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CLASSIFICATION AND CHARACTERIZATION OF FOLLICULAR OOCYTES OF CROSSBRED CATTLE

By
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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that the thesis entitled **"CLASSIFICATION AND CHARACTERIZATION OF FOLLICULAR OOCYTES IN CROSSBRED CATTLE"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Lydia Priscilla, K.

CERTIFICATE

Certified that the thesis, entitled **"CLASSIFICATION AND CHARACTERIZATION OF FOLLICULAR OOCYTES IN CROSSBRED CATTLE"** is a record of research work done independently by **Ms. K. Lydia Priscilla**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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We the undersigned members of the Advisory committee of **Ms. K. Lydia Priscilla**, a candidate for the degree of Master of Veterinary Science in Animal Reproduction, agree that the thesis **"CLASSIFICATION AND CHARACTERIZATION OF FOLLICULAR OOCYTES IN CROSSBRED CATTLE"** may be submitted by Ms. K. Lydia Priscilla, in partial fulfillment of the requirement for the degree.

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Lydia Priscilla, K.

To My
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Introduction

1. INTRODUCTION

Ruminants, especially the dairy cows and buffaloes play a significant role in maintaining a strong agricultural economy in India. Profitability of animal production depends to a large extent on reproductive performance. India is poised to be the global leader in milk production following European Union. With the biggest cattle population, our milk production potential should have been considerably higher, had the genetic potential of our cattle been improved.

Dairy cattle production is likely to play a key role in the economic upliftment of our country and this in turn would be geared up by efficient reproductive management. Controlled breeding and assisted reproductive technologies are expected to contribute substantially in bovine reproductive management in the coming decades.

Embryo biotechnology is predicted to play a major role in ushering in the new era of animal production programmes. Embryo transfer technology introduced into commercial dairy practice from 1970 onwards has enabled cows to produce calves of genetic quality and composition quite different from that of the surrogate mother.

Superovulation and embryo transfer can increase the number of good calves to ten per year and 100 per cow during its lifetime, (Butterworth-Heinemann, 1992) thus placing enormous scope on dairy cattle improvement using these technologies.

Cost effectiveness of embryo recovery after superovulation of donor cattle limits large scale application of this technology, necessitating viable alternate technologies for genetic improvement of dairy cattle.

Success of egg transfer depends on the economic production of large number of high quality embryos demanding a shift from Multiple Ovulation Embryo Transfer (MOET) to viable technologies including *in vitro* maturation and *in vitro* fertilisation programme.

Basic research in the bovine has long been plagued by the inability to acquire competent oocytes in sufficient numbers. This had imposed restriction on detailed studies on oocytes, gametic interactions and early development of embryo (Leibfried-Rutledge *et al.*, 1989).

An efficient exploitation of the female gamete pool and shortening of the generation interval by the advanced reproductive technique namely *in vitro* embryo production requires abundant

availability of developmentally competent cumulus oocyte complexes from valuable donor cows (Bungartz *et al.*, 1995).

In an age in which there is increasing resistance by animal welfare groups to the use of live animal in research, there is obvious advantages to techniques which enable farm animal embryos and oocytes to be made available for research without this involving live animal and superovulation intervention. It may be that the needs of many research programs can be satisfactorily met from slaughterhouse ovaries and a supply of semen rather than involving live animals (Gordon, 1994).

The bovine ovary, as that of other mammals contains thousands of oocytes of which more than 99.9 per cent undergo atresia and they can be rescued before they degenerate (Katska and Rynska, 1998). Standardization of oocyte recovery method, classification and characterization of follicle obtained from abattoir specimens are effective tools in implementation of assisted reproductive programs.

Leibfried and First (1979) have found a direct relationship between morphological characteristics of follicular oocytes and their ability to mature *in vitro*. Therefore, the present study aims to understand the preliminary ovarian factors that would influence the production of embryos on large scale.

Kerala State through its massive cross breeding program has developed 65 per cent of its cattle population as elite crossbred animals. The potential scope of harvesting the follicular oocytes from this valuable germplasm is yet to be standardised. This assumes importance, taking into account of large scale culling and slaughter of even elite animals for meat purpose in the state. In this background this study is undertaken with the following objectives;

1. Observation of ovarian parameters and morphometry in relation to follicular development in crossbred cattle.
2. Development and standardisation of protocols involved in oocyte recovery from the ovaries obtained.
3. Evaluation of follicular oocytes based on developmental pattern of corpus luteum.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Collection of ovaries

The interval between the oocyte recovery and the slaughter of the animal had great impact on the recovery rate of oocytes and degree of subsequent maturation rate.

2.1.1 Medium

For transport of ovaries to the laboratory transportation medium was used. Some of the research workers used Dulbecco's phosphate buffer saline for transport of caprine ovaries (Chauhan and Anand, 1991; Agarwal, 1992; Gall *et al.*, 1993; Martino *et al.*, 1994) and buffalo ovaries (Umadevi and Reddy, 1998).

Sterile normal saline solution (0.9 per cent) was preferred for transport of buffalo ovaries, (Selvaraj *et al.*, 1992; Das *et al.*, 1996a; Nandi *et al.*, 2000; Yadav *et al.*, 2000) cattle ovaries, (Baruha *et al.*, 1998) goat ovaries, (Pawshe *et al.*, 1993;

Chakaravarty *et al.*, 1994; Dutta *et al.*, 1996) and sheep ovaries (Reddy and Shankarappa 1999).

2.1.2 Temperature

Cattle ovaries were transported at a temperature of 35 to 39°C (Lenz *et al.*, 1983), 37°C (Baruha *et al.*, 1998) or 32 to 37°C (Nandi *et al.*, 2000). Selvaraj *et al.* (1992) transported buffalo ovaries at 0°C whereas sheep and caprine ovaries were transported at a range of 30 to 35°C (Das *et al.*, 1996c; Agarwal and Verma 1997).

2.1.3 Duration

In the transport of ovaries of various species there has been observed a wide range in the time taken to reach the laboratory.

Caprine ovaries were transported within either one hour (Chauhan and Anand, 1991) or two hours of slaughter (Pawshe *et al.*, 1993; Chakaravarty *et al.*, 1994; Agarwal and Verma 1997). Sheep ovaries were transported within an hour by

Datta *et al.* (1993) and within 30 to 60 minutes by Reddy and Shankarappa (1999).

Selvaraj *et al.* (1992) transported buffalo ovaries within two hours of slaughter. But Nandi *et al.* (2000) and Yadav *et al.* (2000) transported within four and five hours of slaughter.

2.2 Oestrous cycle

2.2.1 Stages of oestrous cycle

Ireland *et al.* (1980) developed a strategy to classify the oestrous cycle based on the gross changes in the appearance of corpora lutea namely colour, size, vasculature, weight. Stage I (zero to four days) included the interval between ovulation and the time when epithelium grew over the rupture point, forming the apex of a new corpus luteum. During Stage II (five to ten days) the corpus luteum was fully formed with vasculature visible around its periphery. The bisected CL had its apex red or brown coloured and the remainder was orange or yellow. Stage III (11 to 17 days) was marked with the change in colour from red or brown to bright orange or yellow of the whole structure. Later in this stage, vasculature was visible over the apex of the CL. In stage IV (18 to

20 days), the ovary contained at least one large follicle and a regressed CL.

Leibfried-Rutledge *et al.* (1985) classified paired cattle ovaries as follicular, early luteal or luteal based on weight of corpus luteum and appearance of other ovarian structures. The early luteal ovaries contained a developing CL that weighed less than two grams. The corpus albicans of the previous oestrous cycle was identified in one of the ovaries. The luteal phase ovaries had CL that weighed over two grams. The follicular phase ovaries had regressed CL that weighed less than three grams and were yellow and firm in texture.

Gandolfi *et al.* (1997) categorised bovine ovaries into three groups based on presence of a follicle >10mm in diameter, presence of more than 10 follicle of 2 to 5mm in diameter and no follicles in >10mm diameter, presence of less than 10 follicles of 2 to 5mm in diameter and no follicles >10mm. The cumulus oocyte complexes per ovary was 11.0, 12.0 and 6.2 in the above respective groups.

Varisanga *et al.* (1998) classified bovine ovaries into five classes based on presence or absence of corpus luteum and

dominant follicle. Class I had one ovary with CL and the other ovary had neither a CL nor a dominant follicle. Class II had ovaries with CL and dominant follicle. Class III had one ovary with CL but the other ovary lacked a dominant follicle. The class IV had one ovary with dominant follicle and lacked CL in the other. Class V had ovaries, which lacked both structures.

de Witt *et al.* (2000) allocated pairs of ovaries to one of the four groups based on the morphology of the corpus luteum in order to identify the relationship between ovarian phase and cumulus oocyte complex (COC) distribution and development. The CL was identified to be small and quick red in early luteal (day zero to seven) phase and the colour turned to orange or brown in late luteal phase (day 8 to 17). Ovaries were classified as follicular when CL was in regression, which was recognisable, by its pale colour and the presence of dominant follicle. The fourth group of ovaries termed as non-cyclic because of absence of CL or larger follicle (>25mm diameter) in either of the ovaries.

2.3 Ovary

2.3.1 Size and shape

The size and weight of ovary was found to alter during the oestrous cycle according to the development and regression of

Graafian follicles and the formation, development and involution of corpora lutea. (Luktuke and Rao, 1962).

El – Wishy *et al.* (1971) reported a considerable change in the size and weight of the ovaries of Egyptian buffaloes during different phases of sexual cycle. The maximum size and weight were attained when a fully developed corpus luteum (CL) was present in the ovary. Roberts (1986) observed the shape of the ovaries of cows to be oval.

Sane *et al.* (1994) observed that the size and weight of the ovary of cow varied according to the stage of the oestrous cycle, number of follicles and type of functional and regressed corpus luteum.

Guraya (1997) stated that the cattle ovaries underwent conspicuous structural changes during maturation and during different phases of the oestrous cycle.

2.3.2 Weight

Luktuke and Rao (1962) reported that the weight of buffalo ovaries varied according to the changes occurring in them

during the oestrous cycle. The weight ranged between 0.7 and 5.25g and ovary reached maximum size during 10 to 15 days after the oestrus.

El-Wishy *et al.* (1971) recorded the minimum and maximum weights of ovaries of Egyptian buffaloes to be 2.9 and 6.1g respectively. Luktuke *et al.* (1973) recorded the average weight of functional ovaries with a CL to be 4.1g and of ovaries with developing follicles but without luteal activity to be 2.4g. They suggested that the ovaries of buffalo showed conspicuous changes in size and weight as a result of follicle growth and corpus luteum formation with the ovarian cycle.

Ireland *et al.* (1979) observed that the total ovarian weight increased significantly from day one through 17 and then declined which was because of the influence of cyclic changes in the weight of the CL.

Roberts (1986) observed that the weight of cattle ovaries varied from 5 to 15g and the average weight of both ovaries in cattle of all ages was found to be 19.5g.

Chauhan and Adamu (1990) observed the mean weight of left and right ovaries of heifers and cows to be 3.80±0.12g, 4.48g and 3.53±0.10g, 5.48±0.04g respectively.

Chandrahasan (1992) obtained the mean values for the right and left ovaries of buffalo as 2.86±0.06g and 2.77±0.06g respectively. The weight of the ovaries was found to be maximum during S2 and was maintained till the end of S3 stage. S1 and S4 stage had comparatively lower values. Parmar and Mehta (1992) recorded the mean weight of the right and left ovary of Surti buffaloes to be 2.72±0.1g and 2.54±0.09g and concluded that there was no significant difference in the ovarian weights.

Sane *et al.* (1994) reported that the ovaries of cows weighed between 7 to 15g. Napolean and Quayam (1996) recorded the mean weight of the left and right ovaries of non-descript buffalo to be 2.98±0.46g and 1.75 ± 0.05g respectively.

2.3.3 Biometry

Bhalla *et al.* (1964) recorded the average dimensions for the left and right ovary to be 2.44, 2.36 (length in cm); 1.34, 1.37 (width in cm) and 1.55, 1.64 (thickness in cm). The length was

considered to be the distance from the anterior to the posterior extremity; width was considered to be the greatest distance from the medial to the lateral borders and thickness the distance from the attached to the free surface.

El-Sheikh and Hadi (1970) studied the various anatomical parameters in the Egyptian buffaloes namely the length which was the diameter between two extremities of ovary, the thickness, which was considered as the distance from attached to free border; the width which was the greatest distance between lateral and medial surfaces.

Fadle *et al.* (1974) studied ovarian dimensions during different phases of the sexual cycle in 245 nonpregnant, six to eight year old Egyptian buffaloes. The average length, breadth and thickness during proestrus were 22, 11 and 14mm; during oestrus 24, 18 and 12mm; during metestrus 23, 15 and 11mm and during diestrus was 26, 16 and 14mm respectively.

Roberts (1986) recorded that the length of the ovary of cattle varied from 1.3 to 5cm, the width varied between 1.3 to 3.2cm and thickness from 0.6 to 1.9cm.

Parkale and Hukeri (1989) reported the average ovarian length, width and thickness in buffalo to be 2.48cm, 1.67cm, 1.46cm for left and 2.44cm, 1.74cm, 1.47cm for right. Chauhan and Adamu (1990) recorded the mean dimensions of left ovary in heifers as 2.50±0.06, 1.44±0.05, 0.84±0.15cm and corresponding values for left ovary of cows to be 2.81±0.02, 1.56±1.01, 1.04±0.01cm respectively. As for the right ovaries in heifers and cows the values were 2.53±0.05 and 2.84±0.12, 1.45±0.04 and 1.63±0.02, 0.85±0.4 and 1.05±0.01cm. It was also observed that the ovaries of heifers were smaller compared to that of the cows.

The overall mean length of the left and right ovaries of buffaloes was observed to be 2.06±0.03cm and 2.0±0.04cm respectively. The values during Stage I (S1), Stage II (S2), Stage III (S3) and Stage IV (S4) of the oestrous cycle for the right and left ovaries was recorded to be 1.88±0.02, 2.27±0.04, 2.15±0.06, 1.91±0.07 and 1.89±0.02, 2.14±0.05, 1.99±0.07 and 1.89±0.09cm respectively. The length of the ovary was found to be maximum in the S2 phase of the cycle and decreased during the first stage. There was no significant variation in length between sides during different stages of the cycle. The mean height was recorded to be 1.60±0.02cm and 1.51±0.03cm for the right and left ovary. The

ovaries of second stage had maximum height though there was no significant difference between stages. The mean width of the right and left ovaries was observed to be 1.35±0.03 and 1.30±0.03cm and there was no significant variation between stages (Chandrahasan, 1992).

Parmar and Mehta (1992) recorded the mean length, height and width for right ovary to be 20.92±0.82mm, 14.76±0.75mm and 11.76±0.13mm. The same parameters had the following values in the left ovary, 19.63±0.46mm, 13.83±0.47mm and 10.74±0.58mm respectively in Surti buffaloes. The width and height of the right ovary was statistically significant but not the length of the ovary in relation to the season.

Datta *et al.* (1993) recorded the average length and width of sheep ovaries to be 1.61±1.10cm and 1.04±0.28cm respectively. Sane *et al.* (1994) reported that the cattle ovary measured about 4cm in length, 1.25cm in width and stated that the right ovary was slightly larger than the left.

Napolean and Quayam (1996) recorded the mean length, width and height of left and right ovaries as 2.24±0.04,

1.63±0.28, 1.49±0.03 and 2.22±0.04, 1.60±0.09, 1.75±0.05cm in non-descript buffalo.

2.3.4 Corpus luteum

The shape of the fully formed corpus luteum (CL) was either globular or oblong in cow (Mc Nutt, 1924). In cow, the colour of the CL was found to be yellow or orange yellow and sometimes muddy dirty yellow (Mc Nutt, 1924).

Asdell (1955) reported that the colour was first brown to brownish yellow and then gradually turned less brown. But Ireland *et al.* (1980) observed that the colour of CL changed from red to brown, tan to orange and light yellow to white during the oestrous cycle.

de Witt *et al.* (2000) identified that the corpus luteum was small and red in early luteal phase (day zero to day seven) and the colour turned to orange or brown in the late luteal phase (day 8 to 17). Later, during the follicular phase the corpus luteum was in regression, which was recognised by its pale colour and the presence of a dominant follicle.

2.4 Follicle

2.4.1 Follicular dynamics in relation to oestrous cycle

Folliculogenesis was defined as the formation of Graafian (mature preovulatory) follicle from a pool of primordial (non-growing) follicles which remained stable from birth to about fourth year of life in cattle (Erickson, 1966; Driancourt *et al.*, 1993).

At birth, mammalian ovary possessed a high follicular capital, and a part of it was used during the reproductive life span of the female. In the cow, an average of 130,000 primordial follicles was identified for a given individual. This number remained stable until about fourth year of life after which it declined (Erickson, 1966).

Choudary *et al.* (1968) obtained a mean relative proportion of 23.7 per cent normal and 76.3 per cent atretic follicles per pair of ovaries during the cycle. Normal follicles above five mm in diameter were not present during the entire luteal phase. However, they were consistently present during the follicular phase and it was concluded that there was no cyclic

change in the number of vesicular follicles up to five mm during the oestrous cycle.

Ireland *et al.* (1979) reported that there was an increase in the total number of follicles in the surface of the ovary from days one to 20. It was identified that from day one through ten, follicles within the small range decreased in size but not in number, while size of the follicle in the medium and large range remained unchanged. During each stage of the cycle, follicles in the small range (with less than 100μl follicular fluid) were observed in heifers. But follicles in the large range were found to be lowest between days one to four (30 per cent) and highest between days five and ten (88 per cent) and days 18 to 20 (73 per cent). It was inferred that different size follicles responded differently to similar stimuli and that control of turnover of follicles differed among follicles within the small, medium and large categories. It was also stated that there could be no relationship between CL and antral follicles during oestrous cycle.

Matton *et al.* (1981) observed that the mean number of small sized follicles (one to three mm) was greater on days three and eight than on days 13 and 18 of the oestrous cycle. The mean number of medium sized follicles (3 to 6mm) was greater on days

13 and 18 than in the other stages. The medium sized follicles were more numerous (4.9±0.3) at all stages studied on the corpus luteum bearing ovary (CLO) than on the non CLO (3.9±0.3). The number of large follicles (>6mm) was not affected by the day of the cycle or type of the ovary. The large number of medium sized follicles on day 13 of the cycle had resulted from the growth of the large pool of small follicles that was present earlier in the cycle.

Dailey *et al.* (1982) observed more small follicles (≤4mm diameter) on day nine than on day 12 and day 14 of the oestrous cycle. The number of follicles (≥2mm) was not affected significantly by the day of the oestrous cycle. Although the ovary bearing the CL had a great number of large follicles, no positive relationship of the corpus luteum to the diameter of the closest follicle or the distance of the largest was found. It was also reported that the number of follicles increased during the late luteal phase of the cycle.

Staigmiller and England (1982) concluded that the largest follicle on a pair of ovaries influenced the development of the remaining follicles. This effect was found to be more pronounced between days four and eight and between days eight and twelve of the cycle. The fate of single large follicle (F1), from

mid cycle was atresia and degeneration. Evidence of turnover of the largest follicle during the final segment of the follicular phase was provided along with development of a new F1 and degeneration of the prior F1.

During the prepubertal and fertile periods, the buffalo and cattle ovaries showed follicles (both normal and atretic) of variable sizes from primordial to antral follicles with large distended cavities. (Spicer and Echternkamp, 1986; Driancourt, 1991).

Spicer and Echternkamp (1986) stated that number of various sized follicles and mean size of various types of follicles had predominantly been used in cattle to assess follicular growth. It was identified that most of the large follicles (>10mm diameter) persisted on the ovarian surface for five days or more between day three and thirteen of the bovine oestrous cycle. After day 13 most of the large follicles were replaced by the new growing follicles. The rate of growth of small follicles (1 to 3mm) into larger follicle increased from day one to 18 of the oestrous cycle.

They further reported that the number of antral follicles within any specific size category or stage of growth was

found to be regulated by three inter related factors i) the rate of entry of growing preantral follicles into the pool of antral follicles, ii) the rate at which the antral follicles transformed into a large size category and iii) the rate of elimination by atresia of the follicles from a large size type into a smaller size type.

It was also stated that when the above factors remained constant, then the constant number of follicles of all sizes would be maintained in the ovaries throughout the oestrous cycle. But significant variations in the number of various size antral follicles was identified during the oestrous cycle of cattle. (Rajakoski, 1960; Marion *et al.*, 1968; Ireland *et al.*, 1979; Matton *et al.*, 1981; Staigmiller and England, 1982; Pierson and Ginther, 1987b).

Brantmeier *et al.* (1987) categorised follicles of bovine ovaries as small (<6mm), medium (6 to 10mm) and large (11 to 20mm) in order to analyse the estradiol 17β concentration. It was observed that the location of CL and stage of oestrous cycle had no interaction on follicular diameter. The number of large follicles had an effect on the rest of the follicular population. There was an interaction between the number of large follicles and follicular size.

Pierson and Ginther (1987a) stated that though there were minor cyclical variations, it was apparent that the CL could exert mild but positive intra ovarian effect. A greater number of small vesicular follicles (two to three mm diameter) were found in the ovary bearing the CL than in the contralateral ovary during the oestrous cycle and in early pregnancy.

Lonergan (1990) showed that the number of vesicular follicles in the two to six mm diameter size category did not differ significantly during any of the phases of the cycle examined.

Chandrahasan (1992) reported the mean number of follicles to be high during S4 phase (4.18±0.01) than the other stages. There was no significant difference in number between the stages of the oestrous cycle. Follicles of 3 to 5mm diameter were found to be high during S2 phase whereas the < 2mm sized follicles were more during S4 phase that gradually decreased in number during S1 and S2.

Parmar and Mehta (1992) grouped follicles of Surti buffaloes into 1 to 4, 5 to 8 and 9 to 12 and above 12mm and found that distribution of follicles of different diameter was not significantly related to the seasons. Stagewise distribution of

developing follicles exhibited significant difference between right and left ovaries especially in case of follicles above 12mm diameter. The occurrence of large number of follicles (1 to 4mm) seems to be due to their faster turnover between development and atresia.

Datta *et al.* (1993) observed the number of visible surface follicles of sheep ovaries to range between one and 28 with an average of 11.01±6.33. It was also observed that the number of visible follicles as well as the total oocytes recovered increased with the increase in length of ovaries.

Fortune (1993) showed that the development of bovine follicles occurred in the distinct striking and regular patterns. It was evident that two or three follicular waves were exhibited during an oestrous cycle and dominant follicles emerged from every wave on days two, nine and 16. Within each follicular wave there appeared to be three phases. During the recruitment phase three to six follicles from the pool of follicles < 5mm in diameter began to grow. In the selection phase one follicle gradually became larger than the others in the wave and thus was dominant. During this phase the subordinate follicles regressed and no new follicles five mm or more in diameter appeared. The dominance exhibited could be both morphological and functional. During an oestrous

cycle the dominant follicle of the final wave was the ovulatory follicle and therefore both morphologically and functionally dominant.

Machatkova *et al.* (1996) reported that the subordinate follicles were known to undergo atresia in the presence of the growing dominant follicle. This number and growth were inversely proportional to the growth of the dominant follicle present on the ovary during days seven to nine and 18 to two of the oestrous cycle.

Guraya (1997) reported that follicular dynamics in buffaloes and cattle was investigated by following four methods namely i) the follicles in the ovaries were counted ii) the follicles were counted and the steroid level in the follicular fluid was measured iii) individual follicles were marked with dye and studied iv) by real time ultrasonography of the ovaries the follicular growth and regression was observed.

Kumar *et al.* (1997) reported the average number of visible surface follicles in buffaloes to be 5.20±0.97 with mean number of 2.5, 1.2 and 0.82 and 0.62 per ovary for follicles sized 4,

8, 12 and >12 mm respectively. Also a biphasic relationship between oocyte diameter and follicle size was established.

Sarkhel *et al.* (1997) reported that the average number of observed follicles was 9.6 in caprine ovaries. Positive but non-significant correlation between size of follicles and oocytes of Grade IV, III, II without cumulus was recorded. But grade I oocytes revealed positive and significant correlation.

2.4.2 Follicular fluid

The volume of follicular fluid from follicles within small, medium and large ranges varied throughout the cycle. The mean volume of follicular fluid from follicles in medium and large range increased as the time of ovulation neared but decreased for follicles within the small range. The average volume of follicular fluid observed in small range, medium range and large range follicles was 19±0.9µl, 248±9µl and 741±40µl respectively (Ireland *et al.*, 1979).

Spicer and Echternkamp (1986) estimated the volume of follicular fluid by aspiration of the fluid into a graduated syringe

to identify a significant correlation between volume of follicular fluid and follicular diameter in cattle.

The colour of the follicular fluid in the ovaries of buffaloes was straw yellow and slightly viscous. The viscosity was found to reduce as the follicle size increased. A gradual increase in the fluid volume was observed as the diameter of the follicle increased. The mean volume recorded as per follicle size was 0.03±0.001, 0.10±0.009, 0.46±0.049ml for 3 to 5, 6 to 10 and >10mm sized follicle (Chandrahasan, 1992).

2.4.3 Effect of follicle size on oocyte number and quality

Kanagawa (1979) recovered unfertilised ova from cattle namely, small follicles (0.3 to 0.5 cm diameter) and large follicles (0.51 to 1.50 cm). There was no difference in the recovery rate between small and large sized follicles. Moreover large number of ova recovered was surrounded by cumulus cell layers.

Leibfried and First (1979) recorded that follicles of one to three mm diameter had a higher proportion of oocytes with a compact, complete investment than those from large follicles. It

was observed that 50 per cent oocyte recovery could be possible from follicles of one to 3 mm and > 3mm size.

Dalhausen *et al.* (1981) grouped follicles based on the presence or absence and quality of the surrounding cumulus matrix and characterised the prepubertal calf follicular oocytes. It was identified that follicles in < 3mm and 3 to 6mm category contained greater proportion of oocytes with compact or only slightly expanded cumulus cells compared to > 6mm category. Majority of oocytes obtained from follicles measuring > 6mm contained oocytes with fully expanded cumulus mass whereby further use in *in vitro* studies was limited.

Katska and Smorag (1984) obtained 10.22±6.17, 9.88±6.24 and 8.37±5.87 oocytes from ovaries of heifer, younger and older cows respectively that had follicles of two to six mm diameter. The recovery rate was found to be similar for all age groups and about 50 per cent of the oocytes were classified to be morphologically normal.

Brantmeier *et al.* (1987) classified follicles into three size groups; small (< 6mm), medium (6 to 10mm) and large (11 to

20mm) in cows and observed the influence of follicle size and stage of the oestrous cycle on estradiol 17β concentration in the follicles.

Pierson and Ginther (1987b) categorised follicles obtained from ovaries of heifers as 2 to 3mm, 4 to 6mm, 7 to 10mm, 11 to 13mm, >13mm. Haines and Emes (1991) demonstrated that an acceptable outcome could be achieved with oocytes returned from smaller sized follicles.

Lonergan *et al.* (1992) demonstrated a clear relationship between follicle size and oocyte quality in bovine ovaries. It was observed that significantly more oocytes with many layers of cumulus cells were obtained from follicles >6mm in diameter (70.2 per cent) compared to 2 to 6mm follicles (46.8 per cent).

Pavlok *et al.* (1992) classified follicles of bovine ovaries as Group A (4 to 8mm), group B (2 to 4mm) and group C (1 to 2mm); large, medium and small. The percent of intact cumulus oocyte complexes obtained was 40 per cent, 32.7 per cent, 34.7 per cent from 25.2 per cent, 58.3 per cent, 16.5 per cent of respective groups.

Selvaraj *et al.* (1992) recovered 81.36 to 89.87 per cent oocytes from buffalo follicles. Good quality oocytes were 44.7 per cent in 0.5cm, 28.3 per cent in < 0.5 to 0.8cm and 22.5 per cent in 0.8cm follicles. Fair quality oocytes were higher in <0.5cm and 0.5 to 0.8cm follicles than in 0.8cm.

Nebar and Threfall (1993) observed high incidence of non-degenerated investment in oocytes from follicles of >10mm size. It was also reported that as the follicle size increased the per cent cumulus investment decreased. Bruck *et al.* (1996) stated that the oocyte recovery rate was significantly higher for follicles 11 to 20mm in diameter than for follicles ≤10mm (48.7 per cent).

Das *et al.* (1996b) categorised follicles obtained from goat ovaries as ≤ 2mm (small), 2 to 5mm (medium) and large 5mm and the oocyte recovery rate was 88.6, 91.1 and 85.7 per cent. The per cent of good quality oocytes was 21.78, 36.59 and 16.0 respectively.

Sarkhel *et al.* (1997) found that the different grades of oocytes was significantly different between different follicular diameter in goats. A positive but non-significant correlation was established between follicle size and grade IV, III and II oocytes

without cumulus. Grade I oocytes revealed positive and significant correlation.

Naik *et al.* (1999) showed a recovery per cent of 52.31 from buffalo follicles. The recovery per cent as per the follicle size was 51.05 (3 to 5 mm) and 53.96 (>5mm) respectively. 39.05 per cent of good, 39.05 per cent of fair and 19.52 per cent of poor oocytes were obtained from 3 to 5 mm sized follicles. 40.44 per cent good, 47.05 per cent fair and 12.5 per cent poor quality oocytes were obtained from follicles >5mm size.

de Witt *et al.* (2000) showed a relationship between the follicle quality and cumulus oocyte complex (COC) in bovines. Follicles with a low degree of atresia contained a relatively high per cent of COC A, follicles with a high degree of atresia contained higher per cent of COC B and COC C.

2.4.4 Effect of stage of oestrous cycle on oocyte quality and number

Suss *et al.* (1988) reported that the number of oocytes aspirated had no significant correlation between their yield, the age of the animal, oestrous cycle stage or the number of small follicles visible on the ovary.

Chandrahasan (1992) obtained recovery rate of 56.30±2.01, 58.51±4.35, 57.95±7.62 and 56.55±7.63 per cent for grade I, II, III and IV respectively. There was no significant difference in the recovery rate between the stages of the cycle though the per cent of cumulus oocyte complex were more in S2 and S3 stage (66.43±8.63 and 68.01±4.23).

Takagi *et al.* (1992) observed no correlation between yield of oocytes and presence of a large CL or any other measure of the ovarian status. Moreno *et al.* (1993) observed in cow ovaries with CL to have yielded lower number of oocytes than ovaries without a CL.

Boediono *et al.* (1995) observed that the mean number of oocytes per ovary (12 vs 10) was not significantly different between the luteal and follicular phase groups in cattle ovaries. The mean number of oocytes per ovary (15 vs. 15) was not different between the CL bearing and CL non-bearing groups.

Das *et al.* (1996a) reported that the presence of a CL had no effect on the recovery of oocytes by slicing or follicular puncturing from buffalo ovaries. However ovary bearing the CL had a significantly lower mean number of oocytes following

aspiration and pooled data from the three collection methods revealed a significant decrease in the good, poor and total oocyte yield from CL bearing ovary than from non CL bearing ovary. The presence of CL did not affect the fair quality oocyte recovery.

de Witt *et al.* (2000) stated that the ovarian phase had no severe effect on the distribution of cumulus oocyte complexes. Nandi *et al.* (2000) observed that the total number of oocytes and the number of acceptable quality oocytes recovered per ovary were 1.02 and 0.60 in buffaloes. The recovery of total grade A, grade B and acceptable quality (A and B) oocytes was significantly higher in ovaries without a CL than those in which a CL was present.

2.5 Methods of recovery

To utilise efficiently the technology of *in vitro* embryo production from slaughterhouse ovaries it was necessary to develop techniques, which facilitated the recovery of large numbers of oocytes per ovary. The total number of bovine oocytes obtained per ovary varied with different recovery methods and laboratories (Carolan *et al.*, 1994).

2.5.1 Aspiration

Leibfried and First (1979) stated that aspiration was a simple collection method whereby about 30 to 60 per cent of follicular oocytes could be recovered from bovine ovaries. Katska (1984) stated that aspiration was found to be more practical and enabled relatively quick collection of a significant quantity of bovine oocytes. Balasubramanian *et al.* (1991) reported that the recovery rate of oocytes from caprine ovaries was 43.7 to 56.01 per cent.

Chauhan and Anand (1991) aspirated from 3 to 5mm antral follicles of caprine ovaries using 20G needle and syringe and allowed the aspirate to settle in centrifuge tube and further used them for *in vitro* studies. Goswami *et al.* (1992) reported that the recovery rate of 72.22 per cent was obtained by aspiration.

Naqvi *et al.* (1992) aspirated 1.57 oocytes per ovary whereas, Wahid *et al.* (1992) aspirated 4 oocytes per ovary from one to six mm diameter follicles of sheep ovaries using 19G needle and 2ml syringe and number of oocytes were 1.5 (37.9 per cent), 0.9 (23.7 per cent) and 1.5 (38.4 per cent) in grades I, II and III respectively.

The individual sheep ovaries were held between a pair of forceps and the oocyte along with the follicular fluid was sucked out using five ml syringe attached with 21G needle and 2.17 oocytes per ovary was obtained (Datta *et al.*, 1993).

Carolan *et al.* (1994) recovered bovine oocytes from ovarian follicles (2 to 6mm in diameter) by aspiration with a five ml syringe and an 18G needle primed with washing medium. About nine oocytes were recovered from each ovary by aspiration.

Dutta *et al.* (1996) made a comparative study on the number and type of prepuberal goat oocytes recovered by aspiration, dissection and slicing. The oocytes were recovered from 2 to 6mm diameter follicles by aspiration with the help of a 20G needle. This technique was found to yield less number of oocytes in Grade I and II than when compared to the other two techniques.

Barua *et al.* (1997) aspirated bovine oocytes with the help of a 20G needle and a two ml syringe to recover 1.85±0.15, 0.71±0.08, 0.58±0.07 and 0.45±0.06 of type A, B, C and D oocytes. The recovery rate of type D oocyte was significantly higher in this technique.

Sarkhel *et al.* (1997) used five ml glass syringe and 21G needle and aspirated an average of 2.54 oocytes per goat ovary. The per cent of different grades of oocyte recovered showed a lower incidence of Grade II and III but a higher incidence of Grade IV.

Baruha *et al.* (1998) recovered 3.63±0.17 oocytes by aspiration from follicles of 2 to 6mm diameter and 4.89±0.25 oocytes per ovary by dissection. Erice *et al.* (1998) reported that oocyte recovery rates (oocytes per ovary) in mares resulted in a mean of 0.92 oocytes by aspiration and 1.36 oocytes by additional slicing.

Nandi *et al.* (2000) used 20G needle and five ml glass syringe to recover a total of 1.02 oocytes and the number of acceptable quality oocytes per buffalo ovary was 0.60. Yadav *et al.* (2000) aspirated using 18G needle and five ml syringe with collection media and recovered 0.64 oocytes per buffalo ovary. It was also noticed that the rate of recovery of oocytes between right and left ovaries did not have a significant difference.

Gogoi *et al.* (2001) recovered 39.69, 9.16, 10.69 and 17.18 per cent of type A, B, C and D oocytes from goat ovaries by

aspiration. The recovery was less when compared to the dissection method adopted.

2.5.2 Puncturing

Balasubramanian *et al.* (1991) reported that the recovery rate of oocytes from caprine ovaries by puncturing was 74.4 to 87.7 per cent. Agarwal (1992) harvested oocytes from goat ovaries by puncturing the visible follicle (one to three mm diameter) and the total number of oocytes recovered per ovary ranged from 4 to 24 (average 12.4±4.6).

Pavlok *et al.* (1992) reported that on puncturing of bovine follicles they could get 25.23 per cent oocytes from large (4 to 8mm) follicles, 58.30 per cent from medium (2 to 4mm) and 16.47 per cent of oocytes from small (1 to 2mm) follicles.

Pawshe *et al.* (1994) placed the goat ovaries in a petri dish and were held with an artery forceps and the follicles visible on the surface of the ovaries were punctured with the help of 18G needle to yield 0.57±0.06 good, 0.75±0.09 fair and 0.96±0.12 poor oocytes per ovary.

The ovaries were stabilised in petri dish with forceps and the follicles visible on the surface (2 to 6mm diameter) were submerged in normal saline solution and punctured with 18G needle and the fluid was allowed to escape with gentle pressure on adjacent stroma of punctured follicle. 2.6 oocytes per ovary with 16.7 per cent of good, 34.7 per cent of fair and 48.7 per cent of poor grades of oocytes was obtained by this technique. The per cent of good quality oocytes was found to be slightly higher than when obtained by slicing or aspiration in buffaloes. (Das *et al.*, 1996a)

The surface follicles were punctured by an 18G needle and 4.14±0.41 oocytes from goat ovaries were recovered. The yield of respective quality oocytes was found to be 34.47 per cent, 26.70 per cent and 38.83 per cent of good, fair and poor. There was no significant difference in respect to the per cent of quality oocytes obtained by the two techniques adopted (Das *et al.*, 1996b).

The mean oocytes from sheep ovaries recovered was 11.13 by follicular puncture and gradewise recovery was 19.59, 30.93 and 49.48 per cent of good, fair and poor grade respectively. The number of oocytes recovered by puncturing was found to be significantly lower when compared to that recovered by slicing and dissection techniques (Das *et al.*, 1996c).

Agarwal and Verma (1997) treated caprine ovaries in Dulbecco's phosphate buffered saline containing two per cent goat serum and collagenase at the rate of 6mg/ml for 30 minutes at room temperature and then punctured with 18G needle and left for 30 minutes in the same medium; to yield 126 (10.5 oocytes/ovary) in treated and 86 (7.2 oocytes/ovary) in control groups.

2.5.3 Slicing

Arlotto *et al.* (1990) stated that slicing approximately doubled the number of oocytes released from an ovary. Individual ovaries were chopped into small pieces with a scalpel blade and recovered 7.36 oocytes per ovary of which 38.8 per cent were good quality oocytes.

Datta *et al.* (1993) observed that better quality oocyte was recovered per ovary by slicing and also yielded more oocytes per ovary (7.36). Hamano and Kuwayama (1993) observed that significantly more oocytes were recovered from cattle by slicing than aspiration (mean 63.3 vs 22.1 per cent) and proportion of rank A oocytes was also higher for slicing method (84.6 Vs 41.3).

Balakrishnan (1994) observed that slicing method of oocyte recovery yielded 7.36 oocytes per ovary in sheep. Pawshe *et al.* (1994) observed that a significantly higher number of usable oocytes were obtained by slicing (0.9±0.6) than by aspiration (0.5±0.07) in caprine ovaries.

Das *et al.* (1996a) found that slicing yielded more oocytes per buffalo ovary than puncturing or aspiration and also found that better quality was recovered per ovary by slicing. Dutta *et al.* (1996) recovered grade I oocytes 0.75±0.48 by slicing that was significantly higher than aspiration (0.25±0.25).

Slicing yielded highest number of oocytes in buffalo ovaries (Kumar *et al.*, 1997). Reddy and Shankarappa (1999) sliced ovaries into two mm thick slices using sterile sharp razor blade.

2.5.4 Post aspiration slicing

Arlotto *et al.* (1990) adopted slicing of the bovine ovaries, which were already subjected to follicular aspiration. Slicing was done with the help of razor blade in order to release

the oocytes from follicles deep in the cortex. This was found to double the number of oocytes released from an ovary.

Post aspiration slicing was attempted by Naqvi *et al.* (1992) who reported that additional slicing increased the rate of recovery of oocytes from 1.57 to 4.08 per goat ovary.

Choi *et al.* (1993) obtained a recovery rate of 1.75 to 4.14 oocytes per ovary for aspiration and post aspiration slicing respectively. Post aspiration slicing yielded an additional 3.15 and 2.40±0.40 oocytes from sheep (Balakrishnan, 1994) and caprine ovaries (Vijayakumaran, 1995) from follicles below the surface but might not be competent to undergo meiotic maturation in culture.

2.6 Classification and characterisation of oocytes

Oocyte morphology was said to change during follicular growth. Oocyte quality was evaluated based on the presence and state of cumulus cell layers as well as morphology of the oocyte cytoplasm (Rajakoski, 1960; Marion *et al.*, 1968; Katska and Smorag, 1984).

Leibfried and First (1979) developed separate classification schemes to describe the appearance of the three oocyte components (investment, ooplasm and chromatin). Numerical value of one was assigned to oocytes that had finely granulated, non polar ooplasm that filled the zona pellucida with a compact cumulus at least three cell layers thick and a germinal vesicle. Oocytes were further assigned numerical values of two, three and four depending upon the degree of degeneration.

Thompson and Cummins (1984) classified ovine oocytes into six; I-follicular oocytes with no cumulus, surrounded by corona, II-follicular oocytes with corona and expanded cumulus complete, III-follicular oocytes with dense, compact cumulus or corona, IV-oocytes with no corona but with a thick diffuse cumulus and a pale translucent ooplasm, V-naked oocytes with homogenously dense ooplasm and VI- degenerating tubal oocytes with shrunken, pale translucent ooplasm occassionally granular with clumping of organelles and vacoulization.

Xu *et al.* (1986) graded oocytes based on gross morphology and integrity of cumulus cells. No attempts were made to classify ooplasm quality of groups I, II, III due to thick layers of cumulus cells that made proper evaluation impossible.

Shioya *et al.* (1988) classified the bovine oocytes according to the character of cumulus cells as, class A oocytes having compact and dense cumulus cell layers; class B having compact but not dense cumulus cell layers. Some of the class B oocytes were partially naked oocytes while others were partially naked oocytes within cumulus cell layers or with small remnants of cumulus cells; class C oocytes consisted of naked oocytes.

The bovine oocytes were categorised according to the morphology of the surrounding cumulus cells into denuded, expanded cumulus cells, two to three layers of cumulus cells, four to five layers cumulus cells and those that had more than five layers of cumulus cells (Lonergan *et al.*, 1991).

Lonergan *et al.* (1992) observed the effect of the follicle size on the bovine oocyte morphology and embryo yield wherein oocytes were classified according to the morphology of their surrounding cumulus cells as denuded, expanded, with two to three layers, with four to five layers and with many layers of cumulus cells.

Wahid *et al.* (1992) classified ovine oocytes as 1-those with more than three layers of cumulus oophorus cells; 2- those with less than three layers and 3- denuded oocytes.

Datta *et al.* (1993) obtained oocytes with uniform cytoplasm and five to six layers of compact cumulus cells (Good), two to three layers or with fragmented cumulus mass (Average) and denuded oocytes with dark cytoplasm (Poor) from sheep ovaries.

Carolan *et al.* (1994) classified the oocytes obtained either by aspiration or dissection of follicles of bovine ovary into six categories. Type A – contained COCs with an even cytoplasm and four or more cumulus cell layers. Type B – contained COCs with an even cytoplasm and less than four or more cumulus cell layers. Type C – contained COCs with a granular cytoplasm. Type D – contained denuded oocytes with an even cytoplasm. Type E – contained denuded oocytes with a granular cytoplasm. Type F – contained oocytes with an expanded cumulus.

Das *et al.* (1996a) graded the oocytes obtained from buffalo ovaries into good, fair or poor on the basis of their gross morphology, type of cumulus investment and granularity of

ooplasm. Oocytes with more than five layers of compact cumulus cells and an evenly granulated ooplasm were classified as good. Fair oocytes were those with two to five layers of cumulus cells and an evenly granulated ooplasm. Oocytes with fewer than two layers of cumulus cells and either completely or partially denuded with a dark scattered cytoplasm were graded as poor.

Agarwal and Verma (1997) graded oocytes obtained from caprine ovaries as Good, Fair and Poor qualities. Barua *et al.* (1997) categorised bovine oocytes into Type A that had three or more complete layers, Type B with one to two complete layers, Type C which had incomplete layers of cumulus cells and Type D that had no cumulus cells (nude oocytes).

Sarkhel *et al.* (1997) graded oocytes of goat ovaries into Grade I that had complete thick, more than three layers of compact cumulus all around the zona pellucida, Grade II that had partially thick one to two layers of cells around zona pellucida, Grade III oocytes that had broken incomplete cumulus cells and Grade IV naked oocytes enclosed by zona pellucida only.

Naik *et al.* (1999) graded buffalo oocytes as Good, which had complete thick compact cumulus and ooplasm appearing

evenly granular completely filling the zona, Fair, which had partial or incomplete investment and ooplasm showing uneven granulation of black bodies but filling the zona and Poor, which had expanded or absence of cumulus and shrunken or vacuolated ooplasm.

de Witt *et al.* (2000) classified COC according to the compactness and clarity of the cumulus investment into three categories namely, COC A had a compact and bright cumulus investment; COC B had a less compact and obviously darker cumulus investment and COC C had strongly expanded cumulus investment with dark spots of degenerated cumulus cells.

Nandi *et al.* (2000) classified COC as Grade A (good) that had compact COCs with an unexpanded cumulus mass with at least four layers of cumulus cells and homogenous evenly granulated ooplasm, Grade B (fair) oocytes that had COCs with compaction and with two or three layers of cumulus cells and a homogenous evenly granular ooplasm and Grade C (poor) oocytes either partially or completely denuded or with one or two layers of cumulus cells but with an irregular and dark ooplasm.

2.7 Mineral content of follicular fluid

Murty *et al.* (1986) conducted biochemical studies on follicular fluid and secretions from genital tract in adult Murrah buffalo cows. The level of sodium, chloride and calcium in the follicular fluid during oestrous cycle was recorded to be 362±8.70 mg/dl; 470±42.51 mg/dl and 16.18±0.75 mg/dl respectively.

Wise (1987) made biochemical analysis of bovine follicular fluid in relation to follicle size, rank, atresia and day of the oestrous cycle. It was observed that follicular growth was a dynamic process in which follicular development was continuous but accelerated during the later stages of the oestrous cycle. The concentration of sodium was found to be high in growing antral than atretic follicles. Follicular potassium concentration increased as the oestrous cycle progressed.

Henderson and Cupps (1990) estimated the level of phosphatase in bovine antral follicles and observed that phosphatase level was high in healthy small antral follicles and also during early stages (1 to 12 days). Sharma *et al.* (1995) analyzed the changes in electrolytes in six categories of normally

developing antral follicles of goat. It was observed that the concentration of sodium declined (5281.63 ± 1494.53 to 4065.53 ± 781.37 μg/g) and that of potassium increased with the advancement of follicular size. (141.54 ± 56.63 to 211.87 ± 26.16 μg/g of wet weight).

Kaur *et al.* (1997) conducted the study to identify the mineral profile in the follicular fluid of buffalo ovary. The follicular fluid was collected from small (0.1 to 0.5ml), medium (0.5 to 1ml) and large follicles (1 to 1.5ml). It was observed that the concentration of copper and zinc was low in large follicles. The iron content in the follicular fluid varied significantly with the size of the follicle and it decreased significantly with the size of the follicle.

Sharma and Vats (1998) isolated the follicles from goat ovaries at different stages of development and maturation and found the concentration of iron increase upto the preovulatory follicle stage and the concentration of manganese and copper to vary minutely with the increase in follicle size.

Bordoloi *et al.* (2001) measured the concentration of macro and micro minerals in follicular fluid of small (2 to 3mm),

medium (3 to 5mm) and large (>5mm) follicles of goat ovaries. The concentration of micro minerals namely zinc, iron and copper were found to be significantly higher in medium follicles and lower in large follicles.

Materials and Methods

3. MATERIALS AND METHODS

3.1 Source of ovary

The materials for the present study comprised of normal ovaries of crossbred cows, collected randomly from Corporation Slaughter House, Trichur and from the Department of Livestock Products Technology, College of Veterinary and Animal Sciences, Mannuthy.

3.2 Collection of ovaries

The ovaries from crossbred cows of unknown reproductive status, age and body condition were collected in a thermos flask containing sterile normal saline (0.9 per cent) at 37°C. The ovaries were transported to the laboratory within one hour of slaughter.

Before further processing, the extraneous tissues surrounding the ovary were excised and then the ovaries were washed in warm (37°C) fresh normal saline.

3.3 Classification of stages of oestrous cycle

Based on the visual changes in the corpus luteum during the oestrous cycle, (Ireland *et al.*, 1980) the collected ovaries were classified into four stages.

3.3.1 Stage I days 1 to 4 (S1)

Ovaries showing new developing corpus luteum, bright red in appearance indicating a stage between ovulation and epithelial growth over the rupture point forming an apex were classified as Stage I (Plate 3.1)

3.3.2 Stage II days 5 to 10 (S2)

Ovaries with the corpus luteum that was fully formed with vasculature visible around its periphery and when bisected the apex of corpus luteum was red or brown and the remainder orange or yellow with the diameter that varied between 1.6 to 2.0cm were classified as Stage II (Plate 3.2).

3.3.3 Stage III days 11 to 17 (S3)

The vasculature was visible over the apex of corpus luteum and red or brown colour began to disappear leaving entire corpus luteum bright orange or yellow were classified as Stage III (Plate 3.3).

3.3.4 Stage IV days 18 to 20 (S4)

Ovary contained one large follicle and a regressed corpus luteum. Corpus luteum with grey or white colour and no visible vasculature on its surface was classified as Stage IV (Plate 3.4).

Plate 3.1 Ovaries with developing CL, bright red in appearance (S1 stage)

Plate 3.2 Ovaries with fully formed CL having crown-like protrusion (S2 stage)

Plate 3.3 Ovaries with bright orange or yellow coloured CL having vasculature over its apex (S3 stage)

Plate 3.4 Ovaries with well-developed follicle and regressed CL (S4 stage)

Plate 3.1

Plate 3.2

Plate 3.3

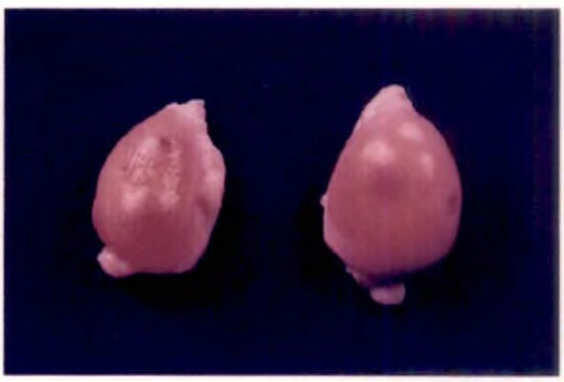

Plate 3.4

3.4 Experimental schedule

The research work was carried out as four different experiments and the parameters studied were as follows;

3.4.1 Biometrical studies (Experiment I)

The various measurements of a total of 197 ovaries were taken according to the technique followed by Luktuke and Rao (1962) and Bhalla *et al.* (1964). Weight (g) of the ovaries was recorded separately using monopan balance after trimming the extraneous tissue of each ovary. Length, width and thickness of the ovaries were measured with the help of vernier callipers.

Weight of the ovaries was recorded separately in grams.

Length (cm) was measured from the anterior to the posterior extremity at the maximum distance.

Width (cm) was measured from the medial to the lateral borders at the greatest distance.

Thickness (cm) was measured as the distance from its attached surface to its free surface.

The influence of the stage of oestrous cycle on the ovarian biometry was statistically analysed as per Snedecor and Cochran (1967).

3.4.2 Effect of the stage of oestrous cycle and follicle size on oocyte quality (Experiment II)

Normal ovaries of crossbred cows at random stage of reproductive cycle were collected from the slaughter house and were transported to the laboratory in normal saline solution. The ovaries were washed in tap water and then fresh normal saline and classified into four groups as per the stages of the oestrous cycle into Stage I, Stage II, Stage III and Stage IV.

The ovaries belonging to each stage were subjected to further study to observe the following;

3.4.2.1 Classification of follicle as per size

The follicles visible on the surface of the ovaries as transparent structures were identified and their diameter was measured using vernier callipers. The follicles were grouped into three categories namely < 4mm (small), 4 to 8mm (medium) and >8mm (large) as per method adopted by Ireland *et al.*, (1979) and Selvaraj *et al.*, (1992). These measurements were recorded separately for each ovary. Also, the corresponding number of follicle in each category was recorded.

The follicular fluid was aspirated by using 18G hypodermic needle attached to a three ml disposable syringe. The volume of follicular fluid in each follicle size was also recorded. The aspirant from each

category of follicle and stage was collected in separate centrifuge tubes with the Tyrode's Lactate (T.L.) HEPES medium (collection and washing media). The contents were allowed to settle down for 15 minutes and then the supernatant was discarded. The remaining solution was transferred to the petri dish (100x15mm square style with 13mm grid, Falcon) for screening and grading of oocytes under stereozoom microscope (25x).

A total of 166 ovaries were utilised to observe the effect of stage on the number of vesicular follicles. A sum of 235 ovaries was allotted to observe the effect of both the stage of the oestrous cycle and follicle size on the oocyte quality and number. The above effects were statistically analysed as per Snedecor and Cochran (1967).

3.4.3 Methods of recovery (Experiment III)

To study the comparative efficiency of various oocyte retrieval techniques a total of 702 ovaries were used. The various methods used for oocyte recovery were as described by Pawshe *et al.*, (1994); Das *et al.*, (1996a); Dutta *et al.*, (1996) with modifications.

3.4.3.1 Aspiration method

The ovaries were washed several times in warm water (37°C) and then with normal saline solution to remove the residual blood

clots on the surface of the ovary. At random 248 ovaries were subjected to oocyte recovery.

Ovaries were secured properly and a three ml disposable syringe attached with 18G hypodermic needle was passed into the follicle through the adjacent area of the ovarian stroma to reach the follicle from the base. The visible individual follicle on the ovarian surface was aspirated. The oocyte along with the follicular fluid was aspirated slowly. This was then collected in a sterile centrifuge tube with T.L. HEPES collection and washing media, kept at 37°C water bath. After the oocytes settled down at the bottom the supernatant was discarded and the remaining solution was transferred into a petri dish with grid (100x15mm square with 13mm grid) for screening and grading under stereomicroscope (25X), (Plate 3.5).

3.4.3.2 Puncturing method

A total of 190 randomly selected ovaries were used for this technique. The ovaries were washed several times in warm water (37°C) and then with normal saline solution to remove the residual blood clots on the surface of the ovary. The ovaries were kept in petri dish (100x15mm square with 13mm grid) with the T.L. HEPES collection media and held by means of artery forceps. The visible follicles over the ovarian surface were punctured with an 18G hypodermic needle whereby oocytes were released into the medium. This was then taken into another

petri dish with grid (100x15mm square with 13mm grid) for screening and grading stereomicroscope (25X), (Plate 3.6).

3.4.3.3 Slicing method

Randomly selected 104 ovaries were utilised for this technique. The ovaries were washed several times in warm water (37°C) and then with normal saline solution to remove the residual blood clots on the surface of the ovary. The ovaries were taken into petri dish containing 10ml of T.L.HEPES medium and secured with an artery forceps. The ovary was finely sliced with the help of a Bard-Parker blade into thin pieces. Large pieces of the ovary were removed. The media with the oocytes released in it was transferred to another petridish (100x15mm square with 13mm grid) and scanned for oocytes under stereomicroscope (25X), (Plate 3.7).

3.4.3.4 Post aspiration slicing method

Among the ovaries utilised for study of aspiration technique, 160 ovaries were subjected to post aspiration slicing technique. The slicing was similar to the one adopted to the previous slicing procedure.

The oocytes thus obtained by respective techniques were screened for grade and the number recovered in each technique was also

Plate 3.5 Aspiration method

Plate 3.6 Puncturing method

Plate 3.7 Slicing method

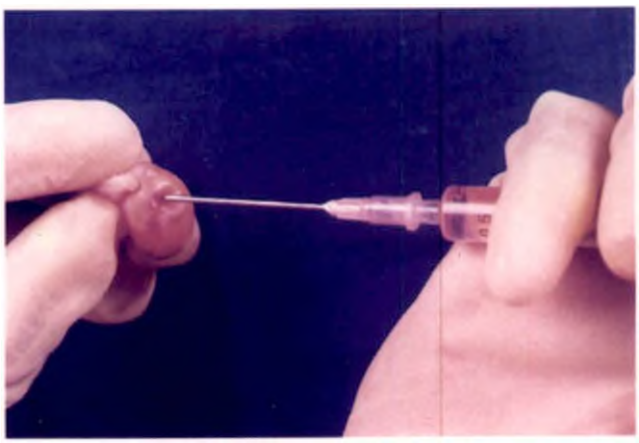

Plate 3.5

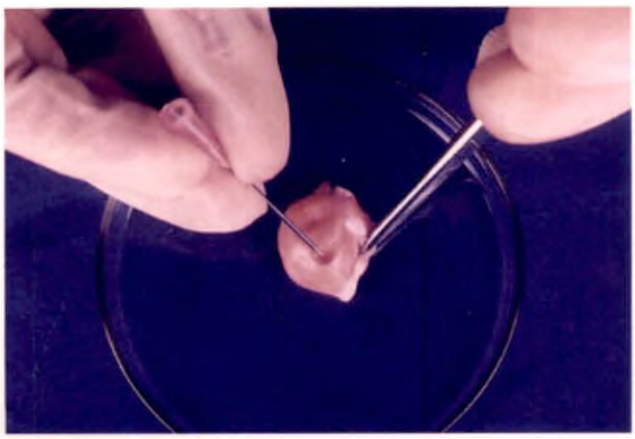

Plate 3.6

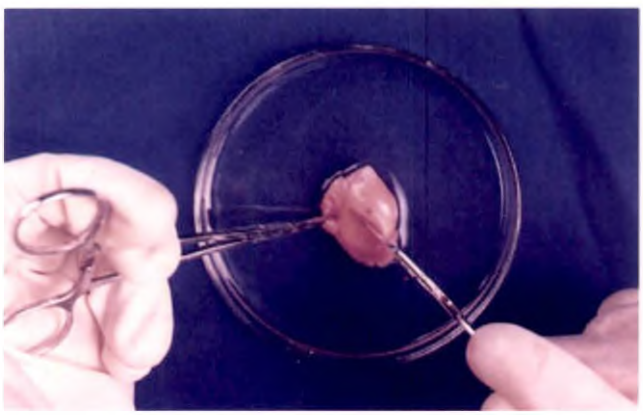

Plate 3.7

noted. The efficacy of the various collection methods in terms of oocyte recovery rate and oocyte quality was statistically analysed as per Snedecor and Cochran (1967).

3.4.4 Classification and characterisation of oocytes

The cumulus oocyte complexes retrieved from the follicles were characterised and classified according to the morphology of the oocyte (Leibfried and First, 1979; Barua *et al.*, 1997).

Grade I	Morphologically normal oocytes with more than five layers of compact cumulus cells and uniform ooplasm (Plate 3.8)
Grade II	Morphologically normal oocytes with 2 to 5 layers and uniform ooplasm (Plate 3.9)
Grade III	Morphologically normal oocytes with partial cumulus or with corona radiata only (Plate 3.10)
Grade IV	Morphologically normal oocytes without cumulus or corona radiata (Plate 3.11)

3.4.5 Estimation of mineral constituents in the follicular fluid (Experiment IV)

A total of 180 ovaries were obtained from the slaughterhouse and transported to the laboratory. The ovaries were washed thoroughly with tap water and then normal saline. The ovaries were categorised as per the stage of the oestrous cycle and also follicle size.

Plate 3.8 Bovine follicular oocytes (Grade I) (25x15x1.25)

Plate 3.9 Bovine follicular oocytes (Grade II) (25x15x1.25)

Plate 3.10 Bovine follicular oocytes (Grade III) (25x15x1.25)

Plate 3.11 Bovine follicular oocytes (Grade IV) (25x15x1.25)

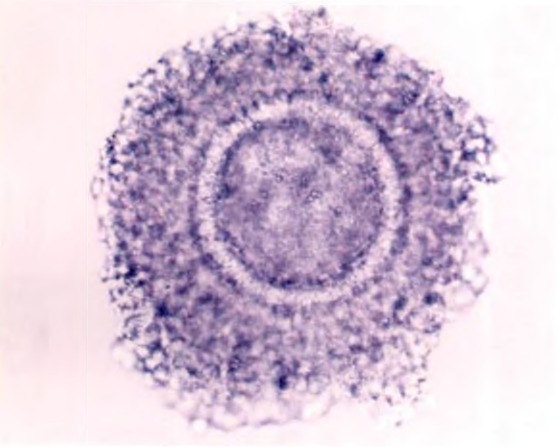

Plate 3.8

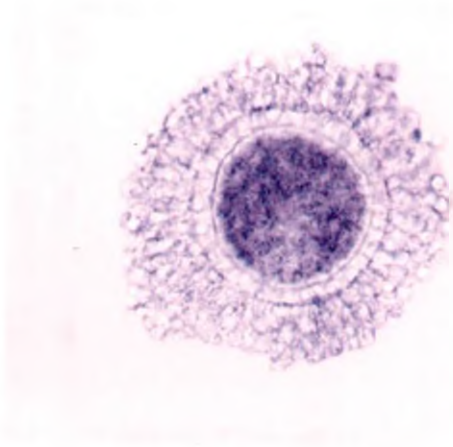

Plate 3.9

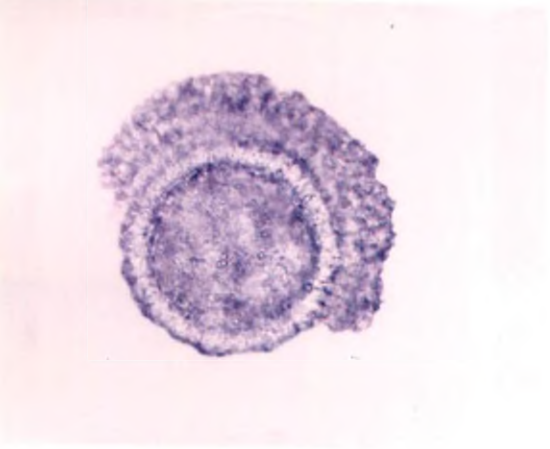

Plate 3.10

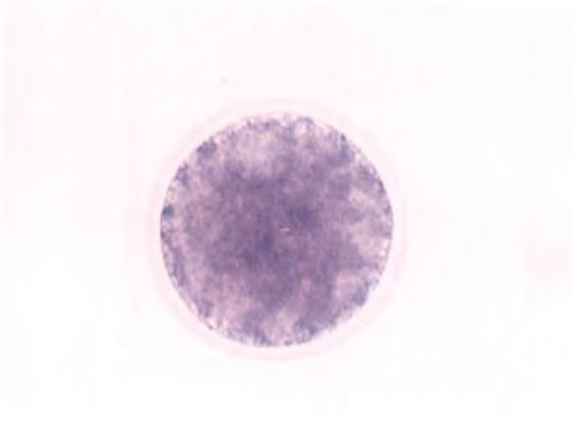

Plate 3.11

The follicular fluid was aspirated with the help of 18G hypodermic needle attached to a three ml disposable syringe. The follicular fluid collected in separate centrifuge tubes as per the follicle size and the stage of the cycle was then centrifuged at 2000 rpm for 15 minutes. The supernatant was kept at -20°C until further use.

The follicular fluid from each category of the follicle was mixed with 10 per cent Trichloro acetic acid (TCA) in the ratio of 1:10. This was then placed in water bath (90°C) for 15 minutes and then cooled. The content was then centrifuged at 3000 rpm for 30 minutes. The supernatant was separated and used for estimating concentration of the respective minerals namely iron, copper and zinc by atomic absorption spectrophotometry (Bordoloi *et al.*, 2001).

In order to estimate the level of sodium and potassium in the follicular fluid the respective samples were diluted in deionised water and then measured by flame photometry.

3.5 Composition of various solutions used in the course of the work

3.5.1. Normal Saline (Transport medium)

For collection and transport of ovaries to the laboratory, normal saline was used. Sterile solution of 0.9 per cent sodium chloride (NaCl, Merck) was prepared in millipore filtered water. This step was done fresh on each day.

3.5.2 Oocyte collection and washing media

3.5.2.1 Modified Tyrode's-Lactate Medium (T.L. Base medium)

The following were measured using electronic precision monopan balance.

Sodium chloride (NaCl)	7010mg
Potassium chloride (KCl)	240mg
Sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$)	62mg
Phenol red	10mg
Sodium lactate (CDH, Bombay)	1860µl

The above were mixed well initially with small volume of millipore water.

Magnesium chloride ($MgCl_2.6H_2O$)	100mg
Calcium chloride ($CaCl_2.6H_2O$)	300mg
Sodium bicarbonate ($NaHCO_3$)	168mg
Gentamicin (Himedia, Bombay)	1000mg were added as last constituents

The volume was made upto 1000 ml with millipore water.

The solution was then sterilised with millipore membrane filter (0.45 µm pore size) and stored at 4°C until use. All the chemicals used for the preparation of this media unless specified were from M/S Merck (India) Ltd., Bombay.

3.5.2.2 Tyrode Lactate (T.L.) HEPES Medium

T.L. Base solution	100 ml
HEPES buffer (4-2-hydroxy ethyl piperazine ethane sulfonic acid, Himedia, Bombay)	120mg was added to it.

After through mixing 300 mg of Bovine Serum Albumin fraction V (BSA), SRL, India was gently sprinkled over the surface of the solution. When BSA was completely dissolved, the pH of the solution was adjusted to 7.3 to 7.4 using sterile 1N NaOH or 1N HCl.

The media was then sterilised by filtration with a millipore membrane filter (0.22μm pore size) and kept in water bath at 37°C. T.L. HEPES media was prepared fresh on each day.

3.5.3 Water quality

Water being the single largest component of the various media used in this study, deionised triple distilled water was used for the purpose.

3.5.4 Plastic ware

Disposable sterile petri dishes, syringes, pipettes were used for this study.

3.5.5 Glassware

All glassware such as volumetric flasks, beakers, media bottles, petri dishes and measuring cylinders used for the study were cleaned thoroughly with triple distilled water and dried before being subjected for sterilization in hot air oven.

3.5.6 Metal wares

Only disposable sterile scalpel blade and needles were used for retrieval of oocytes.

Results

4. RESULTS

4.1 Ovarian biometrics

A total of 197 ovaries were classified as per the stage of the oestrous cycle in order to study the biometrics of ovaries.

4.1.1 Weight

The mean values recorded for weight of the ovaries (g) as per stage was 3.25±0.24 (S1), 3.99±0.24 (S2), 5.6±0.27 (S3), 3.65±0.27 (S4) and the overall mean value of 4.05±0.14. (Table 4.1)

The weight was maximum during third stage (S3) of the cycle and least during first (S1) stage. The weight of the ovary increased from first stage to third stage and then gradually decreased in the fourth stage (S4) of the cycle. (Table 4.1 and Figure 4.1)

On statistical analysis it was observed that ovaries belonging to S3 had a statistically significant higher value ($p<0.01$) when compared to other stages. But S2 ovaries had significantly higher value when compared to S1 though S2 was statistically similar when compared to S4.

4.1.2 Length

The average length (cm) of the ovaries as per the stage was found to be 2.32±0.06, 2.42±0.06, 2.80±0.07, 2.38±0.07 for S1, S2, S3, S4 stages with an overall mean of 2.47±0.03. (Table 4.1)

The length was maximum during third stage (S3) of the cycle and least during S1 stage. The length of the ovary increased from first stage to third stage and then gradually decreased in the fourth stage of the cycle. (Table 4.1 and Figure 4.1)

On statistical analysis it was found that length of the ovary was significantly influenced ($p<0.01$) by the late luteal phase (S3) when compared to the other stages.

4.1.3 Width

The overall mean width (cm) was observed to be 1.81±0.03 with values ranging from 1.70±0.05, 1.72±0.05, 2.16±0.06, 1.73±0.06 for the respective stages (Table 4.1).

The width was maximum during third stage (S3) of the cycle and least during S1 stage. The width of the ovary increased from first stage to third stage and then gradually decreased in the fourth stage of the cycle (Table 4.1 and Figure 4.1).

The statistical interpretation of the data revealed that ovarian width was significantly influenced ($p<0.01$) by the S3 stage.

4.1.4 Thickness

The average thickness (cm) for ovaries of respective stage was observed to be 1.32±0.05 (S1), 1.35±0.05 (S2), 1.66±0.05 (S3), 1.39±0.05 with overall mean of 1.42±0.03 (Table 4.1).

The thickness was maximum during third stage (S3) of the cycle and least during S1 stage. The thickness of the ovary increased from first stage to third stage and then gradually decreased in the fourth stage of the cycle (Table 4.1 and Figure 4.1).

The statistical analysis revealed that the thickness of the ovaries belonging to S3 stage was significantly ($p<0.01$) influenced as observed for the above two parameters.

4.2 Effect of stage of oestrous cycle on the number of vesicular follicles

A total of 105, 244, 287 and 346 follicles irrespective of their size was obtained from ovaries belonging to S1, S2, S3 and S4 stages of the oestrous cycle. Out of the total of 982 follicles; 458 belonged to < 4mm category, 430 belonged to 4 to 8mm category and 94 belonged to > 8mm category (Table 4.2) .

A high number of vesicular follicles of < 4mm size was recorded in S3 than when compared to the rest of the stages. Similarly more number of follicles belonging to 4 to 8mm and > 8mm category was observed in S3 and S4 (Table 4.2).

Statistical analysis (Table 4.2) revealed that the number of follicles belonging to <4mm and >8mm category was not influenced by the stage of the oestrous cycle as it was observed in 4 to 8mm category ($p < 0.05$).

4.3 Effect of follicle size on the oocyte quality

A total of 447, 362 and 82 oocytes were recovered from follicles belonging to < 4, 4 to 8 and > 8mm size irrespective of the stage of oestrous cycle from 166 ovaries (Table 4.3).

From < 4, 4 to 8 and > 8mm sized follicles 53.48 per cent, 35.86 per cent and 10.65 per cent of Grade I oocytes were obtained. The values recorded for Grade II was as follows 47.33, 42.01 and 10.65 and for Grade III oocytes 41.12, 53.27 and 5.60 per cent. In Grade IV the per cent of oocytes recorded as per the follicle size was found to be 48.81, 46.45 and 4.72 per cent (Table 4.4).

Follicles belonging to < 4mm size category yielded significantly more ($p < 0.01$) number of oocytes than 4 to 8

and > 8mm follicles and also the oocytes obtained were of comparatively good quality. It was also observed that 4 to 8mm sized follicles yielded comparatively more number of oocytes that was statistically significant than >8mm sized follicles (Table 4.3 and Figure 4.2).

On statistical interpretation of the data it was identified that irrespective of follicle size a statistically significant ($p < 0.01$) yield of Grade I oocytes was obtained when compared to the other grades. A combined effect of follicle size and grade on the oocytes obtained was recorded.

4.4 Effect of the stage of oestrous cycle on the number of oocytes in each follicle size

A total of 877 oocytes were recovered from 235 ovaries. The number of oocytes was observed to be more from ovaries belonging to the S4 stage than when compared to the other stages of the cycle. It was also noticed that irrespective of the stage, the number of oocytes obtained from follicles of < 4 mm diameter was high (Table 4.5).

On statistical analysis (Table 4.5) it was observed that oocytes from follicles of <4mm category was significantly influenced ($p < 0.01$) by the stage of oestrous cycle but not the oocytes obtained from the other category.

On further analysis it was noticed that S3 stage had significantly higher output of oocytes when compared to S1 and S4 (Table 4.5 and Figure 4.3).

4.5 Effect of stage of oestrous cycle on oocyte quality

A total of 888 oocytes were recovered from 56 ovaries of S1, 58 ovaries of S2, 40 ovaries of S3 and 81 ovaries of S4 respectively. The oocytes were graded as per their morphology into Grades I, II, III, IV.

On statistical analysis of the data it was observed that stage of the oestrous cycle had an influence on the grade of oocytes obtained. The different grades of oocytes was significantly ($p < 0.01$) influenced by S3 stage when compared to other stages. But S1, S2 and S4 had statistically similar effect on oocyte quality (Table 4.6 and Figure 4.4).

Statistically it was shown that oocytes belonging to Grade I had a significant difference ($p<0.01$) than the other grades (Table 4.6). It was also recorded that the stage and grade of oocyte had a significant interactive effect on the oocytes recovered.

4.6 Comparative efficiency of collection methods for oocyte retrieval

The comparative efficacy of various oocyte retrieval techniques was found to be as follows:

4.6.1 Aspiration

A total of 1031 oocytes were recovered by aspiration from 248 ovaries. 2.68 oocytes of Grade I, 0.57 oocytes of Grade II, 0.33 oocytes of Grade III and 0.57 oocytes of Grade IV and on an average 4.16 oocytes per ovary was recovered (Table 4.7).

4.6.2 Puncturing

A total of 697 oocytes were obtained by puncutring 190 ovaries. According to grades, 2.30 of Grade I, 0.58 of Grade II, 0.33 of Grade III and 0.45 of Grade IV ooctyes were recovered. An average of 3.67 oocytes per ovary was obtained by this method (Table 4.7).

4.6.3 Slicing

A total of 655 oocytes were obtained from 104 ovaries and the number of oocytes as per grade was 3.88 of Grade I, 1.22 of Grade II, 0.63 of Grade III and 0.57 of Grade IV. The overall average yield by this technique was recorded to be 6.27 (Table 4.7).

4.6.4 Post aspiration slicing

A total of 501 oocytes were obtained from 160 ovaries in this technique. The average yield as per grade was recorded to be 2.19 of Grade I, 0.46 of Grade II, 0.25 of Grade III and 0.24 of Grade IV. The overall average yield per ovary was recorded to be 3.13 (Table 4.7).

Slicing yielded a higher recovery of oocytes per ovary, (Table 4.7 and Figure 4.5) which was found to be statistically significant when, compared to other methods ($p<0.01$). 69.86 per cent of Grade I oocytes was obtained by post aspiration slicing technique when compared to the other techniques (Table 4.8).

A significantly higher ($p<0.01$) proportion of Grade I oocytes was recovered (Table 4.8) when compared to other grades irrespective of the method employed for the recovery of oocytes from the ovaries. Both the method of recovery and grade of oocyte had a significant effect on the oocyte recovery rate ($p<0.01$).

4.7 Concentration of macro and micro minerals in follicular fluid of different follicular size

The mineral profile of the follicular fluid aspirated from follicles of various sizes in 180 ovaries was recorded and presented in Table 12. The stages of the oestrous cycle had a significant influence

over the level of iron, copper, potassium in all categories of follicles but not over the level of zinc. The concentration of sodium in < 4 and 4 to 8 mm category during different stages of the cycle was significantly different.

The level of iron was high during S2 and S3 phase in 4 to 8mm follicles. The level of copper was significantly high in the ovaries belonging to S2 and S3 phase in all the categories of the follicles. The sodium content in follicles <4mm category that were present in ovary belonging to S1 phase was lower than in S2 and S3 phase of the cycle. Whereas the sodium level in 4 to 8mm category follicles present in S1 phase ovary was significantly higher than in S2 phase ovaries.

The level of potassium was highest in < 4mm sized follicle irrespective of the stage of the cycle. Irrespective of follicle size the level of iron, copper, potassium was high during S2 and S3 phases, whereas the level of Zinc was found to be high in 4 to 8mm sized follicle during S2 to S4 phases compared to the other follicle sizes and stages.

Table 4.1 Ovarian biometrics (Mean ± SE)

Stage of the oestrous cycle (days)	Number of ovaries	Weight (g)	Length (cm)	Width (cm)	Thickness (cm)
S1 (0-4)	56	3.25±0.24 ^{b}	2.32±0.06^{b}	1.70±0.05 ^{b}	1.32±0.05 ^{b}
S2 (5-10)	54	3.99±0.24 ^{bc}	2.42±0.06^{b}	1.72±0.05 ^{b}	1.35±0.05 ^{b}
S3 (11-17)	42	5.63±0.27 ^{a}	2.80±0.07^{a}	2.16±0.06 ^{a}	1.66±0.05 ^{a}
S4 (18-20)	45	3.65±0.27 ^{bc}	2.38±0.07 ^{b}	1.73±0.06 ^{b}	1.39±0.05 ^{b}
Mean		4.05±0.14	2.47±0.03	1.81±0.03	1.42±0.03
CD		0.74	0.19	0.16	0.149

Means bearing different superscript within the same column differ significantly ($p<0.01$).

Table 4.2 Effect of stage of oestrous cycle on the number of vesicular follicles

Stage of the oestrous cycle (days)	Number of ovaries	Follicle size			Total
		<4mm	4-8mm	>8mm	
S1 (0-4)	34	43 (1.26)	52 (1.53)	10 (0.29)	105 (3.08)
S2 (5-10)	43	121 (2.81)	116 (2.69)	7 (0.16)	244 (5.67)
S3 (11-17)	31	153 (4.94)	111 (3.58)	23 (0.74)	287 (9.26)
S4 (18-20)	58	141 (2.43)	151 (2.60)	54 (0.93)	346 (5.96)
Total	166	458	430	94	982
F Value		2.718^{NS}	3.58*	2.58 ^{NS}	

Figures in parenthesis denote the average values
*p (< 0.05) significance
NS- not significant

Table 4.3 Effect of follicle size on the oocyte quality

Oocyte grade	Follicle size			Total	F Value	CD
	<4mm	4-8mm	>8mm			
I	261^{aA}	175^{bA}	52^{cA}	488	13.96**	0.9
II	80^{aB}	71^{bB}	18^{cB}	169		-
III	44^{aB}	57^{bB}	6^{cB}	107		-
IV	62^{aB}	59^{bB}	6^{cB}	127		-
Total	447	362	82	891		
F Value	9.83**					
CD	0.95	0.92	-			-

** Highly significant

Values bearing different superscript (AB) within the same column differ significantly.

Values bearing different superscript (abc) within the same row differ significantly.

Table 4.4 Per cent distribution of different quality oocytes in each follicle size

Oocyte grade	Follicle size		
	<4mm	4 to 8mm	>8mm
Grade I	53.48	35.86	10.65
Grade II	47.33	42.01	10.65
Grade III	41.12	53.27	5.60
Grade IV	48.81	46.45	4.72

Table 4.5 Effect of the stage of oestrous cycle on the number of oocytes in each follicle size

Follicle size (mm)	Stage of the oestrous cycle				F Value	Total
	S1 (56)	S2 (58)	S3 (40)	S4 (81)		
0-4	123 (2.19)	105 (1.81)	99 (2.48)	111 (1.37)	27.06**	438 (7.85)
4-8	71 (1.26)	99 (1.70)	68 (1.70)	122 (1.50)	0.36NS	360 (6.16)
>8	9 (0.16)	6 (0.10)	21 (0.52)	43 (0.53)	1.28 NS	79 (1.31)
Total	203 (3.61)	210 (3.61)	188 (4.7)	276 (3.4)		877 (15.32)

Figures in parenthesis denotes the average values

** Highly significant ($p<0.01$)

NS – Not significant

Table 4.6 Effect of stage of oestrous cycle on oocyte quality

Oocyte Grade	Stage of the oestrous cycle				Total	F Value	CD
	S1	S2	S3	S4			
I	108^{aA} (1.92)	127^{aA} (2.18)	92^{aB} (2.30)	151^{aA} (1.86)	478	15.4**	0.89
II	47^{bA} (0.83)	38^{bA} (0.65)	36^{bB} (0.90)	59^{bA} (0.72)	180		-
III	18^{bA} (0.32)	28^{bA} (0.48)	22^{bB} (0.55)	39^{bA} (0.48)	107		-
IV	31^{bA} (0.55)	26^{bA} (0.44)	37^{bB} (0.92)	29^{bA} (0.35)	123		-
Total	204 (3.64)	219 (3.77)	187 (4.67)	278 (3.42)	888		
F Value	2.49**						
CD	1.29	-	-	-		-	

** Highly significant ($p < 0.01$)

Figures in parenthesis denotes the average values

Values bearing different superscript (ab) within the same column differ significantly.

Values bearing different superscript (AB) within the same row differ significantly.

Table 4.7 Comparative efficacy of oocyte retrieval methods (Mean oocytes/ovary)

Retrieval method	Mean number of oocytes per ovary as per their grade				Total
	Grade I	Grade II	Grade III	Grade IV	
Aspiration	2.68	0.57	0.33	0.57	4.16
Puncturing	2.30	0.58	0.33	0.45	3.67
Slicing	3.88	1.22	0.63	0.57	6.27
Post aspiration slicing	2.19	0.46	0.25	0.24	3.13

Table 4.8 Comparative efficacy of oocyte retrieval methods

Method of collection	Number of ovaries	Oocyte grade				Total
		I	II	III	IV	
Aspiration	248	664^{aB} (64.40)	142^{bB} (13.77)	83^{bB} (8.05)	142^{bB} (13.77)	1031
Puncturing	190	438^{aB} (62.84)	110^{bB} (15.78)	63^{bB} (9.03)	86^{bB} (12.33)	697
Slicing	104	403^{aA} (61.53)	127^{bA} (19.47)	66^{bA} (10.12)	59^{bA} (8.58)	655
Post aspiration slicing	160	350^{aB} (69.86)	73^{bB} (14.17)	40^{bB} (8.38)	38^{bB} (7.58)	501

The values bearing different superscript (ab) within the same column differ significantly.

The values bearing different superscript (AB) within the same row differ significantly.

Figures in parenthesis denotes the per cent values

Table 4.9 Concentration of macro and micro minerals during different stages of the cycle in different follicle sizes (mg%)

Stage of the cycle	Follicle size														
	<4mm					4-8mm					>8mm				
	Sodium	Potassium	Iron	Copper	Zinc	Sodium	Potassium	Iron	Copper	Zinc	Sodium	Potassium	Iron	Copper	Zinc
S1	236.92± 17.72^{a}	25.80± 0.86^{a}	2.42± 0.33^{a}	0.57± 0.05^{a}	4.02± 0.38*	400.1± 20.92^{a}	16.92± 1.03^{a}	3.79± 0.48^{a}	0.76± 0.11^{a}	3.48± 0.15*	360.49± 8.34*	17.30± 0.75^{a}	2.12± 0.23^{a}	0.67± 0.04^{a}	3.39± 0.14*
S2	353.32± 18.32^{b}	42.88± 1.75^{b}	4.43± 0.41^{b}	0.94± 0.08^{b}	3.73± 0.25*	318.8± 16.94^{b}	31.68± 1.28^{b}	4.75± 0.49^{a}	1.25± 0.07^{b}	3.81± 0.37*	351.44± 5.26*	29.76± 0.90^{b}	4.09± 0.30^{b}	1.13± 0.11^{b}	3.36± 0.14*
S3	411.66± 24.49^{c}	43.03± 1.23^{cb}	5.72± 0.69^{b}	0.83± 0.07^{bc}	4.02± 0.2*	379.8± 15.25^{a}	26.66± 0.65^{c}	4.69± 0.42^{a}	0.83± 0.08^{a}	4.17± 0.28*	347.57± 6.32*	27.90± 1.01^{bc}	4.98± 0.61^{bc}	0.86± 0.09^{a}	3.33± 0.14*
S4	245.95± 13.61^{a}	40.46± 1.46^{b}	2.61± 0.35^{a}	0.6± 0.09^{a}	3.48± 0.15*	418.7± 14.02^{a}	21.19± 1.19^{d}	2.69± 0.24^{ab}	0.66± 0.08^{a}	3.64± 0.32^{8}	340.49± 5.97*	17.73± 0.79^{a}	1.92± 0.19^{ab}	0.6± 0.05^{ac}	3.03± 0.15*

Means bearing different superscript within the same column differ significantly.

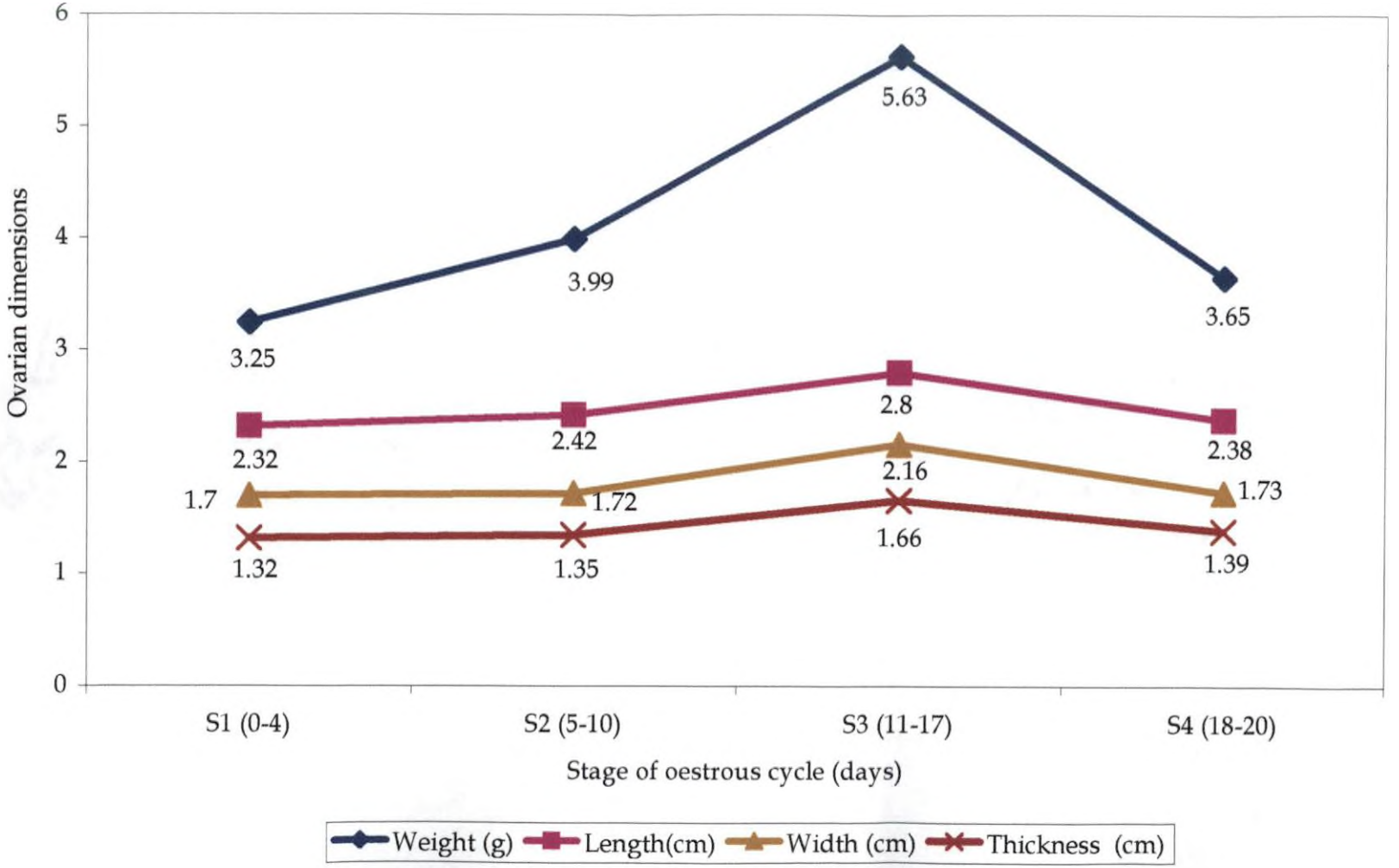

Figure 4.1 Effect of stage of the oestrous cycle on ovarian biometrics

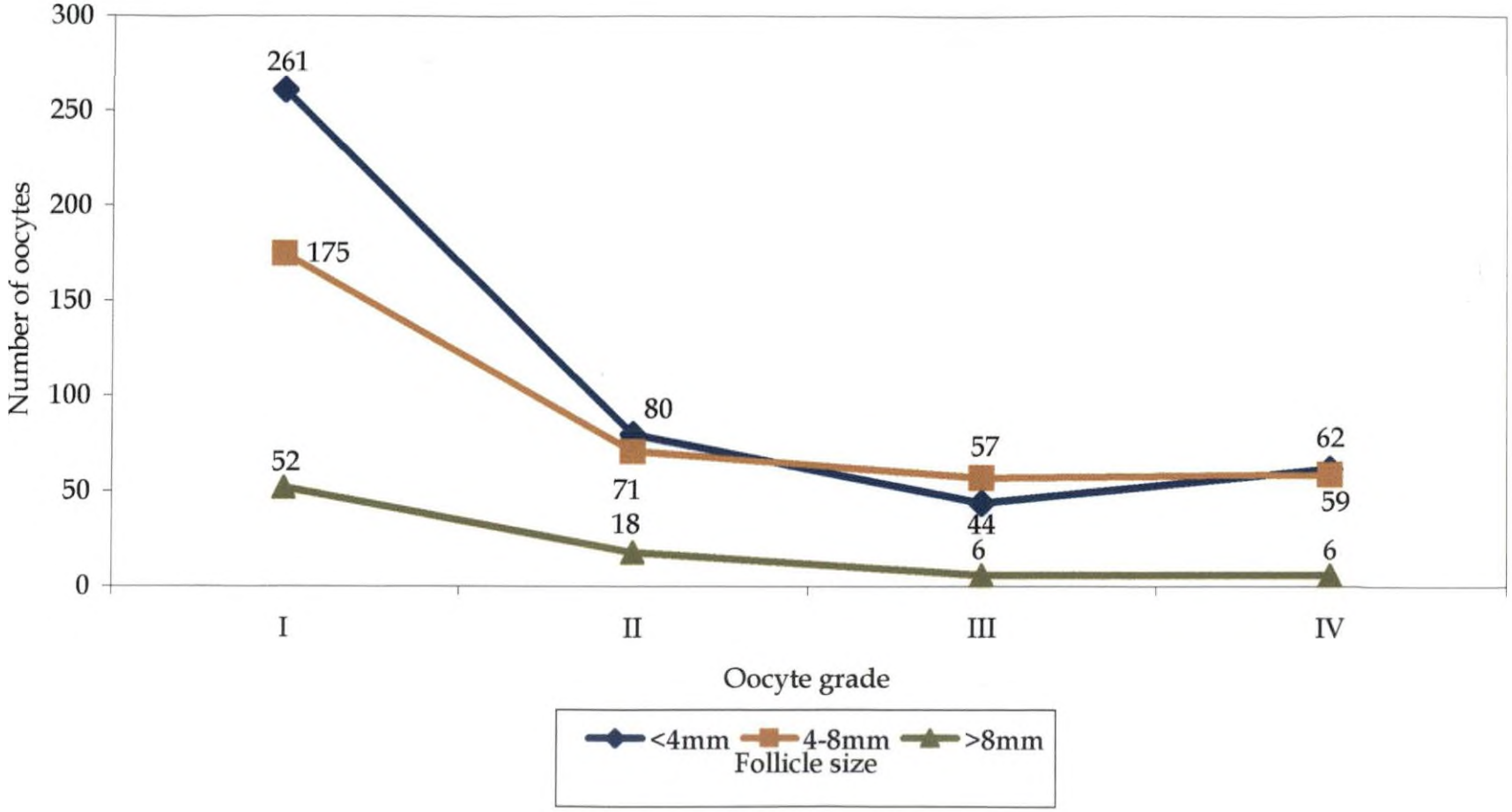

Figure 4.2 Effect of follicle size on the oocyte quality

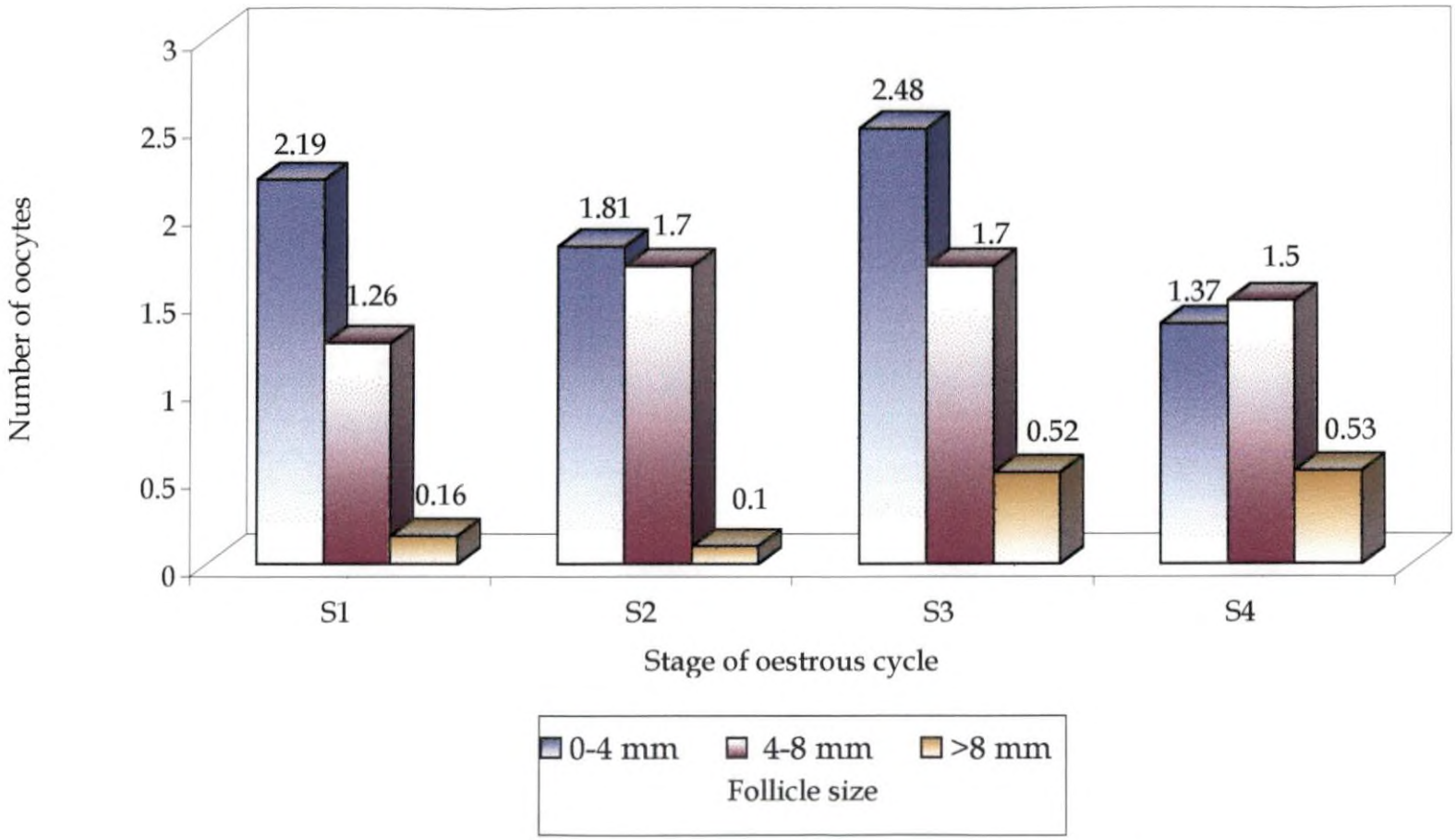

Figure 4.3 Effect of the stage of oestrous cycle on the number of oocytes in each follicle size

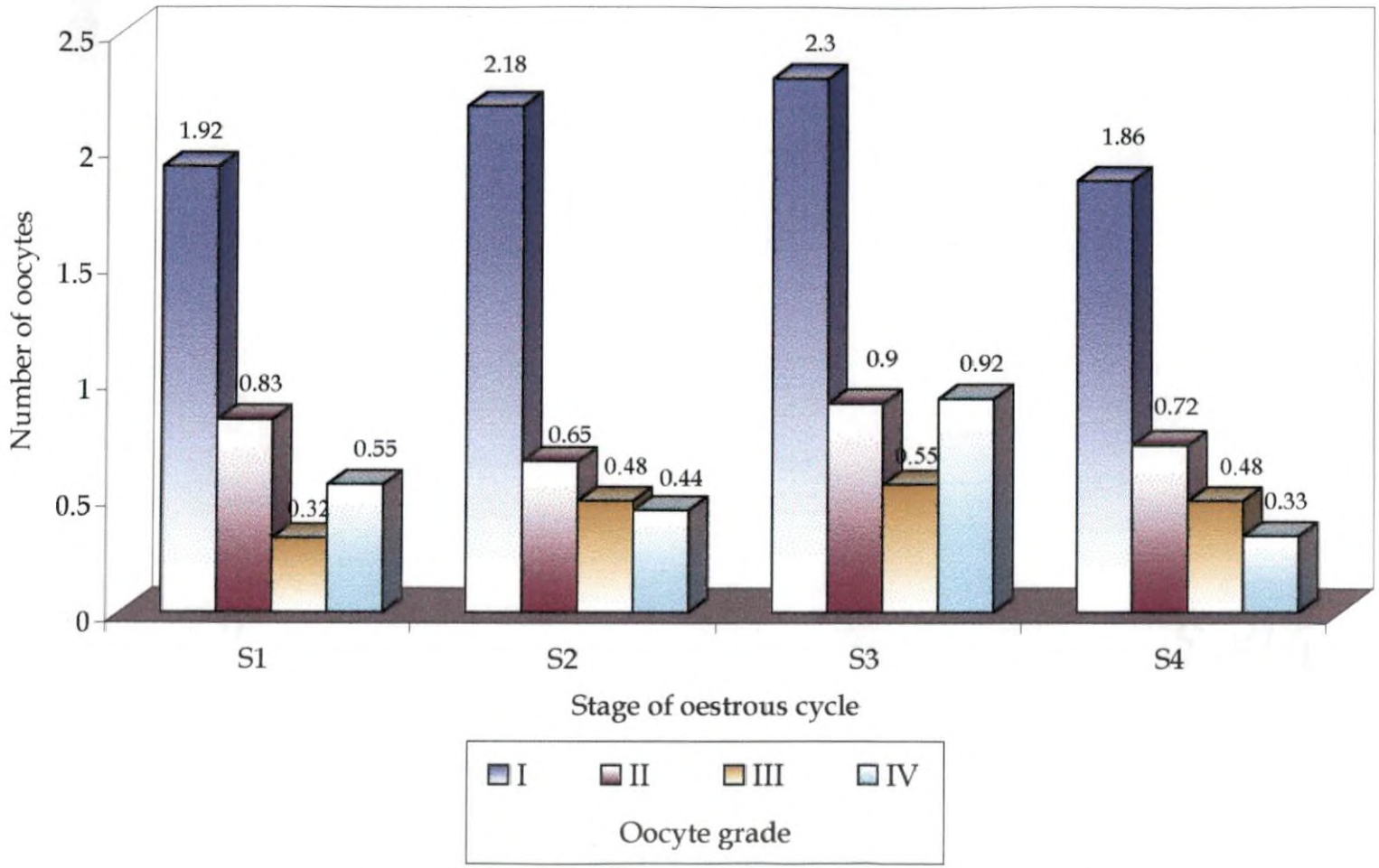

Figure 4.4 Effect of the stage fo oestrous cycle on oocyte quality

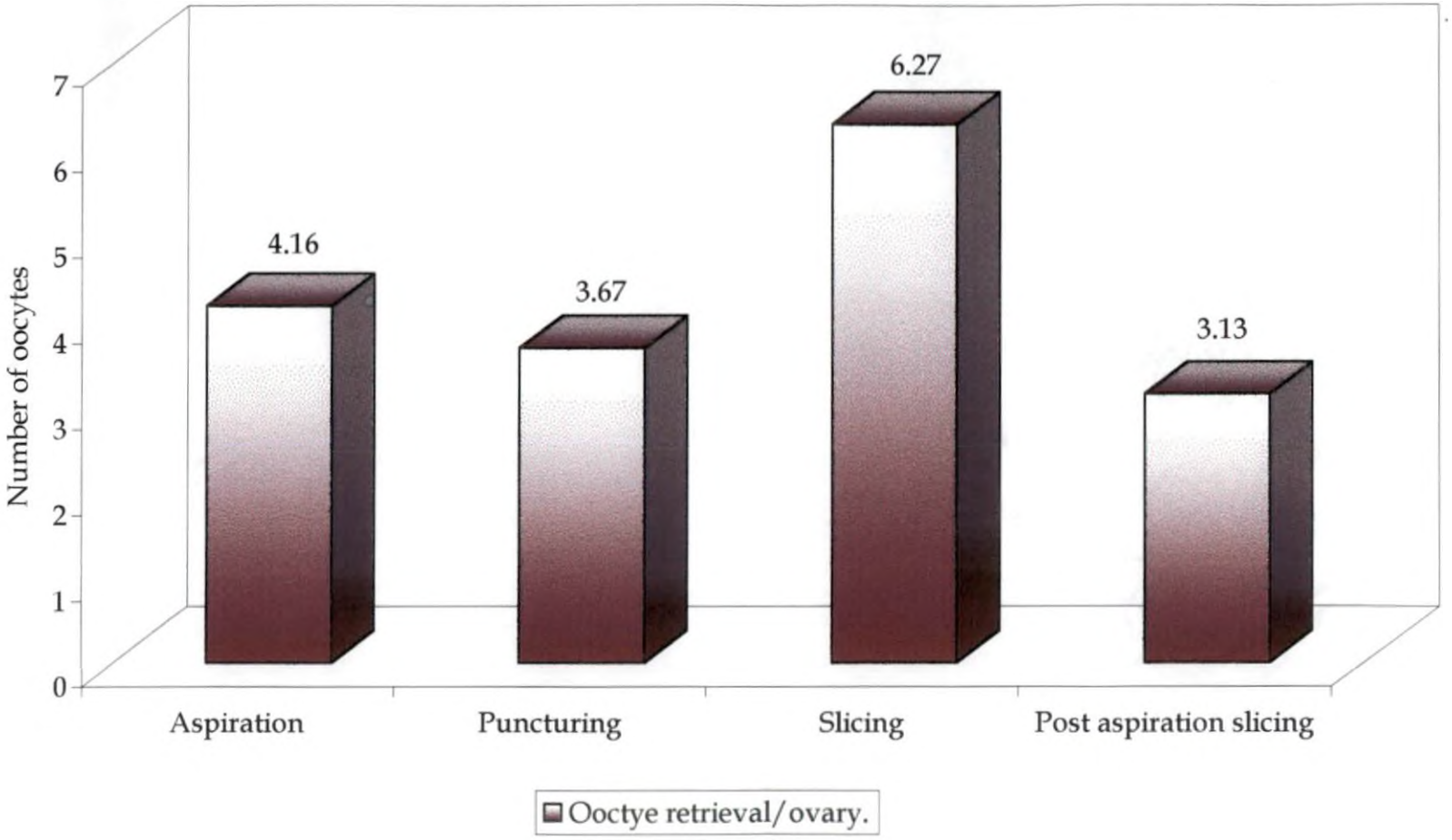

Figure 4.5 Comparative efficacy of oocyte retrieval methods

Discussion

5. DISCUSSION

5.1 Ovarian biometrics

The biometry of a total of 197 ovaries of crossbred cows collected randomly was recorded as per the stage of the oestrous cycle.

5.1.1 Weight

The mean values recorded as per the stages of the oestrous cycle in the present study was 3.25±0.24 (S1), 3.99±0.24 (S2), 5.63±0.27 (S3), 3.65±0.27 (S4) respectively with an overall mean value of 4.05±0.14 g. (Table 4.1)

The average value obtained in the present study was within the range as observed by Luktuke and Rao (1962) and El-Wishy *et al.* (1971). But the average value recorded was lower than that observed by Roberts (1986), Chauhan and Adamu (1990) and Sane *et al.* (1994) for cows.

On the other hand the average value obtained in the study was found to be higher than reported by Chandrahasan (1992),

Parmar and Mehta (1992) and Napolean and Quayam (1996) in buffaloes.

The variations in the weight of the ovary between the stages of oestrous cycle in the study confirmed the findings of Luktuke and Rao (1962) who stated that the variation was according to the changes occurring in the ovary during the oestrous cycle. Ireland *et al.* (1979) opined that the ovarian weight increased from day one to 17 and then declined because of the influence of the cyclic changes in the weight of the CL.

In the present study the ovaries belonging to S3 stage had significantly higher weight when compared to the weight in other stages. The weight of the ovaries gradually increased in S2 and S3 and then declined during S4 and S1 stage (Table 4.1 and Figure 4.1). Chandrahasan (1992) observed maximum ovarian weight during S2 that was maintained till the end of S3. This revealed the fact that during S3 stage there was a well-developed CL that increased the total weight of the ovary. The ovaries without luteal activity were found to be comparatively of lesser weight which was in accordance to Luktuke *et al.* (1973).

5.1.2 Length

The overall mean value for the length of the ovary in the present study was 2.470±0.03cm with average values as per stage of

2.32±0.06, 2.42±0.06, 2.80±0.07, 2.38±0.07 for S1, S2, S3 and S4 respectively (Table 4.1) which was comparable with the observations of Roberts (1986).

The average value obtained in the present study was found to be lower than that reported by Chauhan and Adamu (1990) and Sane (1994) in case of heifers and cows but in accordance with the findings of Bhalla *et al.* (1964), Parkale and Hukeri (1989) in buffalo. The values recorded by Parmar and Mehta (1992) and Napolean and Quayam (1996) for buffalo ovaries were found to be lower than the value obtained in this study.

Fadle *et al.* (1974) studied the ovarian biometrics during various phases of the oestrous cycle in buffaloes and reported that the average length of the ovary was maximum during dioestrus and oestrus. In the present study, the length of the ovary was maximum during S3 (Table 4.1 and Figure 4.1) and similar to that reported by Fadle *et al.* (1974) and least during S1 stage. Chandrahasan (1992) found that the length of the ovary was maximum during S2 phase and least during S1 though there was no significant variation in length at different stages of the cycle.

5.1.3 Width

The overall mean width of ovaries of crossbred cows in the study was recorded as 1.81±0.03cm with values ranging from

1.70±0.05, 1.72±0.05, 2.16±0.06, 1.73±0.06 for the respective stages (Table 4.1).

The average width recorded was found to be within the range as recorded by Roberts (1986) and was higher compared to that reported by Bhalla *et al.* (1964) in buffaloes, Parkale and Hukeri (1989) in buffaloes, Chauhan and Adamu (1990) in cattle, Chandrahasan (1992) and Parmar and Mehta (1992) in buffaloes and Sane *et al.* (1994) in cows.

Chandrahasan (1992) found that width of the buffalo ovary did not show variation in all the four stages of the cycle and there was no statistical difference between stages of the cycle. On the contrary, the present study revealed that the ovarian width increased as the stages of the cycle advanced. The width was found to be high in S3 stage, which could be attributed to the increased ovarian activity during this period (Table 4.1 and Figure 4.1).

5.1.4 Thickness

The average thickness of ovaries of crossbred cows was recorded to be 1.42±0.03cm with values of 1.32±0.05, 1.35±0.05, 1.66±0.05, 1.39±0.05 for the respective stages in the present study (Table 4.1).

The mean value obtained for the thickness of the ovary in the present study was within the range reported by Roberts (1986) and comparable to that of the findings of Bhalla *et al.* (1964), Parkale and Hukeri (1989) and Napolean and Quayam (1996). Chauhan and Adamu (1990), Parmar and Mehta (1992) and Datta *et al.* (1993) reported lower values for the thickness of ovary in heifer and cows, buffaloes and sheep.

5.2 Effect of stage of the oestrous cycle on the number of vesicular follicles

Henderson and Cupps (1990) stated that the average follicle size varied with the physiological state of the cow. Mean follicle size from ovaries containing a preovulatory follicle was reported to be statistically greater than those from ovaries containing a corpus haemorrhagicum, a developing CL and/or a mature CL.

In the present study a total of 3.08, 5.67, 9.26 and 5.96 follicles were obtained from ovaries belonging to S1, S2, S3 and S4 stages respectively (Table 4.2). Irrespective of the stage of the cycle, a total of 458, 430 and 94 follicles belonging to <4 (small), 4 to 8 (medium) and >8mm (large) category was obtained.

The results obtained in the present study suggested that the number of follicles of various size categories was not constant

throughout the oestrous cycle and also indicated constant turnover of follicles. The total number of follicles irrespective of size was recorded to be increasing as the stage of the cycle advanced and was maximum during S3 phase. This was in accordance with Ireland *et al.* (1979) who reported that the total number of follicles increased from day one to 20. Dailey *et al.* (1982) also stated that the number of follicles increased during the late luteal phase. Chandrahasan (1992) found that the total number of follicles in buffalo ovaries was less during second and third phase of the cycle as compared to the fourth phase.

In the present study, <4mm sized follicles were recorded to be more in S3 stage than that in the other stages which was contrary to Choudary *et al.* (1968) who found no cyclic changes in the number of vesicular follicles upto 5mm size during the oestrous cycle.

In the present investigation though the number of follicles in <4mm category was reported to be high in S3 followed by S2 and other stages, there was no significant influence of stage on the number. The decrease in the medium sized follicle during stages S1 and S2, could be because of the suppression of the medium sized follicle on day one to four of the oestrous cycle and its turnover into larger sized one. The dominant follicle of the first follicular wave could also have had a suppressive effect. Dailey *et al.* (1982) observed more < 4mm diameter follicles on day nine than on days 12 and 14.

Similarly, Matton *et al.* (1981) found that on day three and eight the number of follicles (1 to 3mm diameter) was high than when compared to day 13 and 18. Chandrahasan (1992) observed that the mean number of follicles with 2mm diameter decreased during S1 and was least during S2. Again there was rise in the number of <2mm follicles in S3 indicating that there might be two waves of follicular growth.

On the other hand, in the present investigation, 4 to 8mm and >8mm sized follicles were more in S3 and S4 stage. The large number of medium sized follicle on day 13 of the cycle might have resulted from growth of the large pool of small follicles present earlier in the cycle. The growth stimulus might have been the result of systemic or local intraovarian action of estrogens. Matton *et al.* (1981) found that on day 13 and 18, 3 to 6mm follicles were more and also were high in number in the CL bearing ovary and the findings in the present study was akin to this. But Lonergan *et al.* (1990) found no significant difference in the number of follicles in 2 to 6mm category during any of the phases of the cycle.

The follicles in the large range were found to be lowest between day one to four and highest between days five and ten and 18 to 20 (Ireland *et al.*, 1979). In the present study the number of large follicles was maximum during 18 to 20 days (S4 stage) followed by the other stages. This could be due to the relation of this stage to

the rapid regression of CL and growth of large follicle. The high level of oestrogen and small quantity of progesterone might be the cause of high occurrence of large follicle in this stage. Moreover, in the present study the presence of comparatively more number of large follicles during S4 phase had an effect on small follicles.

Matton *et al.* (1981) reported that the number of > 6mm follicles was not affected by day of the cycle or the type of the ovary. Brantmeier *et al.* (1987) found no interaction between location of CL and stage of the oestrous cycle on follicular diameter but found that number of large follicles had an effect on follicular population. Machatkova *et al.* (1996) reported that the number and growth of subordinate follicles was inversely proportional to the growth of dominant follicle that was present in days seven to nine and 18 to two which indicated the low number of the subordinate follicles during these days.

The difference observed could be partly due to the fact that follicular turnover occurred in a wave like pattern during oestrous cycle (two or three wave pattern) and partly due to high variability among individuals. Also the variations observed in the number of vesicular follicles of various sizes during the different stages of the oestrous cycle could be regulated by three inter related factors I) the rate of entry of growing preantral follicles into the pool of antral follicles II) the rate at which the antral follicles transformed

into large size category III) the rate of elimination by atresia of the follicle from a large size type into smaller size type.

5.3 Effect of follicle size on the oocyte quality

The oocytes from follicles of different sizes displayed a disproportionate relationship between the appearance of the investment and ooplasm and with either the pre or post culture chromatin configuration (Leibfried and First, 1979). In the present study a total of 447, 368 and 82 oocytes were recovered from follicles <4, 4 to 8 and >8mm category respectively irrespective of the stages of oestrous cycle (Table 4.3).

The number of oocytes recovered was more from <4mm which was in accordance to the findings reported by Liebfried and First (1979) in bovines, Dalhausen (1981) in prepuberal calves, Selvaraj *et al.* (1992) in buffaloes and Das *et al.* (1996b) in goats. But, Pavlok *et al.* (1992) and Bruck *et al.* (1996) obtained low proportion of intact cumulus oocyte complex from follicles of this category in bovine.

Chandrahasan (1992) recovered low proportion of intact cumulus oocyte complex from <2mm follicles compared to 3 to 5 and 6 to 10mm range in buffalo ovaries. In the present study a

comparatively similar observation was made and was also in accordance with Pavlok *et al.* (1992).

Lonergan *et al.* (1992) obtained good quality oocytes from follicles >6mm diameter. Whereas, Naik *et al.* (1999) reported that >5mm size follicles can yield higher number and good quality oocytes. In the present study >8mm follicles yielded less number of oocytes and comparatively lower quality of oocytes than when compared to other follicle sizes which might be due to the partial or fully expanded cumulus mass which was probably because of loss of connection between cumulus cells and oocyte cytoplasm.

The present study demonstrated a clear relationship between the follicle size and oocyte quality. Leibfried and First (1979) classified the follicles as one to three mm and more than three mm diameter and reported that the follicles of one to three mm diameter tend to have a high proportion of oocytes possessing a compact, complete investment and follicle more than three mm diameter have expanded cumulus cells. However in the present study follicle of <4mm diameter possessed high compact complete investment than follicles of 4 to 8mm diameter which confirm the findings of Nebar and Threfall (1993) who reported that as the follicle size increased the per cent of cumulus oocyte complex investment decreased.

5.4 Effect of the stage of oestrous cycle on the number of oocytes in each follicle size

In the present study a total of 877 oocytes were recovered of which 203, 210, 188 and 276 oocytes belonged to S1, S2, S3 and S4 respectively (Table 4.5).

The output from the third stage (S3) was significantly higher which was akin to the findings of Chandrahasan. (1992) in buffalo cows. The high number of oocytes recorded during the S3 and S2 phase (ovaries with CL) could be correlated with the increased functional activity of the surface of the ovary when functional CL was maximum in size. On the contrary Suss *et al.* (1988) stated that there was no correlation between the yield, age, oestrous cycle, stage or number of small visible follicles. Takagi *et al.* (1992) found no correlation between yield and presence of a large CL or any other measure of the ovarian status. Boediono *et al.* (1995) also found no difference in the mean number of oocytes per ovary between luteal and follicular phase. de Witt *et al.* (2000) also stated that the ovarian phase had no severe effect on the distribution over the cumulus oocyte complex. Nandi *et al.* (2000) recovered significantly high number of acceptable and total number of oocytes from the ovaries without a CL than those in which CL was present.

5.5 Effect of stage of oestrous cycle on oocyte quality

The stage of oestrous cycle was found to have an effect on the quality of oocytes obtained in the present investigation, which was in disagreement with de Witt *et al.* (2000).

The overall number of cumulus oocyte complex was high during S3 stage and S2 stage, which was in accordance with Chandrahasan (1992).

The oocyte quality was significantly influenced by the stage especially S3 in the present study than when compared to S1, S2 and S4, wherein the S3 stage had a significant output of good quality oocytes (Table 4.6 and Figure 4.4). Das *et al.* (1996a) observed that there was a decrease in the yield of good, poor and total oocytes from CL bearing ovaries than those from the non CL bearing ovaries, which could be because of the characteristic feature of the development of CL that it occupies a substantial portion of the ovary.

In the present study the variation in the number of oocytes in four stages of the cycle indicated that the hormone secreted during the oestrous cycle had an influence on the type of oocyte and it was hypothesised that the maximum production of progesterone and prostaglandin by the uterus (Lenz *et al.*, 1983) limit

the maximum diameter of the follicle and oocyte quality (Moor and Warns, 1978).

5.6 Comparative efficacy of collection methods for oocyte retrieval

5.6.1 Aspiration

In the present study the average yield by this method was 4.16 oocytes per ovary. This technique yielded 2.68 grade I, 0.57 grade II, 0.33 grade III and 0.57 grade IV oocytes (Table 4.7 and Figure 4.5).

The average value obtained was in accordance with Wahid *et al.* (1992) and comparable with Baruha *et al.* (1998). Lower values were recorded by Naqvi *et al.* (1992) and Datta *et al.* (1993) for sheep ovaries; Barua *et al.* (1997) in bovine ovaries; Sarkhel *et al.* (1997) in goats, Nandi *et al.* (2000) and Yadav *et al.* (2000) in buffaloes. Lonergan *et al.* (1991) and Carolan *et al.* (1994) recovered a high number of oocytes by this technique.

A high proportion of grade I oocytes was obtained in the present study when compared to the values reported by Wahid *et al.* (1992); Dutta *et al.* (1993); Gogoi *et al.* (2001) but comparable to the findings of Lonergan *et al.* (1992).

Barua *et al.* (1997) and Sarkhel *et al.* (1997) in cattle and goats reported a higher incidence of lower grades though it was not found so in the present study.

The higher incidence of grade I oocytes indicated tight adhering of cumulus cell layers surrounding the zona pellucida in most of the oocytes. Also aspiration of follicular oocytes and subsequent forceful expiration using needle and syringe might cause detachment of loosely adhered cumulus cells from the oocyte. Though the proportion of good quality oocytes obtained was found to be high in the present study when compared to puncturing and postaspiration slicing methods, the recovery rate was low than when compared to slicing which could be due to the difficulty in separating the cumulus cell layer from cumulus oophorus and therefore it is possible that the best oocytes for *in vitro* maturation were not recovered completely.

5.6.2 *Puncturing*

In the present study the average yield obtained by this method was 3.67 with 62.84, 15.78, 9.03 and 12.33 per cent from grade I, II, III and IV oocytes respectively (Table 4.7 and Figure 4.5).

The average value was comparable with the results obtained by Das *et al.* (1996b and 1996c) in goats and sheep. Pawshe *et al.* (1994), Vijayakumaran (1995) and Das *et al.* (1996a) reported lower values for this technique in goats and buffalo. On the contrary, Agarwal *et al.* (1992), Das *et al.* (1996) and Agarwal and Verma (1997) obtained more number of oocytes by this technique. The per cent of grade II oocytes obtained was found to be higher than the values obtained by Das *et al.* (1996a,b,c) for buffalo, goat and sheep ovaries. But the per cent of grade II, III and IV oocytes obtained was low when compared to the values obtained for sheep, buffalo and goat ovaries by Das *et al.* (1996c,a,b). The low recovery rate could be attributed to the restriction of the method to surface follicles during puncturing.

5.6.3 Slicing

This technique yielded the highest average of 6.27 and was significant statistically when compared to the other recovery methods (Table 4.7 and Figure 4.5). The average yield was similar to that reported by Datta *et al.* (1993) and Das *et al.* (1996c) in sheep and comparable to that of Arlotto *et al.* (1990).

The present study confirmed the observations of Das *et al.* (1996a) and Kumar *et al.* (1997) in buffalo ovaries wherein it was identified that slicing yielded more oocytes per ovary than the other techniques. The results of the present investigation indicated

that slicing was far more superior to the other three methods in terms of the recovery rate. Slicing of the ovary released oocytes from two sources, surface follicle and those in deeper cortical stroma, which may be the reason for high oocyte yield in the present study.

5.6.4 Post aspiration slicing

The overall mean obtained by this method was 3.13 (Table 4.7 and Figure 4.5) which was higher than reported by Vijayakumaran (1995). Naqvi *et al.* (1992) and Choi *et al.* (1993) reported comparatively higher values for this technique.

The per cent of poor quality oocyte obtained by this method was lower than that reported by Vijayakumaran (1995). The difference observed in the recovery rate might be due to the differences in the functional status of the ovaries in different age, in different breeds, seasons and/or in the procedures adopted and expertise of the person concerned.

In the present study it can be concluded that slicing technique could be an effective tool for optimum retrieval of culturable quality oocytes within a short period of time for *in vitro* maturation and *in vitro* fertilisation. The selection of ovaries containing a larger number of visible surface follicles can result in a higher yield of usable quality oocytes for *in vitro* culture. However the effect of the presence or absence of corpus luteum on the quality

and quantity of oocytes and their subsequent developmental potential through *in vitro* maturation and *in vitro* fertilisation and culture until birth of viable offspring needs to be determined.

5.7 Mineral profile of follicular fluid

In the present investigation the stage of the oestrous cycle had significant influence over the level of iron, copper, potassium in all categories of the follicles. But the concentration of zinc was not influenced by the stage of the oestrous cycle (Table 4.9). The variation in the level of iron, zinc, copper between classes of follicles was not observed to be significant.

There was significant variation in the level of sodium, potassium in follicles of various diameters. The concentration of sodium in < 4mm and 4 to 8mm category during different stage of the cycle was significantly different. There was decline in the level of sodium in large follicles compared to medium sized follicles which could be due to the less number of granulosa cells per unit volume as the follicle matures (Sharma *et al.* 1995). Kaur *et al.* (1997) observed that the concentration of sodium increased as the follicular diameter increased.

The variation in the level of potassium in different follicle sizes was in agreement with Sharma *et al.* (1995) which reflected a physiological process of follicular maturation. Also the

anoxia and intrafollicular acidosis that resulted from ischaemia before collection of follicular fluid could lead to degeneration of granulosa cells with resultant loss of intracellular potassium into the follicular fluid.

Though statistical significance was not observed in the level of zinc, copper, and iron in different categories of follicles the variations could be correlated to the physiological processes that takes place during follicular growth and could also be due to the synergistic effect of progesterone and oestrogen.

During S1 phase the level of iron, copper, sodium and potassium was found to be low in < 4mm follicles. As the stages of the oestrous cycle progressed the level of the minerals increased in the same category. This could be because of the high follicular turnover during the subsequent phases of the cycle.

The level of macro and micro minerals in 4 to 8mm category follicles during the various phases of the cycle was found to have a significant variation. The macro minerals namely sodium and potassium were found to be in high concentrations in this category in all stages, whereas the micro minerals (iron and copper) were in low levels during S1 and S4 stage but high during S2 and S3 stage of the cycle.

The level of macro minerals namely sodium was low in >8mm category follicles and was not influenced by the stage of the cycle which could be due to the less number of granulosa cells per unit volume as the follicle matures (Sharma *et al.*, 1995).

The level of micro minerals in >8mm category follicles was found to vary significantly between stages wherein the concentration was high in S2 stage but low in the S4 stage of the cycle.

The above differences in the concentration of the minerals during various phases of the cycle depicted the influence of endocrine regulation during the various phases and also when the ovaries were in active phase (luteal phase) the concentration of the minerals was more which could be because of the rich blood supply to the developing and developed CL present during these stages of the cycle.

In the present study the number of small follicles was found to be high (4.94) during the late luteal phase (S3), with a significant output of oocytes of high quality (2.30 of Grade I,) and also with high amounts of sodium, potassium, iron and copper in the follicular fluid (Table 4.9). Hence, the oocytes from the ovary of late luteal phase can be considered as the ideal choice for further utilisation in *in vitro* techniques for better results.

Summary

6. SUMMARY

Ovaries of crossbred cows collected from Corporation slaughterhouse, Thrissur and from the Department of Livestock Products Technology, College of Veterinary and Animal Sciences, Mannuthy formed the material for the present study.

The ovaries were classified into four stages according to its morphological characteristics and the appearance of the CL. The ovarian biometrics, the effect of the stage of the oestrous cycle on follicle number, oocyte number and quality and the effect of follicle size on oocyte number and quality was studied. The comparative efficacy of the various oocyte retrieval methods and the mineral profile of the follicular fluid obtained from follicles of different size categories was estimated in this study.

The mean length, width, thickness and weight of the ovaries of the crossbred cows were observed to be 2.47±0.03cm, 1.81±0.03cm, 1.42±0.03cm and 4.05±0.14g respectively with variations between the stages of the oestrous cycle. The length, width and thickness of the ovary were significantly influenced by the stage of the oestrous cycle especially the late luteal phase (S3) of the cycle ($p<0.01$). The weight of the ovaries of the crossbred cows in the

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present study was also influenced by the stage of the cycle except during the early luteal phase (S1) of the cycle ($p<0.01$).

The mean number of follicles observed during the various stages of the oestrous cycle was recorded to be 3.08, 5.67, 9.26 and 5.96. The S3 phase of the cycle had more number of vesicular follicles in <4mm diameter size than that in S4 phase. The number of follicles in 4 to 8mm and >8mm diameter range was found to be high during S3 and S4 phase of the cycle. It was also observed that the number of vesicular follicles belonging to <4mm diameter and >8mm category were not influenced by the stage of the oestrous cycle. But the number of follicles in 4 to 8mm category was significantly influenced by the stage ($p<0.05$) of the oestrous cycle.

The follicles were categorised as per their size and the effect of the follicle size on oocyte number and quality was studied. It was observed that more number of oocytes (447/891) were recovered from <4mm sized follicles which was statistically significant when compared to the recovery rate in 4 to 8mm and >8mm category follicles ($p<0.01$). Irrespective of the size of the follicle, the yield of grade I oocytes was significantly high ($p<0.01$) when compared to other grades. It was also observed that the quality of oocyte and follicle size had an interactive effect.

The oocytes recovered from ovaries classified as per stage was categorised as per their quality to study the influence of the stage of the oestrous cycle on the quality and number of oocytes obtained. The overall mean output of oocytes was found to be high in S3 stage (4.7) followed by S2 (3.61), S1 (3.61) and S4 (3.4). On statistical analysis it was found that there was a significant higher output of oocytes from S3 stage of the cycle ($p<0.01$) which substantiated the functional status of the surface of the ovary. Irrespective of the stage of the cycle, <4mm sized follicles yielded comparatively more number of oocytes.

The oocytes recovered from ovaries of respective stages were graded and categorised to study the effect of stage on its quality. The S3 phase of the cycle had comparatively higher yield and also the number of good quality oocytes was high (2.30). This was statistically significant ($p<0.01$) when compared to other stages. But the other stages (S1, S2 and S4) were statistically similar in their effect on oocyte quality. Both the stage of the oestrous cycle and the quality of oocyte obtained, had a significant interactive effect on the oocytes recovered.

The recovery of oocytes by Aspiration, Puncturing, Slicing and Post aspiration slicing was compared to study its influence on the number of oocytes and quality of oocytes.

Among the recovery methods employed, slicing was found to be effective in both yield and quality. The mean number of oocytes per ovary obtained by the slicing method was 6.27 with 61.53 per cent of grade I oocytes. The mean number of oocytes obtained by aspiration, puncturing, and post aspiration slicing was recorded to be 4.16, 3.67 and 3.13 respectively. The poor quality oocytes were found to be comparatively high when obtained by aspiration than those obtained by the other recovery methods. The method employed had a significant effect ($p<0.01$) on oocyte recovery rate. The recovery rate was also influenced by oocyte quality.

The stages of the oestrous cycle had a significant influence over the level of iron, copper, potassium in all categories of follicles but not over the level of zinc. The level of sodium in <4mm and 4 to 8mm category follicles was influenced by the stages of the cycle. When the ovaries had a functional corpus luteum the mineral content was comparatively high than in the other phases of the cycle.

Thus the oocytes from ovaries of the late luteal phase (S3) can be considered for further *in vitro* techniques, as in the present study the average number and good quality oocytes recovered during S3 phase were found to be significantly high than when compared to those recovered in the other stages of the oestrous cycle.

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CLASSIFICATION AND CHARACTERIZATION OF FOLLICULAR OOCYTES OF CROSSBRED CATTLE

By
LYDIA PRISCILLA. K.

ABSTRACT OF A THESIS

Submitted in partial fulfilment of the
requirement for the degree of

Master of Veterinary Science

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Kerala Agricultural University

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2001

ABSTRACT

The objective of the present study was to analyse the ovarian factors that would influence the type of oocyte recovered for further use in terms of *in vitro* embryo production. The normal ovaries of crossbred cows collected randomly from the slaughter house were classified into four stages Stage I (S1), Stage II (S2), Stage III (S3) and Stage IV (S4) of the oestrous cycle according to the visual appearance of CL and ovarian morphology.

The length, width, thickness and weight of the ovary were significantly influenced by the stage of the oestrous cycle and the maximum value was recorded during the S3 stage (late luteal phase) of the cycle. The average values recorded irrespective of the stage for length, width, thickness and weight of the ovary was 2.47±0.03cm, 1.81±0.03cm, 1.42±0.03cm and 4.05±0.14g respectively.

The mean number of vesicular follicles belonging to the 4 to 8mm category was significantly influenced by the stage of the oestrous cycle ($p<0.05$). But the number of <4mm and >8mm diameter follicles were not influenced by the stage of the oestrous cycle. The late luteal phase (S3) had more number of follicles in less than 4mm category whereas S3 and S4 stage had more number of follicles in 4 to 8mm and >8mm category.

The size of the follicle had an interactive effect along with the quality of oocyte obtained in each category of the follicle. The number of oocytes obtained from <4mm sized follicle was significantly high ($p<0.01$) than the number observed in the other categories. The good quality oocytes were also found to be significantly higher in number when recovered from <4mm sized follicles.

The recovery rate of oocytes as per the stage was found to have a statistical significance ($p<0.01$). There was a significantly higher output from ovaries belonging to the S3 phase than the number obtained from S1, S2 and S4 stage. Moreover, the quality of the oocyte recovered from S3 stage was significantly influenced ($p<0.01$) than when compared to other stages which were statistically similar in their effect on oocyte quality.

Slicing method yielded the maximum number of oocytes per ovary than when compared to the other techniques employed. There was significant difference in the recovery rate among the methods ($p<0.01$) with slicing yielding high proportion of oocytes both in number and quality (6.27 and 61.53 per cent of grade I). The mean number of oocytes recovered by aspiration, puncturing and post aspiration slicing was 4.16, 3.67 and 3.13 respectively. The method of recovery and the quality of oocyte recovered had an

interactive and significant effect on the oocyte recovery rate ($p<0.01$).

The level of sodium, potassium, iron and copper in the follicles of various diameters present during the different stages of the cycle in the ovaries was found to vary significantly between stages. The level of zinc was not influenced by the stages of the cycle. The variation in the concentration of the minerals was found to be related with the normal physiological changes that occurred during the different stages of the cycle.