	4.0	4.0	3.5
	3.	3.5	4.0	3.0	3.5	4.0	3.5	3.0
	4.	3.0	3.5	1.0	4.0	3.5	3.0	3.5
	5.	3.5	3.0	2.5	3.0	3.5	3.5	3.5
Milk yolk Sod. bicarb.	1.	4.5	4.0	3.5	3.5	3.0	3.5	4.0
	2.	2.5	4.0	0.5	3.5	3.5	3.0	3.5
	3.	4.0	4.0	4.0	3.0	3.5	3.5	3.0
	4.	4.0	3.5	0.5	3.0	3.5	3.5	4.0
	5.	3.5	4.0	3.0	3.5	3.0	3.5	3.0
Milk yolk glycerol.	1.	3.5	2.0	3.5	3.5	2.0	3.5	2.0
	2.	2.0	4.0	1.0	3.0	3.0	4.5	3.0
	3.	2.5	3.0	3.5	3.0	3.5	4.0	3.0
	4.	2.0	3.0	0.5	2.0	3.0	0.5	2.0
	5.	1.0	2.0	1.5	2.5	0.5	2.0	2.0
Milk glycerol.	1.	3.5	1.5	4.0	0.5	1.0	3.0	0.5
	2.	1.0	4.0	0.5	4.5	2.0	2.0	2.0
	3.	0.5	1.0	0.5	0.5	0.5	2.0	0.5
	4.	0.5	0.5	0.5	0.5	0.5	0.0	0.5
	5.	0.5	0.5	0.5	0.5	0.5	0.0	0.5

N. B. - Every figure is followed by + .

Table No. 20.

Motility of spermatozoa in diluted semen samples after 72 hours preservation at 4±1°C.

Diluent	Collection.	Bull Number.						
		11	0/8	38	0/4	H	4	137
Milk yolk.	1.	3.0	3.5	3.0	4.0	3.0	3.5	3.5
	2.	2.0	3.0	0.5	1.0	3.5	3.5	3.0
	3.	3.0	3.0	3.5	3.0	3.5	3.5	2.0
	4.	3.0	3.0	0.5	3.5	3.5	3.5	3.0
	5.	3.0	3.0	2.0	3.5	3.0	1.5	2.0
Milk yolk citrate.	1.	3.5	3.0	3.0	4.0	3.5	3.0	3.0
	2.	3.0	2.0	0.5	3.0	3.5	3.5	3.0
	3.	3.0	3.0	3.0	2.0	4.0	3.5	1.5
	4.	3.0	2.0	3.0	3.0	3.0	3.0	3.0
	5.	3.0	3.0	1.5	2.5	3.0	4.0	2.0
Milk yolk glucose.	1.	3.0	3.0	3.0	4.5	3.0	3.5	2.5
	2.	2.5	3.0	0.5	3.0	0.5	3.5	3.0
	3.	3.5	3.0	2.0	3.0	3.5	3.5	0.5
	4.	3.0	3.5	0.5	3.0	4.0	3.5	3.5
	5.	3.0	2.5	2.0	2.0	3.0	3.0	3.0
Milk yolk Sod. bicarb.	1.	3.5	3.5	3.5	4.0	3.5	3.5	3.5
	2.	2.5	3.0	0.5	3.5	0.5	3.0	3.5
	3.	3.5	3.5	3.5	0.5	3.0	4.0	1.5
	4.	3.0	3.0	0.5	2.5	4.0	3.0	4.0
	5.	3.0	4.0	3.0	2.0	2.0	3.0	3.0
Milk yolk glycerol	1.	4.0	2.0	3.0	2.0	3.5	3.0	2.5
	2.	1.0	3.5	0.5	3.0	3.0	3.5	0.5
	3.	2.0	2.0	2.5	3.0	0.5	3.5	0.5
	4.	1.0	2.0	0.5	2.5	3.0	1.5	1.5
	5.	0.5	2.0	1.5	2.0	3.0	1.0	1.0
Milk glycerol.	1.	3.0	0.5	0.5	0.5	0.5	3.5	0.5
	2.	0.0	0.2	0.0	0.5	2.5	1.0	1.0
	3.	0.5	0.5	0.5	0.5	0.5	2.0	0.5
	4.	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	5.	0.5	0.5	0.5	0.5	0.5	0.5	0.5

N. B. Every figure is followed by + .

Table No. 11

Motility of spermatozoa in diluted semen samples after 96 hours preservation at 4±1°C.

Diluent	Collection.	Bull Numbers.						
		11	0/8	38	0/4	M	4	137
Milk yolk	1.	3.0	3.0	2.0	3.5	3.5	3.5	3.0
	2.	2.0	2.0	0.5	0.5	3.0	2.0	1.0
	3.	3.0	2.5	0.5	3.0	4.0	3.0	1.5
	4.	2.5	3.0	0.5	0.0	3.0	1.5	2.5
	5.	2.0	2.5	0.5	3.0	2.0	0.5	2.0
Milk yolk citrate.	1.	3.0	2.5	2.5	3.5	3.0	3.0	3.0
	2.	2.0	2.0	0.5	0.5	3.0	2.0	2.0
	3.	2.0	0.5	2.0	2.0	4.0	3.5	1.5
	4.	2.0	1.0	3.0	3.0	2.5	3.0	2.5
	5.	2.0	1.5	1.5	2.0	3.0	3.5	1.5
Milk yolk glucose.	1.	3.5	2.0	3.0	4.0	4.5	3.5	2.0
	2.	2.0	2.0	0.5	0.5	4.0	3.0	2.0
	3.	2.0	2.5	0.5	2.0	4.0	3.0	0.5
	4.	2.5	3.0	0.5	3.0	3.5	3.0	3.0
	5.	3.0	2.0	0.5	0.5	2.0	3.0	3.0
Milk yolk Sod. Bicarb.	1.	3.5	3.5	3.0	4.0	3.5	3.0	3.5
	2.	2.0	2.5	0.5	0.5	0.5	3.0	2.5
	3.	3.0	3.5	3.5	0.5	3.5	3.5	0.5
	4.	2.0	3.0	0.5	0.5	4.0	2.0	3.0
	5.	3.0	3.0	2.0	0.5	2.0	3.0	2.0
Milk yolk glycerol.	1.	4.0	1.0	2.0	2.0	3.5	3.0	2.0
	2.	1.0	1.5	0.0	0.5	2.5	3.0	0.5
	3.	1.0	0.5	2.0	2.0	0.5	2.5	0.0
	4.	1.0	0.5	0.0	1.5	4.0	0.5	1.5
	5.	0.5	2.0	0.0	1.0	3.0	0.5	1.0
Milk Glycerol.	1.	3.0	0.5	0.5	0.5	0.5	3.0	0.5
	2.	0.0	1.0	0.5	0.5	2.5	0.5	0.5
	3.	0.5	0.5	0.5	0.0	0.5	2.0	0.0
	4.	0.5	0.5	0.5	0.0	2.0	0.5	0.5
	5.	0.5	0.5	0.5	0.0	0.5	0.0	0.5

N. B. Every figures is followed by + .

Table No. 12

pH of Semen, diluted in various diluents and preserved for 24 hours at 4 ± 1°C.

Diluent	Collection	Bull Numbers						
		11	0/8	38	0/4	M	4	137
Milk yolk	1.	5.9	5.9	5.3	6.1	5.9	6.1	5.8
	2.	6.1	6.1	5.2	5.9	5.7	5.9	6.1
	3.	5.9	5.9	6.0	6.3	5.7	6.2	6.3
	4.	6.1	6.4	6.4	6.2	5.7	6.3	6.1
	5.	6.1	6.2	6.2	6.1	6.2	6.2	6.2
Milk yolk citrate.	1.	5.8	5.9	5.5	6.1	6.1	6.2	5.7
	2.	5.7	5.9	5.1	5.7	5.8	5.8	5.7
	3.	5.9	5.7	5.7	6.1	5.8	5.9	6.0
	4.	5.8	6.3	5.9	6.1	5.7	5.7	6.0
	5.	5.9	6.1	5.9	6.0	5.9	5.7	6.1
Milk yolk glucose	1.	5.9	5.8	5.3	5.7	6.1	6.1	5.5
	2.	5.5	5.6	5.1	5.5	5.9	5.1	5.5
	3.	6.0	5.5	5.3	6.1	5.7	5.7	5.7
	4.	6.1	6.4	5.7	6.1	5.5	5.7	5.9
	5.	6.0	6.2	5.7	6.1	5.7	5.8	6.1
Milk yolk Sod. bicarb.	1.	5.9	6.0	5.5	5.7	6.2	6.0	6.0
	2.	5.7	5.9	5.5	6.2	6.1	5.8	6.3
	3.	5.8	6.1	6.2	6.5	6.2	5.8	6.5
	4.	5.7	6.5	6.6	6.4	5.9	6.5	6.2
	5.	5.7	6.3	6.2	6.1	6.1	6.1	6.1
Milk yolk glycerol.	1.	5.9	5.9	5.3	5.7	6.0	6.1	5.7
	2.	5.6	6.0	5.2	5.3	5.6	5.8	5.7
	3.	5.9	5.1	5.5	6.0	5.7	5.8	5.5
	4.	5.9	5.9	5.9	6.1	5.3	5.5	5.6
	5.	5.9	6.0	5.7	6.1	5.5	5.5	5.7
Milk glycerol.	1.	6.2	6.4	6.1	6.5	6.5	6.4	6.4
	2.	6.7	6.6	6.3	6.5	6.4	6.2	6.5
	3.	6.4	6.5	6.4	6.8	6.5	6.5	6.6
	4.	6.5	6.2	6.8	6.7	6.5	6.7	6.5
	5.	6.4	6.7	6.5	6.6	6.7	6.6	6.5

Table No. 13

pH of semen, diluted in various diluents and preserved for 48 hours at 4 ± 1°C.

Diluent	Collection	Bull Numbers						
		11	0/8	38	0/4	M	4	137
Milk yolk	1.	5.8	5.8	5.2	5.7	5.9	6.0	5.6
	2.	5.9	5.9	5.0	5.5	5.5	5.9	6.1
	3.	5.5	5.7	5.7	6.3	5.5	5.9	5.5
	4.	6.0	6.2	6.3	6.1	5.6	6.1	6.0
	5.	6.1	6.0	6.2	6.1	6.1	6.1	6.1
Milk yolk citrate.	1.	5.7	5.8	5.4	5.3	5.9	6.0	5.7
	2.	5.5	5.4	5.1	5.5	5.6	5.8	5.7
	3.	5.5	5.5	5.4	5.9	5.5	5.8	5.5
	4.	5.8	6.1	6.1	5.9	5.6	5.5	6.0
	5.	5.7	6.0	5.9	6.0	5.8	5.7	6.0
Milk yolk glucose.	1.	5.8	5.7	5.3	5.1	6.9	6.1	5.5
	2.	5.5	5.0	5.4	5.4	5.9	5.9	5.5
	3.	5.6	5.2	5.1	5.9	5.5	5.5	5.5
	4.	6.0	6.1	5.7	5.9	5.5	5.7	5.8
	5.	5.9	6.0	5.6	5.9	5.5	5.7	5.9
Milk yolk Sod. bicarb.	1.	5.8	6.0	5.4	5.5	6.1	6.2	6.0
	2.	5.3	5.9	5.2	5.9	6.0	5.9	6.2
	3.	5.5	5.5	6.1	6.4	5.9	5.6	5.7
	4.	5.6	6.2	6.2	6.3	5.7	6.4	6.1
	5.	5.6	6.0	6.1	5.9	6.0	6.0	5.9
Milk yolk glycerol.	1.	5.9	5.7	5.2	5.4	5.8	6.1	5.7
	2.	5.2	5.9	5.1	5.9	5.5	5.8	5.7
	3.	5.4	5.2	5.5	6.0	5.7	5.5	5.5
	4.	5.8	5.6	5.7	6.0	5.2	5.5	5.5
	5.	5.9	5.8	5.9	6.0	5.4	5.4	5.6
Milk glycerol.	1.	6.1	6.2	6.0	5.4	6.3	6.4	6.3
	2.	6.5	6.6	6.1	6.1	6.2	5.9	6.4
	3.	6.2	6.3	6.1	6.7	6.2	6.4	6.1
	4.	6.4	6.7	6.8	6.6	6.4	6.6	6.4
	5.	6.3	6.5	6.4	6.5	6.6	6.5	6.4

Table No. 14

pH of semen, diluted in various diluents and preserved for 48 hours at 4 ± 1°C.

Diluent	Collection.	Bull Number.						
		11	0/3	38	0/4	3	4	137
Milk yolk	1.	5.7	5.7	5.1	5.6	5.8	5.9	5.9
	2.	5.5	5.5	5.0	5.4	5.4	5.7	5.3
	3.	5.4	5.3	5.4	5.9	5.5	5.7	5.5
	4.	5.9	6.1	6.1	6.0	5.5	6.1	5.9
	5.	5.9	5.9	6.1	6.0	5.9	6.0	5.8
Milk yolk citrate.	1.	5.6	5.8	5.1	5.2	5.7	5.8	5.5
	2.	5.2	5.0	5.5	5.5	5.3	5.7	5.1
	3.	5.5	5.3	5.1	5.6	5.4	5.5	5.5
	4.	5.7	5.9	6.1	5.7	5.5	5.5	5.9
	5.	5.6	5.8	5.8	5.9	5.8	5.5	5.9
Milk yolk glucose.	1.	5.7	5.6	5.0	5.0	5.5	5.9	5.4
	2.	5.0	5.4	5.0	5.2	5.3	5.8	5.1
	3.	5.6	5.1	5.0	5.6	5.4	5.3	5.2
	4.	6.0	5.9	5.6	5.9	5.4	5.6	5.8
	5.	5.9	5.8	5.5	5.8	5.5	5.6	5.1
Milk yolk Sod. bicarb.	1.	5.7	5.9	5.1	5.4	5.8	5.9	5.9
	2.	5.1	5.7	5.1	5.9	5.9	5.9	5.5
	3.	5.4	5.3	5.9	6.3	5.7	5.5	5.6
	4.	5.5	6.2	6.1	6.2	5.6	6.2	6.1
	5.	5.5	6.0	6.1	5.9	6.0	5.9	5.7
Milk yolk glycerol.	1.	5.9	5.6	5.1	5.3	5.5	5.8	5.5
	2.	5.0	5.5	5.0	5.1	5.5	5.7	5.5
	3.	5.3	5.2	5.4	5.9	5.6	5.3	5.4
	4.	5.7	5.5	5.7	5.9	5.1	5.4	5.4
	5.	5.7	5.7	5.8	5.9	5.3	5.3	5.5
Milk glycerol.	1.	6.0	6.0	5.2	6.3	6.1	6.4	6.0
	2.	6.0	6.2	6.0	6.8	6.0	5.9	6.3
	3.	6.0	6.1	6.0	6.6	6.0	6.4	6.0
	4.	6.4	6.6	6.4	6.5	6.3	6.7	6.3
	5.	6.2	6.4	6.4	6.4	6.4	6.4	6.4

Table No. 14.

pH of semen, diluted in various diluents and preserved for 96 hours at 4 ± 1°C.

Diluent	Collection.	Bull Numbers.						
		11	0/8	38	0/4	M	4	137
Milk yolk	1.	5.6	5.6	5.0	5.5	5.5	5.8	5.3
	2.	5.5	5.2	5.0	5.3	5.3	5.6	5.1
	3.	5.3	4.9	5.0	5.7	5.1	5.6	5.4
	4.	5.9	6.0	6.0	5.9	5.4	5.9	5.8
	5.	5.8	5.9	5.9	6.0	5.9	5.9	5.7
Milk yolk citrate.	1.	5.5	5.7	5.0	5.1	5.5	5.7	5.1
	2.	5.0	5.0	5.0	5.5	5.2	5.6	5.9
	3.	5.5	5.0	4.9	5.5	5.2	5.4	5.5
	4.	5.5	5.5	6.1	5.7	5.1	5.1	5.8
	5.	5.5	5.7	5.7	5.8	5.7	5.5	5.9
Milk yolk glucose.	1.	5.6	5.5	5.0	4.9	5.2	5.8	5.1
	2.	5.0	5.1	5.0	5.1	5.3	5.7	5.0
	3.	5.5	5.0	5.0	5.5	5.1	5.3	5.0
	4.	5.9	5.9	9.5	5.9	5.1	5.4	5.7
	5.	5.9	5.7	5.7	5.4	5.5	5.4	5.5
Milk yolk Sod. bicarb.	1.	5.7	5.8	5.0	5.8	5.5	5.8	5.3
	2.	5.0	5.5	5.0	5.8	5.7	5.8	5.4
	3.	5.4	5.2	5.0	6.2	5.5	5.5	5.2
	4.	5.4	6.0	6.0	6.1	5.8	6.1	6.0
	5.	5.3	5.9	5.0	5.9	5.9	5.8	5.7
Milk yolk glycerol.	1.	5.8	5.6	4.9	5.2	5.3	5.7	5.5
	2.	5.0	5.2	5.0	5.0	5.4	5.6	5.3
	3.	5.2	5.0	4.9	5.8	4.9	5.3	5.3
	4.	5.6	5.4	5.6	5.7	5.0	5.3	5.4
	5.	5.6	5.6	5.7	5.7	5.1	5.2	5.5
Milk glycerol.	1.	6.0	5.9	5.1	5.2	6.1	6.2	6.0
	2.	5.9	6.1	6.0	5.9	5.9	5.8	6.0
	3.	5.9	6.0	6.0	6.4	6.0	6.3	6.0
	4.	6.3	6.3	6.3	6.4	6.0	6.6	6.2
	5.	6.1	6.2	6.3	6.3	6.3	6.3	6.3

Table No. 16

Dead percentage of spermatozoa in diluted semen preserved for 24 hours at 4 ± 1°C.

Diluent	Collection	Bull Numbers						
		11	0/8	38	B/4	M	4	137
Milk yolk.	1.	20	30	60	20	19	30	92
	2.	20	20	65	20	30	15	40
	3.	30	35	20	20	21	29	80
	4.	30	95	50	60	20	41	41
	5.	20	20	45	20	40	40	30
Milk yolk citrate.	1.	30	30	25	19	26	30	39
	2.	30	15	39	19	20	20	30
	3.	20	31	40	20	27	19	60
	4.	30	40	30	19	30	15	39
	5.	30	30	30	22	60	30	62
Milk yolk glucose.	1.	10	10	15	18	21	29	25
	2.	30	20	30	21	38	19	32
	3.	45	29	39	20	28	31	40
	4.	42	91	30	25	30	21	35
	5.	35	30	35	20	30	21	40
Milk yolk Sod. bicarb.	1.	30	20	15	30	24	31	29
	2.	30	19	40	21	28	18	27
	3.	15	20	30	21	20	40	40
	4.	20	40	60	22	20	19	21
	5.	20	20	50	18	30	42	30
Milk yolk glycerol.	1.	20	40	29	32	39	38	55
	2.	60	19	39	29	41	20	41
	3.	39	55	40	40	80	40	60
	4.	90	50	40	40	50	60	60
	5.	65	40	40	35	90	41	60
Milk glycerol.	1.	40	50	20	95	61	20	93
	2.	92	14	70	62	92	75	60
	3.	60	90	90	90	80	50	90
	4	80	94	90	80	95	90	90
	5.	95	95	90	90	90	90	91

Table No.17.

Dead percentage of spermatozoa in diluted semen preserved for 48 hours at 4 ± 1°C.

Diluent	Collection.	Bull Numbers.						
		11	0/8	38	0/4	M	4	137
Milk yolk	1.	30	15	35	28	30	20	30
	2.	40	30	90	59	30	15	38
	3.	35	28	20	32	31	39	60
	4.	30	40	80	28	30	35	40
	5.	20	30	30	21	80	60	40
Milk yolk citrate. Mixk	1.	20	25	25	19	40	30	25
	2.	40	20	90	29	20	20	31
	3.	25	19	30	41	29	30	60
	4.	30	40	60	32	50	15	30
	5.	35	50	40	41	80	16	60
Milk yolk glucose	1.	40	38	40	28	29	41	40
	2.	39	21	60	28	19	19	28
	3.	40	20	40	30	12	30	40
	4.	40	31	80	20	30	40	32
	5.	35	30	50	40	30	35	32
Milk yolk Sod. bicarb.	1.	10	20	30	30	40	25	20
	2.	50	19	85	39	30	28	25
	3.	30	15	20	40	30	29	40
	4.	20	32	99	42	30	30	80
	5.	15	30	40	35	20	30	42
Milk yolk glycerol.	1.	30	60	35	30	60	25	60
	2.	60	22	80	39	40	20	41
	3.	50	40	30	91	90	20	41
	4.	60	41	95	40	40	90	62
	5.	82	40	50	70	95	62	60
Milk glycerol.	1.	30	70	20	95	91	40	90
	2.	22	95	71	60	75	61	61
	3.	90	80	10	70	95	62	95
	4.	92	90	91	90	95	99	95
	5.	95	95	60	90	90	90	90

Table No. 18.

Dead percentage of spermatozoa in diluted semen preserved for 72 hours at 4 ± 1°C.

Diluent	Collection	Bull Numbers.						
		11	0/8	38	0/4	M	4	137
Milk yolk.	1.	38	29	90	15	42	29	30
	2.	60	40	95	80	31	30	39
	3.	40	39	30	42	30	30	60
	4.	42	40	40	35	30	29	41
	5.	42	30	40	35	42	70	62
Milk yolk citrate.	1.	29	40	70	29	95	39	40
	2.	35	62	90	41	32	30	35
	3.	40	39	40	62	20	30	80
	4.	45	41	60	42	40	39	42
	5.	45	40	50	50	41	22	60
Milk yolk glucose.	1.	40	40	95	10	42	30	50
	2.	47	41	90	40	95	29	38
	3.	37	39	55	41	25	32	90
	4.	42	32	90	39	20	30	30
	5.	41	20	40	62	40	40	40
Milk yolk Sod. bicarb.	1.	30	29	95	15	40	30	28
	2.	50	41	90	30	90	29	29
	3.	30	30	30	40	40	15	90
	4.	40	30	90	40	20	40	20
	5.	40	20	60	30	60	40	40
Milk yyolk glycerol.	1.	21	60	65	60	30	40	48
	2.	91	30	99	40	35	28	90
	3.	60	60	45	42	90	25	90
	4.	80	60	90	50	40	70	80
	5.	90	40	60	60	40	82	85
Milk glycerol.	1.	40	90	90	90	95	30	95
	2.	97	60	97	90	52	80	90
	3.	90	90	80	99	95	60	90
	4.	92	90	95	98	90	90	95
	5.	92	90	90	99	91	98	90

Table No. 19

Dead percentage of spermatozoa in diluted semen preserved for 96 hours at 4 ± 1°C.

Diluent	Collection	Bull Numbers.						
		11	0/8	38	0/4	M	4	137
Milk yolk	1.	39	40	62	29	25	30	39
	2.	68	62	95	95	40	60	40
	3.	41	40	90	40	20	40	80
	4.	50	50	90	40	60	80	50
	5.	60	60	90	40	60	85	65
Milk yolk citrate.	1.	37	55	80	30	40	40	38
	2.	90	61	90	95	42	38	60
	3.	65	93	55	60	18	30	70
	4.	65	80	40	40	50	40	60
	5.	65	50	75	62	48	39	85
Milk yolk glucose.	1.	25	65	42	20	10	29	60
	2.	65	59	95	90	90	38	60
	3.	63	45	95	62	19	39	90
	4.	63	40	95	42	30	42	40
	5.	42	30	93	90	48	41	42
Milk yolk Sod. bicarb.	1.	29	30	40	19	30	85	27
	2.	60	50	95	95	80	40	50
	3.	40	30	30	95	30	42	90
	4.	60	40	95	95	20	60	40
	5.	41	20	60	95	60	60	60
Milk yolk glycerol.	1.	20	80	60	65	30	39	58
	2.	80	70	99	45	50	30	90
	3.	85	90	60	60	95	50	99
	4.	85	90	99	70	20	92	70
	5.	90	20	95	80	40	90	80
Milk glycerol.	1.	39	90	91	90	90	38	90
	2.	99	80	92	90	47	90	92
	3.	92	90	95	90	95	65	99
	4.	92	95	90	95	60	99	90
	5.	95	95	90	95	99	99	90

APPENDIX - 'C'

Table No. 1

Quantities of fructose in milligrammes in 0.1 ml. of undiluted or 0.2 ml. (1:1) diluted semen samples, fresh and preserved before and after incubation at 37°C. for three hours with phosphate buffer.

Bull No.	Fresh Zero hr.	Diluents						
		*N.S.	MY	MYC	MYD	MYS	MYG	MG
137	-	1.11	1.30	1.11	1.30	1.20	1.30	1.20
M	-	1.04	1.11	1.04	1.11	1.88	1.04	0.85
38	-	0.82	0.81	0.81	0.81	0.78	0.81	0.81
After 3 hrs. incubation.								
137	-	1.04	1.04	0.89	1.04	0.83	1.20	1.07
M	-	0.78	0.96	0.96	0.96	1.04	0.96	0.87
38	-	0.62	0.65	0.65	0.74	0.70	0.70	0.74
Preserved zero hour.								
137	-	-	0.62	0.69	0.78	1.07	0.81	0.85
M	-	-	0.93	0.93	0.93	0.93	0.93	0.93
38	-	-	0.79	0.78	0.73	0.65	0.78	0.73
After 3 hrs. incubation.								
137	-	-	0.62	0.59	0.98	0.62	0.62	0.78
M	-	-	0.85	0.81	0.85	0.81	0.74	0.89
38	-	-	0.74	0.74	0.85	0.59	0.74	0.88

N. B. * Neat semen.

Table No. 2

Quantities of Total Reducing Substances in Mgs. in 0.1 ml. of undiluted or 0.2 ml. (1:1) diluted semen samples, fresh and preserved before and after incubation at 37°C. for three hours with phosphate buffer.

Bull No.	Fresh zero hour.	Diluents						
		N.S.	MY	MYC	MYD	MYS	MYG	MG
137	-	0.94	0.94	0.84	0.89	0.74	0.94	1.03
M	-	0.55	0.58	0.58	0.65	0.58	0.58	0.61
38	-	0.57	0.56	0.54	0.56	0.56	0.58	0.56
	3 hours							
137	-	0.80	0.80	0.75	0.80	0.70	0.80	0.84
M	-	0.45	0.50	0.50	0.58	0.52	0.50	0.58
38	-	0.45	0.50	0.45	0.50	0.50	0.56	0.58
	Preserved zero hour.							
M	-	-	0.65	0.58	0.65	0.65	0.61	0.64
137	-	-	0.94	0.89	0.80	0.84	0.89	0.98
38	-	-	0.61	0.61	0.61	0.63	0.61	0.61
	3 hours.							
137	-	-	0.89	0.80	0.70	7.74	0.84	0.84
M	-	-	0.61	0.56	0.61	0.61	0.58	0.58
38	-	-	0.54	0.46	0.52	0.54	0.48	0.50

Table No. 3.

Quantities of fructose in milligrammes in 0.1 ml. of undiluted or 0.2 ml. (1:1) diluted semen samples, fresh and preserved before and after incubation at 37°C. for three hours without phosphate buffer.

Bull No.	Fresh zero hr.	Diluents					
		I. S.	MY	MYC	MY D	MYS	MYG	MS
137	-	0.89	0.85	0.85	0.93	0.89	0.93	0.89
M	-	0.93	1.00	0.93	0.96	0.93	0.93	1.00
38	-	0.89	1.07	0.89	0.89	0.89	0.96	0.89
	3 hrs.							
137	-	0.81	0.48	0.55	0.70	0.70	0.89	0.81
M	-	0.82	0.93	0.90	0.81	0.81	0.85	0.89
38	-	0.84	0.85	0.81	0.80	0.85	0.78	0.80
	Preserved zero hr.							
137	-	"	0.81	1.11	0.85	0.93	1.04	0.93
M	-	"	0.70	0.74	0.74	0.85	0.89	0.96
38	-	"	1.11	1.48	1.30	1.96	1.11	1.20
	3 hrs.							
137	-	"	0.78	0.81	0.74	0.74	0.83	0.81
M	-	"	0.66	0.71	0.70	0.93	0.78	0.74
38	-	"	0.93	0.96	1.00	1.34	1.04	0.96

N. B. * Neat semen

Table No. 4

Quantities of TRS in milligrammes in 0.1 ml. of undiluted or 0.2 ml. (1:1) diluted semen samples, fresh and preserved before and after incubation at 37°C. for three hours without phosphate buffer.

Bull No.	Fresh zero hr.	Diluents						
		N. S.	MY	MYC	MYD	MYS	MYG	MG
137	-	0.68	0.58	0.58	0.58	0.58	0.58	0.58
M	-	0.61	0.56	0.56	0.61	0.56	0.56	0.5
38	-	0.46	0.61	0.56	0.56	0.56	0.56	0.
	3 hrs.							
137	-	0.58	0.48	0.48	0.52	0.50	0.61	
M	-	0.42	0.48	0.47	0.47	0.48	0.50	
38	-	0.40	0.56	0.48	0.50	0.54	0.50	
Preserved zero hour.								
137	-	-	0.47	0.37	0.45	0.47	0.45	0
M	-	-	0.54	0.56	0.54	0.56	0.46	0
38	-	-	1.13	1.22	1.31	1.03	1.22	1
	3 hrs.							
137	-	-	0.45	0.30	0.37	0.41	0.41	
M	-	-	0.52	0.47	0.57	0.52	0.52	
38	-	-	1.02	0.94	1.22	1.00	0.94	

N. B. * Neat semen.

Table No. 5.

Quantities of fructose and TRS in 0.2 ml. of diluted semen (1:1) in control semen samples before and after preservation and incubation.

Fresh/ Preserved.	Fructose	Bull Numbers		
		137	M	38
Fresh Zero hr.	-	0.18	0.28	0.13
Three hrs. incubation	-	0.17	0.27	0.11
Preserved zero hr.	-	0.17	0.28	0.12
Three hrs. incubation	-	0.17	0.27	0.10
Fresh zero hr.	TRS -	0.45	0.97	0.45
Three hrs. incubation	-	0.44	0.97	0.44
Preserved zero hr.	-	0.45	0.97	0.45
Three hrs. incubation	-	0.44	0.96	0.44