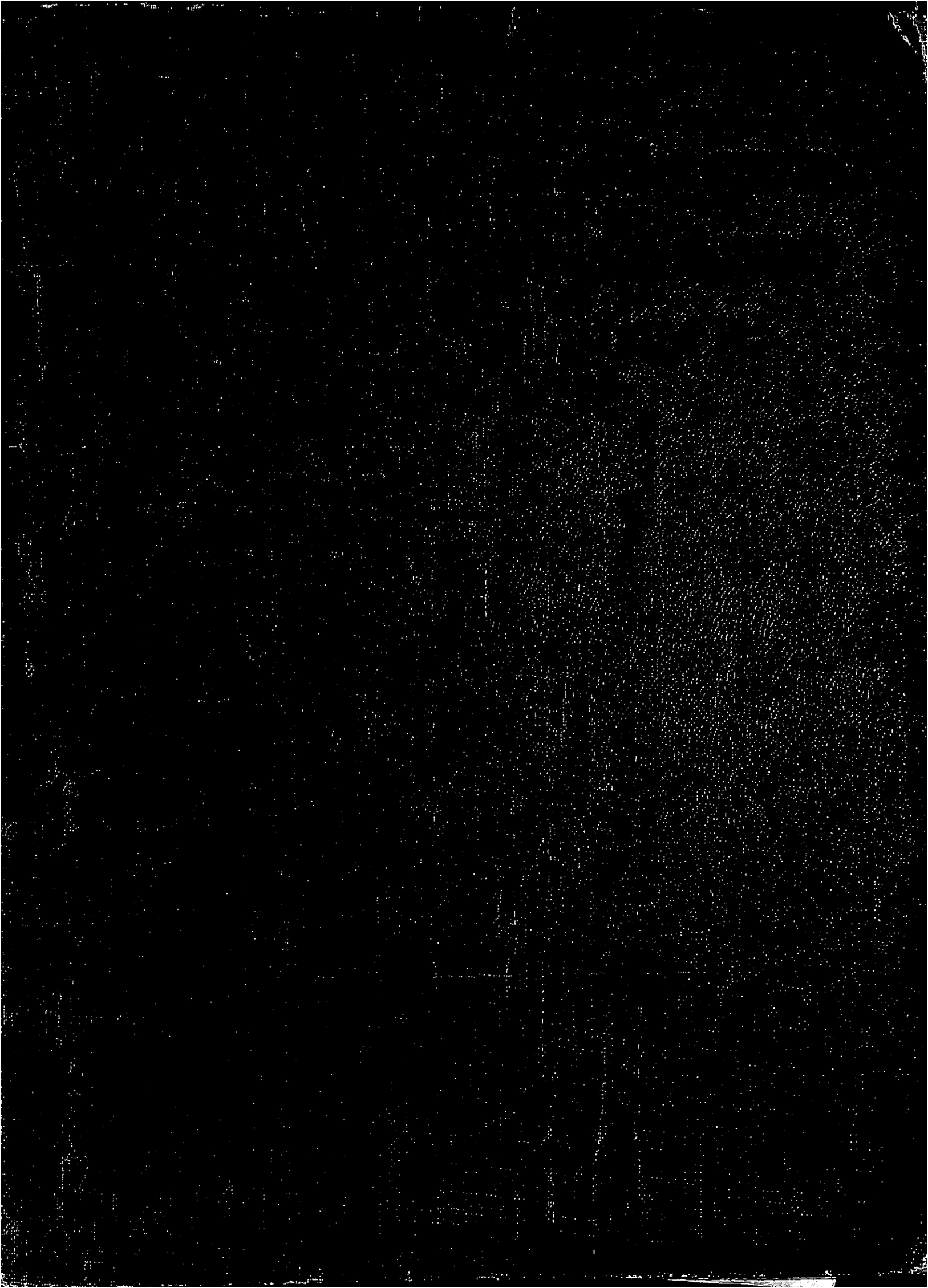

CERTIFICATE

Certified that Shri Arun Chandra Gupta, U.P. College of Veterinary Science & Animal Husbandry, Mathura. a candidate for M.V.Sc, Final examination of 1968 in Animal Genetics & Breeding, has been working under my supervision during the session and that the accompanying thesis entitled "STUDIES ON EFFECT OF VARYING AGES OF EGG YOLK ON SPERM MOTILITY AT 5°C.; KEEPING QUALITY OF SEMEN IN MODIFIED MILK DILUTORS; LIVEABILITY OF SPERMATOZOA IN CERVICAL MUCUS.

which he is submitting is his genuine work.

DATED 29/4/68

B P Singh

Signature in full of Supervisor.

Prof. of ~~Professor of Animal Genetics & Breeding,~~ I/c. Animal Genetics Section, College. L. R. S. U. P. MATHURA.

G/526

EXTERINAL EXAMINER.

STUDIES ON

(A) EFFECT OF EGG YOLK OF VARYING AGES ON SPERM MOTILITY AT 5° C

(B) KEEPING QUALITY OF SEMEN IN MODIFIED MILK DILUTORS

(C) LIVABILITY OF SPERMATOZOA IN CERVICAL MUCUS

THESIS

SUBMITTED TO THE AGRA UNIVERSITY IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE AWARD OF
MASTER OF VETERINARY SCIENCE
DEGREE
IN
ANIMAL GENETICS AND BREEDING

APRIL, 1968

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MATHURA.

The author is highly grateful to Govt. of Nepal for deputing me to undergo M.V.Sc. course and also grateful to the Govt. of India for providing admission and financial assistance for the training.

***

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C O N T E N T S

CHAPTER - I.

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

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

There cannot be two opinions that artificial insemination plays an important role in cattle breeding. It is the reliable, easiest and rapid method to improve the non-descript cows which have occupied a greater percentage of cattle population. Cows belonging to non-descript class are very poor milk yielders, unless and until they are bred by improved bull, it is not possible to fulfil the increasing demand of milk for human population. Under the present economic circumstances, it is quite impossible to keep more animals. Maximum gain from a few animals is the goal of the animal breeder. This goal can only be achieved by artificial insemination by using superior genetic material and by castrating the scrub bulls.

Superior bulls are very few in comparison to the requirement. The breeding material will remain unutilized if every ejaculate of such a bull is not used for a number of cows. Artificial insemination is the only method by which maximum

utilization of semen from superior bulls is possible.

Success of artificial insemination depends on fertilizing capacity of semen which in turn depends upon suitable dilutors to maintain +3 motile life of spermatozoa.

Though fresh egg yolk is an important constituent of the dilutors to maintain fertility and liveability of spermatozoa, it is difficult to obtain fresh egg at every artificial insemination centre easily. Hence it is desirable to study the effect of egg yolk of varying ages on sperm motility. Recently milk dilutors have shown their ability to maintain fertility and liveability of the spermatozoa. More work is needed to study the life of spermatozoa in various modified milk dilutors. Liveability of spermatozoa is said to be dependent mainly on viscosity, elasticity and pH range of cervical mucous and at storage temperature.

The present piece of work was undertaken with a view to find out the effect of varying ages of egg yolk, and the suitability of modified milk dilutors to maintain +3 motility of spermatozoa.

The idea of using extended semen in cervical mucous of oestrous cows and buffaloes was to have a rough estimate of the percentage of progressive motile spermatozoa in different categories of cervical mucous after four hours of incubation at 38°C.

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CHAPTER - II

REVIEW OF LITERATURE

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REVIEW OF LITERATURE

EFFECT OF EGG YOLK OF VARYING AGES ON SPERM MOTILITY:

Although egg yolk is a component of majority of dilutors commonly used, yet very few attempts appear to have been made on knowing the effect of age of egg yolk on motility or keeping quality of spermatozoa in any dilutor. Only report known to author in this respect is that from Morozov (1951) who reported that as long as colour of egg yolk was uniform and not separated from the white, there was no appreciable difference in the viability of spermatozoa if the egg was one to two months old.

MILK DILUENT:

Kolliker (1856) used milk as a bull semen dilutor followed by Underbjerg *et al* (1942) getting good fertility levels with autoclaved milk with egg yolk phosphate.

Michajilov (1950) reported satisfactory results with boiled filtered milk.

Thacker and Almquist (1951) used milk and its products as a successful diluent for bull semen. Spermatozoa remained in the boiled products for periods approximately equal to those for spermatozoa in the egg yolk citrate. Boiling of milk for ten minutes appear to be adequate and no marked differences in sperm liveability were detected between boiling intervals of 1, 5, 10 and 30 minutes.

Sobek (1951) heated milk to 100°C., cooled and again heated to 100°C. 24 hours before dilution and advoated the use of milk-yolk and milk-yolk-citrate for semen dilution.

Flipse et al (1954) reported that lactenin has been concentrated by acetone fraction of Whey and found to be highly toxic to bovine spermatozoa. Lactenin prepared by tryptic digestion of whey dialysis and alcoholic precipitation was also toxic to spermatozoa. Lactoperoxidase when added to heated skim milk and used as a semen diluent exhibited no toxicity for bovine spermatozoa at

the concentration used.

Boyd et al (1954) studied the effect of chemical and heat treated pasturized milk on the liveability of bovine spermatozoa and confirmed that bovine spermatozoa will not survive for more than 1 or 2 days in pasturized milk.

Johnson et al (1955) found no motile spermatozoa in unheated skim milk diluent after storage for one day. Motility was slightly higher in unheated skim milk containing cystine hydrochloride than in the heated skim milk. No beneficial effect was found when cystine hydrochloride was added to protein free milk serum instead of skim milk. It was concluded that cystine hydrochloride act on skim milk and not directly on the spermatozoa.

Flipse et al (1956) in a study on the motility of bovine spermatozoa in milk glycin and egg yolk glycin diluents with or without glycerol found the following results: Fresh skim milk (heated)-0.5 M glycine (1:1) sustained liveability as good as did egg yolk-0.5 glycine (1:1) and significantly better than did heated skim milk alone. Mean liveability at 5°C. over 20 days storage period in

reconstituted NFDMS*-0.5 glycine was superior to reconstituted NFDMS alone. There was an improvement, further in liveability when glycerol or fructose added to NFDMS-0.5M. glycine.

Willett et al (1956) ascertained the progressive motility of spermatozoa in yolk citrate, yolk-citrate-glucose or non-fat milk solids at 5°C. after storage of 7 days. It was found that by increasingly glycerol concentration motility decreased markedly in yolk-citrate and to a lesser extent in yolk-citrate glucose, it increased in non-fat milk solids. Motility was not significantly different in the above diluents when no glycerol was added.

Saacke et al (1956) studied optimum liveability of spermatozoa in skim milk heated to temperatures varying from 81° to 105°C. for 3 d and 9 minutes and 73°C to 97°C. for 27 minutes. Heating to 73°C. for 3 and 9 minutes and 105°C. for 27 minutes resulted in significant decrease in liveability. They further obtained optimum liveability with temperature ranging from 87°C. to 97°C. for one minute and 77 to 97°C. for 10 minutes,

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* NFDMS - Non fat dry milk solid.

skim milk heated for one minute at 77°C. and 82°C. resulted in a significant decrease in liveability.

Jaskowski (1956) found no significant differences in liveability between egg-yolk-citrate, egg yolk-citrate-glucose, skim boiled milk and skim boiled milk plus 10 per cent egg yolk at 1:40 dilution. At 1:80 dilution sperm liveability was much better in skim milk plus 10 per cent egg yolk than in other diluents.

Albright et al (1958) studied the motility of bovine spermatozoa stored at 5°C. extended in a mixture of yolk-citrate, yolk-glycine, whole milk, skim milk and glycerol. Semen samples was divided into a glycerolated (7.5%) and a non-glycerolated group. Each group was extended 1:24 into 10 different extenders at an initial temperature of 32°C. and stored at 5°C. The extenders were egg yolk-citrate (YC), whole milk (WM), skim milk (SM) and egg yolk-glycine (YG), motility on an average were lower after extension at day 3 and day 7 of storage ($P < 0.01$). Whole milk yolk glycine (WMYG) and skim milk yolk-glycine (SMYG) showed higher average

motility on 7th. day ($P \leq 0.01$) than whole milk yolk-citrate (WMYC), skim milk-yolk-citrate (SMYC), (WMSM) or yolk-citrate-yolk glycine (YCYG). YCYG was superior ($P \leq 0.01$) to either YC or YG. Whole milk, skim milk and yolk-milk-yolk-citrate had higher motility after extension ($P \leq 0.05$) than the same extender containing glycerol. In this study 2.9% glycine maintained progressive motility under storage at 5°C. when used with WM, SM or YC.

Albright et al (1958) studied spermatozoal survival in milk diluent with andwithout seminal plasma. Spermatozoa without milk or seminal plasma showed low motility in all tests. Motility during storage was greater as the amount of milk and seminal plasma increased and amount of sod. citrate reduced. Greater quantities of milk were required to maintain motility of the washed spermatozoa. After incubation for three hours in each diluent spermatozoal motility was greater in whole semen than in the other variants. Milk can essentially replace seminal plasma for maintenance of sperm motility.

Fulka et al(1960) studied the spermatozoal survival of bull and ram semen diluted in the milk

preparation, Evika and Sunar (Dried milk). Survival rate in Evika was not much different from that in yolk-citrate, it was lower in Sunar. The conception rate to first insemination of bull semen in milk diluent was 58.9% v/s 58.7% when yolk citrate was used.

Brown et al (1960) in a study of motility of bovine spermatozoa in reconstituted butter milk reported that on motility percentage basis 12% concentration of reconstituted butter milk in distilled water wsa superior to 11% and 13% concentration of butter milks, and on the basis of fertility trial it was superior to yolk-citrate.

Albright et al (1960) extended 16 semen samples in a ratio of 1:50 in 14 extenders with 7.5% glycerol. Direct glycerolation generally had better results as evaluated by progressive sperm motility.

Lunca et al (1961) comparing egg yolk and milk diluents for bull semen reported that the average spermatozoal survival as 83.1, 72 and 84 hours respectively with egg yolk-citrate, egg yolk glucose and milk. Survival was considerably increased

by the addition of antibiotics.

Seving et al (1961) made comparative study of spermatozoal motility in different dilutors. They diluted bull semen in CUE, heated homogenised whole milk (HM), CUE+HM, CUE+ Catalase, HM+Catalase and CUE+HM+Catalase. Samples were stored at 5 C. for 21 days and motility was evaluated on the day of collection, 7, 14 and 21 days later. The highest overall average motility (50%) and motility at 21 days (25%) with CUE+Catalase+HM and lowest with CUE alone (33%) and 7% respectively). CUE+HM was superior to CUE or HM alone.

Almquist et al (1961) further reported the effectiveness of glycerol in maintaining the spermatozoal motility in skim milk diluent containing 5, 10, 20% glycerol. Liveability of spermatozoa was superior ($P < 0.01$) when glycerol was added to partially diluted semen at 5^{o}C. There was a highly significant increase mean liveability with each decrease in glycerol level from 20% to 5%.

Almquist et al (1962) found the following results with the use of glycerol in skim milk diluent

spermatozoal liveability in 10 or 13% glycerol incorporated skim milk was higher (P/_0.01) at 5°C. during 14 days of storage than in the absence of glycerol. Glycerol level of 16-25% did not improve liveability over semen diluted without glycerol.

Crespo Garcia (1964) found higher spermatozoal motility in semen diluted with spermasol milk than in that of diluted with egg yolk-citrate, particularly after 15 days of storage. There was no appreciable difference in the conception rates of 720 cows inseminated with the semen diluted with either of the diluents and stored for 72 hours.

Meding (1964) diluted bull semen with homogenised milk heated to 92-95°C. for 10 minutes and then cooled, homogenised sterilized milk or homogenised sterilized cream. The difference between the diluents were not significant as resulted by the

insemination of cows. Cream diluent was somewhat more favourable for semen from highly fertile bulls. It was concluded that homogenised sterilized milk is a very satisfactory diluent for bull semen.

LIVEABILITY OF SPERMATOZOA IN CERVICAL MUCUS:

C. Vanduijn et al (1942) stated that the total number of spermatozoa moving normally depends on pH but the optimum varies with individual ejaculates.

Anderson (1942) reported that motility should be greater at low pH. He used 221 ejaculates out of which 12 had pH between 6.31 to 6.90 with a mean motility of 38% range (10% to 60%) and 22 ejaculates had a pH between 6.91 and 7.20 with a mean motility of 76%. Higher motility never had been found at a pH greater 7.60.

Lardy and Phillips (1943) found that motility should not be affected much by pH differences between 6.8 to 7.5 but it should decrease greatly at pH $\angle$ 6.7.

Henle and Zittle (1942) studied maximum repiration and motility at pH 7.5 to 8.0.

Romijn (1950) stated that optimum motility could be obtained at pH 7.2 in certain case it had detrimental effects on the liveability of spermatozoa.

Laing, J. (1945) determined the period of survival of spermatozoa in the genital tract of cows. Cows and heifers were inseminated with a known number of spermatozoa. Animals were slaughtered at the end of oestrus and ova were collected from the fallopian tube. It was found that animals inseminated earlier than 16 hours before the end of oestrous were not fertile. It was concluded that period of survival of spermatozoa under the condition of the experiment was about 16 hours plus the interval between the end of heat and ovulation.

According to Roark and Herman (1950) average penetration rate of spermatozoa in mucus collected during oestrus was 2.81 mm. per minute and the range was 0-6 mm. per minute. The average maximum penetration was in mucus collected at 10-13 hours in oestrus. In vitro spermatozoal motility and survival in mucus correlated positively with surface tension and flow of elasticity and

inversely to leucocytic concentration and pH of mucus.

Vandeplaschi and Paredis (1949) inseminated 45 cows and heifers on the tweentieth day after the previous heat to determine the maximum duration of viability of spermatozoa in the genital tract. The cows inseminated 3-24 hours before onset of oestrus conceived and those inseminated 48 hours before did not conceive. 50% animals conceived when inseminated 3-24 hours before onset of heat and that percentage was not markedly lower than average 60% when animals were inseminated during heat. They concluded that fertilizing capacity of spermatozoa was retained upto 51-56 hours.

Blackshaw and Emmens (1951) stated that bull spermatozoa shows optimum motility at pH 7.0 but there is no difference in motility between pH 5.5 and 8.5.

Kinney and Salisbury (1953) investigated motility, oxygen consumption, fructose consumption and carbondioxide production under aerobic condition in the pH range of 5.6 to 7.5. Spermatozoal motility was greater with increasing pH. Motility in

undiluted semen was maintained slightly above that of samples of pH 6.9.

Binello (1954) used a drop of semen to each of 230 samples of mucus collected from uterus and examined motility and viability of spermatozoa microscopically. Motility and viability lasted 5-60 minutes or longer in different samples.

Rickard et al (1957) reported the effects of various levels of pH on semen quality. They increased pH of bovine semen samples by sodium bicarbonate. After incubation at 37°C. - 39°C. liveability was somewhat better at pH 7.1 to 7.4 than at pH 6.5 to 6.8. A reverse trend was seen when samples were stored at 4 - 6°C. Evaluation of refrigerated samples indicated somewhat better liveability at lower pH values 6.5 to 6.8 as compared with 7.1 to 7.4.

Olds and VanDemark (1957) collected cervico-vaginal mucus, uterine fluid, oviduct fluid and follicular fluid from the cows at known stages and unknown stages of oestrus cycle after slaughter. They used the above fluids as semen

diluter. On an average, under anaerobic condition at 37°C., spermatozoa were motile about nine hours in mucus, seven hours in uterine fluid, twelve hours in oviduct fluid and nineteen hours in follicular fluid. Generally spermatozoa lived longer in mucus and uterine fluid from cows in or near oestrus than in the same fluid at other stages of oestrus cycle. They also reported that oxygen uptake of spermatozoa was highest in follicular fluid followed by oviduct fluid, mucus and uterine fluid respectively. These fluids had shown the same decreasing order in maintaining the viability of spermatozoa. Agglutination of spermatozoa was also noted in all of the genital tract fluid but was most frequent in follicular fluid.

According to DeGroot (1958) motility of spermatozoa depended on pH. At pH 6.75 movements were normal, at pH 5.8 movements were slow, at pH 5.5 movements of spermatozoa were slow and wave formation disappeared and at pH 7.3 swiming movements became stuporous with high tail beat frequency.

Norman et al (1958) reported that in a

pH range of 5.58 to 5.80 motility, respiration and glycolysis should be inhibited maximum.

Vasilzeva (1958) measured vaginal mucus of 21 cows in a viscometer and in an elastometer at the beginning of oestrus and six and twelve hours after. They noted that higher conception rate was related to low viscosity and high elasticity of vaginal mucus.

Kalev (1959) performed an experiment, in vitro, with uterine secretions of cows and semen samples from bull. Spermatozoa survived and retained motility for a long period than when they were in glucose phosphate diluent.

Guard (1960) put cirvical mucus on the ruled area of counting chamber covered by cover slip and seminal fluid was fed by pipette from opposite side. As soon as mucus and seminal fluid came in contact the spermatozoa started migrating. The number of living spermatozoa lying in one sq.mm. was counted after the intervals of five and fifteen minutes. Within five minutes large number of spermatozoa migrated to ruled areas and after

fifteen minutes number of spermatozoa were too many with active motility. He also described a new technique utilizing modified haemocytometer chamber in which number of spermatozoa penetrating cervical mucus could be counted.

Tampion and Gibbons (1962) conducted 25 experiments with 10 samples of mucus from 8 cows and 12 samples of semen. They recorded that mean swimming rates of spermatozoa varied from 22.0 to 80.7 U/second and the mean of all the means was 56.2 U/second. The mucus used in all these experiments had been stored for sometime at -10°C. In another experiment with six different samples of mucus fresh from the cows and six samples of semen, the range of mean swimming rate was 37.5 to 70.5 U/second and the mean of all the means was 55.6 U/second. From the above results they came to conclusion that swimming rate of bull spermatozoa in cervical mucus was only about half of the rate of the movement in the female genital tract.

Basic (1962) examined 578 samples of vaginal mucus of 523 cows in oestrus. The conception

rate was 79-83% for cows with clear mucus and reduced by 11-50% for those with pus in the mucus. The conception rate was 10.6-46.4% for cows with traces of mucus in the vagina. The pH of mucus varied from 6.9 to 9.0 with a mean of 7.6, it was optimal for conception at 7.5 to 8.0. Viscosity varied between 11.1 and 73.5 cps., the medium was 39.0. Elasticity varied between 36 and 267 mm., the median was 172 mm. Viscosity was correlated with elasticity (r = 0.59). The movement of the spermatozoa was mainly on the surface of the samples of the mucus without penetrating it.

Tampion and Gibbons (1962) reported that bull sperm swim more slowly in mucus from cows, uterine cervix than they do in saline and that might be due to high pH of mucus. It had been claimed that media above 8.3 pH were very harmful to spermatozoa. Alternatively, it might be due to the presence of inhibitory substances in solution or due to the visco-elasticity imparted to the secretion by very large flexible molecules.

Segling (1966) inseminated 99 cows with semen diluted with yolk citrate to contain

$40X10^6$ - $50X10^6$ motile spermatozoa per ml. The cows were slaughtered 1-53 hours after insemination. Spermatozoa retained motility in the oviduct for 39 hours when recto-vaginal method was used.

Tampion (1966) studied the movement of spermatozoa in the mucus of uterine cervix. Some individual spermatozoa, observed for a distance of 310 U swam at nearly constant rate and other showed vide fluctuation. The fluctuation in swimming rate might be due to nonhomogenity of mucus. Large fluctuation and variation in swimming rate were also observed in more homogenous medium of sperm extract.

Sarapa (1966) while carrying out experiment with cows reported that when the musculature tonus of the uterus was low, the vaginal mucus had high viscosity and increased osmotic pressure. The mucus had a high content of calcium and chlorine. Viability of spermatozoa and its speed were adversely affected by such mucus.

Pattabiraman et al (1967) collected cervical mucus from oestrus cows and studied pH and viscosity

The viability of spermatozoa was studied by mixing equal volume of mucus and semen at 38 C. pH of oestral mucus affected significantly the spermatozoal progressive movement. Progressive movement was maintained at a pH range of 7.0 to 9.0. This finding was in close agreement with the findings of Wales (loc.cit). Extreme alkalinity of cirvical mucus of above pH 9.0 was found to be less deliterious than acidity especially with old semen. With the increase of alkalinity of cervical mucus, the percentage of viable spermatozoa declined in whole as well as in extended semen.

They further pointed out that mucus of medium viscosity maintained motility better than the mucus of thin or thick viscosity. Viscosity of cervical mucus did not significantly effect the viability of spermatozoa either in whole or in extended semen. Mid and late oestral mucus maintained viability better than early oestral mucus.

CHAPTER - III

MATERIAL AND METHODS

CHAPTER - III.

M A T E R I A L AND M E T H O DSS

The experiment was conducted in three parts to study:

(a) The effect of egg yolk of varying ages on sperm motility at $5^{o}C$.

(b) Keeping quality of semen in modified milk extenders.

(d) Liveability of spermatozoa in cervical mucus.

The first part of experiment was conducted during August, 1967 to October, 1967 and the second and third parts during December, 1967 to February, 1968.

(a) Effect of Egg Yolk of Varying Ages on Sperm Motility at $5^{o}C$.

In this experiment 19 split semen samples from 5 different Hariana bulls were utilized.

Collection of semen from a bull was done after every 72 hours as a routine practice. The bulls were maintained under uniform and good managemental practice. Semen samples having +3 or above initial motility were used. The semen was extended in a buffer solution (A_4) of the following composition:

Glucose	- 5%	....	18.750 gms.
Glycine	- 4%	....	12.500 gms.
Sod.Citrate	- 2.9%	....	9.375 gms.
Distilled water			1000 ml.

The dilutor contained 80 parts buffer solution and 20 parts egg yolk of 4 different periods namely yolk from egg preserved:

(i) in refrigerator at 5^{o}C. for 7 days.
(ii) at room temperature for 4 days.
(iii) at room temperature for 2 days.
(iv) yolk from fresh egg.

The dilutors were designated as (i) S_1, (ii) S_2, (iii) S_3 and (iv) S_4

The eggs were taken from the College Poultry Farm. These were washed with tap water

and then kept at 5°C. in the refrigerator or at room temperature. At the time of dilution, the eggs were sterilized with ether and rectified spirit and dried in air. The eggs were broken with sterilized forcep, the albumen was drained off. The yolk was taken with intact membrane on sterilized filter paper. The chalaza and rest of the albumen were removed and by puncturing membrane 2.5 ml. yolk was collected in four different sterilized cylinders from the four eggs of four different periods described above. 10 ml. glucose-glycine-sodium citrate buffer solution was added in each four cylinders. Egg yolk and buffer solution was thoroughly mixed by sterilized glass rod. Penicillin and streptomycin at the rate of 1000 units and 1000 mgs. per ml. of dilutor respectively were also dissolved in each dilutor. 2.5 ml. dilutor was taken from each of the cylinders in four storage tubes of 5x1 cm. size and kept cork fitted at room temperature.

5 ml. from each dilutor was taken in four sterilized test tubes for bacterial test.

Experimental Procedure:-

The semen was collected in the morning from 7 to 8.30. Prior to collection, the bull was

cleaned with water. Collection was done in artificial vagina (Russian model), using cow or buffalo as a dummy for Hariana bulls. Just after collection, semen was protected from sun rays and water of artificial vagina was drained out. The semen was kept in a beaker containing water of room temperature. The volume, initial gross motility, pH and initial sperm concentration were determined.

Motility:-

Motility rating was done under low power objective of undiluted fresh semen without cover slip.

Motility scale was divided for convenience in catagories as below:

Motility 0 = Sperm without any movement.

" +0.5 = Approximately 10% of sperms showing an oscillating movement.

" +1.0 = About 10-20% sperms showing slow movement.

" +1.5 = About 20-30% sperms were motile.

" +2.0 = About 30-40% spermatozoa were motile with progressive motion.

" +2.5 = About 30-50% spermatozoa with progressive movement.

Motility +3.0 = About 40-60% spermatozoa with progressive movement.

" +3.5 = About 50-70% spermatozoa motile, many of them in progressive motion and slow wave formation.

" +4.0 = About 60-80% spermatozoa were motile with progressive motion and moderate wave formation.

" +5.0 = Above 90% motile spermatozoa with vigorous waves.

Hydrogen-ion Concentration (pH):-

pH of semen was determined by B.D.H. narrow range nitrazine paper.

Slide Preparation:-

One drop of semen was taken on a slide and 5 drops of eosin-nigrosin stain at 30°C. was mixed cautiously with the edge of the slide. After two minutes smear was made on clean, grease free dry slide. Smear was dried in air and slide was kept for microscopic examination.

Initial Sperm Concentration:-

The number of spermatozoa per ml. of semen

was determined with the help of photo-electric calorimeter. 9.9 ml. (2.9%) sodium citrate solution was taken in a standardized test tube checked for light transmission and 0.1 ml. semen was added to it with micropipette. The content of the test tube was mixed by inverting slowly for 8-10 times. Calorimeter was adjusted at 0 reading by placing the standardized test tube containing 10 ml. sodium citrate solution (2.9%). The test tube was removed and the tube containing 9.9 ml. sodium citrate solution and 0.1 ml. semen was put in the calorimeter and reading was taken. The calorimeteric reading was multiplied by the factor 1.54 for dtermination of sperm concentration per ml. of semen.

Then with the help of table, required volume of semen was added by 1 ml. bulb pipette to 2.5 ml. of each dilutor.

Percentage of live and abnormal spermatozoa:-

Eosin-nigrosin stain was used for preparation of slide has already been described. Live and dead spermatozoa were counted under oil emersion objective. The slide was focused at low objective

power and then few drops of cidar oil was put and adjusted under oil emerson power. At least total of 100 spermatozoa were counted for each sample. Red coloured and partially stained spermatozoa were counted as dead and white spermatozoa as alive.

Dilution and storage:-

Just after evaluating neat semen dilution was done within fifteen minutes after collection. 100 million of spermatozoa per ml. of dilutor were added in 2.5 ml. dilutor of each tube according to the table given in the Appendix. The tubes were kept in the beaker half filled with water and transferred in the refrigerator for gradual cooling upto 5^{o} - 10^{o}C.

After eight hours of storage in the refrigerator, beaker was taken out. Water of beaker was thrown and tap water was kept in the beaker. The tube containing diluted semen was taken one by one and contents gradually mixed by rotating the tube in between the two palms. The slide was heated at 30 C. on the hot water bag or spirit lamp. A drop of semen was taken on the slide and covered with

cover slip. Motility of spermatozoa was estimated under low objective. The tubes were put in the beaker containing water and transferred in the refrigerator. After every 24 hours motility was tested till motility was maintained at +3.

Bacterial Count:-

After completing dilution, dilutors were taken to bacteriology department for bacterial test. Nutrient agar media of the following composition was prepared either on the day of incubation or a day before.

Nutrient Agar Media:

i) Nutrient broth:

Meat extract	- 1000 ml.	
Peptone (Bacto)	10 gm.	
Sodium chloride	5 gm.	
pH adjusted at	7.6	
Autoclave	- 15 lb.	pressure for 15 minutes.

ii) Nutrient broth - 1000 ml.

Agar - 25 gm.

Autoclave - 15 lb. pressure for 15 minutes.

Nutrient agar was prepared in a sterilized flask. 15. ml. warm nutrient agar was taken in 8 large tubes. The temperature of the agar was noted. When it came to body temperature, 1 ml. was taken from each dilutor and at the rate of 0.5 ml. was added in two test tubes containing 15 ml. nutrient agar. Altogether eight test tubes were taken for mixing dilutors in agar media. The test tubes were well shaked till contents were thoroughly mixed. Dilutor number was put on eight petridishes. Cotton plug of the test tubes were removed infront of bunsen flame. The mouth of the test tubes were heated to destroy any adhering bacteria and content was put in petridishes. The petridishes were rotated gently till the contents were spread. After cooling petridishes were put in incubator for 24 hours. After 24 hours petridishes were examined for bacterial growth (colonies).

(b) Keeping Quality of Semen in Modified Milk Extender:-

In this study in all 20 ejaculates from 8 Hariana bulls were used for dilution. Samples with +3.0 initial motility or above were taken for dilution purpose.

Fresh cow's milk was taken from College Dairy Farm, just after milking a day before dilution of semen. The milk was boiled for 3 to 4 minutes. It was cooled at room temperature and then kept in the refrigerator overnight.

The fresh eggs were taken from the College Poultry Farm, one hour before collection of semen.

5% glucose solution was prepared one day before starting the experiment and used for fifteen days and the fresh solution was again prepared.

Preparation of Dilutors:-

The dilutors were prepared one hour before collection. The egg yolk was collected from fresh egg by the procedure described in the first experiment. From one egg both albumen and egg yolk were taken in a sterilized beaker and mixed thoroughly by a sterilized rod. Previous day, boiled milk was taken out from the refrigerator. With the help of sterilized 10 ml. pipette fat was removed to one side of the beaker. The tip of the pipette was dipped under the surface of milk and sucked up. In this way about 60 ml. milk was taken in a sterilized cylinder.

Eight cork fitted tubes 5 cm. x 1 cm. size were taken. Eight dilutors of the following composition were prepared:

1. Boiled milk (S_1) - 2.5 ml. fat separated milk was taken in storage tube.

2. Boiled milk-whole egg ($S_2/10$) - 1 ml. whole egg was taken in 20 ml. sterilized test tube and 9 ml. fat separated milk was added to make 10 ml. This contained 10% whole egg.

3. Boiled milk-whole egg ($S_2/15$) - This contained 15% whole egg and boiled milk. 1.5 ml. whole egg was taken in 20 ml. sterilized test tube and 8.5 ml. boiled milk was added to make the volume 10 ml.

4. Boiled milk-whole egg ($S_2/20$) - This contained 20% whole egg. 2 ml. whole egg was taken in a 20 ml. sterilized test tube and 8 ml. boiled milk was added to make the volume 10 ml.

5. Boiled milk-glucose ($S_3/10$) - This contained 10 parts glucose solution. Milk was added as in No. 2 dilutor.

6. Boiled milk-glucose ($S_3/15$) - This contained

15 parts glucose solution. 1.5 ml. glucose solution was added to 8.5 ml. boiled milk to make the volume 10 ml.

7. Boiled milk-glucose ($S_3/20$) - This contained 20 parts glucose solution. 2 ml. glucose solution and 8 ml. boiled milk were mixed together to make volume 10 ml.

8. Boiled milk-egg yolk-glucose (S_4) - This dilutor contained 20 parts egg yolk - 20 parts glucose solution and 60 parts boiled milk. 2 ml. egg yolk, 2 ml. glucose solution and 6 ml. boiled milk were taken in 100 ml. cylinder.

In all the dilutors, penicillin and streptomycin were added at the rate of 1000 units and 1000 mgs. per ml. of the dilutor and after mixing 2.5 ml. of the dilutor was taken in storage tubes and kept at room temperature with cork fitted.

Experimental Procedure:-

Semen was collected from 10.30 A.M. to 12.00 Noon. Just after evaluating neat semen and

finding it upto the mark, dilution was done within 15 minutes after collection. The semen was added to the dilutor at the rate of 100 million per ml. of dilutor. After dilution the storage tubes were kept in the beaker, containing half filled with water. The beaker was then kept in the refrigerator.

The motility of the diluted semenwwas tested on the first day after one and half an hour and at the interval of 24 hours at subsequent days till +3 motility was seen.

(C) Liveability of Spermatozoa in Cervical Mucus:-

Thirteen split semen samples from Murrah and 26 split semen samples from Hariana bulls were used in this experiment.

Hariana semen was extended in S_4 dilutor (milk) and Murrah semen in A_4.

For cervical mucus of buffalo, fresh extended semen of Murrah was used in the ratio of 1:10 of mucus. For mucus of cows fresh, two or three days old extended semen of Hariana bulls was used upto +3 motility.

Collection of Mucus:-

Mucus was collected from village and farm cows and buffaloes brought for insemination at the Artificial Insemination Centre of the Department of Animal Genetics and Breeding. T

Technique of Mucus collection:

Prior to mucus collection, vulva was washed with water. Sterilized thick glass pipette about 1/2" diameter was introduced in the vagina by rotating motion. By thumb pressure sterilized rubber bulb was fitted to the other end of the pipette. By gradual movement of the pipette mucus was collected and as soon as pipette was about to be filled up with mucus, the bulb was removed and thumb pressure was applied to the opening of the pipetted. Mucus was taken in a sterilized test tube and cork was fitted.

Test of Mucus:-

The following tests were done prior to adding extended semen.

(i) Viscosity:- It was divided in three classes

either thick, medium or thin.

(ii) pH:- pH of the mucus was tested with the help of B.D.H. narrow range nitrazin paper (ranged from 7.0 to 8.5).

(iii) Elasticity:- A little of mucus was taken on a slide and the test tube raised. Approximate elasticity was note by scale.

Mucus contaminated either with dung or urine and mixed with blood or pus were discarded.

Addition of Semen:-

Prior to adding semen, motility was tested as usual under low power objective. Semen was added 1/10th. of mucus volume. The tubes were kept in the incubator adjusted at 38°C. After four hours incubation mucus was examined under higher power objective. A number of spermatozoa were counted out of which only progressive motile spermatozoa were taken into consideration.

Statistical Calculation:-

All statistical calculations were done according to Snedecor (1956).

CHAPTER - IV

R E S U L T S

CHAPTER - IV.

R E S U L T S

CHARACTERISTICS OF SEMEN USED FOR STUDIES:

The averages of semen characteristics obtained from 8 Hariana bulls during the period of study are given in Table No. 1.

TABLE NO. 1. SEMEN CHARACTERISTICS OF HARIANA BULL SEMEN

Characteristics	Average Value
Volume (ml.)	6.25±0.336
pH	6.42±0.041
Initial gross motility	3.52±0.066
Initial sperm concentration/mm^3	0.766±0.065
Live spermatozoa percentage	81.00±2.676
Live abnormal spermatozoa percentage	18.65±0.660

(a) Studies on Effect of Varying Ages of Egg Yolk on +3 Sperm Motility in Days During Storage in the A_4 Buffer Media at $4^{\circ}C$.

The results were analysed to study the impact of egg yolk quality as affected by storage at room temperature (maximum 81 - $92^{\circ}F$ and minimum 74 - $87^{\circ}F$.) for two to four days and by storage at $4^{\circ}C$. for seven days in comparison with fresh egg yolk. The +3 motile life in days of spermatozoa in A_4 buffer medium comprising of egg yolk of varying ages were analysed according to initial sperm motility groups of fresh semen. For the convenience of analysis the motility data were grouped into (i) +4 and above initial motility group and (ii) +3 to less than +4 group. The analysis for two groups were finally pooled.

Since the semen is considered suitable for insemination upto the period it maintains +3 motility, the analysis of variance of the effect of egg yolk of varying ages in A_4 buffer in maintaining +3 motility in days was performed. The results for two initial sperm motility groups and the pooled results are given in Table No. 2.

TABLE NO. 2. ANALYSIS OF VARIANCE OF THE EFFECT OF VARYING AGES OF EGG YOLK FOR MAINTAINING +3 MOTILITY IN DAYS FOR THE TWO INITIAL SPERM MOTILITY GROUPS.

Source of Variation	+4 & above Initial Motility Group		+3 to less then+4 Initial Group		Pooled Estimate (For the two Groups)	
	d.f	M.S.	d.f	M.S.	d.f	M.S.
Total	27		47		74	
Between Ages of Egg Yolk	3	NS 0.233	3	NS 0.183	6	NS 0.208
Within Ages of Egg Yolk	24	0.655	44	1.305	68	1.075

NS - Not Significant.

From the above Table it is apparent that there was no significant difference in the number of days upto which +3 motility was maintained in the dilutors containing egg yolk of four different periods.

The average number of days upto which +3 motility was maintained in S_1, S_2, S_3 and S_4 dilutors was 4.7, 4.8, 4.8 and 4.8 respectively for the semen of +3.0 to less than +4.0 initial motility groups. Corresponding figures for +4 and above sperm motility

group were 5.3, 5.6, 5.1 and 5.4 days for S_1, S_2, S_3 and S_4 dilutors respectively.

The decline in motility in various dilutors on hours of preservation are given initial motility group wise in Table No. 3.

TABLE NO. 3. REGRESSION OF MOTILITY ON HOURS OF PRESERVATION IN DILUTORS CONTAINING VARYING AGES OF EGG YOLK.

Dilutors	+4 and above Initial Sperm Motility Group (Regression Estimate)	+3 to less than +4 initial Sperm Motility Group (Regression Estimate)	Pooled Estimate (Regression Estimate)
S_1	-0.00958±0.0076	-0.00712±0.0047	-0.0077±0.0037
S_2	-0.00976±0.0072	-0.00679±0.0094	-0.0085±0.0055
S_3	-0.00840±0.0090	-0.00546±0.0089	-0.0032±0.0061
S_4	-0.00812±0.0088	-0.00675±0.0099	-0.0074±0.0062

The decline in motility in all the four dilutors was relatively greater for +4 and above initial sperm motility group in comparison to the +3 to less than +4 initial sperm motility group.

All the regression estimates except the pooled estimate in S_1 dilutor were, however, not significant as given in Table No. 3.

The percentage of samples that maintained +3 motility after different period of storage is given in Table No. 4.

TABLE NO. 4. PERCENTAGE OF SAMPLES MAINTAINING +3 MOTILITY AFTER DIFFERENT PERIODS OF STORAGE.

No.of days	+4 and Above Initial Sperm Motility Group			
	S_1 Dilutor	S_2 Dilutor	S_3 Dilutor	S_4 Dilutor
1	2	3	4	5
1	100.00	100.00	100.00	100.00
2	100.00	100.00	100.00	100.00
3	100.00	100.00	100.00	100.00
4	100.00	100.00	100.00	100.00
5	85.71	85.71	85.71	85.71
6	42.85	57.14	42.85	57.14

Table Contd.....

TABLE No. 4. (Contd...)

1	2	3	4	5
		+3 to Less Than +4 Initial Sperm Motility Group		
1	100.00	100.00	100.00	100.00
2	100.00	100.00	100.00	100.00
3	100.00	100.00	100.00	100.00
4	83.33	91.66	83.33	91.66
5	58.33	58.33	66.66	66.66
6	25.00	33.33	33.33	33.33
		Pooled Estimate		
1	100.00	100.00	100.00	100.00
2	100.00	100.00	100.00	100.00
3	100.00	100.00	100.00	100.00
4	89.47	94.73	89.47	94.73
5	68.42	68.42	73.68	73.68
6	31.57	42.10	36.84	42.10

In both the initial motility groups all the samples maintained +3 motility in the dilutor containing four different ages of egg yolk. The number of samples

maintaining +3 motility declined on the fourth day of storage for +3 to less than +4 initial sperm motility group. The number of samples that maintained +3 motility on 5th. and 6th. day of storage was greater for semen of +4 and above initial sperm motility group as compared to the group having lesser initial sperm motility.

Seventy six samples were used to see the bacterial growth after twenty four hours of incubation in nutrient agar. These samples equally represented the four ages of egg yolk. Out of these seventy six samples bacterial growth was observed in 18 samples, 6 in S_1, 3 in S_2, 3 in S_3 and 6 in S_4. The number of samples in which bacterial growth observed was the same for S_4 where fresh egg was used, as for S_1 in which egg stored in refrigerator for 7 days were used. The number of bacterial growth observed in various samples are given in Table No. 5. The bacterial colonies that were observed were streptococcus, staphylococcus and of non-specific type which could be due to external contamination during preparation of dilutors.

TABLE NO. 5. BACTERIAL COUNT IN VARYING AGES OF EGG YOLK DILUTORS.

Sl.No.	Dilutors			
	S_1	S_2	S_3	S_4
1	16	2	4	2
2	3	1	-	-
3	-	-	-	2
4	14	-	-	139
5	-	-	-	1
6	1	-	-	15
7	5	-	12	7
8	13	25	14	-

(b) Keeping Quality of Hariana Semen in Modified Milk Extenders:-

In order to study the keeping quality of Hariana semen in modified milk extenders the number of days upto which +3 motility was maintained in various extenders

were analysed. Since the initial motility of the semen varied, the keeping quality of semen in modified milk extenders were analysed in two groups according to initial sperm motility.

The average number of days upto which +3 motility of spermatozoa was maintained in various milk extenders are given in Table 6, for the two

TABLE NO. 6. AVERAGE MOTILE LIFE OF SPERMATOZOA IN VARIOUS MILK DILUTORS.

Motility Group	DILUTORS							
	S_1	$S_2/10$	$S_2/15$	$S_2/20$	$S_3/10$	$S_3/15$	$S_3/20$	S_4
Upto +4	3.4	3.9	4.1	4.0	3.3	3.3	3.5	4.2
+4 and above	3.5	4.5	4.7	4.4	4.0	3.8	3.4	4.7

initial sperm motility groups separately. The keeping quality of semen in each dilutor was greater for +4 and above in initial sperm motility group in comparison to +3 to less than +4 initial sperm motility group.

The analysis of variance of the number of days to which +3 motility of the two groups of semen samples (+3 to $\angle$ +4 and +4 and above) was maintained

revealed significant difference among the various modified milk dilutors as shown in Table No. 7,

TABLE NO. 7. ANALYSIS OF VARIANCE FOR NUMBER OF DAYS TO WHICH + 3 MOTILITY WAS MAINTAINED FOR THE INITIAL MOTILITY GROUPS.

Source of Variation	+4 and Above Initial Motility Group		+3 to less than +4 Initial Motility Group		Pooled Estimates	
	d.f.	M.S.S.	d.f.	M.S.S.	d.f.	M.S.S.
Total	55	-	103	-	158	-
Between dilutors	7	1.851**	7	1.902*	14	1.877**
Within dilutors	48	0.345	96	0.795	144	0.645

* - Significant at P/_ 0.05;

** - Significant at P/_ 0.01.

indicating that the various dilutors differed significantly in maintaining +3 motile life of the spermatozoa. On pooling the two initial motility groups (Table No. 7), there was again significant difference among the various dilutors.

Differences between the efficiency of any two dilutors was tested with critical difference in Table No. 8, and 9.

TABLE NO. 8. DIFFERENCES BETWEEN THE EFFICIENCY OF ANY TWO DILUTORS (+4 AND ABOVE IN INITIAL MOTILITY GROUPS, DILUTORS ARRANGED IN ASCENDING ORDER).

Dilutors	$S_3/20$	S_1	$S_3/15$	$S_3/10$	$S_2/20$	$S_2/10$	$S_2/15$
S_4	**	**	**	*	NS	NS	NS
$S_2/15$	**	**	**	*	NS	NS	
$S_2/10$	**	**	*	NS	NS		
$S_2/20$	**	**	NS	NS			
$S_3/10$	NS	NS	NS				
$S_3/15$	NS	NS					
S_1	NS						

* - Significant at P/_ 0.05;

** - Significant at P/_ 0.01

NS - Not Significant.

TABLE No. 9. DIFFERENCES BETWEEN THE EFFICIENCY OF ANY TWO DILUTORS (+3 TO LESS THAN +4 INITIAL MOTILITY GROUPS) - DILUTORS ARRANGED IN ASCENDING ORDER.

Dilutors	$S3/10$	$S_3/15$	S S_1	$S_3/20$	$S_2/10$	$S_2/20$	$S_2/15$
S_4	**	**	*	*	NS	NS	NS
S2/15	*	*	*	NS	NS	NS	
$S_2/20$	*	*	NS	NS	NS		
$S_2/10$	NS	NS	NS	NS			
$S_3/20$	NS	NS	NS				
S_1	NS	NS					
$S_3/15$	NS						

There was highly significant difference between S_4, $S_3/20$, S_1 and $S_3/15$, between $S_2/15$, $S_3/20$, S_1 and $S_3/15$, between $S_2/10$, $S_3/20$ and S_1, between $S_2/20$, $S_3/20$ and S_1 . There was significant difference between S_4 and $S_3/10$, between $S_2/15$ and $S_3/10$, between $S_2/10$ and $S_3/15$ as in Table 8.

There was highly significant difference between S_4, $S_3/10$ and $S_3/15$. There was significant difference between S_4 , S_1 and $S_3/20$, between $S_2/15$, $S_3/10$, $S_3/15$ and S_1, between $S_2/20$, $S_3/10$ and $S_3/15$.(T.*9).

The regression of motility on days was also analysed and the estimate along with S.E. are given in Table No. 10. Since the motility was observed till +3 motility was maintained, the regression estimate for the most of the dilutors was not significant indicating that there was no decline in sperm motility on days.

However, from Table No. 11, it will be observed that the number of samples maintaining +3 motility in various dilutors progressively decline after two days of storage in the +3 to less than +4 initial sperm motility group, and after three days in +4 and above initial sperm motility group. On fifth day in S_1, $S_3/10$, $S_3/15$ and $S_3/20$, no sample was observed

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*T - Table.

having motility of +3 or above, in case of semen of +4 and above initial sperm motility group, similarly in other sperm motility group no sample could maintain +3 motility in S_1 and $S_3/15$ dilutors.

On pooling the two initial sperm motility groups as shown in Table No. 12, it was observed that there was decline from third day onward in maintaining +3 motility in almost all the dilutors except in S_4. The number of samples that are maintaining +3 motility continued to decline with age and no sample on 5th. day in S_1 and $S_3/15$. S_4 dilutor continued to show its efficacy by maintaining +3 motility in 50 per cent of the samples.

TABLE NO. 10. REGRESSION OF MOTILITY ON DAYS OF PRESERVATION IN MODIFIED MILK DILUTORS

Dilutors	+4 and above Initial Sperm Motility Group (Regression Estimate)	+3 to less than +4 Initial Motility Group (Regression Estimate)	Pooled Estimate (Regression Estimate)
S_1	-0.290±0.2646	-0.128±0.2164	-0.1929±0.167
$S_2/10$	-0.342±0.2246	-0.203±0.2213	-0.2714±0.157
$S_2/15$	-0.357±0.2060	-0.179±0.2038	-0.2670±0.144
$S_2/20$	-0.378±0.2466	-0.232±0.2093	-0.2931±0.158
$S_3/10$	-0.324±0.2496	-0.145±0.2755	-0.2433±0.184
$S_3/15$	-0.409±0.2091	-0.222±0.2851	-0.3435±0.168
$S_3/20$	-0.379±0.3360	-0.190±0.2147	-0.2447±0.180
S_4	-0.356±0.1641	-0.270±0.3116	-0.3373±0.145

TABLE NO. 11. PERCENTAGE OF SAMPLES MAINTAINING +3 MOTILITY IN DIFFERENT MODIFIED MILK DILUTORS IN DIFFERENT INITIAL MOTILITY GROUPS.

No.of days	S_1	$S_2/10$	$S_2/15$	$S_2/20$	$S_3/10$	$S_3/15$	$S_3/20$	S_4
	+ 4 AND ABOVE INITIAL SPERM MOTILITY GROUP							
1	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
2	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
3	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
4	42.85	100.00	100.00	100.00	85.71	71.42	28.57	100.00
5	-	28.57	42.85	28.57	-	-	-	57.14
	+ 3 TO LESS THAN + 4 INITIAL SPERM MOTILITY GROUP							
1	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
2	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
3	92.30	92.30	92.30	92.30	92.30	84.61	84.61	100.00
4	38.46	69.23	69.23	69.23	38.46	38.48	46.15	76.92
5	-	30.76	46.15	38.46	7.59	-	15.33	46.15

TABLE NO. 12. PERCENTAGE OF SAMPLES MAINTAINING +3 MOTILITY IN DIFFERENT MODIFIED MILK DILUTORS (POOLED ESTIMATE OF THE TWO INITIAL MOTILITY GROUPS)

No.of days	POOLED ESTIMATE							
	S_1	$S_2/10$	$S_2/15$	$S_2/20$	$S_3/10$	$S_3/15$	$S_3/20$	S_4
1	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
2	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
3	95.00	95.00	95.00	95.00	95.00	90.00	90.00	100.00
4	40.00	80.00	80.00	80.00	55.00	50.00	40.00	85.00
5	-	30.00	45.00	35.00	5.00	-	10.00	50.00

(c) Liveability of Spermatozoa in Cervical Mucus:-

Under this experiment twenty six samples of cervical mucus from oestrus cows and thirteen samples of cervical mucus from oestrus buffaloes were used to study the progressively motile spermatozoa in Hariana and Murrah bull semen extended in S_4 milk dilutor and A_4 dilutor respectively after four hour of incubation at 38^{o}C. The average percentage of progressive motile spermatozoa in different viscosity, elasticity and pH groups are given in Table No. 13, for cervical mucus of oestrus cows. The analysis of variance to study the differences between groups is given in Table 14.

TABLE NO. 13. AVERAGE PERCENTAGE OF PROGRESSIVE MOTILE SPERMATOZOA IN DIFFERENT VISCOSITY, ELASTICITY AND pH GROUPS, AFTER 4 HOURS INCUBATION IN CERVICAL MUCUS OF OESTRUS COWS.

Group	Average Percentage of Progressive Motile Spermatozoa
VISCOSITY	
Thick	15.10±0.74
Medium	15.66±0.74
Thin	20.89±0.58
ELASTICITY	
10 - 12 cm.	14.82±0.47
13 - 15 cm.	17.94±1.08
16 - 18 cm.	21.30±0.79
pH	
7.0 - 7.5	16.33±0.86
7.6 - 8.0	18.36±1.43
8.1 - 8.5	16.70±1.30

The differences were significant for viscosity and elasticity. The percentage of progressively motile spermatozoa increased as the consistency of cervical mucus became thiner. Similarly, the percentage of progressively motile spermatozoa increased with increase in elasticity.

TABLE NO. 14. ANALYSIS OF VARIANCE BETWEEN GROUPS FOR SPERM MOTILITY AFTER INCUBATION IN VISCOSITY, ELASTICITY AND pH GROUPS (HARIANA - S_4 EXTENDED SEMEN)

Source of Variation	d.f.	Viscosity	Elasticity	pH range
		M.S.S.	M.S.S.	M.S.S.
Total	25			
Between groups	2	80.60**	69.74**	11.87 NS
Within groups	23	5.23	6.17	11.20

** - Significant at P/_ 0.01.

NS - Not significant.

The average percentage of progressively motile spermatozoa in cervical mucus of oestrus buffaloes according to viscosity, elasticity and pH groups is given in Table No. 15.

The analysis of variance to study the significant difference between groups is given in Table No. 16.

TABLE NO. 15. AVERAGE PERCENTAGE OF PROGRESSIVELY MOTILE SPERMATOZOA IN DIFFERENT VISCOSITY, ELASTICITY, AND pH GROUPS AFTER 4 HOURS INCUBATION IN CERVICAL MUCUS OF OESTROUS BUFFALOES.

GROUPS	Average Percentage of Progressively Motile Spermatozoa
VISCOSITY	
Thick	12.53±0.83
Medium	14.66±0.95
Thin	17.00±0.55
ELASTICITY	
11 -13 cm.	13.19±0.65
14 - 16 cm.	16.39±0.74
17 - 20 cm.	17.42±1.07
pH	
7.0 - 7.7	16.29±0.62
7.8 - 8.5	13.12±0.86

TABLE NO. 16. ANALYSIS OF VARIANCE BETWEEN GROUPS FOR SPERM MOTILITY AFTER INCUBATION IN VISCOSITY, ELASTICITY AND pH GROUPS OF CERVICAL MUCUS OF OESTROUS BUFFALOES.

Source of Variation	Viscosity		Elasticity		pH	
	d.f	M.S.S.	d.f	M.S.S.	d.f	M.S.S.
Total	12		12		12	
Between groups	2	19.39*	2	19.92*	1	30.93*
Within groups	10	2.87	10	2.76	11	3.32

* - Significant at P/__0.05.

In the progressively motile percentage of spermatozoa there was significant difference between viscosity groups of cervical mucus. There was an increase in the percentage of progressively motile spermatozoa with the decrease of consistency of cervical mucus. The percentage of progressively motile spermatozoa increased with elasticity of cervical mucus. There was significant difference between two pH groups indicating that pH range 7.0 - 7.7 of the cervical mucus was more suited for motility of spermatozoa as compared with 7.8 - 8.5 pH groups (Table No. 15).

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CHAPTER - V

D I S C U S S I O N

CHAPTER - V

D I S C U S S I O N

The averages of semen characteristics for volume, hydrogen ion concentration, initial sperm concentration per mm. , live spermatozoa percentage and live abnormal spermatozoa percentage are given in Table No. 1. There was wide variation in semen volume, sperm concentration between bulls. In rest of the characters there was very little variation between ejaculates.

(a) Effect of Varying Ages of Egg Yolk on Sperm Motility at 4°C.:-

Maintenance of Hariana semen in A_4 medium supplemented with egg yolk of varying ages was found unaffected as a result of quality of egg yolk. In both the groups whether the initial motility of semen preserved was +4 and above or +3 to less than +4, use of egg yolk obtained from eggs, stored for 2 and 4 days at room temperature and for 7 days at refrigerator temperature, and the eggs obtained freshly on the day of laying did not significantly bring about changes in the quality of diluent to maintain spermatozoa upto +3 motile life.

The result thus indicated that the quality of egg yolk did not deteriorate when eggs were maintained, after laying at room temperature ranging from 25° - 34°C. for 2 to 4 days and at 5°C. for 7 days in the refrigerator. Bacteriological examination of the diluent prepared after adding egg yolk did not indicate any association of the age of egg with the number of bacterial colonies obtained after incubation (Table No. 5).

It only appeare that the incidence of micro-organisms in egg yolk was related to the eggs irrespective of the period of storage, obtained from different hens. Since, the quality of semen extenders did not seem to be affected with egg yolk obtained from eggs stored for 2 to 4 days at 25° - 34°C. at room temperature and for 7 days at 5°C. in the refrigerator. There could be sufficient justification for not insisting on egg yolk obtained from freshly laid eggs since the egg yolk is bound to be rendered unsuitable on account of microbial growth or due to enzymic degration of compound when eggs are maintained at about body temperature.

In the present result, storage temperature not exceeding 34°C. appeared ineffective in causing any adverse changes in egg yolk quality. Meglioli et al (1955) used egg yolk from eggs stored upto 25 days at

refrigerator temperature and found no adverse effect on sperm motility. Morozov (1951) reported that egg yolk could be used safely for semen preservation till it was maintained inside yolk-membrane separated from egg-albumen.

Thus, it could be concluded at such places, where eggs cannot be procured freshly, it is not disadvantageous to obtain eggs from outside and maintain at refrigerator temperature for at least 7 days and used in the semen extenders. Not only this, but eggs can suitably be stored upto 34°C. at room temperature for four days for use of semen preservation.

The preservation quality of four types of egg yolk in A_4 buffer medium when studied for day to day maintenance of sperm motility, it was observed that the regression of motility for each day of preservation was nearly similar in all the media when initial motility of semen used was +4 and above and with lower motility grade semen also there was no apparent variation for regression of motility for each day of preservation though the regression estimate was lowest for yolk used from eggs stored for two days at room temperature.(Table No. 3).

The pooled estimates of regression, however, appear to indicate that with yolk, obtained from eggs stored for two days at room temperature, the rate of decline in motility in each day of preservation was least. The slightly beneficial effect of yolk used from eggs stored for two days at room temperature cannot be over estimated from the present experiment.

When the efficiency of yolk was tested on the basis of percentage of semen samples maintained at +3 motility for 6 days of storage period, it was found that fresh yolk always maintained higher percentage of samples.(Table No. 4).

The overall study which was aimed for the maintenance of +3 motile life of spermatozoa at 4°C. however, did not differentiate among the effect of yolk obtained from eggs of varying periods i.e. 2-4 days at 25°C. - 34°C. at room temperature and for 7 days at 4°C.

(b) Keeping Quality of Semen in Modified Milk Extenders:-

The efficiency of the dilutors to maintain +3 motile life of spermatozoa varied highly significantly when initial motility of semen samples taken for study was +4 and above (Table No. 7). When initial motility was less than +4, the efficiency of

the dilutor was significant at 5 per cent level of probability (Table No. 7).

The use of boiled milk for maintenance of Hariana semen proved on an average to maintain +3 motile life for 3.4 to 3.5 days in both the groups (Table No. 6). Addition of glucose solution for 10 parts to 20 parts per 100 ml. of boiled milk did not increase the efficacy of the medium to maintain spermatozoa, where replacemtnt of boiled milk with 20 parts of egg yolk in $S_3/20$ medium highly significantly increased the keeping quality of spermatozoa (Table No. 6).

In S_4 medium 50 per cent of the samples were maintained at +3 motile life for five days, where in $S_3/20$ medium which contained milk-glucose but not egg yolk, only 10 per cent of the samples could maintain +3 motile life on 5th. day of storage period (Table No. 12).

The overall regression estimate of decline in motility for each day of storage was slightly higher in S_4 medium and was because of the fact that very few samples could maintain +3 motile life beyond three days of preservation in comparison to S_4 dilutor.

The role of egg yolk in preservation of semen

is profound, though addition of glucose in milk has some beneficial effect as it provides energy to the spermatozoa which can metabolise glucose and not lactose which is present in milk.

In the present result, no beneficial effect of addition of glucose in boiled milk medium was observed on the maintenance of +3 motile life of spermatozoa.

The role of egg yolk could be through its protective action on spermatozoa during dilution and temperature shock, in addition, the yolk could provide the SH group which are generally absent in boiled milk.

In another set of observations use of whole egg i.e. yolk + albumen together, along with the boiled milk was foundbbeneficial for the preservation of +3 motile life of spermatozoa at $4^{o}C$. In both groups, whether motility below or above +4, replacement of boiled milk with whole egg in proportion, varying from 10-20% level was significantly advantageous. The level of 15% whole egg appear significantly better for preservation of Hariana semen in boiled milk and its efficacy is equivalent to that of medium containing glucose-egg yolk along with boiled milk (Table No. 6).

When the efficacy of the dilutors was compared

in terms of percentage of samples maintained at +3 motile life, the S_4 medium ranked first (Table No. 12), while the whole egg containing media were equally good upto four days of storage period and 80 per cent of the samples were maintained in these media for +3 motile life for four days (Table No. 12).

From the results, it appears that use of albumen along with yolk could be as advantageous as the addition of glucose solution. In the past, use of whole egg for preservation of semen was reported by many workers and they found its use beneficial provided the pH of medium was adjusted to acidic side.

The regression estimate of motility for each day of preservation was nearly similar in whole egg containing media (Table No. 10). The boiled milk media show lowest regression estimate (Table No. 10), as it could maintain +3 motile life of Hariana semen for significantly less period of storage.

(c) Liveability of Spermatozoa in Cervical Mucus:-

Progressive motility of spermatozoa after four hours incubation at 38°C. in cirvical mucus, obtained from oestrus cows varied highly significantly on account of its viscosity and elasticity but there

was no significant variation in progressive motile percentage of spermatozoa on account of pH of cervical mucus (Table No. 14). Thick viscosity and the elasticity below 13 cm. favour lowest rate of progressive motile percentage of spermatozoa (Table No. 13).

In the case of buffalo, progressive motile percentage of spermatozoa after four hours of incubation at 38°C. in cervical mucus on account of all the three characteristics of cervical mucus, was lowest when viscosity was thick, elasticity less than 14 cm. and pH was above 7.7 (Table No. 15).

From the results, it sounds that for better rate of progressive motility of spermatozoa, the cervical secretion should be thin to medium viscosity and with higher elasticity (Table Nos. 13 & 15).

The mucus characteristics mainly viscosity and elasticity represent the stage of oestrus in animals and thus it could appear that better maintenance of progressive motility of spermatozoa in female reproductive tract would be, when mucus secretion is from thin to medium in viscosity and with comparatively higher elasticity. Such mucus is always present in animal with normal oestrus in nearly middle stage of oestrus. In the later stage, viscosity

becomes thick, the elasticity decrease, while in earlier stage viscosity is thinner to thin while elasticity is less. Thus, on the basis of better maintenance effect of cervical secretion on spermatozoal motility, is the stage of oestrus when mucus is thin to medium in viscosity and has higher elasticity as judged by the length of its thread, is more suitable for insemination.

The role of pH though indicated significant effect in buffalo (Table No. 16), but not such effect observed in cows. Generally higher pH i.e. over 8.5 has some adverse effect on spermatozoal motility. In the present study significant effect of pH of cervical mucus on buffalo spermatozoa may not be very much conclusive. It could only be indicative that a pH range from 7.8 - 8.5 would may not be favourable for maintenance of spermatozoal motility (Table No.15).

Blackshaw and Emmens (1951) stated that the spermatozoal motility was not much affected between pH 5.5 to 8.5. Vasilzeva (1958) reported high conception rate with low viscosity and high elasticity of vaginal mucus.

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CHAPTER - VI

S U M M A R Y

CHAPTER - VI

S U M M A R Y

To study the effect of egg yolk of varying ages on sperm motility at 4°C., four dilutors were prepared with the egg yolk, obtained from eggs, after storage for seven days at 4°C., at room temperature for 2-4 days and fresh egg in A_4 buffer medium. The dilutors were designated as S_1, S_2, S_3 and S_4 containing egg yolk from the eggs after storage for 7 days at 4°C., at room temperature for 4 days, at room temperature for 2 days and fresh egg.

Semen varied in initial sperm motility, so it was grouped into +3 to less than +4 and +4 and above initial motility groups. The average number of days to which +3 motility of spermatozoa was maintained in S_1, S_2, S_3 and S_4 dilutors were 4.7, 4.8, 4.8 and 4.8 respectively for the semen of +3 to less than +4 initial motility group and 5.3, 5.6, 5.1 and 5.4 days respectively for +4 and above initial sperm motility groups.

All the four dilutors had more or less the same effect in maintaining +3 motility of spermatozoa of Hariana semen at 4°C. The decline in motility on hour

of preservation was relatively greater for +4 and above initial motility groups in comparison to +3 to less than +4 initial motility groups. All dilutors showed non-significant decline on hour of preservation.

Seventy six samples of the dilutors were checked for bacterial growth. Bacterial growth was observed in 18 samples. Incidence of microorganisms were related to eggs obtained from different hens or due to external contamination during preparation of dilutors, irrespective of storage period.

(b) In order to study the keeping quality of Hariana semen in milk eight modified milk dilutors were used for preservation at 4°C. Dilutors designated as S_1, $S_2/10$, $S_2/15$, $S_2/20$, $S_3/10$, $S_3/15$, $S_3/20$ and S_4 contained boiled milk, boiled milk + whole egg 10%, 15%, 20%, 5% glucose 10 parts, 15 parts, 20 parts and 20 parts egg yolk-5% glucose 20 parts respectively.

Average days to which +3 motility of spermatozoa was maintained in S_1, $S_2/10$, $S_2/15$, $S_2/20$, $S_3/10$, $S_3/15$, $S_3/20$ and S_4 dilutors was 3.4, 3.9, 4.1, 4.0, 3.3, 3.3, 3.5 and 4.2 days for +3 to less than +4 initial motility groups; and 3.5, 4.5, 4.7, 4.4, 4.0, 3.8, 3.4 and 4.7 for +4 and above initial motility groups. The keeping quality of semen in each dilutor was

greater for +4 and above initial motility groups in comparison to +3 to less than +4 initial motility groups.

The milk dilutors differed significantly in maintaining +3 motility in days. Out of eight dilutors S dilutor was best, maintaining +3 motility in 50% of samples on 5th. day of storage period. No beneficial effect was observed by adding 10 to 20 parts of 5% glucose solution in boiled milk to maintain +3 motile life of the spermatozoa. Boiled milk with whole egg had beneficial effect in maintaining +3 motility of spermatozoa in 30 to 45% of the samples on 5th. day of storage at $4^{o}C$. $S_2/15$ dilutor containing 15% of whole egg was equally good as S4 dilutor containing 20 parts egg yolk.

(c) Cervical mucus from oestrus cows and buffaloes were taken to study the effect of viscosity, elasticity and pH groups of cervical mucus on progressive motility of the extended semen after 4 hours incubation at $38^{o}C$. Progressive motility of the spermatozoa was not much affected by the pH groups of cervical mucus of oestrus cows but motility percentage was increased in thin viscosity and high elasticity of cervical mucus.

In case of buffalo, average progressively motile percentage of spermatozoa was maintained better

in low consistency and high elasticity of cervical mucus. pH range 7.0 to 7.7 of the cervical mucus was more suited to maintain the progressive motility of the spermatozoa. From the viscosity and elasticity of cervical mucus, the stage of oestrus in animal could be found out. In middle stage of oestrus mucus secretion is thin to medium in viscosity and comparatively of high elasticity. Such mucus is suitable for maintaining progressive motility of spermatozoa.

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A P P E N D I X

DILUTION CHART TO GET 100 MILLION SPERMATOZOA/Ml. OF THE DILUTOR.

	Per ml. density of semen samples	Volume of semen to be added in		
		2.5 ml. of dilutor	5 ml. of dilutor	10 ml. of dilutor
1	2000	0.12	0.24	0.48
2	1900	0.13	0.26	0.52
3	1800	0.14	0.28	0.56
4	1700	0.15	0.30	0.60
5	1600	0.16	0.32	0.64
6	1500	0.17	0.34	0.68
7	1400	0.18	0.36	0.72
8.	1300	0.19	0.38	0.76
9.	1200	0.21	0.42	0.84
10.	1100	0.23	0.46	0.92
11.	1000	0.25	0.50	1.00
12.	900	0.28	0.56	1.12
13.	800	0.31	0.62	1.24
14.	700	0.35	0.70	1.40
15.	6600	0.41	0.82	1.64
16.	500	0.50	1.00	2.00
17.	400	0.62	1.35	2.70
18.	300	0.82	1.64	3.28
19.	200	1.00	2.00	4.00
20.	100	2.00	4.00	8.00