CHARACTERIZATION AND *IN VITRO* MATURATION OF FOLLICULAR OOCYTES IN BUFFALOES (*BUBALUS BUBALIS*)

THESIS

submitted to the
Tamil Nadu Veterinary and Animal Sciences University
in partial fulfilment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in

OBSTETRICS AND GYNAECOLOGY

by

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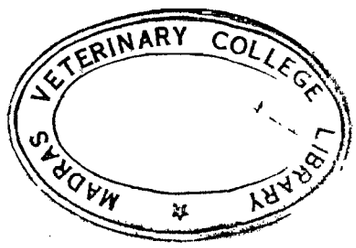

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1992

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CERTIFICATE

This is to certify that the thesis entitled CHARACTERIZATION AND *IN VITRO* MATURATION OF FOLLICULAR OOCYTES IN BUFFALOES (BUBALUS BUBALIS) *submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy (Veterinary) to the Tamil Nadu Veterinary and Animal Sciences University, Madras is a record of bonafide research work carried out by* **Thiru** C.CHANDRAHASAN *under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific popular journal or magazine.*

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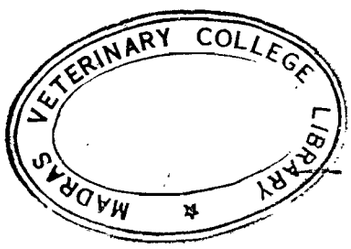

Date : 8. 2. 93

External Examiner

ACKNOWLEDGEMENTS

Words can't express the gratitude that the author owe to **Dr. J.Rajasekaran**, Ph.D. Professor, Department of Obstetrics and Gynaecology, Madras Veterinary College, Madras and Chairman advisry committee who has rendered immense help variable suggestions and criticisms at times need throughout the study.

The author thanks **Dr. R.Venkataraman**, Ph.D., Professor and Head, Department of Clinical Medicine and Therapeutics, Veterinary College and Research Institute, Namakkal, **Dr. K.Parthasarathi**, Ph.D., Professor, Department of Pathology, Madras Veterinary College and **Dr. P.Ranga Reddy**, Ph.D., Professor and Head, Department of Poultry Science, Madras Veterinary College for their valuable guidance as members of the Advisory Committee.

The author place on record his thanks to **Dr. S.L.Goswami**, Ph.D., Department of Biotechnology (Genetics), NDRI, Karnal for his useful suggestion during the course of this study.

The author is indebted to **Dr. P.Thangarasu**, Ph.D., **Dr. V.Mani**, M.Sc., for the help in carrying out the statistical analysis.

The author is thankful to **Dr. D.Kathiresan**, M.V.Sc., **Dr. A.Subramanian**, M.V.Sc., for their cooperation rendered through out the course of this study.

The author thanks to **Dr. R.Prabakaran**, Ph.D., for spending his valuable time in correcting the manuscript.

The author is extremely indebted to **Dr. Natchimuthu**, B.V.Sc., for his support rendered during the course of this study.

The author's thanks are due to **Dr. V.Ramasamy**, Ph.D. and **Dr. K.Kumanan**, Ph.D., for assisting in the cultural work.

The author extends sincere gratitude to Dean, Veterinary College and Research Institute, Namakkal for facilities provided.

The author places on record his thanks to **Dr. A.Maghalinga Nayanar**, Ph.D., for his in help taking the photomicrographs.

Last but not the least the author greatly indebted to his presents and his beloved wife without who's help and support this work couldn't have been impossible.

ABSTRACT

Title	:	**CHARACTERIZATION AND *IN VITRO* MATURATION OF FOLLICULAR OOCYTES IN BUFFALOES (BUBALUS BUBALIS)**
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Year	:	1992

By utilising the ovaries of non-descript pluriparous she buffaloes from slaughter house, the biometry of the ovary, morphology of the corpus luteum and follicles were studied. There was no significant difference in biometry of the ovary on both sides. The corpus luteum of Indian non-descriptive buffaloes were found to be small when compared to other breeds. Length, diameter and weight of the corpus luteum was found to differ significantly between four stages of the cycle.

The mean number of visible follicles on the ovarian surface did not vary between right and left ovaries. Increase in number of ≤ 2 mm diameter follicles twice in a cycle indicated two waves of follicular growth in buffalo ovaries. All the year around, there was present a constant and good number of 3-5 mm diameter follicles on the ovarian surface with a slight variation in other follicular diameter ranges and total number of follicles. The peak ovarian activity of Indian non-descriptive buffaloes in Madras was noticed during the month of November to January.

There was an increase in the amount of follicular fluid as the diameter of the follicle increased. The maximum fluid volume collected was 1.26 ml in a follicle with a diameter of 2.1 cm. The mean diameter of the buffalo follicular oocyte was 157.37 µ with a range of 130 to 161 µ which found to be constant throughout its growth period.

The oocyte recovery rate was almost equal and did not differ much between the stages of the cycle. More number of good quality oocytes were obtained in 3-5 mm follicles and in ovary containing corpus luteum.

Dissection method yielded more number of good quality oocytes than other methods. Though the percentage of maturation was low, total number of oocytes that reached metaphase II was higher in dissection method when compared with other techniques like aspiration and rupture of follicles methods.

Supplements like serum and hormones are essential for buffalo oocyte culture *in vitro*. Addition of 20 percent EBS, 10 percent FCS with 10 µg LH, 1 µg FSH and 1 µg E_2-17ß in TCM 199 were found to be ideal media for culturing good and fair quality oocytes from buffalo ovary. Nude oocytes with degenerated ooplasm completely lost its potentital to mature *in vitro*.

There was no significant difference in the maturation rate of follicular oocytes in media containing either FCS or EBS. The number of cumulus expanded oocytes appeared to reflect cytoplasmic matured oocytes only in media containing FSH, FCS or EBS. A 2 ml culutre media was found to be ideal for culturing large number of oocytes *in vitro* than 100 µl droplets.

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CHAPTER 1

INTRODUCTION

Ruminants especially the dairy cow and buffaloes play a significant role in maintaining a strong agricultural economy in India. Buffalo is a multipurpose animal reared mainly for milk, and is also used for meat production and also as a draft animal. More than half of India's milk production is obtained from buffalo and hence it plays a pivotal role in the dairy development.

Fertility records among buffaloe, however, shows lower level of reproductive efficiency with considerable influence of climatic conditions and condition faced by veterinarian in breeding programmes posing economic stress on the farmers. Available reports are suggestive of a lower number of primordial and Graafian follicular population in buffalo ovaries. Scanty folliculogenesis is also reflected in low estrogen production, poor luteal function and lower FSH responsibilities. A clear and thorough knowledge of the development, structure and function of the buffalo ovary and folliculogenesis and thus the functional anatomy is therefore necessary to optimise the reproductive efficiency of buffaloes.

The goal of genetic improvement by dairy cattle is to produce the best possible genotype which will operate at top efficiency to which it is subjected, in order to bring the greatest possible profit to the dairy man (Bath *et al.*, 1985). The biological limit for buffalo genetic improvement per annum is around 1.8 times, with present breeding methodology. The break through in embryo transfer technology can increase this genetic

progress almost four times (Nagarcenkar, 1987). This can make great thrust in modern dairying.

The success of egg transfer depends on the economical production of large numbers of high quality embryos and to achieve this increased ovulation by superovulation in pubertal and prepubertal animals were used. But the ovarian response on superovulation treatment in terms of follicular development and ovulation has been highly variable under local agro-climatic conditions. This poor superovulatory response in buffaloes could also be due to use of heterologus FSH, since no buffalo FSH is commercially available till now (Madan, 1990).

Furthermore, both recovery and fertilization rates of ovulated eggs have been observed to be low due to an hostile reproductive tract environment. It is a recognised fact that, in animal breeding, the exogenous gonadotrophins initiate the premature activation of the germinal compartment resulting in aged or abnormal oocyte that give rise to many abnormal embryos.

The other disadvantages of this technique being

i. The prohibitive cost and availability of hormones essential for superovulation,
ii. Requirements of sophisticated gadgets and
iii. Presence of highly skilled technical personnel.

Inspite of high inputs and setbacks under best conditions this technique can only produce 10 calves in a year from a single donor. Hence an alternative method of increasing the availability of eggs from cattle is the recovery of follicular oocytes directly

from the ovaries. Such oocytes would have to be matured *in vitro* for subsequent fertilization and successful development of embryos.

This modern technique of *in vitro* maturation and subsequent fertilization can be used to surpass the setbacks of the technique of embryo transfer. This technique is claimed to be a new method of embryo transfer that could reduce the cost by about 90 per cent since large numbers of matured and subsequent fertilized eggs can be obtained at a lesser cost by bypassing the critical process of superovulation. It can be evidently practised in developing countries like India.

It has been predicted that by 2000 A.D. this inovulation technique will dramatically replace artificial insemination and the existing trade in semen for artificial insemination and will give way to commercial availability of embryos and thus will form the establishment of "embryo bank" like that of semen bank.

Although birth of live offspring following embryo transfer with *in vitro* matured, fertilized ovum has been reported in several experimental animals and some domestic animals there have been few published reports of successful *in vitro* maturation and fertilization of follicular oocytes in buffaloes. Comprehending the usefulness of this technique endeavour has been made to investigate a suitable method for *in vitro* maturation of buffalo follicular oocytes with clear basic understanding of folliculogenesis in relation to climate and stage of the estrous cycle. The scope of the present work is:

i. To study the characteristics of the buffao oocyte in relation to ovarian activity and follicular development.

ii. To standardise the technique for large scale oocyte collection for *in vitro* maturation.

iii. To identify a suitable medium required for *in vitro* maturation of buffalo follicular oocytes to have a higher proportion of matured ova.

iv. To find out a simple technique to assess the nuclear maturation of *in vitro* cultured oocytes.

v. To investigate a suitable culture condition for *in vitro* maturation of buffalo follicular oocytes.

CHAPTER 2

REVIEW OF LITERATURE

2.1 BIOMETRY OF OVARIES

2.1.1 Length, width and height of the ovary

The biometry of ovary involving the mean measurements of ovarian length, width and thickness in Murrah buffalo (Polding and Lall, 1945 and Sane *et al.*, 1964), in non-descript buffalo (Damodaran, 1955; Luktuke and Rao, 1962; Bhalla *et al.*, 1964; Jokhio and Pardehi, 1986 and Napolean, 1989), in Jaffri buffalo (Sane *et al.*, 1965), in Egyptian buffalo (El-Sheikh and Abdel-Hadi, 1970), in Surti buffalo (Kodagali *et al.*, 1971), in Berai buffalo (Kaikini, 1974) and in Nili-Ravi buffalo (Khan, 1987) were stuided in detail and it was found that there was no significant difference in biometrical value of right and left ovaries.

The ovaries of Jaffri buffaloes seems to be longer and heavier than those of the other Indian breeds of buffaloes viz. 3.31, 1.44, 1.15 and 3.12, 1.49, 1.19 cm in length, width and thickness of left and right ovaries respectively (Khan, 1987). Though there was highly significant correlation between all the characters of the left and right ovaries during the four stages of oestrous cycle (Napolean, 1989), there were no studies among the literature reviewed in relation to season, eventhough summer sterility is a major cause for buffalo infertility (Sharma, 1987).

2.1.2 Size of the ovary

In the same animal, the size of the ovary varied on different days. Physiological reason for the change in size of the ovary was due to the development and regression of Graafian follicles and formation, development and regression of the corpus luteum (Zemjanis, 1962). In general, there was an increase in ovarian size as the animal became older in cow (Foley *et al.*, 1964) and in buffalo (Usmani *et al.*, 1985). Singh and Singh (1988) reported that the mean size of both the ovaries varied widely and increased with the body weight in pre-puberal buffalo heifers. He calculated the size of the ovary by using the formula: size of the ovary (m_1) = 11/6 x lengh x breadth and height in cms.

2.1.3 Weight of the ovary

Delange (1950) observed that in non pregnant cow, the weight of the ovary was ranging from 3.8 to 14.5 g. The weight difference might be related to the stage of the estrous cycle (Luktuke and Rao, 1962), age, nutrition and body weight (Foley *et al.*, 1964). Elsawaf and Schmidt (1963) reported that, in buffalo both the ovaries were active and ovary weight did not differ significantly due to season or stage of estrous cycle. Variation of 22.7 per cent in weight was found between ovaries of the same animal. Berai buffalo was found to be having the least ovarian weight (Kaikini, 1974) when compared with the Egyptian buffalo which had the heaviest ovarian weight (El-Sheikh and Abdel-Hadi, 1970).

There was no significant difference in weight of the left and right ovaries in buffaloes (Luktuke and Rao, 1962 and Khan, 1987). However, Damodaran (1955), Bhalla *et al.*, (1964), El-Sheikh and Abdel-Hadi (1970), Kodagali *et al.*, (1971), Kaikini (1974)

stated that the left ovary was heavier than right ovary. On the contrary there were some reports stating that the right ovary was heavier than the left (Sane *et al.*, 1964).

2.2 CORPUS LUTEUM

The shape of the fully formed corpus luteum may be either globular or oblong in cow (Mc Nutt, 1924); but in buffaloes, it was oval in shape, occasionally spherical and mostly buried in the ovarian stroma (El-Sheikh and Abdel-Hadi, 1970).

In cow, the colour of corpus luteum was found to be yellow or orange yellow and sometimes muddy dirty yellow (Mc Nutt, 1924). On contrary, Asdell (1955) reported that the colour was first brown to brownish yellow and gradually it turned to less brown. But Ireland *et al.*, (1980) stated that the colour of the corpus luteum was changed from red to brown, tan to orange and light yellow to white during the estrous cycle.

In buffaloes, the colour of the corpus luteum was found to be brown to pale yellow (El-Sheikh and Abdel-Hadi, 1970). Napolean (1989) studied the colour of the corpus luteum externally and on bisection found it to be bright red, red, tan or yellow, grey or white and bright red, reddish brown, tan and grey or white in four stages respectively.

The diameter of the corpus luteum in cow was observed to be 22-30 mm (Mc Nutt. 1924). The young corpus luteum was about 6-8 mm in diameter. By day 8, it increased to 18-20 mm and reached a maximum diameter of 20-25 mm when it fully matured. There was no marked reduction in size until 18-20 days. In a similar study, Dellmann and Brown (1981) reported that the corpus luteum continued to grow until the 20th day when it attained a diameter of approximately 25 mm. Ireland *et al.*, (1980)

reported that the size of the corpus luteum increased from 0.5-1.5, 1.6-2.0, 1.6-2.0 and 1 cm in the four stages of the estrous cycle respectively.

In buffaloes, the diameter of the corpus luteum were 1.2, 1.5 and 1.0 cm respectively for the early diestrous, mid diestrous and late diestrous phase (Hafez, 1955). Napolean (1989) reported that the length and diameter of the corpus luteum was 0.57, 1.45, 1.25, 1.08 and 0.63, 1.05, 0.86 and 0.85 cm respectively in four stages of the estrous cycle. In an another study, Luktuke and Rao (1962) found that the length and breadth of corpus luteum were maximum during 10-15 days of estrous cycle and reduced to 1.22 and 1.23 cm respectively during the 16-20 days of estrous cycle. El-Sheikh and Abdel-Hadi (1970) found that the average diameter of the corpus luteum was 16.85 mm.

Foley *et al.*, (1964) studied the weight of luteal tissues and observed a high relationship between the stage of the cycle and weight of the corpus luteum. The corpus luteum increased rapidly in size between day 2 and 8 and tended to remain relatively constant in weight, until regression began about day 18 to 19 then decreased rapidly in size and weight. There was also a high degree of variability among individuals and various breeds.

Ireland *et al.*, (1980) reported that the mean weight of the corpus luteum was 0.8, 3.4, 3.6 and 3g respectively during the four stages. The vasculature on the surface of the corpus luteum was not always present in stage I, but it was limited to periphery and apex in II and III stages and not visible in the IV stage. In a similar study, Dellmann and Brown (1981) found that the corpus luteum was fully developed and vascularised 9 days post ovulation.

2.3 FOLLICLE

2.3.1 Follicular dynamics in relation to

2.3.1.1 The different stages of the estrous cycle

At birth or slightly afterwards, the ovary contains a large number of primordial follicles, an average of 1,33,000 in cow (Erickson, 1966) and an average of 43,000 in sheep (Cahill, *et al.*, 1979) which were present in non growing or static condition. Through an unknown mechanism, a cohort of follicles gets recruited to grow and a single follicle is selected and becomes a dominant follicle and rupture at ovulation suppressing the growth of other medium follicles, which undergo atresia during the estrous cycle, in ewes (Turnbull *et al.*, 1977) and in heifers (Matton *et al.*, 1981; Roche and Boland, 1991).

The growth of follicle from one size to another appeared to be continuous and independent of the stage of the cycle (Choudary *et al.*, 1968). The mean number of follicles on the ovarian surface in cow was reported to be 8-10 (Moor *et al.*, 1984) and in ewe 4.3 (Dailey *et al.*, 1982). Among this 95 per cent of the follicle underwent atresia, which concurred with the study of Choudary *et al.*, (1968) who stated that the mean relative proportion of normal and atretic follicles during the estrous cycle was 23.7 and 76.3 per cent. The mean total counts for normal and atretic follicle per pair of ovaries during the estrous cycle was 93.9 and 787.9.

Marion *et al.*, (1968) have reported that the development of vesiculation began in the ovarian follicles of cow in secondary follicles of 0.5 mm diameter. Follicular growth to 1.0 mm size was rapid and however, development from 1 to 2 mm appeared to be continuous and without periods of acceleration.

During the 18-24 hrs prior to ovulation, rapid expansion of the follicle occurs. The total number of small follicles were greater on day 19 compared with day 12. But the intermediate follicle were only fewer on day 19 which are more on day 12-14 (Skyer *et al.*, 1987). The medium size non-atretic follicles were more abundant on day 0-5 and from day 9-13 of the cycle while large non-atretic follicles (>10 mm diameter) were found more on day 4-9 and 13-18 (Moor *et al.*, 1984).

Spicer and Echternkamp (1986) reviewed that the rate of growth of small follicles (1 to 3 mm) into large follicles increased as the estrous cycle progressed from day 1 to 18 (day 0 = estrous). Most large follicles of >10mm persisted on the ovarian surface for 5 days or more between day 3 and day 14 of the bovine estrous cycle. After day 13, most of these large follicles were replaced by new growing follicles. On contrary, Kruip (1982) reported that follicles of >10mm in diameter were present in all days of estrous cycle except during the first 3 days and on days 9,10, 11 and 12. On these days, follicles of <1mm and medium size were found.

For several domestic mammals, a positive relationship between luteal and follicular activity has been observed (Clark *et al.*, 1975). Increased functional activity on the surface of the ovary has been observed consistently when function of corpura lutea was maximal in heifers (Rajakoski, 1960) in women (Block, 1951) and in ewes (Brand and de Jong, 1973) and in gilts (Clark *et al.*, 1975).

Further, Driancourt (1991) stated that the number of growing follicles were found to be greater in luteal phase (1.6) than in the follicular phase (1:2). Normal follicles larger than 5 mm were not present in ovaries obtained during the luteal phase of the cycle;

however, atretic follicles larger than 5 mm were present in ovaries during the entire cycle (Choundary *et al.*, 1968).

In Egyptian buffaloes, the largest size of follicles vary from 0.5 to 0.7 cm and 0.8 to 1.3 cm in ovaries with corpus luteum and without functional corpus luteum respectively (Hafez, 1965). Whereas El-Seikh and Abdel-Hadi (1970) recorded the larger diameter of the Graafian follicle to be 18 mm, El-Wishy *et al.*, (1988) reported that large follicles of 10-20 mm were found in the ovaries during the follicular phase of the cycle. They further stated that the mean diameter of the largest follicle during this phase was 13 $\pm$ 3 mm. During metestrous and diestrous phase, the mean diameter of the largest follicles was 8 $\pm$ 3 and 11 $\pm$ 3.6 mm respectively.

In ewes, more number of small follicles were found on day 9 whereas the number of follicles and its diameter were least during the luteal phase. While studying the effect of luteal cells on follicular development in ewes, Dufour *et al.*, (1971), Fogwell *et al.*, (1977) have found that the ovary havings corpus luteum had more large follicles than the ovary bearing the corpus albicans. Further he reported that greater development of follicles occurred in the ovary with the corpus luteum.

2.3.1.2 The different months in a year

Hafez (1955), observed that majority of buffaloes remain in anoestrous condition during the summer months. High environmental temperature and lack of feed may restrict sexual activity during some months of the year in the tropics, but shortly after the onset of rainy season, sexual activity increases, probably due to a change in feeding activity (Jainudeen and Hafez, 1987). The fertility therefore is minimum and it is referred to as

summer infertility in buffaloes which remains from April to June, when the environmental temperature reaches as high as 42-46°C (Sharma, 1987).

Majeed *et al.*, (1961) and Hossain and Ahmed (1971) reported that during summer, characterized by high temperature, the reproductive efficiency of buffalo species is severly hampered. Hafez (1955) reported that the Egyptian buffaloes remained completely anoestrus during hot weather (April-July). In an another study, Hossain and Ahmed (1971) reported that only 2 per cent of buffaloes were joined in heat during summer in Bangladesh. But during autumn and winter when day length and ambient temperature decline, there is enhanced reproductive activity in buffaloes (Hafez, 1955). In a similar study, Majeed *et al.*, (1961) reported that with decline in day-length and ambient temperature, the buffaloes became sexually active.

Kodagali *et al.*, (1973) observed sexual activity in Indian buffaloes to be at its peak during the period from September to February when 69.62 per cent of all the females were served. The highest incidence of estrous was recorded during October and November (Afiefy, 1967). Hossain and Ahmed (1971) also reported that most of the estrous period (66.0 per cent) in buffaloes occurred during the month of October to January.

2.3.2 Follicular contents

2.3.2.1 Follicular fluid

Follicular fluid was a slightly viscus straw coloured solution with pH above 7.0 (Lutwax-Mann, 1954). As the follicle approached pre ovulatory stage, the consistency of the follicular fluid changes more viscus (Bedirian and Baker, 1975). On contrary, Byskov

(1969) stated that the follicular fluid to be more viscous and metachromatic in growing, than in fully grown preovulatory follicle of hamsters and mice.

In cow the mean volume of the follicular fluid collected from large follicles was 1.0 ml and then greatest volume was 2.2 ml (Henderson *et al.*, 1982).

Since follicles can be located at various depths in the ovarian stroma, reliable measurements of the diameter of antral follicles are not obtained unless follicles are removed from the ovary. Therefore, the relationship between diameter and fluid volume was examined by Spicer and Echternkamp (1986) who found that both were significantly correlated with each other. A similar curvilinear realtionship was obtained between diameter and volume in human follicles (Mc Natty *et al.*, 1979) and in ovine follicles (England *et al.*, 1981). This relationship between diameter and volume was analysed and it was reported that the follicular volume (V_{ml}) could be related to follicular diamter (D_{mm}) by the equation $V = 0.3xD^3$ (Henderson *et al.*, 1982).

Napolean (1989) studied the volume of follicular fluid in different size of follicles and found that the volume increased as the diameter increased and at each range of diameter, the increase being from 0.026 ± 0.002 to 0.155 ± 0.008 in follicles of 2.1 - 4.0 to more than 10 mm in diameter respectively.

2.3.2.2 Diameter of the oocyte

Pederson and Peters (1968) classified oocyte into (a) the small oocyte, a cell with a diameter of less than 20 μ, (b) the growing oocyte, a cell which had begun to grow but not reached its final size yet (diameter between 20 and 70 μ) and (c) the large oocyte, a cell which had reached its final size (diameter 70 μ).

El-Sheikh and Abdel-Hadi (1970) found that in buffalo the average diameter of the primary follicle was 35-48 μ and of the ovum was 21-28 μ. The average diameter of the secondary follicle and the ovum inside were 43-60 and 24-38 μ respectively. It appeared that the ovum of the primary follicle increased in size, reaching a diameter of 145 μ in the 8-celled fertilized ovum. The average diameter of the corresponding ovum including the zona pellucida was 160 μ.

In juvenile female, maximum oocyte size was much smaller than that in prepubertal or adult female in mouse (Szybek, 1972; Sorensen and Wassarman, 1976), in hamster (Iwamatsu and Yanagimachi, 1975) and in man (Lintern-Moore *et al.*, 1974). Linder *et al.*, (1980) has reported that the oocyte completed their I meiotic division when they attained the diameter of 77-78 μm.

Sato *et al.*, (1990) reported that in bovines, oocytes < 90 μm diameter did not resume meiosis. However, germinal vesicle breakdown was observed in oocytes whose diamter exceeded 91 μ. Polar body formation was observed in oocytes with diameter exceeding 101 μm. About 80 per cent of the oocytes with diameter ≥ 121μm were able to extrude the polar body.

2.3.2.3 Oocyte recovery rate

Asakawa *et al.*, (1982) studied the percentage of oocytes recovery in different classes of follicles at 37 and 47h post hCG treatment in squirrel monkey. It showed that the percentage of oocytes recovery at 37 h and 47 h in >3, 2-3, 1-2 and < 1 mm diameter follicles were 33.3, 43.0, 29.7 and 20.8 and 43.3, 36.0, 31.9 and 24.6 respectively.

In cow the oocytes recovery rate was 66 per cent (Mc Natty *et al.*, 1984) and 65 per cent (Leibfried-Rutledge, *et al.*, 1987) by aspiration of follicles of ≥2mm diameter. Schnurrbusch *et al.*, (1990) studied the recovery rate during early or late diestrous, pro-estrous or early; mid or late estrous in pigs and the recovery rate was 70.0, 75.5, 43.5, 65.4, 78.0 and 92.1 per cent respectively.

2.3.3 Characterization of the oocytes in relation to

Size of the follicle and stage of the cycle greatly influenced the characterization of the oocyte since oocytes remain dependent on the follicle almost upto the moment of follicle rupture.

2.3.3.1 Size of the follicle

The first healthy follicle was about 3 mm in cattle (Rajakoski, 1960) and 2 mm in sheep (Driancourt *et al.*, 1990). Follicles with the diameter of 3-5 mm yielded more (81 per cent) oocytes with germinal vesicle stage, while follicles > 10 mm diameter yielded more degenerated oocytes (Bedirian and Baker, 1975). In a similar study Dahlhausen *et al.*, (1981) reported that the follicles with <3 and 3-6 mm diameters contained a greater proportion of oocytes with compact or only slightly expanded cumulus cells (42/48 and 31/36 respectively) as compared with follicles in the >6mm category (4/12). In contrast, Leibfried and First (1979) stated that a high proportion of non degenerated investments was found in follicles of 1-3 mm in size; but the incidence of intact chromatin was greater in follicles of >3mm in size.

2.3.3.2 Stage of the cycle

Moor *et al.*, (1984) studied the state of the oocytes at different cyclic stages and in different follicular classes. All oocytes in non atretic follicles and almost all follicles undergoing early atresia remained in the normal germinal vesicle stage; with the progression of atresia, the percentage of oocytes showing degenerative changes increased. Leibfried and First (1979) compared the chromatin nature to the stage of estrous cycle and concluded that there was a greater incidence of intact chromatin present in the follicular stage.

In a similar study, Schnurrbusch et al.,(1990) stated that the rate of oocyte degeneration was high (50 per cent) in late diestrous than other stages and least in late estrous period.

2.4 ISOLATION OF OOCYTES AND ITS PERCENTAGE OF RECOVERY AND MATURATION RATE

Though the ovary contained hundreds of antral follicles, only 8-10 follicles of 2 mm diameter were visible on the ovarian surface in cow (Moor *et al.*, 1984). Most of the follicles were deep in the cortex. The mean s.e.m. number of 2mm diameter follicles per cow was 52.4 $\pm$ 4.1 in a dissected ovary (Mc Natty *et al.*, 1984).

A variety of means have been used to acquire oocytes from the mammalian ovary. These have included aspirating oocytes from individual follicle, rupturing of isolated follicles or dissecting of the upper surface of the follicle.

Lonergan *et al.*, (1991) studied the effect of recovery rate of oocytes by aspiration and dissection method and found that the recovery of oocyte per ovary was 18.7 by dissection method, whereas it was 14.6 by aspiration method. Further, he stated that there was significantly more good quality oocytes recovered by dissection when compared to aspiration. Similarly, Arlotto *et al.*, (1990) reported that slicing method approximately doubled the number of oocytes released from the ovary, than the aspiration technique. Further, he stated that the size distribution, proposition of cumulus intact oocytes selected for culture from the ovarian surface and deep in the cortex were similar.

On contrary, Bottcher *et al.*, (1989) reported that the percentage of first quality oocytes were more by rupture of isolated follicles than by aspiration and dissection methods (60, 14, 0 per cent). The percentage of second and third quality oocytes was 43, 30, 60 and 43, 10, 40 by rupture of isolated follicles, aspiration and dissection methods respectively.

Sato *et al.*, (1990) compared the recovery of oocytes by needle puncture and dissection method and found that the dissection method gave more number of oocytes (16.0 ± 7.1), than needle puncturing method (10.0 ± 4.4). The oocytes with cumulus cells were more (3.7 ± 1.7) in razor blade method than needle puncture method (2.0 ± 0.8). The mean number of oocytes liberated from each ovary by combination of needle and razor blade method was 26.2 ± 7.4.

The obvious advantage of aspiration is in terms of speed of operation, which is especially important in *in vitro* culture in an embryo production line system (Gordon and Lu, 1990).

Wahid *et al.*, (1992) studied the effect and efficiency of recovery methods for obtaining ovine follicular oocytes for *in vitro* procedure and reported that a significantly higher number of cumulus enclosed oocytes/ovary could be obtained if dissection was employed. Recovery of oocytes by aspiration was 43 (4.48/1.03) times faster than dissection.

Pre-Inberge *et al.*, (1989) stated that the number of oocytes obtained per ovary averaged 16.0 $\pm$ 0.9 and did not differ significantly between right and left ovaries.

2.5 *IN VITRO* MATURATION

The term "maturation" has been used in various ways by different researchers. In order to limit the implication of the word, Scheutz (1969) proposed the term "meiotic maturation" to refer to the meiotic process following the release of the oocyte from prophase arrest. In many species, however meiosis is again arrested at MI and MII meiotic divisions at which time the oocytes are fully fertilizable.

Oocyte maturation is traditionally defined as those events associated with the initiation of germinal vesicle breakdown (GVBD) and completion of first meiotic division (Leibfried-Rutledge *et al.*, 1987). The process of oocyte maturation however also includes changes within the cytoplasm (Thibault *et al.*, 1975).

2.5.1 Condition required for maturation of oocytes *in vitro*

2.5.1.1 Time interval from slaughter to oocyte collection

Time interval from slaughter until oocyte aspiration is a critical period for subsequent germinal vesicle breakdown *in vitro* (Motlik *et al.*, 1978). Oocytes collected

within 30 minutes after slaughter have the highest success rate (Xu *et al.*, 1987). On contrary, Shea *et al.*, (1976) found the oocytes collected one or two hours after death had no significant difference in the maturation process. Similarly, oocytes collected 1.30 to 2 h (Trounson *et al.*, 1977), 2 h (Fukui *et al.*, 1987 and Kim *et al.*, 1990b) and 2.5 h (Fukuda *et al.*, 1990) after the death of the animal had no significant effect on the maturation rate.

2.5.1.2 Size of the follicle

When fully grown immature oocytes removed from antral follicles are placed in culture, they undergo spontaneous meiotic maturation, but oocytes removed from preantral follicles are incapable of maturation (Eppig and Schroeder, 1986). Motlik *et al.*, (1984) observed that no GVBD took place after 24 h of culture of pig oocyte if taken from 0.3-0.7 mm follicle size. Bovine oocytes from follicles below 1.6 mm did not mature spontaneously (Motlik *et al.*, 1986).

Asakawa *et al.*, (1982) stated that there was a significant difference between oocyte maturation from follicles less than 1 mm diameter and from large size follicles. Oocytes from small follicles could resume meiosis but only a small number of such oocytes reached M II. The majority of the oocytes were found degenerated during culture. In a similar study, Schellander *et al.*, (1989) reported that the percentage of oocytes maturing *in vitro* from follicles measuring <2 mm was low or non existent versus maximum percentage was attained in oocytes collected from 6-10 mm follicles. In Goat, Veronique de Smedt *et al.*, (1991) reported that 86 per cent of oocytes from follicles of 2-6 mm diameter progressed to M II whereas only 24 per cent of oocytes with the diameter of 1-2 mm completed meiotic maturation after 27 h in culture.

Pig oocytes from follicles ≤ 5 mm matured faster than those from larger ones (Fukui and Sakuma, 1980). They suggested that oocytes from small follicles were well surrounded by cumulus cells which helped maturation of oocytes *in vitro*.

It indicated that the oocytes for *in vitro* maturation should not be collected from too small follicle. However, a few studies have shown that size of the follicle does not influence the maturational ability *in vitro* in mice (Erickson and Sorensen, 1974), hamster (Iwamatsu and Yanagimachi, 1975), pig (Mc Gaughey, 1977) and bovines (Fukui and Sakuma, 1980).

2.5.1.3 Age of the donar

The ability of the mammalian oocyte to resume meiosis and complete nuclear maturation was acquired in a stepwise fashion and which has been shown to increase with age of the females (Leibfried-Rutledge *et al.*, 1987). The ability to mature spontaneously *in vitro* was not acquired until about 15 days of age in mice (Szybek, 1972; Sorensen and Wassarman, 1976) and 20 days of age in rats (Bar-Ami and Tsafriri, 1981). The oocytes inside the follicles in young female were in GV stage as in adult one, but it was observed that in Juvenile female the resumption of meiosis *in vitro* occurs less frequently. Katska and Smorag (1984) examined the ovaries of three age group of animals and found that the animals within the age group of 3-8 years were having more number of follicles and normal oocytes than the age group of 18-24 months and 9-17 years. On contrary, Onuma and Foote (1969) stated that the potential of *in vitro* maturation of oocytes obtained from young calves were equivalent to those from sexually matured cattle. Cytogenetic study on mouse oocytes revealed that oocytes of older females had fewer chiasmata per cell than did oocytes from young females (Chandley, 1971).

2.5.1.4 pH

pH is a vital factor for survivability of oocyte in media and was maintained by bicarbonate ions. A high pH in medium reduced the proportions maturing often to nil (Edwards, 1965). For each species there was an optimum pH where maturation rate was maximum. oocytes can grow in a fairly wide range of pH eg. 5.87 to 7.78 in mouse (Brinster, 1965a) and 6.64 to 7.91 in rabbit. (Kane, 1975). Bagger *et al.*, (1987) reported that alkalization beyond 7.45 resulted in increased rate of degeneration and decreased rates of GVBD and polar body formation and they stressed the importance of a stable pH during the period of isolation of oocytes for *in vitro* culture.

HEPES buffer (N-2 hydroxy ethyl piperazine N-2 ethanesulphoric acid) in culture medium maintains proper pH level for a longer culture period in 5 per cent carbondioxide although TCM 199 contains 2.20 mg/ml $NaHCo_3$ (Fukuda *et al.*, 1990).

Though the oocytes matured in media with pH ranging from 6.70 - 7.59, the highest percentage (69 per cent) was reached in media at pH 7.00 to 7.29 (Shea *et al.*, 1976). In general, the range of pH in which oocyte can grow well was 7.0 to 7.5 (Iritani and Niwa, 1977).

2.5.1.5 Temperature

Shea *et al.*, (1976) transported the ovaries from slaughter house to the laboratory without any precautions to maintain transport media temperature and found that 76 per cent of the oocyte resumed meiosis; but only 34 per cent reached M II. It revealed that temperature before setting of culture was an important factor for completing of meiosis (Fukui *et al.*, 1984). The original protocol in bringing the ovaries from the abattoir in

medium held at 30-37°C (Cheng *et al.*, 1986) was disproved and now it has been cleared that the ovaries can be held at 20°C for atleast 8 h before oocytes recovery (Yang and Lu, 1990). This permits much greater freedom in arranging production line work.

The incubation temperature has been found to play an important role in *in vitro* oocyte maturation. The most commonly used temperature for culturing mammalian cells was at 37°C eventhough body temperature varies among species. Cattle have an average rectal temperature of 38°C-39.3°C with a slightly higher core body temperature (Anderson, 1970). The temperature during the period of fertilization may be higher though basal temperature rises as ovulation approaches in women (Lenz *et al.*, 1983).

Glycosaminoglycan which is highly essential for induction of acrosomal reaction produced by cumuli was thought to be temperature dependent (Lenz *et al.*, 1983). Further, he stated that temperature ranging between 35-39°C had no deleterious effect on resumption and completion of meiosis, but at 41°C the frequency of oocytes that progressed to M II was significantly reduced. Ova matured at 39°C had significantly higher rates of fertilization than at 35°, 37°, or 41°C.

Moor and Crosby (1985) lowered the incubation temperature to 20°C at different stages of meiotic maturation and found that cooling induced chromosomal abnormalities. The time of GVBD (8-11 h after start of culture) was the most sensitive to cooling. Similarly, increased culture temperature also had deleterious effect on nuclear maturation. He stressed the point that temperature should be constant throughout the culture period. Eng *et al.*, (1986) stated that 39°C was the optimum temperature for pig oocyte *in vitro* culture. But there was no difference in the maturation rate of buffalo follicular oocytes cultured *in vitro* at 37°C and 39°C (Singh *et al.*, 1989).

2.5.1.6 Gas phase and humidity

The pH of the egg white saline mixture was found to rapidly rise from 7.00 to 7.8 during measurement with glass electrode or when exposed in shallow vessels, and the change was attributed to loss of carbondioxide contained in the egg white (Whittan, 1957).

Bagger *et al* (1987) controlled the pH by gassing the culture media with carbondioxide. Majority of the workers have used different combinations of gases eg. air, 5 per cent carbondioxide in air or 5 per cent carbondioxide plus 5 per cent oxygen plus 90 per cent nitrogen, of which 5 per cent carbondioxide plus 5 per cent oxygen plus 90 per cent nitrogen was found to be the best (Trounson *et al.*, 1977, Schellander, *et al.*, 1986; Berg and Brem, 1990). On contrary Shea *et al.*, (1976), Schellander *et al.*, (1989), Fukuda *et al.*, (1990), Kim *et al.*, (1990a) and Sato *et al.*, (1990) used a gaseous phase of 5 per cent carbondioxide in air and attained the maximum maturation rate.

Oocytes could convert pyruvate into carbondioxide (Eppig, 1977) which was important for normal maturation of pig oocytes (Eng *et al.*, 1986) and bovine oocytes (Fukui *et al.*, 1986).

Fukui *et al.*, (1987) used a gaseous phase of 5 per cent carbondioxide 45 per cent oxygen and 50 per cent nitrogen to culture intrafollicular oocytes and found that 57.9 per cent of the oocytes reached M II. Akufo *et al.*, (1988) exposed the culture media with oocytes to a mixture of 50 per cent oxygen, 45 per cent air and 5 per cent carbondioxide during culture and found that majority of the cultured oocytes had resumed meiosis as indicated by GVBD or chromatin changes, but only a small proportion reached M II.

Skyer *et al.*, (1987) cultured intact follicle in an atmosphere of 5 per cent carbondioxide, 40 per cent oxygen and 55 per cent nitrogen and stated that the gas mixture selected was superior to 5 per cent carbondioxide mixture. Similarly Fukui *et al.*, (1987) used a gas phase of 5 per cent carbondioxide 45 per cent oxygen 50 per cent of nitrogen for culture of intrafollicular oocytes in bovines.

High humidity of 90-100 per cent is suitable for oocyte maturation since it maintained the osmolarity by preventing evaporation. However, highest rate of maturation of oocytes was seen at 95 per cent humidity (Fukui *et al.*, 1986).

2.5.1.7 Osmolarity

Work with laboratory animals indicated that ova grow over a wide range of osmolarity. Mouse ova could be matured in media varying in osmolarity from 200 to 354 mOSm (Brinster, 1965,a) and rabbit ova in media varying from 230 to 339 mOSm (Naglee *et al.*, 1969; Bae and Foote, 1980). The osmolarity of the media can be adjusted by Na^{++} and Cl^{-} ions. Though there was a wide range of osmolarity, oocytes grow best at a particular osmolarity respective of the species eg. 270 mOSm in rabbit (Bae and Foote, 1980), 285 mOSm in pig (Mc Gaughey, 1977), 290 mOSm in sheep and cattle (Tervit *et al.*, 1972) and 294 mOSm in human (Soupart and Morgenstern, 1973). Maturation rate was found to reduce if osmolarity was varied.

2.5.1.8 Culture time

When the other culture conditions were provided, the time required for maturation varied depending upon the species. Edwards (1965) stated that oocytes reached M II after culturing in the media for 46 h in sheep, 31 h in cow, 43 h in pig and 30 h in Rhesus

monkey. Thibault (1977) reviewed that in cow, 60-90 per cent oocytes were in M II after 29-30 h of culture and in sheep culture time for maturation (91 per cent) was 40 h.

Though a high percentage of oocytes completed nuclear maturation *in vitro* after 22 to 24 hr in culture media (Trounson *et al.*, 1977; Fulka *et al.*, 1982), increased culturing period upto 28 h improved the maturation rate (Iritani *et al.*, 1984); but further incubation did not increase the proportion of maturation (Shea *et al.*, 1976). This was complemented by the study of Sirad *et al.*, (1988) stating that the percentage of oocytes maturation (M II) was increased from 28.6 to 55.4 per cent as the culture time increased from 18 to 28 h. After 28 h, there was no improvement in the maturation rate. In a similar study the percentage of maturation was increased from 74 to 84 per cent as the culture time increased from 24 to 28 h (Fukuda *et al.*, 1990). On contrary, Goto *et al.*, (1988) attained 82.9 per cent maturation rate within 19-22 h culture time.

2.5.1.9 Antibiotics

To prevent bacterial growth, antibiotics, penicillin and streptomycin were used in ova culture medium. However, Yablonski *et al.*, (1987) observed that antibiotics inhibited oocyte maturation.

2.6 CHARACTERIZATION OF FOLLICULAR OOCYTES AND *IN VITRO* MATURATION RATE

The percentage of bovine oocytes to undergo nuclear maturation *in vitro* was dependent on the presence of compact, complete cellular investment of the oocyte in mice, rabbit, pig, cattle and humans (Donahue and Stern, 1968; Kennedy and Donahue; 1969; Mc Gaughey, 1977; Fukui and Sakuma 1980; and Shioya *et al.*, 1988). Uniform

granules in the cytoplasm (intact germinal vesicle) were the only obvious indicators on oocytes potential to mature *in vitro* when removed from follicles (Leibfried and First, 1979; Jinghe *et al.*, 1989).

Intact cumulus cells have been found to facilitate oocyte maturation inhibitors (Schulz *et al.*, 1985) and increase the time of maturation (Xu *et al.*, 1986a) It was generally felt that a compact cumulus mass around the oocytes an important factor for maturation of oocytes *in vitro*.

On contrary, Cross (1973) in mouse and Zeilmaker and Verhamme (1974) in rat clearly indicated that cumulus free oocytes also undergo maturation to M II.

According to Donahue and Stern (1968), oocytes are dependent upon cumulus cells for their nourishment and essential nutrients and therefore compact oocyte cumulus was a must for oocyte maturation *in vitro*. It has been observed that if the connection between cumulus cells and oocyte was disintegrated, oocyte undergoes degeneration. When the oocyte matures, it does not need any support from cumulus cells and cumulus cells expand as a result of loss of connection between cumulus cells and oocyte cytoplasm. It was important that cumulus cells are with the oocyte for oocyte maturation (Eppig, 1977).

The oocytes were classified based on cumulus cells and the maturation rates of class A oocytes with compact dense cumulus cells, class B partially naked oocytes with thin cumulus layers or small remnants of cumulus cells and class C naked oocytes were 97.4, 89.9 and 59.9 per cent respectively (Shioya *et al.*, 1988). In an another study Kim and Park, (1990) attained 92.8, 84.7 and 55.2 per cent of maturation in A, B and C grade oocytes respectively.

Similarly the *in vitro* maturation rate was 85.2 per cent in A grade oocytes which decreased to 29.6 per cent in C grade oocytes (Garcia *et al.*, 1988).

Jinghe *et al.*, (1989) classified the oocytes based on cytoplasmic granules as 1) compact, complete oocyte with even cytoplasmic granulation, (2) fewer number of cumulus cells and speckled cytoplasm, (3) expanded cumulus with atretic bodies, (4) few or no cumulus with even granulation of cytoplasm. The maturation rate was 77, 30, 68 and 36 per cent respectively.

Leibfried and First (1979), Berg and Brem (1990) attained a high 71 and 65 per cent of maturation in good quality oocytes, where as nude oocytes attained only 44 per cent and 10 per cent maturation rate (Leibfried and First, 1979; Tsafriri and Channing, 1975). On contrary, Dahlhausen *et al.*, (1981), Moor *et al.*, (1984) reported that no oocyte from any size follicle group which were devoid of cumulus cells matured *in vitro* and opined that this might probably be due to poor cytoplasmic conditions of the oocytes which were subjected for maturation.

2.7 EFFECT OF SUPPLEMENTS ON OOCYTE MATURATION RATE *IN VITRO*

2.7.1 Culture medium

Bovine oocytes resume and complete the first meiotic division in a variety of media ranging from simple balanced salt solution (Leibfried-Rutledge *et al.*, 1987) to complex media (Edwards, 1965; New comb *et al.*, 1978 and Leibfried and First, 1979). Early attempts to culture eggs in various natural media such as blood serum (Brock and Rowson, 1952) and follicular fluid (Sreenan 1978) were not of much success. On contrary, Akufo *et al.*, (1988) showed 98 per cent of oocytes resumed meiosis in a media

containing 50 per cent of bovine follicular fluid, but only 45 per cent attained M I and 9 per cent completed maturation. He further stated that there was apparently slight increase in inhibition of meiosis resumption by increasing the percentage of follicular fluid to 100 per cent. This maturational block may be avoided by supplementing gonadotropin hormones or granulosa cells.

The studies of Natio *et al.*, (1988) indicated that the male pronuclei formation rate was elevated significantly to 59.5 per cent when the oocytes were cultured with porcine follicular fluid and this action was further enhanced by addition of follicular stimulating hormone. In a similar study addition of cumulus cells in a medium containing 50 per cent cow follicular fluid enhanced the maturation rate 90.5 per cent than 50.7 per cent in a medium containing only cow follicular fluid (Yoon *et al.*, 1989). In an another study by Ayoub and Hunter (1990), it was found that the culture of cumulus enclosed oocytes with 36, 50, 70 and 68 per cent GVBD. This inhibition of GVBD was reversed by removal of follicular fluid and culture in TCM 199. He further stated that heating of follicular fluid to 100°C for 15 minutes did not destroy its GVBD activity and suggested that the meiotic arrest at the germinal vesicle stage in the bovine oocytes was sustained by heat stable polypeptide factors existing in follicular fluid.

Tervit *et al.*, (1972) cultured sheep eggs successfully in medium based on the composition of sheep oviduct as described by Restall and Wales (1966). More than 65 per cent of bovine follicular oocytes in fetal calf serum matured to M II (Trounson *et al.*, 1977). Recently, a large number of media have been developed and used for culturing oocyte *in vitro* viz. Tyrode's medium (Hunter *et al.*, 1972 and Ball *et al.*, 1983; Leibfried-Rutledge *et al.*, 1987 and Berg and Brem, 1990) Eagles minimum essential medium (Bagger *et al.*, 1987) Hams F-10 medium (Shea *et al.*, 1976 and New comb *et al.*, 1978).

Hams F-12 medium (Fukui *et al.*, 1987) Way mouth's medium MB 752/1 (Edwards, 1965), TCM 199 (Lu *et al.*, 1987; Leibfried-Rutledge, 1986, Sirad *et al.*, 1988; Schellander, *et al.*, 1989 and Younis *et al.*, 1991). Brackett's defind medium (Sirad et al., 1986) and modified KRB (Choi *et al.*, 1987).

Zhiming *et al.*, (1990) compared the effect of different media on *in vitro* maturation rate of mouse and goat follicular oocytes and found that WM was the best culture media for *in vitro* maturation of mouse oocytes and the mD-PBS was the worst for the goat oocytes. On contrary Chauhan and Anand (1991) stated that there was a similar result on the three basal media like Dulbecco's phosphate buffer, Kreb's - Ringer bicarbonate and Hams F-12, with Hams F-12 being slightly better.

In other studies Singh *et al.*, (1989) in buffalo and Garcia *et al.*, (1988) in cow compared the efficiency of Hams F-10 and TCM 199 media on the maturation rate of follicular oocytes *in vitro* and found that there was no significant difference between the Hams F-10 and TCM 199 media. On contrary Totey *et al.*, (1991) stated that Hams F-10 has better maturation rate 63.3 per cent than TCM 199 medium (39.6 per cent).

Kim *et al.*, (1990b) reported that the highest *in vitro* maturation rate (88 per cent) was obtained in medium TCM 199 containing 10 per cent ECS than Kreb's-Ringer buffer and Hams F-10 media. But, for Pig oocyte maturation, Kreb's Ringer bicarbonate buffer had higher rate of maturation than TCM 199 (Koppany *et al.*, 1990).

Sreenan (1978) discussed the major inorganic ions like sodium, potassium, calcium, magnesium, chloride, phosphate, sulphate and bicarbonates needed for ova culture media. Principal function of Na^{++} and Cl^{-} was to maintain the osmolarity of the culture media. The effect of all these major inorganic salt constituents except bicarbonates

have been systematically examined by Wales (1970) in the case of mouse ova. Potassium concentration in female reproductive tract fluid was considerably higher than that in plasma (Restall and Wales, 1966; Borland *et al.*, 1977). Two media designed for culture of cattle ova have contained high level of potassium (Tervit *et al.*, 1972). Calcium ions play an indispensable role in the initiation of oocyte maturation (Wales, 1970 and Wassarman and Letcwineau. 1976).

Quinn and Wales (1973) and Kane (1975) have stated that the main role of HCO_3-in culture media was to function as part of a pH buffer system and it was also involved in the metabolism and maturation of oocytes.

2.7.2 Serum

Serum was found necessary in the culture media not for resumption of meiosis, but to promote developmental capacity of oocytes for fertilization (Cross, 1973). It was observed that a macro molecule present in the serum prevents the hardening of zona pellucida and helps in cumulus expansion by promoting retention of hyaluronic acid synthesized *in vitro* in response to FSH (Eppig, 1980).

Different authors used different source and concentration of serum viz; 1g vegetable protein in 100 ml of culture media (Bagger *et al.*, 1987), blood serum (Leibfried-Rutledge *et al.*, 1986), 0.6 per cent bovine serum albumin (BSA) (Rosenkranz and Lipp, 1989), 3 per cent BSA (Bondioli and Wright, 1983 and Zhiming *et al.*, 1990), 10 per cent BSA (Chauhan and Anand, 1991), 5 per cent Neonatal calf serum (Goto *et al.*, 1988), Neonatal Pig serum (Nakanishi *et al.*, (1990), 1 per cent Foetal Calf Serum (FCS) (Nakanishi *et al.*, 1990), 10 per cent FCS (Shea *et al.*, 1976; Agrawal and Polge, 1989, Singh *et al.*, 1989; Berg and Brem, 1990; Nakanishi *et al.*, 1990; Sato *et al.*, 1990;

Fukui *et al.*, 1991 and Totey *et al.*, 1991), 15 per cent FCS (Nakanishi *et al.*, 1990), 20 per cent FCS (Rosenkranz and Lipp, 1989 and Schellander *et al.*, 1989), 10 per cent estrus cow serum (ECS) (Kim *et al.*, 1989; Kim *et al.*, 1990a; Kim *et al.*, 1990b), 20 per cent Estrous buffalo serum (EBS) (Rosenkranz and Lipp, 1989 and Schellander *et al.*, 1989), 10 per cent ECS (Kim *et al.*, 1989; Kim *et al.*, 1990a), 30 per cent ECS (Sanbuissho and Threfall, 1986; Lu *et al.*, 1987, Kim et al., 1989) and 20 per cent EBS (Totey *et al.*, 1991) were used for *in vitro* culture of follicular oocyte and variable percentage of maturation was attained.

Menezo *et al.*, (1984) in human and Saeki *et al.*, (1990) in bovine reported that serum was not necessary for *in vitro* maturation and fertilization. In an another study, Wright and Bondioli (1981), stated that no media or protein supplement was superior in promoting *in vitro* fertilization of cattle, sheep and swine oocytes. On contrary, Sanbuissho and Threfall (1986) attained a high rate of early embryonic development by addition of serum from an estrus cow to the oocyte maturation. When such serum was added to the medium, the most striking morphological change was the complete expansion of the cumulus cells, including the corona radiata. This was supplemented by the findings of Epping and Schroeder (1986) that the presence of a high molecular weight compound of serum was essential during oocyte maturation in cattle culture in order that the ova become fertilizable. Kajihara *et al.*, (1987) studied the effects of various serum concentrations in the culture medium on *in vitro* fertilized bovine embryos and found that 2.5 per cent of the serum was optimal.

This was confirmed by the findings of Nakao and Nakatsuji (1990) who showed that 2 per cent FCS was better than 10 per cent during early phase of culture. Xu *et al.*, (1987) observed that one of the reasons for this high success rate of bovine oocyte

maturation rate might be due to the addition of serum from estrous cow to the maturation medium.

Mouse oocytes matured *in vitro* in chemically defined medium were not penetrated by spermatozoa. Addition of fetal calf serum to the medium for maturation of oocytes improved the incidence of sperm penetration and shortened the time of enzymic dissolution of zona pellucida. Without the supplement of the foetal calf serum, the zona pellucida and/or vitellus was penetrated in only 12 per cent of the oocytes, while the addition of 10 per cent FCS increased this rate to 79 per cent (Choi *et al.*, 1987).

2.7.3 Hormones

Foote and Thibault (1969) reported that steroids and gonadotropins did not influence resumption of meiosis in *in vitro* oocyte maturation. Similarly, Cross and Brinster (1970) stated that steroids were not necessary for the resumption of meiosis, but were probably involved in its completion; the incidence of chromosomal abnormalities at telophase I and M II significantly increased in the absence of steroids. The importance of steroids during maturation was however much more evident in regulating the biosynthetic events in the cytoplasm and membrane.

The effect of gonadotropins and steroids *in vitro* was explained by Hillensjo *et al.*, (1976). It was observed that by acting on the cumulus cells, the gonadotropins change their metabolic pathway resulting in enhanced progesterone production. But the report on role of hormones for *in vitro* maturation seemed to be conflicting, making it difficult to define a generalised mode of hormone action. In an *in vitro* study in Pig, oocytes from follicles with low estrogen level failed to undergo normal nuclear and cytoplasmic maturation and nuclear maturation of oocytes. In sheep in the presence of gonadotropin,

oocytes from small atretic follicles attained high percentage of maturation rate than in non-atretic follicles in *in vitro* follicular culture (Moor and Trounson, 1977).

Eppig and Koide (1978) found that the hormones in increased dose decreased the rate of GVBD and polar body production *in vitro*.

In sheep, supplementation of gonadotropins and estradiol in maturing sheep oocytes had proved to be essential (Staigmiller and Moor, 1984). Moor *et al.*, (1985) found that 46 out of 47 oocytes from sheep follicle cultured without hormonal addition contained intact dictyate nuclei; but inclusion of FSH and LH in the culture medium induced germinal vesicle breakdown in 30 out of 39 oocytes (79 per cent).

In bovines, oocytes cultured in the presence of FSH induced cumulus expansion (Thibault *et al.*, 1975) which increased the incidence of oocytes penetration when compared to untreated control (Ball *et al.*, 1983). On contrary, Dahlhausen *et al.*, (1981) and Sirad *et al.*, (1988) stated that the addition of FSH, LH and E_2 or FSH, LH alone (Sanbuissho and Threlfall, 1990) to maturation culture do not alter the frequency of nuclear maturation.

Although supplementation of FSH/LH and E_2 in maturation media has shown a favourable effect (Younis *et al.*, 1991). Oocytes were routinely matured in the absence of hormones capable of yielding more than 90 per cent oocytes at M II (Gordon and Lu, 1990). Schellander *et al.*, (1989) studied the effect of hormones FSH, LH and E_2 and found that 95 per cent of the cultured oocytes displaced a marked expansion of cumulus cells characteristic of maturation of ova whereas without hormones, only the outer layer of the cumulus expanded.

Younis *et al.*, (1991) reported that the presence of LH or FSH during goat oocytes maturation improved both IVM and IVF results over those of the control. Agrawal and Polge (1989) tested a medium without hormones for *in vitro* maturation of pig oocytes and found that 38.73 per cent of oocytes progressed to M II, whereas 65.2 per cent of the pig oocytes attained M II in a medium supplemented with foetal calf serum and hormones (Cheng, 1985).

Mattioli *et al.*, (1990) indicated that both LH and FSH can influence the membrane potential of follicular somatic cells and the oocyte.

Zuelke and Brackett (1990) found that luteinizing hormone was shown to enhance maturation of immature oocytes. Further, he reported that enhancement of viability of oocytes with 50 µg LH/ml.

Saeki *et al.*, (1990) stated that there was no difference in maturation rate of bovine oocytes in media containing commercially available FSH than standard FSH (NIADDKO FSH-1). Rosenkranz (1991) studied the effect of 17-ß estradiol on the *in vitro* maturation of follicular pig oocytes and found that media with 17ß estradiol caused a light microscopically detectable better cumulus expansion. The viability of the ooplasm was proved to be better in the experimental group than in the control group with no influence on the nuclear malturation. (Rosenkranz and Lipp, 1989). The fertilization rate of oocyte cultured in the medium supplemented with FCS was higher than that of oocytes cultured in medium supplemented with ECS or in no serum (Mochizuki, 1991). However, while examining FCS versus ECS, it was found that higher proportion of oocyte fertilization after maturation in media containing 20 per cent ECS than 20 per cent FCS. On contrary, Kim *et al.*, (1990b) stated that 10 per cent ECS in TCM 199 promoted a higher

proportion of penetrated oocytes than 15 and 20 per cent ECS. Sanbuissho and Threlfall (1990) found that the addition of both ECS and FCS to the culture medium promoted oocyte maturation. Similarly, Totey *et al.*, (1991) stated that there was not much of difference in maturation rate of buffalo follicular oocytes cultured in 10 per cent FCS and 20 per cent EBS (66.8 and 64.9 per cent respectively).

Sanbuissho (1989) studied the influence of bovine serum albumin, estrous cow serum and foetal calf serum on *in vitro* bovine oocyte maturation and found that all serum supplements increased the proportion of oocytes undergoing cumulus expansion.

The effect of various other hormones like progesterone (Shea *et al.*, 1976; Prokofev *et al.*, 1986), testosterone (Prokofev *et al.*, 1986) and $PGF_2\alpha$ (Yablonski *et al.*, 1987) have been found to have stimulatory effect on *in vitro* oocyte maturation and fertilization.

2.7.4 Follicular cells

Experiments by Tsafriri and Channing (1975) demonstrated that a gradual addition of cumulus cells to a culture of follicule free oocytes inhibits their maturation in a dose dependent manner. In a similar study, presence of 7 day culture granulosa cell monolayer (Sirad and Bilodeau, 1990) in a culture media reduced the maturation rate. This inhibitory effect may depend upon the source and age of the follicular cells. The follicular cells from medium size follicles were having higher inhibitory effect than cells from large size follicles (Park *et al.*, 1990). This transient delay in meiotic resumption probably result in the presence of an oocyte maturation inhibitor was first reported by Tsafriri and Channing (1975). If the addition of supplementary cells during oocyte culture was delayed until after a period of more than 45 minutes of meiotic commitment occurs in bovine, then the

problem of meiotic delay can be avoided, because if once meiotic commitment occurs spontaneously, nuclear maturation becomes irreversible by currently known method of meiotic inhibition (Gordon and Lu, 1990). Yoon *et al.*, (1989) reported that addition of matured granulosa cells had resulted in higher maturation rate than control.

The specific changes in protein synthesis and protein phosphorylation of the cumulus cells during maturation (Kastrop *et al.*, 1990) was by the soluble factor produced by the follicular cells in the culture media required a direct cell oocyte contact. Oocytes surrounded by cumulus cells for more than one third of their surface and ovary yielding more than 30 oocytes were effectively matured *in vitro* by utilising a co-culture system with bovine cumulus cells (Goto *et al.*, 1988; 1991). Since the formation of perivitelline space with loss of contact between the cumulus cells and oocytes followed by the resumption of meiosis as defined by germinal vesicle breakdown (Kruip *et al.*, 1983). Presence or absence of cumulus cells *in vitro* culture did not affect the fertilization rate (Whittingham, 1977).

2.8 CUMULUS CELLS EXPANSION AND OOCYTE MATURATION RATE

When the oocyte matures, it does not need any support for cumulus cells and cumulus cells expand as a result of loss of connection between cumulus cells and oocyte cytoplasm (Donahue and Stern, 1968). Hence the morphological appearance of the cumulus mass surrounding the oocyte had a significant relation to the maturation state of the oocyte. Bedirian and Baker (1975) found that fewer than one per cent of oocytes surrounded by a compact cumulus underwent nuclear maturation, whereas 81 per cent of these with an expanded cumulus underwent nuclear maturation. A similar findings was made by Loos *et al.*, (1989) that the cumulus investment of the oocytes in M II was

always expanded, oocytes with a compact cumulus investments remained in GV stage of development *in vitro*. This cumulus expansion was induced with FSH (Thibault *et al.*, 1975a) and serum (Sanbuissho and Threfall, 1986).

Sanbuissho and Threfall (1986) stated that the most striking morphological change was the complete expansion of the cumulus cells including the corona radiata, when serum was added to media.

A similar expansion of cumulus and corona cells in *in vitro* matured oocytes were made by several authors (Motlik, *et al.*, 1986; Xu, *et al.*, 1986b and Loose, *et al.*, 1989).

Dahlhausen *et al.*, (1981) reported that <3 mm and 3-6 mm categories follicles contained a greater proportion of oocytes with compact or only slightly expanded cumulus as compared with follicles in the >6mm category. Follicles of >6mm size contained a majority of oocytes with a fully expanded cumulus mass.

Ball *et al.*, (1982) stated that hyaluronic acid is produced by bovine cumulus cells during the expansion process and this prepares the sperm for fertilization. This was confirmed by his another study (Ball *et al.*, 1983) that the percentage of FSH or cAMP increased the incidence of oocyte penetration. This might relate to the physical barrier present to spermatozoa by expanded cumulus cells.

Sirad *et al.*, (1986) found that the percentage of oocytes with expanded cumulus cells were varied according to the types of serum added to the media, 56, 45, 57, 34 and 33 per cent of oocytes with expanded cumulus oocytes were attained in media containing 10 per cent serum from heifers 2 days after superovulation, serum collected from cow 2 days after natural service, FCS, tubal secretions and rabbit tubal secretions respectively.

Olson *et al.*, (1990) stated that the percentage of cumulus expansion and cleaved rate was not correlated. The percentage of cumulus expansion and cleaved was 3, 64, 2 and 52 per cent and 52, 8, 13 and 25 per cent in control, media containing FSH, heparin and heparin plus FSH respectively.

2.9 SETTING OF CULTURE

The volume of the culture media and number of oocytes in the media had great influence on oocyte maturation rate. New Comb *et al.*, (1978), Lu *et al.*, (1987) and Goto *et al.*, (1988) cultured 10-90 oocytes in 1-2.5 ml of culture media which was covered by paraffin oil or without cover and the same attained variable percentage of maturation. In another method, Shioya (1988), Leibfried-Rutledge *et al.*, (1989) Schellander *et al.*, (1989) and Olson *et al.*, (1990) used drop of culture media varying from 50 µl to 0.2 ml and attained the same results as above.

Leibfried-Rutledge *et al.*, (1989) studied the effect of number of oocytes/drops of culture media on its maturation rate and found that the maturation rate was reduced from 81 to 71, 49 and 32 per cent in drops containing 10, 20, 30 and 40 oocytes respectively. In an another study, the oocytes kept in small drops of media under paraffin oil in petridishes failed to achieve germinal vesicle breakdown when kept in the ordinary incubator, because of the insufficient amount of carbondioxide necessary for maturation of proper pH.

CHAPTER 3

MATERIALS AND METHODS

3.1 EXPERIMENT I

3.1.1 Experimental material

Normal ovaries of non-descript pluriparious buffaloes at a random stage of reproductive cycle were collected from slaughter house for the study.

One hundred and eighty one pairs of ovaries were collected from July 1988 to June 1989, transported to the laboratory in physiological saline within 30 minutes after slaughter at 30-38°C temperature. Biometry of the ovaries, corpus luteum and follicles of four stages of the estrous cycle were studied.

3.1.2 Classification of the estrous cycle

Based on the corpus luteum (Ireland *et al.*, 1980) and characteristics of the ovary during cycle (Zemjanis, 1962), the estrous cycle was fixed into the four stages.

3.1.2.1 Stage I : Days 1 to 4

The corpus luteum appeared red due to the filling of blood with loosely arranged cells and presented a rough surface. The corpus albicans were identified in one of the ovaries.

3.1.2.2 Stage II : Days 5 to 10

A fully formed brown coloured corpus luteum with vascularity visible around its periphery and on bisecting, the lobulation was not well formed. The apex appeared red or brown and the reminder part flesh in colour. The diameter of the corpus luteum varied from 0.5 to 2.0 cm.

3.1.2.3 Stage III : Days 11 to 17

The vascularity was visible over the apex of the corpus luteum and on bisecting, the entire corpus luteum was flesh coloured with good lobulation. The diameter appeared to be unchanged.

3.1.2.4 Stage IV : Days 18 to 20

The ovary contained one large follicle and a regressed corpus luteum. The corpus luteum was grey or white in colour with no vasculature visible on its surface. The diameter became less than 1 cm.

3.1.3 Biometrical studies

3.1.3.1 Ovary

Length in cm (Pole to pole)

Width in cm (Surface to surface)

Height in cm (Hilus to free border)

Weight in g

Length, width and height of the ovaries were measured as mentioned above with the help of vernier callipers with an accuracy of 0.02 mm. Weight (g) of right and left ovaries were recorded in a monopan balance after trimming the extraneous tissue of each ovary. The size of ovary was calculated by using the formula of Singh and Singh (1988).

3.1.3.2 Corpus luteum

After noting the blood supply and colour, the externally visible portion of corpus luteum was gently squeezed out from the ovarian surface and the weight was taken by using a monopan balance. After making mid-line section of corpus luteum mass, the colour inside was observed and the final length was measured by vernier callipers, reading corrected to 0.02 mm. The diameter of the corpus luteum at the widest portion was measured. The fluid filled cavities (Lacunae) were noted, if present, in the cut surface of the corpus luteum.

3.1.3.3 Follicles

The number of follicles visible as transparent were identified and counted in each ovary and their diameters were measured, using the average of three measurements taken with a dial vernier calliper and grouped according to size into groups of ≤ 2, 3-5, 6-10 and > 10 mm in diameter.

Follicles with more than 1 ml of fluid on visual inspection, were aspirated by using 2 ml glass syringes with 18G hypodermic needle, whereas the follicles which contained less than 1 ml of fluid were aspirated by 1 ml tuberculin syringes with 21G hypodermic needle. Volume and colour of the fluid were recorded then fluid taken in a grease free glass slide and examined under a stereomicroscope (10 x for isolation, 25 x

for character of cellular investment). The same slide was focussed under binocular microscope in which the stage micrometer was fixed to observe ooplasm characterisation and oocyte diameter. The number of oocyte recovered and their characterization in relation to the stage of the cycle and their ipsilateral or contralateral relationship to the most recent corpus luteum were recorded. The catagories possible for investment and ooplasm and score assigned to each category are presented in Table 1 and Plate 2 to 7 (Leibfried and First, 1979; Mahadevan and Fleetham, 1990).

Table 1

Classification schemes for evaluating oocyte components

Components	Description	Score
Investment	a) Compact, complete, dense cumulus cells around the zona	3
	b) Thin, expanded cumulus layer or partially naked oocytes	2
	c) Nude oocytes enclosed by Zona Pellucida only	1
Ooplasm	a) Uniform distribution of ooplasm granules giving a dusty appearance	3
	b) Evenly filled ooplasm with uneven distribution of granules coalesce into black bodies leaving clear periphery	2
	c) Degenerated ooplasm indicating uneven filling in the Zona, vacuolation, fragmentation or presence of remnants.	1

Plate 2 : Oocyte with compact complete cellular investment and even ooplasm granulus

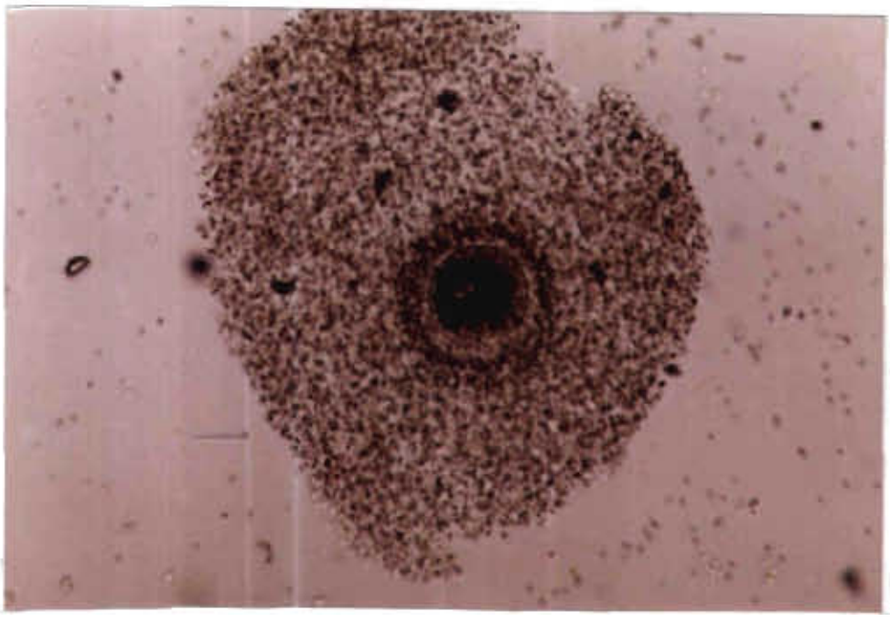


Plate 3 : Oocyte with compact complete cellular investment and clumped ooplasm granulus

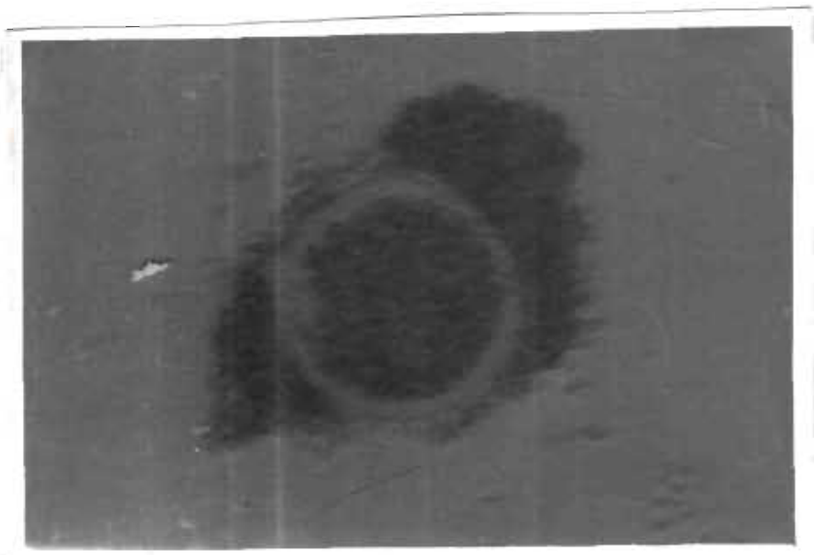


Plate 4 : Oocyte with partial cellular investment and even granulation

Plate 5 : Oocyte with partial cellular investment and clumped ooplasm granulus

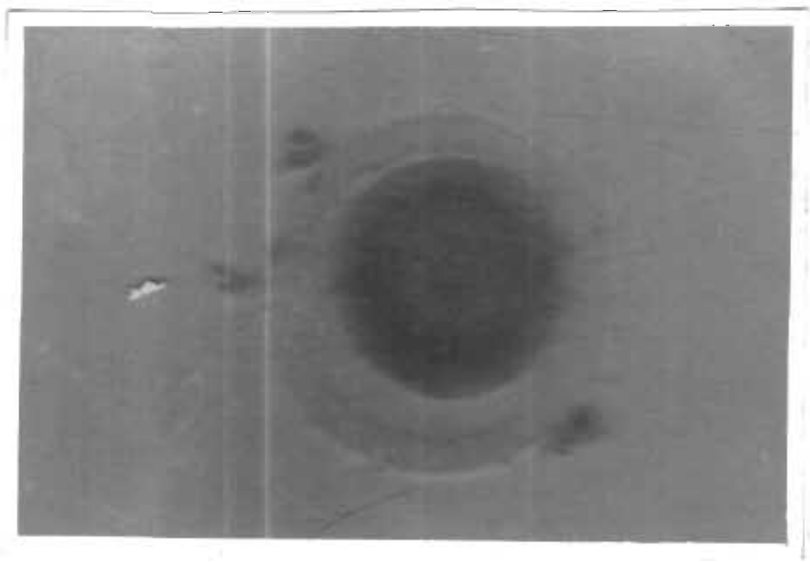


Plate 6 : Nude oocyte with even granulation

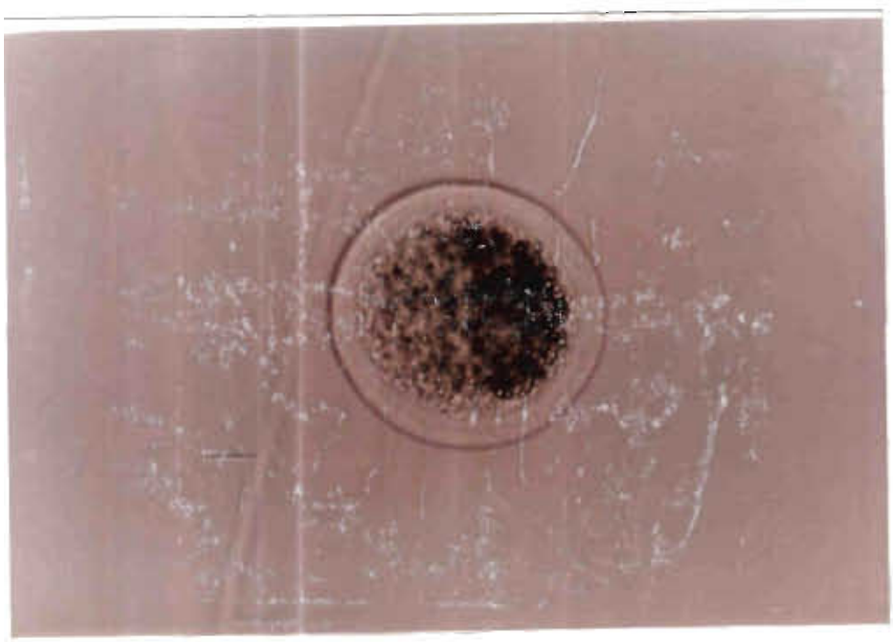


Plate 7 : Nude oocyte with clumped ooplasm granulus

The total score will be maximum of 9 for a complete compact dense cumulus oophorus around the zona with uniform distribution of granules in the evenly filled ooplasm of the oocyte and a minimum of 1 for a nude oocyte with degenerated ooplasm.

3.2 EXPERIMENT II

Two hundred and thirty five ovaries were brought to the laboratory adopting standard method of Leibfried and First, (1979). Materials were transported in a thermocole box within 1 hour after slaughter. The ovaries were kept in water bath at 37°C temperature. Collection and processing of oocyte was completed within 4 hour after death of the animals.

3.2.1 Collection of oocytes

The ovaries were washed twice with phosphate buffer saline (PBS) to remove the residual blood clots on the surface of the ovary and oocytes were collected by methods outlined by Bottcher *et al.*, (1989). The rate of recovery, percentage of oocytes in different scores and its maturation ability were recorded.

3.2.1.1 Aspiration method

The visible follicles on the ovarian surface were aspirated through the ovarian tissue with the help of sterile 5 ml syringes partially filled with heparinized PBS using 18G hypodermic needle. The ova along with follicular fluid were aspirated slowly. The content were dropped according to their size in a sterile glass tubes that were kept at 38°C water bath. After the oocytes settled at the bottom, they were taken out using finely drawn pasteur pipette into a petridish containing fresh PBS without heparin.

3.2.1.2 Rupture method

The visible follicles on the ovarian surface were punctured and the contents expelled into a disposable petridish (100 x 15 mm Nuclon, Intermed, Denmark) containing 10 ml PBS supplemented with heparin to prevent clotting of the follicular fluid.

3.2.1.3 Dissection method

The ovarian surface was dissected with a razor blade into small pieces in a petridish containing heparinized PBS. Harvested ovarian components were filtered on a membrane with a mesh of 200 μm and the filtrate was collected in a disposable petridish.

3.3 COMPOSITION AND PREPARATION OF VARIOUS SOLUTIONS USED IN THE STUDY

3.3.1 Medium

3.3.1.1 Normal saline (Transport medium)

A total of 9g of sodium chloride (AR) was dissolved in 1000 ml of deionised triple glass distilled water and supplemented with 50 μg streptomycin and 50 I.U. penicillin per ml. This was prepared fresh each time.

3.3.1.2 Phosphate buffered saline (working medium)

Modified Dulbecco's phosphate buffered saline powder (ca^{++}, Mg^{++} free Sigma Chemical Co, U.S.A.) was dissolved in one litre of deionised triple distilled water and 30 grams of bovine serum albumin (Fraction V) was sprinkled over the PBS solution, allowed to dissolve and sterilized through filtration by using a millipore membrane filter

(0.45μ). It was made into 100 ml aliquotes in a sterile glucose bottle containing antibiotics (Penicillin and streptomycin) and one unit of sodium heparin/ml.

3.3.1.3 Culture medium (TCM 199, Earle's salt)

The nutrient mixture TCM 199 (Earle's salt - Sigma Chemical Co, USA), was dissolved in 900 ml of deionised triple glass distilled water and stirred gently. The original package was rinsed with 50 ml of deionised triple glass distilled water to remove all the traces of powder and poured into 900 ml solution. The pH was adjusted to 7.2 using sterile sodium bicarbonate and osmolarity was adjusted to 290 mOSm using sodium chloride solution; 50 ml of deionised triple glass distilled water was added to make up the volume to 1000 ml.

The media was sterilized by filtration using a millipore membrane filter (0.45μ). The media was aseptically dispensed into sterile containers in which antibiotics (Penicillin and Streptomycin), 1μg FSH, 10μg LH and 1μg estradiol 17 ß/ml with 10, 15, 20 per cent of foetal calf serum (FCS) or estrous buffaloes serum (EBS) were present and kept at 4°C temperature until use.

3.3.2 Antibiotics

3.3.2.1 Penicillin stock solution : 300 mg (1670 unit/mg)

Three hundred mg (1670 unit/mg) of penicillin G (Sigma) was dissolved in 10 ml double distilled water and kept frozen at -20°C and 0.1 ml of the solution added to 100 ml of media to yield a concentration of 50 I.U/ml.

3.3.2.2 Streptomycin stock solution

A total of 500 mg of streptomycin sulphate (Sigma Co., U.S.A.) was dissolved in 10 ml double glass distilled water and kept frozen at -20°C and 0.1 ml of this solution added to 100 ml of media to yield a concentration of 50 ug/ml.

3.3.3 Serum

3.3.3.1 Collection of estrous buffalo serum (EBS)

Blood was collected from the jugular vein of standing estrous buffalo to the sterile glass tubes in an aseptic manner. The tubes were kept slanting overnight at 4°C and serum was taken out into centrifuge tubes in a laminar flow hood. This was centrifuged at 3000 rpm for 20 minutes and the supernatent serum was collected and kept at -20°C.

3.3.3.2 Heat inactivation of serum

Estrous buffalo serum was kept in a beaker and was heated at 56°C for 30 minutes in a water bath; 10, 15 and 20 ml of heat inactivated serum was added to 90, 85, and 80 ml of culture media respectively to yield a 10, 15 and 20 per cent serum supplementation in culture media.

3.3.3.3 Foetal calf serum (FCS)

Foetal calf serum supplied by Sigma Chemical Co, USA was thawed and added as above to get 10, 15 and 20 per cent serum supplementation in culture medium in a sterile manner.

3.3.4 Liquid Paraffin

Liquid paraffin was washed twice with water to purify as much as possible. Extra water contained in the paraffin oil was evaporated by heating directly over a spirit lamp and autoclaved at 15 lbs for 15 minutes. The paraffin was used to cover the media during culture to prevent water loss from the media.

3.3.5 Fixative (Carnoy's fixative)

Methanol and glacial acetic acid in a ratio of 3:1 were mixed thoroughly and kept in a refrigerater before use. This was prepared fresh each time.

3.3.6 Stain

One g of orcine powder was dissolved in 45 ml of boiling glacial acetic acid; 55 ml of distilled water was added after cooling and stirred well and filtered by using Whatman No.1 filter paper. The filtrate was centrifuged at 3000 rpm for 15 minutes to remove the thin particles and the supernatant was kept at 4°C before use.

3.3.7 Paraffin wax-vaseline mixture

Ninety g of vaseline and 10 g of paraffin (60°C melting point) were melted together to get a medium softness. The mixture was loaded in 2 or 5 ml plastic disposable syringes, cooled and stored at 4°C.

3.3.8 Preparation of glasswares

All the glasswares were cleaned with water and rinsed thrice in deionised triple glass distilled water, dried and sterilized in hot air oven at 160°C for two hours.

3.4 *IN VITRO* MATURATION

3.4.1 Preparation and scoring of the oocytes

The medium which contained the oocytes from all the three methods were viewed under a stereomicroscope and oocytes were transferred to petridishes (60x15mm) containing 10-15 ml of TCM 199 culture medium by using a finely drawn pasteur pipette which was connected to a rubber tube and a mouth pipe (Plate 1) to minimize the volume of fluid from working medium to culture medium.

The oocytes were graded according to the characterization and scoring was done based on the investment and ooplasm condition as per the score card developed. The maximum score of 9 was given to a complete compact dense cumulus cells with even ooplasm granules (3 x 3), oocyte, a minimum of 1 for a nude with degenerated ooplasm (1 x 1) and intermedia score of 6-2 was given to oocytes of compact complete dense cumulus cells with clumped ooplasm granules (3 x 2), degenerated ooplasm (3 x 1), partial or expanded cumulus cells with even granulation (2 x 3), clumped ooplasmic granules (2 x 2), degenerated ooplasm (2 x 1), nude oocytes with even ooplasmic granules (1 x 3) and clumped ooplasm granules (1 x 2). They were stained with trypan blue to determine cell viability. Live unstained oocytes were used for further culture.

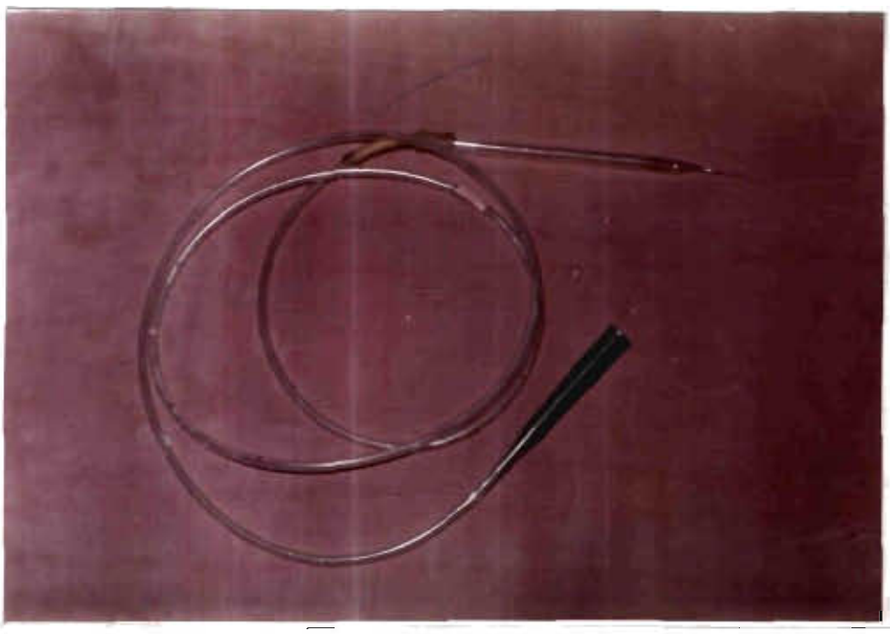


Plate 1 : Microtipped pasture pipette with connection tube

3.4.2 Oocyte culture

TCM 199 culture medium supplemented with antibiotics (penicillin and streptomycin) and 10 per cent FCS, 1 µg FSH, 10 µg LH and 1 µgE_2-17ß/ml was passed through a disposable microfilter (0.22 µ millipore filter) and placed under incubation of 37°C with a gas phase of 5 per cent carbondioxide in air for a minimum period of two hours before use. The preincubation of the culture medium was to allow equilibrium of 37°C and gas phase of 5 per cent carbondioxide in air.

After washing the oocytes thrice in the culture medium in order to remove the trypan blue, the classified oocytes were cultured for 27 h in 6 well mutli dishes (Nune, Intermed, Denmark). The culture was done in 2 ml of medium for 10-20 oocytes (Lu *et al.*, 1987) at 37°C and 5 per cent carbondioxide in air with 100 per cent humidified incubator. After 27 hour culture, the oocytes were processed and the number of oocytes that reached metaphase II (M II) under various scoring system recorded.

3.4.3 Denudation of oocytes

Preparation of fine tip glass pipette : Using a low flame, a series of micropipettes of graded sized tips were hand-pulled from sterile 9 inch pasteur pipettes. Using a sterile microforceps the tip of the drawn pipette is broken back until the pipette diameter was slightly larger than that of egg/corona mass. Care was taken so that the tip of the micro pipette was not too small, because distortion of the egg could result.

The egg and corona mass were aspirated in and out of the pipette until the corona cells were mechanically dislodged to allow a clear view of the cytoplasm of the egg.

3.4.4 Preparation for chromosomal studies

3.4.4.1 Wet mount slide preparation

In between the two lengthy rides of paraffin wax vaseline mixture prepared over a grease free clean glass slide, the PBS washed denuded oocytes were transferred with a minimum volume of media and covered with a rectangular coverslip. Gentle and uniform pressure was given over the cover slip, so that it just touched the oocytes and the coverslip was placed in position by using melted wax along the lengthy sides of the coverslip and glass slide. Media was passed in between the coverslip and glass slide to keep the oocytes in position and prevent damage.

3.4.4.2 Fixing and Staining

The processed slide was dipped gently in methanol and acetic acid (3:1) fixative and allowed for 24 hours to fix the oocytes and then stained by gentle stream of 1 per cent acetoricine stain in between the coverslip and glass slide for about a minute to study nuclear changes.

Studies on chromatin changes such as germinal vesicle (GV, Plate 8), Metaphase I (MI, Plate 9), Metaphase II (M II (Plate 10) and degenerated (Plate 11) were conducted by the following classification.

3.4.4.3 Stages of oocyte maturation

3.4.4.3.1 Germinal vesicle

The large nucleus was located eccentrically or peripherally. The nuclear membrane was intact, and the nuclear area quite distinct. The chromatin strands were arranged in the form of a ring of horse shoe around a single large nucleolus again eccentrically placed.

3.4.4.3.2 First metaphase

There was no trace of the nuclear membrane. The chromosomes were formed and arranged in an orderly sequence on the equator of a bipolar spindle. The long axis of the spindle was oriented radially to the surface of the egg.

3.4.4.3.3 Second metaphase

The first polar body has been abstricted from the egg (second oocyte) and the chromosomes of the latter were once more arranged on the equator of the spindle.

3.5 EXPERIMENT III

3.5.1 Classification of different types of oocyte and preparation of media with various supplements

Five thousand six hundred and twenty eight oocytes were classified into 9 types based on cumulus cells (compact, partial and nude) and ooplasm condition (even, clumped ooplasm granules, and degenerated ooplasm) as follows.

Type 1 : Compact, complete dense cumulus cells around the zona with even distribution of ooplasm granules.

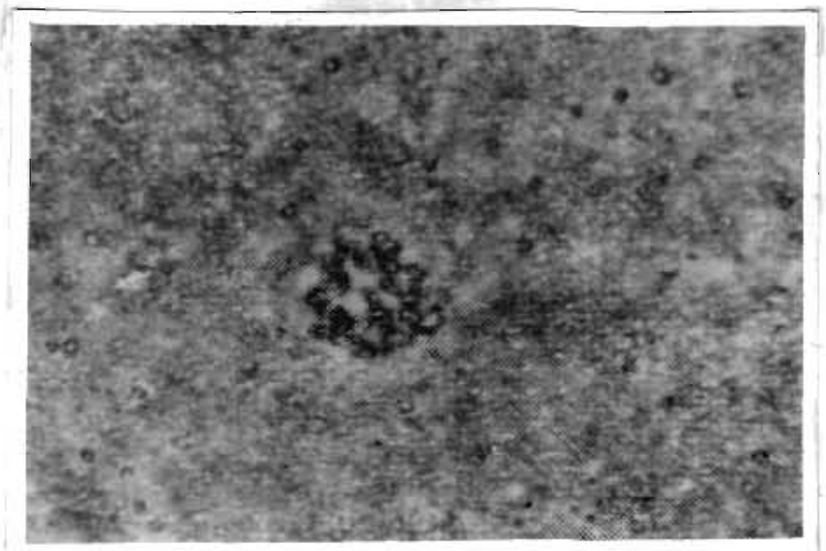


Plate 8 : GV stage

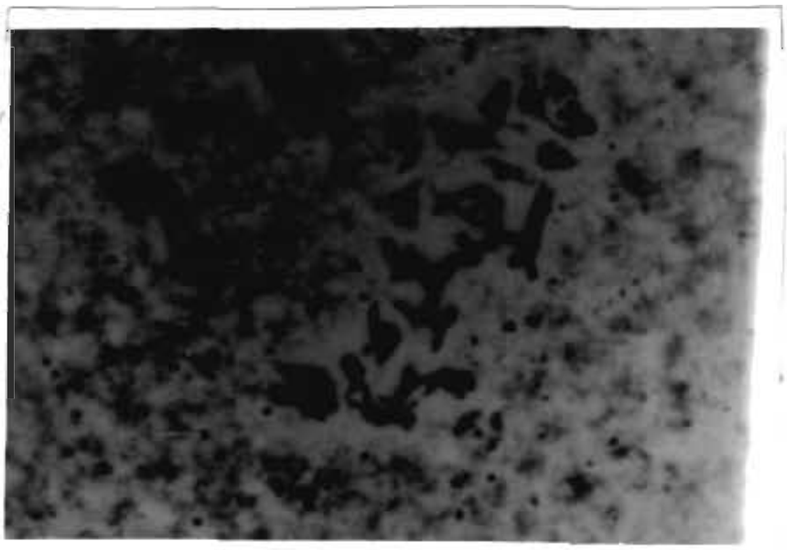


Plate 9 : MI stage

Plate 10 : MII stage

Plate 11 : Nude oocyte with degenerated ooplasm

Type 2 : Compact complete dense cumulus cells around the zona with clumped ooplasm granules leaving clear periphery.

Type 3 : Compact complete dense cumulus cells around the zona with degenerated ooplasm.

Type 4 : Partial cumulus cells with even distribution of ooplasm granules.

Type 5 : Partial cumulus cells with clumped ooplasm granules leaving clear periphery.

Type 6 : Partial cumulus cells with degenerated ooplasm.

Type 7 : Nude oocytes with even distribution of ooplasm granules.

Type 8 : Nude oocytes with clumped ooplasm granules leaving clear periphery.

Type 9 : Nude oocyte with degenerated ooplasm granules.

All the nine types of oocytes were subjected to the following five treatments (T) in two types of sera (group I and II) with a common control for both the groups.

GROUP I

T_1 TCM 199 + 10 per cent FCS

T_2 TCM 199 + 10 per cent FCS + 10 µg LH/ml

T_3 TCM 199 + 10 per cent FCS + 1 µg FSH/ml

T_4 TCM 199 + 10 per cent FCS + 1 µg E_2 - 17 ß/ml

T_5 TCM 199 + 10 per cent FCS + 10 µg LH + 1 µg FSH + 1 µg E_2 -17 ß/ml

GROUP II

T_1 TCM 199 + 10 per cent EBS

T_2 TCM 199 + 10 per cent EBS + 10 µg LM/ml

T_3 TCM 199 + 10 per cent EBS + 1 µg FSH/ml.

T_4 TCM 199 + 10 per cent EBS + 1 µg E_2 - 17 ß/ml

T_5 TCM 199 + 10 per cent EBS + 10 µg LH + 1 µg FSH + 1 µg E_2 - 17 ß/ml

The culture was done in 2 ml of media with 10-20 oocytes in 6 well multidishes for 27 h under 5 per cent carbondioxide in air with 100 per cent humidity at 39°C. At the end of the culture period, the oocytes were examined for maturation (M II).

3.5.2 Different concentration of serum

In part second of this experiment, seven hudnred and twenty six oocytes were aspirated from 2-5 mm diameter follicles with an intact unexpanded cumulus and evenly granulated ooplasm were included for study. Maturation *in vitro* was accomplished in TCM 199 media supplemented with 10 ug LH/ml, 1 ug FSH/ml, 1 ug E_2ml and with 10, 15 or 20 per cent of FCS or EBS and studied their maturation rate. The culture condition was similar to that of experiment III.

3.6 EXPERIMENT IV

3.6.1 Cumulus cells expansion and oocyte maturation rate

Six hundred and fifty one cumulus oocyte complexes (COC) aspirated from 2-5 mm diameter follicles were subjected to maturation in the following media.

1. TCM 199 + 10 per cent ECS
2. TCM 199 + 20 per cent EBS
3. TCM 199 + 1 ug FSH/ml
4. TCM 199 + 1 ug E_2 - 17 ß/ml

Culture conditions were similar to that of Experiment III.

After 27 h of culture, the oocytes were reexamined under the stereomicroscope at 50 x and rolled during examination. The number of expanded cumulus oophorus oocytes under different media and the percentage of germinal vesicle breakdown (GVBD) were recorded.

3.6.2 Setting of culture

Ten, 20, 30 and 40, COC were either distributed in 2 ml of culture media or in the 100 µl drops of culture media under paraffin oil in 6 well multidishes. The petridishes were partially opened to facilitate the air circulation. The culture media was TCM 199 supplemented with 10 µg LH, 1 µg FSH and 1 µg E_2 - 17ß/ml. The culture was done at 37°C in a gas phase of 5 per cent carbondioxide in air with 100 per cent humidified incubator.

Each individual combination was replicated 3 times with proper control for each replicate. After 27 h, the colour of the media and percentage of the maturation of the oocytes were recorded.

3.7 STATISTICAL METHODS

The data collected were analysed as per standard procedure prescribed by Snedecor and Cochran (1967).

CHAPTER 4

RESULTS

4.1 BIOMETRY OF OVARIES

The average length, width, height, weight and volume of right and left ovaries in the four stages of estrous cycle are presented in Table 2. The preliminary analysis showed no significant variation in the biometry of ovaries between sides.

The mean length of right and left ovaries in four stages of the cycle were 1.88 ± 0.05, 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 ± 0.09 cm respectively. In both the ovaries, the length was maximum in the second stage of the cycle and was the least during the first stage. The length increased from first to second stage of the cycle and gradually decreased to the fourth stage. Analysis of variance (Table 3) however, revealed no significant variation in length at different stages of the cycle. The overall mean length of right and left ovary was 2.06 ± 0.03 and 2.0 ± 0.04 cm respectively. There was no siginificant difference in length of the ovaries between sides.

The mean height of the right and left ovaries noted was 1.60 ± 0.02 and 1.51 ± 0.03 cm respectively. On right side, the maximum height was 1.79 ± 0.04 cm during the second stage of the cycle and it gradually decreased to 1.64 ± 0.05, 1.52 ± 0.04 and 1.43 ± 0.02 cm in III, IV and I stages of the cycle. For the left ovary, the height was maximum during the III stage of the cycle with 1.61 ± 0.06 followed by

TABLE 2

BIOMETRY OF OVARIES OF NON-DESCRIPT SHE BUFFALOES

Side	Stage of the cycle	Length (cm)	Width (cm)	Height (cm)	Weight (g)	Volume (ml)
Right	I (44)	1.88 ± 0.05	1.32 ± 0.04	1.43 ± 0.02	2.67 ± 0.09	7.52 ± 0.63
	II (46)	2.27 ± 0.04	1.39 ± 0.04	1.79 ± 0.04	2.97 ± 0.12	10.79 ± 0.97
	III (46)	2.15 ± 0.06	1.38 ± 0.04	1.64 ± 0.05	3.01 ± 0.11	9.20 ± 0.63
	IV (45)	1.91 ± 0.07	1.31 ± 0.05	1.52 ± 0.04	2.79 ± 0.10	7.56 ± 0.76
	Overall Mean (181)	2.06 ± 0.03	1.35 ± 0.03	1.60 ± 0.02	2.86 ± 0.06	8.78 ± 0.40
Left	I (44)	1.89 ± 0.02	1.29 ± 0.04	1.41 ± 0.06	2.66 ± 0.08	7.21 ± 0.05
	II (46)	2.14 ± 0.05	1.31 ± 0.04	1.51 ± 0.05	2.86 ± 0.10	8.59 ± 0.69
	III (46)	1.99 ± 0.07	1.35 ± 0.05	1.61 ± 0.06	2.89 ± 0.13	9.51 ± 1.24
	IV (45)	1.89 ± 0.09	1.25 ± 0.06	1.49 ± 0.04	2.65 ± 0.13	8.00 ± 0.82
	Overall Mean (181)	2.00 ± 0.04	1.30 ± 0.03	1.51 ± 0.03	2.77 ± 0.06	8.34 ± 0.44

Figures in parentheses are the number of specimens.

TABLE 3

ANALYSIS OF VARIANCE FOR LENGTH, WIDTH, HEIGHT, WEIGHT AND VOLUME OF OVARIES OF SHE BUFFALOES

Source of Variation	df	Length	Width	Height	Weight	Volume
		MS	MS	MS	MS	MS
Between stages of the cycle	3	0.4902^{NS}	0.0856^{NS}	0.1601^{NS}	0.6689^{NS}	39.3193^{NS}
Error	177	0.2354	0.1162	0.2707	0.6247	34.1737

NS - Not signficant

II stage of the cycle (1.51 ± 0.05) and the same was the least during the first stage of the cycle (1.41 ± 0.06). Hence, in both the ovaries the height was the least during the I stage of the cycle. Tough there was a slight variation in the height of both sides of the ovaries, the statistical analysis revealed no significant difference in height between the stages of the cycle (Table 3).

The slight variation in length and height of the ovary between the stages of the cycle might be due to the presence of cyclic corpus luteum or the follicles in the ovary as the case may be.

The mean width of the right and left ovaries in four stages of the cycle was 1.32 ± 0.04, 1.39 ± 0.04, 1.38 ± 0.04, 1.31 ± 0.05 and 1.29 ± 0.04, 1.31 ± 0.04, 1.35 ± 0.05 and 1.25 ± 0.06 cm respectively. The mean width of the right and left ovaries was found to be 1.35 ± 0.03 and 1.30 ± 0.03 cm respectively. The width was almost equal in all the four stages of the cycle and between the sides. Statistical analysis (Table 3) revealed no significant difference in width between the four stages of the cycle.

The average weight of the right and left ovaries were 2.86 ± 0.06 and 2.77 ± 0.06 gm respectively with a range of 0.55 to 4.92 g. The mean weight of the right and left ovaries in the four stages of the cycle was 2.67 ± 0.09, 2.97 ± 0.12, 3.01 ± 0.11, 2.79 ± 0.10 and 2.66 ± 0.08, 2.86 ± 0.10, 2.89 ± 0.13 and 2.65 ± 0.13 g, respectively. The weight was almost equal in II and III stages which was higher than the weight in I and IV stages of the cycle. But on critical analysis between I-II, I-III and IV-II, IV-III stages, the same revealed no significant difference. This showed that the ovarian weight was maximum during the second stage of the cycle and maintained as such till the end of the III stage of the cycle and decreased in the subsequent stages of the cycle, because the

TABLE 4

CORRELATION COEFFICIENTS BETWEEN LENGTH, WIDTH, HEIGHT, WEIGHT AND VOLUME OF THE LEFT AND RIGHT OVARIES DURING FOUR STAGES OF THE ESTROUS CYCLE IN SHE BUFFALOES

Characters correlated	Stage I (44)		Stage II (46)		Stage III (46)		Stage IV (45)	
	Left	Right	Left	Right	Left	Right	Left	Right
Length : width	0.653**	0.673**	0.401**	0.608**	0.792**	0.406**	0.807**	0.675**
Length : height	0.901**	0.818**	0.223NS	0.548**	0.893**	0.214NS	0.827**	-0.762**
Length : weight	0.954**	0.389**	0.934**	0.853**	0.832**	0.771**	0.912**	0.726**
Length : volume	0.913**	0.689**	0.652**	0.872**	0.891**	0.623**	0.848**	0.914**
Width : height	0.818**	0.798**	0.681**	0.666**	0.852**	0.271*	0.721**	0.786**
Width : weight	0.561**	0.650**	0.332*	0.691**	0.714**	0.723**	0.724**	0.486**
Width : volume	0.952**	0.715**	0.791**	0.715**	0.890**	0.760**	0.972**	0.755**
Height : weight	0.681**	0.940**	0.634**	0.603**	0.801**	0.423*	0.588**	0.555**
Height : volume	0.883**	0.839**	0.852**	0.884**	0.762**	0.981**	0.831**	0.919**
Weight : volume	0.706**	0.877**	0.481*	0.702**	0.811**	0.552**	0.761**	0.638**

NS - Not significant ($P > 0.05$)

* - Significant at 5% level ($p < 0.05$)

** - Significant at 1% level ($p < 0.01$)

Figures in parentheses are the number of specimens.

subsequent results of this study showed that the corpus luteum weight decreased in the IV stage of the cycle. This decrease in weight of the corpus luteum was partially compensated by the developing follicle so that there was no significant difference between the III and IV and I stages of the cycle.

The mean volume of right and left side ovaries in four stages of the cycle were 7.52 ± 0.63, 10.79 ± 0.97, 9.20 ± 0.63, 7.56 ± 0.76 and 7.21 ±0.05, 8.59 ± 0.69, 9.51 ± 1.24 and 8.00 ± 0.82 ml respectively. The overall mean volume of right and left side ovary was 8.78 ± 0.40 and 8.34 ± 0.44 ml respectively. Statistical analysis (Table 3) revealed that there was no significant difference between the sides and stages of the ovary.

The correlation co-efficients between different biometrical parameters of both the sides of the ovary in four stages of the cycle studied are presented in Table 4. Correlation coefficients between length and height of both right and left ovaries at IIand III stages alone were not found to be significant while all others were found to be significant at 5 per cent or 1 per cent level.

4.2 CORPUS LUTEUM

The measurments and weight of the corpus luteum during the four stages of the cycle are given in Table 5. The average length of the corpus luteum during the four stages of the cycle were 0.68 ± 0.02, 1.47 ± 0.03, 1.27 ± 0.2 and 0.94 ± 0.02 cm in I, II, III and IV stages respectively. The overall mean length was 1.09 ± 0.01 cm. The length was maximum during the II stage of the cycle and tended to remain relatively constant in the III stage of the cycle and reduced during the IV stage of the cycle.

TABLE 5

BIOMETRY OF CORPUS LUTEUM DURING FOUR STAGES OF THE ESTROUS CYCLE IN SHE BUFFALOES

Stage of the Cycle	Length (cm)	Diameter (cm)	Weight (g)
I (44)	0.68 ± 0.02	0.62 ± 0.02	0.75 ± 0.03
II (46)	1.47 ± 0.03	1.01 ± 0.03	1.13 ± 0.01
III (46)	1.27 ± 0.02	1.04 ± 0.02	1.11 ± 0.01
IV (44)	0.94 ± 0.02	0.87 ± 0.02	0.77 ± 0.03
Mean	1.09 ± 0.01	0.89 ± 0.02	0.93 ± 0.01

Figures in parentheses are the number of pairs of ovaries examined.

TABLE 6

ANALYSIS OF VARIANCE FOR LENGTH, DIAMETER AND WEIGHT OF THE CORPUS LUTEUM IN SHE BUFFALOES

Source of variation	Length		Diameter		Weight	
	df	MS	df	MS	df	MS
Between Stages	3	9.546**	3	2.381**	3	3.033**
Error	290	0.047	274	0.039	275	0.038

** Highly Significant ($p < 0.01$)

The diameter and weight in I, II, III and IV stages of the cycle were 0.62 ± 0.02, 1.01 ± 0.03, 1.04 ± 0.02 and 0.87 ± 0.02 cm and 0.75 ± 0.03, 1.13 ± 0.01, 1.11 ± 0.01 and 0.77 ± 0.03 g respectively. Similar to the length, the diameter and weight were maximum during the second and third stages and decreased at IV and I stages of the cycle. Analysis of variance of length, diameter and weight of the corpus luteum revealed that differences between stages were highly ($p < 0.01$) significant as shown in table 6. Critical analysis of the data between II and III stage of the cycle revealed no significant difference in diameter and weight while the same between I-II, I-III, IV-II, IV-III stages significantly differed. The overall mean diameter and weight of the corpus luteum was 0.89 ± 0.02 cm and 0.93 ± 0.01 g respectively.

Externally the colour of the corpus luteum was observed as bright red in the first stage of the cycle, flesh colour with reddish tip in the second stage, tan in the third stage and grey or white in the fourth stage of the cycle.

4.3 FOLLICLE

4.3.1 Follicular dynamics in relation to

4.3.1.1 The different stages of the cycle

Follicles at different stages of the estrus cycle are shown in table 7. The mean number of follicles on the ovarian surface was 3.83 and 3.45 in the right and left ovaries respectively. Total number of follicles in right and left side of the ovary was not significantly different. The mean number of follicles was more viz., 4.18 ± 0.11 during the IV stage of the cycle than in other stages of the oestrous cycle. In the second and third stages, numbers of follicles of 3.41 ± 0.11 and 3.39 ± 0.12 respectively were almost

TABLE 7

MEAN NUMBER OF FOLLICLES UNDER DIFFERENT DIAMETER RANGES IN FOUR STAGES OF THE ESTROUS CYCLE

Stage of the Cycle	Side	Follicular diameter ranges (mm)				No. of follicles/ovary
		≤ 2	3 - 5	6 - 10	> 10	
I (21)	Right	0.91 ± 0.18	1.86 ± 0.19	0.91 ± 0.18	0.13 ± 0.01	3.78 ± 0.17
	Left	1.00 ± 0.12	1.62 ± 0.14	0.67 ± 0.13	0.14 ± 0.01	3.39 ± 0.12
	Mean	0.96 ± 0.12	1.74 ± 0.13	0.79 ± 0.13	0.14 ± 0.08	3.59 ± 0.13
II (26)	Right	0.42 ± 0.14	1.96 ± 0.16	1.00 ± 0.14	0.16 ± 0.02	3.54 ± 0.12
	Left	0.35 ± 0.12	1.85 ± 0.15	0.89 ± 0.13	0.19 ± 0.02	3.28 ± 0.14
	Mean	0.39 ± 0.11	1.91 ± 0.11	0.95 ± 0.12	0.18 ± 0.07	3.41 ± 0.11
III (42)	Right	0.72 ± 0.11	1.62 ± 0.13	0.95 ± 0.10	0.33 ± 0.01	3.62 ± 0.11
	Left	0.62 ± 0.12	1.48 ± 0.11	0.81 ± 0.06	0.24 ± 0.10	3.15 ± 0.09
	Mean	0.67 ± 0.08	1.55 ± 0.09	0.88 ± 0.09	0.29 ± 0.05	3.39 ± 0.12
IV (32)	Right	1.37 ± 0.16	1.67 ± 0.13	1.00 ± 0.16	0.40 ± 0.11	4.38 ± 0.14
	Left	1.22 ± 0.12	1.40 ± 0.09	1.13 ± 0.19	0.23 ± 0.11	3.98 ± 0.12
	Mean	1.27 ± 0.09	1.54 ± 0.10	1.07 ± 0.11	0.31 ± 0.06	4.18 ± 0.11

Figures in parentheses are the number of specimens.

equal and little less than the I stage of the cycle with 3.59 ± 0.13. Statistical analysis revealed that the difference in four stages of the cycle was not significant (Table 8).

Mean number of follicles with (≤ 2 mm diameter) in four stages of the cycle were 0.96 ± 0.12, 0.39 ± 0.11, 0.67 ± 0.08 and 1.27 ± 0.09 respectively. Mean number of ≤ 2 mm follicles was more during the IV stage of the cycle which gradually decreased to I and II stage of the cycle. There was again an increase in the number of follicles in the III stage of the cycle indicating that there was two waves of follicular activity in buffalo ovary. The difference in total number of ≤ 2 mm diameter follicles in four stages of the cycle was statistically highly significant ($p < 0.01$) (Table 8).

Mean number of 3-5 mm diameter follicles in four stages of the cycle were 1.74 ± 0.13, 1.91 ± 0.11, 1.55 ± 0.09 and 1.54 ± 0.10 respectively. The changes in the diameter during different reproductive stages were not significant. However, the number of 3-5 mm diameter follicles was more during the second stage of the cycle.

Mean number of 6-10 mm diameter follicles in four stages of the cycle were 0.79 ± 0.13, 0.95 ± 0.12, 0.88 ± 0.09 and 1.07 ± 0.11 respectively. Though there was slight variation in the number of 6-10 mm diameter follicles in four stages of the cycle the same was not found to be significant. Similarly the mean number of follicles of > 10 mm diameter in four stages of the cycle were 0.14 ± 0.08,0.18 ± 0.07, 0.29 ± 0.05 and 0.31 ± 0.06 with the difference being not significant. The diameter of the largest follicle observed was 2.1 cm.

TABLE 8

ANALYSIS OF VARIANCE FOR THE MEAN NUMBER OF FOLLICLES UNDER DIFFERENT RANGES IN FOUR STAGES OF THE ESTROUS CYCLE

		Follicular diameter ranges			
Source of Variation	**df**	**≤ 2**	**3 - 5**	**6 - 10**	**> 10**
Between different stages of the cycle	3	8.42^{**}	1.44^{NS}	0.73^{NS}	0.49^{NS}
Error	238	0.60	0.65	0.70	0.24

NS - Not significant ($p > 0.05$)

** - Highly significant ($p < 0.01$)

4.3.1.2 The different months of a year

Total number under different diameter ranges of follicles observed in 16 pairs of ovaries per month for a period of one year from July 1988 to June 1989 are shown monthwise in table 10.

Follicles with different diameter ranges were present on the ovarian surface throughout the year, but the total number of follicles per ovary and number of follicles in each diameter ranges were variable in different months of a year. The mean number of follicles per ovary from the month of July 1988 to June 1989 was 3.94, 4.15, 4.06, 4.25, 4.72, 4.69, 4.53, 3.78, 3.41, 3.22, 3.03 and 3.81 respectively. Total number of follicles per ovary was maximum during the month of November and was the least during the month of May. It was almost constant during the months of October to January. This revealed that the ovarian activity was peak during this period of the year. After January, there was a steady decline in the mean number of follicles per ovary upto the month of May. This might be due to the rise in temperature and low rain fall during this period as shown in table 9. From June onwards, there was a slow increase in the total number of follicles per ovary. Though there was a variation in the mean number of follicles throughout the year, it was not statistically significant (Table 11).

Total number of follicles in follicular diameter range of $\leq$ 2 mm was found steadily increasing from the month of August to January to 26, 31, 37, 29, 36 and 27 respectively in 32 ovaries indicating that more number of follicles were recruited for development because of reduction in ambient temperature and increased rainfall (Table 9) during this period. Similarly mean number of follicles in follicular diameter in the range of 6-10 and > 10 mm also increased from August to January.

TABLE 9

MONTHLY MEAN AND RANGE OF TEMPERATURE AND RAIN FALL FROM JULY 1988 TO JUNE 1989 IN MADRAS (STUDY PERIOD)

Months		Temperature (°C)		Rain fall (mm)
		Mean	Range	
July	1988	34.8	37.7 - 29.0	126.3
August	1988	33.7	37.7 - 29.0	316.8
September	1988	33.1	36.3 - 29.1	165.3
October	1988	33.6	35.9 - 31.5	133.5
November	1988	29.2	32.4 - 26.9	527.6
December	1988	29.0	30.1 - 27.7	100.7
January	1989	29.0	31.2 - 26.7	014.6
February	1989	30.9	33.9 - 29.1	000.0
March	1989	33.2	37.0 - 28.9	030.8
April	1989	35.9	40.8 - 31.9	000.0
May	1989	38.8	41.6 - 35.8	002.8
June	1989	36.8	40.0 - 32.4	109.8

Source : National Meteorology Lab, Madras.

TABLE 10

MONTHWISE MORPHOMETRY OF FOLLICLES UNDER DIFFERENT DIAMETER RANGES IN SHE BUFFALOES

Month and Year		Follicular diameter range (mm)				Total no. of follicles	Mean no. of follicles/ovary
		≤ 2	3-5	6-10	> 10		
July	1988	20	64	31	3	118	3.94
August	1988	26	67	34	6	133	4.15
September	1988	31	61	30	8	130	4.06
October	1988	37	64	39	4	144	4.25
November	1988	29	72	46	4	151	4.72
December	1988	36	69	42	5	150	4.69
January	1989	27	73	40	5	145	4.53
Feburary	1989	18	76	25	2	121	3.78
March	1989	19	69	19	2	109	3.41
April	1989	13	67	22	1	103	3.22
May	1989	11	64	20	2	97	3.03
June	1989	19	69	32	2	122	3.81

TABLE 11

ANALYSIS OF VARIANCE FOR DIFFERENT DIAMETER RANGE OF FOLLICLES IN 12 MONTHS PERIOD

Source of variance	D.F.	S.S.	M.S.	F
Between different diameter ranges	3	9239.79	3079.93	179.18**
Between months	11	17.78	1.62	0.094^{NS}
Error	33	567.23	17.19	1.00

N.S. - Not significant

** - Highly significant ($p < 0.01$).

The follicles of 3-5 mm diameter range was almost constant throughout the year. Statistical analysis revealed that various months in a year significantly affect the development of various follicles of different diameter ranges, but with in eeach range the months had no effect (Table 11).

Fluid filled cavities (Lacunae) were seen in 35 out of 327 dioestrous corpura lutea (10.7 per cent) examined and none of the corpus luteum of follicular phase was found with a lacunae.

4.3.2 Follicular contents

4.3.2.1 Follicular fluid

The colour of the follicular fluid was straw yellow and slightly viscous. The viscosity was not constant in follicles of different sizes and was observed to reduce as follicle size increased. The mean volume of follicular fluid in different size is shown in fig.1 which indicates that there was a gradual increase in the volume of fluid as the diameter of the follicle increased. The fluid volume was constant in follicular diameter of 6 and 7 and thereafter there was a steep rise in the volume of the fluid. The maximum fluid volume collected during this study was 1.26 ml in a follicle with a diameter of 2.1 cm. The mean volume of the follicular fluid in 3-5, 6-10 and > 10 mm diameter follicular ranges were 0.033 ± 0.001, 0.103 ± 0.009, 0.464 ± 0.049 ml respectively. The volume of the fluid in ≤ 2 mm diameter follicles was negligible.

4.3.2.2 Diameter of the oocyte

The mean diameter of the buffalo follicular oocyte was 157.37 ± 1.82 μ with a range of 130-169 μ. The mean diameter of the oocyte in different follicular ranges of ≤

FIG.1
VOLUME OF FOLLICULAR FLUID IN RELATION TO FOLLICULAR DIAMETER

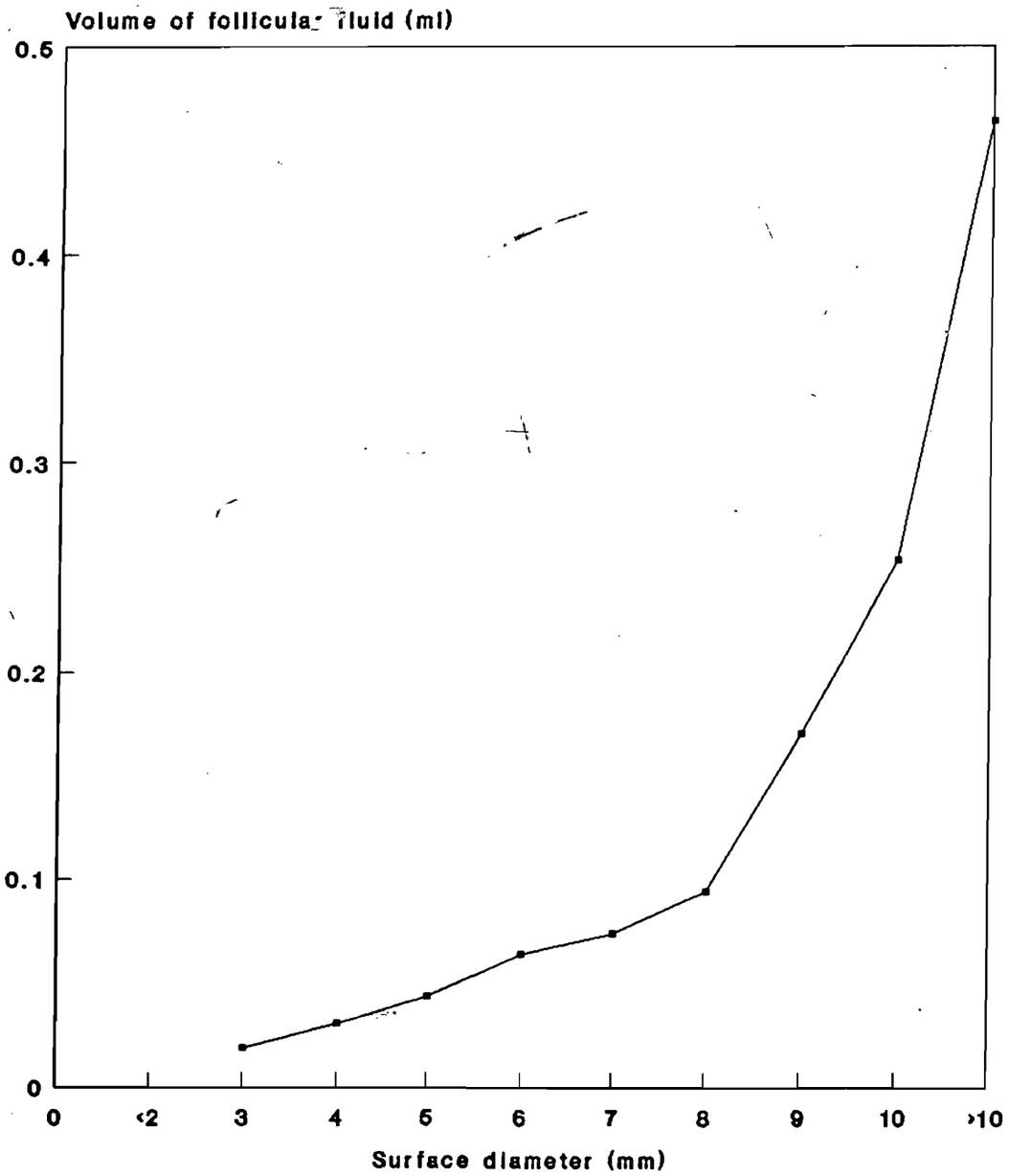


TABLE 12

MEAN DIAMETER OF OOCYTE IN RELATION TO DIFFERENT FOLLICULAR DIAMETER RANGES

	Follicular diameter range (mm)				Mean
	≤ 2	3-5	6-10	> 10	
Ooplasm diameter (μ)	130.89 ± 2.59	130.39 ± 0.60	132.81 ± 0.94	131.55 ± 1.71	131.41 ± 1.67
Zona pellucida diameter (μ)	26.15 ± 0.28	26.05 ± 0.34	26.15 ± 0.28	26.28 ± 0.16	26.16 ± 0.27
Total oocyte diameter (μ)	156.87 ± 2.59	156.53 ± 0.61	158.96 ± 0.95	157.13 ± 1.94	157.37 ± 1.82

2, 3-5, 6-10 and > 10 mm were 156.87 ± 2.59, 156.53 ± 0.61, 158.96 ± 0.95 and 157.13 ± 1.94 μ respectively (Table 12). Analysis of variance showed that there was no signifcant difference in oocyte diameter betweeen different diameter ranges.

Mean diameter of the zona pelluceda was 26.16 ± 0.27 μ. The same in different follicular diameter ranges were almost constant with 26.15 ± 0.28, 26.05 ± 0.34, 26.15 ± 0.28 and 26.28 ± 0.16 μ. This indicating that there was no change in the diameter of the zona from follicles of ≤ 2 mm to the preovulatory size. In only one oocyte out of 1672 oocytes examined, the diameter of the zona pelluceda was 39 μ. In an another oocyte the diameter of the zona pellucida was normal but the diameter of the ooplasm was 169 μ. The mean diameter of ooplasm in different follicle ranges of ≤ 2, 3-5, 6-10 and > 10 mm sizes were 130.89 ± 2.59, 130.39 ± 0.60, 132.81 ± 0.94 and 131.55 ± 1.71 respectively.

4.3.2.3 Oocyte recovery rate

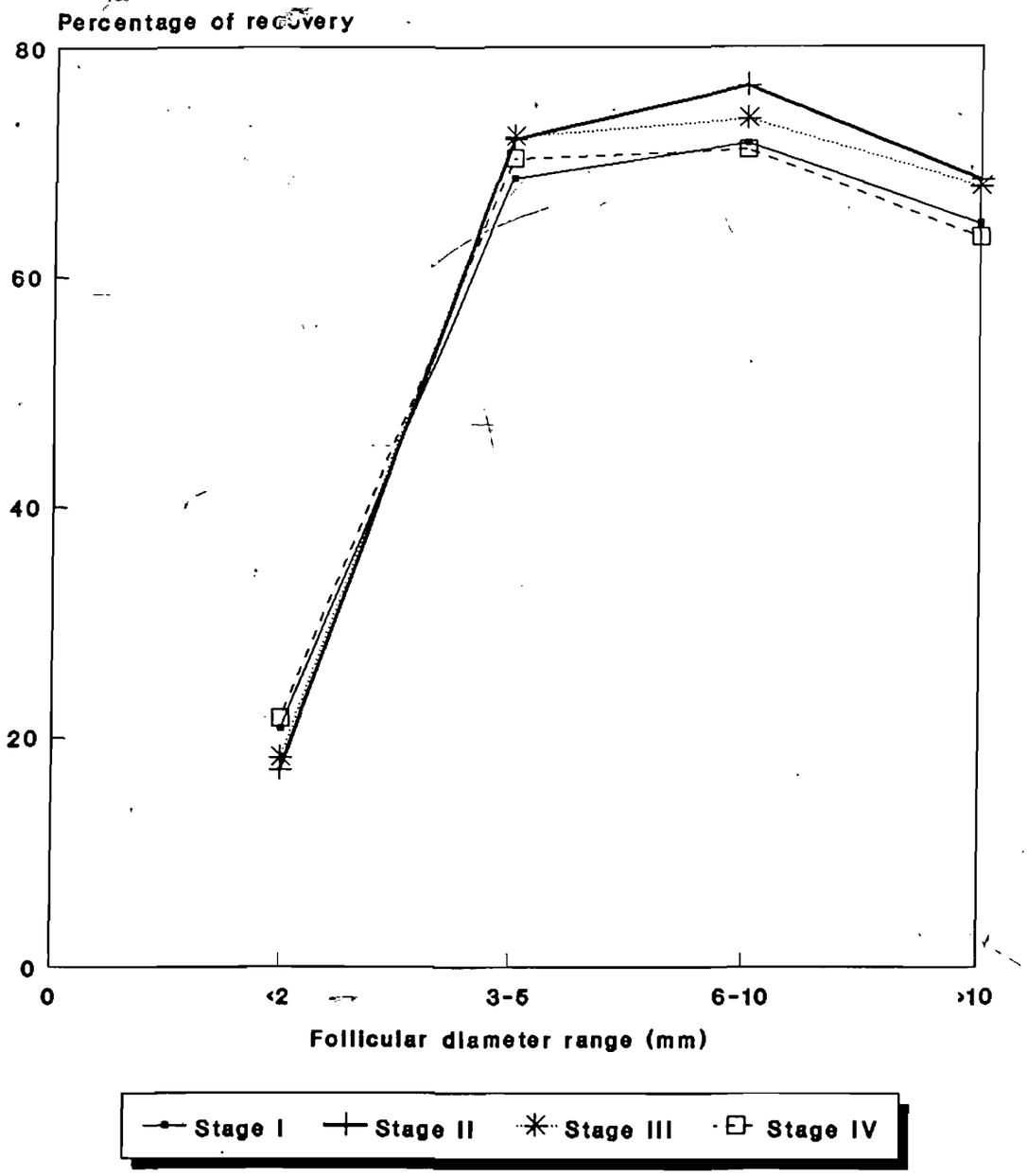
The mean percentage of oocyte recovery was 57.33 ± 5.54. The recovery rate in different stages of the cycle were 56.30 ± 2.01, 58.51 ± 4.35, 57.95 ± 7.62 and 56.55 ± 7.63 per cent in I, II, III and IV stages of the cycle respectively (Table 13). The recovery rate was almost equal and not much difference was noticed between the stages of the cycle, but the recovery rate was low in follicles ≤ 2 mm diameter (19.43 ± 5.31) and moderate in follicles above 10 mm diameter (65.95 ± 7.38). There was no difference in the recovery rate of follicular diameter range of 3-5 and 6-10 mm (70.71 ± 7.65 and 73.24 ± 6.28 per cent respectively (Fig.2).

TABLE 13

PERCENTAGE OF OOCYTE RECOVERY RATE IN RELATION TO FOLLICULAR DIAMETER RANGE IN FOUR STAGES OF THE ESTROUS CYCLE

Stages of the cycle	Follicular diameter range (mm)				Mean
	≤ 2	3-5	6-10	> 10	
I	20.72 ± 1.18	68.47 ± 3.23	71.52 ± 4.32	64.48 ± 3.21	56.30 ± 2.01
II	17.14 ± 4.30	71.98 ± 8.60	76.63 ± 2.60	68.27 ± 2.42	58.51 ± 4.35
III	18.18 ± 2.62	72.14 ± 8.32	73.74 ± 5.67	67.72 ± 8.30	57.95 ± 7.62
IV	21.62 ± 7.10	70.24 ± 8.66	71.01 ± 9.78	63.32 ± 9.62	56.55 ± 7.63
Mean	19.43 ± 5.31	70.71 ± 7.65	73.24 ± 6.28	65.95 ± 7.38	57.33 ± 5.54

FIG.2
EFFECT OF FOLLICULAR DIAMETER ON
OOCYTE RECOVERY RATE



4.3.3 Characterization of the oocyte in relation to

4.3.3.1 Size of the follicle

Follicles of 3 to 5 mm diameter tended to have high proportion of oocytes (63.92 per cent) possessing compact, complete investment than 6-10 and > 10 diameter follicles. As the follicle size increased, it was observed that the percentage has gradually decreased to 54.59, 36.17 per cent in 6-10 and > 10 mm diameter follicles respectively (Table 14). But it was not significantly different from ≤ 2 mm follicles.

Partial cumulus oophorus oocytes were more i.e. 40.43 per cent in the follicles of > 10 mm diameter which were highly significant ($P < 0.01$) from those of the other three groups. But between the first three groups, the percentage of partial cumulus oophorus oocytes were not significantly different. The percentage of nude oocytes were more i.e. 23.40 in follicular diameter range of > 10 mm. However, statistical analysis revealed that there was no significant difference in between follicles of different sizes. It clearly indicated that the percentage of degeneration has increased as the follicle size increased.

The percentage of even ooplasm granules oocytes of ≤ 2, 3-5, 6-10 and > 10 mm follicular diameter ranges were 57.14, 83.14, 81.19 and 65.96 per cent respectively (Table 14). The percentage between 3-5 and 6-10 mm follicular diameter ranges were not significantly different but with other two groups, the percentage was highly significant. The clumped oocytes granules in different follicular diameter ranges were 36.02, 14.76, 15.14 and 29.76 per cent. The percentage was more in follicles of ≤ 2 and > 10 mm diameter follicles than other two groups. The percentage of degenerated oocytes in all the four groups were not statistically different.

TABLE 14

EFFECT OF FOLLICULAR DIAMETER RANGE ON OOCYTE TYPE

Types of characterization	Oocyte type	Follicular diameter range (mm)				Mean
		≤ 2	3-5	6-10	> 10	
Cellular Investment	Compact	62.73 (101/761)	63.92 (163/255)	54.59 (119/218)	36.17 (17/47)	54.35 (400/681)
	Partial	19.88 (32/101)	21.18 (54/255)	26.61 (58/218)	40.43 (19/47)	27.25
	Nude	17.39 (28/161)	14.90 (38/255)	18.80 (41/218)	23.40 (11/47)	18.17
Ooplasm granules	Even	57.14 (92/161)	83.14 (212/255)	81.19 (177/218)	65.96 (31/47)	71.86
	Clumped	36.02 (58/161)	14.76 (35/255)	15.14 (34/218)	29.76 (14/47)	23.92
	Degenerated	6.83 (11/161)	2.10 (8/255)	3.21 (7/218)	4.25 (4/47)	4.09

Tendency for effect of size of the follicle on investment : 16.9

Tendency for effect of size of the follicle on Ooplasm granules : 43.11

4.3.3.2 Stage of the cycle

Microscopical examination of the oocytes aspirated from follicles in ovaries at different stages of the cycle revealed characteristic changes. The observed oocytes were classified into three groups based on the cellular investment as cumulus-oocyte-complex (COC), Partial and nude oocytes.

The percentages of COC complexes were more i.e. 66.43 ± 8.63 and 68.01 ± 4.23 per cent in II and III stages of the cycle respectively. On critical analysis, the percentage of COC complexes between third and second stages were not significantly different, but the same at first stage (57.11 ± 11.27 per cent) was significantly different in comparison to that of II and III stages. The percentage of COC complexes of 58.40 ± 5.63 per cent in the IV stage of the cycle was not significantly different from the I stage of the cycle, but significantly different from that of II and III stages. The mean percentage of COC complex was 62.45 ± 6.54 per cent (Table 15).

The percentage of partial cumulus oophorus oocyte or thin corona radiata cell oocytes were low, 14.55 ± 1.14 and 19.0 ± 4.52 in II and III stages of the cycle, where as it was high in IV and I stages of the cycle with 29.1 ± 3.67 and 25.32 ± 1.28 per cent respectively. The percentage of nude oocytes were low in III and IV stages (13.03 ± 1.32 and 12.5 ± 3.12) which gradually increased in I and II stages (17.57 ± 2.75 and 19.01 ± 1.48 per cent). The different types of oocytes in four stages of the cycles were highly significant ($p < 0.01$).

The first group oocytes had uniform distribution of ooplasm granules in evenly filled ooplasm. In the second group, there were clumped ooplasm granules in the evenly filled ooplasm. Oocytes with degenerated ooplasm were observed to be in the third group.

TABLE 15

EFFECT OF FOUR STAGES OF THE ESTROUS CYCLE ON OOCYTE TYPE

Stages of the cycle	Total No. of Oocytes	Cellular Investment						Condition of Ooplasm					
		Compact		Partial		Nude		Even granuglation		Clumped		Degenerated	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
I	387	221	57.11 ± 11.27	98	25.32 ± 1.28	68	17.57 ± 2.75	263	67.96 ± 16.32	112	28.88 ± 3.12	12	3.16 ± 1.12
II	426	283	66.43 ± 8.63	62	14.55 1.14	81	19.01 ± 1.48	352	82.63 ± 11.18	62	14.55 ± 2.74	12	2.82 ± 0.87
III	422	287	68.01 ± 4.23	80	19.0 ± 4.52	55	13.03 ± 1.32	346	81.99 ± 9.01	61	14.45 ± 2.67	15	2.56 ± 0.92
IV	488	285	58.40 ± 5.63	142	29.1 ± 3.67	61	12.5 ± 3.12	392	80.33 ± 11.12	82	16.59 ± 4.42	14	3.08 ± 0.34
Mean	1723	1076	62.45 ± 6.54	382	22.17 ± 1.82	265	15.38 ± 4.81	1353	78.53 ± 13.61	317	18.38 ± 3.74	53	3.09 ± 0.17

Tendency for effect of stage on investment $\chi^2 = (p < 0.01)$

Tendency for effect of stage on ooplasm granuls $\chi^2 = 33.92$ $(p < 0.01)$

Except first stage, the percentage of even granulation oocyte was more in other three stages as shown in table 15. The percentage of even cytoplasmic granular oocytes in I, II, III and IV stages of the cycle were 67.96 ± 16.32, 82.63 ± 11.18, 81.99 ± 9.01 and 80.33 ± 11.12 respectively. Proportionately the clumped ooplasm granules' oocyte percentage was more in first stage (28.88 ± 3.12) than in other three stages (14.55 ± 2.74, 14.45 ± 2.67, 16.59 ± 4.42). The percentage of degenerated ooplasm oocytes were almost constant in all the four stages of the oocyte. The mean percentage of even ooplasm granules, clumped and degenerated ooplasm oocytes were 78.53 ± 13.16, 18.38 ± 3.74 and 3.09 ± 0.17 per cent respectively.

4.3.3.3 Presence or absence of corpus luteum

There was a significant difference in good quality oocytes collected from ovaries with corpus luteum or without corpus luteum. The percentage of COC and even ooplasm granules of ovaries with corpus luteum and without corpus luteum were 76.19 and 64.36 per cent respectively (Table 16). The percentage of COC with even granulation was more in ovaries with corpus luteum than without corpus luteum. Statistical analysis reveals that the difference was not significantly different. However the difference between the even granulation was significant ($p < 0.05$) between ovaries with and without corpus luteum. The percentage of partial and nude oocytes in ovaries with and without corpus luteum were 12.14, 11.67 and 24.12 and 11.57 respectively. The percentage was almost equal in ovaries with corpus luteum, but ovaries without corpus luteum had higher percentage of partial oocytes than nude which was highly significant ($p < 0.01$). The percentage of nude oocytes was almost equal both in ovaries with corpus luteum or without corpus luteum which was not statistically different.

TABLE 16

EFFECT OF CORPUS LUTEUM ON OOCYTE TYPE

	Oocyte type	With corpus Luteum		Without corpus luteum	
		No	%	No	%
Cellular Investment	Compact	496	76.19	419	64.36
	Partial	79	12.14	157	24.12
	Nude	76	11.67	75	11.57
Ooplasm granules	Even	579	88.93	443	68.05
	Clumped	57	8.76	184	28.26
	Degenerated	15	2.31	24	3.68

Tendency for effect of CL on Investement χ^2 32.266^{**} ($p < 0.01$)

Tendency for effect of CL on Ooplasm χ^2 25.389^{**} ($p < 0.01$)

The percentage of clumped ooplasm granules was lesser in ovaries with corpus luteum i.e. 8.76 per cent than from ovaries without corpus luteum with 28.26 per cent. The percentage of oocytes with degenerated ooplasm was almost similar in both the types of ovaries. The overall effect of the corpus luteum on the ooplasm condition was highly significant ($p < 0.01$).

4.4 ISOLATION OF OOCYTES AND ITS Percentage OF RECOVERY AND MATURATION RATE

Mean number of oocytes liberated from each ovary by aspiration, rupture and dissection methods were 2.71, 3.09, and 5.57 respectively (Table 17). The total number of oocytes recovered by dissection method was almost doubled when compared to the aspiration method. In rupture method, the number of oocytes liberated were slightly more than the aspiration method which was, however, not significant. The difference between aspiration rupture with that of dissection method was more. Statistical analysis showed that the difference was highly significant.

Three major types of oocytes were encountered after aspiration, rupture and dissection methods. Those oocytes completely devoid of cumulus cells with degenerated ooplasm was third quality oocyte and oocytes with COC and even granulation was first quality oocyte. The remaining was second quality oocytes.

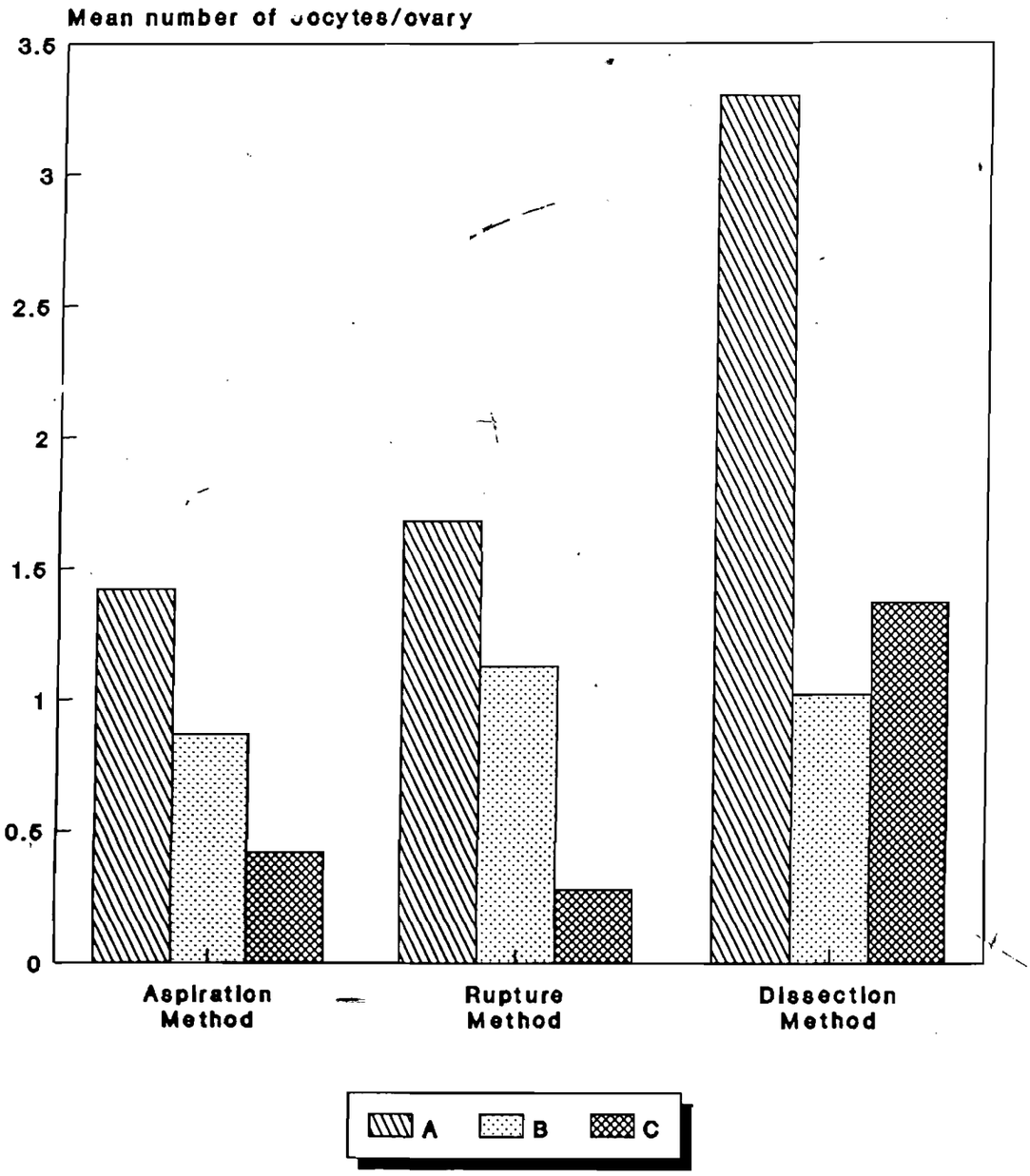
Mean number of first quality oocytes recovery was 1.42, 1.68 and 3.30 in aspiration, rupture and dissection methods respectively. This clearly indicated that total number of first quality oocytes recovered by dissection method was doubled (Fig.3). When compared to the aspiration or rupture method and the percentage of COC was gradually increased from aspiration to dissection methods (52.38, 54.21 and 58.02 per

TABLE 17

EFFECT OF DIFFERENT METHODS ON OOCYTE RECOVERY RATE

Method of Collection	No. of ovaries	Oocyte score			Total No. of Oocytes
		9	6-2	1	
Aspiration	85	121	74	86	231
Recovery (%)		52.38	32.04	15.58	100
Mean Oocytes/Ovary		1.42	0.87	0.42	2.71
Rupture method	104	174	118	29	321
Recovery (%)		54.21	36.76	9.03	100
Mean Oocytes/Ovary		1.68	1.13	0.28	3.09
Dissection	46	152	47	63	262
Recovery (%)		58.02	17.94	24.05	100
Mean Oocytes/Ovary		3.30	1.02	1.37	5.57

FIG.3
EFFECT OF DIFFERENT METHODS ON
OOCYTE RECOVERY RATE



A,B & C - Grade of the oocytes

TABLE 18

EFFECT OF DIFFERENT RECOVERY METHODS ON OOCYTE MATURATION RATE

Method of Collection	Total No. of oocytes collected	Stage of Maturation			Degenerated
		GV	MI	MII	
Aspiration	279	56 (20.07)	23 (8.24)	193 (69.18)	7 (2.51)
Rupture of follicles	261	44 (16.86)	26 (9.96)	187 (71.65)	4 (1.53)
Dissection	392	46 (11.73)	58 (14.79)	206 (52.56)	82 (20.92)

cent). The number and percentage of oocytes in second and third quality oocyte was 0.87 (32.04), 1.13 (36.76), 1.02 (17.94) and 0.42 (15.58), 0.28 (9.03) and 1.37 (24.05) by aspiration, rupture and dissection methods respectively. Though the percentage of first quality oocytes were not much different in all the three methods, the third quality oocytes was high in dissection method than in other two methods. This high percentage of third quality oocyte in dissection method might be due to the release of some of the oocytes from deeper part of the cortex.

The mean percentage of maturation of first quality oocytes to M II was 69.18, 71.65 and 52.56 in aspiration, rupture and dissection methods respectively. Though majority of the cultured oocytes had returned meiosis as indicated by GVBD or chromatic changes in dissection methods, only a moderate proportion reached M II (Table 18). The percentage of oocytes completing II stage of meiosis in aspiration and rupture methods were almost similar. In dissection method after the germinal vesicle breakdown, most of them were degenerated. However, total number of oocytes that reached M II per ovary by dissection method was higher than other two methods; however, the efficiency of further growth in *in vitro* of oocytes collected by dissection method was not clear. This requires further research to prove the efficiency of maturation rate of COC oocytes collected by dissection methods beyond maturation II.

4.5 EFFECT OF SUPPLEMENTS ON OOCYTE MATURATION RATE IN *IN VITRO*

Influence of foetal calf serum and oestrous buffalo serum and their interaction with gonadotropins and steroids on the *in vitro* maturation rate of nine types of buffalo oocytes

TABLE 19

EFFECT OF SERUM GONADOTROPINS AND STEROID IN *IN VITRO* MATURATION OF DIFFERENT TYPES OF BUFFALO FOLLICULAR OOCYTES

Investment		Compact			Partial			Nude		
Cytoplasm score		3	2	1	3	2	1	3	2	1
Oocyte type		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Group I	T_1	22.22 (28/126)	9.21 (7/76)	0.0 (0/12)	18.84 (13/69)	1.92 (1/52)	0.0 (0/8)	11.32 (6/53)	0.0 (0/13)	0.0 (0/8)
	T_2	63.64 (91/143)	45.91 (28/61)	0.0 (0/16)	42.30 (44/104)	36.17 (17/47)	0.0 (0/12)	17.11 (13/76)	11.11 (2/18)	0.0 (0/3)
	T_3	63.50 (87/137)	44.58 (37/83)	11.11 (2/18)	41.23 (40/97)	28.21 (11/39)	0.0 (0/6)	18.75 (12/64)	0.0 (0/15)	0.0 (0/7)
	T_4	70.13 (108/154)	62.50 (40/64)	0.0 (0/8)	61.45 (51/83)	50.70 (36/71)	5.88 (1/17)	15.39 (8/52)	0.0 (0/23)	0.0 (0/12)
	T_5	67.81 (80/118)	61.86 (60/97)	4.55 (1/22)	58.00 (62/107)	51.85 (28.54)	0.0 (0/12)	22.92 (11/48)	5.88 (1/17)	0.0 (0/8)
	T_6	74.13 (106/143)	67.16 (45/67)	13.04 (3/23)	67.76 (63/94)	49.25 (33/64)	0.0 (0/13)	18.75 (12/64)	0.0 (0/16)	0.0 (0/12)
Group II	T_1	21.28 (17/93)	13.04 (6/46)	0.0 (0/16)	20.34 (12/59)	6.25 (1/16)	0.0 (0/11)	16.08 (6/23)	0.0 (0/21)	0.0 (0/14)
	T_2	59.84 (76/127)	61.90 (39/63)	0.0 (0/9)	59.72 (43/72)	35.42 (17/48)	0.0 (0/9)	18.92 (7/37)	0.0 (0/9)	0.0 (0/10)
	T_3	61.61 (69/112)	59.26 (32/54)	8.7 (2/23)	57.81 (37/64)	35.63 (31/87)	8.33 (1/12)	19.64 (11/56)	0.0 (0/7)	0.0 (0.12)
	T_4	69.23 (72/104)	68.96 (66/93)	0.0 (0/6)	68.00 (60/87)	40.50 (32/79)	0.0 (0/8)	16.67 (8/48)	0.0 (0/16)	0.0 (0/12)
	T_5	65.29 (79/121)	65.08 (41/63)	0.0 (0/7)	59.68 (37/62)	47.30 (25/67)	0.0 (0/16)	13.88 (5/36)	9.52 (2/21)	0.0 (0/8)
	T_6	74.22 (72/97)	70.10 (61/87)	0.0 (0/10)	72.3 (68/94)	42.86 (30/70)	0.0 (0/8)	21.43 (9/42)	0.0 (0/17)	0.0 (0/14)

are given in table 19. The rate of oocyte maturation was examined by the analysis of chromosomes in the M II stage.

Only 22.22 per cent of type I oocytes could reach M II when cultured in TCM 199 without addition of serum or hormones. The percentage of maturation to M II was reduced to 18.84 and 11.32 per cent in type 4 and 7 oocytes. The maturation rate was further reduced to 9.21, 1.92 and nil per cent in type 2, 5, and 8 oocytes respectively. This indicates that, as the cellular investment around the oocytes was disturbed, the percentage also got reduced.

In type 2 oocytes, though the cellular investment was complete and compact, the maturation rate was decreased when compared to the type 1 oocytes. This showed that the maturation rate was not only influenced by the cellular investment, but also by ooplasm condition. The reduction in the maturation rate of type 2 oocytes was statistically significant. Hence the potency of oocyte maturation was more influenced by the ooplasm condition than the cellular investment. In type 5 and 7, the maturation rate was least indicating that combination of cellular investment and ooplasm condition further reduced the potency of oocyte to undergo *in vitro* maturation.

In type 3, 6 and 9, none of the oocytes reached M II, clearly indicating that oocytes with degenerated ooplasm completely lost its potency to undergo *in vitro* maturation.

There was a singificant increase in the proportion of oocytes' maturation rate in media supplemented with 10 per cent FCS or EBS than control. It revealed that media alone did not permit marked increase in the maturation; but addition of FCS or EBS is necessary to increase the maturation of buffalo follicular oocytes. The percentage of

maturationof type 1,4 and 7 oocytes in 10 per cent FCS and EBS was 63.64, 42.30, 17.11 and 59.84, 59.72 and 18.92 respectively.

In both the groups, there was a singificant increase in the maturation rate of follicular oocytes in type 1 and 4; but in type 7 the maturation rate did not differ. It indicated that for the beneficial action of the serum on oocyte maturation, it requires compact cellular investment or atleast 2/3 of the cellular investment. In the absence of cellular investment, the maturation rate was not altered even in the presenece of serum.

In type 2, 5 and 8 the rate of maturation of oocytes in media containing 10 per cent FCS or EBS was 45.91, 36.17, 11.11 and 61.90, 35.42 and nil per cent respectively. In type 2 and 5, there was a marked increase in the maturation rate in both groups. It showed that though the ooplasm granules were clumped, complete compact cellular investment or partial cellular investment (atleast 2/3) evoke the oocyte maturation equal to that of the even granulation oocyte. In type 8, the rate of maturation was not significantly different with that of control indicating that, for action of serum on the oocyte maturation, the same requires attachment of the follicular cells to the oocyte.

In group second, the percentage of maturation was not significantly different from that of first group of type 1 oocytes, but in type 5, there was an increase in the percentage of maturation rate in second group than in I group.

In the second treatment, the media was supplemented with LH at a concentration of 10 µg/ml. The oocytes in type 1, 2, 4 and 5 in group I and II which completed M II was 63.50, 44.58, 41.23, 28.21 and 61.61, 59.26, 57.81 and 35.63 respectively. There was no significant difference in the maturation rate of oocytes between treatments in first and second group. In second group in type 2,4 and 5, the percentage of maturation was

increased which was highly significant from that of first group in treatment second than in treatment first. It showed that addition of LH to media containing FCS did not increase the maturation rate significantly in all the above types of oocyte except in media containing EBS, in which LH increased the maturation rate in 2, 4th and 5th types of oocytes.

In the fourth treatment with FSH concentration of 1 µg/ml increased the rate of maturation. The percentage of maturation in type 1,2, 4 and 5 in I and II groups were 70.13, 62.50, 61.45, 50.70 and 69.23, 68.96, 68.00 and 40.50, respectively. Though there was slight increase in the percentage of maturation in different types, statistical analysis revealed that the difference was not significant between groups. Hence the increase in the maturation rate was uniform in all the types of oocytes in both the groups which was not similar to that in 3rd treatment, where only the maturation rate was increased in media containing EBS and LH in 2,4 and 5th types of oocytes alone.

The rate of maturation in media containing 1 µg/ml of E_2 17 with FCS or EBS in type 1,2,4 and 5 oocytes were 67.81, 61.86, 58.00, 51.85 and 65.29, 65.08, 59.68 and 47.30 per cent respectively. The difference in the maturation rate was not significant between groups and between treatments 3 and 4. It showed that E_2 in media containing either FCS or EBS increased the maturation rate similar to that of FSH in media.

The maturation rate in combination of all the hormones in two groups of oocyte in type 1,2,4 and 5 were 74.13, 67.16, 67.76, 59.25, and 74.22, 70.10, 72.3 and 42.86 per cent respectively. In all the types, the maturation rate was increased but the difference was not significant between treatment groups. There was also no significant difference in the maturation rate of oocytes between the two groups (Table 20).

TABLE 20

ANALYSIS OF VARIANCE OF THE EFFECT OF DIFFERENT TREATMENTS BETWEEN GROUPS ON OOCYTE TYPE

Source of variation	df.	Group I	Group II
		M.S	M.S
Between treatments	5	510.41**	496.55**
Error	20	34.17	29.46

** - Highly significant ($p < 0.01$)

TABLE 21

EFFECT OF DIFFERENT CONCENTRATION OF FCS AND EBS ON *IN VITRO* MATURATION OF BUFFALO FOLLICULAR OOCYTES

Kind of serum	Concentration of serum									Mean
	10%			15%			20%			
	No. of oocytes	MII	%	No. of oocytes	MII	%	No. of oocytes	MII	%	
FCS	114	26	66.67	132	94	71.21	123	87	70.73	69.65
EBS	182	113	62.08	82	56	68.29	76	59	79.63	67.06

TABLE 22

ANALYSIS OF VARIANCE FOR DIFFERENT CONCENTRATION OF FCS AND EBS ON *IN VITRO* MATURATION OF BUFFALO FOLLICULAR OOCYTES

Source of variation	df	S.S.	M.S.	F
Between treatments	5	375.71	75.14	1.57^{NS}
Error	33	1583.12	47.97	

NS - Not significant (P > 0.05)

The mean rate of maturation in FCS and EBS was 69.65 and 67.06 per cent respectively (Table 21). Oocyte maturation rate for this was comparable to those for the first part of the experiment III. Though there was slight decrease in the rate of maturation in EBS, it was not significantly different. The rate of maturation in 10, 15 and 20 per cent FCS was 66.67, 71.21 and 70.73 per cent respectively. The rate of maturation was slightly increased in 15 per cent concentration of FCS and there was no further improvement in 20 per cent. The difference between these two was not significant (Table 22). The rate of maturation in 10, 15 and 20 per cent EBS was 62.08, 68.29 and 79.63 per cent respectively. There was a gradual increase in the percentage of maturation. The increase in the percentage of maturation was more in media containing 20 per cent EBS than in 10 and 15 per cent EBS. This indicated that increase in the concentration of FCS did not improve the percentage of maturation but upon increase in the concentration of EBS to 20 per cent level, the percentage of maturation attained the maximum level.

4.6 CUMULUS CELLS EXPANSION AND OOCYTE MATURATION RATE

The percentage of expanded cumulus cell complexes in media containing 10 per cent FCS, 20 per cent EBS, 1 µg FSH/ml, 1 µg E_2. 17ß/ml after 27 h of culture were 76.87, 84.76, 79.64 and 52.70 per cent respectively. Expanded cumulus cells complexes were more in media containing EBS than other media. The difference between the first three media were not significantly different. In the fourth media the rate of expanded cumulus cell oocytes was significantly different with that of other three gorups. The mean percentage of expanded cumulus cells in different media was 73.45 per cent.

The expanded cumulus cells oocytes were processed and examined for their maturation. The number of oocytes that reached M II was 117 (72.52 per cent) of 182

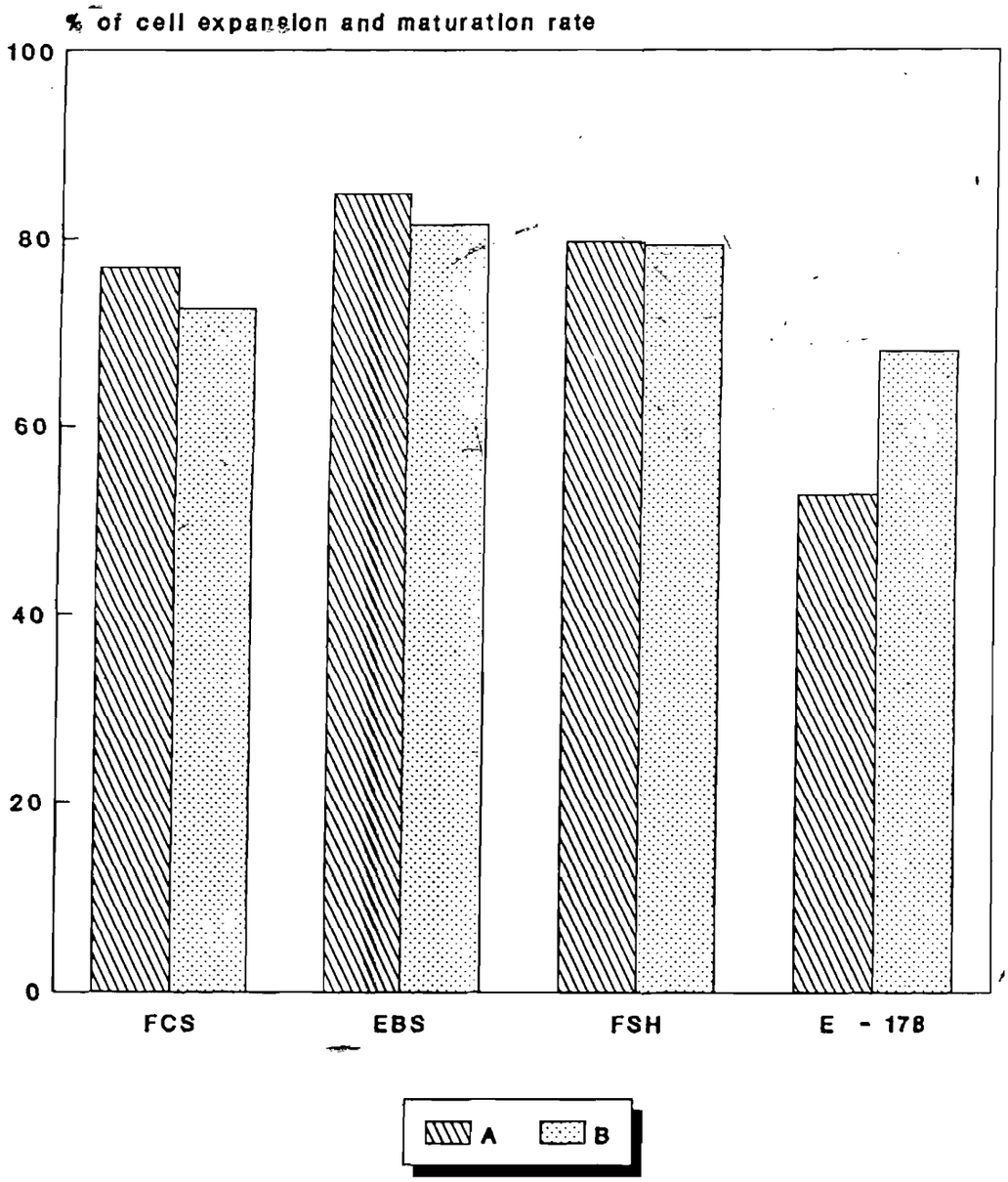
TABLE 23

RELATIONSHIP OF CUMULUS CELLS EXPANSION AND OOCYTE MATURATION RATE

Media with different supplements	No. of Oocyts cultured	COC complexes with expanded cumulus cells			
		No	%	MII	
				No	%
FCS	182	149	76.87	117	72.52
EBS	164	139	84.76	116	81.45
FSH	157	125	79.64	99	79.20
E2	148	78	52.70[a]	53	67.95[b]
Mean	162.15	122.75	73.45	96.25	73.78

Percentage bearing different superscripts differ significantly within the character ($p<0.01$).

FIG.4
RELATIONSHIP OF CUMULUS CELLS EXPANSION
AND OOCYTE MATURATION RATE



A - % of cumulus cells expansion
B - % of Maturation

oocytes in FCS, 116 (81.45 per cent) of 164 in EBS, 99 (79.20 per cent) of 157 in FSH and 53 (67.95 per cent) of 148 in E_2 - 17ß. (Table 23). Though there was a slight difference in the percentage of cumulus cell expansion and their maturation rate (M II) (Fig.4) the difference was not statistically different. But the difference between the cumulus cell expansion and the maturation rate in media containing E_2 was statistically significant ($p < 0.5$). This indicated that oocytes cultured in media containing FCS, EBS, and FSH directly reflect the oocyte maturation on the morphological appearance of the cumulus cells surrounding the oocyte.

4.7 SETTING OF CULTURE

The colour of 2 ml media containing 10,20,30 and 40 oocytes in four experiments respectively was not changed even after 27 h of culture. This indicated that the circulating CO_2 in the incubator was sufficient to maintain the pH in the above culutre condition. The number of oocytes that reached M II was 36 (72 per cent) of 50 in media containing 10 oocytes, 68 (68 per cent) of 100 in media containing 20 oocytes, 109 (72.67 per cent) of 150 in media containing 30 oocytes and 147 (73.51 per cent) of 200 in media containing 40 oocytes respectively (Table 24). The difference in the maturation rate was not found to differ siginificantly in all the treatments in the first culture condition. It indicated that the number of oocytes upto 40 in 2 ml of culture media did not affect the maturation rate significantly.

In second culture condition (100 µl) the number of oocytes that reached M II was 37 (75 per cent) of 50 oocytes in media containing 10 oocytes in the 100 µl drops of media which was not significantly different from that of the first culture conditions. But in other experiments in the second culture condition, the number of oocytes that reached

TABLE 24

EFFECT OF NUMBER OF COC COMPLEXES AND VOLUME OF MEDIA ON *IN VITRO* MATURATION RATE

Culture condition	Percentage of Maturation				Mean percentage of maturation
No. of Oocytes	10	20	30	40	
2 ml of media	72 (36/50)[a]	68 (68/100)[a]	72.67 (109/150)[a]	73.5 (14/200)[a]	72 (360/500)
100 µ ml	75 (37/50)[a]	58 (58/100)[ab]	22.60 (34/150)[ab]	8 (16/200)[ab]	29 (145/500)

Numbers bearing different superscripts differ significantly ($p<0.01$) within the given culture condition

M II was 58 of 100 oocytes (58 per cent), 34 of 150 (22.60 per cent) oocytes, 16 of 200 (8 per cent) oocytes differe significantly ($p < 0.01$). This indicated that the increasing number of oocytes in 100 µl of culture media significantly affected the maturation rate of oocytes. The colour of the media was changed from orange to plae, pale yellow and yellow colour in the last three experiments respectively.

CHAPTER 5

DISCUSSION

5.1 BIOMETRY OF OVARIES

The biometry of the ovaries of non-descript buffaloes deserves much attention since the most important native germplasm comes out of it. Literature available on this aspect of non descript Indian buffaloes are scanty. Hence many observations in the present study do not have comparative references as published works.

The various measurements of the ovary were comparable with the other studies in non-descript Indian buffaloes. The mean length of both the ovaries were found to be shorter in the present study when compared to observations of Damodaran (1955); Luktuke and Rao (1962) and Napolean (1989). This might have been due to the variations in the climatic and management conditions, since the first two studies were conducted in the Northern part of India.

Though the third and the present study were carried out in the same place, shorter duration, smaller sample size and consequent sampling variance would have accounted for the variation. For the present study, the ovaries were collected from July 1988 to June 1989. Hafez (1955), Hossain and Ahmed (1971) and Majeed *et al.*, (1961) reported the correlation between ovarian activity and temperature and stated that majority of buffaloes remain in anoestrus condition during the summer months (April-July). Further Zemjanis (1962) stated that in the same animal, the size of the ovary varied during different days of the week because of the changes in development and regression of the corpus luteum. Hence, the difference could also be attributed to ovarian activity.

The width and height of the ovary of non-descript Indian buffaloes were almost similar to the observations made by Luktuke and Rao (1962).

There was no significant difference in weight of the left and right ovaries in buffaloes in the present study which agrees the findings of Luktuke and Rao (1962) and Khan (1987). Mean weights were 2.86 ± 0.06 and 2.77 ± 0.06g for the right and left ovaries respectively. The mean weight reported by Luktuke and Rao (1962) was more by a gram than the weight observed in this study for both left and right ovaries, while those reported by Damodaran (1955) was slightly less in non-descript buffaloes. The results of the present investigations are in agreement with the findings of Napolean (1989).

The non-significant variations in the volume of both the ovaries in different stages of the cycle noted in this study might have been due to the change in size of the ovary due to the formation, development and regression of Graafian follicles and regression of corpus luteum (Zemjanis, 1962). The highest volume of the ovary during the II and III stages of the cycle on both the sides might be due to the maximum development of the corpus luteum during the II stage of the cycle which was maintained as such until the end of the III stage of the cycle. This confirms the findings of earlier workers (Asdell, 1955 and Foley *et al.*, 1964).

The existence of highly significant correlations between different biometrical parameters of ovaries like length, width, height and volume indicated that even any one such measurement would firmly be representative of other measurements, all of which, hence, need not be measured to define the biometry of such ovaries in non-descript Indian buffaloes.

5.2 CORPUS LUTEUM

The colour of the corpus luteum externally and on bisection in buffaloes observed in the present investigation agrees with the findings of Napolean (1989).

The length, diameter and weight of the corpus luteum were the least in the first stage of the estrous cycle and it grew significantly in the subsequent stages. The maximum size was reached during the II stage of the cycle. There was no marked reduction in size until the end of the III stage of the cycle. Similar observations were made by several authors (Mc-Nutt, 1924; Ireland *et al.*, 1980 and Dellmann and Brown, 1981). On contrary, Napolean (1989) stated that the decline in the length of the corpus luteum had started by the end of the second stage of the cycle itself. However, the slight decline noticed in this study during the third stage of the cycle was not found to make significant difference in the length of the same.

Luktuke and Rao (1962) reported that the weight of the corpus luteum varied from 1.30 to 4.40g during different stages of the estrous cycle which is confirmative with the present study. The mean diameter and weight of the diestrous corpus luteum noted by El-Wishy *et al.*, (1988) as 16 ± 4.2mm and 1.9 ± 0.48g, were much higher than the values observed in the present study. Similar values were given by El-Sheikh and Abdel-hadi (1970) also in Egyptian buffaloes. This indicated that the corpus luteum of Indian buffaloes seemed to be smaller as reported by Luktuke and Rao (1962). The percentage of lacunae in the corpus luteum during the diestrum (10.7 per cent) was almost similar to the findings of El-Wishy *et al.*, (1988) which was, however much lower than 39.0 and 25 per cent reported by Mylrea (1962) and Arthur *et al.*, (1982) respectively in cows.

5.3 FOLLICLE

5.3.1 Follicular dynamics in relation to

5.3.1.1 The different stages of the estrous cycle

The mean number of follicles on the ovarian surface was found to be less in the present study than those reported by Kruip (1982) and Moor *et al.*, (1984) in cows. This less number of follicles per ovary in buffalo might be due to the presence of low number of primordial and Graafian follicular population in buffalo ovaries (Madan, 1990; Totey *et al.*, 1991).

The distribution of total number of follicles during the different stages of the cycle was comparable to the observations made by Skyer *et al.*, (1987) in cow. Total number of follicles were less during the second and third phase of the cycle as compared with the IV stage in which the total number of follicles were relatively more. In I stage, the mean number of follicles per ovary was slightly lower when compared with the IV stage of the cycle; however, the same was significantly more than the numbers at II and III stages of the cycle atleast for follicles of ≤ 2mm size. Henderson and Cupps (1990) stated that the average follicle size varied ($P < 0.001$) with physiological state of the cow. Mean follicle size from ovaries containing a preovulatory follicles was reported to be statistically greater ($P < 0.05$) than those from ovaries containing a corpus hemorrhagicum, a developing corpus luteum and or a mature corpus luteum.

In this study, there was significant variation in number of various size of the antral follicles during the entire cycle. Ireland *et al.*,(1979) and Skyer *et al.*, (1987) also made similar observations. These changes in number of follicles within different size categories during an estrous cycle could result from any combination of the three factors as follows:

a. rate of entry of progression of growing preantral follicles into the pool of antral follicles

b. the rate of growth of these antral follicles into a large size category and

c. the rate of loss (atresia) of these follicles from a large size category into small size category (Spicer and Echternkamp, 1986).

The present investigation confirmed the findings of Matton *et al.*, (1981) and Skyer *et al.*, (1987) who reported that a large pool of small antral follicles developed during the pre-ovulatory and early luteal periods, and might be associated with the rise in both FSH and LH prior to ovulation. The mean number of follicles with 2mm diameter was slightly decreased during the I stage of the cycle and was the least during the II stage. Again there was a rise in the number of ≤ 2mm follicles in the III stage of the cycle indicating that there might have been two waves of follicular growth taking place in buffalo ovaries.

The available literature showed that the transformation characterised by an increase in the rate of growth of small antral follicles into large antral follicles takes place as the estrous cycle progresses towards ovulation. Present investigation showed that the mid luteal phase was a period of diminished ovarian activity and there was significantly fewer follicles of smaller sizes compared with follicular phase when statistically analysed. Similar observations were made by Matton *et al.*, (1981) and Ireland and Roche (1988) in cows.

In several domestic mammals, a positive relationship between ovarian, luteal and follicular activity has been observed (Clark *et al.*, 1975). In the present study, the ovary bearing corpus luteum had more larger follicles than the ovary bearing the corpus

albicans. Several authors have reported similar findings in cows (Dufour *et al.*, 1971; Fogwell *et al.*, 1977 and Dailey *et al.*, 1982) and in buffaloes (Hafez, 1955).

In the present investigation, there was no significant difference in the distribution of follicles between the sides of the ovary. This clearly indicated that both the ovaries were active which is in agreement with the findings of El-sawaf and Schmidt (1963) who reported that, in buffaloes both the ovaries were active.

The largest diameter of the Graafian follicle of 2.1 cm recorded in this study is supported by the findings of El-Sheikh and Abdel-Hadi (1970) and El-Wishy *et al.*, (1988), where as Hafez (1955) recorded the diameter of the largest follicle to be only 0.5 to 0.7 cm and 0.8 to 1.3 cm in ovary with corpus luteum and without corpus luteum respectively in Egyptian buffaloes. The difference might have been due to the breed variation and environmental differences between places of study.

5.3.1.2 The different months in a year

From the month of June onwards the total number of follicles especially that of follicles of ≤ 2 and 6-10mm diameter ranges were found to gradually increase upto December. Thereafter, the number of above category follicles started to decline. Similarly, the ambient temperature started to decrease and rainfall to increase during the month of June. From the observations, it can be concluded that decrease in ambient temperature and increase in energy intake by grazing more lush grass have brought in greater ovarian activity. This confirms the findings of Kodagali *et al.*, (1973) who observed that the sexual activity of Indian buffaloes was in peak during the period from September to February. Similarly, Afiefy (1967) also found that the highest incidence of estrous was recorded during October and November. Hossain and Ahmed (1971) too

reported that most of the estrous period (66.0 per cent) in buffaloes occurred during the months of October to January. The minor differences found in the present study might be due to variation of maximum temperature between adjacent months during that particular period of study.

From January onwards, the total number of follicles started declining and became the least during the month of May. The same was mostly due to the decrease in number of follicles of ≤ 2mm and 6-10mm diameter ranges. This reduction in ovarian activity might have been due to higher temperature and lower rain fall recorded during the period.

Hafez (1955) reported that majority of buffaloes remained in anestrous condition during summer months (April-June) because of high environmental temperature (42-46°C) which caused reduction in Gn-RH release. Consequently, reduced recruitment of small follicles and high atresia resulted in low secretion of estrogen and thus bringing "Silent heat" in buffaloes.

Further, when ambient temperature is more (photoperiodism) prolactin levels are the highest (Kakar *et al.*, 1982) and plasma progesterone levels were found to be the lowest (Rao and Pandey, 1982) which aggravate the silent heat condition.

A very low nutritional level during winter might be the main reason for the delay in animal returning to cyclicity. It has been noted that when cows are turned out on pasture, the percentage of cycling cows rises abruptly.

Though the ambient temperature showed a decrease and the rain fall an increase during the month of June, the total number and number of ≤ 2mm diameter follicles

remained almost constant during the first three months. Increase in follicles of ≤ 2mm diameter was noticed from month of August onwards. Lussier *et al.*, (1983) indicated that two estrous cycles are required for a follicle to grow from preantral size to an antral size of 8.5mm. Hence during the months from June to August, increase in recruitment of the smaller follicles and prevention of atresia of such follicles might have led to appreciable increase in total number of follicles of ≤ 2mm diameter range from August onwards.

5.3.2 Follicular contents

5.3.2.1 Follicular fluid

Follicular fluid was found to be composed partly of exudates from plasma (Edwards, 1974). In the present study, there was an increase in the volume of follicular fluid as the follicular diameter increased and this concurs with the findings of Napolean (1989). There was a steep rise in the volume of fluid in follicles of > 8mm diameter. This might be due to the increase in blood flow in absolute terms (ml/min) and increased capillary permeability of large follicles than the smaller ones (Moor and Trounson, 1977). Spicer and Echternkamp (1986) found that follicular diameter and fluid volume of 243 follicles were significantly correlated (r=86) with each other. A similar curvi linear relationship was observed between diameter and volume in human follicles (Mc Natty *et al.*, 1979) as in the buffaloes in the present study.

The fluid was straw coloured and slightly viscous and the viscosity was found to be more in the growing follicles than in fully-grown pre ovulatory follicles (Byskov, 1969 and Norman and Greenwald, 1972). These findings were in agreement with the results of the present study in buffalo cows. Edwards (1974) reported that the changes in the viscosity of fluid occurred towards ovulation as the mucopolysaccharides were depolarized. The mean volume of fluid collected from large size follicles in the study

was less than those reported by Henderson *et al.*, (1982) in cows. This might be due to the species variation.

5.3.2.2 Diameter of the oocyte

The mean diameter of the oocyte in the present study was 157.37 μ with a range of 130 to 161 μ which was slightly higher than the findings of Sato *et al.*, (1990) in cows. However, the values were close to the findings of El-Sheikh and Abdel Hadi (1970) who reported that the diameter of the oocyte in Egyptian buffalo was 160μ.

5.3.2.3 Oocyte recovery rate

The percentage of recovery rate of 57.33 per cent recorded in this study was lower when compared to the findings of Mc Natty *et al.*, (1984) and Leibfried-Rutledge *et al.*, (1987) in cows and the percentage of recovery rate in relation to the size of the follicle was in agreement with the findings of Asakawa *et al.*, (1982) in squirrel monkey. There was no significant difference in the percentage of recovery rate between different stages of the cycle. On contrary, Schnurrbusch *et al.*, (1990) stated that the percentage of recovery was more, i.e., 92 per cent in late estrous and the least 43.5 per cent in proestrous stage.

5.3.3 Characterization of the oocyte in relation to

5.3.3.1 Size of the follicle

The characteristics of oocytes from non atretic bovine vesicular follicles has been adequately described (Rajakoski, 1960; Lobel and Levy, 1968 and Marion *et al.*, 1968). Bovine oocytes from follicles of various sizes displayed a disparate relationship between the appearance of the investment and ooplasm and either pre or post culture chromatin

configuration. The observations in the present study ≤ 2mm and 3-5mm categories contained almost equal and a greater proportion of oocytes with compact complete cumulus oocytes, which was higher than those in the follicles in ≥ 6mm categories. Follicles ≥ 6mm contained relatively more number of oocytes with partial or fully expanded cumulus mass. This expansion of cumulus mass in follicles of ≥ 6mm diameter was probably due to loss of connection between cumulus cells and oocyte cytoplasm.

Since matured oocytes did not require any connection between the cytoplasm of the oocytes to cumulus cells for their nourishment, the same had resulted in expansion of cumulus mass (Donahue and Stern, 1968). The findings of Bedirian and Baker (1975) that the 81 per cent of oocytes with an expanded cumulus underwent nuclear maturation. A similiar finding was made by Loos *et al.*, (1989) that the cumulus investment of the oocyte in M II was always expanded.

This study demonstrates a clear relationship between follicle size and oocyte quality. Leibfried and First (1979) classified the follicles as 1-3mm and > 3mm in diameter and reported that follicles of 1-3 mm diameter tended to have a high proportion of oocytes possessing a compact complete investment and follicles > 3mm diameter had expanded cumulus cells. However, in the present study both follicles of ≤ 2mm and 3-5mm diameter ranges had almost equal proportion of compact complete cumulus oocytes and the difference might be due to the difference in the classification of the follicles. On contrary, Lonergan *et al.*, (1992) stated that significantly more oocytes with many layers of cumulus cells which had a significantly higher proportion of morula/blastocyst stage embryos were obtained from follicles > 6mm in diameter compared to 2-6mm follicles.

However, the observation that the percentages of ≤ 2mm diameter in this study is in confirmity with the findings of Lonergan *et al.*, (1992) who reported that the follicles

of 2mm contain more percentage of oocytes with clumped ooplasm granules which might have altered the maturation rate *in vitro*. Attainment of a high success rate in follicles of above 6mm diameter might be due to the high percentage of evenly distributed cytoplasmic granules in oocytes in more than 3 mm diameter follicles. A similar finding was noted in the present investigation. More oocytes from follicles of 3-5 and 6-10mm diameter follicles were found to have even ooplasm granules and oocytes from follicles > 10mm diameter tended to have low percentage of even granulation and this observation was in agreement with the findings of Bedirian and Baker (1975), who stated that follicles of > 10mm diameter tended to yield more degenerated chromatin, compared to oocytes with a compact investment or oocytes showing preovulatory expansion of the cumulus.

5.3.3.2 Stage of the cycle

The variation in percentage of oocytes in four stages of the cycle in this study indicates that hormones secreted during the cycle had an influence on the type of oocytes and it was explained as follows by the earlier workers. It has been hypothesized that the maximal production of progesterone and increased production of prostaglandin $F_2 \alpha$ by the uterus (Ottober *et al.*, 1980) limits the maximum diameter of the follicle and this enhances atresia of follicles (Brand and deJong, 1973 and Daily *et al.*, 1976) and hence the preovulatory follicle is followed by a group of medium sized (2-4mm) follicles in advanced atresia (Driancourt, 1991). Moor *et al.*, (1984) stated that all oocytes in non-atretic follicles and almost all follicles undergoing early atresia remain in the normal germinal vesicle stage and with the progression of atresia the percentage of oocytes showing degenerative changes increases. In general, atresia increased the percentage of degenerated oocytes.

From the above observations, they concluded that the percentage of degenerated oocytes were more during the follicular and early luteal phases. The present study concurred with the findings that the percentage of oocytes was reduced in the fourth stage of the cycle and further reduction took place during the first stage of the cycle. On contrary, Leibfried and First (1979) reported a greater incidence of oocytes with intact chromatin in the follicular stage as compared to the luteal and early luteal stages. The question whether the degeneration of oocytes starts first on the cellular investment or on cytoplasmic granules would need more investigation before a definite answer could be given and hence no conclusion was drawn.

5.3.3.3 Presence or absence of corpus luteum

Increase in percentage of COC with even granulation oocytes in ovary containing corpus luteum in this study might have been due to the increased functional activity of the surface of the ovary when functional corpus luteum was maximum in size (Block, 1951; Rajakoski, 1960, Brand and deJong, 1973; and Clark *et al.*, 1975). It can be inferred that by acting on the cumulus cells, gonadotropins change their metabolic pathway resulting in enhanced progesterone production. This enhanced progesterone with estrogen was intimately involved in cytoplasmic and nuclear maturation of oocytes (Thibault, 1977), which results in good quality oocytes in ovaries with corpus luteum.

5.4 ISOLATION OF OOCYTES AND ITS PERCENTAGE OF RECOVERY AND MATURATION RATE

The mean number of oocytes liberated from each ovary was incomparable between buffalo cows and cows. The mean number of oocytes liberated from each ovary was 26.2 ± 7.4 in cow (Sato *et al.*, 1990). In buffalo it was 2.71 in the present study which

is much lower and the same might be due to the low number of primordial and Graafian follicular population in buffalo ovaries.

However, the number of good quality oocytes per ovary in buffalo was high 1.42 in the present study when compared to the observation of Totey *et al.*, (1991) who found the same to be 0.46 oocytes/ovary by aspiration method in buffaloes.

The proportion of total number of oocytes and good quality oocytes between dissection and aspiration method in cow was 1:2 (Arlotto *et al.*, 1990) which was similar to the present study, that dissection method yielding double the number of oocytes of 5.57/ovary while the same by aspiration method was 2.71/ovary. The number of good quality oocytes in the present study was 1.42/ovary by aspiration method and 3.30 oocytes/ovary by the dissection method, which also simulates the findings of Lonergan *et al.*, (1991) who reported that there was significantly more good quality oocytes and conversely significantly fewer poor quality oocytes recovered by dissection when compared to the aspiration method. On contrary, Bottcher *et al.*, (1989) reported that the percentage of first quality oocytes were more by rupture of isolated follicles than by aspiration and dissection methods in cows.

Although follicular dissection and needle puncturing were initially employed in recovering more good quality oocytes from follicles, for routine use, aspiration has been recognised now as the method of choice because of the obvious advantage, in terms of speed of operation, which was especially important *in vitro* culture of follicular oocytes (Lonergan *et al.*, 1992).

The percentage of *in vitro* maturation of good quality oocytes to M II by aspiration, rupture of follicles and dissection methods in this study was concomitant with

the findings of Arlotto *et al.*, (1990) in which he stated that intact oocytes from follicles located deeper in the cortex had a lesser probability of completing meiosis I in *in vitro* compared to similar oocytes from visible follicles on the surface. In an another study, Sato *et al.*, (1990) found that poor meiotic competance of oocytes from deeper cortex was due to the absence of some factors necessary for oocyte maturation or at least to its presence of insufficient quantity in the cytoplasm and/or nucleus of smaller oocytes from deeper cortex.

Erickson and Sorensen (1974) observed that larger oocytes taken from late and middle antral follicles were more likely to undergo maturation *in vitro* than were oocytes taken from pre-antral follicles in mice. He concluded that oocytes of differing sizes released from the ovary might exhibit unequal maturation potentials. Further, Veronique de Smedt *et al.*, (1991) stated that 86 per cent of oocytes from follicles of 2-6mm diameter progressed to metaphase II and only 24 per cent of oocytes from follicles of 1-2mm diameter completed meiotic maturation after 27h of goat oocytes cultured *in vitro*.

5.5 EFFECT OF SUPPLEMENTS ON OOCYTE MATURATION RATE IN *IN VITRO*

High variation in the maturation rate has been observed in culturing different types of oocytes *in vitro* in TCM 199 with or without the addition of supplements. The percentage of maturation of COC with even granuation in TCM 199 alone was high in this study when compared to the findings of Singh *et al.*, (1989) who attained only 2.1 per cent of maturation in culturing of buffalo follicular oocytes in TCM 199 without any supplements. This increase in maturation rate in this study was due to the subjecting to live oocytes (dead oocytes were removed by using the vital staining technique) with even granulation. The percentage of maturation of oocytes with clumping cytoplasmic granules

and compact or partial cellular investment was low in this study and this confirms the findings of Garcia *et al.* (1988), Shioya *et al.*, (1988) and Kim and Park (1990).

The percentage of maturation of nude oocytes with even granulation was low in this study and was confirmed by the findings of Tsafriri and Channing (1975) who reported that only 10 per cent of porcine oocytes lacking investment had matured. However, in cows 44 per cent (Leibfried and First, 1979), 55 per cent (Kim and Park, 1990) of nude oocytes were found to mature to M II. On contrary Dahlhausan *et al.*, (1981) reported that no oocytes from any of the different follicular size groups which were devoid of cumulus cells matured *in vitro*. This poor response might probably be due to the poor cytoplasmic condition of the oocytes which were subjected for maturation. The rate of maturation of partial cellular investment oocytes with even granulation stood in between the COC and nude oocytes which was in agreement with the findings of Shioya *et al.*, (1988) and Kim and Park (1990).

Addition of FCS alone to the maturation media had a significant difference in maturation rate. Ninty one oocytes (63.64) per cent out of 143 attained MII in this study. Several authors also reported similar findings in cows and buffaloes (Singh *et al.*, 1989; Sanbuissho and Threlfall, 1990 and Totey *et al.*, 1991). This was supplemented by the findings of Eppig and Schroeder (1986) that the presence of a high molecular weight compound of serum was essential during oocyte maturation in culture. In contrast, Menezo *et al.*, (1984) reported that serum was not necessary for *in vitro* maturation and fertilization of follicular oocytes.

The rate of maturation was not significantly different between groups I and II. However, in 10 per cent EBS, there was a striking difference in the percentage of COC with clumped cytoplasmic granules and partial oocytes with even and clumped granules.

The increase in the percentage of maturation in these types of oocytes was almost equal to the percentage of oocyte maturation in media containing FSH or combination of all hormones, suggesting that estrous buffalo serum contains certain substances which increase the maturation rate compared to FCS. The exact substance for this high rates of maturation in the present study was difficult to be elucidated.

Sanbuissho and Threlfall (1990) reported that there was no significant difference in the maturation rate of oocytes cultured in EBS or FCS. On contrary, Schellander *et al.*, (1989) stated that the fertilization and maturation rates were increased in media containing EBS than FCS. Xu *et al.*, (1987) stated that one of the reasons for his success rate of bovine oocytes maturation might be due to the addition of serum from estrous cow to the maturation medium.

In the present study, the percentage of maturation was increased in media containing 15 per cent or 20 per cent EBS. On contrary, Totey *et al.*, (1991) stated that there was not much of difference in maturation rate of buffalo follicular oocytes cultured in 10 per cent FCS or 20 per cent EBS. In an another study, Kim *et al.*, (1990 a) reported that there was a gradual decrease in the fertilization rate of cow oocytes cultured in 10, 15 and 20 per cent ECS.

Oocytes cultured in media containing gonadotropins had a significant increase in the maturation rate than the control. This increase in maturation rate might have been due to change in the membrane potential of follicular somatic cells and the oocyte by both FSH and LH (Mottioli *et al.*, 1990).

The electrical coupling between somatic cells and oocyte may represent a means by which the gonadotropin message gets passed on to the germinal cells by the somatic compartment, for which a direct cell oocyte contact was essential (Kastrop *et al.*, 1990).

Oocytes surrounded by cumulus cells to more than one third of their surface was effectively matured *in vitro* (Goto *et al.*, 1988), From this, it was proved that oocytes are dependent upon cumulus cells for their nourishment, essential nutrient, passing of certain signal from outside to inside of the germinal cells. Hence, cell-oocyte contact is considered as a must for oocyte maturation.

This was amply proved in the present experiment since the percentage of maturation was significantly increased upon addition of FCS or FCS with FSH in COC or partially cumulus cells oocytes with even or clumping granulation. There was no change in the percentage of maturation in nude oocytes with even granulation. This was confirmed by the findings of Moor *et al.*, (1984) who stated that oocytes denuded of their follicular cells do not acquire developmental competence irrespective of the hormonal support. This may due to lack of connection to the oocyte from outside to pass the signals elicited by the FSH or FCS in the culture media. When oocytes were mature, it does not need any support from cumulus cells and cumulus cell expanded as a result of loss of connection between cumulus cells and oocyte cytoplasm (Donahue, 1968).

Though clumping of cytoplasmic granules was found to be a sign of degeneration, addition of FCS plus FSH increased the percentage of maturation from 9.21 to 62.50 and 1.92 to 50.70 per cent. This indicated that even the oocytes with clumping ooplasm granules does not loose the meiotic maturation potential and this was confirmed by the findings of Leibfried and First (1979) who stated that the ability of bovine follicular

oocytes to undergo spontaneous nuclear maturation *in vitro* may not be lost until late in the process of atresia.

The size of the follicle, whether or not it was atretic, the concentration of FSH, LH and E_2 percentage during the culture period are critically influenced by the development potential of oocyte matured *in vitro*.

The striking point noticed in this study was that the percentage of maturation of type 2,4,5 oocytes were almost equal to the type 1 oocytes indicating that the former oocytes were responded more than the later. Thus it is postulated that oocytes in small non-atretic follicles respond poorly to gonadotropin treatment *in vitro*, probably because of the overriding inhibitory effect of the follicular fluid, (Chang, 1955) and granulosa cells (Foote and Thibault, 1969). During atresia, granulosa cell involvement may well be accompanied by a reduction in the inhibitory factors secreted by those cells and as a result, oocytes in large and small atretic follicles might then respond more readily to administration of gonadotropins (Moor and Trounson, 1977).

Thibault (1977) reported that the ovarian steroids, estrogen and progesterone are intimately involved in cytoplasmic and nuclear maturation of oocytes and further it was confirmed that synchronization of cytoplasmic, nuclear and membrane events in the maturation of oocytes was of atmost importance for normal fertilization and development. On contrary, the percentage of maturation was not much different by the addition of E_2 to the media with 10 per cent FCS in all types of oocytes in this study. This is in confirmation of the findings of Singh *et al.*, (1989) who stated that addition of E_2 did not increase the maturation rate.

Smith and Tenney (1980) stated that estradiol - 17 B was to block maturation after the resumption of meiosis and before completion of the first meiotic division in mouse oocyte. At higher concentration, it prevented germinal vesicle breakdown without causing oocyte degeneration. The inhibitory actions of all steroids were reversible in oocytes exposed for 4 to 18 hr. However, Hunter *et al.*, (1976) reported that ovulated oocytes induced by HCG in the follicles with low E_2 level failed to undergo normal nuclear and cytoplasmic maturation and hence were unable to became normally fertilized. No conclusion could be drawn based on the present study and to confirm these findings, further research on *in vitro* and *in vivo* maturation of buffalo oocytes may provide the answer.

Increased percentage of oocyte maturation was noticed in media containing 10 per cent FCS with steroids and gonadotropins which was however, not significantly more within individual hormones. The percentage of maturation was 74.13 in COC complexes with even granulation studied in this work was in agreement with the findings of Chuangsoongneon and Kamonpatna (1991) and slightly lower when compared to the findings of Singh *et al* ., (1989).

In contrary, Sirad *et al.*, (1988) reported that addition of FSh, LH and E_2 - 17 ß to maturation culture do not alter the frequency of the nuclear maturation (90 per cent Vs 80 per cent) with and without hormone supplement.

5.6 CUMULUS CELL EXPANSION AND OOCYTE MATURATION RATE

All media containing serum and hormones caused marked expansion of COC. This was confirmed by the findings of Ball *et al.*, (1983), Thibault *et al.*, (1975a) and Sanbuissho and Threlfall (1986). Percentage of COC expansion was poor (52.7 per cent)

in media containing E_2 and only 62.95 per cent of the expanded COC underwent nuclear maturation. The COC expansion and maturation rate were not significantly correlated in media containing E_2 than in other groups. In other groups, about 80 per cent of expanded coc underwent nuclear maturation. A similar findings was made by Bedirian and Baker (1975). It showed that the COC expansion in media containing FCS, EBS, FSH directly reflected the percentage of oocyte maturation whereas the same in media containing E_2 did not directly reflect the oocyte maturation.

In first three media, only 8 per cent of the unexpanded COC complexes showed nuclear maturation and in the fourth media, about 20 per cent of the unexpanded COC had underwent nuclear maturation.

5.7 SETTING OF CULTURE

The relationship between culture medium, volume and the number of COC complexes were examined. The maturation rate was similar in all the trials in first culture condition. Similar findings were made by several authors (New Comb *et al.*, 1978; Lu *et al.*, 1987 and Xu *et al.*, 1987). There was no change in the colour of the media indicating that the change in the pH during the maturation process was maintained by the excess $NaHCo_3$ present in 2 ml of media under 5 per cent carbondioxide in air.

In the first trial of II culture condition, the percentage of maturation and colour of the media were not changed to that of the I culture condition. In II trial the rate of maturation was reduced to 58 per cent and colour was changed into orange. This indicated that the change in the pH during the maturation process of 20 oocytes was not able to be maintained by $NaHCo_3$ in a small volume of media under 5 per cent CO_2

condition, and in III and IV trials the colour of the media was pale yellow indicating a further change in the pH.

Another reason for high maturation rate in COC complexes in 2 ml media was that the inhibitory signals present in the COC complexes itself was diluted *in vitro* by the addition of large volume of culture media, thereby permitting the resumption of meiosis (Sirard *et al.*, 1982).

Raising the number of COC complexes to 25 or 40 per drop resulted in a significant inhibition of oocyte maturation compared to controls containing 10 coc's per drop in the present study.

From this, it was concluded that in 2 ml of media even upto 40 number of oocyte complex were matured without any deleterious effect. This finding confirms the observation of Goto *et al.*, (1988) who attained a maximum maturation.

CHAPTER 6

SUMMARY

Ovaries from non-descript pluriparous she buffaloes collected from slaughter house were grouped into four stages based on the appearance of the corpus luteum and the follicles.

The biometry of the ovary and corpus luteum and morphometry of the ovary were done and variations were recorded during different stages of the estrous cycle. Follicles were grouped according to the diameter range as ≤ 2, 3-5, 6-10 and > 10 mm. Percentage of recovery rate of oocytes and their characterization in relation to size of the follicle, stage of the cycle and ipsilateral or contralateral relationship to recently formed corpus luteum were studied. Efficacy and efficiency of different recovery methods of oocytes were also studied. Different kinds of supplementations with different percentages of serum were made and their influence on various types of oocyte maturation was assessed. Percentage of maturation in two different culture conditions and the morphological changes in relation to their nuclear maturation were studied.

Biometry of ovaries on left and right side was not found to differ significantly and also between stages of the cycle; however length, diameter and weight of the corpus luteum was highly significantly different during the four stages of the cycle. The corpus luteum of Indian non-descriptive she buffalo was found to be smaller than other breeds in India.

The mean number of visible follicles of 3.83 and 3.45 in right and left side ovaries also did not significantly differ. The number of follicles of 2 and less than 2 mm

diameter size rose at two times of the cycle indicating two waves of follicular growth in buffalo ovary.

Total number of follicles especially those in ≤ 2 mm diameter range gradually increased from the month of June. Constant and considerable number of 3-5 mm diameter follicles were present in ovary throughout the year, indicating that ovaries from slaughter house can be utilized to collect the oocytes for *in vitro* maturation throughout the year. There was a slight variation in the number of follicles and follicles in different diameter ranges, which was, however, not significant. The maximum ovarian activity was observed from the month of October to January in Madras.

A curvi linear relationship of follicular diameter and fluid volume was noticed. The maximum fluid volume collected was 1.26 ml in a follicle with a diameter of 2.1 cm. The mean diameter of the buffalo oocytes (zona pelluceda plus ooplasm) was 157.37 μ, almost constant from follicles of antral size to preovulatory size. The mean diameter of the zona pelluceda was 26.16 μ which is constant in all size follicles.

The mean percentage of oocyte recovery was 57.33 which is not significantly differ between stages of the cycle. The recovery rate was low in follicles of ≤ 2 mm diameter, maximum in follicles of 3-5 and 6-10 mm diameter range and moderate in follicles of > 10 mm diameter.

Size of the follicle, stage of the cycle and presence or absence of corpus luteum on the ovary significantly affected the quality of the oocyte. More number of good quality oocytes were obtained in 3-5 mm follicles and in ovary containing corpus luteum. Hence oocytes were collected from 3-5 mm diameter follicles during the II and III stages of the cycle to get more number of good quality oocytes for *in vitro* maturation.

Dissection method found to yield more number of compact, complete cumulus oophorus oocytes than the other two methods viz., aspiration and rupture of follicles. Though the percentage of oocyte from dissection method was low, total number of oocytes that reached metaphase II was more in dissection method. The study revealed that dissection method is the method of choice for getting more number of matured oocytes.

Addition of serum and hormones were found to be essential for the *in vitro* maturation of buffalo follicular oocytes. Supplements like 10 percent FCS, 20 percent EBS, 10 µg LH, 1 µg FSH, and 1 µg E_2-17ß had beneficial effect on the good anf fair quality oocytes. Maturation *in vitro* supplements had no effects on nude oocytes indicating that for the action of the above, oocyte cumulus cell contact was must. Oocytes with degenerated ooplasm with any degree of cellular investment was found to be unfit for *in vitro* maturation.

The percentage of maturation was not significantly different between FCS and EBS. The proportion of expanded cumulus cells was found to directly reflect the oocyte maturation in culture media containing FCS, EBS or FSH. However in the media containing E_2-17ß the same was not significantly reflected in the maturation rate of oocytes.

It was found out that 2 ml culture media to an ideal volume for culturing 10 to 40 oocytes. Culturing more than 10 oocytes in 100 µl drops adversely affected the oocyte maturation rate *in vitro*.

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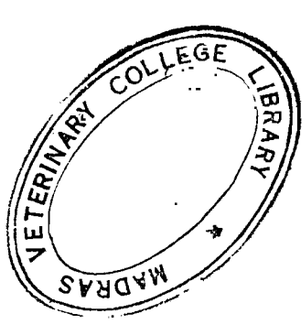
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