CHAPTER III

MATERIALS AND METHODS
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A. Experimental Animals:

The present study under report was based on three breeds of bulls namely, (i) Jersey, (ii) Holstein Cross (Holstein X Sahiwal) and (iii) one Indian breed (Sahiwal). Thus there were three groups of different breeds of animals each group, comprising four bulls. Altogether, the observations were made on twelve bulls.

The bulls were randomly selected for the purpose. They belonged to the age groups of 6 to 7 years. They were healthy and were almost free from worm burden. They were not only dewormed before inclusion in the experiment but they were dewormed at regular internal during the period of experiment.

The bulls of all the groups were maintained and kept under identical conditions of managerial practice housing, watering and feeding. They were fed rations as recommended under I.C.A.R. Research bulletin No. 25 (1964).

To find out the effect of precollection stimulation on semen characters i.e., quantity and quality, the period was divided into four weeks in each month.

The procedure for accomplishing the levels of sexual excitement for treatment, restraint, one false mount and two false mounts were similar as those described by Collins et al.
The procedure for collection of semen from the bulls consisted of allowing the bulls to be near a dummy and allowing them a mount without restraint and serve into the artificial vagina. In this case, only one ejaculate was collected from each bull at weekly collection period. In the next week, the procedure for accomplishing the increased sexual excitement of the bulls consisted of allowing the bulls to be near the dummy and restraining them for a period of 2 to 3 minutes. Then, they were allowed to make a false mount, i.e., they were allowed to mount but were not allowed to serve the artificial vagina. After this false mount, they were allowed to serve the artificial vagina at the next attempt.

Similarly, in case of two false mounts, the same procedure was adopted. If they failed to do so they were allowed one more false mount.

The procedure for minimising sexual excitement consisted of bringing the bulls directly from the shed to the collection floor, allowing them to mount immediately, serve the artificial vagina and ejaculate. No effort was made to restrain them at the initial collection. Occasionally, two Sahiwal and one Jersey bulls would not mount immediately and ejaculate. In these cases, the semen was collected immediately the bulls mounted and ejaculated. As far as possible, all of the bulls on a particular treatment (restraint or unrestraint) were brought to about the same level of sexual excitement.
All the bulls were put to collection in the morning between 6 to 8 a.m. at least 2 to 3 hours before feeding.

B. Materials and methods in connection with study of undiluted semen:

As already stated all the bulls of different breeds were allowed to ejaculate once a week.

Study of volume of semen per ejaculate, colour and consistency, pH, initial motility, percentage of live spermatozoa, sperm density or concentration ( million ) per ml, percentage of abnormal spermatozoa, fructose concentration, and percentage of conception rate of semen of the experimental bulls under different treatments were based on the ejaculates obtained.

Volume of Semen:

Semen was collected in graduated tube by means of artificial vagina and the volume obtained was recorded as per technique adopted by Melrose and Laing (1970).

Colour and Consistency:

Colour and consistency of semen was noted immediately after collection and visually studied and graded according to Roberts (1956) and Singh (1965).
The pH of semen was ascertained immediately after collection by pH metre (Systronic pH metre) according to Tomar (1976).

Initial motility:

Initial motility was studied in place of mass activity and individual motility as most of the workers in this field have put more emphasis on initial motility rather than mass activity and individual motility.

Initial motility was studied on a 0 to 5 scale according to the method adopted by Walton (1933) and Rao and Hart (1948).

Counting of live spermatozoa in fresh semen:

Living spermatozoa in terms of percentage in fresh semen were calculated according to the method of Bishop et al. (1954).

The composition of the staining material used for the purpose was as follows:

- Negrosin (G.T. Gurr) ................ 30 g.
- Eosin Y water soluble (G.T. Gurr) .. 5 g.
- Distilled water ....................... 300 ml.
After mixing one drop of semen and 8 drops of eosin-negrosin stain in a test tube, the latter was incubated at 30°C for 5 minutes. A drop of semen stain mixture was then put on a glass slide and smear prepared. The prepared stain was used for 14 days after which period a fresh eosin-negrosin stain was prepared.

Sperm density or concentration:

Sperm concentration (million) per ml was determined with the help of Neubauer haemocytometer ruled slide according to the method described by Melrose and Laing (1970) which was as follows:

\[
\frac{n \times 4000 \times d \times 1000}{80} = \text{number of spermatozoa in million per ml}.
\]

where \(n\) is the number of spermatozoa counted in 5 large squares (80 small squares) and \(d\) is the rate of dilution. The rate of dilution is 1 : 200.

The average of three successive calculations was taken as a final one.

Counting of morphological abnormalities of spermatozoa in fresh semen:

Morphological abnormalities of spermatozoa the numbers of which were studied in terms of percentage in fresh semen and calculated according to the method advocated by Roberts (1971) & Rao and Rao (1978).
The composition of the stain was as follows:

- Rose Bengal (BDH) ................. 3 g.
- 40% Formalin ......................... 1 ml.
- Distilled water ....................... 99 ml.

Semen was diluted 1:10 with 3% sodium citrate dehydrate solution. Smears were prepared from the diluted semen on clean and dry glass slides. The smears were dried in air. Dried smears were stained for 15 minutes in Rose Bengal stain. The slides were washed carefully in tap water and were dried in air and mounted in DPX. (DPX mountant, BDH).

Fructose content in semen:

The initial fructose content of semen was estimated according to the conventional method followed by Mann (1948) as modified by Bishop et al. (1954).

Soon after collection of semen 0.1 ml of semen was dropped in a tube containing 0.9 ml of distilled water. For deproteinisation of semen, 2 ml of each of 2% Zinc sulphate and N/10 sodium hydroxide were added. The sample was heated in a boiling water bath. It was then cooled and filtered. Two ml of this filtrate was taken in a 15 ml tube. In another tube 2 ml of fructose solution containing 0.2 mg of fructose (0.1 mg/ml) was taken. Two ml of distilled water were taken in a third tube labelled as "blank". Two ml of 0.1% alcoholic
resorcinol and 6 ml of 30% HCl were added to each tube. All the three tubes were kept in the water bath at 80-85°C for 10 minutes and then cooled. The samples were compared against blank to find out the optical density of solution in the other two test tubes with the help of photo electric colorimeter.

Calculation:

\[
\text{Optical density (O.D.) of unknown} \times 0.2 \times 5 \times 100
\]
\[
\text{O.D. of standard} \times 0.1 \times 2
\]

= mg of fructose / 100 ml of semen.

Dilution rate of semen for insemination:

Semen after proper evaluation was diluted in the ratio of 1:15 in conventional egg yolk citrate buffer (Salisbury et al. 1941) and insemination was done as per recto-vaginal method.

Acrosomal Study:

Study on acrosomal make up (free loose head) of spermatozoa was also studied using Giemsa stain according to Hancock (1962) and modified by Mukherjee (1979). Suspension of semen in normal saline (0.1 ml semen with 9.9 ml normal saline) was made and smeared on slides. The slides were then completely dried at 37°C, fixed in buffer formal saline for 30 minutes, washed in running water for 15 minutes, rinsed with neutral distilled water and stained for 48 hours in Giemsa stain.
containing 5 ml of Giemsa, 8 ml of buffer and 87 ml of glass distilled water. The slides were then dried and mounted in DPX mountant. The compositions of buffered formal saline and the buffer used with Giemsa stain were as follows:

<table>
<thead>
<tr>
<th>Buffered formal saline</th>
<th>Buffer solution</th>
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<tbody>
<tr>
<td>3.82 ml of 0.1 M Citric Acid</td>
<td>176.40 ml of 0.1 M Citric acid</td>
</tr>
<tr>
<td>41.18 ml of 0.2 M Na$_2$HPO$_4$</td>
<td>823.60 ml of 0.2 M Na$_2$HPO$_4$</td>
</tr>
<tr>
<td>125.00 ml of 1.2% NaCl</td>
<td>31.25 ml of Commercial formalin (40%)</td>
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<tr>
<td>Glass distilled water up to 250 ml</td>
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Statistical analysis:

For statistical analyses of data methods described by Snedecor and Cochran (1969) were followed:

(a) Mean values together with standard error of different items of semen were calculated.

(b) Analyses of variance were made to find out whether the different items differed significantly amongst individual bulls and groups of different breeds. The differences between bulls and groups were further analysed by the test of critical difference. The critical differences were found out at 1% and 5% level of probability. For analyses of variance percentage values were transferred into angle values.