# STUDIES ON *IN VITRO* MATURATION OF PORCINE FOLLICULAR OOCYTES

**DEEPA. S.**

**Thesis submitted in partial fulfilment of the requirement for the degree of**

## Master of Veterinary Science

**Faculty of Veterinary and Animal Sciences**
**Kerala Agricultural University, Thrissur**

**2007**


**Department of Animal Reproduction, Gynaecology and Obstetrics**
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# DECLARATION

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

Mannuthy
30.6.2007

**Deepa S.**

# CERTIFICATE

We, the undersigned members of the Advisory Committee of **Deepa, S.,** a candidate for the degree of Master of Veterinary Science in Animal Reproduction agree that the thesis entitled **"STUDIES ON *IN VITRO* MATURATION OF PORCINE FOLLICULAR OOCYTES"** may be submitted by Deepa, S., in partial fulfilment of the requirement for the degree.

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# *ACKNOWLEDGEMENT*

*I find myself on look out for words as I place on record my sincere thanks and heartfelt gratitude to the chairman of the Advisory Committee,* ***Dr. V. Vijayakumaran,*** *Associate Professor, Department of Animal Reproduction Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Mannuthy, for the meticulous guidance, personal attention, keen interest, affectionate encouragement, persuasion and help offered to me during the course of study. Without his strong support and co-operation, the successful completion of this work would not have been possible.*

*I am in short of words to express my respect and deep sense of gratitude to* ***Dr. T. Sreekumaran,*** *Associate Professor and Head, Department of Animal Reproduction Gynaecology and Obstetrics and member of the advisory committee for his inspiring advice, valuable help, constructive suggestions and support throughout the course of my study.*

*I am deeply indebted to* ***Dr. Metilda Joseph,*** *Associate Professor, Department of Animal Reproduction Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Pookot and member of the advisory committee for her never ending help, meticulous guidance and constant encouragement.*

*I acknowledge the support and suggestions given to me by* ***Dr. P. Kuttinarayanan,*** *Associate Professor and Head, Department of Livestock Products Technology and member of advisory committee, for the successful completion of my work.*

*I am also grateful to other teachers in the Department of Animal Reproduction Gynaecology and Obstetrics,* ***Dr. Joseph Mathew, Dr. K.V. Athman, Dr. K.N. Aravind Gosh, Dr. G. Ajitkumar*** *and* ***Dr. C Ibraheem Kutty*** *and teachers of other Departments for their help rendered during this period of study.*

*I am grateful to* ***Dr. E. Nanu****, Dean, College of Veterinary and Animal Sciences, Mannuthy, Kerala Agricultural University for the facilities provided for research work.*

*I wish to express my sincere thanks to* ***Dr. Jabeena Martha Philip****, Ph.D. Scholar, University of Saskatchewan, Canada, for her valuable and wholehearted help during my research work by sending articles through e-mail.*

*I am very much obliged to* ***Mrs. Sujatha and Mrs. Mercy****, Assistant Professors, Department of Statistics for their help and wholehearted suggestions offered in the statistical analysis of the data.*

*I express my sincere thanks to* ***Sri. Chandrasekharan****, Assistant Professor, Instrumentation Lab,* ***Mr. Anil*** *and* ***Mrs. Annie*** *for the help and co-operation during the course of my work.*

*I express my sincere thanks to* ***Dr. G. Jeba Sujana Dhas, Dr.Magnus paul, Dr. Ratheesh Babu, Dr. Asha Marina Kuriyakose, Dr. Jegaveerapandian, Dr. Kishore Mathew*** *and* ***Dr. Alex*** *for their help and support.*

*My special thanks to my colleagues* ***Dr. Safna Isaac, Dr. Sandhya Achuthan, Dr. Sathyaraj, Dr.Deepthi, Dr. Hari Narayanan, Dr.Rajeswari, Dr. Seena,*** *and* ***Dr. Juliet.*** *for the help and support rendered to me at all times.*

*With exquisite pleasure and gratitude I acknowledge the help and co-operation rendered to me by my friends* ***Dr.Ranjini, Dr. Jimcy, Dr. Reshmi, Dr. Nisa, Dr.Naseera, Dr. Jinesh, Dr. Tessy, Dr.Raji, Dr. Sany, Dr.Preetha, Dr. Dinker ,Dr.Shijin, Dr.Bibu , Dr. Rajajopal Dr. Jesto, Dr. Kavitha, Dr. Nisha, Dr.Kishore, Dr. Surya Narayanan, Dr. Anees and Dr. Surya***

*The service of all the labourers of AI Centre, Mannuthy and Kokkalai are greatly acknowledged.*

*I thank all workers of Dept. of Livestock products technology, College of Veterinary and Animal Sciences, Mannuthy and Slaughterhouse, Ollur for their help in collection of ovaries.*

*I have no words to express my gratitude to my beloved Parents and my brother,* ***Deepu****, for their unfailing patience, support, encouragement and valuable prayers. Without their blessings, it would not have been possible for me to complete this study in a successful manner.*

***Above all, I bow before the Divine love and blessings that helped me in this endeavour and throughout my life.***

***Deepa. S***

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# LIST OF ABBREVIATIONS USED

| | | |
|---|---|---|
| ATP | - | Adenosine tri phosphate. |
| bFGF | - | Bovine fibroblast growth factor. |
| BSA-V | - | Bovine serum albumin fraction -V. |
| cAMP | - | Cyclic adenosine monophosphate. |
| CDOs | - | Cumulus denuded oocytes |
| $CO_2$ | - | Carbon dioxide |
| COCs | - | Cumulus oocytes complexes. |
| CGs | - | Cortical granules |
| CL | - | Corpus luteum |
| cm | - | Centimetre |
| DNA | - | Deoxy ribo nucleic acid |
| ECS | - | Estrous cow serum. |
| EGF | - | Epidermal growth factor. |
| FCS | - | Foetal calf serum. |
| FDA | - | Fluorescein diacetate. |
| GSH | - | Glutathione |
| GJC | - | Gap junction communication. |
| GV | - | Germinal vesicle. |
| GVBD | - | Germinal vesicle breakdown. |
| h | - | Hour |
| hCG | - | Human chorionic gonadotrophin |
| hnRNA | - | Heterogeneous nuclear ribo nucleic acid |
| IGF | - | Insulin like growth factor. |
| IU | - | International units. |
| IVF | - | *In vitro* fertilization. |
| IVM | - | *In vitro* maturation. |
| IVP | - | *In vitro* production. |
| kg | - | Kilogram |
| MAP | - | Mitogen activated protein |
| MEM | - | Minimum essential medium. |
| mg | - | Milligram |
| MI | - | Metaphase I |
| MII | - | Metaphase II. |
| ml | - | Millilitre |
| mM | - | Millimole |
| MPF | - | Maturation promoting factor |
| mSER | - | Mitochondrial smooth endoplasmic reticulum |
| NCSU-23 | - | North carolina state university medium-23 |
| NIH | - | National institute of health. |
| nm | - | Nanometre |
| OMI | - | Oocyte maturation inhibitor |
| PAS | - | Post aspiration slicing |

| | | |
|---|---|---|
| PB | - | Polar body |
| PBS | - | Phosphate buffered saline. |
| pFF | - | Porcine follicular fluid |
| pFSH | - | Porcine follicle stimulating hormone. |
| pLH | - | Porcine luteinizing hormone. |
| PRL | - | Prolactin |
| RER | - | Rough endoplasmic reticulum |
| RNA | - | Ribo nucleic acid |
| rRNA | - | Ribosomal ribo nucleic acid |
| SER | - | Smooth endoplasmic reticulum |
| SOF | - | Synthetic oviduct fluid. |
| SS | - | Steer serum. |
| TCA | - | Tri carboxylic acid. |
| TCM | - | Tissue culture medium. |
| TGF | - | Transforming growth factor. |
| TL-HEPES | - | Tyrode's lactate-hydroxy ethyl piperazine ethane sulfonic acid. |
| UV | - | Ultraviolet |
| °C | - | Degree Celsius |
| µg | - | Microgram |
| µm | - | Micrometre |

# *Introduction*

# 1. INTRODUCTION

Compared to ruminants, pig embryo transfer has been used only to a limited extend for commercial and breeding applications. This is due to the high fecundity of this species and complexity in the procedure of surgical embryo collection and transfer. Until recently the most important application of embryo transfer technology in pig was disease control that provides greater flexibility in the introduction of new genetic material into closed herds. Guidelines laid down by the Veterinary Animal Health authorities in various countries indicates that international movement of pig as embryos is much safer than any other means of movements of pigs.

Recently, pigs have become increasingly important in the field of biomedical research and interest has grown in use of transgenic pigs as potential xenograph donors in future.

Clinical use of organ transplantation has become a major feature in dealing with many forms of terminal organ failure in human patients. According to Prather *et al.* (2003) more than 80,000 patients are waiting for organs in USA, but with less than one third receiving transplants. None of the current human possibilities offers a solution to this acute shortage of organs for transplantation. Pigs are believed to be an appropriate species for xenotransplantation because their organs are similar in size to human organs. The disease free embryo transfer pigs could be used to supply transplant material such as heart, heart valves, pancreas islets transplantation etc. as per requirement (Gordon, 2004).

The commercial application of embryo transfer in pigs has been restricted by the requirement for tedious and costly surgical interventions in the recovery and transfer of embryos. The development of *in vitro* maturation (IVM) and *in vitro*

fertilization (IVF) techniques in pigs is of considerable importance because of the possible application of this technology in different areas of research. Since most studies that attempt to produce transgenic pigs by nuclear transfer, cloning or pronuclear microinjection, use matured oocytes and early embryos, it is becoming more important to produce large number of developmentally competent oocytes and embryos for research. IVM and IVF made the production of transgenic animal economically feasible, even though the overall efficiency is low. *In vitro* produced embryos are good for research on advanced technique like sexing of embryo, embryo splitting, cloning etc. which can produce excellent models in various areas of medical and biological research. The development in cryopreservation of pig oocytes and embryos can augment the impact of these technologies on pig breeding programme in future.

As per 1995 census, pig population in India is 11.78 million, which represent 1.31 per cent of World population (897.1 million). Lot of *in vitro* maturation studies on follicular oocytes have been carried out in India on buffalo, goat, sheep and cattle. On perusal of literature it was found that no work of this type has been carried out in India in porcine species.

Only limited number of research articles are available on the effect of retrieval techniques on oocyte yield and its quality in porcine, probably because researches were mainly concentrating on *in vitro* maturation, *in vitro* fertilization and *in vitro* production of embryos. Available literature shows the existence of a wide range of variation in the yield of oocytes per ovary. Stancic *et al.* (1992) recovered 7.9 and 45.3 oocytes per ovary by aspiration and dissection procedures respectively. *In vitro* culture conditions required for the maturation of porcine oocytes are comparable to that of other species with the exception of culture duration (Prather and Day, 1998). As per the references available the culture duration of porcine oocytes shows

extensive variation ranging from 24 to 44h. The incidence of polyspermy was another problem found in porcine species, but recent advances in IVM and IVF culture systems reduced this defect to certain extent and increased the yield of *in vitro* produced embryos.

In the Department of Animal Reproduction, Gynaecology and Obstetrics of Kerala Agricultural University many research works have been carried out in the area of synchronization of oestrum, multiple ovulation, embryo transfer, cryopreservation of oocytes and embryos and *in vitro* maturation of oocytes in different species. But no such studies were carried out in porcine species. It is in this context that the present study was undertaken in porcine species with the following objectives.

1.Assess the efficiency of oocyte retrieval systems on oocyte yield and its quality.

2.Assess the effect of retrieval systems, quality grades of oocytes and culture durations on *in vitro* maturation of oocytes.

# *Review of Literature*

# 2. REVIEW OF LITERATURE

## 2.1 *IN VITRO* MATURATION

In most mammalian species, some hours before rupture of the follicle and ovulation, the fully-grown oocyte resumes meiosis, progressing from prophase of the first meiotic division to metaphase of the second division. This complex biological process, which transforms the primary oocyte into an unfertilized egg, is known as oocyte maturation. In live animal, the oocyte that has been arrested in the dictyate stage of the first meiotic division, resumes meiosis in response to the signals associated with preovulatory luteinizing hormone (LH) surge (Gordon, 2003). Mammalian ovarian oocytes can mature *in vitro* when liberated from graafian follicles and placed in appropriate culture media (Yanagimachi, 1974); this phenomenon is exploited in *in vitro* maturation (IVM) culture systems.

## 2.2 SOURCE OF OOCYTES

### 2.2.1 Ovary Collection

Ovaries obtained from local abattoir are usually used for the collection of porcine oocytes for *in vitro* maturation, *in vitro* fertilization and *in vitro* production of embryo (Agrawal and Polge 1989; Mattioli *et al.*, 1989; Hirao *et al.*, 1994). Oocytes could also be recovered from live animal by follicle aspiration, using either endoscopy or the ultra sound guided transvaginal approach (Gordon, 1997; Neglia *et al.*, 2001).

### 2.2.2. Ovary Transportation

Sterile normal saline supplemented with antibiotics (Yoshida *et al.*, 1993; Xia *et al.*, 2000; Gil *et al.*, 2003 and Wongsrikeao *et al.*, 2005) or Dulbecco's phosphate buffered saline (Funahashi and Day, 1993; Martinez *et al.*, 1993; Grupen *et al.*, 1995 and Suzuki *et al.*, 2000) was used commonly as transportation media for ovaries. In most of the studies porcine ovaries were transported in thermally insulated bags containing transportation media. The temperature of transportation media can vary from 25 degree celsius (Kikuchi *et al.*, 1993) to 35 degree celsius (Kikuchi *et al.*, 1999) with a maturation rate of 75 per cent and 70 per cent respectively. Effect of storage temperature on IVM of porcine oocyte has been studied extensively and it was found that exposing porcine ovaries to a low temperature of 25 degree celsius or less before aspiration of oocytes has adversely affected their subsequent *in vitro* maturation (Yuge *et al.*, 2003). Transient exposure to elevated temperature during slaughter did not have any detrimental effects on nuclear maturation, but resulted in extensive cytoskeletal damage, which in turn drastically decreased the developmental competence of porcine oocytes (Tong *et al.*, 2004).

Wang *et al.* (1995) found that storage of ovaries at either 4 degree celsius or at room temperature results in considerable loss in developmental capacity of bovine oocytes, even with storage time as short as 24h. Long transport time from the slaughterhouse to the laboratory before culture may adversely affect mare oocyte quality which might reduce the developmental competence of embryos after *in vitro* maturation and fertilization (Guignot *et al.*, 1999). There was no significant difference in the percentage of oocytes undergoing germinal vesicle breakdown (GVBD) and maturing to M II stage for porcine oocytes obtained from ovaries stored for zero and three hour (Wongsrikeao *et al.*, 2005). The percentage of degenerated oocytes increased with increasing storage time. The percentage of oocytes with normal morphology after

*in vitro* maturation was 61.1 and 41.6 per cent after 0h and 6h storage time respectively (Kim *et al.*, 2006).

### 2.2.3 Pre-Retrieval Processing of Ovary

Wang *et al.* (1995) used PBS containing antibiotics to wash the ovaries collected from slaughterhouse. Removal of extraneous tissues, repeated washing in tap water and 2-3 times washing in normal saline solution at 37 degree celsius reduced the chance of contamination in the oocyte culture (Qian *et al.*, 2003).

## 2.3 OOCYTE RETRIEVAL

### 2.3.1 Retrieval Media

Commonly used oocyte retrieval media are TL-HEPES, HEPES or Bicarbonate buffered TCM-199, Dulbecco's phosphate buffered saline and synthetic oviduct fluid (SOF). TL-HEPES (Tyrode's Lactate-2-hydroxy ethyl-piperazine-N'-2-ethanesulfonic acid) media supplemented with 0.3 per cent BSA fraction V and antibiotics was used for oocytes retrieval in many studies (Bavister *et al.*, 1983 and Grupen *et al.*, 1995). HEPES buffer, without a $CO_2$ atmosphere maintain the pH to 7.4, used for porcine embryonic development (Hagen *et al.*, 1991). Hyun *et al.* (2003) used HEPES-buffered TCM-199 or HEPES buffered NCSU-23 as oocyte retrieval media.

### 2.3.2 Retrieval Methods

Several methods are available for the retrieval of oocytes from porcine ovaries. But the commonly used methods are aspiration, slicing, puncture and post aspiration slicing.

*2.3.2.1.Aspiration*

Aspiration of oocytes from 3-6mm follicles with disposable 18-21-gauge needle with 10ml syringe was a common oocyte retrieval method (Mori *et al.*, 2000; Sturmey *et al.*, 2003; Algriany *et al.*, 2004; Sherrer *et al.*, 2004 and Kim *et al.*, 2006). Bols *et al.* (1996) reported that use of thicker needle (18G) yielded the highest oocyte recovery rate (75 per cent) but number of naked oocytes was more with this needle than with thinner gauge (21G) needle (10-20 per cent). With thinner needle oocyte recovery rate was low (50 per cent) but proportion of oocytes with compact cumulus was higher (35 per cent) than with thicker needle (20 per cent). They opined that the lower rate of naked oocytes following the use of thinner needle might be due to the movement of fluid and the COCs more laminar with less turbulence in thinner needle. Dell'Aguila *et al.*, (2001) found that the rate of abnormal fertilization was significantly higher for equine oocytes collected by aspiration (19 VS.6 per cent for dissection.). These results demonstrated that the collection method might contribute to differences in results observed among laboratories working with horse oocytes. By aspiration, oocytes from the surface follicles were released and at the same time it has the advantage of high-speed recovery of oocytes when compared to dissection or slicing (Gordon, 2003).

*2.3.2.2.Slicing*

Slicing produced a three-fold increase in the yield of oocytes over aspiration (Carolan *et al.*, 1992). High yield of COCs by slicing was due to recovery of oocytes located in cortical position in addition to follicles visible on surface (Arlotto *et al.*, 1996). The method involved slicing of ovaries repeatedly with a sharp sterile surgical blade while keeping ovaries in petridish containing the retrieval medium (Das *et al.*, 1996; Bols *et al.*, 1997 and Prisclla, 2001).

### *2.3.2.3.Post Aspiration Slicing*

Naqui, *et al.* (1992) reported that additional slicing increased the rate of recovery of oocytes from 1.57 to 4.08 per goat ovary. Post aspiration slicing yielded an additional 3.15 and 2.40±0.40 oocytes respectively from sheep (Balakrishnan, 1994) and caprine ovaries (Vijayakumaran, 1995). Post aspiration slicing approximately doubled the number of oocytes released from bovine ovary (Priscilla, 2001and Gordon, 2003).

### *2.3.2.4 Puncturing*

Didion *et al.* (1990) used puncturing method using 18-gauge needle for the collection of oocyte from pig ovaries. Ovaries were stabilized in a petridish containing retrieval media with forceps and visible follicles ranging from 2.0 to 6.0 mm in diameter were punctured with an 18-gauge needle. Follicular fluid was encouraged to escape with gentle pressure on the adjacent stroma of the punctured follicle (Vijayakumaran, 1995; Das *et al.*, 1996 and Priscilla, 2001).

## 2.4 OOCYTE YIELD PER OVARY

Studies with the ovaries of slaughtered gilts, showed the recovery of 7.9 and 45.3 oocytes per ovary respectively for aspiration and dissection methods (Stancic *et al.*, 1992). For *in vitro* fertilisation and *in vitro* embryo production from slaughtered animal, an efficient oocyte recovery method is absolutely essential. The recovery of oocyte using slicing technique yielded more oocytes per ovary (6.05) than dissection (1.71) or aspiration (1.27) from goat ovaries (Martino *et al.*, 1992). But in a study conducted by Pawshe *et al.* (1994) in goat ovaries, revealed that the number of oocyte recovered per ovary by aspiration (2.78) was significantly more than that of slicing (2.40) and puncturing (2.28). Jain *et al.* (1995) found that the yield of oocytes per

buffalo ovary was 7.02 and 1.84 oocytes respectively for slicing and aspiration. Das *et al.* (1996) found that the number of oocytes per buffalo ovary recovered by slicing (5.7) was significantly higher than that achieved by follicle puncture (2.6) or aspiration (1.7). Abdoon and Kandil (2001) reported that slicing of buffalo ovaries yielded 8.08 oocytes per ovary compared to 3.34 oocytes by aspiration.

## 2.5 OOCYTE PROCESSING PRIOR TO *IN VITRO* CULTURE

Rinsing of oocytes in fresh drops of medium was performed to avoid contamination with by-products of recovery process. The washing medium usually used were Dulbecco's phosphate buffered saline (Marchal *et al.*, 2001; Qian *et al.*, 2003 and Kim *et al.*, 2006), TL-HEPES (Hagen *et al.*, 1991;Yamauchi and Nagai, 1999 and Algriany *et al.*, 2004), TCM-199 (Hirao *et al.*, 1994; Sturmey and Leise, 2003 and Gil *et al.*, 2004) or in HEPES- buffered TCM-199 (Hyun *et al.*, 2003). Hirao *et al.* (1994) used modified Krebs-Ringer bicarbonate (mKRB) solution for washing of oocytes. Advantage of HEPES or phosphate buffered media for short term processing was that these media do not require a $CO_2$ controlled gas phase to maintain constant pH (Gordon, 2003).

## 2.6 GRADING OF CUMULUS OOCYTE COMPLEXES

There are various systems used in the grading of oocytes. Most of them depended on a visual subjective appraisal by laboratory personnel and as such could vary with the individual and the laboratory (Gordon, 2003). Oocytes can be graded based on its cumulus and ooplasm characters.

### 2.6.1 Grading Based on Cumulus Character

Agrawal (1992) graded caprine oocytes as good (morphologically normal with complete layers of cumulus cells), fair (morphologically normal with incomplete or no cumulus cell layers) and poor (morphologically abnormal with or without cumulus cell layers). Palomo *et al.* (1992) graded and classified oocytes into two groups as oocytes having more than or equal to three layers of cumulus cells and oocytes having one to two layers of cumulus cells. Pawshe *et al.* (1994) classified oocytes into three categories as good, fair and poor according to the character of the cumulus cells. Oocytes with more than four layers of compact cumulus cells and uniform cytoplasm were classified as good quality oocytes, oocytes with less than four layers or with a partial cumulus mass were classified as fair quality oocytes and oocytes without cumulus cells with a uniform cytoplasm were classified as poor quality oocytes. Das *et al.* (1996) graded oocytes with more than five layers of compact cumulus cells as good. Fair oocytes were those with two to five layers of cumulus cells. Oocytes with fewer than two layers of cumulus cells and either completely or partially denuded were graded as poor. Good and fair quality culturable grade oocytes had compact multilayered cumulus cells and an evenly granulated cytoplasm.

According to Abdoon and Kandil (2001) good quality oocytes were surrounded by more than 6 layers of cumulus cells. Oocytes having three to five and one to two layers of cumulus cells were graded as fair and poor quality oocytes respectively. Naked oocytes were assigned as denuded. Gupta *et al.* (2006) graded the recovered cumulus oocytes complexes from Murrah buffaloes with more than or equal to 5 layers of cumulus cells as grade A, those with two to four layers as grade B, partially denuded oocytes as grade C, and completely denuded oocytes as grade D.

### 2.6.2 Grading Based On Ooplasm Character

Oocytes, which have vacuolated or fragmented ooplasm and not evenly filling the zona pellucida or shrunken away from zona pellucida were, classified as degenerated oocytes (Leibfried and First, 1979). Das *et al.* (1996) graded oocytes with evenly granulated ooplasm as good and with a dark scattered ooplasm were graded as poor. Nashta *et al.* (1998) selected good quality oocytes with homogenous evenly distributed cytoplasm for maturation.

## 2.7 FUNCTIONAL ROLE OF CUMULUS CELLS

Cumulus cells were considered to be a subtype of granulosa cell, and were believed to retain some of the physiological properties of granulosa cells. The oocytes and the entire mass of surrounding cumulus cells in the follicle can be regarded as a structural and functional syncytium. The metabolic cooperation between oocyte and cumulus cells serves an important nutritive role during maturation. Any undue disturbance of the intimate connections between cumulus cells and the dependent oocyte is likely to adversely affect its subsequent developmental capacity (Gordon, 2003). Germinal vesicle stage oocytes were coupled with cumulus cells by gap junctions that were found at the areas of contact between the oolemma and cytoplasmic process of the cumulus cells that traverse the zona pellucida. A quantitative or qualitative change in the signal communication from the cumulus to the oocyte results in oocyte maturation (Eppig, 1982). Oocytes not surrounded by the cumulus cell will have aberrant protein synthesis pattern (Kastrop *et al.*, (1990)

Zona free and cumulus enclosed bovine oocytes were cultured *in vitro* in a study by Bilodeau *et al.* (1993) on cAMP and the resumption of meiosis; increases in cAMP in cumulus cells may activate a stimulatory signal for maturation and such a signal may overcome the inhibitory effect of a high content of cAMP in the oocyte

itself. The gap junction communication (GJC) between the oocyte and the cumulus cell was thought to play an important role in regulating ooplasmic factors involved in the removal of sperm nuclear envelops as well as in GSH (glutathione) transportation (Mori, 2000). Geshi *et al.* (2000) demonstrated that blastocysts obtained from cumulus denuded oocytes (CDOs) matured in a serum free medium supplemented with sodium pyruvate were developmentally competent to become calves, although the percentage of embryos that developed to the blastocyst stage was low.

## 2.8 EFFECTS OF RETRIEVAL SYSTEMS ON OOCYTES QUALITY

### 2.8.1 Grade A Oocytes

Oocytes with multiple layers of cumulus cells more than or equal to five and uniform granulation of ooplasm were considered as grade A oocytes (Gupta *et al.*, 2006). Retrieval methods like aspiration, slicing, puncture and post aspiration slicing were adopted for COC recovery.

Mongas *et al.* (1992) obtained 0.27 (11.2 per cent) and 4.6 (4.32 per cent) A grade oocytes per ovary by aspiration and slicing technique respectively from goat ovaries. Aspiration technique yielded 4.3 ±0.9 (30.9 per cent) A grade oocytes from bovine ovaries (Carolan *et al.*, 1994). Jain *et al.* (1995) obtained 0.80±0.15 (11.39 per cent), 0.20±0.06 (11.11 per cent) and 0.52±0.11 (18.71 per cent) A grade oocytes per ovary by slicing, aspiration and puncturing method respectively from buffalo ovaries. Das *et al.* (1996) obtained 0.7±0.09 (11.5 per cent), 0.4±0.08 (16.7 per cent) and 0.2 ±0.05 (11.3 per cent) A grade oocytes per ovary by slicing, puncturing and aspiration of buffalo ovaries. Abdoon and Kandil (2001) reported that the recovery of good quality oocytes by aspiration and slicing method was1.98±0.14 and 2.15±0.16 respectively from buffalo ovaries. Gogoi *et al.* (2001) recovered 39.69 per cent A grade oocytes by aspiration method from buffalo ovary.

### 2.8.2 Grade B Oocytes

Oocytes with 2-4 layers of cumulus cells were considered as grade B oocyte (Gupta *et al.* 2006). The aspiration, slicing and post aspiration slicing technique yield 24.49, 16.73 and 17.29 per cent B grade oocyte respectively (Vijayakumaran, 1995) from goat ovaries. Jain *et al.* (1995) obtained 2.32±0.37 (33.05 per cent), 0.69±0.14 (37.78 per cent) and 0.90±0.16 (32.37 per cent) B grade oocytes per ovary by slicing, aspiration and puncturing method respectively from buffalo ovaries. Das *et al.* (1996) obtained 1.9±0.19 (32.7 per cent), 0.9±0.12 (34.7 per cent) and 0.7 ±0.10 (39.1 per cent) B grade oocytes per ovary by slicing, puncturing and aspiration of buffalo ovaries. Gogoi *et al.* (2001) recovered 9.16 per cent B grade oocyte by aspiration technique from buffalo ovary.

### 2.8.3.Grade C Oocytes

Oocytes with <2 layers of cumulus cells and partially denuded oocytes were graded as C grade oocyte (Gupta *et al.*, 2006). By adopting aspiration, slicing and post aspiration slicing techniques in goat ovaries Vijayakumaran (1995) could obtain 16.74, 11.80 and 14.33 per cent C class oocytes respectively. Priscilla (2001) obtained 0.3-0.9 (8-13 per cent) C grade oocytes per ovary by aspiration technique and slicing technique yielded 0.4-0.6(10.12 per cent) C grade oocytes per ovine ovary. Gogoi *et al.* (2001) got 10.69 per cent C grade oocyte by aspiration technique from buffalo ovary. Paul (2005) recovered 18.3, 20.66 and 19.13 per cent C grade oocytes by aspiration, slicing and puncturing respectively from bovine ovary.

### 2.8.4.Grade D Oocytes

Gupta *et al.* (2006) graded completely denuded oocytes as D grade oocyte. Vijayakumaran (1995) recorded 18.96, 18.16, 17.48 and 14.58 per cent D grade oocytes

by slicing, puncturing, aspiration and post aspiration slicing techniques respectively from goat ovaries. Puncturing method yielded 48.7 per cent poor quality oocytes from buffalo ovaries (Das *et al.*, 1996). Gogoi *et al.* (2001) got 17.18 per cent D grade oocyte by aspiration technique from buffalo ovary. Priscilla (2001) obtained 0.57, 0.45, 0.57 and 0.24 oocytes per ovary by aspiration, puncturing, slicing and post aspiration slicing techniques respectively from bovine ovaries. Paul (2005) obtained 3.7, 12.1 and 6.01 per cent D grade oocytes by aspiration, slicing and puncturing respectively from bovine ovary.

## 2.9 STAGES OF OESTROUS CYCLE

Dailey *et al.* (1982) found that the number of follicles was increasing during the late luteal phase of the cycle in ewes. Babalola and Shapiro (1988) found that the mean diameter of the predominating follicles were directly related to the stage of ovarian cycle in sow, the early luteal to mid follicular stage ovaries had essentially small to medium sized follicles (2-6mm diameter) while late follicular and ovulatory ovaries contained large follicles (6-11mm diameter). The criteria for categorizing ovaries into different stages (follicular and luteal) were developed based on the morphological change which was shown to be characteristic of pig ovaries during the oestrous cycle (Mc Donald, 1989). Follicular development in the bovine is a highly coordinated event involving successive waves of dominant follicles in both the oestrous cycle and early pregnancy. But there was no evidence of follicular waves in either the non-pregnant or pregnant sows. In the non-pregnant sows, individual follicles were identified and there was no evidence of a change in size until day 15. The proportion of small follicles decreased while medium and large follicle increased from day 15 through day 20 of the oestrous cycle in sow (Ryan *et al.*, 1994). In pig the follicles destined to ovulate was increase growth between day 14 and 16 of the cycle; follicles grew from about 4-5mm diameter on day 15 to an ovulatory diameter of 9-11mm by the end of the cycle (Gordon, 2004)

### 2.9.1 Effect of Stages on Ovarian Biometry

Nair (1970) reported the mean measurements of length, width and thickness of the right and left porcine ovaries were 2.43×1.69×1.24 cms and 2.52× 1.73 ×1.28 respectively. Roberts (1971) reported the weight of sow ovaries varies from 3.5 to 10 gms. Bagg *et al.* (2004) found that corpus luteum (CL) bearing ovaries were significantly larger than non-CL bearing ovaries, in both width (22.3±0.9 mm versus 15.9 ±0.4 mm) and length (33.2± 1 mm versus 24.1±0.4mm) in porcine species and the follicles of CL bearing ovaries were significantly larger in diameter than those of non-CL bearing ovaries (4.5±0.1mm versus 3.3±0.02mm).

### 2.9.2 Effect of Stages on Oocyte Yield

Follicular numbers and oocyte quality were recorded during different stages of oestrous cycle. Al-gubory and Martinet (1987) found that the CL had no effect on oocyte growth and cellular development of the granulosa in sheep ovaries. Agrawal (1992) reported that the oocyte recovery rate from caprine ovaries containing CL was very poor and a positive relationship was observed in oocyte recovery rate and size of ovary. Moreno *et al.* (1993) observed that the number of oocytes per ovary was significantly greater in pregnant cows vs. open cows and ovaries from pregnant cows with out a CL have a greater percentage of grade I oocytes than with a CL. Boediono *et al.* (1995) opined that higher quality oocytes can be obtained from ovaries in the luteal phase. Das *et al.* (1996) found a significant decrease in good, poor and total yield of oocyte from CL bearing ovaries than from non-CL bearing ovaries and the average oocyte recovery rate was higher from ovaries containing larger number of follicles in buffaloes. Development to blastocyst was greater in oocytes collected during phases of follicular dominance in bovine (Hagemann *et al.*, 1999). Studies conducted in Egyptian buffaloes revealed that the number of small and large follicles was more in ovaries without a CL and also the total yield of good quality oocytes was more from CL

bearing ovaries (Abdoon and Kandil, 2001). Priscilla, (2001) observed that stage of the oestrous cycle had an influence on the grade of oocytes obtained in bovine species and the ovaries bearing a functional CL yielded more number of grade I oocytes per ovary.

### 2.9.3 Effect of Surface Follicle on Oocyte Quality

Katska (1984) obtained a recovery rate of 43.2 per cent by aspiration from surface follicles from bovine ovaries. A positive correlation exists between follicle diameter and oocyte quality. Pavlok *et al.* (1992) reported that blastocysts from follicles ranging from more than two to eight millimetres in size had a similar developmental potential but oocytes from one to two millimetre follicles had a significantly lower competence to undergo *in vitro* maturation. Lucas *et al.* (2002) pointed out that the meiotic maturation of porcine oocytes isolated from antral follicles progressed as the follicular size increased. Marchal *et al.* (2002) reported that the porcine oocytes from medium and large follicles were competent for maturation (77 and 86 per cent respectively) than those from small follicles (44 per cent). Bagg *et al.* (2004) studied the correlation between ovarian follicle and oocyte morphology immediately after the onset of puberty and the developmental competence of *in vitro* matured oocytes in pigs. The average number of antral follicles per ovary was greater on the surface of non-CL ovaries than on CL ovaries (46±2.2mm versus 21.1±1.8mm).

## 2.10 FACTORS CONTROLLING *IN VITRO* MATURATION

### 2.10.1 Oocyte Quality

Oocytes with highest developmental competence possessed a compact and complete cumulus mass and uniform appearance and an even, smooth, finely granulated cytoplasm (Gordon, 2004). Tsafriri and Channing (1975) found that porcine oocytes lacking investments when removed from follicles had a low rate (10 per cent)

of maturation *in vitro*. Leibfried and First (1979) found that the ability of bovine oocytes to undergo nuclear maturation *in vitro* after removal from vesicular follicles was not dependent on either size of follicle or stage of the estrous cycle, the presence of cellular investment, intact cytoplasm and dictyate stage chromatin may be the only determinants of the bovine oocytes's ability to mature *in vitro*. Data reported by Vassena *et al.* (2003) suggested that oocytes collected during the static or regressing phases of the follicular wave were preferable to those collected in the growing phase in bovine species, and that the effect of early follicular atresia were beneficial to oocyte competence.

Hawk *et al.* (1992) demonstrated that the bovine oocytes selected for good quality could be stripped of almost all surrounding cells before IVM and still retain their developmental potential. Fertility in the young pig might be lower at the first oestrus than at subsequent heat periods. Oocytes recovered from gilts at the pubertal and third oestrous and found that the percentage of immature oocytes was significantly greater (22 VS. 12 per cent) at the first than at the third oestrus; such results indicated that oocytes of first oestrous gilts are inferior to those of third oestrous animals (Koenig and Stormshak, 1993). Marchal *et al.* (2001) reported that although prepubertal gilt oocytes appeared less meiotically and developmentally competent than their adult counter parts, they could be used to produce blastocysts, which might be able to develop to term. The incomplete differentiation of prepubertal oocytes was reflected by decrease sensitivity to EGF, FSH and FF compared to adult oocytes. Similar findings were also reported by Bagg *et al.* (2004), which suggested that the higher developmental competence usually observed in adult oocytes is acquired gradually and required exposure to multiple oestrous cycles.

### 2.10.2 Maturation Media

The culture media employed in the extra follicular maturation of the bovine oocyte can be broadly divided into simple and complex. Simple media are usually bicarbonate buffered systems containing basic physiological saline with pyruvate, lactate, glucose and supplemented with serum or albumin with trace amounts of antibiotics. The complex media contain in addition to the basic components of simple media, amino acids, vitamins, purins and other substances, mainly at levels at which they were found in serum (Gordon, 2003).

These media were designed originally for cell culture and were not capable of supporting high level of oocyte maturation and hence supplemented with sera (Gasparrini, 2000). The basic media commonly used were TCM-199 (Hyun *et al.*, 2003). BSA-free North Carolina state university (NCSU-37) medium (Suzuki *et al.*, 2006), Ham's F-10, Ham's F-12, Minimum essential medium-α (MEM- α), RPMI-1640, Waymouth medium etc. Among these TCM-199 was most widely used.

#### *2.10.2.1 TCM-199 medium*

TCM-199 without any additives could support maturation of oocytes and on fertilization and culture *in vitro* could result in formation of hatched blastocyst (Bevers *et al.*, 1997). The basic culture medium most commonly used for the maturation of porcine oocytes was tissue culture medium-199, supplemented hormones, amino acids and other substances found in the concentrations as in serum (Mori *et al.*, 2000; Gordon, 2003; Isobe and Terada, 2001; Hyun *et al.*, 2003)

### *2.10.2.2 North Carolina State University medium (NCSU-23)*

NCSU-23 medium supplemented with porcine follicular fluid and other hormonal supplements were used for *in vitro* maturation of porcine oocytes. Hyun *et al.* (2003) reported that NCSU-23 medium was as effective as TCM-199 for *in vitro* maturation of porcine oocytes. Suzuki *et al.* (2006) opined that *in vitro* maturation of porcine oocyte in NCSU-37 supplemented with fetal bovine serum (FBS) had reduced the maturation ability, but once oocyte have matured, they have the same ability to develop to term after *in vitro* fertilization and embryo transfer as those matured with porcine follicular fluid (pFF).

## 2.10.3Components of Maturation Media

### *2.10.3.1 Hormones*

The supplementation of maturation media with hormones is based on the presumptive involvement of these hormones in the *in vivo* maturation process. Many of the reports on cattle IVM have dealt with the direct addition of gonadotropins (FSH, LH) and steroids (estradiol) to the maturation medium. (Wang *et al.*, 1992; Choi *et al.*, 2001; Gordon, 2003). Gonadotropin stimulate cumulus granulosa cells to secrete hyaluronic acid that disperses the cumulus cells, a process called expansion or mucification (Dekel and Kraicer, 1978). LH and FSH accelerated and facilitated meiotic progression, and LH selectively improved cytoplasmic maturation, which is required to promote the formation of a male pronucleus (Mattioli, *et al.*, 1991a). Funahashi *et al.* (1994) reported that two different hormonal conditions during *in vitro* maturation results in improved cytoplasmic maturation of pig oocytes, namely presence of PMSG during the first 20 h of culture and the absence of PMSG and oestradiol during the second 20h of culture. The oocyte maturation rate was significantly

increased on supplementation of maturation medium with a combination of FSH, LH and oestradiol (Sachan *et al.*, 1999).

Ball *et al.* (1985) recorded that more than 90 per cent of the porcine COCs exhibited partial or complete expansion when exposed to FSH at a concentration of 10μg/ml. Ding and Foxcroft (1994) observed that addition of FSH at a concentration of 2.5μg /ml enhanced the oocyte maturation in pigs. FSH used at a concentration of 0.5 μg /ml in many studies improved the *in vitro* maturation process in buffaloes (Totey *et al.*, 1993 and Gasparrini *et al.*, 2000). Supplementation of pFSH at a concentration of 5 μg /ml in TCM-199 resulted in significantly higher maturation rates in goat oocytes after 30h of culture (Katiyar *et al.*, 1997).

Luteinizing hormone (LH) used at a concentration of 5 μg /ml for *in vitro* maturation of buffalo oocytes (Totey *et al.*, 1993; Gasparrini *et al.*, 2000). Ding and Foxcroft (1994) used LH and prolactin (PRL) at a concentration of 2.5 μg /ml and 20 ng/ml respectively for *in vitro* maturation of porcine oocytes.

Estradiol supplemented to the maturation medium at a concentration of 1 μg /ml was found to improve *in vitro* maturation of oocytes from Bovine (Harper *et al.*, 1993), buffalo (Sachan *et al.*, 1999) and pig (Bing *et al.*, 2001). Liu *et al.* (2003) reported that eCG (PMSG) promoted porcine follicular development, probably by inhibiting granulosa-cell apoptosis. Li *et al.* (2004) observed that estradiol (E2) added to protein free medium inhibited nuclear and cytoplasmic maturation of cumulus enclosed pig oocytes, possibly by inhibiting progesterone (P4) synthesis by cumulus cells through the estradiol receptor mediated pathway.

Funahashi and Day (1993) suggested that there was at least two different phases during in vitro maturation of porcine oocytes, meiotic maturation was controlled by stimulation from hormone supplements in the primary phase (20h) and cytoplasmic

maturation was enhanced by the absence of some or all the hormone supplements in the second 20h. Pig oocytes completed germinal vesicle breakdown (GVBD) in vivo and in vitro about 20 h after hCG injection. It appeared therefore that the requirement of hormone supplementation was limited to the GVBD phase. The placement of COCs in hormone free medium at about 20h after culture enhanced the secretary potential of cumulus cells.

### *2.10.3.2 Growth factors*

Growth factors were agents that can enhance the efficiency of maturation. Major growth factors that can enhance tha oocyte maturation was epidermal growth factors (EGF), insulin like growth factors (IGF-I), transforming growth factor-α (TGF-α) and bovine fibroblast growth factor ( b FGF ).These factors exert their biological action by binding to membrane bound receptors of either thecal cells and /or granulosa cells and seems strongly related to quality and size of the follicle . The effects of these growth factors were absent when denuded oocytes were used (Bevers *et al.*, 1997). For sow oocytes, EGF was effective even at the lowest dose tested, which was sufficient to obtain the maximum rate of nuclear maturation, where as gilt oocytes needed at least 10 ng /ml to reach the maximum level of nuclear maturation (Abeydeera *et al.*, 2000; Marchal *et al.*, 2000). Gupta *et al.* (2002) observed that 20ng/ml was the optimum concentration of both EGF and FGF for buffalo oocyte maturation. EGF and IGF-I increased granulosa cell proliferation, decreased cellular apoptosis, and promoted follicular antrum formation (Mao *et al.*, 2004).

### *2.10.3.3 Serum*

It was a common practice to include macromolecular constituent from various sources like foetal calf serum (FCS), estrus cow serum (ECS), steer serum (SS) or bovine serum albumin (BSA) to various culture media. Serum was known to contain a

wide range of compounds including hormones, growth factors, amino acids and binding proteins (Gordon, 2003). Suzuki *et al.* (2006) used fetal bovine serum instead of porcine follicular fluid and the result showed that the proportions of porcine oocyte with expanded cumulus cells were lower but the efficiency of subsequent *in vitro* embryo culture and blastocyst formation were not affected.

In many of the studies 10 per cent fetal calf serum (Yoshida *et al.*, 1992 and Coy *et al.*, 1999) or 10 per cent fetal bovine serum (Katiyar *et al.*, 1997) was used as protein source.

#### *2.10.3.4 Other factors*

The presence of organic osmolytes, such as sorbitol and taurine, reduced the detrimental effect of high NaCl concentration in media used for the maturation of porcine oocytes (Funahashi *et al.* 1996). Male pro-nuclear formation in porcine oocytes was promoted by the addition of amino acids in simple maturation medium (Ka *et al.*, 1997). Funahashi *et al.* (1997) found that supplementation of dibutyryl cyclic adenosine monophosphate during the first 20h of culture for maturation induced a more synchronous meiotic progression of porcine oocytes and improved the rate of early embryonic development to the blastocyst stage after IVF. Addition of amino acids was found to be most beneficial for IVM when gonadotropin and EGF were also present in the maturation media. Non-essential amino acids added to a defined medium promoted oocyte maturation and subsequent development to the blastocyst stage (Hong *et al.*, 2004). Addition of Pyruvate to maturation media provided the necessary energy for oocyte's primary metabolism and also acted as an antioxidant, protecting oocytes against the stress of the *in vitro* maturation environment (Gonzales-Figueroa and Gonzales- Molfino, 2005).

Cysteamine added to culture media was reported to increase glutamine synthesis in oocytes, which promoted male pro-nucleus formation in porcine (Grupen *et al.*, 1995; Bing *et al.*, 2001and Jeong and Yang, 2001) and Buffalo (Gasparrini *et al.*, 2000). Sawai *et al.* (1997) reported that the presence of cysteine in serum free medium was required only between 42 and 48 h after initiation of maturation of porcine oocytes. Wongsrikeao *et al.* (2006) studied the effect of fructose as an alternative to glucose for supporting IVM of porcine oocytes and found that fructose was superior to glucose for producing high quality porcine embryos *in vitro.*

### 2.10.4 Culture Conditions

#### *2.10.4.1 Temperature*

The success of *in vitro* embryo production is temperature dependent. Eng *et al.* (1986) found that pig oocytes cultured at 39 degree celsius had a higher percentage of polar body formation than did those cultured at 37 degree celsius. Conventional *in vitro* maturation systems for pig oocytes were maintained at 38.5-39 degree celsius, which closely resembled body temperature. Microtubule assembly in pig oocytes cultured at 35 degree celsius may be retarded resulting in lower MII rates. Pig oocytes could complete maturation when cultured at 35 degree celsius with reduced degree of developmental competence to the blastocyst stage (Abeydeera *et al.*, 2001). Changes in temperature, exposes oocytes to temperature shock, which induced chromosome abnormalities. The temperature range of 38-39 degree celsius was found to be beneficial for both cattle and buffalo oocytes (Gordon, 2003).

#### *2.10.4.2 Gas Phase*

Development of oocyte was found to be dependent on the gas phase environment during *in vitro* culture. The best gas phase observed was 5 per cent $CO_2$, 5

per cent $O_2$ and 90 per cent $N_2$ (Boone and Shapiro, 1990; Nagao *et al.*, 1993). Eng *et al.* (1986) suggested that $CO_2$ /bicarbonate might be important for the normal maturation of pig oocytes for that the bicarbonate buffering system was maintained by incubation in an atmosphere of 5 per cent $CO_2$ in air. Low oxygen tension during *in vitro* maturation of porcine oocyte was beneficial for supporting their subsequent development to the blastocyst stage (Karja *et al.*, 2004). Iwamoto *et al.* (2005) found that when oocytes were matured under 5 per cent $O_2$ tension the rate of normal oocyte activation was higher than when oocytes were matured under 20 per cent $O_2$ tension.

### *2.10.4.3 Humidity*

Maximum humidity was used in order to prevent medium evaporation during *in vitro* culture (Gordon, 1994). Humidified atmosphere was used in many maturation studies (Harper and Brackett, 1993; Eng *et al.*, 1986 and Coy *et al.*, 1999).

### *2.10.4.4 Culture Duration*

The duration required for the completion of *in vitro* maturation of porcine oocyte is more than that of cattle (24h). The basic physiological difference in interval between standing oestrus and ovulation explains why *in vitro* maturation of oocytes show species difference in duration of culture. Wang *et al.* (1992) found that cleavage rates of porcine oocytes cultured for 32-36h were significantly higher than those of oocytes culture for 24-28h or 40-44h after *in vitro* fertilization. Various time periods ranging between 36h and 48h were used among laboratories for IVM of porcine oocytes with some preference given for 40-44h duration (Prather and Day, 1998). The culture duration used for *in vitro* maturation of porcine oocyte varied from 42h(Yuge *et al.*, 2003 and Hyun *et al.*, 2003), 44h (Coy *et al.*, 1999,Suzuki *et al.*, 2000, Li *et al.*, 2004 and Suzuki *et al.*, 2006) 44-47h (Kim *et al.*, 1999) 48h (Hirao *et al.*, 1994, Mori *et al.*, 2000 and Qian *et al.*, 2003) 44-48h (Techakumpher and Srianan, 2000). Abeydeera

*et al.* (2001) opined that extension of culture time upto 68h could stimulate nuclear maturation but the further developmental competence was reduced.

## 2.11 OOCYTE MATURATION

In most mammalian species, some hours before rupture of the follicle and ovulation, the fully-grown oocyte resumed meiosis, progressing from prophase of first meiotic division to metaphase of the second division. This complex biological process, which transformed the primary oocyte into an unfertilized egg, was known as oocyte maturation (Gordon, 1994). Hyttel *et al.* (1997) described how the oocyte from the dominant follicle underwent further ultra structural modifications and attained full developmental competence through a process that might be termed capacitation. Capacitation involved two components that is, nuclear and cytoplasmic maturation.

### 2.11.1 Cytoplasmic Maturation

The cytoplasmic maturation is referred to as the process of oocytes obtaining factors required for full developmental potential (Gordon, 2004). Maturation was required to acquire the conditions to block polyspermy in case of fertilization and to decondense penetrated spermatozoa to form pronuclei after fertilization which included redistribution of cell organelles, migration of mitochondria to a perinuclear position and accumulation of granules along the oolemma (Prather and Day, 1998; Van den Hurk and Zhao, 2005).

The changes associated with maturation-included resumption of meiosis marked by GVBD; GVBD in pig required protein synthesis at a specific stage of maturation ie, 8-14h of culture. By 32h the layer of corona radiata cells was less compact and inter cellular coupling between the oocyte and cumulus cells decreased. In pig the initiation of meiosis correlated with the appearance of active maturation promoting factor (MPF)

in ooplasm at 8-12h of culture (Motlik and Fulka, 1986). Other changes include disappearance of rough endoplasmic reticulum (RER) and formation of clusters of mitochondria in association with lipid droplets and smooth endoplasmic reticulum (SER). These mitochondrial smooth endoplasmic reticulum (mSER) aggregates could be involved in the production of a reservoir of energy prior to fertilization (Torner *et al.*, 2004). Intensive clustering of mitochondria in association with lipid droplets and elements of SER and appearance of ribosome in cytoplasm were characterized during the period from 8-19 h. At the end of maturation the clusters of mitochondria dispersed, and majority of organelles distributed in the central region of oocytes (Gordon, 2004).

Yoshida *et al.* (1993) based on confocal and fluorescence microscopic study using lectins of the distribution of cortical granules during *in vitro* maturation of porcine oocytes found that between 24 to 36h of *in vitro* maturation cortical granules (CGs) migrate centrifugally and come to lie next to the plasma membrane.

### 2.11.2 Nuclear Maturation

Bilodeau *et al.* (1993) classified nuclear status of oocytes were as GV stage or intermediate stage (GVBD, chromosome condensation, MI) or mature (Anaphasel, Telophase I and mataphase II). Nuclear maturation refers to acquisition of the ability to undergo dissolution of germinal vesicle (GV), GVBD, condensation chromosomes, release of 1$^{st}$ polar body and subsequent arrest at metaphase II (Rodriguez and Farin, 2004). Ellederova *et al.* (2004) found that porcine oocytes released from follicles at the GV stage initiated GVBD at 24h, and by 28h most of the oocytes reached MI, following this phase the oocytes progressed through anaphaseI/telophaseI stage and by 45h most reached the MII stage with the extruded first polar body.

## 2.12 ASSESSMENT OF OOCYTE MATURATION

### 2.12.1 Cumulus Cell Expansion

Maturation of the oocytes *in vitro* involves well-defined changes in the cumulus cell that surround the oocyte. The cumulus cells expand to form a spherical mass in three dimensions and the COC appears to float in the culture medium drops. In the live animal, such changes were part of the process where by the COC separated itself from the follicle wall just prior to ovulation; the stickiness might be important in ensuring the pick up of the COC by the ciliated epithelium of the fimbriated infundibulum of the oviduct (Gordon, 2003).

The morphological transformation of the cumulus oophorus, referred to as expansion or mucification, may lead to a decreased integrity of junctional complexes between the cumulus cell processes and the oolema. Pig oocytes retain functional intercellular communication with adjacent follicle cells for a longer period during maturation than any other species. The oocyte and corona radiata remained tightly coupled in pig and other species, even though the oocyte had already resumed meiosis (Eppig, 1982). Cumulus expansion and cumulus cell oocyte coupling during *in vitro* maturation studies of porcine oocyte showed that at 16 and 24h of culture, cumulus expansion was induced, although the cells of the corona radiata were still closely attached to the oocytes and at 32 and 40h of culture the cumulus was completely expanded and partly detached from the oocyte. At the time of GVBD in pigs, 20-24 h after hCG, the outer layer of the cumulus cells were very expanded (Motlik *et al.*, 1986). The coupling index was high in oocytes at metaphase I (M I) as it was in GV oocytes; significant uncoupling was not observed until about 32h after the induction of maturation (Moor *et al.*, 1990). Cumulus cells were stimulated by gonadotropin and growth factor to produce and secrete hyaluronic acid (HA), which resulted in expansion process. The placement of cumulus oocyte complexes in hormone free medium at about

20h after culture enhanced the secretary potential of cumulus cells (Funahashi and Day, 1993). Vanderhyden (1993) found that pig oocytes could secrete a cumulus expansion-enabling factor similar to that of mouse and rat, but expansion of pig cumulus oocyte complexes did not depend upon this factor. Suzuki *et al.* (2000) found that cumulus expansion and mucification profoundly occured after 24h IVM in pigs coinciding with events the tranzonal cumulus cell projections were disconnected. Danfour *et al.* (2002) found cumulus cell mass and its expansion level could be good predictors of blastocyst potential after IVF.

### 2.12.2 Nuclear Changes

Oocytes with a polar body were regarded as matured (Yoshida *et al.*, 1993). Porcine oocytes released from follicles at the GV stage initiated GV breakdown at 24 h, and by 28h most of oocytes reached M I stage. By 42h most of the oocytes reached M II stage with the extruded first polar body (Ellederova *et al.*, 2004).

#### *2.12.2.1 Germinal Vesicle Stage*

Ultra structural studies revealed that the oocyte collected from 3-8mm diameter follicles have a diameter more than 100μm. The nucleus displayed moderate auto radiographic labelling excluding the nucleolus, and the fibrillo-granular nucleolus develops a large central vacuole and marginalized fibrillar centers. The organelles dislocated to the periphery, the number of lipid droplets and vesicles have increased (Hyttel *et al.*, 1997). The germinal vesicle of fully-grown porcine oocyte was characterised by a prominent, compact nucleolus formed by an electron dense fibrillar material and rRNA and hnRNA synthesis was extremely stable (Motlik and Fulka, 1986).

### *2.12.2.2 Germinal Vesicle Breakdown Stage*

The perivitelline space developed further and in the oocyte the mitochondria tend to arrange around the lipid droplets and the nuclear envelope was dissolved into tubules of smooth endoplasmic reticulum and microtubules appeared adjacent to the condensing chromosomes (Hyttel *et al.*, 1997). Cyclic AMP could block the spontaneous meiotic maturation of oocytes in culture. A drop in oocyte cAMP had been shown to precede GVBD (Xia *et al.* 2000). GVBD coincided with an appearance of active maturation promoting factor (MPF) molecules in ooplasm. Protein synthesis, which was necessary for GVBD of pig oocytes was initiated later than 6h and earlier than 12h of culture (Motlik and Fulka, 1986).

### *2.12.2.3 Metaphase I Stage*

The number and size of the lipid droplets has increased and mitochondria had assembled around the droplets. Numerous ribosomes appeared especially around the chromosomes and the size of the golgi complexes decreased further (Hyttel, 1997). In metaphase I diploid set of chromosomes were fully condensed and were arranged on equatorial plane (Hyun *et al.*, 2003).

### *2.12.2.4 Metaphase II Stage*

The cortical granules were distributed at solitary positions along oolemma. The lipid droplets and mitochondria attained a more central location in the ooplasm leaving a rather orgenelle free peripheral zone in which the most prominent features were large clusters of smooth endoplasmic reticulum (Hyttel *et al.*, 1997). M II stage was observed in various configurations like presence of a pair of condensed chromatin mass representing the oocyte and polar body chromatin or presence of a full set of haploid chromosomes along with a polar body chromatin mass (Hyun *et al.*, 2003)

## 2.13. EFFECT OF RETRIEVAL METHOD AND OOCYTES QUALITY ON IVM

### 2.13.1. Aspiration

Funahashi and Day (1993) collected oocyte cumulus complexes with uniform ooplasm and a compact cumulus cell mass and after 40h of culture got a maturation rate of 88 per cent. Rabahi *et al.* (1993) aspirated bovine oocytes with more than five to six layers of cumulus cells, and for 24-26h of culture 85-96 per cent cumulus expansion rate was observed. Wang *et al.* (1994) got a maturation rate of 73 per cent after culturing of compact porcine oocytes, collected by aspiration, for 36h inTCM-199B medium. Pawshe *et al.* (1994) in goats obtained 77 to 79 per cent maturation rate when oocytes with more than five layers of cumulus cells. Dell'Aguila *et al.*, (2001) found that the rate of abnormal fertilization was significantly higher for oocytes collected by aspiration. Aspiration resulted in greater disruption of surrounding cumulus cells; this may be due to the cumulus oophorus being firmly attached to the stratum granulosum. Paul (2005) observed a maturation rate of 83.08, 68.29 and 44.74 per cent respectively for A, B and C grade oocytes by aspiration technique in bovine oocytes.

### 2.13.2.Puncturing

When puncturing method was applied for collection of oocytes from bovine ovaries, after maturation a metaphase II rate of 60 per cent was obtained for Iritani and Niwa (1977), whereas for Schellander *et al.* (1990), it was 78-88.4 per cent.

### 2.13.3.Slicing

A higher maturation rate in A grade caprine oocytes was also reported by Pawshe *et al* (1994). In slicing method the oocyte with more layers of cumulus cells

showed high maturation rate. The observations of the present study indicate the importance of cumulus cells for maturation. Vajta *et al.* (1996) reported that the number of oocytes recovered and number of blastocysts obtained was approximately doubled by slicing ovaries compare to aspiration. Slicing method releases oocytes from the surface and cortical follicular population. Arlotto *et al.* (1996) found that the oocytes from cortical follicles were still in the growth phase, so they did not acquire complete competency for maturation and development. Paul (2005) recorded a maturation rate of 69.70 per cent for A grade oocytes 53.01 per cent for B grade and 35.29 per cent for C grade oocytes from bovine ovaries.

### 2.13.4. Post aspiration slicing

Motlik (1989) pointed out that reduced maturation for oocytes collected by post aspiration slicing due to the absence of some facts necessary for oocyte maturation. Ellederova (2004) found that transcripts and proteins synthesized and stored earlier, during the period of oocyte growth and completion of all the metabolic steps, allows acquisition of a full meiotic and developmental competence. These findings reveal that the oocytes collected from cortical follicles are immature and developmentally incompetent.

## 2.14 MATURATION CHANGES OF OOCYTE AT DIFFERENT TIME INTERVALS

### 2.14.1 At Zero Hour of Culture

Wang *et al.* (1994) observed that at zero hour culture cent percentage of the porcine oocytes were in GV stage and GVBD started at 6h of culture, the morphology of cumulus mass was very compact at the start of culture and at 6h of culture the cumulus mass slightly expanded. Isobe *et al.* (1998) reported that 99.6 per cent of

porcine oocytes were at GV stage upto zero to eight hour of culture. At the initial stage of culture all culture grade COCs showed a tightly packed cumulus cell with no observable expansion (Isobe and Terada, 2001). Torner *et al.* (2004) found that 60.4 per cent porcine oocytes collected showed compact cumulus and 39.6 per cent showed dispersed cumulus, of this 76.9 per cent and 57.1 per cent respectively showed GV stage.

### 2.14.2 At 24h of culture

After 24h of culture, 80-85 per cent of oocytes were at the germinal vesicle stage (Mattioli *et al.*1991b). Funahashi and Day (1993) recorded that 85 per cent of oocytes showed cumulus expansion limited to outer layer of cumulus cells (second degree) and 98 per cent of them were in GVBD stage. The study of Meinecke and Meinecke-Tillmann (1993) using α-amanitin (hnRNA synthesis inhibitor) during porcine oocyte maturation showed that 33.9 per cent of oocytes cultured for 24h reached Metaphase I and 3.5 per cent of them were in the process of GVBD. Wang *et al.* (1994) observed that at 24h culture almost 95 per cent of the porcine oocytes completed GVBD and the degree of expansion progressed to moderate. Isobe *et al.* (1998) reported that after 16h of culture, GVBD had occurred 22.7 per cent of porcine oocytes and the incidence increased significantly after 24h (66.7 per cent).

### 2.14.3 At 36h of culture

Funahashi and Day (1993) found that 48 per cent of COCs showed third degree cumulus expansion (cumulus expansion except the corona radiate) and 52 per cent showed second-degree cumulus expansion. Wang *et al.* (1994) observed that full expansion of the cumulus mass was observed at 36h of culture, the corona radiata were still compacted at that time. Isobe *et al.* (1998) noticed 84 per cent GVBD at 32h of culture of porcine oocytes. Torner *et al.* (1998) studied the morphology of porcine

COCs and found that 72.9 per cent of COCs aspirated two hour before hCG were compact or slightly expanded but 34 h after hCG injection 98.3 per cent of COCs showed cumulus expansion.

### 2.14.4 At 42h of culture

Agrawal and Polge (1989) recorded 38.73 per cent of porcine oocytes progressed to Metaphase II, 17.57 percent remained GV stage 32.89 percent oocytes progressed to M I stage and 10.81 percent oocytes exhibited degenerative changes after 44h of culture. After 42h of culture the percentage of M II stage porcine oocytes was significantly higher ie, 76-86 per cent (Mattioli *et al.* 1991b). Funahashi and Day (1993) recorded 90 per cent porcine COCs in maximum degree of cumulus expansion and 88 per cent of them showed M II oocytes. Kikuchi *et al* (1993) found that more than 75 per cent of the porcine oocytes examined had matured to the M II stage after 42h.

## 2.15 FERTILITY STATUS OF IVM OOCYTES

Nagai *et al.* (1988) aspirated oocytes from two to five millimeter diameter follicles at slaughter and matured these oocytes *in vitro* for 32h in 0.1 ml hormone supplemented TC-199 medium at 39°C. These oocytes were incubated with epididymal and ejaculated spermatozoa sample and obtained sperm penetration rate of 0-40 per cent and zero per cent respectively. Mattioli *et al.* (1988) aspirated oocytes from 3-6mm diameter follicles and cultured in TCM-199 medium for 44h at 39°C has obtained 78 per cent fertilization rate. Suzuki *et al.* (1994) obtained 81.8 percent and 61.6 percent *in vitro* maturation rate and *in vitro* fertilization rate respectively. Kikuchi *et al.* (1999) studied the developmental competence of *in vitro* matured oocytes in porcine and found that 70 per cent of examined oocytes were identified as matured and 30 per cent showed penetration and 14 per cent of inseminated oocytes reached to the morula or blastocyst stage. Abeydeera *et al.* (2000) obtained 86.8 per cent M II oocytes, 7.5 per

cent M I oocyte, 3.1 per cent GVBD and 2.5 per cent GV stage oocytes on culture of porcine oocytes in a protein free medium supplemented with epidermal growth factor for 40 to 44 h and showed 80.9 per cent sperm penetration rate. Ellederova *et al.* (2004) got 85 per cent sperm penetration for the *in vitro* matured oocyte and the monospermic fertilization varied between 20-60 per cent and approximately 10-30 per cent developed to the blastocyst stage.

Yoshida *et al.* (1993) documented maturation rate of 93 per cent and fertilization rate of 78 per cent in porcine species out of which 53 per cent showed cleavage after 36h of insemination and 24 per cent cleaved to 2-4 cell stages with blastomeres of regular size. Bagg *et al.* (2004) recorded 92.4 per cent nuclear maturation of *in vitro* cultured oocytes and the parthenogenetically activated oocytes cleaved (90.7 per cent) or developed to the four to eight cells (35.5 per cent) morula (14.5 per cent) and blastocyst stage (23.7 per cent). Incidence of polyspermy was a problem in porcine species, but recent advances in IVM and IVF culture systems reduced this defect to certain extent and increased the yield of *in vitro* produced embryos.

## 2.16 STAINING OF OOCYTES

### 2.16.1 Aceto Orcein

Aceto-orcein stain used for staining the oocytes in many of the studies but the concentration might be one per cent (Sawai *et al.* 1997; Kikuchi *et al.*, 2002 and Wongsrikeao *et al.*, 2006) or two per cent (Torner *et al.* 2004) or three per cent (Meinecke and Meinecke-Tillmann, 1993). Martinez *et al.* (1993) and Isobe *et al.* (1998) used one per cent lacmoid for staining of oocytes. Abeydeera *et al.* (2000) fixed the porcine oocytes after culture in ethanol acetic acid (3:1) for 48-72h at room temperature and then stained with one per cent orcein in 45 per cent acetic acid and

examined under a phase contrast microscope (400 X). Maturation was assumed with the presence of metaphase II chromosomes and first polar body.

### 2.16.2 bis Benzimide (Hoechst 33342)

Wongsrikeao *et al.* (2005) stained oocytes with one microgram per millilitre bis Benzimide (Hoechst 33342) for two to five minutes immediately after cumulus cells were removed. Oocytes displaying a disassembled nuclear membrane, chromatin condensation and first polar body were considered to be mature. Paul (2005) used Hoechst 33342 (bis Benzimide) stock solution of five microgram per millilitre for staining the nucleus of oocyte and obtained good results.

### 2.16.3 Fluorescein Diacetate (FDA) Staining

Didion *et al.*, (1990) used 3'6'-Fluorescein diacetate (FDA) for viability assessment of bovine oocytes. The basic staining solution contained five microgram FDA per one millilitre of acetone. Immediately before use, it was diluted, giving a final concentration of 1.7 µg FDA/ml in modified Dulbecco's phosphate buffered saline. The author reported that the degree of fluorescein accumulation was a reflection of quality of oocyte cytoplasm. Ratheeshbabu (2006) used FDA staining for the viability assessment of vitrified goat embryos.

# *Materials and Methods*

# 3. MATERIALS AND METHODS

## 3.1 SOURCE OF OVARY

Ovaries were collected from pigs that were slaughtered at the Department of Livestock Products Technology, College of Veterinary and Animal Sciences, Mannuthy and Pig slaughter unit, Ollur. Large White Yorkshire, Duroc and Landrace breeds of pigs are mainly slaughtered here.

## 3.2 TRANSPORTATION OF OVARY

Ovaries were dissected out within 30-60 minutes of slaughter from 38 animals of unknown reproductive status, age and body condition and transported to the laboratory within 2-3 h of slaughter at 36-38°C in a thermo flask containing freshly prepared normal saline solution fortified with 100 IU/ml Benzyl penicillin and 100 µg/ml Streptomycin sulphate. Details of media, hormone, chemicals, glassware equipments etc. used in this study are shown in Table 1.

## 3.3 PROCESSING OF OVARY

The ovaries were washed in clean tap water repeatedly to remove clotted blood and tissue debris. After trimming off the extraneous tissue, the ovaries were washed several times in sterile normal saline solution supplemented with penicillin and streptomycin at 37°C. Final washing was done with TL-HEPES media warmed to 37°C. The composition of TL-HEPES media is shown in Table 2.

## 3.4 CLASSIFICATION OF OVARY

The collected porcine ovaries were classified into follicular and luteal stage ovaries as per the criteria laid down by Mc Donald (1989).

### 3.4.1 Follicular Stage Ovary

The ovary that contained large follicle with o without regressing corpora lutea was categorised as follicular stage ovary (Plate.1). Forty number of follicular stage ovaries were utilized for this study.

### 3.4.2 Luteal Stage Ovary

Ovary that contained functional corpus luteum and small sized follicles came under this group (Plate.2). Thirty-six number of luteal stage ovaries were utilized for this research.

The research work were carried out as three experiments and the parameters studied are as follows.

**Experiment I**

## 3.5 BIOMETRIC STUDY

Ovarian biometry, distribution of surface follicle and follicular size were assessed under this experiment.

### 3.5.1 Ovarian Biometry

Fifty ovaries selected at random and categorized into follicular and luteal stage groups in equal proportion were subjected to biometrical studies as per Bagg *et al.* (2004). Length, width and thickness of the ovaries were measured with vernier calliper and the weight using electric monopan balance. The influence of the stages of estrous cycle on ovarian biometry was analysed.

### 3.5.2 Distribution of Surface Follicle

Randomly selected 24 follicular stage and 18 luteal stage ovaries from a total of 40 follicular stage and 36 luteal stage ovaries were utilized for this purpose.

*3.5.2.1 Follicle number*

All follicles that could be identified by visual observation were counted as surface follicle.

*3.5.2.2 Follicle size*

The diameter of all surface follicles found on randomly selected 24 follicular and 18 luteal stage ovaries were measured using a vernier calliper and classified into three size groups as 1) Small follicle (<3mm diameter), 2) Medium follicle (3-5mm diameter) and 3) Large follicle (>5mm diameter) as per Liu *et al.* (2003).

**Experiment II**

3.6 RETRIEVAL OF FOLLICULAR OOCYTES

Oocytes were retrieved from 40 follicular stage and 36 luteal stage ovaries by applying any one of the following retrieval methods. The retrieval process was carried out in HEPES buffered Tyrode's lactate medium enriched with BSA at the rate of 0.6 per cent and maintained at 37°C. Heparin was supplemented to this medium at the rate of 0.1 mg/ml. The ovaries used for aspiration method of retrieval were again subjected to post aspiration slicing to obtain additional number of oocytes.

**3.6.1 Aspiration**

All the ovaries used to study the distribution pattern of surface follicles and its size categories were used for aspiration. Oocytes from all the three size groups were aspirated separately using sterile 20 gauge disposable needle connected to a 10 ml sterile plastic syringe (Plate.3). The aspirates were then kept at 37 °C in separate sterile centrifuge tubes until further processing.

### 3.6.2 Slicing

Oocytes were collected from eight follicular and ten luteal stage ovaries by slicing method. Ovaries were held with an artery forceps in a petridish containing 5 ml TL-HEPES medium during the slicing process. All the visible surface follicles and the whole ovary were gently sliced with a Bard-Parker blade No: 22. (Plate.5) Large sized ovarian tissue debris was removed from the medium prior to further processing.

### 3.6.3 Puncture

Puncturing method was applied on eight follicular and eight luteal stage ovaries. During puncturing ovaries were kept in a petridish containing 5ml TL-HEPES medium and held in position with an artery forceps. All the visible follicles and ovarian surface were repeatedly punctured with an 18-G needle until antrum from all surface follicles were exteriorised. (Plate.4)

### 3.6.4 Post Aspiration Slicing

The same ovaries used for aspiration were utilised for post aspiration slicing. The procedure adopted for this was almost same as that of the slicing technique.

## 3.5 POST RETRIEVAL PROCESSING OF OOCYTES

After harvesting by all the four methods the medium containing oocytes were centrifuged for three minutes at 500 rpm. The supernatant fluid was removed with a pipette aid and the sediments was reconstituted with 10ml of TL-HEPES. Contents from each centrifuge tube was transferred into separate sterile petridish having grid (100×15mm) and examined under the zoom stereomicroscope (10X Magnification) for identification of oocytes. The identified oocytes were transferred a into sterile 60 mm petridish containing fresh TL-HEPES medium by means of unopette. The total yield of oocytes from different retrieval systems and their morphological grades were recorded separately. In case of aspiration technique, the number and grade of oocytes obtained from small, medium and large follicles were recorded separately.

## 3.6 CHARACTERIZATION AND CLASSIFICATION OF OOCYTES

All oocytes obtained from different retrieval systems were examined separately under 40X magnification of zoom stereomicroscope and their morphological characters were assessed based on the number of layers of cumulus cells and ooplasm character. The oocytes were graded for their quality as per Bols *et al.* (2004).

Grade A: Oocytes having more than five complete layers of cumulus cells with uniform granulation of ooplasm (Plate.6&7)

Grade B: Oocytes having three to five complete layers of cumulus cells with uniform granulation of ooplasm (Plate.8)

Grade C: Oocytes having one or two complete layers of cumulus cells with uniform granulation of ooplasm (Plate.9)

Grade D: Completely/partially denuded oocytes and degenerated /deformed oocytes (Plate.10&11)

A representative sample of the oocytes was stained with fluorescein diacetate (Plate 12, 13& 14) and hoechst 33342 stains (Plate 15, 16 &17) to assess the viability of the oocytes before culture.

Grade A, B and C oocytes from different retrieval systems were selected for *in vitro* maturation and put separately in labelled drops of TL-HEPES medium maintained at 37°C.

**Experiment III**

## 3.7 *IN VITRO* MATURATION OF OOCYTES

A total of 525 oocytes collected by aspiration, slicing, puncturing and post aspiration slicing were subjected to *in vitro* maturation process. The number of oocytes used for *in vitro* maturation process in each grade category and retrieval method is shown below (Table.3)

**Table.3 Category of oocytes used for *in vitro* maturation.**

| S.No. | Retrieval Techniques | No. of oocytes subjected to IVM | | | Total No. of oocytes subjected to IVM |
|---|---|---|---|---|---|
| | | A grade | B grade | C grade | |
| 1 | Aspiration | 53 | 59 | 54 | 166 |
| 2 | Slicing | 57 | 62 | 68 | 187 |
| 3 | Puncturing | 33 | 24 | 25 | 82 |
| 4 | Post aspiration slicing | 37 | 25 | 28 | 90 |
| 5 | Total | 180 | 170 | 175 | 525 |

### 3.7.1 Media

Medium used for *in vitro* maturation of oocytes was freshly prepared TCM-199, enriched with FSH 0.5 µg/ml, LH – 5 µg/ml, Oestradiol 1 µg/ml, sodium pyruvate 10µl/ml and foetal calf serum (FCS) 10 per cent and filtered using 0.22µm membrane filter. Required numbers of 100 µl maturation drops were prepared with this media in 35 x 10 mm sterile Petri dishes to accommodate each category of oocytes. Sterile mineral oil was layered over these drops and equilibrated in $CO_2$ incubator for at least two hour before introducing the oocytes. Two small dishes of

maturation media were also placed in the incubator for the purpose of pre and post maturation washing of oocytes.

### 3.7.2 Culture Conditions

Culture condition set for this study was 38.5°C temperature, 5 per cent carbon dioxide tension with maximum humidity. Standard water-jacketed type $CO_2$ incubator was used to get this culture environment during incubation up to 42 h.

### 3.7.3 Pre Culture Washing of Oocytes

Each category of oocytes selected from different retrieval systems were washed repeatedly in TL-HEPES medium and final washing was done with already acclimatized maturation media of TCM-199.

### 3.7.4 *In vitro* Culture of Oocytes

On completion of final washing, each category of oocyte was loaded gently into separate maturation drops using unopette. Depending on the availability, a maximum of 10 oocytes were introduced into each maturation drop. The oocytes were allowed to complete incubation for a period of 24 h without any disturbance in the culture environment. After this incubation period the oocytes were again washed two to three times in freshly prepared maturation media and transferred into another freshly prepared and acclimatized maturation media drops without the hormonal supplements (FSH, LH and Oestradiol) and incubated for another 18h. Cumulus expansion rate was recorded at 24h, 36h and 42h of incubation by observing oocytes in maturation drops under zoom stereomicroscope without disturbing the drops.

## 3.8 ASSESSMENT OF MATURATION

### 3.8.1 Cumulus Cell Expansion

All oocytes in the culture drops were examined under zoom stereomicroscope for maturation changes such as degree of cumulus expansion and mucification of cumulus cells at an interval of 24h, 36h and 42h.The degree of cumulus expansion was assessed and recorded as per Table.4

**Table. 4 Score card for cumulus expansion.**

| S.No. | Score | Degree of cumulus expansion |
|---|---|---|
| 1 | 0 | No detectable response |
| 2 | 1 | Minimum observable response |
| 3 | 2 | Expansion limited to the outer layer of cumulus cells |
| 4 | 3 | Maximum degree of cumulus expansion |

Those oocytes showing maximum degree of cumulus expansion were graded as matured oocytes (Plate.21&25) and confirmed the changes by examining under phase contrast microscope (Plate 18, 19&20).

### 3.8.2 Assessment of Nuclear Maturation of Oocytes

A representative sample of 52 oocytes (10 per cent) out of 525 oocytes were checked for nuclear changes after maturation using aceto-orcein staining and examined under phase contrast microscope for assessing nuclear changes.(Plate 22, 23 &24)

## 3.9 STATISTICAL ANALYSIS

The data obtained were subjected to statistical analysis as per Snedecor and Cochran, 1996.

**Table 1. Details of Media, Hormones, Chemicals, Glassware and Equipments**

| Sl. No. | Name of Item | Patent Name | Manufacturer |
|---|---|---|---|
| (1) | **Media, Hormones and Chemicals** | | |
| 1. | TCM-199 | Medium 199 | Sigma Chemicals St. Louis, USA |
| 2. | p FSH (FSH from Porcine Pituitary) | Folltropin – V | Vetrepharm Canada Inc. |
| 3. | p LH (LH from porcine pituitary) | Lutropin — V | Vetrepharm Canada Inc. |
| 4. | 17-β-Estradiol | β-Estradiol | Sigma Chemicals St. Louis, USA |
| 5. | Foetal Bovine Serum | Fetal bovine serum | Sigma Chemicals St. Louis, USA |
| 6. | Sodium Pyruvate Solution(100 mM) | Sodium Pyruvate | Sigma Chemicals St. Louis, USA |
| 7. | Gentamicin Sulphate | Gentamicin solution | Hi media Laboratories Ltd. Mumbai |
| 8. | Streptomycin sulphate | Ambistrin | Alembic Ltd. Vadodra |
| 9. | Benzyl penicillin sodium | Benzyl penicillin injection | Alembic Ltd., Vadodra |
| 10. | Sodium Chloride | Sodium Chloride | Sigma Chemicals St. Louis, USA |
| 11. | Sodium bicarbonate | Sodium bicarbonate | Sigma Chemicals St. Louis, USA |
| 12. | D- (+) – Glucose | D- (+) – Glucose | Sigma Chemicals St. Louis, USA |
| 13. | Mineral Oil | Mineral oil | Sigma Chemicals St. Louis, USA |
| 14. | Orcein | Natural red | Hi media Laboratories Ltd. Mumbai |
| 15. | Bis Benzimide (Hoechst-33342) | Bis Benzimide | Sigma Chemicals St. Louis, USA |

| | | | |
|---|---|---|---|
| 16. | Methanol | Methanol | Merck, Germany |
| 17. | Acetic acid | Acetic acid | BDH Laboratories England |
| 18. | Potassium chloride | Potassium chloride | Sigma Chemicals St. Louis, USA |
| 19. | Sodium phosphate mono basic | Sodium phosphate mono basic | Sigma Chemicals St. Louis, USA |
| 20. | Phenol red | Phenol red | Sigma Chemicals St. Louis, USA |
| 21. | Sodium lactate | Sodium lactate (60%) | Central Drug House (P) Ltd. |
| 22. | Magnesium chloride | Magnesium chloride | Sigma Chemicals St. Louis, USA |
| 23. | $CaCl_2-2H_2O$ | Calcium chloride | Sigma Chemicals St. Louis, USA |
| 24. | HEPES | Hepes | Sigma Aldrich, USA |
| 25. | BSA fraction V | Albumin, Bovine Faction V | Sigma Chemicals St. Louis, USA |
| 26. | Heparin sodium salt | Heparin sodium salt | Sigma Chemicals St. Louis, USA |
| 27 | Fluorecein diacetate | Fluorecein diacetate | Sigma Chemicals St. Louis, USA |

| **(2)** | **Disposable Wares** | **Patent Name** | **Manufacturer** |
|---|---|---|---|
| 1. | Petridish (35 mm) | Falcon | Becton Dickinson Labware,NewJersy, USA |
| 2. | Petridish (60 mm) | Falcon | Tarson Products, India |

| | | | |
|---|---|---|---|
| 3. | Petridish (90 mm) | Falcon | Tarson Products, India |
| 4. | Syringe Filter Units (0.22 µm) 25 mm | Millex – GS | Millipore Corporation USA |
| 5. | Disposable pipette tips 10 µl, 200 µl, 1000 µl | Brand | BRAND Germany |
| 6. | Plastic syringe (2 ml, 5 ml, 10ml) | Dispovan | Hindustan Syringes and Medical Devices Ltd., India |
| 7. | 20 g needle 20 x 1 ½ 1.20 x 38 mm | Dispovan | Hindustan Syringes and Medical Devices Ltd., India |
| 8. | Conical centrifuge tubes | Falcon | Becton, Dickinson, Labware NEW Jersy USA |
| 9. | Capillary pipettes | Unopette | Becton Dickinson vacutainer systems USA |
| 10. | Microcentrifuge tubes (1.5 ml) | Tarson | Tarson Products, India |
| 11. | Serum vials (2 ml) | Tarson | Tarson Products, India |

| (3) | **Glasswares** | **Manufacturer** |
|---|---|---|
| 1. | Conical flask with stopper 100 ml, 500 ml, 1000 ml | Schott Duran, Germany |
| 2. | Measuring Cylinders 100 ml, 250, 500 ml, | Borosil Glass Works, Mumbai |
| 3. | Standard flasks 100 ml, 250 ml, 500 ml, 1000 ml | Borosil Glass Works, Mumbai |
| 4. | Petridish 60mm, 90mm | Borosil Glass Works, Mumbai |
| 5. | Glass slides (75 x 25) and cover slip (22 x 22) | Blue Star, India |

| (4) | Equipments | Patent Name | Manufacturer |
|---|---|---|---|
| 1. | $CO_2$ Incubator | Lab Line | Lab line instruments inc, USA |
| 2. | Zoom stereomicroscope | Leica M Z 6 | Leica micro system, Germany |
| 3. | Phase contrast microscope | Leica DMIL | Leica micro system, Germany |
| 4. | Streamline vertical Laminar flow Cabinet | Esco | ESCO, India |
| 5. | Millipore ultra filtration water filtration unit. | Milli Q UF Plus | Millipore Corporation USA |
| 6. | Digital Camera | Leica I M 50 | Leica micro system, Germany |
| 7. | Stage Warmer | Linkam MC 60 | Linkam, England |
| 9. | Micro pipettes 0.5 – 10 µl,100 – 1000 µl | Labopette | Hirschmann Laborgerate, Germany |
| 10. | Micropipette 10 – 200 µl | Transferpetter | Brand, Germany |
| 11. | Micropipetter helper | Brand | Brand, Germany |
| 12. | Water Distillation Unit | | Vensil, india |
| 13. | Digital pH meter | Cyber Scan 2500 | Cyber Scan Eutech Instrument, Singapore |
| 14. | Electronic Analytical precision Balance | | Sartorius, Germany |
| 15. | Thermal Flask | Eagle | Eagle, India |
| 16. | $CO_2$ gas (Medical grade) | | PSROL, Thrissur |

**Table 2. Composition of modified TL HEPES medium**

| Ingredient | mM | mg per 100 ml |
|---|---|---|
| NaCl | 114.0 | 666.0 |
| KCl | 3.2 | 23.8 |
| $NaHCO_3$ | 2.0 | 16.8 |
| $NaH_2PO_4$ $2H_2O$ | 0.4 | 6.2 |
| Sodium lactate | 10.0 | 112.1 |
| Mg $Cl_2$ $6H_2O$ | 0.5 | 10.0 |
| Ca $Cl_2$.$2H_2O$ | 2.0 | 29.4 |
| HEPES | 10.0 | 240.0 |
| Phenol red | - | 1.0 |
| Sodium pyruvate | 0.5 | 5.5 |
| Bovine Serum Albumin (Fraction V) | - | 300.0 |
| Glucose | 5.6 | 100.0 |
| Triple distilled water | | 100.0ml |
| pH | | 7.4 |
| mOsmol | | 270-290 |

Plate 1-Porcine ovaries- Follicular stage

Plate 2- Porcine ovaries-Luteal Stage

# Porcine Ovaries

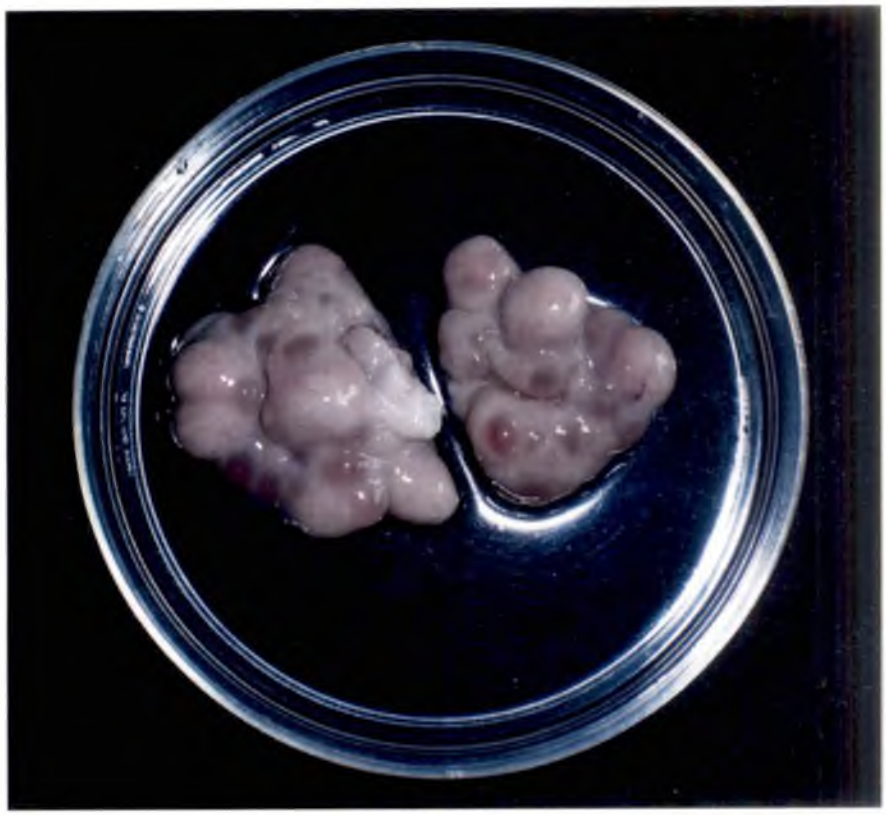


Plate. 1

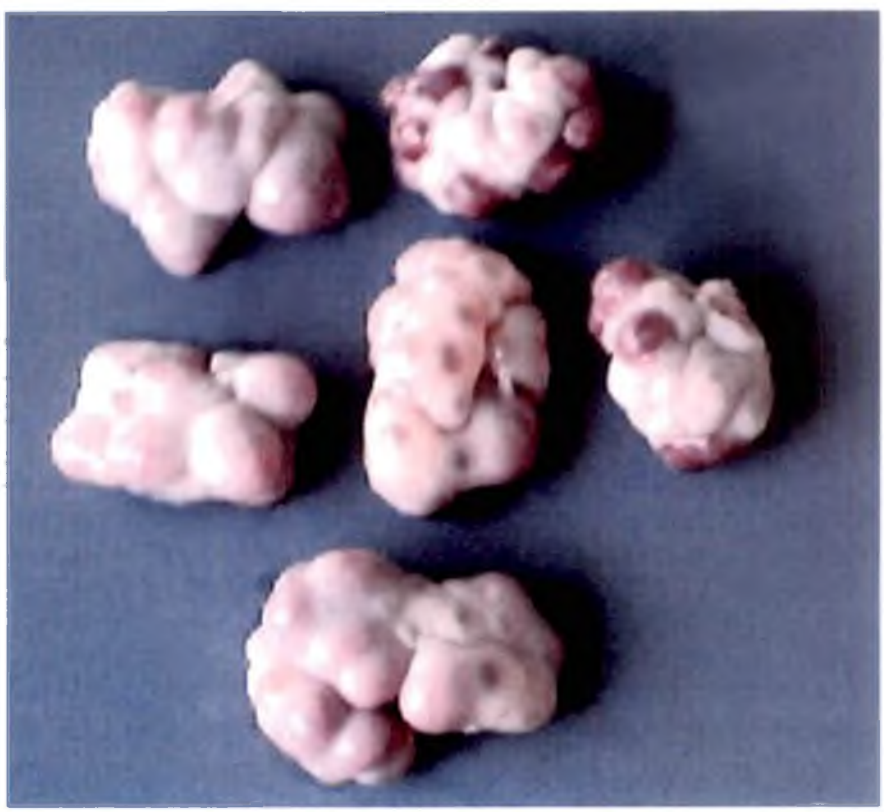


Plate. 2

Plate 3- Oocyte Retrieval methods-Aspiration

Plate 4- Oocyte retrieval method-Puncturing

Plate 5- Oocyte retrieval method- Slicing

# Oocyte Retrieval Methods

Plate. 3

Plate. 4

Plate. 5

Plate 6&7- A Grade oocyte (100 x)

Plate 8- B Grade oocyte (200 x)

Plate9- C Grade oocyte (200 x)

Plate10- D Grade oocyte (400 x)

Plate11 -Degenerated oocytes (400 x)

# Grades of Cumulus-Oocyte Complexes

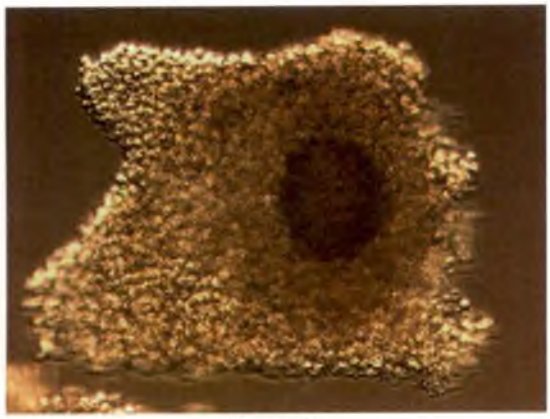


Plate. 6

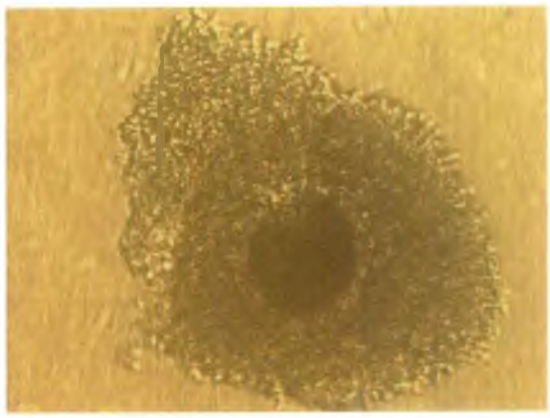


Plate. 7

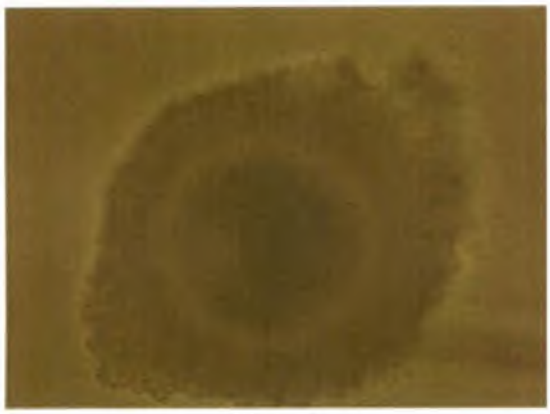


Plate. 8

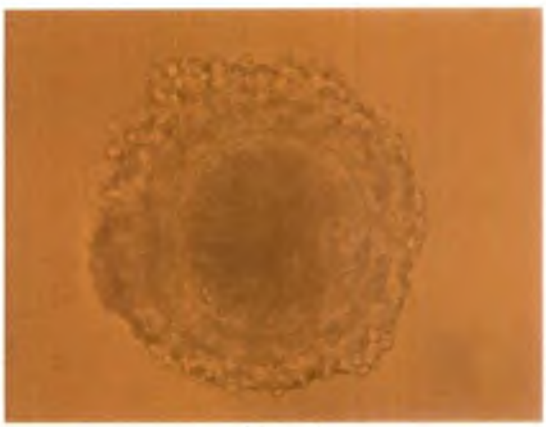


Plate. 9

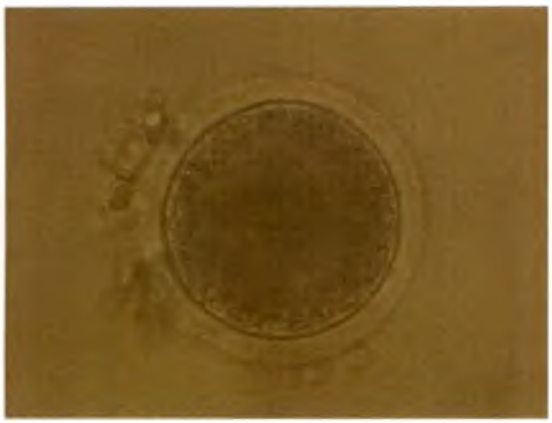


Plate. 10

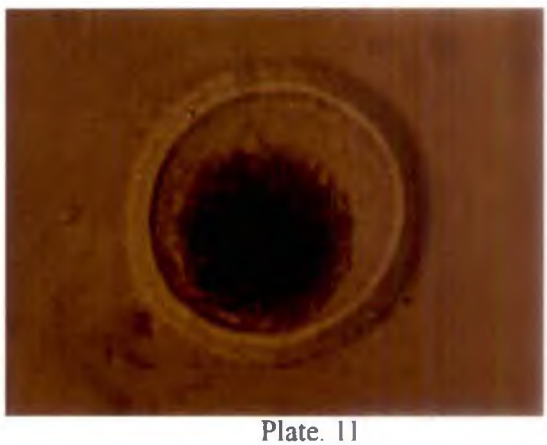


Plate. 11

Plate12- Viability assessment of cumulus oocytes complexes using FDA staining-Viable COCs

Plate13- Viability assessment of cumulus oocytes complexes using FDA staining- Viable oocytes ,Viability of cumulus mass decreased.

Plate14- Viability assessment of cumulus oocytes complexes using FDA staining-Viable oocytes, non-viable cumulus

# Viability Assessment of Cumulus Oocyte Complexes

Plate. 12

Plate. 13

Plate. 14

Plate15- Viability assessment of cumulus oocytes complexes using Hoechst stain-Viable COCs

Plate 16- Viability assessment of cumulus oocytes complexes using Hoechst stain- Viable oocytes ,Viability of cumulus mass decreased.

Plate 17-Viability assessment of cumulus oocytes complexes using Hoechst stain-Viable oocytes with uneven ooplasm

# Viability Assessment of Cumulus Oocyte Complexes

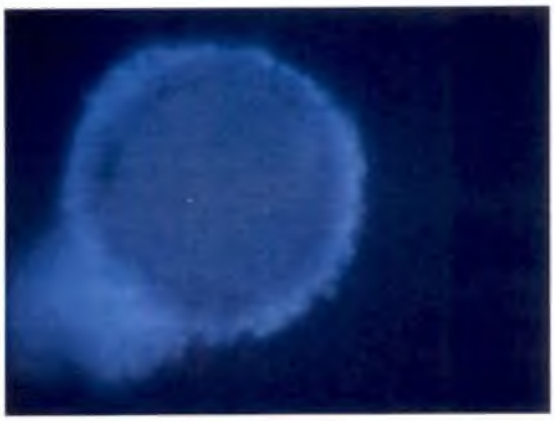


Plate. 15

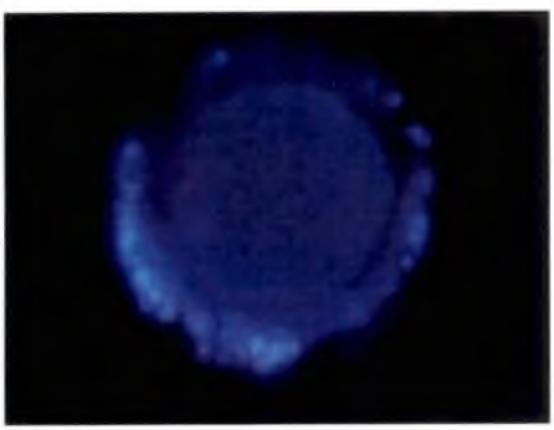


Plate. 16

Plate. 17

Plate18- Degree of cumulus expansion- Score 0

Plate19- Degree of cumulus expansion- Score 1

# Degree of Cumulus Expansion

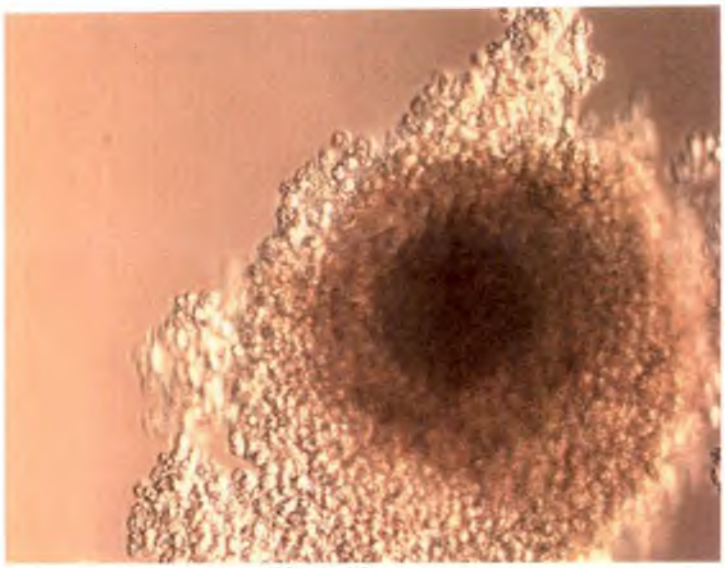


Plate. 18

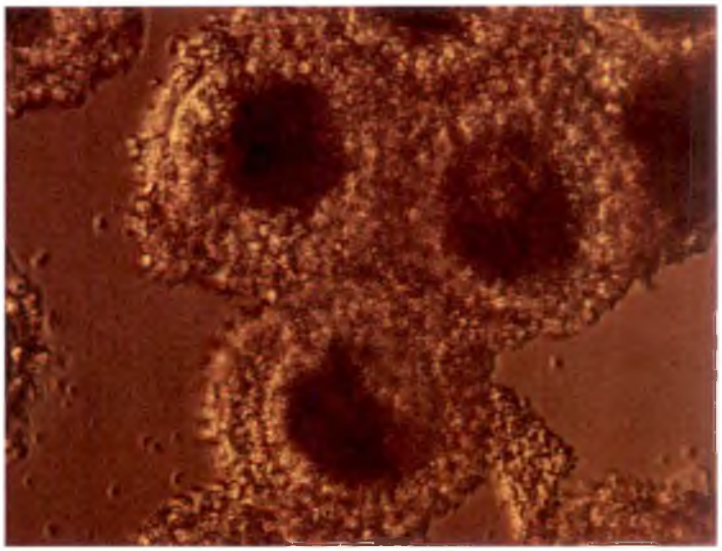


Plate. 19

Plate 20- Degree of cumulus expansion- Score 2

Plate21- Degree of cumulus expansion- Score 3

# Degree of Cumulus Expansion

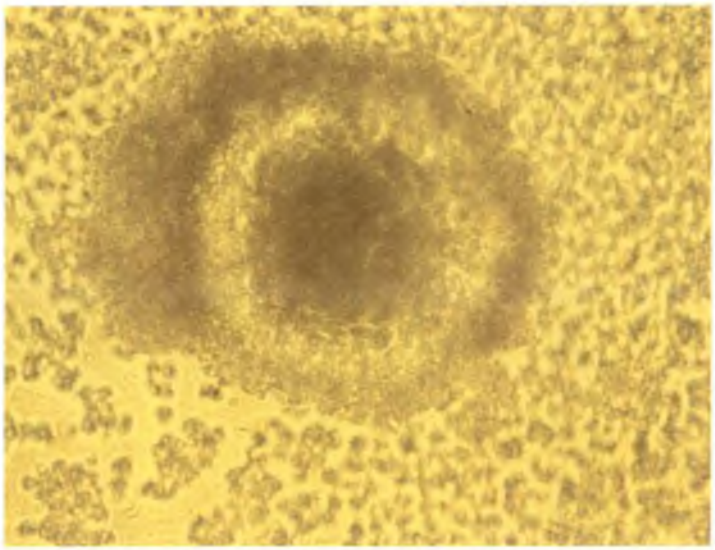


Plate. 20

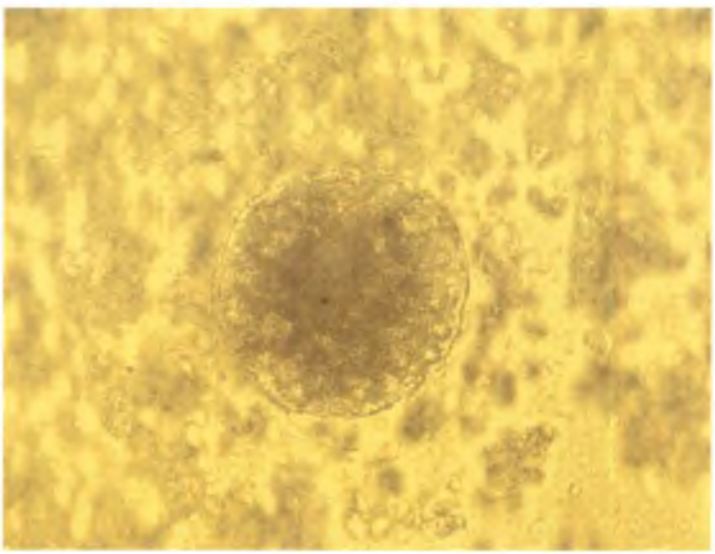


Plate. 21

Plate 22- Oocyte maturation –nuclear changes-GV stage oocytes

Plate 23- Oocyte maturation –nuclear changes-Metaphase I stage oocytes

Plate24- Oocyte maturation –nuclear changes- Metaphase II stage oocytes

# Oocyte Maturation - Nuclear Changes

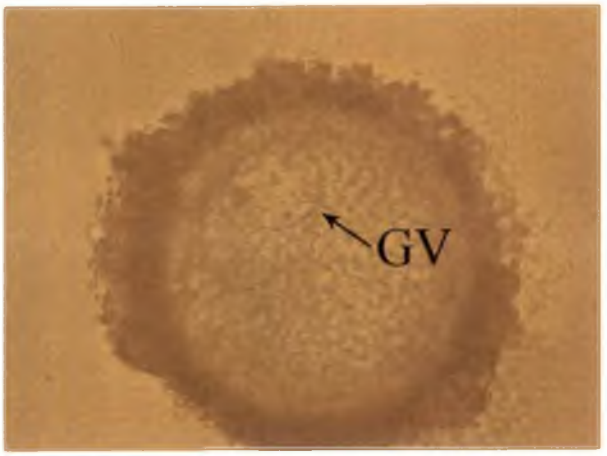



Plate 22

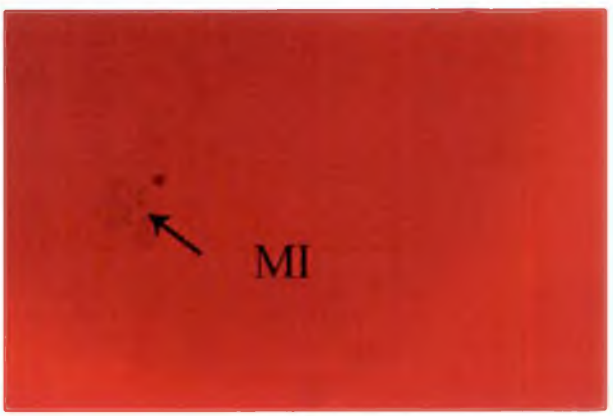



Plate 23

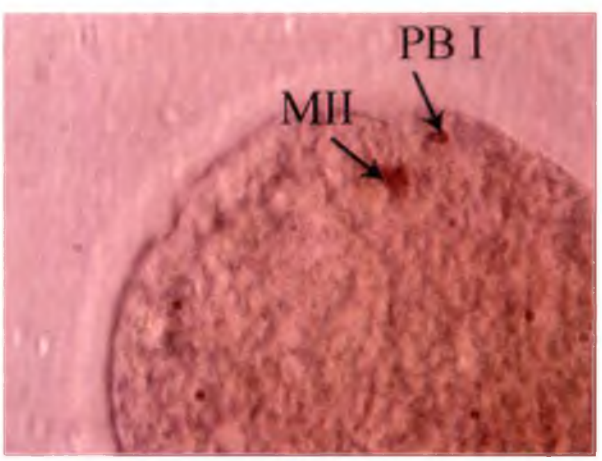



Plate 24

Plate25- Degree of cumulus expansion

# Degree of Cumulus Expansion

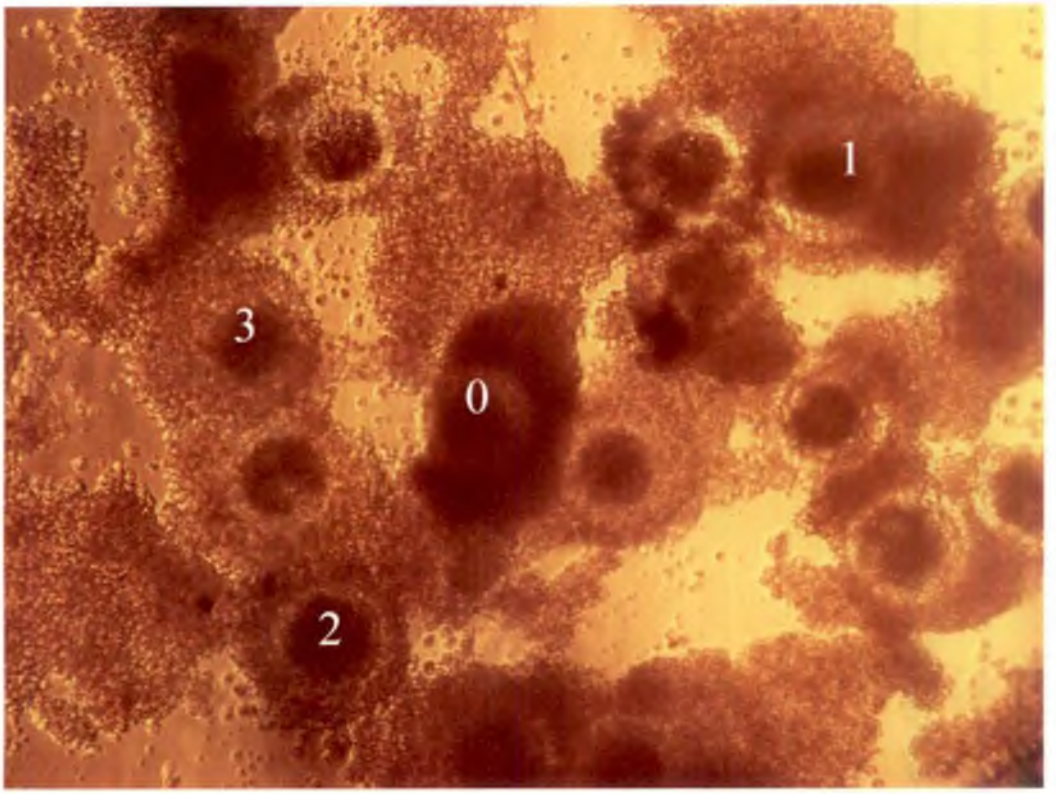



Plate. 25

Score 0 - No detectable response

Score 1 - Minimum observable response

Score 2 - Expansion limited to outer layer of cumulus cells

Score 3 - Maximum degree of cumulus expansion

# *Results*

# 4. RESULTS

A total of 40 follicular and 36 luteal stage ovaries obtained from slaughtered pigs were utilised for this study. Oocytes 2533 were collected from 76 ovaries by applying four retrieval techniques. (Table.9)

EXPERIMENT I

## 4.1 OVARIAN BIOMETRY

A total of 25 follicular and 25 luteal stage ovaries were used for this study. The mean weight, length, width and thickness of the follicular stage ovaries were found to be 3.88± 0.39g, 2.5±0.10cm, 1.2±0.09cm and 1.4±0.05cm respectively and the luteal stage ovaries were 5.42±0.60g, 2.74±0.12cm, 1.3±0.05cm and 1.70±0.07cm respectively. On statistical analysis the weight of the luteal stage ovaries was found to be higher than that of follicular stage ovaries. There was no significant difference in dimensions of luteal stage and follicular stage ovaries. The over all mean weight, length, width and thickness of 50 ovaries were found to be 4.65±0.50g, 2.62±0.11cm, 1.25±0.07cm and 1.55±0.06cm respectively. (Table 6& Fig.1)

## 4.2 DISTRIBUTION OF SURFACE FOLLICLE

A total of 770 surface follicles were recorded in 24 follicular stage ovaries of which 130 were small sized, 399 were medium sized and 241were large sized follicles. Out of 371 total surface follicles recorded in 18 luteal stage ovaries, 248 were small and 123 were medium follicles and it was found that large follicles were absent in luteal stage ovaries. The data were analysed by using multiple linear regression for finding the correlation of small, medium and large follicle on the total surface follicle and it was found that maximum contribution on total surface follicle in follicular stage ovary

was by medium sized follicle (51.81 per cent) followed by large (31.29 per cent) and small (16.88 per cent). Maximum contribution on total surface follicle in luteal stage ovary was by small follicle (66.84 per cent) followed by medium follicle (33.15 per cent). The mean number of small, medium, large and total surface follicle in follicular stage ovary was found to be 5.41, 16.62, 10.04 and 32.08 respectively. In case of luteal stage ovary the mean number of small, medium, large and total surface follicle was found to be 13.77, 6.83, zero and 20.61respectively. (Table.7, 8 &Fig.2).

## 4.3 EFFECT OF FOLLICLE SIZE AND STAGE OF OVARY ON OOCYTE QUALITY

A total of 191 oocytes were collected from 770 surface follicles of follicular stage ovaries, out of which 32 oocytes were from 130 small sized follicles, 98 oocytes from 399 medium sized follicles and 61 from 241 large sized follicles. A, B, C and D grade oocytes obtained from small follicles was found to be one (3.12 per cent), six (18.75 per cent), 13(40.62 per cent) and 12(37.50 per cent) respectively and from medium sized follicles were 30(30.61 per cent), 34(34.69 per cent), 22(22.44 per cent) and 12 (12.24 per cent) respectively and from large sized follicles were 21(34.42 per cent), 19(31.14 per cent), 14(22.95 per cent) and 7 (11.47 per cent) respectively. The percentage yield of total oocytes in relation to surface follicle of follicular stage ovary was found to be 24.61, 24.56 and 25.31 respectively for small, medium and large sized follicles. On statistical analysis it was found that the yield of good quality oocyte was significantly more from medium and large sized follicles compared to small sized follicles and there was no much significant difference in oocytes recovery rate from small, medium and large sized surface follicles. (Table.10& Fig.3)

A total of 130 oocytes were collected from 371surface follicles of luteal stage ovaries, out of which 84 oocytes were from 248 small sized follicles and 46 from 123 medium sized follicles. A, B, C and D grade oocytes obtained from small follicles were

found to be 18 (21.42 per cent), 17(20.23 per cent), 32 (38.09 per cent) and 17(20.23 per cent) respectively and from medium sized follicles were 15(32.60 per cent), 18(39.13 per cent), nine (19.56per cent) and four (8.69) respectively. The percentage yield of total oocytes in relation to surface follicle of luteal stage ovary was found to be 33.87 and 37.39 respectively for small and medium sized follicles. On statistical analysis it was found that the yield of good quality oocyte was significantly more from medium sized follicles compared to small sized follicles and there was no significant difference in oocytes recovery rate from small and medium sized surface follicles. The mean oocyte recovery rate was found to be 28.13 per cent, a total of 321 oocytes were collected from 1141 surface follicles. (Table.10&Fig.3)

EXPERIMENT II

## 4.4 EFFECT OF RETRIEVAL TECHNIQUE AND STAGE OF OVARY ON TOTAL OOCYTE YIELD

A total of 1460 oocytes were obtained from 40 follicular stage ovaries and 1073 oocytes from 36 luteal stage ovaries by applying aspiration, slicing, puncturing and post aspiration slicing techniques. 191 and 130 oocytes were obtained from 24 follicular stage and 18 luteal stage ovaries respectively by aspiration technique. Slicing method yielded 341 and 268 oocytes from 8 follicular and 10 luteal stage ovaries respectively. Puncturing technique yielded 301 and 234 oocytes from 8 follicular and 8 luteal stage ovaries respectively. Post aspiration slicing yielded 627 and 441 oocytes from 24 follicular and 18 luteal stage ovaries respectively (Table.9).

The mean oocytes yield per follicular stage ovary was found to be 7.91±0.84, 42.62±0.21, 37.62±2.05 and 26.12±2.37 for aspiration, slicing, puncturing and post aspiration slicing respectively. The mean oocyte yield per luteal stage ovary was found to be 7.22±0.52, 26.8±1.04, 29.25±0.61 and 24.5±1.16 for aspiration, slicing,

puncturing and post aspiration slicing respectively. The combined mean of oocyte yield per ovary was found to be 7.64±0.50, 33.83±2.14, 33.43±1.45 and 25.42±1.43 for aspiration, slicing, puncturing and post aspiration slicing respectively. On statistical analysis it was found that slicing and puncturing yielded more oocytes per ovary compared to other methods. Aspiration method yielded significantly less oocyte than other methods. (Table.11&Fig.4)

## 4.5 EFFECT OF RETRIVAL TECHNIQUE AND STAGE OF OVARY ON OOCYTE QUALITY

### 4.5.1 Grade A Oocytes

The mean yield of A class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were 2.12±0.29, 5.5±0.73,4.12±0.71 and 2.79±0.35 respectively and from luteal stage ovaries were1.83±0.29, 2.7±0.26, 3.5±0.32 and 2.38±0.61 respectively. (Table.12)

The percentage of A class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were 27.22, 12.9, 10.96 and 10.68 per cent respectively and from luteal stage ovaries 25.38, 10.07, 11.96 and 9.75 per cent respectively. The percentage yield of A class oocyte by aspiration technique was significantly different from other methods. (Table.12&Fig.5)

### 4.5.2 Grade B Oocytes

The mean yield of B class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were 2.45±0.35, 12.5±1.01, 9.75±0.72

and 4.45±0.34 respectively and from luteal stage ovaries 1.94±0.24, 4.6±0.30, 4.25±0.61 and 4.83±0.38 respectively.

The percentage of B class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were 30.89, 29.32, 25.91 and 17.06 per cent respectively and from luteal stage ovaries were 26.92, 17.16, 14.52 and 19.72 per cent respectively. The percentage yield of B class oocyte by aspiration technique was significantly different from other methods. (Table.12&Fig.5)

### 4.5.3 Grade C Oocytes

The mean yield of C class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were 2.04±0.47, 13.75±0.75, 11.37±0.82 and 10.5±2.57 respectively and from luteal stage ovaries were 2.27±0.28, 10.4±0.58, 10.12±0.54 and 7.83±0.40 respectively.

The percentage of C class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were 25.65, 32.25, 30.23 and 40.19 per cent respectively and from luteal stage ovaries were 31.53, 38.80, 34.61 and 31.97 per cent respectively. (Table.12&Fig.5)

### 4.5.4 Grade D Oocytes

The mean yield of D class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were1.29±0.27, 10.87±0.69, 12.37±0.80 and 8.37±0.40 respectively and from luteal stage ovaries were1.16±0.21, 9.1±0.50, 11.37±0.53 and 9.44±0.40 respectively.

The percentage of D class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were16.23, 25.51, 32.89 and 32.05 per cent respectively and from luteal stage ovaries were16.15, 33.95, 38.89 and 38.54 per cent respectively. The percentage of D class oocytes obtained by aspiration method was significantly different from other methods. (Table.12&Fig.5)

EXPERIMENT III

## 4.6 EFFECT OF RETRIEVAL TECHNIQUE, OOCYTES QUALITY AND CULTURE DURATION ON IVM

A total of 525 oocytes collected by aspiration, slicing, puncturing and post aspiration slicing were subjected to *in vitro* maturation process. Each grade of oocytes (A, B and C grades) collected by four retrieval techniques were kept in separate maturation drops and assessed the *in vitro* maturation changes at zero, 24, 36 and 42 hours respectively.

### 4.6.1 Effect of Retrieval Technique on Maturation

#### *4.6.1.1 Aspiration*

The percentage of A, B and C grade oocytes showing maximum degree of cumulus expansion under aspiration method was 73.5, 71.18 and 59.25 per cent respectively. On statistical analysis there was no significant difference between A and B grade. But C grade was found to be different from other grades. (Table. 13&Fig.6)

*4.6.1.2 Puncturing*

The percentage of A, B and C grade oocytes showing maximum degree of cumulus expansion under puncturing method was 69.69, 66.67 and 56.00 per cent respectively. On statistical analysis there was no significant difference between A and B grade. But C grade significantly different from other grades. (Table. 13&Fig.6)

*4.6.1.3 Slicing*

In slicing method the percentage of A, B and C grade oocytes showing maximum degree of cumulus expansion was 68.42, 66.12 and 58.82 per cent respectively. On statistical analysis there was no significant difference between A and B grade. But C grade significantly different from other grades. (Table. 13&Fig.6)

*4.6.1.4 Post aspiration slicing*

In post aspiration-slicing method the percentage of A, B and C grade oocytes showing maximum degree of cumulus expansion was 51.35, 48.00 and 35.71 per cent respectively. On statistical analysis there was no significant difference between A and B grades. But C grade significantly different from other grades. (Table. 13&Fig.6)

*4.6.1.5 Overall*

When overall maturation rate was taken into account, aspiration, puncturing, slicing and post aspiration slicing methods gave a maturation rate of 68.07, 64.63, 64.17 and 45.56 respectively. Statistical analysis revealed that there was no significant difference between aspiration, puncturing and slicing. But post aspiration slicing significantly different from other methods. (Table. 13&Fig.6)

### 4.6.2 Effect of Oocyte Grade on Maturation

#### *4.6.2.1 A Grade oocyte*

The percentage of A grade oocytes showing maximum degree of cumulus expansion under aspiration, puncturing, slicing and post aspiration slicing was 73.5, 69.69, 68.42 and 51.35 per cent respectively. On statistical analysis there was no significant difference in aspiration, puncturing and slicing even though maximum maturation was observed in aspiration followed by puncturing and slicing. Post aspiration slicing was significantly different from other methods. (Table.14&Fig.7)

#### *4.6.2.2 B Grade oocyte*

The percentage of B grade oocytes showing maximum degree of cumulus expansion under aspiration, puncturing, slicing and post aspiration slicing was 71.18, 66.67, 66.12 and 48.00 per cent respectively. Maximum maturation was observed in aspiration followed by puncturing and slicing. Post aspiration slicing was significantly different from other methods. (Table.14& Fig.7)

#### *4.6.2.3 C grade oocyte*

The percentage of C grade oocytes showing maximum degree of cumulus expansion under aspiration, puncturing, slicing and post aspiration slicing was 59.25, 56.00, 58.82 and 35.71 per cent respectively. Post aspiration slicing was significantly different from other methods. Maximum maturation was observed in aspiration followed by puncturing and slicing. (Table.14& Fig.7)

*4.6.2.4 Overall*

The overall maturation rate for A, B and C grade oocytes were found to be 66.67, 65.29 and 54.85 per cent respectively. On statistical analysis there was no significant difference between A and B grades. But C grade significantly different from other grades. (Table.14& Fig.7)

**4.6.3Effect of Culture Duration on Maturation**

A total of 525 oocytes collected by four retrieval techniques were subjected to *in vitro* maturation. The same oocytes that were observed at zero hour were examined again at 24, 36 and 42h for observing the maturation changes. The results are furnished in Table.15&Fig.8.

*4.6.3.1 At Zero Hour Culture*

At 0h culture all the oocytes (100 per cent) showed no detectable response (Degree of cumulus expansion, Score-0). The cumulus expansion index was found to be zero.

*4.6.3.2 At 24 h Culture*

At 24 h of culture 98 oocytes (18.67 percent) showed no detectable response and 427 oocytes (81.33 per cent) showed minimum observable response (Degree of cumulus expansion, Score -1). The cumulus expansion index was found to be 0.81.

*4.6.3.3 At 36 h Culture*

At 36h of culture 147 oocytes (28 per cent) showed minimum observable response and 378 (72 per cent) showed expansion limited to the outer layer of cumulus cells (Degree of cumulus expansion, Score- 2). The cumulus expansion index was found to be 1.72.

*4.6.3.4 At 42h Culture*

At 42h of culture 327 oocytes (62.28 per cent) showed maximum degree of cumulus expansion (Degree of cumulus expansion, Score -3) and 182 oocytes (34.72 per cent) showed expansion limited to the outer layer of cumulus cells and 16 oocytes (three per cent) showed minimum observable response. Cumulus expansion index after 42h found to be 2.57.

*4.6.3.5 Overall*

The morphology of cumulus mass found to be changing during the entire culture period. The overall maturation rate after 42 h was found to be 62.28 per cent with a cumulus expansion index of 2.57.The data shows that 42h was required for complete maturation of porcine oocytes.

5.7 ASSESSMENT OF NUCLEAR MATURATION OF OOCYTES

It was found that out of 52 oocytes examined for assessing the nuclear maturation 32 oocytes showed metaphase II stage.

Table. 5 Distribution of ovaries for the study

| Sl.No. | Parameters | Follicle stage ovary | Luteal stage ovary | Total |
|---|---|---|---|---|
| 1 | Ovarian biometry | 25 | 25 | 50 |
| 2 | Surface follicle | 24 | 18 | 42* |
| 3 | Aspiration | 24 | 18 | 42* |
| 4 | Post aspiration slicing | 24 | 18 | 42* |
| 5 | Slicing | 8 | 10 | 18 |
| 6 | Puncturing | 8 | 8 | 16 |

* Same ovaries were used

Table.6 Effect of stage of oestrous cycle on ovarian biometry (Mean±SE)

| Sl.No. | Stage of ovary | Total Ovary | Weight (g) | Length (cm) | Width (cm) | Thickness (cm) |
|---|---|---|---|---|---|---|
| 1 | Follicular | 25 | $3.88\pm0.39^{a}$ | $2.50\pm0.10^{a}$ | $1.20\pm0.09^{a}$ | $1.40\pm0.05^{a}$ |
| 2 | Luteal | 25 | $5.42\pm0.60^{b}$ | $2.74\pm0.12^{a}$ | $1.30\pm0.05^{a}$ | $1.70\pm0.07^{a}$ |
| 3 | Mean | 50 | 4.65±0.50 | 2.62±0.11 | 1.25±0.07 | 1.55±0.06 |

Values bearing different superscript in the same column differ significantly ($p<0.01$)

Table.7 Distribution of surface follicle on follicular stage ovary

| Sl.No. | Total surface follicle | Small follicle (<3mm diameter) | Medium follicle (3-5mm diameter) | Large follicle (>5mm diameter) |
|---|---|---|---|---|
| 1 | 21 | 7 | 9 | 5 |
| 2 | 25 | 6 | 13 | 6 |
| 3 | 22 | 2 | 11 | 9 |
| 4 | 28 | 4 | 16 | 8 |
| 5 | 35 | 6 | 17 | 12 |
| 6 | 23 | 3 | 12 | 8 |
| 7 | 38 | 6 | 19 | 13 |
| 8 | 41 | 5 | 21 | 15 |
| 9 | 26 | 6 | 11 | 9 |
| 10 | 42 | 7 | 22 | 13 |
| 11 | 36 | 5 | 20 | 11 |
| 12 | 29 | 4 | 16 | 9 |
| 13 | 51 | 5 | 28 | 18 |
| 14 | 31 | 8 | 15 | 8 |
| 15 | 35 | 6 | 18 | 11 |
| 16 | 29 | 4 | 17 | 8 |
| 17 | 31 | 8 | 14 | 9 |
| 18 | 38 | 5 | 21 | 12 |
| 19 | 39 | 5 | 23 | 11 |
| 20 | 29 | 5 | 16 | 8 |
| 21 | 29 | 5 | 15 | 9 |
| 22 | 23 | 3 | 13 | 7 |
| 23 | 26 | 8 | 10 | 8 |
| 24 | 43 | 7 | 22 | 14 |
| Total | 770 | 130 | 399 | 241 |
| Mean | 32.08 | 5.41 | 16.62 | 10.04 |
| Percentage | 100 | 16.88 | 51.81 | 31.29 |

Table.8 Distribution of surface follicle on luteal stage ovary

| Sl.No | Total surface follicle | Small follicle (<3mm diameter) | Medium follicle (3-5mm diameter) | Large follicle (>5mm diameter) |
|---|---|---|---|---|
| 1 | 18 | 12 | 6 | 0 |
| 2 | 21 | 15 | 6 | 0 |
| 3 | 22 | 17 | 5 | 0 |
| 4 | 20 | 15 | 5 | 0 |
| 5 | 18 | 13 | 5 | 0 |
| 6 | 23 | 15 | 8 | 0 |
| 7 | 19 | 13 | 6 | 0 |
| 8 | 18 | 11 | 7 | 0 |
| 9 | 23 | 15 | 8 | 0 |
| 10 | 21 | 16 | 5 | 0 |
| 11 | 18 | 12 | 6 | 0 |
| 12 | 19 | 11 | 8 | 0 |
| 13 | 23 | 15 | 8 | 0 |
| 14 | 25 | 15 | 10 | 0 |
| 15 | 21 | 12 | 9 | 0 |
| 16 | 18 | 11 | 7 | 0 |
| 17 | 19 | 13 | 6 | 0 |
| 18 | 25 | 17 | 8 | 0 |
| Total | 371 | 248 | 123 | 0 |
| Mean | 20.61 | 13.77 | 6.83 | 0 |
| Percentage | 100 | 66.84 | 33.15 | 0 |

Table.9 Number and source of oocyte studied

| Sl.No. | Retrieval techniques | Oocyte yield(follicular stage ovary) | | Oocyte yield (luteal stage ovary) | | Total | |
|---|---|---|---|---|---|---|---|
| | | Number of ovary | Number of oocytes | Number of ovary | Number of oocytes | Number of ovary | Number of oocytes |
| 1 | Aspiration | 24 | 191 | 18 | 130 | 42 | 321 |
| 2 | PAS | | 627 | | 441 | | 1068 |
| 3 | Slicing | 8 | 341 | 10 | 268 | 18 | 609 |
| 4 | Puncturing | 8 | 301 | 8 | 234 | 16 | 535 |
| 5 | Total | 40 | 1460 | 36 | 1073 | 76 | 2533 |

Table.10 Effect of follicle size and stage of oestrous cycle on oocyte quality.

| Sl.No: | Size category of surface follicle | Stage of ovary | Total No. of Surface follicle | Grade of oocytes (%) | | | | Total oocytes | % of oocytes to total surface follicle |
|---|---|---|---|---|---|---|---|---|---|
| | | | | A | B | C | D | | |
| 1 | Small | Follicular | 130 | 1 (3.12) | 6 (18.75) | 13 (40.62) | 12 (37.50) | 32 | 24.61[a] |
| | | Luteal | 248 | 18 (21.42) | 17 (20.23) | 32 (38.09) | 17 (20.23) | 84 | 33.87[b] |
| 2 | Medium | Follicular | 399 | 30 (30.61) | 34 (34.69) | 22 (22.44) | 12 (12.24) | 98 | 24.56[a] |
| | | Luteal | 123 | 15 (32.60) | 18 (39.13) | 9 (19.56) | 4 (8.69) | 46 | 37.39[b] |
| 3 | Large | Follicular | 241 | 21 (34.42) | 19 (31.14) | 14 (22.95) | 7 (11.47) | 61 | 25.31[a] |
| | | Luteal | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | Total | Follicular | 770 | 52 (27.22) | 59 (30.89) | 49 (25.65) | 31 (16.23) | 191 | 24.80[a] |
| | | Luteal | 371 | 33 (25.38) | 35 (26.92) | 41 (31.53) | 21 (16.15) | 130 | 35.04[b] |
| | | | 1141 | 85 (26.47) | 94 (29.28) | 90 (28.03) | 52 (16.19) | 321 | 28.13[ab] |

Percentage bearing different superscript within same column differs significantly ($p<0.05$).

Table.11 Effect of retrieval technique and stage of ovary on total oocyte yield

| Sl.No. | Stage of ovary | Retrieval techniques | | | |
|---|---|---|---|---|---|
| | | Aspiration | Slicing | Puncturing | Post aspiration slicing |
| 1 | Follicular | 7.91±0.84[a] | 42.62±0.21[b] | 37.62±2.05[d] | 26.12±2.37[c] |
| 2 | Luteal | 7.22±0.52[a] | 26.80±1.04[c] | 29.25±0.61[c] | 24.50±1.16[c] |
| 3 | Mean | 7.64±0.50 | 33.83±2.14 | 33.43±1.45 | 25.42±1.43 |

Values bearing different superscript in same column differ significantly ($p<0.01$) among retrieval methods.

Table. 12 Effect of retrieval techniques and stage of ovary on oocyte quality

| Sl.No. | Retrieval techniques | Stage of ovary | Grade of oocytes | | | | Total |
|---|---|---|---|---|---|---|---|
| | | | A | B | C | D | |
| 1 | Aspiration | Follicular | 2.12±0.29 (52) | 2.45±0.35 (59) | 2.04±0.47 (49) | 1.29±0.27 (31) | 7.91±0.84 (191) |
| | | % | 27.22 | 30.89 | 25.65 | 16.23 | 100 |
| | | Luteal | 1.83±0.29 (33) | 1.94±0.24 (35) | 2.27±0.28 (41) | 1.16±0.21 (21) | 7.22±0.52 (30) |
| | | % | 25.38 | 26.92 | 31.53 | 16.15 | 100 |
| 2 | Slicing | Follicular | 5.5±0.73 (44) | 12.5±1.01 (100) | 13.75±0.75 (110) | 10.87±0.69 (87) | 42.62±0.21 (341) |
| | | % | 12.9 | 29.32 | 32.25 | 25.51 | 100 |
| | | Luteal | 2.7±0.26 (27) | 4.6±0.30 (46) | 10.4±0.58 (104) | 9.1±0.50 (91) | 26.8±1.04 (268) |
| | | % | 10.07 | 17.16 | 38.80 | 33.95 | 100 |
| 3 | Puncturing | Follicular | 4.12±0.71 (33) | 9.75±0.72 (78) | 11.37±0.82 (91) | 12.37±0.80 (99) | 37.62±2.05 (301) |
| | | % | 10.96 | 25.91 | 30.23 | 32.89 | 100 |
| | | Luteal | 3.5±0.32 (28) | 4.25±0.61 (34) | 10.12±0.54 (81) | 11.37±0.53 (91) | 29.25±0.61 (234) |
| | | % | 11.96 | 14.52 | 34.61 | 38.89 | 100 |
| 4 | Post aspiration slicing | Follicular | 2.79±0.35 (67) | 4.45±0.34 (107) | 10.5±2.57 (252) | 8.37±0.40 (201) | 26.12±2.37 (627) |
| | | % | 10.68 | 17.06 | 40.19 | 32.05 | 100 |
| | | Luteal | 2.38±0.61 (43) | 4.83±0.38 (87) | 7.83±0.40 (141) | 9.44±0.40 (170) | 24.5±1.16 (441) |
| | | % | 9.75 | 19.72 | 31.97 | 38.54 | 100 |

Figures in parenthesis indicates the number of oocytes

Table.13 Effect of retrieval technique on *in vitro* maturation.

| Sl.No. | Retrieval Techniques | Grade of oocytes | Number of oocytes examined | Number of oocytes showing Maturation changes | Percentage of oocytes showing Maturation changes |
|---|---|---|---|---|---|
| 1 | Aspiration | A | 53 | 39 | 73.50[a] |
| | | B | 59 | 42 | 71.18[a] |
| | | C | 54 | 32 | 59.25[b] |
| | | Total | 166 | 113 | 68.07 |
| 2 | Puncturing | A | 33 | 23 | 69.69[a] |
| | | B | 24 | 16 | 66.67[a] |
| | | C | 25 | 14 | 56.00[b] |
| | | Total | 82 | 53 | 64.63 |
| 3 | Slicing | A | 57 | 39 | 68.42[a] |
| | | B | 62 | 41 | 66.12[a] |
| | | C | 68 | 40 | 58.82[b] |
| | | Total | 187 | 120 | 64.17 |
| 4 | Post aspiration slicing | A | 37 | 19 | 51.35[c] |
| | | B | 25 | 12 | 48.00[c] |
| | | C | 28 | 10 | 35.71[d] |
| | | Total | 90 | 41 | 45.56 |

Percentage bearing different superscript within same column differs significantly ($p<0.05$).

Table 14. Effect of oocyte quality on *in vitro* maturation

| Sl.No. | Grade of oocytes | Retrieval Techniques | Number of oocytes examined | Number of oocytes showing Maturation changes | Percentage of oocytes showing Maturation changes |
|---|---|---|---|---|---|
| 1 | A | Aspiration | 53 | 39 | 73.50[a] |
| | | Puncturing | 33 | 23 | 69.69[a] |
| | | Slicing | 57 | 39 | 68.42[a] |
| | | Post aspiration slicing | 37 | 19 | 51.35[b] |
| | | Total | 180 | 120 | 66.67 |
| 2 | B | Aspiration | 59 | 42 | 71.18[a] |
| | | Puncturing | 24 | 16 | 66.67[a] |
| | | Slicing | 62 | 41 | 66.12[a] |
| | | Post aspiration slicing | 25 | 12 | 48.00[b] |
| | | Total | 170 | 109 | 65.29 |
| 3 | C | Aspiration | 54 | 32 | 59.25[c] |
| | | Puncturing | 25 | 14 | 56.00[c] |
| | | Slicing | 68 | 40 | 58.82[c] |
| | | Post aspiration slicing | 28 | 10 | 35.71[d] |
| | | Total | 175 | 96 | 54.85 |

Percentage bearing different superscript within same column differs significantly ($p<0.05$).

Table. 15 Effect of culture duration on cumulus oocyte complexes expansion

| Sl.No. | Time interval (h) | Number of oocyte examined | Degree of cumulus expansion (%) | | | | Cumulus expansion index |
|---|---|---|---|---|---|---|---|
| | | | 0 | I | 2 | 3 | |
| 1 | 0 | 525 | 525(100)$^{a}$ | 0(0)$^{a}$ | 0(0)$^{a}$ | 0(0)$^{a}$ | 0.00 |
| 2 | 24 | 525 | 98(18.67)$^{b}$ | 427(81.33)$^{b}$ | 0(0)$^{a}$ | 0(0)$^{a}$ | 0.81 |
| 3 | 36 | 525 | 0(0)$^{c}$ | 147(28)$^{c}$ | 378(72)$^{b}$ | 0(0)$^{a}$ | 1.72 |
| 4 | 42 | 525 | 0(0)$^{c}$ | 16(3)$^{d}$ | 182(34.72)$^{c}$ | 327(62.28)$^{b}$ | 2.57 |

Percentage bearing different superscript within same column differs significantly ($p<0.05$).

Table. 16 Nuclear maturation status of oocytes on *in vitro* culture for 42 h

| Sl.No. | No. of COCs examined | Nuclear changes | | | |
|---|---|---|---|---|---|
| | | GV | GVBD | M I | M II |
| 1 | 52 | 1 | 5 | 14 | 32 |

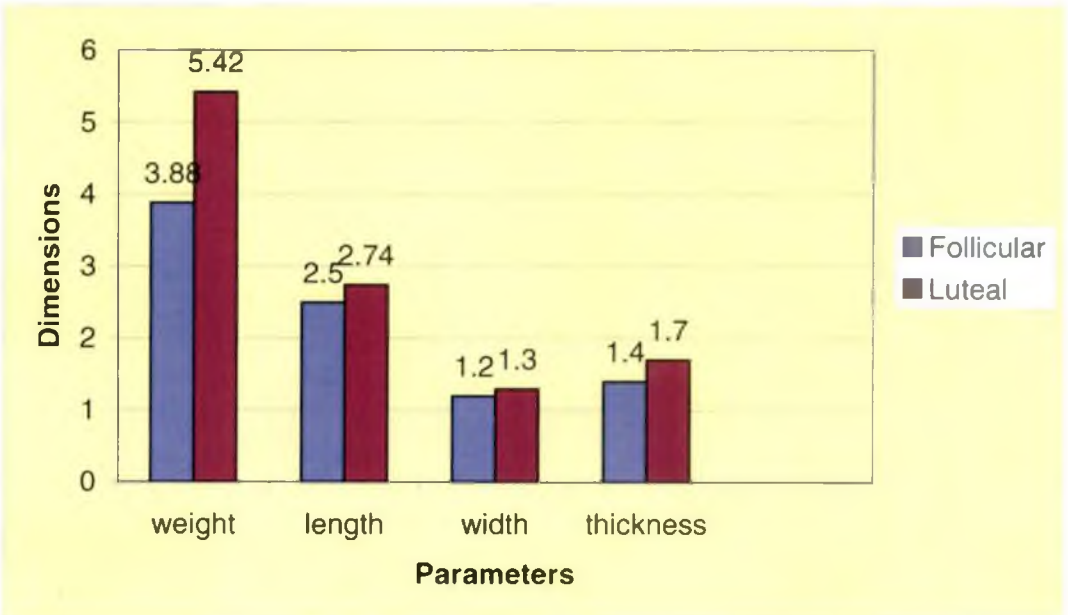
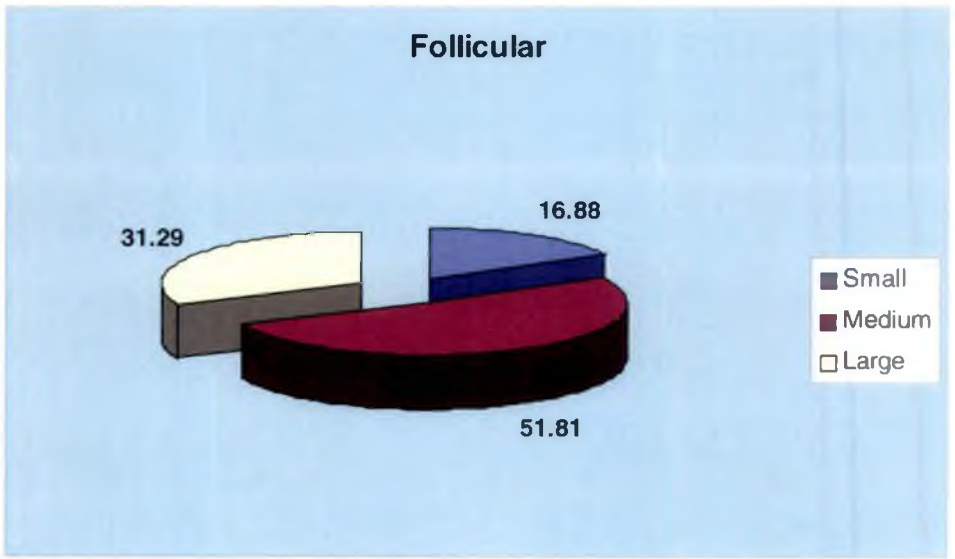



**Fig.1 Effect of stage of oestrous cycle on ovarian biometry**



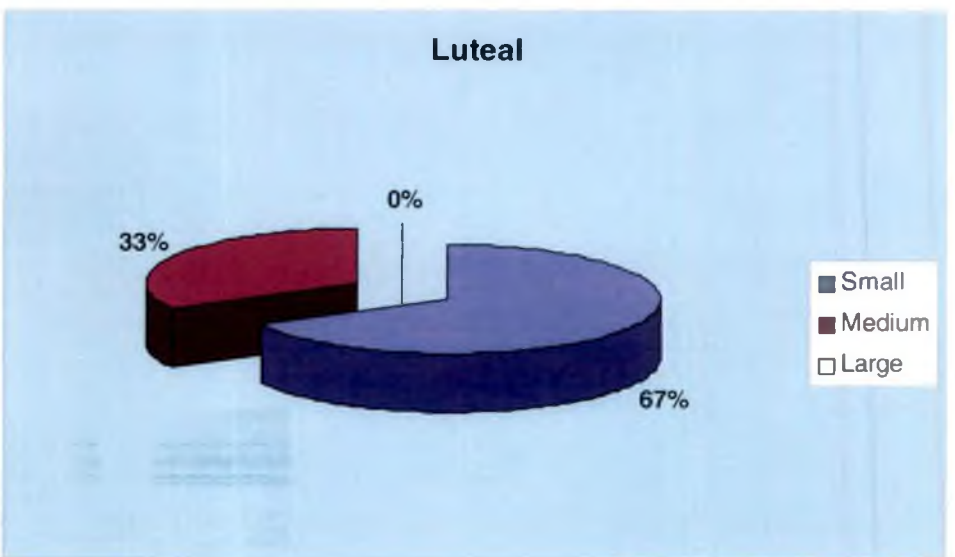




**Fig.2 Distribution of surface follicle on follicular and luteal stage ovary**

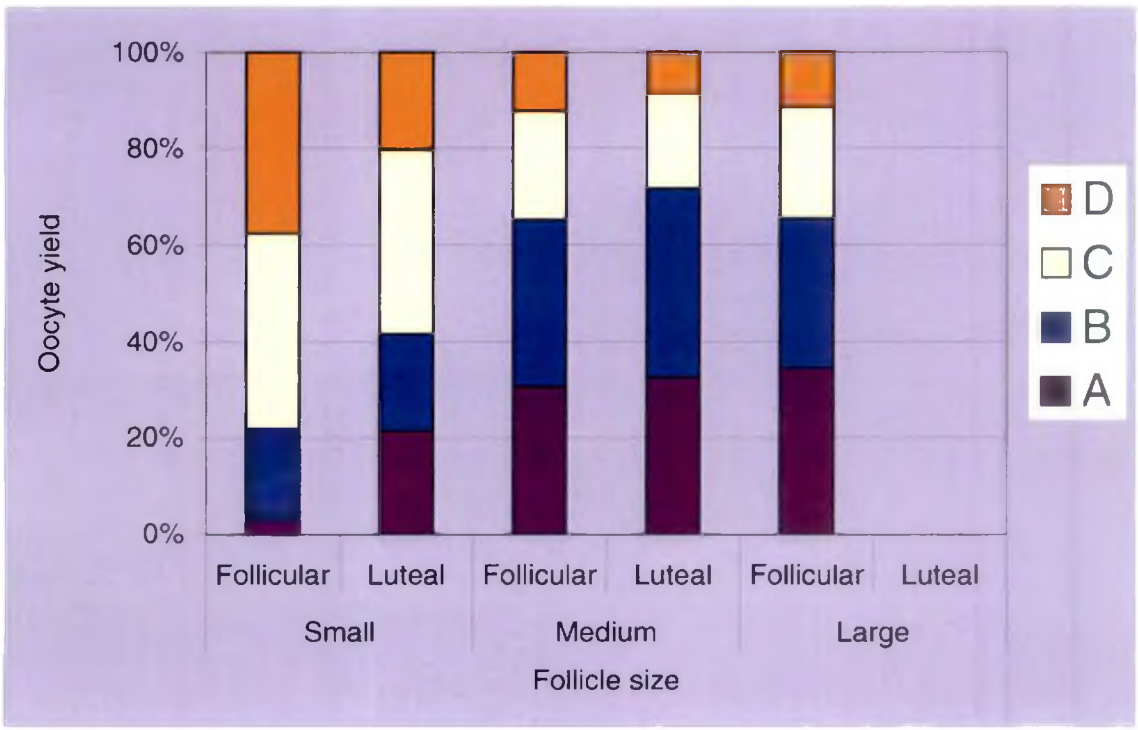



**Fig.3 Effect of follicle size and stage of oestrous cycle on oocyte quality**

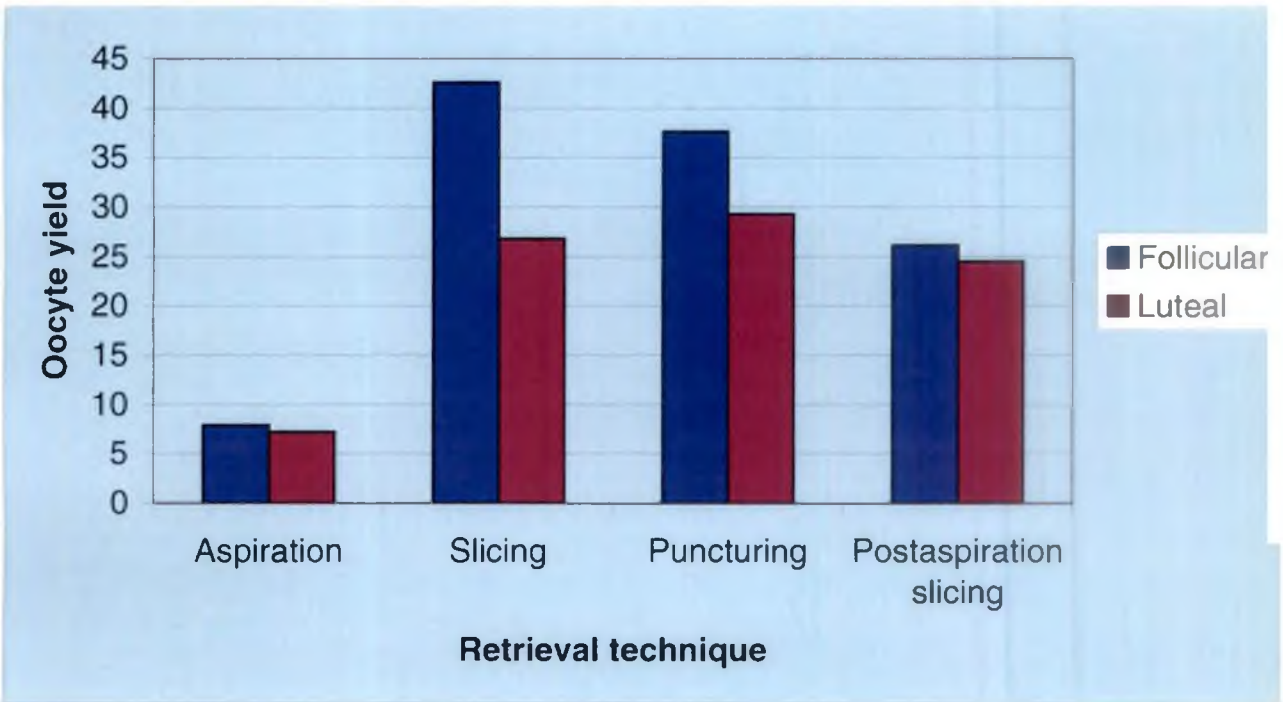



**Fig.4 Effect of retrieval technique and stage of ovary on total oocyte yield**

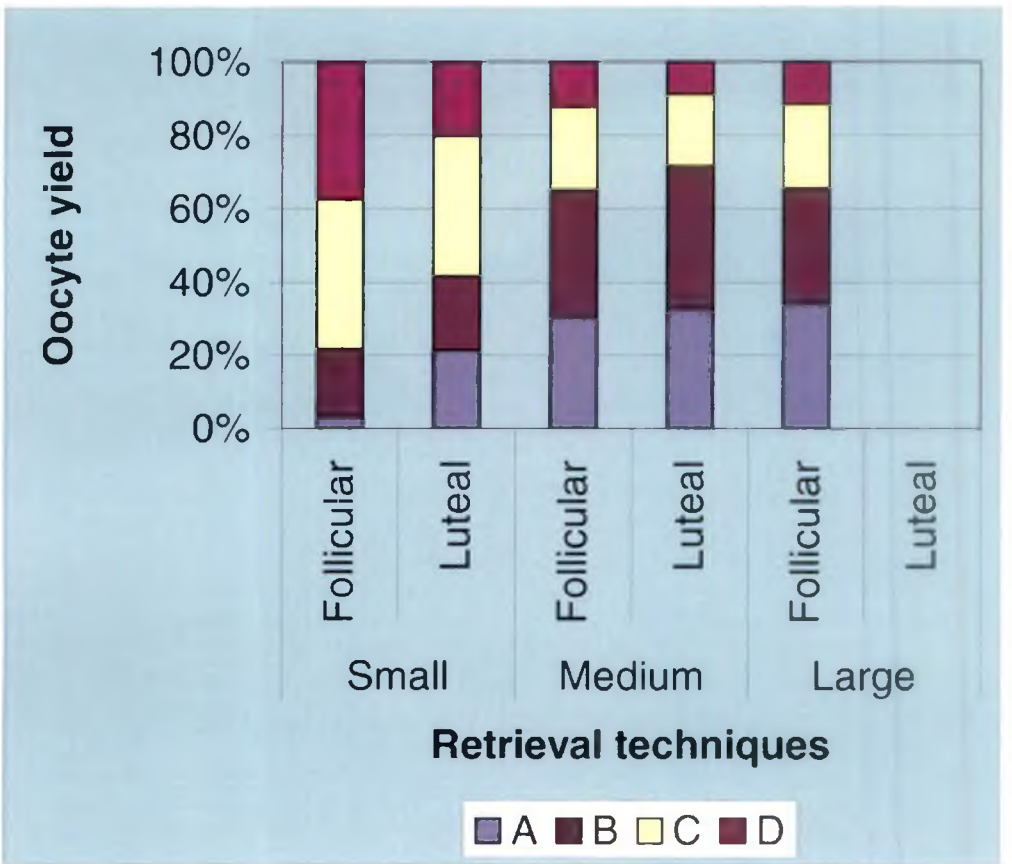



**Fig .5 Effect of retrieval technique and stage of ovary on oocyte quality**

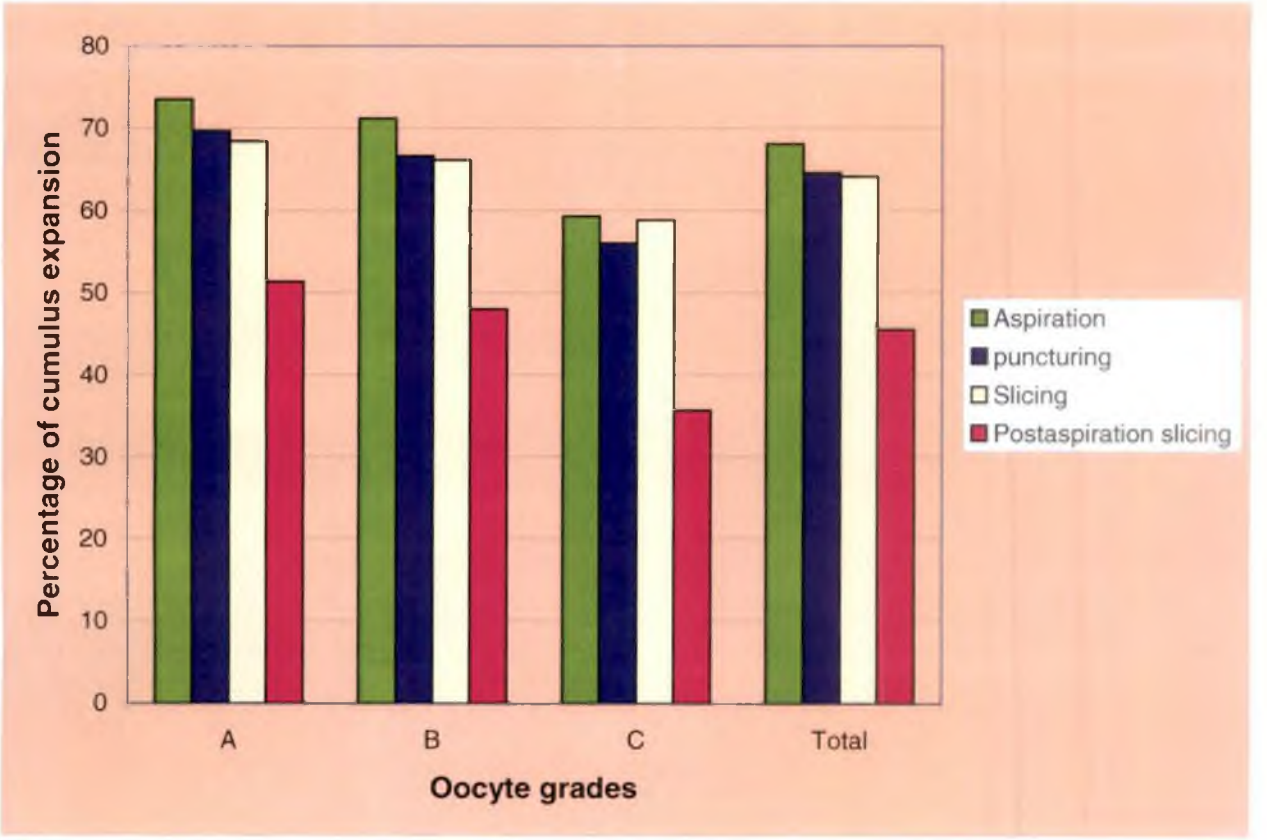



**Fig.6 Effect of retrieval technique on IVM**

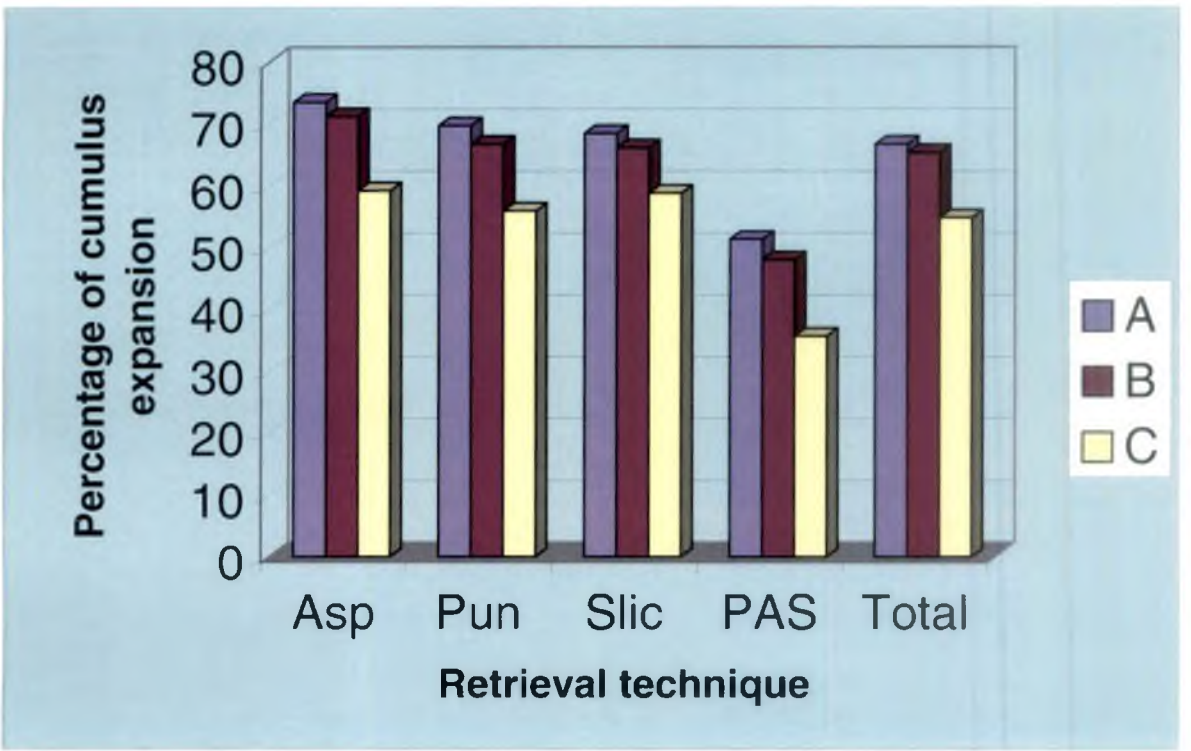



**Fig .7 Effect of oocyte quality on IVM**

Asp- Aspiration
Pun-Puncturing
Slic-Slicing
Pas-Post aspiration slicing

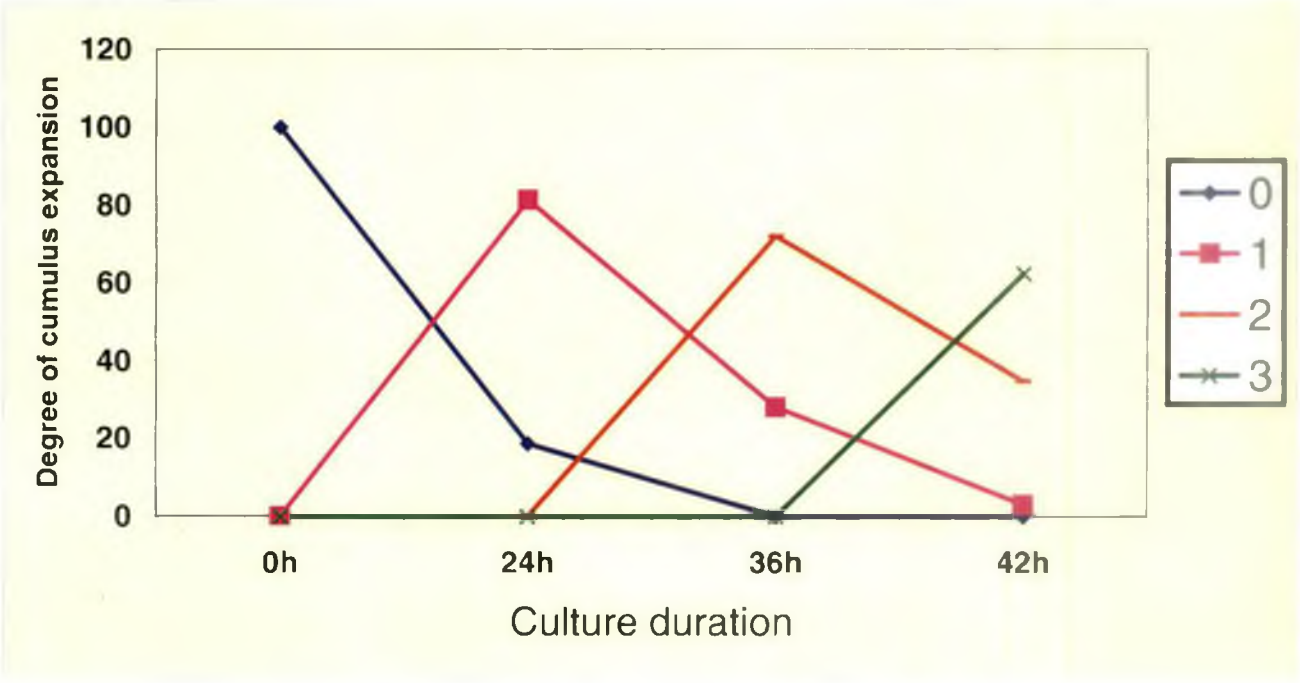



**Fig.8 Effect of culture duration on cumulus expansion**

# *Discussion*

# 5. DISCUSSION

The present study was under taken to assess the efficiency of oocyte retrieval methods on total yield of oocytes, yield of different quality grades of oocytes and the effect of these retrieval systems, quality grades and culture durations on *in vitro* maturation of oocytes of porcine species.

EXPERIMENT I

## 5.1 OVARIAN BIOMETRY

The mean weight, length, width and thickness of the follicular stage ovaries were found to be 3.88± 0.39g, 2.50±0.10cm, 1.20±0.09cm and 1.40±0.05 cm respectively and the luteal stage ovaries were 5.42±0.60g, 2.74±0.12cm, 1.30±0.05cm and 1.70±0.07cm respectively. The weight of luteal stage ovary was found to be more than follicular stage ovary. The variation in the weight of ovary between stages of oestous cycle in this study are in agreement with the findings of Bagg *et al.* (2004). The ovarian weight varies according to the changes occurring in the ovary during the oestrous cycle because of the influence of the cyclic changes in the weight of the CL. There was no significant difference between the dimensions of follicular and luteal stage ovaries.

The over all mean weight, length, width and thickness of 50 ovaries were found to be 4.65±0.50g, 2.62±0.11cm, 1.25±0.07cm and 1.55±0.06cm respectively. (Table.6). Roberts (1971) reported that the weight of sow ovaries varied from 3.5 to 10 gms. Nair (1970) observed that the length, width and thickness of the right and left porcine ovaries were 2.43×1.69×1.24 cms and 2.52× 1.73 ×1.28 cms respectively. The result of the present study is comparable with those reported by the above workers in the relevant aspects.

## 5.2 DISTRIBUTION OF SURFACE FOLLICLE

The data (Table.7&8, Fig.2) shows that maximum distribution of total surface follicle in follicular stage ovary was medium sized (51.81 per cent), followed by large sized (31.29 per cent) and small sized (16.88 per cent) follicles. Whereas maximum contribution on total surface follicle in luteal stage ovary was by small follicle (66.84 per cent) followed by medium follicle (33.15 per cent). These observations of the present study regarding the relationship between follicle size and ovarian status agree with the findings of Babalola and Shapiro (1988), Mc Donald (1989) and Ryan *et al.* (1994). Babalola and Shapiro (1988) found that the mean diameter of the predominating follicles were directly related to the stage of ovarian cycle in sow, the early luteal to mid follicular stage ovaries had essentially small to medium sized follicles (2-6mm diameter) while late follicular and ovulatory ovaries contained large follicles (6-11mm diameter). The proportion of small follicles decreased while medium and large follicles increased from day 15 to day 20 of the oestrous cycle in sow (Ryan *et al.*, 1994). The greatest increase in mean follicular size occurred in mid to late follicular phase of swine ovaries (Mc Donald, 1989). The major criticism on the use of slaughterhouse materials concerns the difficulty in accurately determining the day of the oestrous cycle of the donor animal. So the findings of the present study after observing the ovary that the major contribution of follicular population in follicular stage ovary is by medium and that of luteal stage ovary is by small sized follicles, give an indication of the stage of oestrous cycle of the animal .

The proportional shift in follicle populations from day 15 in the non-pregnant sows is consistent with the elevation of utero-ovarian vein concentrations of $PGF_2\alpha$ between day 12 and 16 of the oestrous cycle, leading to luteolysis. Lysis of the corpus luteum by $PGF_2\ \alpha$ reduces progesterone concentration and allows stimulation of the pool of small follicles by gonadotropins and the largest follicle was greater in diameter on day 18-20 (Ryan *et al.*, 1994). Foxcroft *et al.* (1987) proposed that dominant

follicles promoted the maturation of small follicles in the same ovary because of the observed asynchrony in both morphological and steroidogenic development of follicles in sows.

## 5.3 EFFECT OF FOLLICLE SIZE AND STAGE OF OVARY ON OOCYTE QUALITY

The oocytes from follicles of different sizes displayed a disproportionate relationship between the appearance of the investment and ooplasm and with either the pre or post culture chromatin configuration (Leibfried and First, 1979). In the present study, in case of follicular stage ovary 40.62 per cent C grade and 37.50 per cent D grade oocytes were obtained from small follicles whereas yield of A and B grade oocytes were comparatively more from medium and large sized follicles (Table.10). Marchal *et al.* (2002) reported that the porcine oocytes from medium and large sized follicles were competent for maturation than those from small follicles. Sirard *et al.* (1989) demonstrated the relationship between follicle size and oocyte's developmental competence and observed a lack of developmental competence of oocytes from small follicles. The study further indicated that all categories of follicles above one millimetre undergo atresia at an almost similar rate. The higher frequency of atretic follicles in medium and small follicles could have been caused by the regression of non-ovulatory follicles from large follicles and medium follicles respectively.

The percentage of good quality oocytes obtained from small follicles (21.42 per cent) is not significantly different from medium sized follicles (32.60 per cent) in case of luteal stage ovary. Abdoon and Kandil (2001) reported that total yield of good quality oocytes was more from CL bearing ovaries. Higher fertilization abnormalities reported in oocytes that originated from small follicles may be due to insufficiencies in cytoplasmic maturation and may be partly related to the degree of atresia of the follicle. These findings may be explained by an incomplete synthesis of maternal RNA hence the lack of synthesis of essential proteins required for oocyte maturation. Other

investigators find a higher percentage of mature oocytes in follicles with a fluid more than 0.3 to 0.6ml compared with follicles of less volume. The lager follicles provide the oocytes with a microenvironment that improves its quality. The intra follicular environment comprises an ever-changing environment of steroid and peptide hormones, growth factors, cytokines and other molecules, each of which may act alone or in concert with one or more of the other factors in influencing oocyte and follicle development. In this context the above observations strongly support the findings of relationship between follicle size and oocyte quality; that is the highest-grade oocytes come from large follicles.

The mean oocyte recovery rate per ovary was found to be 28.13 per cent (Table.10). This observation is not in agreement with Katska (1984), who obtained 43.2 per cent oocyte recovery rate from bovine ovary by aspiration of surface follicles. No significant difference is observed in oocyte recovery rate from different size follicles of follicular and luteal stage ovaries while significant difference was observed on oocyte recovery rate from follicular and luteal stage ovaries. As perusal of literature does not reveal any reference on this aspect of study in porcine, relevant observations from related species were used for comparison. The oocyte recovery rate in bovine by ovum pick up method, is influenced by numerous factors such as hormonal pre-treatment of animals, puncture frequency, time of puncture with in the oestrous cycle, aspiration vacuum and needle diameter, and the operator's experience. (Bols *et al.*, 1996)

EXPERIMENT II

## 5.4 EFFECT OF RETRIEVAL TECHNIQUE AND STAGE OF OVARY ON TOTAL OOCYTE YIELD

The observations of the present study (Table.11&Fig.4) clearly indicate that slicing, puncturing and post aspiration slicing yielded more oocytes from follicular stage ovaries than aspiration. It was found that the stage of ovary (follicular or luteal)

has no effect on mean yield of oocytes by different retrieval technique. Boediono *et al.* (1995) found no difference in the mean number of oocytes per ovary between luteal and follicular phase bovine ovaries. Takagi *et al.* (1992) observed no correlation between yield and presence of a large CL or any other measure of the ovarian status. Priscilla (2000) observed that the output of oocytes from CL bearing ovaries was found to be more. The lower yield of oocyte from luteal stage ovaries in the present study may be due to the interference of luteal tissues in the extraction of oocytes from luteal stage ovaries.

The different retrieval technique has an effect on mean oocyte yield from follicular and luteal stage ovaries. Total yield of oocyte was more by slicing followed by puncturing, post aspiration slicing and aspiration. Studies conducted by Martino *et al.* (1992) in caprine species and, Jain *et al.* (1995) and Das *et al.* (1996) in buffalo indicate that slicing method was more superior than other methods in terms of the recovery rate. Slicing of the ovary released oocytes from two sources, surface follicles and those in deeper cortical stroma, which may be the reason for high oocyte yield in the present study. Pawshe *et al.* (1994) found that oocyte recovered per ovary by aspiration was significantly more than that of slicing or puncturing from goat ovaries.

## 5.5 EFFECT OF RETRIVAL TECHNIQUE AND STAGE OF OVARY ON THE OOCYTE QUALITY

### 5.5.1Grade A Oocytes

The percentage of A class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were 27.22, 12.9, 10.96 and 10.68 per cent respectively and from luteal stage ovaries were 25.38, 10.07, 11.96 and 9.75 per cent respectively. It was found that the percentage yield of A grade oocytes is high for aspiration method even though the number is less (Table.12& Fig.5). Aspiration of

follicular oocytes and subsequent forceful expiration using needle and syringe might cause detachment of loosely adhered cumulus cells from the oocyte. Though the proportion of good quality oocytes obtained was found to be high, the recovery rate was low because of difficulty in separating the cumulus cell layer from cumulus oophorus. No significant difference was observed between follicular and luteal stage ovaries on the yield of A grade oocytes. Vijayakumaran (1995) obtained higher percentage of A grade oocytes from goat ovaries by slicing method. Carolan *et al.*, (1994) obtained 30.9 per cent A grade oocytes from bovine ovaries by aspiration technique. Gogoi *et al.* (2001) recovered 39.69 per cent A grade oocytes by aspiration method from buffalo ovary. Jain *et al.* (1995) got 11.11 per cent A grade oocytes from buffalo ovary by aspiration technique.

The observations of the present study conclude that the stage of oestrous cycle has no effect on the yield of A grade oocytes, but the retrieval technique has an effect on the percentage yield of A grade oocytes and it shows that aspiration technique yielded higher percentage of A grade oocytes compared to other techniques. Even though no references are available in porcine species, the research work in other species mentioned above support the present findings.

### 5.5.2 Grade B Oocytes

The percentage of B class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were 30.89, 29.32, 25.91 and 17.06 per cent respectively and from luteal stage ovaries were 26.92, 17.16, 14.52 and 19.72 per cent respectively (Table. 12 & Fig.5). Significantly no difference was observed between follicular and luteal stage ovaries on the yield of B grade oocytes. The percentage yield of B class oocyte by aspiration technique was significantly more from other methods. No references are available in porcine species to compare this study. Vijayakumaran (1995) obtained, higher percentage of B grade oocytes from puncturing

method followed by slicing, aspiration and post aspiration slicing from goat ovaries. Gogoi *et. al.* (2001) recovered 9.16 per cent B grade oocytes by aspiration technique from buffalo ovary.

### 5.5.3 Grade C Oocytes

The percentage of C grade oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were 25.65, 32.25, 30.23 and 40.19 per cent respectively and luteal stage ovaries were 31.53, 38.80, 34.61 and 31.97 per cent respectively. Slicing technique yielded more C grade oocytes per ovary and also the percentage yield of C grade oocyte was more by post aspiration slicing and slicing technique. No references are available in porcine species to compare this study. Higher percentage of low-grade oocytes found in the post aspiration slicing technique may be due to the release of sub cortical oocytes from porcine ovaries. Paul (2005) recovered 20.66 per cent C grade oocyte by slicing method compared to aspiration (18.3 per cent) and puncturing (19.13 per cent) from bovine ovaries. From the observations of the present study, it was found that more number of C grade oocytes was obtained by slicing method from porcine ovaries; in this context the above findings are comparable with the findings of Paul (2005).

### 5.5.4 Grade D Oocytes

The mean yield of D class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were1.29±0.27, 10.87±0.69, 12.37±0.80 and 8.37±0.40 respectively and from luteal stage ovaries were1.16±0.21, 9.1±0.50, 11.37±0.53 and 9.44±0.40 respectively. The percentage of D class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were16.23, 25.51, 32.89 and 32.05 per cent respectively and from luteal stage ovaries were16.15, 33.95, 38.89 and 38.54 per cent respectively (Table. 12 & Fig.5). The mean

yield and percentage of D grade oocytes were more from puncturing technique and least from aspiration technique. No references are available in porcine species to compare this study. Das *et al.* (1996) reported that puncturing method yielded 48.7 per cent poor quality oocytes from buffalo ovaries. Priscilla (2001) obtained 0.57, 0.45, 0.57 and 0.24 oocytes per ovary by aspiration, puncturing, slicing and post aspiration slicing techniques respectively from bovine. Paul (2005) obtained 3.7, 12.1 and 6.01 per cent D grade oocytes by aspiration, slicing and puncturing respectively from bovine ovary.

The variation in yield can be attributed to the individual variation of the slaughtered animals, breed, nutritional status, age of animal and season of the year (Dominguez, 1995). Aspiration yielded higher percentage of good quality oocytes and by aspiration speedy recovery of oocyte possible compared to slicing or puncturing. But the oocyte yield per ovary by aspiration method was very low compared to other methods. Slicing and puncturing yielded comparatively equal proportion of good and poor quality oocytes. The oocytes yield per ovary was very high in these methods because it released sub cortical oocytes also. The disadvantage of slicing method was that it resulted in more debris and interfered with the isolation of oocytes from medium. In this aspect puncturing method is good but it is a time consuming procedure. The quality of oocyte obtained by post aspiration slicing was poor. These results clearly indicate that slicing and puncturing are equally good for the collection of cumulus oocyte complexes for *in vitro* maturation purpose from porcine ovaries.

## EXPERIMENT III

## 5.6 EFFECT OF RETRIEVAL METHOD, OOCYTES QUALITY AND CULTURE DURATION ON IVM

A total of 525 oocytes collected by aspiration, slicing, puncturing and post aspiration slicing were subjected to *in vitro* maturation process and maturation changes

of each grade oocytes were assessed at zero, 24, 36 and 42 hours intervals. Each retrieval system was also considered separately in the process. Parameters used for identification of maturation of oocytes were the rate of cumulus expansion and a representative proportion of oocytes were subjected to assessment of nuclear maturation.

### 5.6.1 Effect of Retrieval Technique on Maturation

#### *5.6.1.1 Aspiration*

The percentage of A, B and C grade oocytes showing maximum degree of cumulus expansion under aspiration method was 73.5, 71.18 and 59.25 per cent respectively. On statistical analysis, there was no significant difference between A and B grade. However, the percentage of cumulus expansion for the C grade oocytes was found to be low compared to the other two grades. Wang *et al.* (1994) got a maturation rate of 73 per cent after culturing of compact porcine oocytes, collected by aspiration, for 36h inTCM-199B medium. Paul (2005) reported a higher maturation rate for A and B grade oocyte collected by aspiration from bovine ovaries. The metabolic cooperation between oocyte and cumulus cells serves an important nutritive role during maturation. Dell'Aguila *et al.*, (2001) found that the rate of abnormal fertilization was significantly higher for oocytes collected by aspiration. Aspiration resulted in greater disruption of surrounding cumulus cells; this may be due to the cumulus oophorus being firmly attached to the stratum granulosum. The advantage of follicle aspiration in terms of speed of operation, which may be particularly important in a commercial embryo production unit.

It is known that the cumulus cells remain intact, via gap junctions, during the entire maturation period. It is possible that an important role of follicular cells is to compensate for deficiencies in the oocyte's membrane, which is poorly equipped to

meet the metabolic requirements of the germ cell. The spherical oocyte has the minimum possible surface/ area volume ratio of any cell shape. It is also known that carrier systems in the oolemma are often either totally absent or poorly developed (Gordon,1994).

### *5.6.1.2 Puncturing*

The percentage of A, B and C grade oocytes showing maximum degree of cumulus expansion under puncturing method was 69.69, 66.67 and 56.00 per cent respectively. On statistical analysis there was no significant difference between A and B grade. But the maturation of C grade was significantly low compared to A and B grade oocytes. Oocytes not surrounded by the cumulus cell will have aberrant protein synthesis pattern (Kastrop *et al.*, 1990). The gonadotropin surge may act directly on COCs; FSH modifies their metabolism *in vitro*. More likely, this effect of the gonadotropin surge may be delayed by the granulosa cells, which increase the secretion of factors able to act on COC metabolism. In the preovulatory follicle, after the LH surge, the secretion by granulosa cells decreasing the amount of a 28 k Da protein in the COC would be particularly enhanced. Hence the control of COC metabolism by granulosa cells would not be exclusively dependent on intercellular contacts but could also be exerted via substances secreted in follicular fluids acting on a paracrine way (Rabahi *et al.*1993).The above finding clearly indicate that cumulus cells were required for the maturation of oocytes. Aspiration is a method, which yield more number of good quality oocytes. Therefore, the method indirectly increases the maturation rate of COCs.

### *5.6.1.3 Slicing*

In slicing method the percentage of A and B grade oocytes showing maximum degree of cumulus expansion was 68.42 and 66.12 and which differ significantly from

C grade (58.82 per cent). The amount of cumulus cells that surrounds the A and B grade oocytes support the maturation of these oocytes *in vitro*. Paul (2005) recorded a maturation at of 69.70 per cent for A grade oocytes 53.01 per cent for B grade and 35.29 per cent for C grade oocytes from bovine ovaries. A higher maturation rate in A grade caprine oocytes was also reported by Pawshe *et al.* (1994). In slicing method the oocyte with more layers of cumulus cells showed high maturation rate. The observations of the present study indicate the importance of cumulus cells for maturation. Vajta *et al.* (1996) reported that the number of oocytes recovered and blastocysts obtained after IVF was approximately doubled by slicing ovaries compared to aspiration.

Slicing method releases oocytes from the surface and cortical follicular population. Arlotto *et al.* (1996) found that the oocytes from cortical follicles were still in the growth phase, so they did not acquire complete competency for maturation and development. In the present study more amount of poor quality oocytes were obtained form slicing method but that was (D grade) not used for maturation process. Only the good quality oocytes were used for maturation. So the percentage of maturation of porcine oocytes by slicing method is almost comparable with aspiration and puncturing.

#### *5.6.1.4 Post aspiration slicing*

In post aspiration slicing method the percentage of A, B and C grade oocytes showing maximum degree of cumulus expansion was 51.35, 48.00 and 35.71 per cent respectively. On statistical analysis there was no significant difference between A and B grades. But C grade was significantly different from other grades. In post aspiration slicing method the main yield of oocytes were from cortical follicles, the surface follicle will have been already taken out by aspiration method. The oocytes that were released from cortical follicle are small and they are in their growth phase and may not have acquired complete competency for maturation. The findings of the present study

also reveals that the maturation rate of oocytes collected from post aspiration slicing method was significantly low compared to other methods. Motlik (1989) pointed out that reduced maturation for oocytes collected by post aspiration slicing due to the absence of some factors necessary for oocyte maturation. Ellederova (2004) found that nuclear transcripts and proteins synthesized and stored earlier, during the period of oocyte growth and completion of all the metabolic steps, allows acquisition of a full meiotic and developmental competence. These findings reveal that the oocytes collected from cortical follicles are immature and developmentally incompetent.

#### *5.6.1.5 Overall*

When overall maturation rate was taken into account, aspiration, puncturing, slicing and post aspiration slicing methods gave a maturation rate of 68.07, 64.63, 64.17 and 45.56 respectively. Statistical analysis revealed that there was no significant difference between aspiration, puncturing and slicing. But post aspiration slicing showed significantly lower maturation rate compared to other methods.

### 5.6.2 Effect of Oocyte Grade on Maturation

#### *5.6.2.1 A Grade oocyte*

The percentage of A grade oocytes showing maximum degree of cumulus expansion after aspiration, puncturing, slicing and post aspiration slicing was 73.50, 69.69, 68.42 and 51.35 per cent respectively. Cumulus expansion rate was more for A grade oocytes collected from aspiration technique. But statistical analysis revealed that there was no significant difference in maturation rate of A grade oocytes between aspiration, slicing and puncturing. No references are available in porcine species to compare this study. Paul (2005) reported 83.08, 69.70 and 70.37 per cent cumulus expansion rate for A class bovine oocytes collected by aspiration, slicing and

puncturing. Martino *et al.* (1992) observed that oocytes collected from caprine ovaries with three or more layers of cumulus cells showed only slight superiority in their capacity for maturation than oocytes with one or two layers. Presence of intact cumulus cells for at least 24 h was necessary for the normal cytoplasmic maturation of porcine oocytes and the corona radiate remained intact upto metaphase I stage (Motlik *et al.*, 1986). It is evident from many reports that the metabolic cooperation between oocyte and cumulus cell are important for oocyte maturation. Cumulus cells are linked to one another by gap junctions and these permits the passage of small molecules from one cell to another. Carolan *et al.* (1994) showed that the over all maturation rates was significantly low for oocytes obtained from cutting compared to aspiration, while selected oocytes with more than three layers of cumulus cover showed no significant difference in maturation rate. This observation explains the importance of cumulus cells for oocyte maturation and retrieval method does not have any direct effect on oocyte maturation.

#### *5.6.2.2 B Grade oocyte*

The percentage of B grade oocytes showing maximum degree of cumulus expansion under aspiration, puncturing, slicing and post aspiration slicing was 71.18, 66.67, 66.12 and 48.00 per cent respectively. Maximum maturation was observed in aspiration followed by puncturing and slicing. Post aspiration slicing showed a significantly low maturation rate compared to other methods. Cumulus expansion rate obtained in B class bovine oocytes by aspiration, slicing and puncture was 68.29, 53.0 and62.07 per cent (Paul, 2005). The above finding revealed that there was no significant difference between aspiration, slicing and puncturing in bovine oocyte maturation. The observations of the present study agree with the above observations.

*5.6.2.3 C grade oocyte*

The percentage of C grade oocytes showing maximum degree of cumulus expansion under aspiration, puncturing, slicing and post aspiration slicing was 59.25, 56.00, 58.82 and 35.71 respectively. Post aspiration slicing showed significantly low maturation rate compared to other methods. Maximum maturation was observed in aspiration followed by puncturing and slicing. Paul (2005) obtained a oocyte maturation rate of 44.74, 35.29 and 38.46 per cent respectively by aspiration, slicing and puncturing from bovine.

*5.6.2.4 Overall*

The overall maturation rate for A, B and C grade oocytes were found to be 66.67, 65.29 and 54.85 per cent respectively. On statistical analysis, there was no significant difference between A and B grades. However, C grade showed significantly lower maturation rate than other grades. These results indicate that oocytes with more than three layer of cumulus cells (A and B grade) have good maturation capability. These results clearly showed the importance of cumulus cells for oocyte maturation. The existence of a healthy population of cumulus cells surrounding the oocytes was mandatory to facilitate the transport of nutrients and to give signals into and out of the oocytes.

**5.6.3Effect of Culture Duration on Maturation**

The duration required for the completion of *in vitro* maturation in porcine oocyte is more than that of cattle (24h). Cumulus expansion in pig proceeds from the superficial layers to the deeper ones, with the corona radiata layer being reached only at the end of maturation. The loss of gap junction with in cumulus cell triggers the resumption of

meiosis in pig oocytes (Isobe and Terada, 2001). So the degree of cumulus expansion is a good indicator to assess the maturation status of pig oocytes at different intervals.

*5.6.3.1 At Zero hour of Culture*

At zero hour of culture all the oocytes (100 per cent) were devoid of any detectable response to maturation (0-Degree cumulus expansion). Wang *et al.* (1994) observed that at zero hour culture cent percentage of the porcine oocytes was in GV stage and the morphology of cumulus mass was very compact at the start of culture. Isobe *et al.* (1998) reported that 99.6 per cent of porcine oocytes were at GV stage upto zero to eight hour of culture, which indicate that a compact cumulus mass around the oocyte. The observations of the present study agree with the above findings. Cumulus expansion depends upon the contact with the oocyte or oocyte secreted factors (Vanderhyden, 1993). The nuclear changes of oocytes inhibited by inhibitors produced by cumulus cells. Blocking of cumulus cell expansion by GVBD inhibiting factors.

*5.6.3.2 At 24 h of Culture*

At 24 h of culture 98 oocytes (18.67 percent) showed no detectable response and 427 oocytes (81.33 per cent) showed minimum observable response (Score1-Degree cumulus expansion). Funahashi and Day (1993) reported that 85 per cent of oocytes showed cumulus expansion limited to outer layer of cumulus cells (second degree) and 98 per cent of them were in GVBD stage. Wang *et al.* (1994) observed that at 24h culture, almost 95 per cent of the porcine oocytes completed GVBD and the degree of expansion progressed to moderate. The observations of the present study ie, most of the COCs showed a detectable change in cumulus cells, agree with the above findings. Cumulus expansion results when cumulus cells secrete hyaluronic acid, which expands the spaces between the cumulus cells.

*5.6.3.3 At 36 h of Culture*

At 36h of culture 28 per cent oocyte showed minimum observable response and 72 per cent showed expansion limited to the outer layer of cumulus cells (Score 2- Degree cumulus expansion). Funahashi and Day (1993) found that 48 per cent of COCs showed third degree cumulus expansion (cumulus expansion except the corona radiata) and 52 per cent showed second-degree cumulus expansion. Wang *et al.* (1994) observed that full expansion of the cumulus mass was observed at 36h of culture, the corona radiata were still compacted at that time.

*5.6.3.4 At 42h of Culture*

At 42h of culture 62.28 per cent oocytes showed maximum degree of cumulus expansion (Score 3- Degree cumulus expansion) and 34.72 per cent showed expansion limited to the outer layer of cumulus cells and three per cent showed minimum observable response. Cumulus expansion index after 42h was found to be 2.57. (Table.16& Fig.8). Funahashi and Day (1993) recorded 90 per cent porcine COCs in maximum degree of cumulus expansion.

*5.6.3.5 Overall*

The morphology of cumulus mass was found to be changing during the entire culture period. The overall maturation rate after 42 h was found to be 62.28 per cent with a cumulus expansion index of 2.57.The data shows that 42h is required for complete maturation of porcine oocytes and the degree of cumulus expansion is a good indicator of assessing the *in vitro* maturation rate.

## 5.7 ASSESSMENT OF NUCLEAR MATURATION OF OOCYTES

The formation of the second meiotic spindle and expansion of the surrounding cumulus cells are the good indicators of an *in vitro* matured oocyte. (Godron, 1994). In the present study 62 per cent of oocytes showed nuclear progression to metaphase II which is comparable to the rate obtained for cumulus expansion. So for the assessment of maturation rate of *in vitro* cultured oocytes, cumulus expansion is found to be a good indicator.

## CONCLUSION

The present study revealed that there was no significant difference in oocyte yields from follicular and luteal stage ovaries even though the surface follicle distribution was different significantly. Slicing and puncturing method were found to be good for the collection of oocytes from porcine ovaries. These two methods yielded higher number good quality oocytes and the maturation rate was also found to be satisfactory. Among A, B and C grade oocytes, A and B grade oocytes showed a higher maturation rate which indicate that oocytes with more than three layer of cumulus cells are having better potential for maturation. To complete maturation process in porcine oocytes *in vitro* a minimum period of 42h is found to be very essential. TC-199 medium satisfactory for the *in vitro* maturation of porcine oocytes however, a hormone freed medium is preferred during later half of the culture period. In the present study using TCM-199, 64 per cent of porcine oocytes showed cumulus expansion and 62 per cent showed nuclear progression to metaphase II stage at 42h of culture.

# *Summary*

# 6. SUMMARY

A study was carried out on *in vitro* maturation of follicular oocytes in porcine using oocytes obtained by various harvesting techniques from ovaries of recently slaughtered animals. The major objective of this study was to find out the efficiency of various oocyte retrieval systems on the yield of oocytes and its quality and to assess the *in vitro* maturation potentiality of these oocytes on culture for different durations.

Ovaries used for the study were of recently slaughtered pigs belonging to Large White Yorkshire, Landrace and Duroc breeds. Ovaries were dissected out and transported to laboratory within 30-60 minutes in freshly prepared normal saline fortified with antibiotics such as Penicillin and Streptomycin. A total of 40 follicular and 36 luteal stage ovaries obtained from recently slaughtered animals were used for this study. Randomly selected 25 follicular and 25 luteal stage ovaries were used for the study of ovarian biometry. Twenty four follicular and 18 luteal stage ovaries were used for the study for distribution of surface follicle and effect of follicle size and oocyte quality. After repeated washing of ovaries in normal saline and TL-HEPES media kept at 36-38°C, oocytes recovery was performed by aspiration, slicing, puncture and post aspiration-slicing method. Twenty four follicular and 18 luteal stage ovaries were used for aspiration and post aspiration slicing technique. Eight follicular and 10 luteal stage ovaries were used for slicing method. Eight follicular and eight luteal stage ovaries were used for puncturing technique. Recovery of oocytes was performed while keeping ovaries in TL-HEPES medium enriched with BSA at the rate of 0.6 per cent and maintained at 37°C.

Recovered oocytes were rinsed several times in fresh drops of TL-HEPES. These oocytes were classified into four quality grades based on number of cumulus

cell layers and ooplasm character as class A, B, C and D. Grade A COCs were characterised by more than five complete layers of cumulus cells and uniform granulation of ooplasm . Grade B had three to five complete layers of cumulus cells and uniform granulation of ooplasm and grade C with one to two complete layers of cumulus cells and uniform granulation of ooplasm. Grade D were completely/ partially denuded and degenerated or deformed oocytes. A representative sample of the oocytes was stained with aceto-orcein, Fluorescein Diacetate (FDA) and Hoechst 33342 stains to assess the viability and nuclear status of the oocyte before culture.

Each grade of oocytes obtained by different recovery method was cultured separately in 100 µl maturation drops. Medium used for *in vitro* maturation of oocytes was freshly prepared TCM-199, enriched with FSH 0.5 µg/ml, LH – 5 µg/ml, Oestradiol 1 µg/ml, sodium pyruvate 10µl/ml and foetal calf serum (FCS) 10 per cent and filtered using 0.22µm membrane filter. Culture conditions were set in a standard water-jacketed type of $CO_2$ incubator with a temperature of 38.5°C, $CO_2$ tension of 5 per cent and maximum humidity. The oocytes were allowed to complete incubation for a period of 24 h without any disturbance in the culture environment. After this incubation period the oocytes were again washed two to three times in freshly prepared maturation media. These oocytes were then transferred into freshly prepared acclimatized and labelled maturation media drops having no hormonal supplements (FSH, LH and Oestradiol) and incubated for another 18h. Cumulus expansion rate was recorded at 24h, 36h and 42h of incubation by observing oocytes in maturation drops under zoom stereomicroscope without disturbing the drops. Degree of cumulus expansion was confirmed by examining under phase contrast microscope. A representative sample of oocytes was assessed for nuclear maturation.

The mean weight, length, width and thickness of the follicular stage ovaries were found to be 3.88± 0.39g, 2.5±0.10cm, 1.2±0.09cm and 1.4±0.05cm respectively and the luteal stage ovaries were 5.42±0.60g, 2.74±0.12cm, 1.3±0.05cm and

1.70±0.07cm respectively. The over all mean of weight, length, width and thickness of 50 ovaries were found to be 4.65±0.50g, 2.62±0.11cm, 1.25±0.07cm and 1.55±0.06cm respectively. A total of 770 surface follicles was recorded in 24 follicular stage ovaries of which 130 were small sized, 399 were medium sized and 241were large sized follicles. Out of 371 total surface follicles recorded in 18 luteal stage ovaries, 248 were small and 123 were medium follicles and it was found that large follicles were absent in luteal stage ovaries. The mean number of small, medium, large and total surface follicle in follicular stage ovary was found to be 5.41, 16.62, 10.04 and 32.08 respectively. In case of luteal stage ovary the mean number of small, medium, large and total surface follicle was found to be 13.77, 6.83, zero and 20.61 respectively. Good quality oocyte was significantly more from medium and large sized follicles compared to small sized follicles and there was no significant difference in oocyte recovery rate from small, medium and large sized surface follicles. The mean oocyte recovery rate was found to be 28.13 per cent.

The mean oocytes yield per follicular stage ovary was found to be 7.91±0.84, 42.62±0.21, 37.62±2.05 and 26.12±2.37 for aspiration, slicing, puncturing and post aspiration slicing respectively. The mean oocyte yield per luteal stage ovary was found to be 7.22±0.52, 26.8±1.04, 29.25±0.61 and 24.5±1.16 for aspiration, slicing, puncturing and post aspiration slicing respectively. The combined mean of oocyte yield per ovary was found to be 7.64±0.50, 33.83±2.14, 33.43±1.45 and 25.42±1.43 for aspiration, slicing, puncturing and post aspiration slicing respectively. It was found that slicing and puncturing yielded more oocytes per ovary than other methods and the yield in aspiration method was significantly less.

The mean yield of A class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were 2.12±0.29, 5.5±0.73,4.12±0.71 and 2.79±0.35 respectively and from luteal stage ovaries were1.83±0.29, 2.7±0.26, 3.5±0.32 and 2.38±0.61 respectively. The mean yield of B class oocytes from

follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were 2.45±0.35, 12.5±1.01, 9.75±0.72 and 4.45±0.34 respectively and from luteal stage ovaries 1.94±0.24, 4.6±0.30, 4.25±0.61 and 4.83±0.38 respectively. The mean yield of C class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were 2.04±0.47, 13.75±0.75, 11.37±0.82 and 10.5±2.57 respectively and from luteal stage ovaries were 2.27±0.28, 10.4±0.58, 10.12±0.54 and 7.83±0.40 respectively. The mean yield of D class oocytes from follicular stage ovaries by aspiration, slicing, puncturing and post aspiration slicing were1.29±0.27, 10.87±0.69, 12.37±0.80 and 8.37±0.40 respectively and from luteal stage ovaries were1.16±0.21, 9.1±0.50, 11.37±0.53 and 9.44±0.40 respectively.

Slicing, puncturing and post aspiration slicing did not differ significantly in the yield of total number of oocytes per ovary. But the yield from aspiration was significantly lower than other methods. The percentage yield of good quality (A and B) oocytes was more from aspiration than slicing, puncturing or post aspiration slicing. The percentage yield of D grade oocyte was more from post aspiration slicing method.

At zero hour culture all the oocytes showed zero degree cumulus expansion. At 24h, 18.67 per cent of oocytes were in score '0' (no detectable response) degree cumulus expansion and 81.33 per cent oocytes showed score '1' (minimum observable response) degree cumulus expansion. At 36 h, 28 per cent oocytes showed score '1' cumulus expansion and 72 per cent showed score '2' (expansion limited to the outer layer of cumulus cells) degree cumulus expansion. At 42h, about 62.28 per cent COCs showed score '3'(maximum degree of cumulus expansion) degree cumulus expansion, 34.72 per cent showed score '2' and three per cent showed score '1'degree cumulus expansion respectively.

The percentage of maturation for aspiration, puncturing, slicing and post aspiration slicing were 68.07, 64.63, 64.17 and 45.56 per cent respectively. It was found that the maturation rate for post aspiration slicing was significantly lower than other methods. The maturation rate for A grade, B grade and C grade oocytes were found to be 66.67, 65.29 and 54.84 per cent respectively. A and B grade oocytes were not significantly different in the maturation rate. The over all nuclear maturation rate was found to be 62 per cent.

Analysis of the result revealed that there was no significant difference in oocyte yields from follicular and luteal stage ovaries even though the surface follicle distribution was different significantly. Slicing and puncturing method were found to be good for the collection of oocytes from porcine ovaries. In these two methods higher number of good quality oocytes and the maturation rate were found to be satisfactory. Among A, B and C grade oocytes A and B grade oocytes showed a higher maturation rate which indicates that oocytes with more than three layer of cumulus cells are better for maturation. For the completion of maturation process in porcine oocytes a maturation period of 42h is essential.

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# STUDIES ON *IN VITRO* MATURATION OF PORCINE FOLLICULAR OOCYTES

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**Abstract of the thesis submitted in partial fulfilment of the requirement for the degree of**

## Master of Veterinary Science

**Faculty of Veterinary and Animal Sciences**
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**2007**

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# ABSTRACT

A study was designed and carried out to evaluate the effect of different retrieval methods like aspiration, slicing, puncturing and post aspiration slicing on yield of different grades of oocytes and their *in vitro* maturation potential. The effect of cumulus oocyte complex morphology and culture duration on *in vitro* maturation of porcine oocyte was also studied. A total of 40 follicular and 36 luteal stage ovaries obtained from Large White Yorkshire, Landrace and Duroc pigs were used for the study. Each quality grade of oocyte obtained through four retrieval methods was subjected to maturation for 42 h in TCM-199 medium supplemented with LH, FSH, estradiol, pyruvate and foetal calf serum. Culture environment was set as 38.5°C temperature, 5 per cent $CO_2$ and maximum humidity in standard water-jacketed $CO_2$ incubator. Maturation changes were assessed by observing the degree of cumulus expansion at 24h, 36h and 42h of incubation.

There was no significant difference in oocyte yield from follicular and luteal stage ovaries even though the surface follicle distribution was different significantly. Slicing, puncturing and post aspiration slicing did not differ significantly in the yield of total number of oocytes per ovary. But the yield from aspiration was significantly lower compared to other methods (7.64 vs.33.83, 33.43, 25.42). The percentage yield of good quality (A and B) oocytes was more from aspiration than slicing, puncturing or post aspiration slicing. The percentage yield of D grade oocyte was more from post aspiration slicing method.

The percentage of maturation for aspiration, puncturing, slicing and post aspiration slicing were 68.07, 64.63, 64.17 and 45.56 per cent respectively. It was found that the maturation rate for post aspiration slicing was significantly lower than other methods. The maturation rate for A grade, B grade and C grade oocytes were found to be 66.67, 65.29 and 54.84 per cent respectively. A and B

grade oocytes were not significantly different in the maturation rate. At zero hour culture all the oocytes were devoid of any detectable response of maturation. At 24h, 81.33 per cent oocytes showed minimum observable response. At 36 h, 72 per cent oocytes showed expansion limited to the outer layer of cumulus cells. At 42h, about 62.28 per cent COCs showed maximum degree of cumulus expansion. The over all cumulus expansion rates was found to be 64 per cent and nuclear maturation rate was 62 per cent.

Slicing and puncturing were found to be good methods for the collection of oocytes from porcine ovaries. These two methods yielded higher number good quality oocytes with satisfactory level of maturation rate. Among A, B and C grade oocytes A and B grade oocytes showed a higher maturation rate indicating that the oocytes with more than three layer of cumulus cells are better for *in vitro* maturation. For the completion maturation process in porcine oocytes a minimum period of 42h was found essential.