EFFECT OF OVUM RETRIEVAL METHODS AND CUMULUS-OOCYTE COMPLEX MORPHOLOGY ON *In vitro* MATURATION OF BOVINE OOCYTES

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

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DECLARATION

I hereby declare that the thesis entitled **"EFFECT OF OVUM RETRIEVAL METHODS AND CUMULUS-OOCYTE COMPLEX MORPHOLOGY ON *IN VITRO* MATURATION OF BOVINE 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.

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Certified that the thesis entitled ""**EFFECT OF OVUM RETRIEVAL METHODS AND CUMULUS-OOCYTE COMPLEX MORPHOLOGY ON *IN VITRO* MATURATION OF BOVINE OOCYTES**" is a record of research work done independently by **Magnus Paul .K.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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MAGNUS PAUL .K.

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LIST OF ABBREVIATIONS USED

AC	-	Adenyl cyclase .
ATP	-	Adenosine tri phosphate.
bFGF	-	Bovine fibroblast growth factor.
BSA-V	-	Bovine serum albumin fraction V.
cAMP	-	Cyclic adenosine monophosphate.
CO_2	-	Carbon dioxide
COCs	-	*Cumulus oocytes complexes.*
CSF	-	Cytostatic factor
Cx	-	Connexin.
DNA	-	deOxy ribo nucleic acid
ECS	-	Estrous cow serum.
EGF	-	Epidermal growth factor.
FCS	-	Foetal calf serum.
FDA	-	Fluorescent diacetate.
GSH	-	Glutathione
GV	-	Germinal vesicle.
GVBD	-	Germinal vesicle break down.
h	-	Hour
hCG	-	Human chorionic gonadotrophin
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	-	MetaphaseI
MII	-	Metaphase II.
MIS	-	Mullarian inhibitory substance
ml	-	Milliliter
mM	-	Millimole
MPF	-	Maturation promoting factor
MZT	-	Material to zygote transition.
NIH	-	National institute of health.
nm	-	Nanometer
OMI	-	Oocyte maturation inhibitor
PB	-	Polar body
PBS	-	Phosphate buffered saline.
pFSH	-	Porcine follicle stimulating hormone.

pLH	-	Porcine luteinizing hormone.
RER	-	Rough endoplasmic reticulum
RNA	-	Ribo nucleic acid
SER	-	Smooth endoplasmic reticulum
SFRE	-	Serum free
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
χ^2	-	Chi square
°C	-	Degree centigrade
µg	-	Microgram
µm	-	Micrometer

Introduction

1. INTRODUCTION

The main objective of reproductive biotechnological research in animals is to augment production from livestock industry. Twenty first century is heading towards a revolution in the application of biotechnological procedures such as nuclear transfer cloning, sperm and embryo sexing, production of transgenic animals etc. to farm animals. The success of application of these techniques is critically dependent on the expertise in the basic reproduction techniques such as *in vitro* maturation of oocytes, IVF and *in vitro* culture of embryos (Kane, 2003). In addition, the conservation of endangered species and rare livestock breeds are also possible by IVM and IVF techniques (Neglia *et al.*, 2003). It is a known fact that an ample supply of oocytes and embryos of any stage of development can be generated only by *in vitro* production technique. It is possible to produce progenies even from pre-pubertal and dead animals by IVM and IVF technique. But most of these research works are at fundamental level and need lot of refining to apply them in the field in a cost effective manner. Fruitful application of these highly advanced technologies necessitates an expertise in the areas of *in vitro* maturation and *in vitro* fertilization of oocytes. Among this, *in vitro* maturation is the starting point of these complex series of procedures (Merton *et al.*, 2003, Nandi *et al.*, 2003).

Maturation of oocytes means the process of acquisition of competence by oocytes to achieve fertilization and to develop into an embryo. This includes nuclear maturation and cytoplasmic maturation (Bevers *et al.*, 1997). During the maturation the meiotic arrest at diplotene is removed and meiosis resumes and progress to metaphase II. Cytoplasm undergoes changes like m-RNA and protein synthesis and its accumulation (Rodriguez and Farin, 2004). In *in vivo* maturation, these processes start after LH surge (Choi *et al.*, 1998) and get arrested at metaphase II (M II) before fertilization.

In laboratory under suitable culture conditions maturation of immature oocytes can be induced successfully. Lot of work on *in vitro* maturation has been

carried out in *Bos taurus*. In India, *in vitro* maturation studies are carried out in buffalo (Totey *et al.*, 1996; Chauhan *et al.*, 1998; Gupta *et al.*, 2001), goat (Vijayakumaran, 1995; Pawshe *et al.*, 1996; Katiyar *et al.*, 1997) and sheep (Chauhan *et al.*, 1997). Ban on cow slaughter in many states of India made it difficult to carry out such extensive studies in Indian cattle.

In Kerala Agricultural University, Department of Animal Reproduction has carried out many research works in the field of multiple ovulation, *in vivo* embryo production, cryopreservation and its transfer (Benjamin, 1994; Ramachandran, 2000; Joseph, 2003). But only preliminary work has been carried out in the field of *in vitro* production of embryos in this university. Priscilla (2001) had carried out a study on retrieval and characterization of bovine oocytes from slaughterhouse ovaries. The process that comes next to it in *in vitro* production of embryos is *in vitro* maturation of oocytes, which form the basis for the present study.

But the efficiency of *in vitro* production of embryos is less than that of *in vivo* production techniques. *In vivo* matured oocytes showed a higher percentage of blastocyst formation than *in vitro* matured ones, 36.6 ± 9.8 per cent and 23.1 ± 4.4 per cent respectively (Bevers *et al.*, 1997). So improvement in the efficiency of *in vitro* maturation procedure could improve the quality and quantity of IVF/ IVP embryos (Merton *et al.*, 2003). Lot of factors starting right from medium to quality of oocytes, decide the efficiency of IVM. It was in this context the present study was undertaken in cattle with the following as major objective.

1) Compare the efficiency of three oocyte recovery methods on the yield of oocytes.
2) Compare the effect of recovery methods on the quality of yielded oocytes.
3) Compare the effect of retrieval method and quality of oocytes on the IVM success rate.
4) Identify a suitable protocol for IVM of follicular 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 first meiotic division to the metaphase of second meiotic division. This complex biological process, which transforms the primary oocyte into an unfertilized egg, is known as oocyte maturation. In live animal, the oocyte which 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). Oocyte maturation involves both cytoplasmic and nuclear maturation (Mikkelsen, 2004). *In vitro* oocyte maturation could be initiated by removal of the fully-grown (competent) oocyte from its follicular environment (Buccione *et al.*, 1990). This phenomenon is exploited in *in vitro* maturation (IVM) culture systems.

2.2. COLLECTION OF OVARIES

2.2.1 Source of Ovaries

Ovaries obtained from local abattoirs are usually used for the collection of oocytes for *in vitro* maturation and *in vitro* production of embryos (Harada *et al.*, 1997; Abdoon *et al.*, 2001; de Wit and Kruip, 2001; Lequarre *et al.*, 2005). Ultrasound guided ovum pick-up technique via transvaginal follicle aspiration is widely used at present for the collection of oocytes from live animals (Galli and Lazzari, 1996; Goodhand *et al.*, 2000; Reis *et al.*, 2002; Hendriksen *et al.*, 2004). Laparoscopic procedure, though rarely used, is another technique for oocyte harvest from cattle (Stubbings *et al.*, 1988).

2.2.2 Time Interval from Slaughter to Retrieval

Time interval from slaughter to retrieval of oocyte influenced the success rate of *in vitro* maturation (Ali *et al.*, 2004). The usual time interval ranged from one hour (Ectors *et al.*, 1995; Lechniak *et al.*, 1996), 1-3 h (Vassena *et al.*, 2003; Matsushita *et al.*, 2004) 3-5 h (Wu *et al.*, 1997) and >5 (Ali *et al.*, 2004).

Matsushita *et al.* (2004) stored ovaries at 0, 10 and 20°C for 24 h before the retrieval of oocytes in normal saline and obtained maximum (67.0 per cent) maturation rate at 10°C. They assessed effect of long duration storage (24 h, 48 h and 72 h at 10°C) of ovary on subsequent maturation rate of oocyte and observed that the rate was maximum (67.5 per cent) at 24 h group. According to them, the reduction in maturation rate on long duration storage of ovary could be due to the degeneration of some of the accumulated mRNA and oocyte protein that were necessary for early developmental competence of oocytes. Ali *et al.* (2004) opined that ovaries maintained at 35°C for 4 h after slaughter yielded higher frequencies of blastocyst development than those collected 2, 6 or 7 h post slaughter, which indicated that some developmental competence might be acquired shortly prior to *in vitro* maturation.

A longer sojourn within follicle may permit greater storage of maternal m-RNA and protein within the oocytes, making oocytes more capable of developing to blastocyst stage (Vassena *et al.*, 2003).

2.2.3 Temperature and Media

Effect of transportation and storage temperature of ovary on IVM of cattle oocyte has been studied extensively. In most of the studies ovaries were transported in thermally insulated bags containing normal saline with antibiotics at different temperatures like >20°C (Matsushita *et al.*, 2004) with 45.2 per cent maturation rate, 20-30°C (Ectors *et al.*, 1995) with above 86 per cent maturation rate and 30-37°C (Lechniak *et al.*, 1996) with approximately 75 per cent maturation rate. Aman and Parks (1994) observed increased depolymerization

rate of microtubules of oocytes when kept below 20°C resulting in abnormal meiosis. Sterile normal saline supplemented with antibiotics (Ali and Sirard, 2002; Cetica *et al.*, 2002; Bormann *et al.*, 2003) or Dulbecco's phosphate buffered saline with antibiotics (Abdoon *et al.*, 2001; de Wit and Kruip, 2001) were the media usually used for pre-retrieval processing of ovaries.

2.2.4 Pre-retrieval Processing of Ovaries

The pre retrieval processing like removal of extraneous tissues, repeated washing etc. of ovaries reduced the chance of contamination in the oocyte culture (Sachan *et al.*, 1999).

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) HEPES.

2.3.1.1 TL-HEPES Media (Tyrode's Lactate – Hydroxy Ethyl piperazine Ethane Sulfonic Acid)

HEPES buffered Tyrode's medium (pH 7.4) supplemented with 0.3 per cent BSA fraction V and antibiotics was used for oocyte retrieval by many scientists (Aman and Parks., 1994; Azambuja *et al.*, 1998). Arlotto *et al.*, 1996 obtained 62 to 72 per cent fertilization rate after IVF, when this medium was used for oocyte retrieval from bovine ovaries. Chian *et al.*, 1996 obtained up to 100 per cent sperm penetration rate in *in vitro* fertilization (IVF) when this medium was used in bovine oocyte retrieval.

2.3.1.2 TCM-199 (Tissue Culture Medium – 199)

TCM-199 buffered either with HEPES or bicarbonate, 10 per cent FCS and antibiotics was used for oocyte retrieval and washing by Lechniak *et al.*,

1996; Armstrong *et al.*, 1996; deMatos *et al.*, 1997 and Harada *et al.*, 1997. When this medium alone was used for bovine oocyte retrieval Calder *et al.*, 2001 obtained 50-90 per cent cumulus expansion and Silva and Knight 1998 obtained 54-60 per cent cleavage rate.

2.3.2 Retrieval Methods

Several methods were used for oocyte retrieval from bovine ovaries. Common methods are aspiration, slicing, puncture and dissection for collecting oocytes from slaughterhouse specimens and ultrasound guided ovum pick-up via transvaginal follicle aspiration and laparoscopic technique are being used for oocyte retrieval from live animals.

2.3.2.1 Aspiration

Aspiration of oocytes from 2-8 mm follicles with disposable 18-21 gauge needle with syringe or vacuum pump was a common method of oocyte retrieval (Dominguez, 1995; Wu *et al.*, 1997; Abdoon *et al.*, 2001; de Wit and Kruip, 2001; Ali and Sirard, 2002).

Bols *et al.* (1996) reported that thicker needle (18 gauge) yielded the highest oocyte recovery rate (75 per cent) but number of naked oocytes were more with this needle than with thinner gauge (21 gauge) 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 COC's more laminar with less turbulence in thinner needle.

Das *et al.* (1996a) in buffaloes and Pawshe *et al.* (1994) in goat reported that by slicing oocytes from surface follicles as well as follicles of deeper cortical stroma were released, where as by puncture and aspiration, only oocytes from surface follicles were released. Aspiration had the advantage of high speed of oocyte recovery when compared to dissection or slicing (Gordon, 2003).

2.3.2.2 Slicing

Ovaries were sliced repeatedly with a sharp sterile surgical blade keeping ovaries in Petri dish containing the medium .(Das *et al.*, 1996a; Bols *et al.*, 1997; Priscilla, 2001). High number of COC's by slicing was due to recovery of oocytes located in cortical position in addition to follicles visible on surface (Arlotto *et al.*, 1996). More poor quality oocytes were obtained by slicing since oocytes were obtained from all kinds of follicles in the ovary. So a heterogeneous population of oocytes were obtained, which also included oocytes which were not fully grown (Das *et al.*, 1996a). Slicing resulted in more debris and interfered with recovery of oocytes in sheep (Wani *et al.*, 1999). Slicing produced three-fold increase in yield of oocytes over aspiration (Gordon, 2003).

2.3.2.3 Puncture

Repeated puncturing of ovaries with 18-gauge needle to release oocytes into the medium kept in a Petri dish was the method used for oocyte retrieval (Sharma *et al.*, 1996; Wani *et al.*, 1999; Priscilla, 2001). Since oocytes remain firmly attached to cumulus granulosa, before maturation in small and medium sized follicles, they were not easily obtained by aspiration, but pressure exerted by puncturing could hasten the recovery rate (Das *et al.*, 1996a).

2.3.3. Oocyte Yield per Ovary

For *in vitro* fertilization and *in vitro* production of embryos from a slaughtered animal, an efficient oocyte recovery method is absolutely essential. Commonly employed techniques for harvesting oocytes from abattoir derived ovaries are aspiration, slicing and puncture. Various scientists obtained different ranges like 3-10, (Katska and Smorag, 1984; Mermillod *et al.*, 1992; deOliveira *et al.*, 1994; Iwasaki *et al.*, 1987; Baruha *et al.*, 1998; Priscilla, 2001) 11-15 (Takagi *et al.*, 1989; Hamno and Kawayama, 1993; Moreno *et al.*, 1993; Carolan *et al.*, 1994; Arlotto *et al.*, 1996) and >20 COCs (Stringfellow *et al.*, 1993; Thonon *et al.*, 1993) from cattle ovaries by employing aspiration technique.

Number of COC's obtained by slicing method ranged between 5-10 (Priscilla, 2001), 10-15 (Rieger and Loskutoff, 1994; Arlotto *et al.*, 1996), 15-25 (Iwasaki *et al.*, 1987; Sato *et al.*, 1990), 25-50 (Takagi *et al.*, 1998; Carolan *et al.*, 1994), 50-100 (Carolan *et al.*, 1992; Hamno and Kuwayama, 1993) and more than 100 (Xu, *et al.*, 1992), from bovine ovaries.

When puncture method was employed for oocyte retrieval 3-10 oocyte per ovary was obtained for Sato *et al.*, (1990) and Priscilla (2001). When puncture method was performed in buffaloes Das *et al.* (1996a) obtained 2.6 oocyte per ovary. Das *et al.* (1996_b) and Das *et al.* (1996c) obtained 4.14 and 11.13 oocyte per ovary in goat and sheep respectively by puncture method.

Iwasaki *et al.* (1987), Takagi *et al.* (1998), Carolan *et al.* (1994), Hamno and Kuwayama (1993) and Priscilla (2001) obtained maximum oocytes in slicing method than aspiration method in cattle. Similar observations were made by Das *et al.* (1996_a), Gupta and Sharma (2001) in Buffaloe. Sato *et al.* (1990) and Priscilla (2001) obtained more oocyte from cattle by slicing than puncture. Similar finding in buffaloe was reported by Das *et al.* (1996_a). Comparison of aspiration and puncture had shown that aspiration yielded more oocytes than puncture in cattle (Priscilla, 2001). But Das *et al.* (1996_a) obtained more oocyte by puncture method than aspiration method in buffaloe. Oocyte recovery rate was low in buffaloes when compared to cattle (Das *et al.*, 1996_a and Gupta and Sharma, 2001). The low recovery of oocyte per ovary in buffaloe can be attributed to the low average number of primordial follicles in the ovaries (Totey *et al.*, 1992).

2.4. PRE MATURATION PROCESSING

2.4.1 Rinsing of Oocytes

Rinsing of oocytes in fresh drops of medium was performed, because by-products of the recovery process such as blood, urine and follicular fluid, which can impair development of reproductive cells can be eliminated (Boone and

Shapiro, 1990). Identified oocytes in the retrieval media should be rinsed separately in medium like PBS, (Dominguez, 1995; Romero-Arredondo and Seidel, 1996). TCM-199 with 0.3 per cent BSA, 0.2 mM Pyruvic acid and 50 µg/ml Gentamycin sulphate (Abdoon *et al.*, 2001) or TL-HEPES medium with 3 mg BSA/ml and antibiotics (Long *et al.*, 1994). 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).

It was very important to keep the interval short between isolation of oocytes from follicular fluid and placing them in maturation drops (Romero-Arredondo and Seidel, 1996).

2.4.2 Classification of Cumulus Oocyte Complexes (COCs)

COCs are usually classified under stereomicroscope with 40X – 60X magnification (Dominguez, 1995). Most predictive morphological criteria for selection of COCs continue to be an intact cumulus cells and homogeneous cytoplasm (Abdoon *et al.*, 2001).

2.4.2.1 Cumulus Cell Character

In most of the IVM/IVF studies, COCs with more than 3-4 layers of unexpanded, dense and compact cumulus cell layers, completely surrounding oocytes were considered as good quality and healthy and hence selected for further processing. (Romero-Arredondo and Seidel (1996); Tatemoto and Terada., 1996; Ikeda *et al.*, 2000; Cetica *et al.* 2002; Vassena *et al.*, 2003; Lequarre *et al.*, 2005). Blondin *et al.* (1997) aspirated COCs with 18 g needle, who obtained mostly COCs with more than 5 layers of cumulus mass (90 per cent) of this 49 per cent was contributed by oocyte with slight granulation of ooplasm and also procured five per cent COC with only corona radiata and 4 per cent denuded oocytes.

Datta and Goswami (1999) classified buffaloe COCs obtained by aspiration of follicles as good with a compact cumulus mass and having evenly

granulated ooplasm. Those with only few layers of cumulus, often not completely encircling oocytes were graded as average and those oocytes with little cumulus mass and pyknotic ooplasm were graded as poor quality oocytes. In good quality oocytes, 77.27 per cent were observed to have M II plates, but in average and poor quality oocytes, this was 60.00 per cent and 22.38 per cent respectively.

deWit and Kruip (2001) classified bovine COC based on their morphology and level of atresia as A, B1, B2, B3 and C class. A class COCs were with compact cumulus investment and glassy look. B1 class was having uniform cell density for cumulus mass. In B2 class, uniform cell density was there, but corona radiata appeared to dissociate from rest of cumulus investment. B3 class had almost black cumulus and corona radiata almost completely dissociated from rest of cumulus investment with dark spots of degenerated cells. After fertilization and embryo culture for 9 days morulae and blastocyst yield from A, B1, B2, B3 and C class were 13.9 per cent, 14.7 per cent, 17.4 per cent, 19.1 per cent and 11.5 per cent respectively, indicating that A, B1, B2 and B3 classes were very good for *in vitro* embryo production.

2.4.2.2 Ooplasm Character

Oocytes with condensed and vacuolated ooplasam were considered abnormal (Dominguez, 1995). Oocyte with even, smooth and finely granulated ooplasm was having highest developmental competence (Vassena *et al.*, 2003).

Ooplasm shrunken away from zona pellucida and not evenly filling zona, ooplasm vacuolated, fragmenting or just left in remnants are all signs of degeneration of oocyte (Leibfried and First, 1979). In IVM/IVF studies majority of scientists selected oocytes with homogenous and evenly granulated cytoplasm (Romero-Arredondo and Seidel, 1996; Tatemoto and Terada, 1996; Ikeda *et al.*, 2000; Lequarre *et al.*, 2005).

2.4.3 Effect of Retrieval Systems on Oocytes Quality

2.4.3.1 A class Oocytes

Oocytes with multiple layers of cumulus cells (>5) and uniform granulation of ooplasm were considered as class A oocytes. Retrieval methods like aspiration, slicing and puncture were adopted for COC recovery.

By resorting to aspiration technique, Madison *et al.* (1992) and Carolan *et al.* (1994) obtained 20-30% of A class oocytes. Iwasaki *et al.* (1987), Arlotto *et al.* (1990) and Hamno and Kuwayama (1993) obtained 38-41 per cent A class oocytes by the same method. Katska (1984) and Katska and Smorag (1984) obtained 45-50% oocytes with compact and multiple layers of cumulus cells by employing aspiration technique. A class oocytes obtained by aspiration method was 64.40% for Priscilla, 2001.

Percentage of A class oocytes varied between 35-40% when aspiration was performed in buffaloe (Naik *et al.*, 1999), goat (Gogoi *et al.*, 2001) and sheep (Wahid *et al.*, 1992).

Number of oocytes yielded per bovine ovary by practicing aspiration technique ranged between 2-4 (Iwasaki *et al.*, 1987; Priscilla, 2001) 4-5 (Katska and Smorag, 1984 and Carolan *et al.*, 1994).

By resorting to slicing method for oocyte recovery from bovine ovaries, Carolan *et al.* (1994) obtained 38.7% A class oocytes. Whereas Priscilla (2001) obtained 61.53% and Hamno and Kuwayama (1993) obtained 84.3% A class ($\geq$ 4-5 cumulus layers) oocytes by adopting same method.

Average number of A class oocyte per ovary ranged between 2-4 when slicing was employed for oocyte recovery (Priscilla, 2001 and Goswami *et al.*, 2004). When Carolan *et al.* (1994) sliced ovaries for oocyte recovery, 17.1 A class oocyte per ovary was obtained.

By puncturing method 62.84% oocytes were of Grade A oocytes in bovine (Priscilla, 2001) and 54.7% in sheep ovaries (Wani *et al.* 1999).

Das *et al.* (1996_b) and Das *et al.* (1996_c) yielded 34.47% and 19.59% good quality oocytes from sheep and goat ovaries respectively. By puncturing method Priscilla, (2001) obtained 2.3 A class oocyte per ovary in cattle. Wani *et al.* (1999) in sheep and Das *et al.* (1996a) in buffaloe obtained 5.2 and 0.4 A class COC per ovary respectively by puncture method. Puncturing of goat ovaries recovered 0.57 A class oocytes per ovary (Pawshe *et al.*, 1994).

Carolan *et al.* (1994) yielded proportionately more A class oocytes by slicing method. Hamno and Kuwayama (1993) obtained significantly higher number of A class oocytes by slicing method than aspiration method. Priscilla (2001) observed no significant difference between aspiration slicing and puncture in yield of A class oocytes.

Number of A class oocyte obtained per ovary was significantly high for Carolan *et al.* (1994) by slicing method than aspiration method. Gupta and Sharma (2001) obtained more oocyte per ovary by slicing method than aspiration technique. Same result was obtained for Priscilla (2001) and number of oocytes per ovary was almost similar by aspiration and puncture technique.

2.4.3.2 B class Oocytes

Oocytes with 3-5 layers of cumulus cell layers were considered as B class oocytes. When aspiration technique was performed, Carolan *et al.* (1994) obtained 18% and Priscilla (2001) obtained 13.77% B class bovine oocytes. Gogoi *et al.* (2001) obtained 9.16% B class goat oocytes. Aspiration yielded 32-42% B class oocytes from bovine ovaries (Moreno *et al.*, 1993) and buffaloe ovaries (Naik *et al.*, 1999).

By aspiration technique, Carolan *et al.* (1994) obtained 2.5 B class bovine oocyte per ovary and Priscilla (2001) obtained 0.57 B class bovine oocytes.

A proportion of 7% and 19.47% oocytes were of B class when oocytes were recovered by slicing method from bovine ovaries by Carolan *et al.* (1994) and Priscilla (2001) respectively.

Priscilla (2001) and Goswami *et al.* (2004) yielded 1-2 B class oocytes per ovary by slicing method. Whereas Carolan *et al.* (1994) obtained 3.1 B class oocyte per ovary by slicing of bovine ovaries.

By puncture method, Priscilla (2001) obtained 15.78% B class oocytes from cattle. 25-30% B class oocytes were yielded from goat and sheep ovaries by puncture method for Das *et al.* (1996_b) and Das *et al.* (1996c) respectively. Puncture of ovaries yielded 0.58 B class COC per ovary (Priscilla, 2001). Puncture of goat ovaries yielded 0.75 fair quality (B class) oocytes (Pawshe *et al.*, 1994). Puncture of ovaries in buffaloe yielded 0.9 B class COC per ovary (Das *et al.*, 1996a).

2.4.3.3 C class Oocytes

Aspiration was performed in bovine ovaries and obtained 8-13% C class COCs (Katska, 1984; Katska and Smorag, 1984; Carolan *et al.*, 1994 and Priscilla, 2001). Gogoi *et al.* (2001) yielded 10.69% C class oocytes from goat ovaries.

Number of C class oocytes or oocytes with remnants of cumulus cells per ovary was 0.3-0.9 for Katska and Smorag (1984) and Priscilla (2001). Moreno *et al.* (1993) and Carolan *et al.* (1994) obtained 1.7- 4.4 C class oocytes/ovary.

Priscilla (2001) when employed slicing technique yielded 10.12% C class oocytes, whereas Carolan *et al.* (1994) yielded 17.0% C class oocytes.

Slicing of bovine ovaries yielded 0.4-0.6 C class oocytes for Priscilla. (2001) and Goswami *et al.* (2004). But for Carolan *et al.* (1994) yield was substantially large (7.5/ovary).

Puncture method when adopted for recovery 9.03% of oocytes were of C class for Priscilla (2001) in cattle and 21.5% for Wani *et al.* (1999) in sheep.

Oocyte yield per ovary was 0.33 and 2.0 per ovary for Priscilla (2001) and Wani *et al.* (1999) in cattle and sheep respectively when puncture method was adopted.

2.4.3.4 D class Oocytes

Exploitation of aspiration technique in bovine ovaries resulted in 7-14% denuded/D class oocytes (Katska and Smorag, 1984; Priscilla, 2001; Vassena *et al.*, 2003). Whereas Carolan *et al.* (1994) and Moreno *et al.* (1993) obtained 20-27% D class oocytes. Aspiration yielded 16-17% D class oocytes in buffaloe (Naik *et al.*, 1999) and goat (Gogoi *et al.*, 2001).

Technique of aspiration recovered 0.5-0.9 D class bovine oocytes for Katska and Smorag (1984) and Priscilla (2001). Moreno *et al.* (1993) and Carolan *et al.* (1994) obtained 1.7-3 denuded COC/ovary by aspiration technique. Recovery of denuded oocytes upto 6.2/ovary was reported in bovines (Katska,1984).

Practice of slicing of bovine ovaries by Carolan *et al.* (1994) and Priscilla (2001) resulted in 11.5% and 8.58% denuded/D class oocytes.

Priscilla (2001) and Goswami *et al.* (2004) recovered 0.5-0.9 denuded/D class oocytes per bovine ovary by employing slicing technique. With the same technique, Sato *et al.* (1990) and Carolan *et al.* (1994) yielded 3-5 denuded/D class oocytes from bovines.

By adoption of puncture method D class oocytes were recovered at the rate of 12.33% in bovine (Priscilla, 2001) and 38.83% in goat (Das *et al.*, 1996_b)and 23.8 and 49.48% for Wani *et al.* (1999) and Das *et al.* (1996c) in sheep respectively.

Puncturing of bovine ovaries recovered 0.45 denuded oocyte per ovary (Priscilla, 2001). Sato *et al.* (1990) and Wani *et al.* (1999) recovered 2 denuded oocyte per ovary by puncture method in cattle and sheep respectively. Pawshe *et al.* (1994) and Das *et al.* (1996a) when performed puncture method obtained 0.9-1.3 denuded oocyte per ovary in goat and buffaloe respectively.

2.4.3.5 Culture Grade Oocytes

Oocytes with multiple layers of cumulus cells (≥3) were included in culture grade of cumulus oocyte complexes. Moreno *et al.* (1993) by adopting aspiration method 25-35 per cent of oocytes recovered were of this class. Whereas a recovery rate of 40-57 % was obtained for Katska (1984), Dominguez (1995) in bovine and for Naik *et al.* (1999) in buffaloes. Vassena *et al.* (2003) when employed aspiration technique 62% of COCs where of culture grade. Hamno and Kuwayama (1993) observed that more than 90% of COCs obtained were of A and B class when aspiration method was adopted for oocyte harvesting.

By adopting aspiration method Moreno *et al.* (1993) obtained 1-4 cumulus oocyte complexes with 3 or more layers of cumulus from bovines. Katska and Smorag (1984) obtained 4-5 bovine oocytes with multiple layers of cumulus. Takagi *et al.* (1992) obtained a range of 3-37 multilayered bovine oocytes and an average of 17.8 oocyte per ovary.

When slicing method was used for oocytes recovery, Takagi *et al.* (1992) obtained 4-38 oocyte per ovary when A and B groups were combined and the average being 14.3 oocyte per ovary. Whereas Sato *et al.* (1990) obtained 12.3 multilayered oocyte per ovary.

Sato *et al.* (1990) when employed puncture method obtained eight oocyte per ovary with multiple layers of cumulus cells.

Das *et al.* (1996_a) reported that number of pooled culture grade oocyte/ovary was very high after slicing than puncture or aspiration, and largest

recovery of morphologically poor oocytes was by slicing due to recovery of oocytes from a heterogeneous population of follicles. Pawshe *et al.* (1994) in goats observed that most of the oocytes recovered by aspiration method were of poor quality. Recovery of good quality oocytes with more than four layers of cumulus cells occurs significantly more in slicing method than aspiration or puncture method.

Dominguez (1995) opined that larger follicles were more in number in European breeds than zebu or crossbred cattle. Cows with poor body condition exhibited less number of follicles. Factors like age, presence of subclinical disease, managemental factors, reproductive disease etc. affect follicles and oocytes.

2.5 FACTORS CONTROLLING *IN VITRO* MATURATION

2.5.1 Maturation Media

Maturation is carried out in complex tissue culture media with addition of gonadotrophins, steroid hormones, pyruvate and serum of various sources, Growth factors and antibiotics. Complex tissue culture media like TCM-199, Ham's F-10, Ham's F-12, Minimum Essential Medium-α (MEM-α), RPMI – 1640, Waymouth medium etc. were commonly used for maturation of oocytes. Among these, TCM-199 was most widely used. 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, 2002).

2.5.1.1 TCM-199

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). Two different buffering agents like bicarbonate and HEPES were used in TCM-199. Either bicarbonate buffered TCM (Bilodeau *et al.*, 1993; Neglia *et al.*, 2003) or HEPES buffered TCM-199 (Hashimoto *et al.*, 1998; Liu *et al.*, 1999) were used for *in vitro* maturation of bovine oocytes. TCM-199 is a

complex culture medium. It contains vitamins, amino acids, purines, and other substances mainly in the concentrations found in serum (Gordon, 2003).

2.5.1.2 Ham's-F-10 medium

Bavister *et al.* (1992) used Ham's-F-10 medium for maturation of bovine oocytes. Ham's-F-10 medium was used in many Indian works for maturation of buffaloe oocytes (Totey *et al.*, 1993a; Totey *et al.*, 1993b; Totey *et al.*, 1996).

2.5.1.3 Other Tissue Culture Media

In addition to TCM-199 and Ham's F.10 media (Bavister *et al.*, 1992) used many other media like Ham's F-12, SFRE-199-2 medium, Eagles minimum essential medium α (MEM-α), MEM-α with deoxy and ribonucleosides. Waymouth medium MB 752/1 for the IVM of bovine oocytes. They obtained 76-82 per cent cleavage rate in SFRE, TCM-199 and three MEM containing media. But in Waymouth and Ham's-F-12 it was 50 per cent and 35 per cent respectively. In all media except Ham's-F-12 and Waymouth media, after IVF 25-32 per cent zygotes developed to morulae stage and 12-19 per cent developed to Blastocyst stage. In Waymouth and Ham's-F-12 media the yield of morulae was 14 and 3 per cent and blastocyst was 3 and 1 per cent respectively suggesting that these media were less suitable for IVM/IVF. Lim *et al.*, 1999 performed IVM in modified Tyrode's medium and obtained in 37-74 per cent oocytes matured to MII stage.

2.5.2 Major Components of Maturation Media

2.5.2.1 Hormones and Growth Factors

Hormones like FSH, LH, Growth hormone, Estrogen and growth factors like EGF, IGF-I, TGF-α can influence the *in vitro* maturation of bovine oocytes (Bevers *et al.*, 1997). Hormonal stimulation of oocytes during the course of *in vitro* maturation was of paramount importance in achieving nuclear and

cytoplasmic maturation, which was essential for preparation of oocytes for fertilization (Sachan *et al.*, 1999).

2.5.2.1.1 Follicle Stimulating Hormone

Addition of Follicle stimulating hormone alone in maturation medium resulted in higher rate of *in vitro* maturation (Sachan *et al.*, 1999). FSH from different sources like bovine FSH, (Long *et al.*, 1994), Ovine FSH (Chian *et al.*, 1996; He *et al.*, 1997) Porcine FSH (Armstrong *et al.*, 1996; Calder *et al.*, 2001) and recombinant bovine FSH (Barnes *et al.*, 1993) were used for *in vitro* maturation of bovine oocytes. Bevers *et al.* (1997) reviewed and reported that concentration of FSH and LH in most of the IVM protocols varied between 1-10 μg/ml. In most of the *in vitro* maturation studies the concentration of FSH used was in the range of 0.5 – 1 μg/ml (Kobayashi *et al.*, 1994; Chian *et al.*, 1996; He *et al.*, 1997; Calder *et al.*, 2001; Dode and Adona, 2001. Long *et al.* (1994) and Azambuja *et al.* (1998) used FSH at the rate of 0.01 NIH units/ml. Barnes *et al.* (1993) used recombinant bovine FSH at the rate of 45 μg/ml.

Gupta *et al.* (2001) reported that among gonadotrophins, FSH was most essential for cumulus expansion and maturation *in vitro*. But Pawshe *et al.*, 1996 opined that FSH enhanced early embryonic development rather than meiotic maturation. When FSH was added to *in vitro* maturation medium, it appeared to stimulate cumulus cells of oocyte cumulus complex to secrete a positive factor that could over ride arrest due to hypoxanthine and that could trigger meiotic resumption. In addition, FSH stimulated an increase of cAMP concentrations and cumulus expansion (Choi *et al.*, 2001).

2.5.2.1.2 Luteinizing Hormone (LH)

LH was having significant effect on COCs to produce maturation of oocytes. Addition of LH in medium for IVM enhanced the quality of oocytes that was reflected in increased embryo yield after *in vitro* fertilization and *in vitro* culture (Pawshe *et al.*, 1996).

Luteinizing hormone used in *in vitro* maturation of bovine oocytes were from various sources like bovine LH (He *et al.*, 1997; Chian *et al.*, 1996) Ovine LH (Calder *et al.*, 2001), Purified porcine LH (Armstrong *et al.*, 1996), recombinant human LH (Gordon, 2003) and recombinant bovine LH (Barnes *et al.*, 1993). LH was regularly used in *in vitro* maturation medium for bovine oocytes at a concentration of five μg/ml (Long *et al.*, 1994; Kobayashi *et al.*, 1994; Gasparrini, 2002; Neglia *et al.*, 2003). Long *et al.*, 1994 observed 90 per cent M II rate and Gasparrini (2002) obtained 94 per cent maturation rate when LH was used at 5 μg/ml rate in maturation media.

Gupta *et al.* (2001) suggested that combined action of FSH and LH on cumulus cells to synthesize pyruvate, stimulated TCA cycle and thus led to increased production of ATP for the energy requirement of the oocyte. LH surge activated adenylcyclase (AC) promoting a rapid rise in cAMP and stimulate rapid increase in intracellular calcium in granulosa cumulus cells through activation of phospholipase-C, evoking a rapid turnover in phosphoinositol hydrolysis and thus the production of inocitol triphosphate. Intra cellular calcium mobilization was followed by a calcium influx from extracellular environment. This increase in calcium triggers a series of changes resulting ultimately in germinal vesicle breakdown (GVBD) (van den Hurk and Zhao, 2005).

2.5.2.1.3 Estradiol 17-β

Estradiol had a significant role in cytoplasmic maturational changes that was necessary for fertilization and early post fertilization development (Bevers *et al.*, 1997). Estradiol was added at a concentration of 1 μg/ml in *in vitro* maturation media of bovine oocytes (Barnes *et al.*, 1993; Lim *et al.*, 1999; Gasparrini, 2002; Neglia *et al.*, 2003), which was comparable to concentration in follicular fluid of preovulatory follicles shortly after LH peak. Gasparrini (2002) obtained 94 per cent maturation rate and Liu *et al.* (1999) obtained 88.9 per cent M II rate when this concentration of estradiol was used in maturation medium.

2.5.2.1.4 Growth Factors

Growth factors were agents that can enhance the efficiency of maturation. Major growth factors that can enhance the oocyte maturation was Epidermal Growth Factor (EGF), Insulin Like Growth Factor (IGF-I), Transforming Growth Factor-α (TGF-α) and Bovine Fibroblast Growth Factor (bFGF). When these growth factors were added to medium more than 90 per cent of oocytes exhibited cumulus expansion (Kobayashi *et al.*, 1994). He reported that EGF and TGF-α stimulated cumulus expansion and oocyte fertilizability in maturation medium. Avery *et al.*, 2000 added EGF to maturation medium at a concentration of 50 ng/ml and obtained a cleavage rate of 71-79 per cent. Kobayashi *et al.*, 1994 added EGF (10 ng/ml), TGF-α (10 ng/ml) and bovine FGF (10 ng/ml) separately to maturation media and obtained cleavage rate of 70.3 per cent with EGF, 73.6 per cent with TGF-α and 40 per cent with bovine FGF.

Khatir *et al.*, 1998 used 10 ng EGF/ml of TCM-199 for cow oocyte maturation and obtained MII plates and polar body extrusion in 86 per cent of oocytes after 24 h culture. Gupta *et al.*, 2002 reported positive effect of EGF on buffaloe oocytes. When it was added to TCM-199 with 10 per cent FCS at the rate of 20 ng/ml, resulted in improved cumulus expansion (74 per cent) and maturation rate (80 per cent). Lorenzo *et al.*, 1994 reported that EGF in absence of hormone or serum stimulated resumption of meiosis. EGF stimulated oocyte maturation like LH by disrupting it's communication with cumulus cell and it created a positive maturation signal. Bevers *et al.*, 1997 reviewed the induction of cumulus expansion and promotion of nuclear maturation by EGF in several species including cow and sow. Lorenzo *et al.*, 1994 reported both EGF and IGF-I alone or together stimulated nuclear maturation in immature bovine oocytes and opined that their beneficial effect was mediated through cumulus cells.

2.5.2.2 Serum

It was common practice to include macromolecular constituents from various sources like Foetal Calf Serum (FCS), Estrous Cow Serum (ECS), Steer Serum (SS) or Bovine Serum Albumin to various cell culture media. Serum was known to contain a wide range of compounds including hormones, growth factors, amino acids and binding proteins (Gordon, 2003). Mikkelsen (2004) reported that serum contain growth factors such as epidermal growth factor and IGF-I, which were important for cytoplasmic maturation. Raghu *et al.* (2002) reported that serum prevents sticking of oocytes to syringe surface and needle. Gasparrini (2002) opined that serum prevents zona hardening and thereby improves the ability of oocytes to be fertilized.

Pawshe *et al.* (1996) reported that FCS was necessary for FSH induced cumulus expansion. Estrous cow serum was not having any advantage over FCS. FCS was superior to BSA in promoting *in vitro* maturation and fertilization of bovine oocytes. BSA-V at all concentrations in maturation media reduced the developmental rate to the blastocyst stage.

2.5.2.3 Pyruvate and Aminoacids

Pyruvate had physiologically important role in the IVM of bovine oocytes. Cumulus cells metabolize glucose to pyruvate or Krebs cycle intermediates, that can be passed to the oocyte and enhance it's quality (Geshi *et al.*, 2000). Nagai (2001) reported that without cumulus cells cattle oocyte require sodium pyruvate for nuclear maturation in a protein free medium. Sodium pyruvate was commonly added to IVM medium at the rate of 0.2 mM (Barnes *et al.*, 1993; Silva and Knight, 1998; Avery *et al.*, 2000).

Another common ingredient in IVM medium was aminoacids like cysteine, glutamine, taurine, glycein etc. Ali *et al.*, 2003 added cysteine, which could be metabolized by cells during IVM and IVC in defined conditions containing low glucose and improved bovine embryo development.Addition of

cysteamine, cysteine and β-mercapto ethanol to maturation medium increased glutamine synthesis in bovine oocytes during *in vitro* maturation (de Matos *et al.*, 1997). Glutathione was the major non-protein sulphhydryl compound in mammalian cells and played an important role in protecting the cell from oxidative damage. Glutamine added at the rate of 0.68 mM (Choi *et al.*, 2001) or 0.4 mM (Bols *et al.*, 1996; Silva and Knight, 1998) in the maturation media. Silva and Knight (1998) obtained 54-60 per cent cleavage rate. Abdoon *et al.* (2001) added glutamine, taurine, Glycein to the culture medium and proved beneficial for developing bovine embryos. These amino acids could be acting as energy substrates, pH regulators or as a pool for *de novo* protein synthesis.

2.5.3 Culture Environment

2.5.3.1 Temperature

Until 1980's research studies in cattle IVM were carried out at a temperature of 37°C. Since late 1980's however most laboratories carried out IVM of cattle oocytes at 38-39°C as this was close to rectal temperature of cow. But preovulatory follicles were 2°C cooler than their adjacent stroma in cow. So it was suggested that lower temperature was beneficial during IVM in cattle (Gordon, 2003).In the last decade most of the workers were using a temperature range of 38.5 to 39°C for cattle IVM (Barnes *et al.*, 1993; Long *et al.*, 1994; Avery *et al.*, 2000; Neglia *et al.*, 2003). Shi *et al.* (1998) reported that when bovine oocytes were matured at 36°, 38°, 39° and 40° optimal embryo development was achieved between 38°C and 39°C. The temperature changes studied did not affect nuclear maturation.

2.5.3.2 Gas Phase

Optimal gas phase for IVM oocytes were 2.5-5 per cent CO_2 and 20 per cent oxygen (Suzuki *et al.*, 1999; Gordon, 2003). Barnes *et al.* (1993), Long *et al.* (1994) and Kobayashi *et al.* (1994) used 5 per cent CO_2 in atmospheric air for *in vitro* maturation and obtained good results. Khatir *et al.* (1998) used 5 per cent

CO_2 for IVM and obtained 86 per cent oocytes with MII and polar body extrusion on 24 h of IVM.

2.5.3.3 Humidity

Humidified air was used for *in vitro* maturation purpose (Azambuja *et al.*, 1998). Chian *et al.* (1996); de Matos *et al.* (1997) used maximum humidity in maturation environment. Maximum humidity is used in order to prevent medium evaporation (Gordon, 1994).

2.6 PHYSIOLOGY OF OOCYTE MATURATION

2.6.1 Physiologic Role of Cumulus Cells in Oocyte Maturation

Buccione *et al.* (1990) reviewed that in the antral follicles two specific populations of granulose cells existed-one was cumulus granulose and the other mural granulose. Cumulus granulosa organized as a pseudo stratified epithelium that encloses oocytes. Mural granulose comprising the inner layer of follicle wall and form two pseudo stratified epithelium in contact with the basal lamina, which was adjacent to the external theca.

Hoshi *et al.* (2001) cumulus cells are potentially important in the acquisition of full developmental competence during oocyte maturation. In absence of cumulus cells oocyte exhibit no maturation or reduced maturation (Kobayashi *et al.*, 1994). Rieger and Loskutoff (1994) reported that cellular process involved in GVBD and late diakinesis were especially dependent on cumulus cells. Hashimoto *et al.* (1998) demonstrated that proper cumulus cell density (1.6 to 3.2 x 10^6 cells/ml) during oocyte maturation was essential for continuous development of corona-enclosed oocytes.

2.6.1.1 Gap Junctions

Corona radiata cells and part of the cumulus cells surrounding oocyte, penetrate through the zona pellucida and communicate with oocyte via the gap junctions. These intracellular communications allow metabolite transfer as

molecules of low molecular weight and nutrition to the oocyte, which play a vital role in oocyte growth and maturation (Buccione *et al.*, 1990; Armstrong *et al.*, 1996; de Matos, 1997; Datta *et al.*, 1999; Geshi *et al.*, 2000).

Bols *et al.* (1996) reported that surrounding cumulus cells were connected to the oocyte via canals in the zona pellucida. This close connection was important for nutrition and growth of the oocyte. Presence of intact cumulus cells for at least 12 h was necessary for normal cytoplasmic maturation.

Atef *et al.* (2005) reported that high developmental rate of bovine oocytes could be achieved with a short exposure to recombinant human FSH. This was believed to be mediated through gap junctions as developmental competence of oocyte was compromised by the inhibition of their function.

Connexin 43 (Cx43) plays an important role in inter cellular communication mediated through gap junctions. Gap junctions are composed of proteins belonging to Connexin (Cx) family and can mediate the exchange of metabolites between neighbouring cells. It is important that gap junctional network is essential for the induction of oocyte meiotic maturation (Gordon, 2003).

2.6.1.2 Hormones and Growth Factors

IGF-II with FSH synergistically enhanced DNA synthesis, protein synthesis and steroidogenesis in the presence of granulose cells. The synergistic effect was mainly caused by increase of IGF-II receptors in granulose cells by FSH (Pawshe *et al.*, 1998).

Receptors for EGF have been demonstrated in bovine cumulus and small antral granulosa cells and the number of EGF binding site has been reported to be influenced by gonadotrophins. EGF exerted its stimulatory effect by binding to granulose cells since this phenomenon was seen only in cumulus-enclosed oocyte not in denuded oocyte (Chauhan *et al.*, 1999b).

Guoliang *et al.* (1994) reported that oocyte cumulus connections were crucial as far as initiating production of a meiosis inducing substance was concerned. Oocyte cumulus connections were not needed for transferring these substances to the oocyte. Another important substance needed for maturation, and transported through cumulus cell junction was glutathione.

Byskov *et al.* (2002) reported that junctions between the cumulus cells and the oocyte remain intact until shortly after GVBD. This indicated that the cumulus cells were needed for producing maturation promoting factor (MPF).

Chauhan *et al.* (1997) reported that beneficial effect of foetal bovine serum on oocyte maturation was mediated through cumulus cells and FBS contain gonadotropins, oestrogen and a number of growth factors like EGF and TGF-α.

Bilodeau *et al.* (1993) reviewed that when cAMP was high in COC, cAMP content was also high in oocytes derived from these complexes. They reported increase of cAMP in cumulus cells could activate a stimulatory signal for maturation and that their signal could overcome the inhibitory effect of high content of cAMP in oocyte itself. They argued that increasing cyclic AMP in cumulus cells induces maturation by promoting oocyte cAMP hydrolysis.

Staigmiller and Moor (1984) reported that granulose cells provide energy substrates to oocyte, some amino acids, nucleotides and phospholipid precursors to the oocyte, that generate some interactional signals which influence the nucleus and direct the synthesis of certain structural proteins and maturation specific proteins.

2.6.1.3 Fertilization and Cleavage

Abdoon *et al.* (2001) reported essential role of cumulus cells in promoting normal cytoplasmic maturation of oocytes necessary for fertilization, cleavage and subsequent development of buffaloe embryos.

Bols *et al.* (1996) reported that the cumulus improved fertilization by providing a capacitation inducing mechanism, hence the removal of cumulus cells even after maturation but before fertilization decreased developmental potential substantially. The rate and degree of polyspermy was significantly increased in denuded oocytes. Kane (2003) reported that *in vitro* matured oocytes collected by ovum pick-up procedures and from slaughterhouse material, show a high percentage of cleavage and blastocyst development after IVF when four or more layers of cumulus cell surrounded it.

2.6.2 Physiologic Role of Ooplasm in Meiotic Progression and Cumulus Expansion

Oocyte acquire meiotic competence when it had accumulated cell cycle regulating molecules in sufficient amount to enable resumption of meiosis (Armstrong, 2001). The oocyte nuclear maturation programme was automatically turned on after removal of oocyte from its follicle. The resumption of meiosis induces a transcription arrest and immediately limited the oocyte molecular programming required to go through the maternal to zygote Transition (MZT) i.e., the process of embryonic genome taking the control of early embryonic development from oocyte genome (Sirard, 2001). He also reported that ability of an oocyte to become an embryo depends on the accumulation of specific information in the form of mRNA and protein.

Miyano (2003) reported that cell cycle regulatory molecules such as Cdc2 kinase and MAP kinase (Mitogen activated protein kinase) controlled meiotic maturation in mammalian oocyte. Cdc2 kinase composed of a regulatory subunit cyclin B and a catalytic subunit P34Cdc2, and had been found to be a key regulator of G2/M transition in mitosis as well as meiosis. It was activated at the onset of oocyte maturation and its activity peaks at metaphase I and Metaphase II. MAP kinase was phosphorylated and activated oocyte maturation in cattle.

2.7 ASSESSMENT OF OOCYTE MATURATION

In most mammalian species, some hours before rupture of the follicle and ovulation, the fully grown oocyte resumes meiosis, progressing from prophase of 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 (Gordon, 1994). Oocyte maturation involves both cytoplasmic and nuclear maturation (Mikkelsen, 2004).

2.7.1 Cytoplasmic Maturation

van den Hurk and Zhao (2005) opined that maturation was required to acquire the conditions to block polyspermy in case of fertilization and the 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.

Gasparrini (2002) and Kruip *et al.* (2005) reported that changes associated with maturation happening in 1-8 h of LH peak in oocyte *in vivo* and *in vitro* included formation of periviteline space with loss of contact between the cumulus cell and the oocyte, and roughening of nuclear membrane. These changes followed by resumption of meiosis marked by GVBD, disappearance of rough endoplasmic reticulum (RER), and formation of clusters of mitochondria in association with lipid droplets and smooth endoplasmic reticulum (SER). Period from 8-19 h was characterized by intensive clustering of mitochondria in association with lipid droplets and elements of SER and appearance of ribosomes in cytoplasm. After 19 h polar body extruded, mitochondria dispersed, and majority of organelles located towards centre of the cell. Relatively organelle free cortical region contained cortical granules immediately adjacent to the plasma membrane together with aggregates to tubular SER.

Mizushima and Fukui (2001) stated that the concentration of intracellular glutathione (GSH) after IVM might be a valuable marker to assess the degree of

cytoplasmic maturation in pig and bovine oocytes. The GSH was a major nonprotein sulphhydryl compound in mammalian cells. The concentration of intracellular GSH increased as cytoplasmic maturation was completed.

2.7.2 Nuclear Maturation

Nuclear maturation refers to acquisition of the ability to undergo dissolution of germinal vesicle (GV) (nuclear membrane), condensation of chromosomes, release of 1st polar body and subsequent arrest at metaphase II (Rodriguez and Farin, 2004). Oocytes were classified as GV stage or at intermediate stage (GVBD, chromosome condensation, MI) or mature (Anaphase I, Telophase I and metaphase II) (Bilodeau *et al.*, 1993).

van den Hurk and Zhao (2005) reported that nuclear maturation last about 24 h in cow and sheep, about 44 h in pig and about 36 h in horse and comprises several steps, that include 2 consecutive divisions (M-phase) in the absence of DNA replication. Oocytes then become arrested at MII until fertilization, when an active stimulus provided by sperm penetration triggers completion of meiotic cycle and initiates embryonic development. Wehrend and Meinecke (2001) reported that nuclear maturation was characterized by chromosome condensation (CC), nuclear envelope dissolution (GVBD) spindle assembly and chromosome separation.

2.7.2.1 Meiotic Arrest or GV Stage

Khatir *et al.* (1998) reported that oocytes isolated from follicles and immediately fixed contained a GV in all cases (100 per cent). Presence of darkly stained nucleus with very distinct and intact nuclear membrane was observed in GV stage (Datta *et al.*, 1999). Aktas *et al.* (2003) opined that maintenance of meiotic arrest was regulated by the interplay of cAMP, cumulus and granulosa cells. Buccione *et al.* (1990) reported the meiosis arresting effect of the follicular environment being probably mediated by the passage of substances from granulose cells that comprise thin follicle wall to oocyte through the gap

junctions. Cyclic AMP and other purines such as hypoxanthine, guanosine, adenosine, cyclic guanosine peptide known as oocyte maturation inhibitor (OMI), endorphins and mullarian inhibitory substances (MIS) had been implicated in the maintenance of meiotic arrest.

2.7.2.2 GVBD

First step in resumption of meiosis is dissolution of nuclear membrane (GVBD) chromosome condensation and assembly of spindle apparatus (Krischek and Meinecke, 2002; Byskov *et al.*, 2002). Fully grown mammalian oocyte when isolated from antral follicle and placed in culture underwent spontaneous GVBD, but growing follicles from preantral follicles did not (Buccione *et al.*, 1990).

In conjugation with GVBD which occurred 6-8 h after beginning of culture,(Sirard *et al.*, 1989) marked changes in protein synthesis and phosphorylation were observed in bovine oocytes (Khatir *et al.*, 1998). Tatemoto and Terada (1996) reported that dephosphorylation of p34Cdc2 kinase followed by activation of histone II kinase, after the onset of culture played a key role in the meiotic resumption elicited by the MPF activity (Wu *et al.*, 1997) in bovine oocytes. The new protein necessary for inducing GVBD was synthesized in the first 8 h of culture.

Krisher (2004) stated that glucose metabolism particularly pentose phosphate pathway are involved in normal mechanism regulating the resumption of meiosis and maintenance of developmental competence. He *et al.* (1997) observed that Ca2+ was important for resumption of meiosis in bovine oocytes and found that low Ca2+ affected progression of meiosis by inhibiting the activation of kinases (He *et al.*, 1997 and Krisher, 2004).

2.7.2.3 Metaphase – I

In metaphase I diploid set of chromosomes were fully condensed and were arranged on equatorial plane (Datta *et al.*, 1999). Khatir *et al.* (1998) observed that by 12 h of IVM most of the oocytes were at metaphase I (84 per

cent). A reduction in MPF activity between 12 h and 20 h was noticed and this period corresponded to MI and MII stage. Reduction in MPF triggers transition from M I to M II stage of maturation in bovine oocytes (Anas *et al.*, 2000).

2.7.2.4 Metaphase – II

Khatir *et al.* (1998) reported that by 20 h of IVM 89 per cent of oocytes were at MII stage and at 24 h 86 per cent exhibited MII to extrusion of 1st polar body. Dode and Adona (2001) observed that time required to reach metaphase II and maturation rate was similar for Zebu and Taurine cattle. Conti *et al.* (2002) reviewed a cytostatic factor (CSF) of which the mos kinase is an essential component and was responsible for maintaining the metaphase II arrest. Only a metaphase II arrested secondary oocyte was fertilized by spermatozoa.

A criterion for maturation of oocyte was selected as observation of MII. MII 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 (Datta *et al.*, 1999).

2.7.3 Staining

2.7.3.1 Aceto Orcein

Oocytes were denuded either by vortexing for 2 minutes (Palaz *et al.*, 2000) or by repeated pipetting (Li *et al.*, 2002). Cumulus free oocytes were then mounted on glass slides. A cover slip supported by four droplets of a mixture of Vaseline and paraffin oil (9:1 v/v) was placed on to the oocytes, and gentle pressure was applied to the coverslip to hold the oocytes. Oocytes were then fixed in a fixation solution (acetic acid: ethanol 1:3 v/v) for 24-48 h and stained with 1 per cent (w/v) orcein in 40 per cent (v/v) acetic acid. (Li *et al.*, 2002). Choi *et al.* (1998) fixed oocytes in ethanol acetic acid (3:1), after 24 h culture, stained with 1 per cent orcein and examined under a phase contrast microscope (400 X).

Maturation was assumed with the presence of metaphase II chromosomes and first polar body.

2.7.3.2 bis Benzimide (Hoechst 33342)

Li *et al.* (2002) stained oocytes with 10 µg/ml bis Benzimide (Hoechst 33342) for 2-5 minutes immediately after cumulus cells were removed. Oocytes displaying a disassembled nucleus membrane, chromatin condensation and first polar body were considered to be mature. Simon (2005) used Hoechst 33342 (bis Benzimide) stock solution of 5 µg/ml for staining the nucleus of oocyte and obtained good results.

2.7.3.3 Fluorescein Diacetate (FDA) Staining

Katska and Smorag (1985) used 3'6'-Fluorescein diacetate (FDA) for viability assessment of bovine oocytes. The basic staining solution contained 5µg FDA per one ml of acetone. Immediately before use, it was diluted, giving a final concentration of 1.7 µg FDA/ml of modified Dulbecco's phosphate buffered saline. Denuded oocytes were maintained in staining solution for 3-5 minutes after washing in Krebs-Ringer buffered saline. After staining oocytes were washed in Dulbecco's medium and observed under Fluorescent microscope. They reported that the degree of fluorescein accumulation is a reflection of quality of oocyte cytoplasm. Noto (1991) used 2.5 µg/ml Fluorescein diacetate prepared freshly in Dulbecco's phosphate buffered saline containing 4 mg BSA/ml for staining of human embryos, which was washed free of phenol red.

Embryo viability can be assessed by using probes that reveal both membrane integrity and cytoplasmic enzyme activity, include fluorescein diacetate and calcemin flurochromes. These probes document cytoplasmic esterase activity and have been used as intracellular markers of embryo viability in cattle, rabbit and mouse embryos. These dyes readily permeate living blastomeres, where active cytoplasmic esterases cleaves acetate moieties from

fluorescein which then emits green fluorescence when exposed to UV excitation (Church and Raines 1980; Mohr and Trounson 1980; Overstrom, 1996).

2.8 MATURATION RATE OF DIFFERENT QUALITY GRADES OF COCS

2.8.1 A class

Several workers selected oocytes with more than 4-5 layers for *in vitro* maturation studies. Rabahi *et al.* (1993) aspirated bovine oocytes with more than 5-6 layers of cumulus cells, and on 24-26 h of culture 85-96% cumulus expansion rate was obtained. When aspirated bovine oocytes with more than 4-5 layers of cumulus cells on 24 h maturation in TCM-199 with 17-β Estradiol exhibited a percentage cumulus expansion rate of 91 ± 10.5 (Romero-Arredondo and Seidel, 1996).

Leibfried and First (1979) aspirated bovine oocytes and in A class oocytes a maturation rate of 61.8% was obtained. Tatemoto and Terada (1995) and Konishi *et al.* (1996) when performed *in vitro* maturation in bovine oocytes, oocytes with more than 4 layers of cumulus cells, exhibited M II rate of 84-85% on *in vitro* culture. Choi *et al.* (1998) on maturation of aspirated bovine oocytes with compact unexpanded cumulus cell layers, a maturation rate of 91.7% was obtained. Rodriguez *et al.* (2004) reported that oocytes with several layers of cumulus cells exhibited a maturation rate of 99% in TCM-199 on 20 h of maturation culture.

Pawshe *et al.* (1994) in goats obtained 87.1% MII rate in oocytes with more than 4 layers of cumulus cells, collected by aspiration. In buffaloe when Chauhan *et al.* (1998a) conducted *in vitro* maturation in TCM-199, with 10% FCS and FSH, oocytes with more than five layers of cumulus cells exhibited a maturation rate of 77-79%. A similar maturation rate was also obtained for Datta and Goswami (1999) when aspirated good quality oocytes were subjected for maturation.

Carolan *et al.* (1992) reported 87% MII rate in A class/good quality bovine oocytes obtained by slicing after culture in TCM-199 for 24 h . Arlotto *et al.* (1996) performed slicing in bovine ovaries for oocyte retrieval, and on 24 h culture of oocytes with compact multiple layers of cumulus cells in TCM-199, a Metaphase II rate of 13-57% was obtained.

Pawshe *et al.* (1996) by slicing of goat ovaries oocytes were collected. Among these, oocytes with more than 4-5 layers of cumulus cells on culture in TCM-199, with gonadotrophins and Estrogen, a metaphase II rate of 90.0% was obtained. Slicing of caprine ovaries and maturation of compact multilayered COCs with even granulated ooplasm in TCM-199 for 30 h resulted in a MII rate of 81.4% (Katiyar *et al.*, 1997).

Schellander *et al.* (1990) collected bovine oocytes by puncture method and subjected oocytes with multiple layers of compact cumulus cells for maturation treatment in TCM-199 at 39°C a MII rate of 78-88.4% was obtained after 26 h. Whereas oocytes with compact multiple layers of cumulus cells and even granulation of ooplasm was obtained by puncture method, on 32 h of culture with a MII rate of 71.6% (Sharma *et al.*, 1996).

2.8.2 B Class

Oocytes with 3-5 layers of cumulus cell layers were generally considered as B class. Aspiration was performed in bovine oocytes, and on maturation treatment oocytes with more than 3-4 layers exhibited a MII rate of 76%, (Konishi *et al.*, 1996) whereas for Chauhan *et al.* (1998a) when performed maturation in Grade II buffaloe oocytes an MII rate of 54% was obtained.

2.8.3. C Class

Leibfried and First (1979) performed maturation in bovine oocytes with partial cumulus investment. They obtained a metaphase II rate of 46.2%. In grade III oocytes with 1-2 layers of cumulus cells, obtained through aspiration yielded metaphase II rate of 77% on 24 h maturation culture (Konishi *et al.*, 1996).

Warriach and Chohan (2004) when performed aspiration for oocyte recovery and on culture, oocytes with 1-2 layers of cumulus cells exhibited an MII rate of 51.4%. In average quality oocytes obtained through aspiration, when maturation was performed 60.0% metaphase II rate was obtained in cattle (de loose *et al.*, 1989) and buffaloe (Datta and Goswami, 1999).

Oocytes with 1-2 layers of cumulus cells, obtained by slicing of goat ovaries exhibited a metaphase II rate of 53.68% (Martino *et al.*, 1992).

2.8.4 D Class

Denuded bovine oocytes obtained through aspiration exhibited a metaphase II rate of 19-23% on maturation treatment (Leibfried and First, 1979, de loose *et al.*, 1989; Geshi *et al.*, 2000). For Lorenzo *et al.* (1994) and Konishi *et al.* (1996) after aspiration, denuded oocytes obtained exhibited a metaphase II rate of 42-48%. Tatemoto and Terada (1995) when performed maturation in denuded bovine oocytes obtained by aspiration MII rate of 80% was obtained.

2.8.5 Culture Grade

Most of the scientists used oocytes with multiple layers of cumulus investment (≥3 layers) (Tornesi *et al.*, 1995; Leibfried-Rutledge *et al.*, 1986b). On 24 h of maturation culture of bovine oocytes obtained by aspiration, Lorenzo *et al.* (1994) and Olson *et al.* (1990) obtained 58.8-71.4% cumulus expansion on 24 h culture in TCM-199 with gonadotrophins. But when Quero *et al.* (1994) carried out maturation in aspirated oocytes with more than 3 layers of cumulus cells, a cumulus expansion rate of 78% was obtained. When aspirated culture grade buffaloe oocytes were subjected for maturation culture in TCM-199 with gonadotrophins a cumulus expansion rate of 64-84% was obtained (Nandi *et al.*, 2002; Raghu *et al.*, 2002).

When culture grade oocytes obtained by aspiration of follicles on maturation culture in TCM-199, a metaphase II rate of 42-62% was obtained after 24 h. (Leibfried and First, 1979; Lorenzo *et al.*, 1994 and Nakagawa *et al.*, 1994).

A metaphase II rate of 63-70% was obtained for Tornesi *et al.* (1995), Leibfried-Rutledge *et al.* (1986b); Ali and Sirard (2002) when A and B class oocytes obtained by aspiration method were subjected to maturation culture. Arlotto *et al.* (1990), Quero *et al.* (1994) and Geshi *et al.* (2000) obtained 71-80% MII rate in aspirated oocyte with more than 3 layers of cumulus cells on maturation treatments. Some scientists obtained 81-91% metaphase II rate in oocytes with more than 3 layers of cumulus cells, obtained by aspiration on 22-24 h of maturation treatment in TCM-199 (Wiemer *et al.*, 1991; Jiang *et al.*, 1991; Tatemoto and Terada, 1995; Tatemoto and Terada, 1996; Gandolfi *et al.*, 1997). Totey *et al.* (1993b) and Nandi *et al.* (2002) obtained 64-84% Metaphase II rate in A and B class buffaloe oocytes obtained by aspiration on 24 h culture in TCM-199.

Arlotto *et al.* (1990) sliced bovine ovaries to obtain oocytes. These oocytes belong to culture grade when subjected to maturation treatment 56-65 per cent metaphase II rate was obtained whereas Rieger and Loskutoff (1994) sliced bovine ovaries to collect oocytes and when oocytes with ≥3 layers of cumulus cells were subjected to maturation treatment an MII rate of 90.0% was obtained on 24 h culture. Kobayashi *et al.* (1994) collected oocytes by mincing of bovine ovaries and on maturation culture in TCM-199 with gonadotrophins, culture grade oocytes exhibited a MII rate of 99.2 ± 1.0%.

Wahid *et al.* (1992) when sliced sheep ovaries oocytes with compact cumulus investment exhibited a metaphase II rate of 98.9-91.7%. Katiyar (1997) subjected culture grade goat oocytes obtained by slicing of bovine ovaries for maturation treatment in TCM-199 for 32 h, and obtained a maturation (MII) rate of 81%.

Sato *et al.* (1990) performed puncturing for collection of oocytes from bovine ovaries. When these oocytes were matured in TCM-199 with 10% FCS for 24 h 78.7-91.4% MII rate in oocytes with a diameter of 121-130 μm.

2.8.6 Overall Maturation Rate of COCs

When maturation was performed in bovine oocytes without any oocyte grading, different scientists got various maturation rates. An overall cumulus expansion rate of 90.3 ± 2.5 was obtained in aspirated bovine oocytes on 24 h culture in TCM-199 with gonadotrophins, estrogen and 10% serum (Calder *et al.*, 2003).

Kobayashi *et al.* (1994) minced bovine ovaries to obtain COCs. On 24 h culture in TCM-199 with gonadotrophins, an overall cumulus expansion rate of 99.2 ± 1.0 per cent was obtained.

Baruha *et al.* (1998) obtained an overall MII rate of 36.12% in aspirated bovine oocytes on maturation at 39°C in TCM-199. Bovine oocytes collected by aspiration method, when subjected to maturation treatment in Ham's F-10 medium after 26-28 h a metaphase II rate of 51-62% was obtained (Sanbuissho and Threlfall1985, Sanbuissho and Threlfall 1990, de Oliveira *et al.*, 1994, Palasz *et al.*, 2000).

Arlotto *et al.* (1990), Arlotto *et al.* (1996) and Lechniak *et al.* (2002) when aspirated oocytes overall metaphase II rate of 63.0-76.0% was obtained after culture in TCM-199 for 24 h.

When aspirated oocytes were matured in TCM-199 maturation rate in the range of 75-87% was obtained for Monaghan *et al.* (1993), Chian and Niwa (1994), Chian *et al.* (1996), Khatir *et al.* (1998), Dode *et al.* (2001), Mizuzhima and Fukui (2001) and Krischek *et al.* (2002). Ikeda (2003) obtained 76.6% MII rate when aspirated COCs were cultured in modified SOF medium. A maturation rate of 87-91% was obtained for Larocca *et al.* (1993) and Calder *et al.* (2003) when aspiration was the oocyte collection method.

When aspiration was performed in goat ovaries 83% MII rate was obtained for Tajik and Esfandabadi (2003).

By slicing method, Arlotto *et al.* (1990) obtained overall maturation rate in the range of 16.2-64.9% in bovine oocytes. Carolan *et al.* (1994) performed surface dissection to retrieve oocytes from bovine ovaries and obtained overall MII rate of 75.8%. Martino *et al.* (1992) obtained an overall maturation rate of 56.47% when oocytes were collected by slicing of goat ovaries.

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

No significant difference was observed in the maturation rate of oocytes collected by retrieval methods like aspiration, slicing and puncture of ovaries in cattle (Carolan *et al.*, 1992), sheep (Wahid *et al.*, 1992), and goat (Pawshe *et al.*, 1994). But for Iwasaki *et al.* (1987), Arlotto *et al.* (1990), Sato *et al.* (1990), and Carolan *et al.* (1994) maturation rate of oocytes obtained from surface dissection was significantly lower than that obtained by aspiration in bovines. Martino et al. (1992) noticed a similar observation in goat oocytes with less than three layers of cumulus cells. One of the reason for this might be slicing yielded smaller oocytes and smaller oocytes had smaller mass of cumulus (Carolan *et al.*, 1994). For Martino *et al.* (1992) there was no significant difference in maturation of goat oocytes obtained by cutting compared to aspiration when cumulus layers were more than three.

Cumulus intact oocytes from follicles located deep in the ovarian cortex had a lesser probability of completing meiosis I *in vitro*, when compared to visible follicles on surface (Arlotto *et al.*, 1990). Oocyte diameter increased with increase in follicle diameter. Oocytes of larger diameter have high developmental competence. Oocyte follicle units in the deeper cortex of ovary were less advanced in growth and development than oocyte follicle units on surface (Arlotto *et al.*, 1996).

2.9 MATURATION RATE IN DIFFERENT RETRIEVAL TECHNIQUES – A COMPARISON BETWEEN QUALITY GRADES

For successful *in vitro* fertilization procedure high quality oocytes are required. Methods of oocyte recovery is an important factor in determining the quality of oocytes. Oocytes with more number of cumulus cell layers exhibited greater maturation rate than oocyte without/with less number of cumulus cell layers (Leibfried and First, 1979; Martino *et al.*, 1992; Konishi *et al.*, 1996; Chauhan *et al.*, 1998). Raghu *et al.* (2002) opined that the COC's were developmentally competent as long as they had cumulus, but when some cumulus cells were removed before IVM, cytoplasmic maturation is perturbed and hence developmental competence. IVM rates for buffaloe oocytes with $\geq$3 layers (64.5%) and 1-2 layers of cumulus cells (51.4%) were significantly higher than oocytes with partial remnants or without cumulus cells (8.6%) (Warriach and Chohan, 2004). Geshi *et al.* (2000) reported that a continuing presence of cumulus cells during maturation is important for subsequent development of zygotes to the blastocyst stage.

Materials and Methods

3. MATERIALS AND METHODS

3.1 SOURCE OF OVARY

Ovaries required for the harvest of oocytes were obtained directly from Corporation slaughterhouse, Kuriachria, Thrissur. South Indian breeds like Kangayam, Khillari, Hallikar and crossbred cattle of Kerala were mainly slaughtered here.

3.2 COLLECTION AND TRANSPORTATION OF OVARY

Ovaries were dissected out from animals within 30-60 minutes of slaughter and transported to the laboratory within 2-4 h in freshly prepared normal saline fortified with 100 IU/ml Benzyl penicillin and 100 µg/ml Streptomycin sulphate maintained at 36-38°C.

Details of media, hormone, chemicals, glassware and equipments used in this study are shown in Table 1.

3.3 PRE RETRIEVAL PROCESSING OF OVARIES

The ovaries were washed in clean tap water repeatedly to remove excess 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 RETRIEVAL OF FOLLICULAR OOCYTES

Oocytes were retrieved from ovaries by applying any one of the following three retrieval methods in equal proportion. The retrieval process was carried out in HEPES buffered Tyrode's lactate medium enriched with BSA @ 0.6% and maintained at 37°C. Heparin was supplemented to this medium @ 0.1 mg/ml.

3.4.1 Aspiration

Surface follicles measuring 2-8 mm in size were aspirated with a sterile 18 gauge disposable needle connected to a 10 ml disposable plastic syringe. The procedure followed is as per Priscilla, (2001) (Plate 1).

3.4.2 Slicing

Slicing of the ovaries for oocyte collection was carried out as per the procedure of Das *et al.* (1996a) (Plate 2).

3.4.3 Puncture

The procedure followed in this method was as per Das *et al.*,(1996a). (Plate 3).

3.5 POST RETRIEVAL PROCESSING OF OOCYTES

After harvesting by all the three methods the medium containing oocytes were transferred into separate sterile 90 mm Petri dishes having grid and examined under the zoom stereomicroscope. Identified oocytes were collected by means of unopette and transferred into 60 mm Petri dishes containing fresh TL-HEPES.

3.6 CHARACTERIZATION AND CLASSIFICATION OF OOCYTES

All the oocytes obtained under different retrieval systems were examined separately with 40X magnification of zoom stereomicroscope for their morphological character. (Plate 4) Based on number of layers of cumulus cells and ooplasm character, the oocytes were graded into four classes (Plate 4).

Class A : More than 5 complete layers of cumulus cells and uniform granulation of ooplasm (Plate 8 and 9)

Class B : 3-5 complete layers of cumulus cells and uniform granulation of ooplasm (Plate 10)

Class C : 1-2 complete layers of cumulus cells and uniform granulation of ooplasm (Plate 11)

Class D : Denuded oocytes with uniform granulation of ooplasm (Plate 12 and 13)

Class A and B were combined together to form a new class namely Culture grade oocytes, since these oocytes were considered as high quality. (Quero *et al.,* 1994). Each class of selected oocytes under different retrieval systems were put separately in labelled TL-HEPES drops and further processing for *in vitro* maturation was done separately.

3.7 *IN VITRO* MATURATION OF OOCYTES

3.7.1 Media

Medium used for maturation of oocyte was freshly prepared TCM-199, enriched with FSH 0.5 µg/ml, LH – 5 µg/ml, Oestrogen 1 µg/ml, sodium pyruvate 0.2 mM and Foetal Calf Serum (FCS) 10%. Sufficient 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 (Plate.6) and equilibrated in CO_2 incubator for at least 2 h before introducing the oocyte. Two small dishes of maturation media were also put in the incubator for the purpose of pre and post maturation washing of oocytes.

3.7.2 Culture Conditions

Culture conditions set for this study was 38.5°C temperature, 5% carbondioxide tension with maximum humidity. Standard water-jacketed type CO_2 incubator was used to get this culture environment (Plate.5).

3.7.3 Pre Culture Washing of Oocytes

Each category of oocyte was washed repeatedly in TL-HEPES medium and then subjected to final washing with already aclimatized 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 and allowed to complete incubation for a period of 24 h without any disturbance in the culture environment. Depending on the availability, a maximum of 10 oocytes were introduced into each maturation drop.

3.8 ASSESSMENT OF MATURATION

3.8.1 Cumulus Cell Expansion

After 24h of culture, all oocytes in the culture drops were examined under zoom stereomicroscope for maturation changes such as expansion and mucification of cumulus cells (Plate.7) Those oocytes showing expansion of cumullus cells in a radiating fashion(Plate.15 and 16) with mucification (Plate.14) were graded as matured oocytes.

3.8.2 Nuclear Changes

From the oocytes showing cumulus expansion a minimum of 10 percentage of oocytes were taken out of the culture drops and washed again with acclimatized maturation media to free it off from the traces of residual mineral oil, denuded by vortexing, stained with one per cent aceto-orcein, and then examined for nuclear changes associated with maturation. Examination of oocytes for the polar body extrusion was done before and after the staining procedure.

Nuclear stages were identified as Germinal vesicle stage (GV), Germinal vesicle breakdown stage (GVBD), Metaphase I (MI) and Metaphase II (M II). GV stage was characterized by the presence of an outline of nuclear membrane and decondensation of the Chromatin (Plate 20). Oocyte under GVBD stage had highly condensed chromatin and fragmented nuclear membrane. (Pro-metaphase) (Plate 21). Metaphase I (MI) was characterized by condensed chromosomes aligned in a large spindle and non-extrusion of first polar body (Plate 23). Chromosomes aligned in equatorial plane, called metaphase plates (Plate 25) with presence of 1st polar body were identified as Metaphase II (Plate.24 and 25) (He *et al.*, 1997).

3.8.3 Denudation of Oocytes

Denudation of oocytes was achieved by vortexing the cumulus oocyte complex in maturation media in a microcentrifuge tube for two minutes in a vortex machine.

3.8.3.1 Polar Body Extrusion

Denuded oocytes were examined under Bright field of inverted phase contrast microscope under 200X and 400X magnification. While doing so the oocytes were taken on a glass slide in a droop of TCM-199 and rolled gently with a needle to identify the extruded polar body (Plate.17,18 and 19).

3.8.4 Whole Mount Fixation

Denuded oocytes (6-7 in a group) were placed on a clean glass slide in a small drop of maturation medium. Then whole mount fixation of oocytes were performed as per Malenko, 1994 using vaseline paraffin jelly to hold the cover slip and methanol as fixative.

3.8.5 Staining and microscopic examination

3.8.5.1 Aceto Orcein Staining Technique

The slides taken out from fixative were examined under 20x magnification of zoom stereo microscope to locate the oocyte. A minimum of ten percentage of fixed oocytes were stained as per the procedure of Malenko, 1994 with 1% orcein in 45% acetic acid. All the oocytes stained as above were examined under bright field of inverted phase contrast microscope at 200- 400X magnification and assessed the level of meiotic progression.

In aceto-orcein staining Germinal vesicle appeared like a circular ring with a small dark spot at the centre representing nucleolus (Plate.20). Germinal vesicle breakdown (GVBD) stage was characterized by nuclear membrane expanding, fragmenting (Plate.21) and at the same time chromosomes becoming more condensed (Plate.22). Metaphase I appeared like a single set of chromosome in the equatorial plane.(Plate). Metaphase II appeared like two sets of chromosome in two different planes of focus, one for polar body and one for MII chromosomes (Plate.24 and 25). Aceto-orcein stain revealed chromosome structure in much more detail (Plates 26 and 27).

3.8.5.2 Hoechst 33343 Staining Technique.

A cross checking of oocytes for maturation changes were performed with Hoechst 33342 staining technique. After denudation 18 oocytes were incubated in bicarbonate buffered TCM-199 with 5 µg/ml Hoechst 33342 stain (bis Benzimide trihydrochloride,) for 15 minutes. After washing, these oocytes were stained and viewed under fluorescent microscope with excitation filters of 340-380 nm and suppression filter of 425 nm (Smith, 1993).

In Hoechst 33342 staining technique under fluorescent microscope, nuclear material appeared fluorescent. Germinal vesicle was viewed as a round uniform bright disk (Plate.29), GVBD characterized by more condensed nuclear

material with dissolution of nuclear membrane (Plate.30). MI appeared like a single set of chromosomes in equatorial plane (Plate.31). M II appeared as two sets of chromosomes moving away from each other one as polar body and other as oocyte nucleus arrested at Metaphase II (Plate.32).

3.8.5.3 Fluorescein Diacetate (FDA) Staining

Some of the matured oocytes were denuded and stained with Fluorescein Diacetate to assess the viability. FDA reveals both membrane integrity and cytoplasmic enzyme activity of cell by emitting apple green fluorescence (Plate.33). 1.7 µg FDA/ml of Dulbeco's phosphate buffered saline were used for staining. Ten stained oocytes were observed under fluorescent microscope with excitation filters of 490 nm and suppression filter of 515 nm.(Katska and Smorag 1985).

3.9 EXPERIMENTAL DESIGN

3.9.1 Experiment –1: Effect of three harvesting technique on the yield of oocytes

Ovaries collected from slaughterhouse after processing were subjected to different oocyte retrieval methods like Aspiration, Slicing and Puncture and yield of oocytes under each retrieval method was assessed. A total of 150 ovaries were collected, and 50 ovaries each were processed under three retrieval methods

3.9.2 Experiment – 2: Effect of retrieval technique on the percentage yield of different grades of oocytes

Yield of different grades of oocytes derived by aspiration, slicing and puncture were classified into four quality grades based on morphology by observing under a zoom stereomicroscope.

3.9.3 Experiment 3: Effect of retrieval method and COC morphology on cumulus expansion

Each quality grade of oocytes collected by different methods of retrieval like aspiration, slicing and puncture were subjected to *in vitro* maturation in TCM-199 with 10% FCS, LH, FSH and Estradiol 17-β under standard incubation conditions. After 24 hours incubation the COCs were evaluated for cumulus expansion by observing under zoom stereomicroscope.

3.9.4 Experiment 4: Effect of Retrieval Method and COC morphology on Nuclear Maturation

After 24 hours of *in vitro* culture a minimum of 10% oocytes showing cumulus expansion from each quality grade under different retrieval system were evaluated for their nuclear status by staining with 1% aceto-orcein and a few with Hoechst 33342 stain. Before staining, the denuded oocytes were also subjected to bright field microscopy to locate the extruded polar body. The stained oocytes were observed under phase contrast microscope to evaluate nuclear maturation. Oocytes showing the two sets of chromosome mass or extruded polar body were considered as mature. Very few matured oocytes were studied for their viability with vital stain Fluorecein diacetate (FDA)

3.10 STATISTICAL ANALYSIS

Data on oocyte yield, classification, cumulus expansion and nuclear maturation was analysed with Chi-square analysis. Group of data showing significant difference was subjected to pair wise data analysis with chi-square test. P-value of less than 0.05 was considered statistically significant.

Table 1. Details of Media, Hormones, Chemicals, Glass ware and Equipments

Sl. No.	Name of Item	Patent Name/ Catalogue No.	Manufacturer
(1)	**Media, Hormones and Chemicals**		
1.	TCM-199	M – 4530	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 E-8875	Sigma Chemicals St. Louis, USA
5.	Foetal Bovine Serum	F – 2442	Sigma Chemicals St. Louis, USA
6.	Sodium Pyruvate Solution(100 mM)	S – 8636	Sigma Chemicals St. Louis, USA
7.	Gentamycin Sulphate	RM – 461	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	S –5886	Sigma Chemicals St. Louis, USA
11.	Sodium bicarbonate	S – 5761	Sigma Chemicals St. Louis, USA
12.	D-(+) – Glucose	G – 7021	Sigma Chemicals St. Louis, USA
13.	Mineral Oil	Mineral oil M 8410	Sigma Chemicals St. Louis, USA
14.	Orcein	RM – 277	Hi media Laboratories Ltd. Mumbai
15.	Bis Benzimide (Hoechst-33342)	B – 2261	Sigma Chemicals St. Louis, USA
16.	Methanol	Methanol	Merck, Germany

17.	Acetic acid	Acetic acid	BDH Laboratories England
18.	Potassium chloride	P – 5405	Sigma Chemicals St. Louis, USA
19.	Sodium phosphate mono basic	S – 5011	Sigma Chemicals St. Louis, USA
20.	Phenol red	P– 0290	Sigma Chemicals St. Louis, USA
21.	Sodium lactate	Sodium lactate (60%)	Central Drug House (P) Ltd.
22.	Magnesium chloride	M – 2393	Sigma Chemicals St. Louis, USA
23.	$CaCl_2$ –$2H_2O$	C – 7902	Sigma Chemicals St. Louis, USA
24.	HEPES	H – 3784	Sigma Aldrich, USA
25.	BSA fraction V	A– 9418	Sigma Chemicals St. Louis, USA
26.	Heparin sodium salt	H – 3149	Sigma Chemicals St. Louis, USA
27	Fluorecein diacetate	F– 1397	Sigma Chemicals St. Louis, USA

(2)	**Disposable Wares**	**Patent Name/ Catalogue No.**	**Manufacturer**
1.	Petridish (35 mm)	353801 Falcon	Becton Dickinson Labware,NewJersy, USA
2.	Petridish (60 mm)	460060	Tarson Products, India
3.	Petridish (90 mm)	460090	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.	18 g needle 18 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.	Petri dishes 60mm, 90mm	Borosil Glass Works, Mumbai
5.	Glass slides (75 x 25) and cover slip (22 x 22)	Blue Star, India
6.	Beakers 200 ml, 100 ml, 50 ml, 10 ml	Borosil Glass Works, Mumbai

(4)	Equipments	Patent Name/ Catalogue No.	Manufacturer
1.	CO_2 Incubator	Lab Line	Lab line instruments inc, USA
2.	Zoom steriomicroscope	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	X	Vensil, india
13.	Digital pH meter	Cyber Scan 2500	Cyber Scan Eutech Instrument, Singapore
14.	Electronic Analytical precision Balance	CP – 2245	Sartorius, Germany
15.	Thermal Flask	Eagle	Eagle, India
16.	CO_2 gas (Medical grade)		PSROL, Thrissur

Table 2. Oocyte washing medium (modified HEPES – buffered Tyrode's medium) composition as per Gordon (2003)

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
pH		7.4
mOsmol		270-290

Plate 1. **Oocyte retrieval method – Aspiration**

Plate 2. **Oocyte retrieval method – Slicing**

Plate 3. **Oocyte retrieval method – Puncture**

Oocyte Retrieval Methods

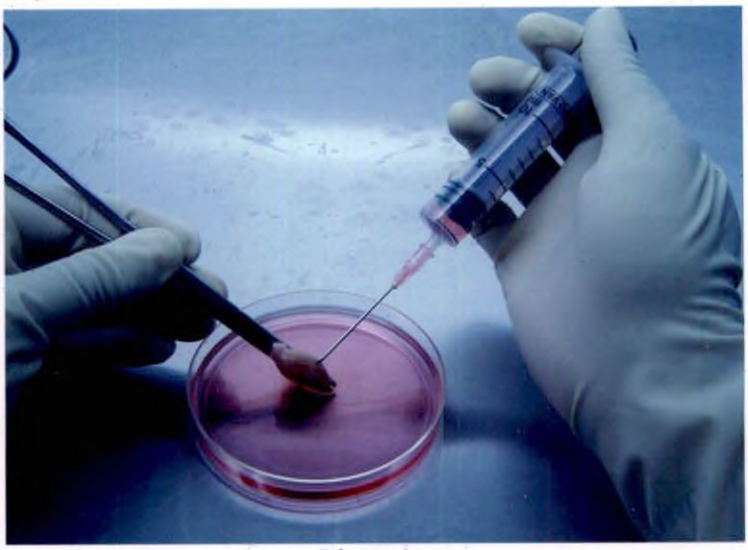

Plate. 1

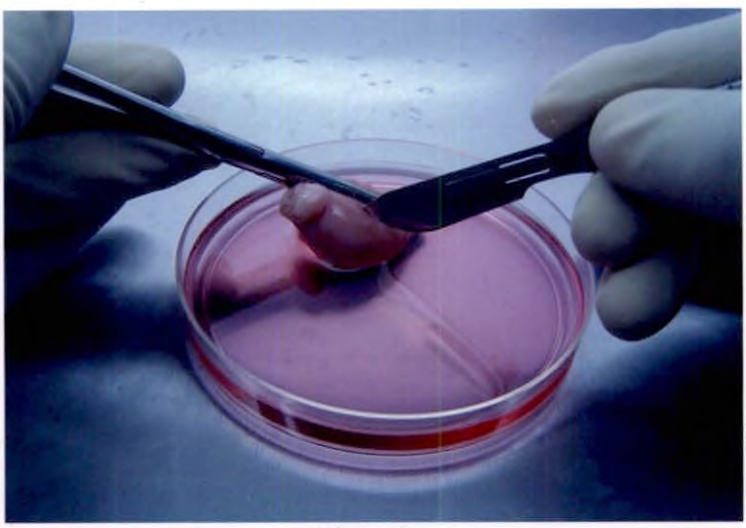

Plate. 2

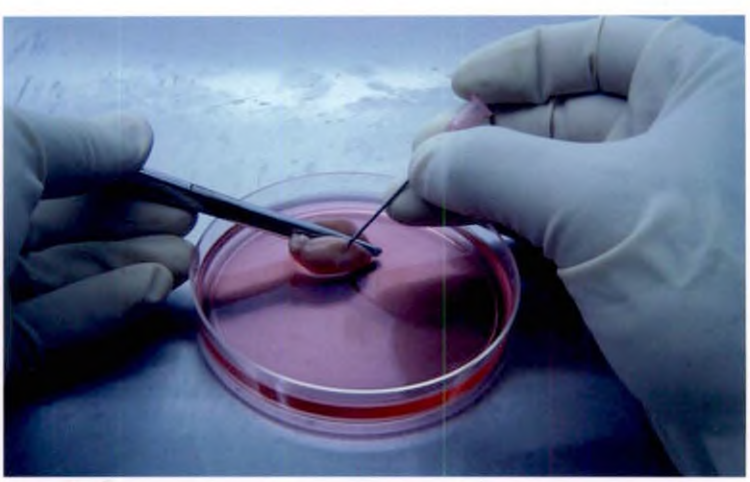

Plate. 3

Plate 4. **Collection of oocytes from oocyte retrieval media by observing under zoom stereo microscope**

Plate 5. ***In vitro*** **culture of oocytes in CO_2 incubator**

Plate 6. **Maturation media (TCM – 199) as 100 µl drop under oil overlay**

Plate 7. **Assessment of maturation under phase contrast microscope**

in vitro Maturation - Different Steps in Laboratory

Plate. 4

Plate. 5

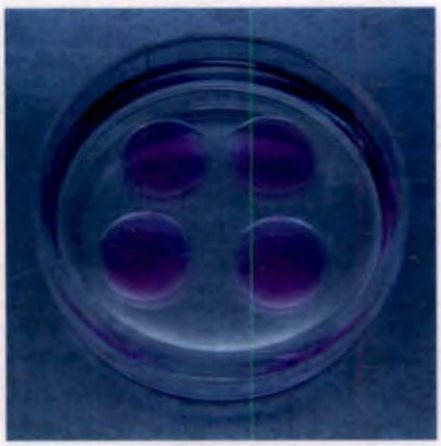

Plate. 6

Plate.7

Plate 8 & 9 **A class oocytes (200 x)**

Plate 10 **B class oocyte (200 x)**

Plate 11. **C class oocyte (200 x)**

Plate 12&13 **D class oocytes (400 x)**

Classification of Cumulus-Oocyte Complexes.

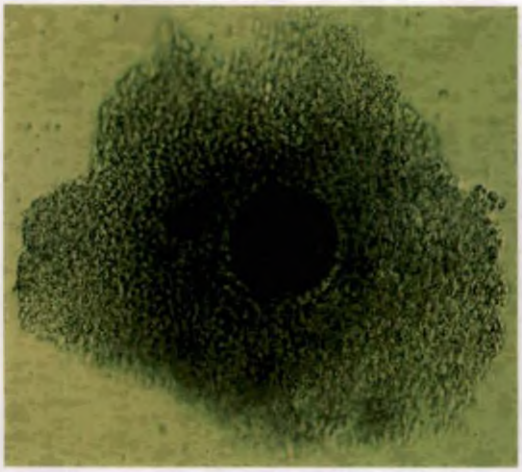

Plate. 8

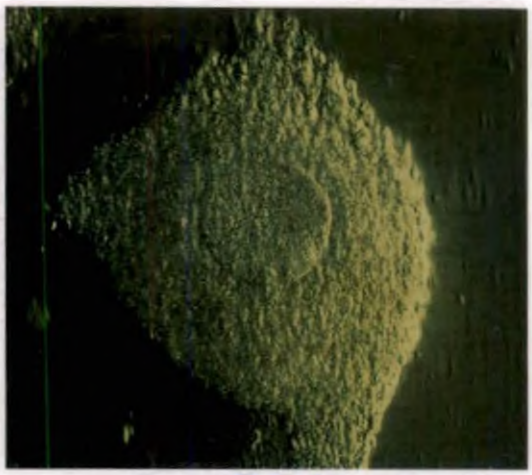

Plate. 9

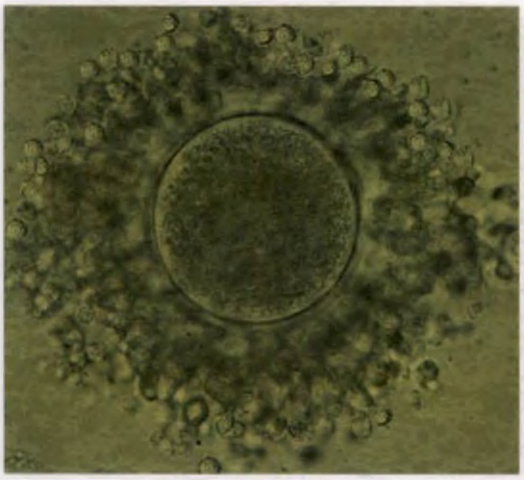

Plate. 10

Plate. 11

Plate. 12

Plate. 13

Results

4. RESULTS

This study was carried out on *in vitro* maturation of follicular oocytes in cattle using oocytes obtained by three harvesting techniques from ovaries of recently slaughtered animals. A total of 150 ovaries from local slaughter house, 639 oocytes and three retrieval techniques were used for the purpose.

4.1 EXPERIMENT 1— EFFECT OF THREE HARVESTING TECHNIQUE ON THE YIELD OF OOCYTES PER OVARY

A total of 150 ovaries were subjected to the study which on harvest by aspiration, slicing and puncture yielded a total of 639 oocytes. Under each retrieval method, 50 ovaries were processed and the yield of oocytes under each method was 185 by aspiration, 271 by slicing and 183 by puncture. The mean yield of oocytes per ovary was found to be 3.7 ± 0.43, 5.42 ± 0.56 and 3.66 ± 0.43 for aspiration, slicing and puncture respectively (Table 3 and Fig.1). On statistical analysis, it was found that aspiration and slicing differed very significantly in the mean yield of oocyte. Similarly puncture and slicing were also found to differ very significantly (P>0.01). But aspiration and puncture did not differ significantly.

4.2 EXPERIMENT 2 — EFFECT OF RETRIEVAL TECHNIQUE ON THE QUALITY OF COCs

4.2.1 Comparative Efficiency between Retrieval Techniques

4.2.1.1 A Class

The yield of Class A oocytes by Aspiration, slicing and puncture were 44.86 per cent, 37.27 per cent and 43.17 per cent respectively (Table 4 and Fig.3). Even though they did not differ significantly, class A oocyte yield was higher in aspiration followed by puncture and slicing.

The maximum number of A class oocyte per ovary was obtained in slicing followed by aspiration and puncture as 2.02 ± 0.25, 1.66 ± 0.25 and 1.58 ± 0.25, respectively (Table 4, Fig 2).

4.2.1.2. B Class

Class B oocyte obtained by aspiration, slicing and puncture was 32.79 per cent, 29.89 per cent and 31.69 per cent respectively (Table 4 and Fig.3). There was no significant difference between these methods in yield of B class oocytes. However, maximum percentage of B class oocytes were obtained by aspiration, followed by puncture and least by slicing.

Number of B class oocyte per ovary was found to be 1.22 ± 0.12, 1.62 ± 0.20 and 1.16 ± 0.16 on aspiration, slicing and puncture respectively. (Table 4, Fig 2). Maximum B class oocyte per ovary was obtained by slicing followed by Aspiration and puncture.

4.2.1.3. C Class

Aspiration, slicing and puncture yielded 18.30 per cent, 20.66 per cent and 19.13 per cent class C oocytes respectively (Table and Fig.3). There was no significant difference between these three methods in yield of class C oocytes. But it was observed that maximum percentage class C oocytes were obtained by slicing followed by puncture and aspiration.

Mean number of C class oocytes per ovary was calculated. Maximum number of C class oocytes per ovary was 1.12 ± 0.15 for slicing followed by 0.70 ± 0.07 for puncture and 0.68 ± 0.20 for aspiration. (Table 4, Fig 2).

4.2.1.4. D Class

D class oocytes were maximum obtained by slicing (12.10 per cent) followed by puncture (6.01 per cent) and aspiration (3.7 per cent) (Table 4 Fig.2). On analysis, there was significant difference between these methods in yield of D

class oocytes (P<0.05). It was observed that slicing yielded significantly higher percentage of class D oocytes than aspiration and puncture, and that puncture and aspiration did not differ significantly.

When number of D class oocytes per ovary was calculated, it was found that by aspiration, slicing and puncture 0.14 ± 0.06, 0.66 ± 0.09 and 0.22 ± 0.03 oocytes were obtained. (Table 4, Fig 2).

4.2.1.5 Culture Grade Oocytes

When Culture grade oocytes were combined together, aspiration, slicing and puncture yielded 76.22%, 67.16% and 74.86% oocytes. Statistical analysis revealed no significant difference between these methods in yield of culture grade oocytes. (Table 4, Fig 2).

Mean number of Culture grade oocyte per ovary was found to be 2.88 ± 0.19, 3.64 ± 0.23 and 2.74 ± 0.21 by aspiration, slicing and puncture respectively. (Table 4, Fig 2).

4.2.2 Comparative Efficiency with in Each Retrieval Technique

4.2.2.1 Aspiration

A total of 185 oocytes obtained from 50 ovaries were subjected to the processing. Of this 83 (44.86 per cent) were of A class oocytes, 61 (32.97 per cent) were B class oocytes, 34 (18.30 per cent) were C class oocytes and 7 (3.7 per cent) were D class (Table 5 and Fig 4.). On statistical analysis it was found that there was significant difference in the yield of each class of oocytes (P<0.05). Yield of A class was higher than B class oocytes, even though they did not differ significantly. It was also observed that yield of A and B class oocytes were significantly higher than those of C and D class COCs. Yield of C class oocyte was significantly higher than that of D class oocytes.

The mean number oocytes/ovary was found to be 1.66 ± 0.25, 1.22 ± 0.15, 0.68 ± 0.2, 0.14 ± 0.06 for Class A, B, C and D respectively (Table 4 and Fig.2).

4.2.2.2 Slicing

On slicing of 50 ovaries, 271 oocytes were obtained. Among these Class A, B, C and D were 101 (37.27 per cent), 81 (29.89 per cent), 56 (20.66 per cent) and 33 (12.10 per cent) respectively (Table 5 and Fig 4.). It was found that A and B classes did not differ significantly from each other in yield of oocytes and their yield was significantly higher in comparison to C and D class oocytes. Yield of C class oocyte was found to be significantly higher than D class oocytes.

Oocyte yield per ovary on slicing was found to be 2.02 ± 0.25, 1.62 ± 0.20, 1.12 ± 0.15 and 0.66 ± 0.09 for A, B, C and D class of oocytes respectively (Table 5 and Fig.2).

4.2.2.3 Puncture

When puncture was performed in 50 ovaries, 183 oocytes were obtained. Among these A, B, C and D class were 79 (43.17 per cent), 58 (31.69 per cent), 35 (19.13 per cent) and 11 (6.01 per cent) respectively (Table 5 and Fig 4.). A and B class oocytes did not differ significantly in percentage yield, but the yield was significantly higher than C and D class of oocytes. C class oocytes yielded significantly higher percentage than class D oocytes.

Yield of mean number of oocyte per ovary by puncturing was 1.58 ± 0.25, 1.16 ± 0.16, 0.70 ± 0.07 and 0.22 ± 0.03 for class A, B, C and D oocytes respectively (Table 5 and Fig.2).

4.3 EXPERIMENT 3 – EFFECT OF RETRIEVAL METHOD AND COC MORPHOLOGY ON CUMULUS EXPANSION

After 24 hours of culture in standard culture conditions and media oocytes were examined for cumulus expansion. Oocytes showing cumulus expansion, (Plate.15 and 16) mucification, (Plate.14) and cumulus monolayer formation were evaluated as mature oocytes.

4.3.2. Comparison Between Methods of Retrieval Under Same Class.

4.3.2.1. A class COCs

The cumulus expansion rate of oocytes obtained by aspiration, slicing and puncture was 83.08 per cent, 69.70 per cent and 70.37 per cent respectively (Table 6 & Fig 5). Even though no significant difference was observed between these methods, maximum maturation rate was obtained by aspiration, followed by puncture and then slicing.

4.3.2.2. B class COCs

Cumulus expansion rate of 68.29 per cent, 53.01 per cent and 62.07 per cent respectively were exhibited by B class oocytes retrieved by aspiration, slicing and puncture on *in vitro* culture (Table 6 & Fig 5). No significant difference was observed between these methods. But percentage of oocytes exhibiting cumulus expansion was maximum in aspiration, followed by puncture and slicing.

4.3.2.3. C class oocytes

C class oocytes harvested by aspiration, slicing and puncture, when subjected to *in vitro* culture, 44.74 per cent, 35.29 per cent and 38.46 per cent respectively have exhibited cumulus expansion (Table 6& Fig 5). Even though there was no significant difference between these methods, maximum cumulus expansion was obtained in aspiration, followed by puncture and least in slicing.

4.3.2.4 Culture grade oocytes

When oocytes with more than 3 layers of cumulus cells were taken, they exhibited a cumulus expansion rate of 77.36 per cent, 62.36 per cent and 66.91 per cent by aspiration, slicing and puncture method (Table 6 & Fig 5). On statistical analysis, there was no significant difference between methods of retrieval.

4.3.2.5 Overall

When overall cumulus expansion rate was taken into account aspiration, slicing and puncture exhibited a cumulus expansion rate of 68.75, 56.33 and 60.67 per cent respectively. Statistical analysis revealed no significant difference between these methods (Table 6 & Fig 5).

4.3.3. Comparison Between Morphological Grades Under Each Retrieval Method

4.3.3.1. Aspiration

When all the 144 oocytes obtained by aspiration were kept for maturation, 99 (68.75 per cent) oocytes exhibited moderate to good cumulus expansion. Among the 65 class A oocytes, 41 class B oocytes and 38 class C oocytes, 54 (83.08 per cent), 28 (68.29 per cent) and 17 (44.74 per cent) respectively had exhibited cumulus expansion (Table 7 and Fig 6). On analysis, it was found that A class was having no significant difference in maturation rate than B class oocytes, but significantly lower maturation rate was observed for C class oocytes in comparison to A and B classes ($P<0.05$).

4.3.3.2. Slicing

Out of 229 oocytes kept for oocyte maturation 129 (56.33) exhibited cumulus expansion. Among 99 A class, 79 B class and 51 C class oocytes, 69(69.70 per cent), 42 (53.01 per cent) and 18 (35.29 per cent) respectively exhibited cumulus expansion (Table 7 and Fig 6). A class was having significantly higher expansion rate than B and C class oocytes. Similarly, B class was having significantly higher expansion rate than C class oocytes ($P<0.05$).

4.3.3.3. Puncture

When 178 oocytes obtained by puncture method were kept for maturation, 108 (60.67 per cent) exhibited cumulus expansion. Out of 81 class A, 58 class B and 39 class C oocytes, 57 (70.37 per cent), 36 (62.07 per cent) and 15 (38.46 per cent) respectively have exhibited cumulus expansion (Table 7 and Fig. 6). No significant difference was observed between A and B class oocytes in cumulus expansion. But A and B class was having significantly higher maturation rate in comparison to C class oocytes.

4.4 EXPERIMENT 4 — EFFECT OF RETRIEVAL METHOD AND COC QUALITY GRADE ON NUCLEAR MATURATION

4.4.1. Comparison Between Methods of Retrieval Under Same Class

4.4.1.1. A class

By aspiration, slicing and puncture 81.8 per cent, 78.94 per cent and 80 per cent of class A oocytes exhibited nuclear maturation. (Table 8 & Fig 7). On chi square analysis, no significant difference in the maturation rate was observed between methods. However, maximum maturation rate was observed in aspiration followed by puncture and slicing.

Polar body extrusion rate in aspiration, slicing and puncture was 45.5%, 42.1% and 44% respectively. Statistical analysis revealed no significant difference between these methods in rate of polar body extrusion.(Table 8)

4.4.1.2. B class

Nuclear maturation rate of 57.1 per cent, 41.67 per cent and 52.63 per cent was obtained by aspiration, slicing and puncture respectively for B class oocytes. (Table 8 & Fig 7). Statistical analysis revealed no significant difference between these methods in nuclear maturation rate. But maximum maturation rate was observed for aspiration (57.1 per cent) and minimum for slicing method (41.67 per cent).

In B class oocytes 28.6%, 25.0% and 26.3% were the polar body extrusion rate by aspiration, slicing and puncture. No significant difference was noticed between these methods in polar body extrusion rate when statistical analysis was carried out.

4.4.1.3 C class

C class oocytes after staining revealed a nuclear maturation rate of 38.46 per cent, 28.57 per cent and 30.77 per cent respectively for aspiration, slicing and puncture (Table 8 & Fig 7). No significant difference between these methods were observed on statistical analysis. Maximum maturation rate was observed in Aspiration (38.46 per cent) and least in slicing (28.57 per cent).

By aspiration, slicing and puncture a polar body extrusion rate of 15.4%, 14.3% and 15.4% was noticed in C class oocytes. But no significant difference between these methods in polar body extrusion rate was noticed.

4.4.1.4. D class

Among the D class oocytes, only one oocyte obtained by aspiration method have exhibited MII plates. None of the D class oocytes exhibited polar body extrusion.

4.4.1.5 Culture grade oocytes

Nuclear maturation rate of culture grade oocytes were found to be 72.22, 64.52 and 68.2 for aspiration, slicing and puncture respectively (Table 8 & Fig 7). Statistical analysis revealed no significant difference between these methods.

Polar body extrusion rate of culture grade oocytes by, aspiration, slicing and puncture were 38.9%, 35.5% and 38.6% respectively. However statistically there was no significant difference between these methods.(Table 8)

4.4.1.6 Overall

When overall maturation percentage was taken into account, the aspiration, slicing and puncture methods revealed a maturation rate of 63.27, 53.66 and 59.65% respectively (Table 8 & Fig 7). Statistical analysis revealed no significant difference between these methods.

Overall polar body extrusion rate was 32.7%, 29.3% and 33.3% respectively. But no significant difference was noticed on statistical analysis.(Table 8)

4.4.2 Comparison Between Morphological Grades Under Each Retrieval Method.

4.4.2.1 Aspiration

In aspiration method, 57 oocytes examined for nuclear maturation. Among them 22 class A oocytes on staining 18 (81.8 per cent) oocytes exhibited nuclear maturation with presence of M II plates. Among these ten oocytes

(45.5%) exhibited extruded polar body. In B class, out of 14 oocytes examined, on staining eight (57.1 per cent) oocytes exhibited M II plates and four (28.6%) exhibited extruded polar body. On examination of 13 class C oocytes, staining revealed M II plates in five (38.46 per cent) oocytes. Among these two (15.4%) oocyte revealed polar bodies. Similarly, out of eight D class oocytes examined only one oocyte exhibited MII plates and none of the oocytes exhibited polar body (Table 9 and Fig 8). On statistical analysis no significant difference was observed between oocytes of class A and B in nuclear maturation and polar body extrusion ($P<0.05$). But nuclear maturation rate and polar body extrusion in A and B class was significantly higher than C class oocytes ($P<0.05$).

4.4.2.2 Slicing

Among 47 oocytes denuded and examined for nuclear changes, 15 (78.94 per cent) out of 19 A class oocytes revealed Metaphase II plates and eight (42.1%) extruded polar bodies. Out of 12 class B oocytes, five (41.67 per cent) oocytes on staining exhibited MII plates and three (25%) revealed polar bodies. On examination of seven C class oocytes, two have shown MII plates (28.57 per cent) and only one (14.3%) has shown extruded polar body. With respect to class D oocytes, none out of nine oocytes has exhibited neither polar body nor MII plates (Table 9 and Fig 8). A class oocytes exhibited significantly higher maturation rate than B and C class oocytes. But no significant difference was observed between B and C class oocytes.

4.4.2.3 Puncture

A total of 67 oocytes were examined for nuclear changes which comprises of Class A 25, class B 19, class C 13 and class D 10 oocytes. Out of 25 A class oocytes 20 (80 per cent) exhibited MII plates and 11 (44%) exhibited polar bodies. Nine (52.63 per cent) B class oocytes exhibited MII plates and Six (26.3%) exhibited polar bodies. Four (30.77 per cent) C class oocytes exhibited MII plates and Two (15.4%) exhibited polar bodies. (Table 9 and Fig 8). No

nuclear maturation was exhibited by D class oocytes. On statistical analysis, A class and B class oocytes did not differ significantly in nuclear maturation. But maturation rate of A class oocytes were significantly higher than C class oocytes. B and C class also did not differ significantly in nuclear maturation rate.

4.4.3 Hoechst 33342 and Fluorescein Diacetate (FDA) Staining

In Hoechst 33342 staining nuclear material appeared as bright blue fluorescent spots. Out of 18 oocytes stained 12 (66.67 %) exhibited Metaphase II plates (Plate 28 to 32).

Fluorescein diacetate staining of matured oocytes revealed uniform apple green fluorescence in the entire surface of oocytes, representing viability of oocytes (Plate 33). Eight oocytes out of ten stained (80%) were viable.

Table 3. Effect of three harvesting technique on the yield of oocytes

Sl. No.	Retrieval methods	Number of ovaries	Total yield of oocytes	Mean number of oocytes
1.	Aspiration	50	185	3.70 ± 0.43^{a}
2.	Slicing	50	271	5.42 ± 0.56^{b}
3.	Puncture	50	183	3.66 ± 0.43^{a}
4.	Total	150	639	4.26 ± 0.46

Values bearing different superscript in same column differ significantly ((P>0.01)

Table 4. Effect of retrieval technique on the quality grade of COCs — Comparative efficiency between the retrieval techniques

Sl. No.	Morpho-logical grade of COCs	Oocyte retrieval system								
		Aspiration			Slicing			Puncture		
		Number of oocytes studied	Percentage of total yield	Mean number per ovary	Number of oocytes studied	Percentage of total yield	Mean number per ovary	Number of oocytes studied	Percentage of total yield	Mean number per ovary
1	A	83	44.86^{a}	1.66 ± 0.25	101	37.27^{a}	2.02 ± 0.25	79	43.17^{a}	1.58 ± 0.25
2	B	61	32.97^{b}	1.22 ± 0.12	81	29.89^{b}	1.62 ± 0.20	58	31.69^{b}	1.16 ± 0.16
3	C	34	18.30^{c}	0.68 ± 0.20	56	20.66^{c}	1.12 ± 0.15	35	19.13^{c}	0.70 ± 0.07
4	D	7	3.7^{d}	0.14 ± 0.06	33	12.10^{e}	0.66 ± 0.09	11	6.01^{d}	0.22 ± 0.03
5	Culture grade	141	76.22	2.88 ± 0.19	152	67.16	3.64 ± 0.23	137	74.86	2.74 ± 0.21

Percentage bearing different superscript within same row differ significantly ($P<0.05$)

Table 5. Comparative efficiency of retrieval technique on quality grades of COCs

Sl. No.	Retrieval method	Number of ovaries studied	Total yield of oocytes	Morphological grades of oocytes	Number of oocytes in each grade	Total yield (%)	Mean number per ovary
1.	Aspiration	50	185	A	83	44.86^{a}	1.66 ± 0.25
				B	61	32.97^{a}	1.22 ± 0.12
				C	34	18.30^{b}	0.68 ± 0.20
				D	7	3.70^{d}	0.14 ± 0.06
2.	Slicing	50	271	A	101	37.27^{e}	2.02 ± 0.25
				B	81	29.89^{e}	1.62 ± 0.20
				C	56	20.66^{g}	1.12 ± 0.15
				D	33	12.10^{h}	0.66 ± 0.09
3.	Puncture	50	183	A	79	43.17^{k}	1.58 ± 0.25
				B	58	31.69^{k}	1.16 ± 0.16
				C	35	19.13^{l}	0.70 ± 0.07
				D	11	6.01^{m}	0.22 ± 0.03

Percentage bearing different superscript differ significantly ($P<0.05$) within each retrieval method

Table 6. Efficiency of oocyte retrieval systems based on comparison of cumulus mass expansion potentiality of each grade of oocytes

Sl. No.	Grade of COCs	Oocyte retrieval methods								
		Aspiration			Slicing			Puncture		
		Oocytes kept for maturation	Oocyte showing cumulus expansion	Expanded COCs (%)	Oocytes kept for maturation	Oocyte showing cumulus expansion	Expanded COCs (%)	Oocytes kept for maturation	Oocyte showing cumulus expansion	Expanded COCs (%)
1	A	65	54	83.08^{a}	99	69	69.70^{a}	81	57	70.37^{a}
2	B	41	28	68.29^{b}	79	42	53.0^{c}	58	36	62.07^{c}
3	C	38	17	44.74^{d}	51	18	35.29^{e}	39	15	38.46^{e}
4	Culture grade	106	82	77.36^{f}	178	111	62.36^{f}	139	93	66.91^{f}
5	Overall	144	99	68.75^{g}	229	129	56.33^{g}	178	108	60.67^{g}

Percentage bearing different superscripts in same column differ significantly among retrieval method ($P<0.05$)

Table 7. Effect of grade of COCs on cumulus expansion rate within each retrieval method on IVM

Sl. No.	Retrieval technique	Grade of oocyte	Oocyte kept for maturation	Oocyte showing cumulus expansion	Expanded COCs (%)
1	Aspiration	A	65	54	83.08[a]
		B	41	28	68.29[a]
		C	38	17	44.74[b]
		Overall	144	99	68.75
2	Slicing	A	99	69	69.70[c]
		B	79	42	53.01[d]
		C	51	18	35.29[e]
		Overall	229	129	56.33
3	Puncture	A	81	57	70.37[f]
		B	58	36	62.07[f]
		C	39	15	38.46[h]
		Overall	178	108	60.67

Percentage bearing different superscripts in same column differ significantly among retrieval method ($P<0.05$)

Table 8 Efficiency of oocyte retrieval method based on comparison of nuclear maturation rate under each quality grade of oocyte

Sl. No.	Grade of oocyte	Methods of oocytes retrieval														
		Aspiration					Slicing					Puncture				
		No. of oocytes examined	MII	Polar body	Per cent of nuclear maturation	Per cent of polar body extrusion	No. of oocytes examined	MII	Polar body	Per cent of nuclear maturation	Per cent of polar body extrusion	No. of oocytes examined	MII	Polar body	Per cent of nuclear maturation	Per cent of polar body extrusion
1	A	22	18	10	81.80^{a}	45.5^{1}	19	15	11	78.94^{a}	42.1^{1}	25	20	16	80.0^{a}	44.0^{1}
2	B	14	8	4	57.10^{b}	28.6^{2}	12	5	3	41.67^{b}	25.0^{2}	19	10	8	52.63^{b}	26.3^{2}
3	C	13	5	2	38.46^{c}	15.4^{3}	7	2	1	28.57^{c}	14.3^{3}	13	4	4	30.77^{c}	15.4^{3}
4	Culture grade	36	26	14	72.22^{d}	38.9^{4}	31	20	14	64.52^{d}	35.5^{4}	44	30	24	68.2^{d}	38.6^{4}
5	Overall	49	31	16	63.27^{e}	32.7^{5}	41	22	15	53.66^{e}	29.3^{5}	57	34	28	59.65^{e}	33.3^{5}

Percentage bearing different superscripts in same row differ significantly ($P<0.05$)

Table 9. Effect of oocyte grades under each retrieval method on nuclear maturation rate

Sl. No.	Retrieval technique	Grade of oocyte	Number of oocytes examined	MII	PB	Percentage of nuclear maturation	Percentage of polar body extrusion
1.	Aspiration	A	22	18	10	81.80^{a}	45.5^{a}
		B	14	8	4	57.10^{a}	28.6^{a}
		C	13	5	2	38.46^{b}	15.4^{b}
2.	Slicing	A	19	15	8	78.94^{c}	42.1^{c}
		B	12	5	3	41.67^{d}	25.0^{d}
		C	7	2	1	28.57^{d}	14.3^{d}
3.	Puncture	A	25	20	11	80.00^{e}	44.0^{e}
		B	19	10	6	52.63^{e}	26.3^{e}
		C	13	4	2	30.77^{f}	15.4^{f}

Percentage bearing different superscripts in same column differ significantly among retrieval method ($P<0.05$)

Fig.1. Effect of three harvesting techniques on the yield of oocytes.

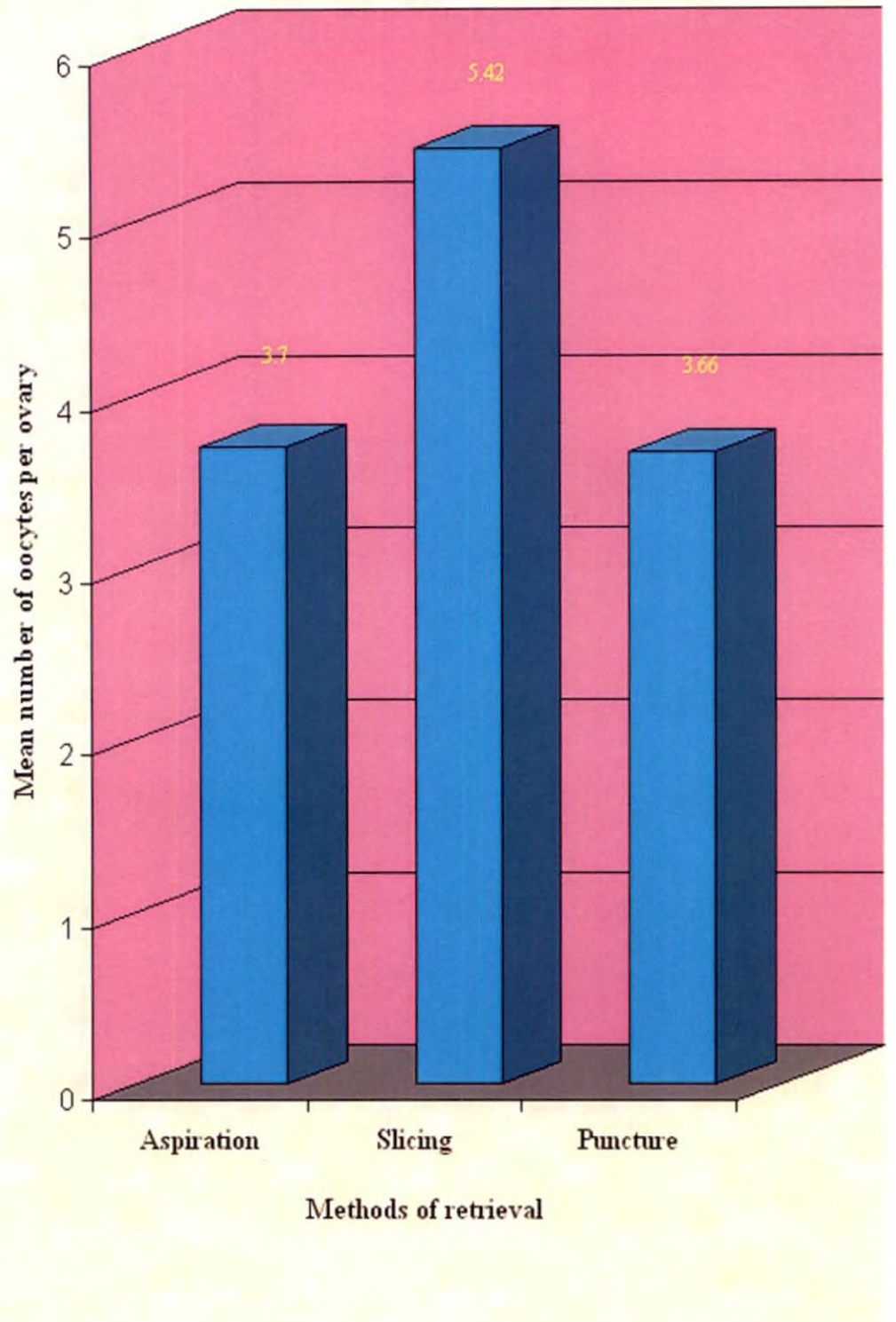

Fig.2.Effect of retrieval technique on the yield of different grades of oocytes.

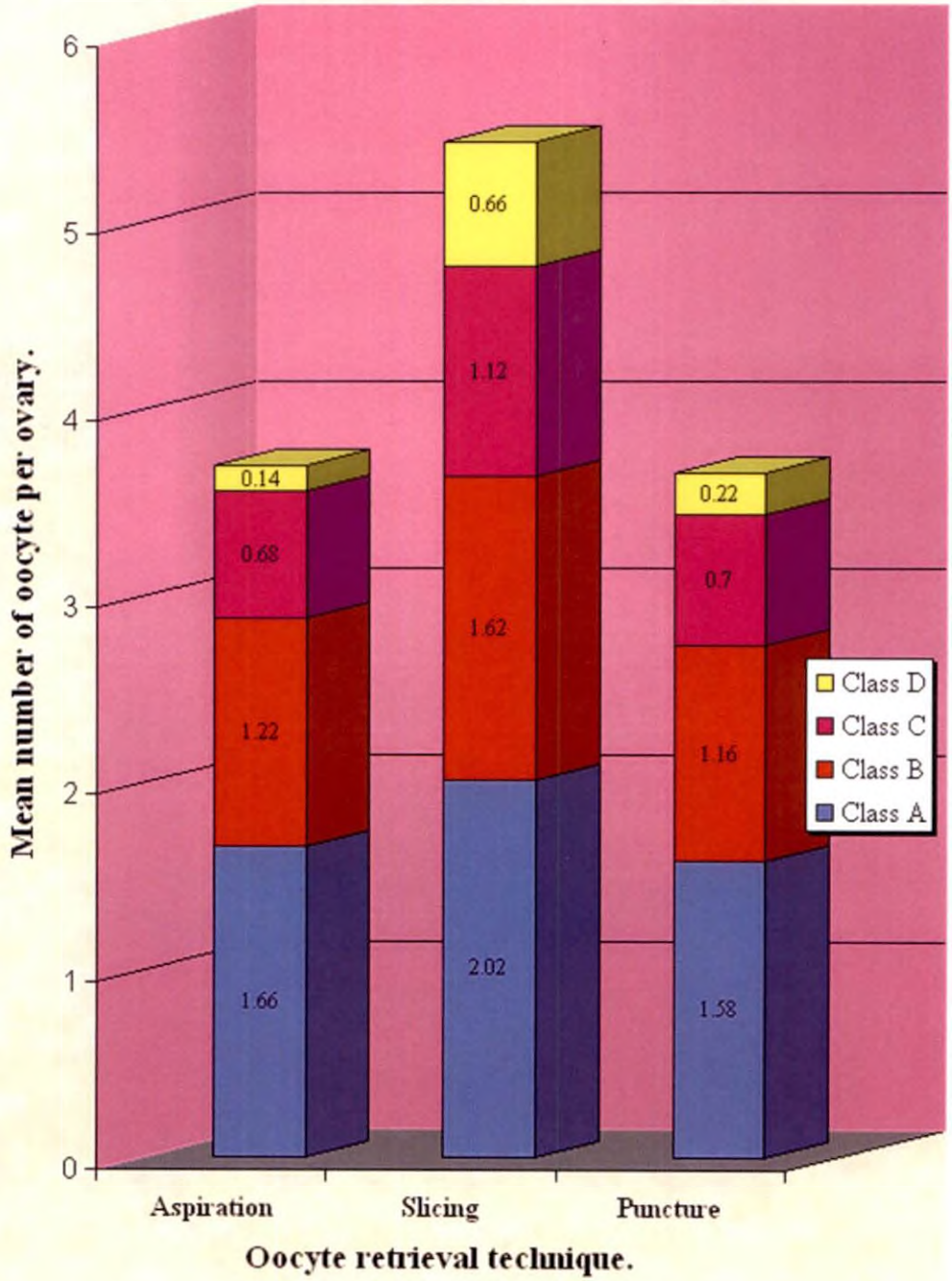

Fig.3. Comparitive efficiency of retrieval technique on quality grades of COCs

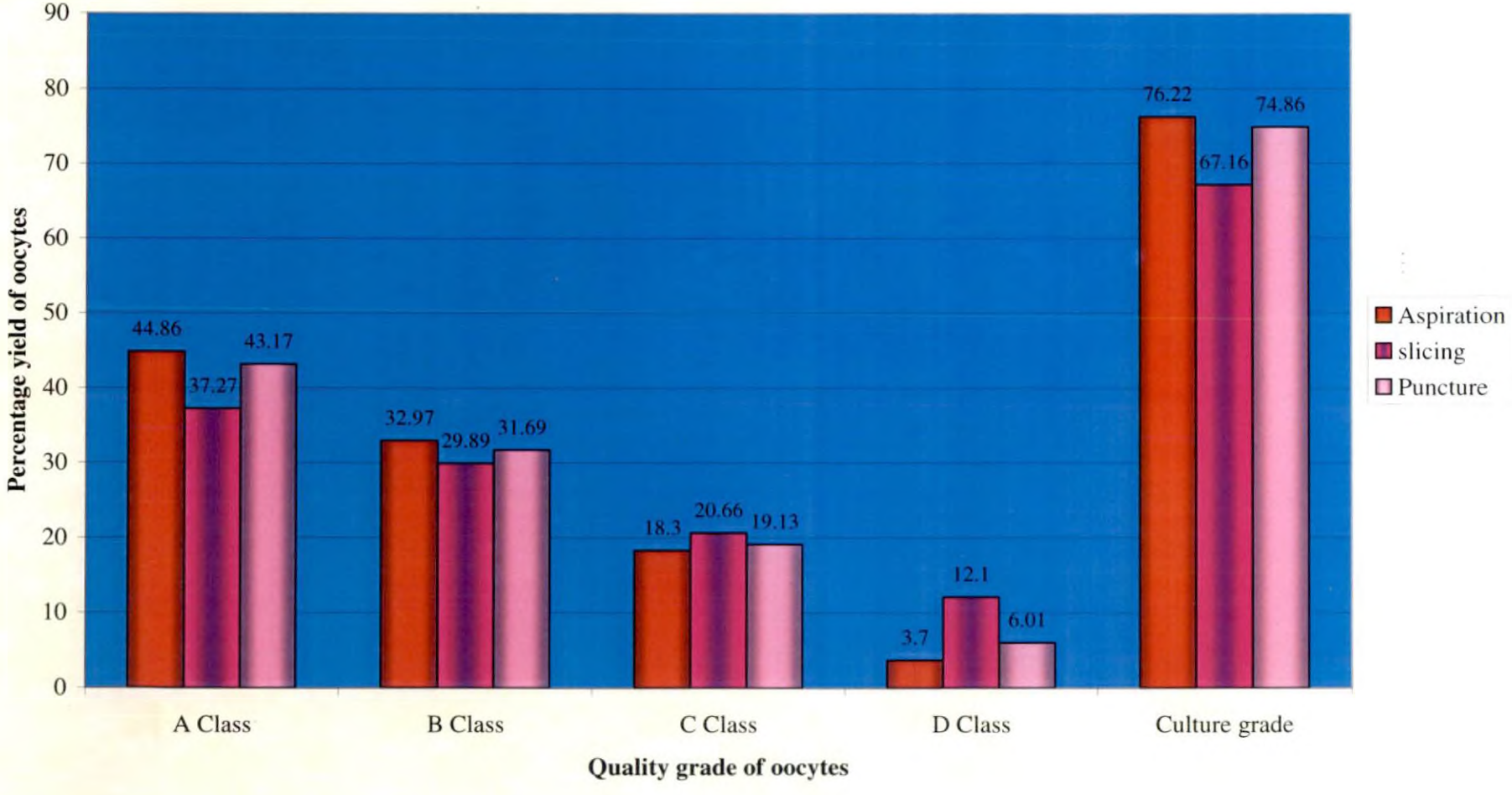

Fig.4. Comparitive efficiency of retrieval technique on quality grades of COCs.

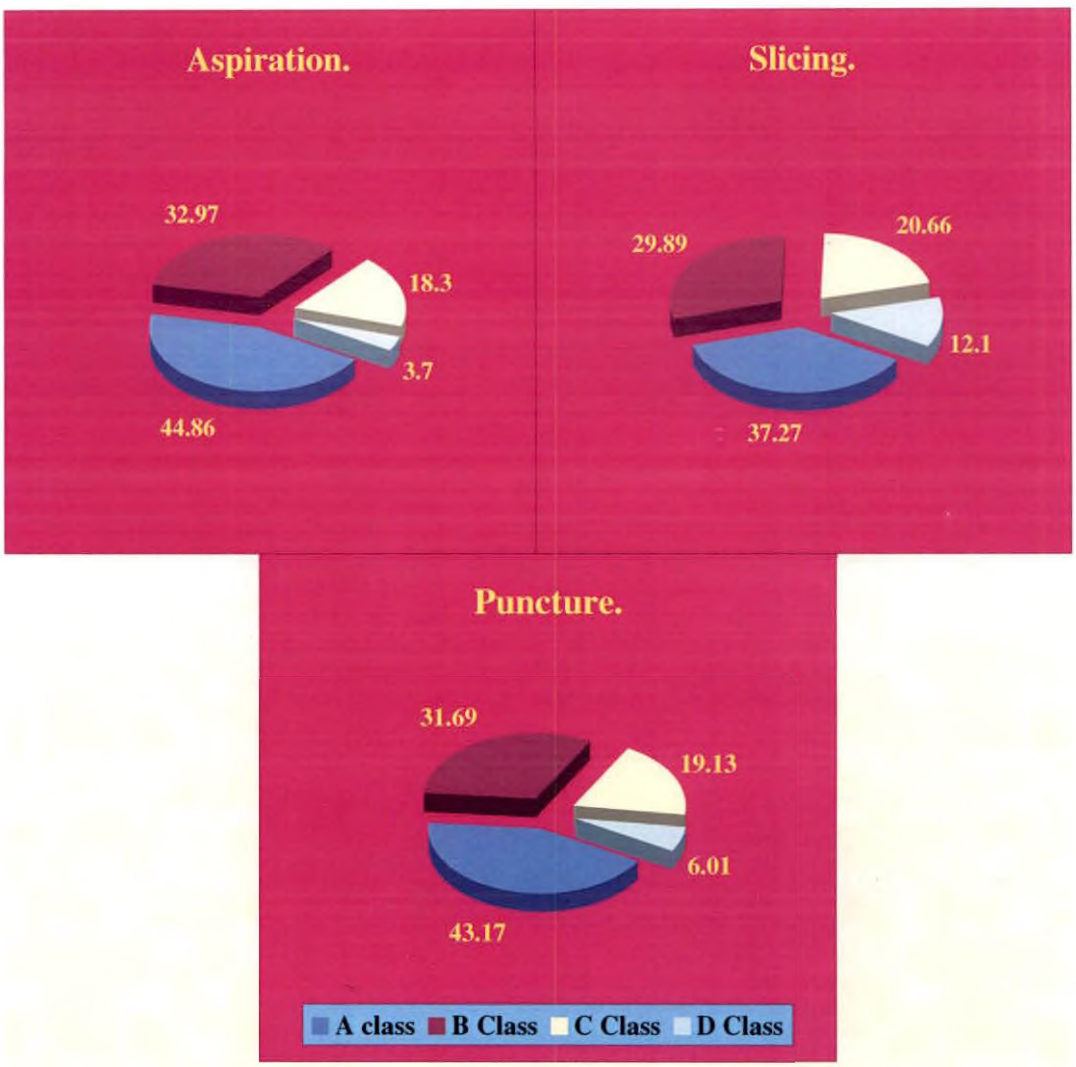

Fig.5. Efficiency of oocyte retrieval systems based on comparison of cumulus mass expansion potentiality of each grade of oocytes

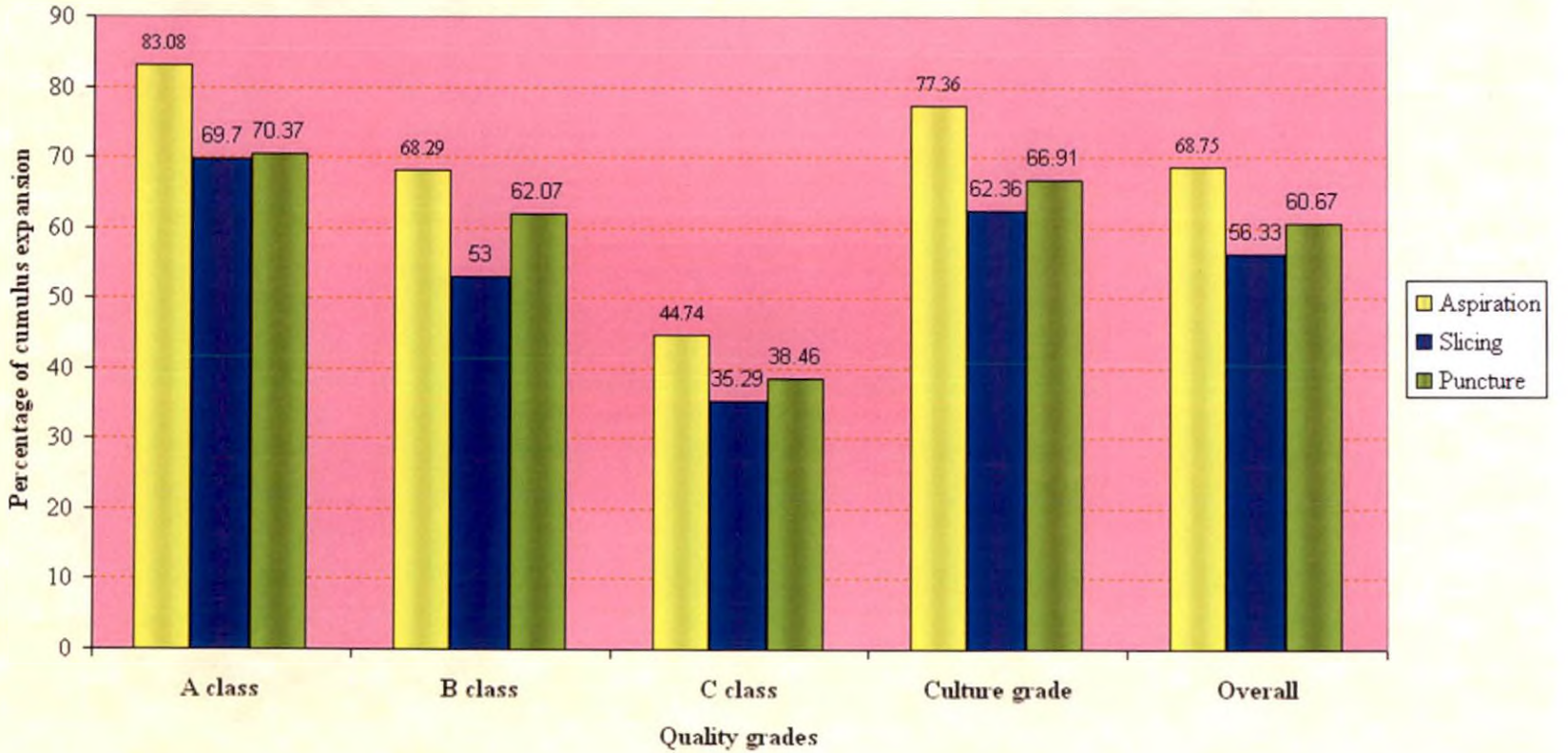

Fig.6. Effect of COCs morphology on cumulus expansion rate within each retrieval method on IVM

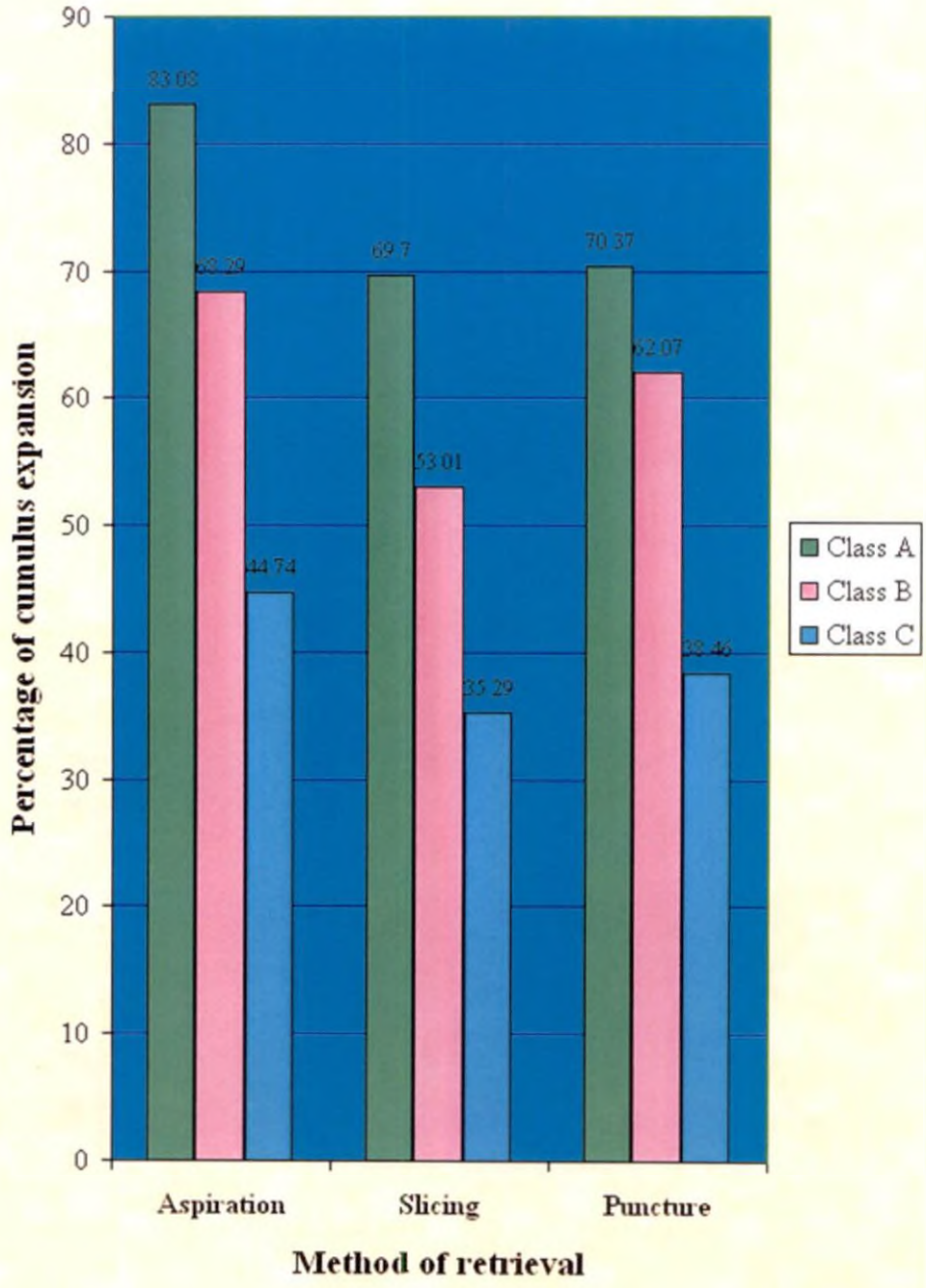

Fig.7.Comparatiive effect of oocyte retrieval methods on nuclear maturation of different quality grades of oocytes.

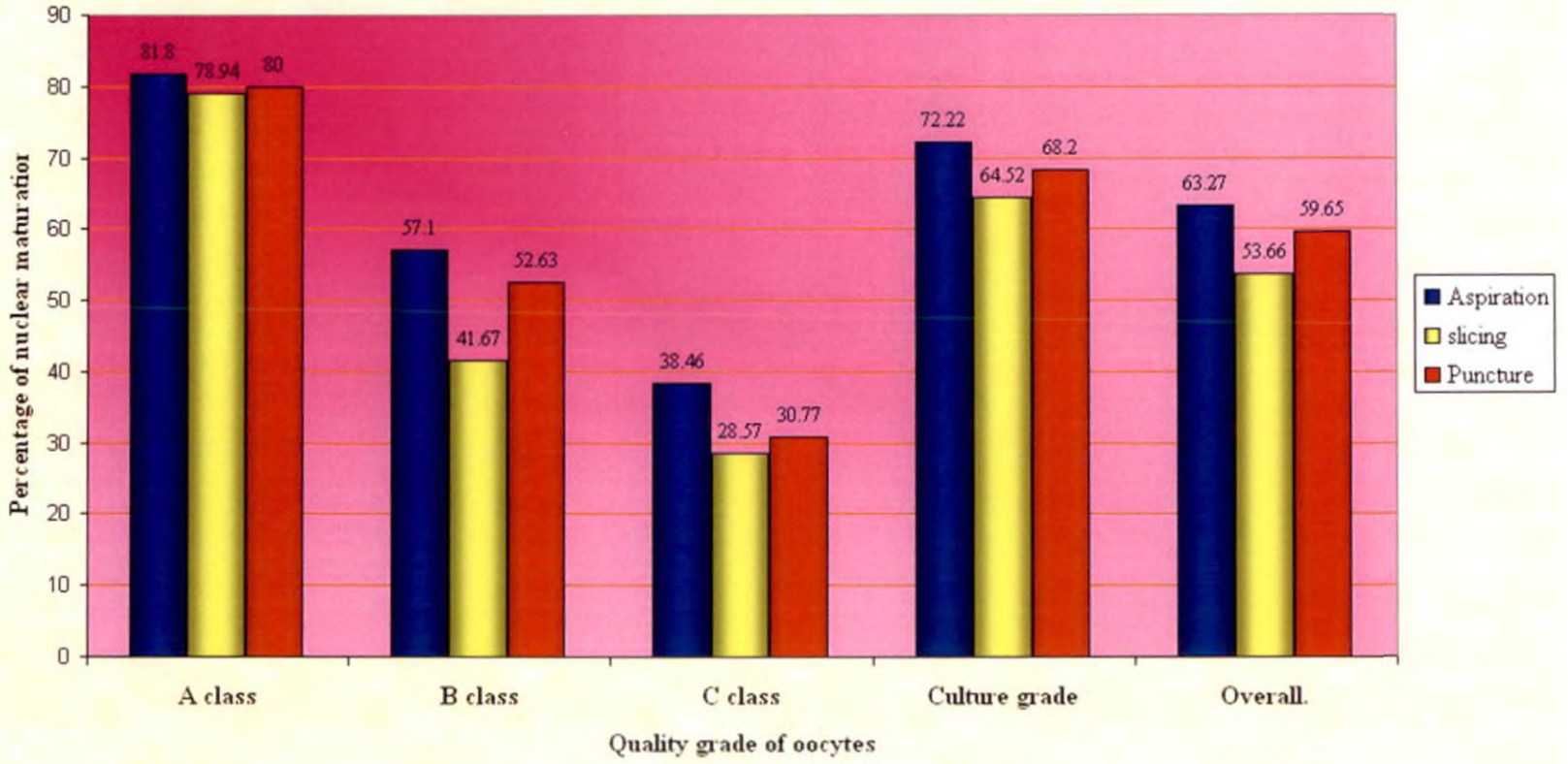

Fig .8. Effect of oocyte grades under each retrieval method on nuclear maturation rate.

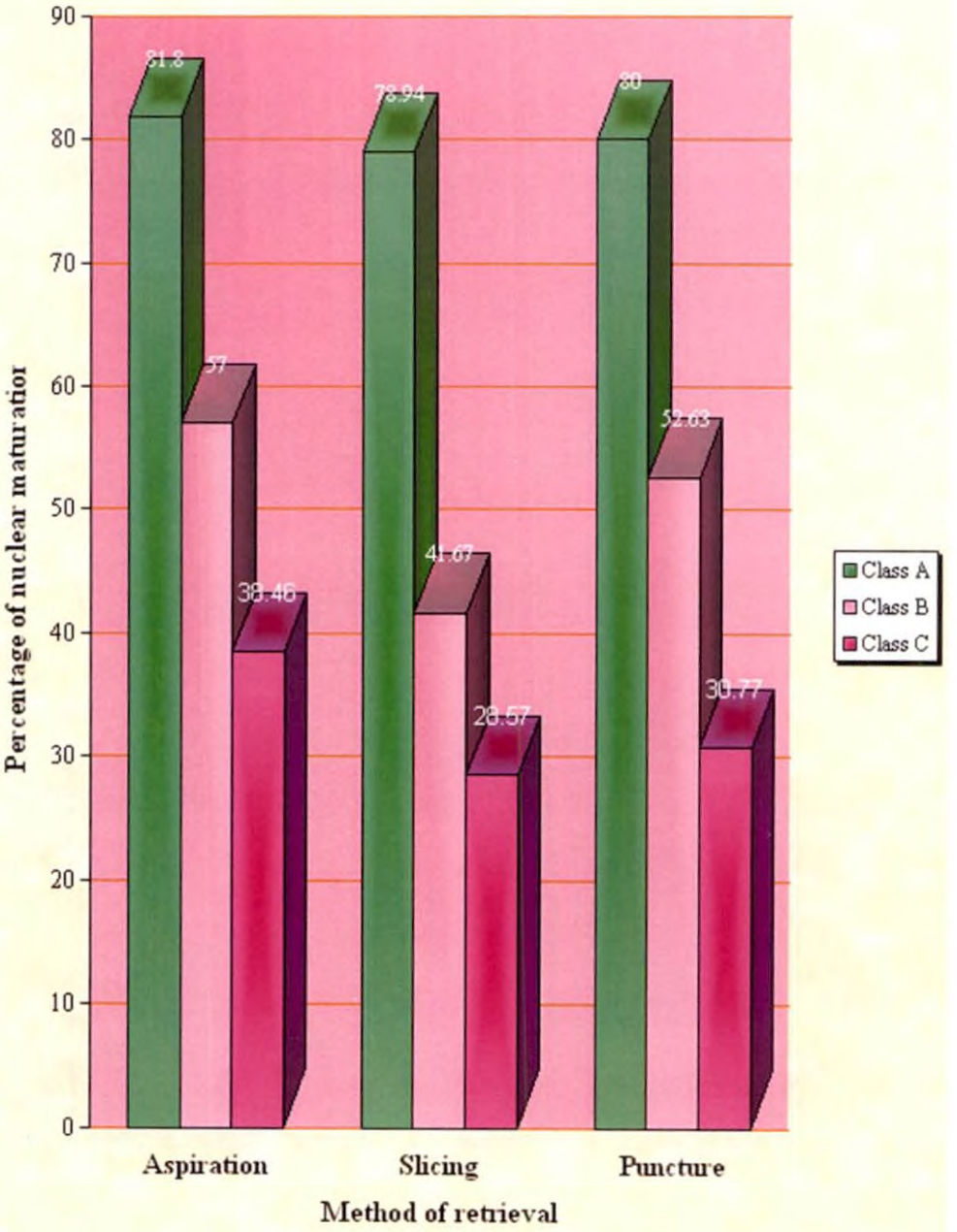

Plate 14. **Matured oocyte showing cumulus monolayer formation and mucification (200 x)**

Plate 15. **A matured oocyte showing cumulus expansion (200 x)**

Plate 16. **Two matured oocytes showing cumulus expansion (100 x)**

Matured Oocytes - Mucification and Expansion of Cumulus Cells.

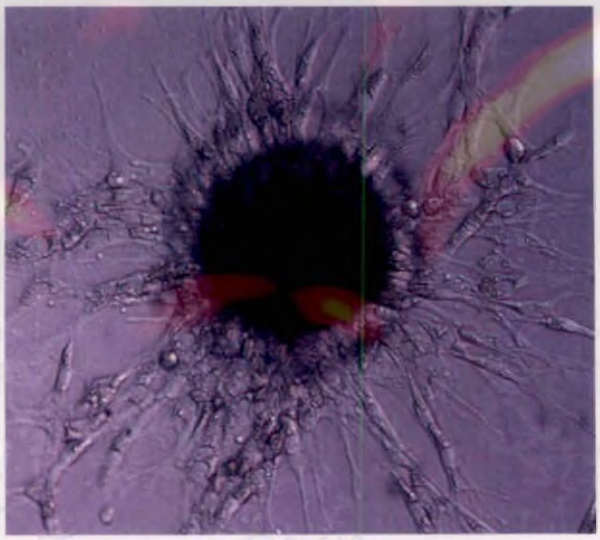

Plate. 14

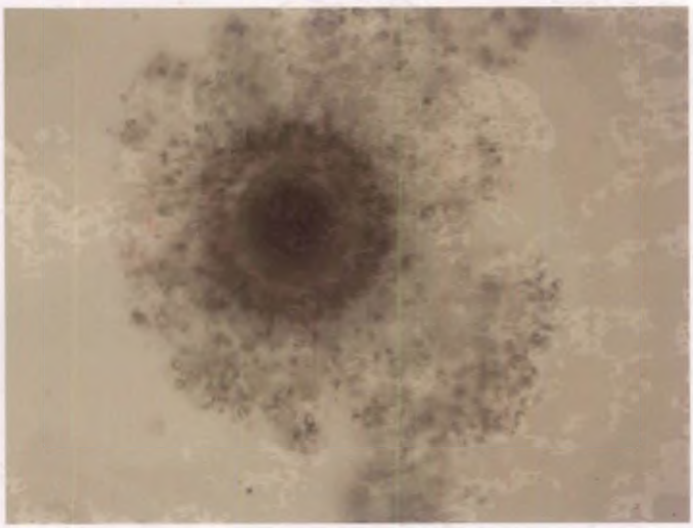

Plate. 15

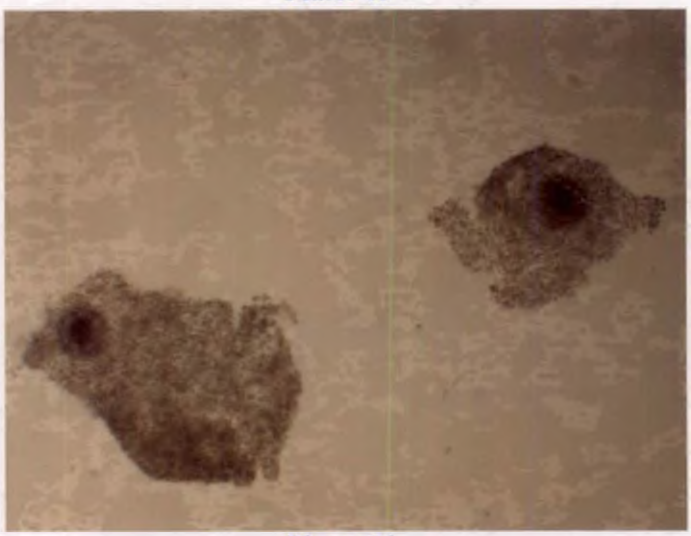

Plate. 16

Plate 17&18 **Matured oocytes with extruded polar body (400 x) PB – Polar Body, Z – Zona Pellucida**

Plate 19. **One mature oocyte with extruded polar body and increased perivitelline space (400 x). PB – Polar Body, PV – Perivitelline space, Z – Zona pellucida**

Matured Bovine Oocytes - Extruded First Polarbody

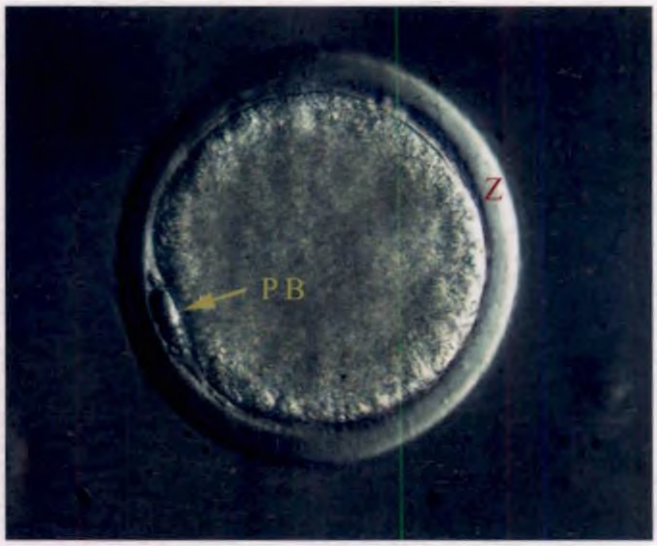

Plate. 17

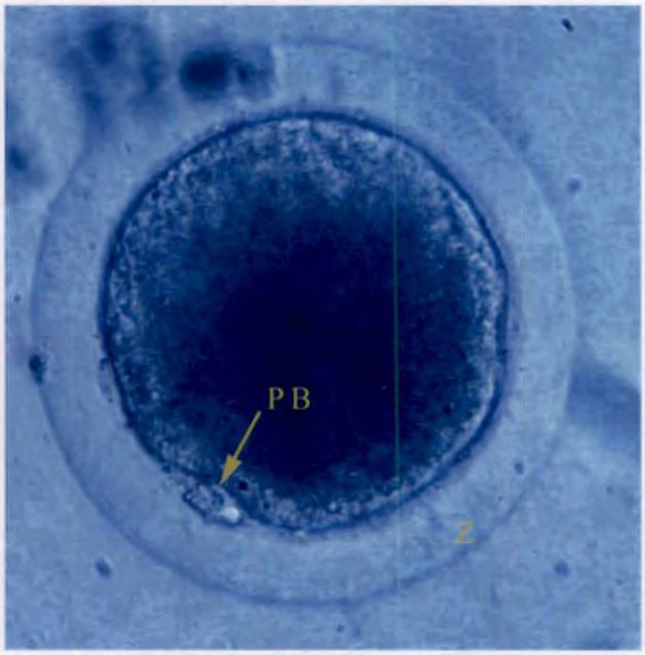

Plate. 18

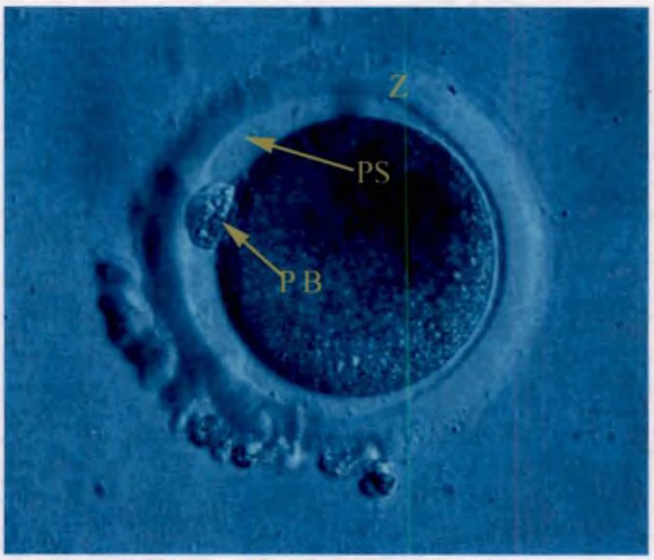

Plate. 19

Plate 20. **Oocyte showing Germinal vesicle stage. Nuclear membrane is visible (400 x)**

Plate 21. **Oocyte showing Germinal vesicle Break down stage (GVBD) (400 x)**

Plate 22. **Oocyte after GVBD, with condensed chromatin mass (400 x)**

Plate 23. **Oocyte with chromosomes arranged in equatorial plane at metaphase I (MI) stage (400 x)**

Plate 24. **Oocyte showing Metaphase II plates and (400 x). PB – Polar Body, M II – Metaphase II**

Plate 25. **Oocyte showing Metaphase II plates and extruded polar body under aceto orcein staining (400 x). PB – Polar Body, M II – Metaphase II**

Nuclear Maturation - Sequential Changes.

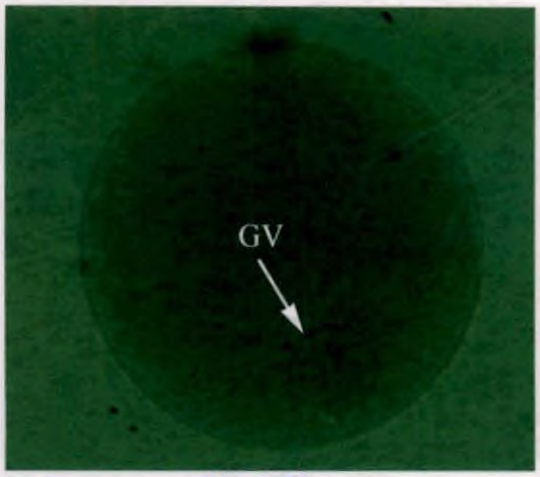

Plate. 20

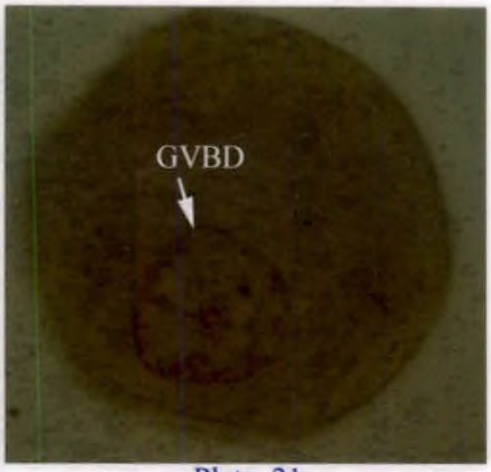

Plate. 21

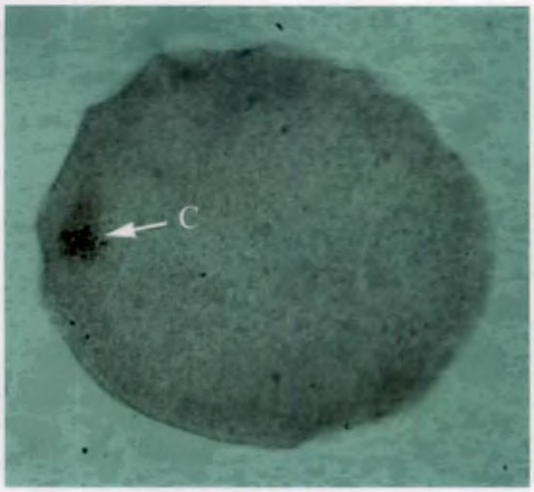

Plate. 22

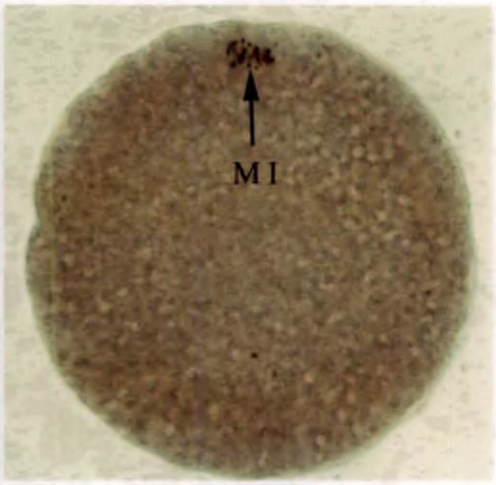

Plate. 23

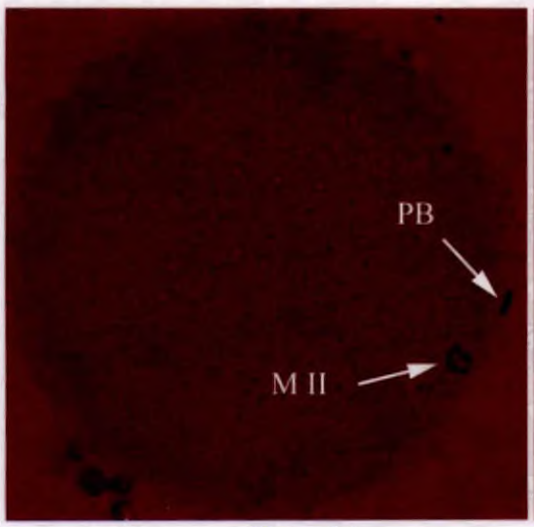

Plate. 24

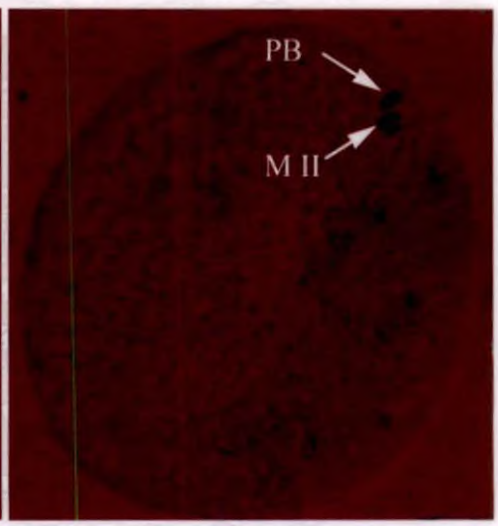

Plate. 25

Plate 26&27 **Chromosomes of maturing bovine oocytes (MI stage) – 1% aceto-orcein stain (400 x with digital zooming)**

Chromosomes of Bovine Oocyte - Aceto Orcein Stain

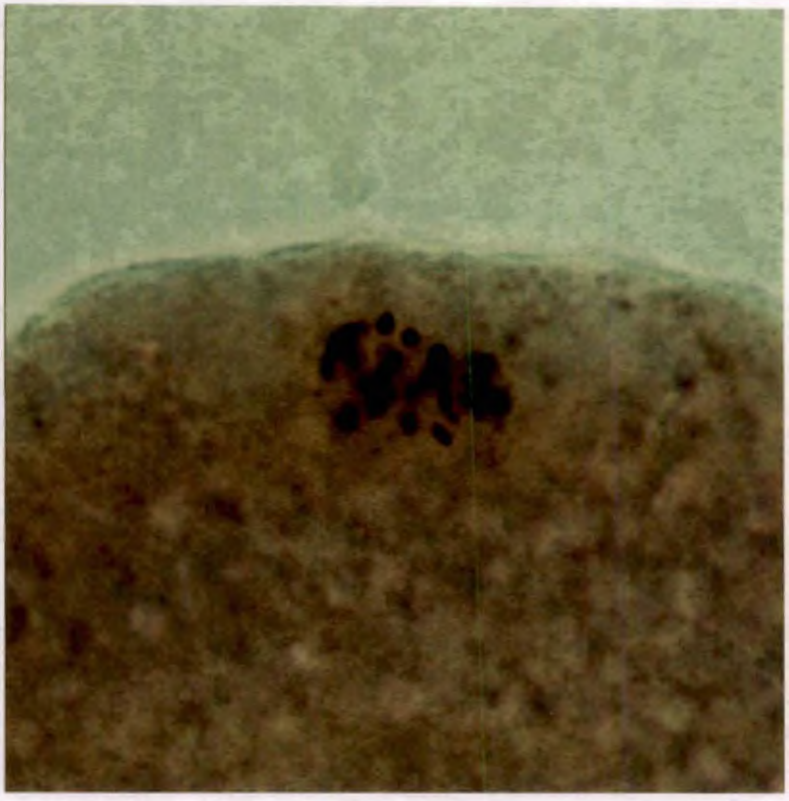

Plate. 26

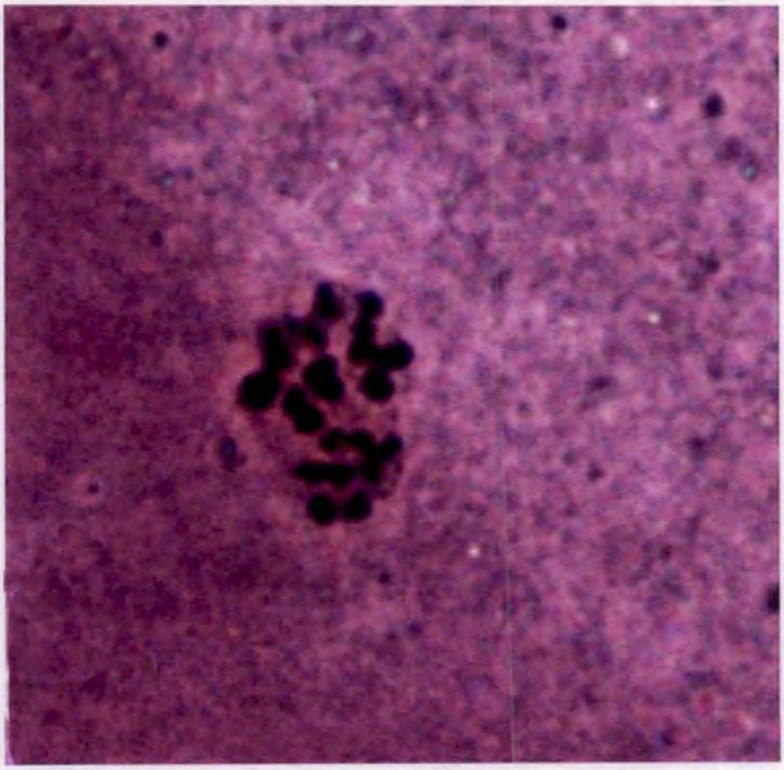

Plate. 27

Plate 28. **B class oocyte with cumulus cells under Hoechst 33342 staining-Observed under fluorescent microscope (200 x)**

Plate 29. **Oocyte at GV stage under Hoechst 33342 staining (400 x). GV – Germinal Vesicle.**

Plate 30. **Oocyte under Hoechst 33342 stain showing GVBD stage (400 x)**

Plate 31. **Oocyte under Hoechst 33342 stain showing condensed chromatin after GVBD (400 x)**

Plate 32. **Mature oocyte under Hoechst 33342 stain with Metaphase II chromosomes and polar body nucleus (400 x)**

Plate 33. **Oocyte under Fluorescein Diacetate stain – Showing a viable oocyte after maturation (400 x)**

Oocytes at Different Stages Stained with Hoechst 33342 and FDA

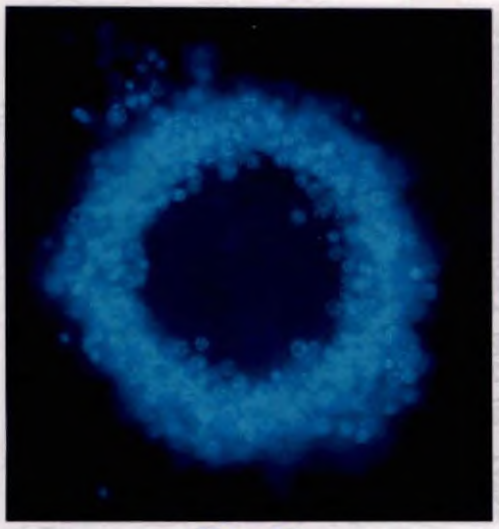

Plate. 28

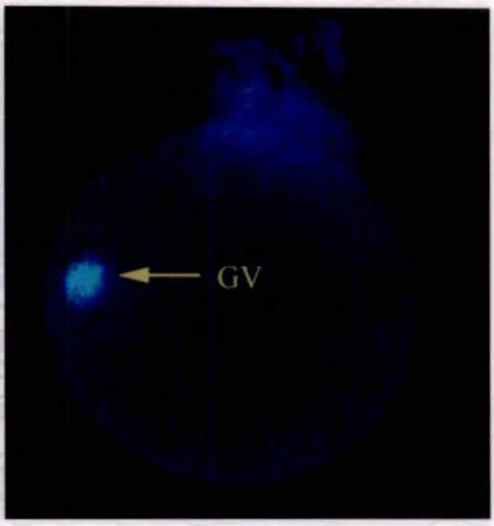

Plate. 29

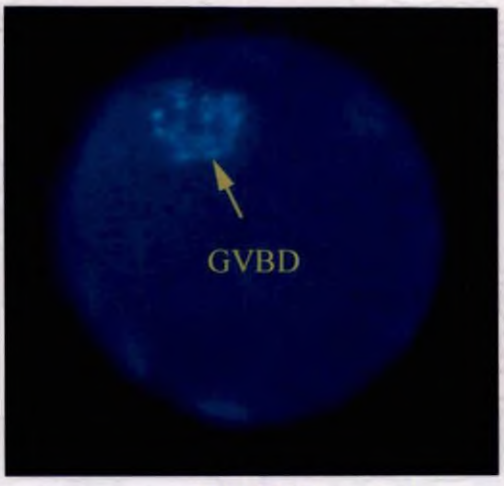

Plate. 30

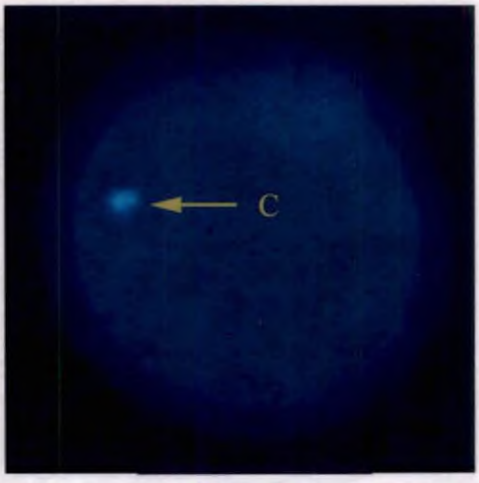

Plate. 31

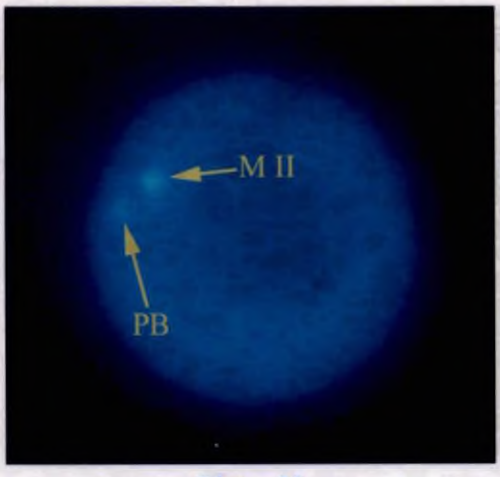

Plate. 32

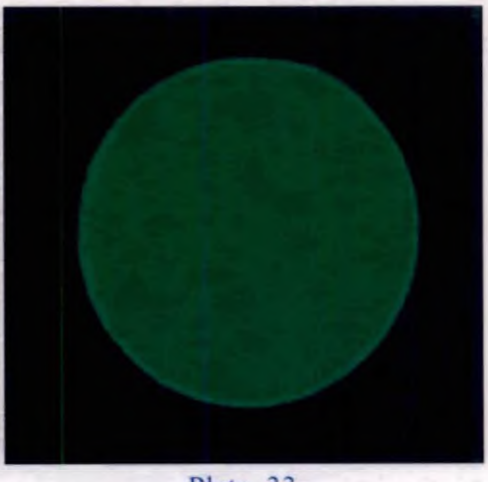

Plate. 33

Discussion

5. DISCUSSION

These experiments were designed to envisage the effect of different retrieval methods on yield of different quality grades of oocytes, and to understand the effect of retrieval methods and quality grades on the *in vitro* maturation of bovine oocytes.

5.1 EXPERIMENT 1 — EFFECT OF THREE HARVESTING TECHNIQUE ON THE YIELD OF OOCYTES PER OVARY

Yield of COCs under different retrieval system is shown in the Table 3 and Fig 1. Aspiration, slicing and puncture yielded 3.7 ± 0.43, 5.42 ± 0.56 and 3.66 ± 0.43 COC per ovary. Slicing yielded significantly higher number of COC/ovary. Aspiration and puncture not differed significantly in yield of COCs per ovary.

In aspiration method the yield of oocyte per ovary was in agreement with the results obtained for Katska and Smorag (1984); Mermillod *et al.* (1992); de Oliveira *et al.* (1994); Iwasaki *et al.* (1987); Baruha *et al.* (1998) and Priscilla (2001). Takagi *et al.* (1998), Hamno and Kuwayama (1993), Moreno *et al.* (1993), Carolan *et al.* (1994) and Arlotto *et al.* (1996) obtained a higher oocyte yield per ovary by aspiration technique. Yield of oocyte per ovary by aspiration technique was very high for Stringfellow *et al.* (1993) and Thonon *et al.* (1993) when compared to the results of present study.

In slicing method, results were in agreement with Priscilla (2001). A higher yield was obtained for Rieger and Loskutoff (1994) and Arlotto *et al.* (1996). When compared to the results of present study, some scientists obtained very high yield of oocyte per ovary by slicing method in bovines (Iwasaki *et al.*, 1987; Takagi *et al.*, 1998; Sato *et al.*, 1990; Carolan *et al.*, 1992,Xu *et al.*, 1992; Hamno and Kuwayama, 1993 and Carolan *et al.*, 1994).

Results obtained for Sato *et al.* (1990) and Priscilla (2001) in cattle was in agreement with the present results in puncture method. Puncturing of buffalo ovaries resulted a lower yield of COC/ovary when compared to the present study in cattle (Das *et al.*, 1996a). Puncturing of goat ovaries yielded a comparable oocyte recovery rate to the present study (Das *et al.*, 1996b). But the yield of oocyte in sheep was very high when compared to present result in cattle (Das *et al.*, 1996c).

This experiment yielded significantly higher COC per ovary in slicing method when compared to aspiration. This was in agreement with the observations of Iwasaki (1987); Takagi *et al.* (1998); Hamno and Kuwayama (1993); Carolan *et al.* (1994), Priscilla (2001) and Gordon (2003) in cattle. Slicing method also yielded high number of oocyte per ovary than puncture method, which agreed with the observations of Sato *et al.* (1990) and Pricilla (2001) in cattle. Aspiration and puncture methods not differed significantly in the present study. These results disagreed with results of Priscilla (2001) in cattle. Priscilla (2001) obtained more COC per ovary in aspiration than puncture.

The reason for more COC yield per ovary in slicing method can be attributed to the fact that, by slicing oocytes from surface follicles as well as follicles of deeper cortical stroma were released, whereas by puncture and aspiration oocytes from surface follicles alone were released (Das *et al.*, 1996a; Pawshe *et al.*, 1994). So this experiment point to the fact that as far as oocyte recovery technique is concerned maximum efficiency is in the slicing method than aspiration or puncture.

5.2 EXPERIMENT 2 — EFFECT OF RETRIEVAL TECHNIQUE ON THE QUALITY GRADE OF COCS

5.2.1 Comparative efficiency between Retrieval Techniques

5.2.1.1 Class A

Yield of grade A oocyte by aspiration slicing and puncture methods are shown in Table 4 and Fig.3. By aspiration method 44.86% COCs obtained were

belonging to A class. This was in agreement with the results obtained for Katska (1984) and Katska and Smorag (1984). Slightly lower percentage of A class oocytes were obtained for Iwasaki *et al.* (1987); Arlotto *et al.* (1990) and Hamno and Kuwayama (1993). Whereas Madison *et al.* (1992) and Carolan *et al.* (1994) obtained much lower percentage of A class oocytes by aspiration method when compared to the results of present study. Much higher percentage of A class oocytes were obtained for Priscilla (2001) by aspiration method than present results.

Average yield of A class oocyte by aspiration method was 1.66 ± 0.25 per ovary. This result was comparable to the result obtained for Iwasaki *et al.* (1987) and Priscilla (2001). This is much lower than the result of Katska and Smorag (1984) and Carolan *et al.* (1994).

When slicing method was employed, 37.27 % A class oocytes were obtained in the present study. This agreed with the results of Carolan *et al.* (1994). For Hamno and Kuwayama (1993) and Priscilla (2001) very high yield of A class oocytes were obtained by adopting slicing technique in cattle.

Average number of A class oocyte per ovary was 2.02 ± 0.25. This was in agreement with the work of Priscilla (2001) and Goswami *et al.* (2004). Results of Carolan *et al.* (1994) was much higher than this.

On puncturing bovine ovaries for oocyte retrieval 43.17% of oocytes obtained were of A class oocytes. This result disagreed with the results of Priscilla (2001) in cattle and Wani *et al.* (1999) in sheep who obtained 62.84% and 54.7%, A class oocytes respectively by puncturing method. Slightly lower recovery rate of A class oocytes were reported in goats by Das *et al.* (1996b) adopting puncture method. Das *et al.* (1996c) when punctured sheep ovaries very low yield of A class oocytes were obtained compared to the present study results.

By puncturing method 1.58 ± 0.25 A class COC/ovary was obtained. This was lower than the results of Priscilla (2001) in cattle when same method was used.

On statistical analysis, there was no significant difference between these methods in yield of A class oocytes. This agreed with the findings of Priscilla (2001). This disagreed with the results of Hamno and Kuwayama (1993) and Carolan *et al.* (1994). They obtained proportionately more oocytes in slicing method than aspiration method.

When mean yield of A class COCs per ovary was analyzed, more number of COCs were obtained by slicing method. This was in agreement with the results of Carolan *et al.* (1994) and Priscilla (2001) in cattle. Slicing method recovered more number of oocyte per ovary (Experiment 1). So proportionately more A class oocytes were yielded by slicing technique. Aspiration and puncture method yielded almost equal number of A class COC per ovary.

Results of experiment 2 clearly indicate that there was no significant difference between the methods of retrieval in proportionate yield of A class oocytes. But when mean number of A class oocytes per ovary was analyzed more oocytes were obtained in slicing method. So aspiration, slicing and puncture method are equally good for retrieval of bovine oocytes, but slicing produced maximum number of oocyte per ovary. So slicing has an advantage when the numbers of ovaries are limited, since it yielded maximum number of oocyte per ovary. This was an important observation as far as conservation of endangered species or breeds were concerned. Maximum good quality oocytes are produced by slicing method from a single dead animal.

5.2.1.2 B Class

Aspiration, slicing and puncture yielded 32.97%, 29.89% and 31.69% B class oocytes from bovine ovaries. Results are shown in table 4 and Fig 3. Yield of B class oocytes were higher than that obtained for Carolan *et al.* (1994) and

Priscilla (2001). Yield of B class oocytes were slightly lower than the results of Naik *et al.* (1999) in buffaloe. Gogoi *et al.* (2001) obtained very low percent of B class oocyte by slicing.

Mean number of B class COC obtained by aspiration in the present study was 1.22 ± 0.12 COC/ovary. This was higher than the result obtained for Priscilla (2001), whereas a result of Carolan et al. (1994) was slightly higher.

Slicing of bovine ovaries yielded 29.89% B class oocytes. This result was slightly higher than the result obtained for Priscilla (2001). Result of Carolan (1994) was very low when compared to this result. These variations in yield can be attributed to the individual variation in the slaughtered animals, its breed, nutritional status, age of animal and season of the year (Dominguez, 1995).

When slicing was adopted for oocyte recovery, mean number of oocyte per ovary was 1.62 ± 0.20. This was in agreement with the results of Priscilla (2001) and Goswami *et al.* (2004). Result of Carolan *et al.* (1994) was higher than the present study results.

Proportion of COCs obtained by puncture method was 31.69 per cent. This was higher than the proportion of oocytes obtained for Priscilla (2001) by puncture method. But the results of Das *et al.* (1996b) and Das *et al.* (1996c) in goat and sheep agreed with these results.

Number of B class COC per ovary by puncture method was 1.16 ± 0.16. This was higher than the observation of Priscilla (2001) in cattle.

5.2.1.3 C class

When aspiration was the method of oocyte recovery, 18.3% of oocytes were of C class oocytes. This was slightly higher than the result of Katska (1984), Katska and Smorag (1984), Carolan *et al.* (1994) and Priscilla (2001). However when Gogoi *et al.* (2001) aspirated goat oocytes a slightly lower

percentage of C class oocytes were obtained. Moreno *et al.* (1993) obtained a higher percentage of C class oocytes than the present study.

Aspiration of bovine ovaries yielded 0.68 ± 0.20 C class COC per ovary. This was in agreement with the results of Katska and Smorag (1984) and Priscilla (2001). Moreno *et al.* (1993) and Carolan *et al.* (1994) yielded slightly higher percentage of C class oocyte per ovary by aspiration technique.

Proportionate yield of C class oocyte by slicing method in present study was 20.66%. This was in agreement with the result of Carolan *et al.* (1994) who obtained 17% C class oocyte by slicing method, whereas Priscilla (2001) recorded lower values than the present study.

Slicing yielded 1.12 ± 0.15 C class COC per ovary. This was higher than the results of Priscilla (2001). But this result was much lower than the result of Carolan *et al.* (1994) who obtained 7.5 C Class COC per ovary.

In present study by puncture method 19.13% of oocytes were of C class. This was higher than the results obtained for Priscilla (2001). But a comparable result was obtained in sheep for Wani *et al.* (1999).

Puncturing of ovaries released 0.70 ± 0.07 C class COC per ovary. This was slightly higher than the results of Priscilla (2001).

Aspiration, slicing and puncture methods did not exhibit significant difference in the yield of C class oocytes. Average yield of oocyte per ovary was maximum by slicing method. Aspiration and puncture exhibited similar yield of C class oocyte per ovary.

5.2.1.4 D class

When aspiration method was employed for oocyte collection, 3.7% oocyte were of D class oocytes. This was slightly lower than the results of Katska and Smorag (1984); Priscilla (2001) and Vassena *et al.* (2003). Carolan

et al. (1994) and Moreno *et al.* (1993) obtained a higher percentage of D class oocyte by aspiration method. Naik *et al.* (1999) in buffaloe and Gogoi *et al.* (2001) in goat also obtained higher percentage of D class oocytes.

By employing aspiration technique, mean number of oocyte per ovary was 0.14 ± 0.06. This was lower than the yield of Katska and Smorag (1984) and Priscilla (2001). Moreno *et al.* (1993) and Carolan *et al.* (1994) obtained still higher number of D class oocytes by aspiration. Katska (1984) obtained very high number of D class oocytes when compared to the present results.

Slicing method yielded 12.10 per cent D class oocytes. This agreed with Carolan *et al.* (1994) who obtained 11.5% D class oocytes by slicing method, whereas Priscilla (2001) obtained a lower percentage of D class oocytes by slicing method (8.58%) in cattle.

Mean yield of D class oocytes per ovary by slicing method was 0.66 ± 0.09. This agreed with results of Priscilla (2001) and Goswami *et al.* (2004). But Sato *et al.* (1990) and Carolan *et al.* (1994) yielded a higher number of D class oocytes per ovary by slicing method.

Puncture method recovered 6.01% D class oocytes in this study. This was lower than the results of Priscilla, (2001) in cattle, Das *et al.*, (1996b) in goat and Wani *et al.* (1999) in sheep.

Puncturing of bovine ovaries recovered 0.22 ± 0.03 D class oocyte per ovary. This was comparable to the results of Priscilla (2001). Sato *et al.* (1990) and Wani *et al.* (1999) recovered high number of denuded oocyte per cattle and sheep ovary respectively (2 oocyte/ovary). Pawshe *et al.* (1994) and Das *et al.* (1996a) when performed puncture in goat and buffaloe ovaries respectively slightly high yield of D class oocyte per ovary was obtained.

By slicing method, recovery of denuded oocytes was significantly higher than the aspiration or puncture method. This was in agreement with the observation of Das *et al.* (1996a). This is because slicing recovers oocytes from a

heterogeneous population of follicles, which includes surface follicles, follicles located deep in cortical stroma, fully grown follicles and growing follicles, small and large follicles.

5.2.1.5 Culture Grade Oocytes

In culture grade oocytes, aspiration yielded 76.22% COCs. This was comparable to the results of Hamno and Kuwayama (1993) and Vassena *et al.* (2003). Lower recovery rate was obtained for Katska (1984) and Dominguez (1995) in bovines. Moreno et al. (1993) observed very low recovery rate in bovine when compared to the present results. Naik *et al.* (1999) also obtained a lower recovery rate in buffaloe.

On aspiration 2.88 ± 0.19 were of culture grade oocytes. This was in agreement with the results of Moreno *et al.* (1993) in cattle. Katska and Smorag (1984) obtained slightly higher number of culture grade oocytes in cattle. Takagi *et al.* (1992) obtained very high number of bovine oocytes with more than 3 layers of cumulus cells in comparison to present study.

By slicing method 67.16 per cent COCs were of culture grade. By slicing method 3.64 ± 0.23 COCs belonging to culture grade were obtained. This was in agreement with the range of culture grade oocytes obtained for Takagi *et al.* (1992) in cattle but lower than the mean yield. Sato *et al.* (1990) also obtained higher number of multi-layered oocytes by slicing method in cattle.

When puncturing was the method of oocyte retrieval 74.86% of oocytes were of culture grade. Mean number of oocytes belonging to culture grade was 2.74 ± 0.21. Sato *et al.* (1990) obtained higher number of oocytes belonging to culture grade by puncture method.

Aspiration slicing and puncture did not differ significantly in proportionate yield of culture grade oocytes. By slicing method, maximum numbers of culture grade oocytes were obtained. This agreed with the

observation of Das *et al.* (1996a), who obtained maximum number of pooled culture grade oocytes by slicing method in buffaloe.

Aspiration, slicing and puncture did not differ significantly in the proportionate yield of A, B, C and culture grade of oocytes. But D class oocytes were proportionately more in slicing method. This was because of the fact that slicing recovered oocytes from surface follicles as well as from deeper cortical stroma, whereas by puncture and aspiration only oocytes from surface follicles were released (Das *et al.*, 1996a; Pawshe *et al.*, 1994). So slicing released oocytes, which were not fully grown (Das *et al.*, 1996a).

It was observed that the mean number of oocytes/ovary in various quality grades were less in this study, when compared to most of the work in cattle done abroad. One of the reasons for this can be attributed to the fact that the present work was done in slaughtered animals, which were culled due to various reasons like advanced age, reproductive problems, etc. Most of these animals were maintained on low plane of nutrition for a prolonged period. This adversely affects the reproductive performance and number of follicles on the ovary. In addition to that, the numbers of follicles are less in the ovary of *Bos indicus* cattle compared to *Bos taurus* cattle (Dominguez, 1995).

By aspiration method speedy recovery of oocytes were possible compared to slicing or puncture. This was in agreement with the report of Gordon (2003). One of the disadvantages of slicing method was that it resulted in more debris and interfered with the isolation of oocytes from medium. Wani *et al.* (1999) made a similar observation in sheep.

5.2.2 Comparative Efficiency Between Yield of Different Quality Grades of COCs

5.2.2.1 Aspiration

When aspiration was the method of retrieval, yield of different quality grades of COC are shown in Table 5 and Fig 4. A, B, C and D grade oocyte yield was 44.86, 32.97, 18.30 and 3.7 per cent respectively. Yield of A class oocytes

were comparable to the results of Katska, (1984), Katska and Smorag, (1984). Slightly lower percentage of A class oocytes were obtained for Iwasaki *et al.*, 1987, in cattle.

Mean number of A class COCs obtained in aspiration technique was 1.66 ± 0.25 COCs per ovary. This was lower than the values obtained for Katska and Smorag, (1984), Carolan *et al.*, (1994) and Iwasaki *et al.*, (1987).

Proportionate yield of B class oocytes were comparable to that of Moreno *et al.* (1993) in cattle. A lower proportion of B class oocytes were yielded for Carolan *et al.* (1994), Priscilla (2001) and Gogoi *et al.* (2001), in cattle.

By aspiration technique, mean number of oocyte per ovary was 1.22 ± 0.15. This was in between the results obtained for Carolan *et al.* (1994) and Priscilla (2001).

Proportionate yield of C class oocytes were lower than the results obtained for Moreno *et al.* (1993). But this was higher than the results of Katska (1984), Katska and Smorag (1984), Carolan (1994), Priscilla (2001) and Gogoi *et al.* (2001).

Yield of C class oocyte per ovary by aspiration technique was 0.68 ± 0.2. This was in agreement with the results of Katska and Smorag (1984) and Priscilla (2001), however a higher C class COC yield was obtained for Moreno *et al.* (1993) and Carolan *et al.* (1994) in cattle.

Percentage yield of D Class oocytes by aspiration technique was slightly lower than the results of Katska and Smorag (1984), Priscilla (2001) and Vassena *et al.* (2003) in bovines, Moreno *et al.* (1993) and Carolan *et al.* (1994) obtained higher percentage of D class oocyte by aspiration in cattle.

Mean yield of D class oocyte per ovary was slightly lower than Katska and Smorag, 1984 and Priscilla (2001). For Moreno *et al.*, 1993 and Carolan *et*

al. (1994) yield of D class oocyte per ovary was still higher. Katska (1984) yielded very high number of D class oocyte per ovary by aspiration technique.

Statistical analysis revealed that A and B Class oocytes yielded significantly higher than C & D class oocytes. A and B grade exhibited no significant difference in yield. C class oocytes were significantly higher than D grade oocytes.

In aspiration method, largest proportion of oocyte and highest number of oocyte per ovary was A class oocyte which was in agreement with the results of Carolan *et al.* (1994) but this disagreed with Carolan *et al.* (1994) in yield of D class oocytes. They obtained second largest proportion of oocytes as D class oocytes but here it was B class. Proportionate yield of different grades of oocytes were in agreement with the result of Katska and Smorag (1984). For Katska and Smorag (1984) maximum number of oocyte per ovary was obtained in A class which was in agreement with this study. Numbers of C and D class oocytes were almost equal for Katska and Smorag (1984), but in this study C class oocytes were higher than D class oocytes. Observation of Naik *et al.* (1999) disagreed with these results, who obtained C class oocytes in maximum percentage followed by A and D class oocytes.

Result show that by aspiration method maximum yield was for A and B class oocytes. Yield of C and D class was significantly lower than A and B. D class oocytes were least in yield when compared to A, B and C class oocytes.

5.2.2.2 Slicing

When slicing was employed for oocyte retrieval percentage of A, B, C and D grade oocyte obtained was given in table 5 and Fig 4. The percentage of A grade oocyte was 37.27%. This was in agreement with the result obtained for Caroloan *et al.* (1994). Hamno and Kuwayama (1993) and Priscilla (2001) obtained very high percentage of A class oocytes by employing slicing method.

The mean number of A class COC per ovary by slicing method was 2.02 ± 0.25. This was in accordance with the results of Priscilla (2001) and Goswami *et al.* (2004). Mean number of A class oocyte per ovary for Carolan *et al.* (1994) was 17.1 when slicing was the method of retrieval.

Recovery rate of B class oocytes were 29.89% when slicing was employed. This was higher than the results of Carolan *et al.* (1994) and Priscilla (2001).

Slicing yielded 1.62 ± 0.20 B Class oocyte from bovine ovaries. Priscilla (2001) and Goswami *et al.* (2004) obtained similar results in bovines. Carolan *et al.* (1994) reported a slightly higher oocyte recovery rate when compared to the present study.

C class COC recovery rate was 20.66% when slicing was the method of recovery. This was similar to the results of Carolan *et al.* (1994) when slicing was employed for oocyte recovery. Priscilla (2001) obtained a lower proportion of C class oocyte by slicing method.

Number of C class oocyte per ovary by slicing method was 1.12 ± 0.15. This was higher than the results of Priscilla (2001) and Goswami *et al.* (2001). But was very low when compared to the results of Carolan *et al.* (1994).

Proportionate yield of D class oocyte by adopting slicing method was 12.10 per cent. A similar result was obtained for Carolan *et al.* (1994) in cattle. But Priscilla (2001) reported a value slightly lower than this in cattle.

Yield of D class COC per ovary was 0.66 ± 0.09. This result was similar to the results of Priscilla (2001) and Goswami *et al.*(2004). But Sato *et al.* (1990) and Carolan *et al.* (1994) obtained a higher number of D class oocyte per ovary by slicing method.

On statistical analysis A grade oocytes were yielded maximum proportion by slicing. This was in agreement with the results of Carolan *et al.* (1994) and

Priscilla (2001). Second largest proportion was B class oocytes followed by C class. Least proportion of oocytes yielded was D class. This was in agreement with the results of Priscilla (2001). Results of Carolan *et al.* (1994) differed from this. Carolan *et al.* (1994) obtained A class oocytes in maximum proportion followed by C, D and B class oocytes.

When mean number of oocyte per ovary was analyzed maximum oocytes were of A class followed by B, C and D. This was in agreement with the results of Carolan *et al.* (1994), Priscilla (2001) and Goswami *et al.* (2004).

5.2.2.3 Puncture

Yield of A, B, C and D class oocytes after puncture method for oocyte recovery are given in Table 5 and Fig 4. A class oocyte yield was 43.17% by puncture method. This was lower than the results of Priscilla (2001) in cattle and Wani *et al.* (1999) in sheep. Proportionate yield of A class oocytes were lower than this result when compared to observations of Das *et al.* (1996b) in goat and Das *et al.* (1996c) in sheep.

Mean number of A class oocytes obtained per ovary was 1.58 ± 0.25. This was comparable to the result of Priscilla (2001) in cattle. From sheep ovaries by puncture method, a larger number of A class oocytes were obtained for Wani *et al.* (1999). In goat (Pawshe *et al.*, 1994) and buffaloe (Das *et al.*, 1996a) ovarian puncture method yielded lower number of A class oocyte per ovary when compared to present results.

Percentage yield of B class oocytes by puncture method was 31.69%. Priscilla (2001) obtained only a lower percentage of B class oocyte by puncture method in cattle. Whereas the results of Das *et al.* (1996b) in goat and Das *et al.* (1996c) in sheep was comparable to this study.

Yield of B class oocyte by puncture method was 1.16 ± 0.16. A lower number of oocytes per ovary were obtained for Priscilla (2001) in cattle and

Pawshe *et al.* (1994) in goat. Das *et al.* (1996a) in buffaloe obtained a comparable result to the present study.

C class oocytes were yielded at the rate of 19.13% by puncture method, which was similar to the observations of Wani *et al.* (1999) in cattle. A lower proportion of C class oocytes were yielded in puncture method in cattle by Priscilla (2001).

Puncture method yielded 0.7 ± 0.07 C class oocyte per ovary. Compared to this a lower yield of C class oocyte were obtained for Priscilla (2001) in cattle. But Wani *et al.* (1999) obtained a higher yield of C class oocyte by same method.

Present study yielded 6.01% D class oocyte by puncture method. This was slightly lower than the result of Priscilla (2001). Proportionately high D class oocyte yield was obtained in goat (Das *et al.*, 1996b) and sheep (Das *et al.*, 1996c; Wani *et al.*, 1999).

Puncturing method recovered 0.22 ± 0.03 D class oocyte per ovary. This was comparable to the result of Priscilla (2001) in cattle. Sato *et al.* (1990) yielded higher number of D class oocyte by puncture method in cattle.

Statistical analysis revealed that the percentage yield of A class oocytes by puncture method was significantly higher than C and D class oocytes. This was similar to the observations of Wani *et al.* (1999) in sheep and Priscilla (2001) in cattle. But disagreed with results of Das *et al.* (1996b) in goat and Das *et al.* (1996c) in sheep, where they obtained minimum yield in A class oocytes. B and C class oocytes yielded significantly higher than D class in present study. This disagreed with results of Das *et al.* (1996b) in goat, Das *et al.* (1996b) and Wani *et al.* (1999) in sheep, and Priscilla (2001) in cattle. Wani *et al.* (1999) and Priscilla (2001) obtained no significant difference in yield of C and D class oocytes. But Das *et al.* (1996b) and Das *et al.* (1996c) yielded maximum percentage of oocyte in D class.

Average number of oocyte per ovary was also high in A grade in present study, which agreed with results of Wani *et al.* (1999) and Priscilla (2001) in sheep and cattle respectively, but the present values disagreed with Pawshe *et al.* (1994) in goat and Das *et al.* (1996a) in buffaloe, where they obtained A class oocytes proportionately minimum. Second largest rate of recovery was for B class, which was similar to result of Priscilla (2001). Least number of oocyte per ovary was in D class. This disagreed with Priscilla (2001) in cattle, Pawshe *et al.* (1994) in goat, Das *et al.* (1996a) in buffaloe and Wani *et al.* (1999) in sheep. Priscilla (2001) in cattle and Wani *et al.* (1999) in sheep obtained D class oocytes slightly more than C class oocytes. Whereas Pawshe *et al.* (1994) in goat and Das *et al.* (1996a) in buffalo obtained maximum number of oocyte per ovary in D class.

5.3 EXPERIMENT 3&4

5.3.1. Maturation Rate of Different Quality Grades of COCs – A Comparison Between Retrieval Systems

Maturation rate of bovine COCs were assessed by cumulus expansion and nuclear maturation. Nuclear maturation was assessed by observing oocytes for extruded 1st polar body (Plate.17,18 and 19) and staining of oocytes with aceto-orcein and a few oocytes with bis Benzimide fluorescent dye (Hoechst 33342). Aceto-orcein revealed chromosome structure in much detail (Plate.26 and 27) Fluorescent stain was also useful in assessment of nuclear maturation. Both methods required completely denuded oocytes, as the chromatin of cumulus cells interfered with the assessment of stage of maturation. In this study, one criterion selected for assessment of maturation of bovine oocyte was extruded polar body, but no comparable work was noticed in this aspect on perusal of literature.

5.3.1.1 A grade COCs

Cumulus expansion rate of oocytes obtained by different methods are shown in Table 6 and Fig.5. Cumulus expansion rate of A class oocytes obtained

by aspiration was similar to the results obtained for Rabahi *et al.* (1993) in cattle. A slightly higher cumulus expansion rate was reported by Romero-Arredondo and Seidel (1996) in cattle when same method was used for oocytes retrieval. Cumulus expansion rate of A class oocytes obtained by slicing and puncture was slightly lower than aspiration method, but statistical analysis reveled no significant difference between these methods in cumulus expansion rate.

Nuclear maturation rate of A class oocytes obtained by aspiration, slicing and puncture are given in Table 8 and Fig.7. By aspiration method A class oocytes exhibited a nuclear maturation rate of 81.8% which was comparable to the results of Konishi *et al.* (1996) and Tatemoto and Terada (1995). A lower nuclear maturation rate was reported by Leibfried and First (1979). A higher percentage of nuclear maturation was obtained for Choi *et al.* (1998) and Rodriguez and Farin (2004) in same class of oocytes obtained by aspiration of bovine ovaries. Results obtained for Chauhan *et al.* (1998a) in buffaloe, and Datta and Goswami (1999) in goat in A class oocytes by aspiration was in agreement with the present study. Pawshe *et al.* (1994) obtained a slightly higher percentage of nuclear maturation in A class goat oocytes recovered by aspiration. Polar body extrusion rate of A grade oocytes obtained by aspiration, slicing and puncture are shown in Table 8. Polar body extrusion rate in A class oocytes obtained by aspiration method was 45.5%.

By slicing method, A class oocytes exhibited a nuclear maturation rate of 78.94%. This was higher than the results obtained for Arlotto *et al.* (1996) but lower than the results of Carolan *et al.* (1992) in bovines. A comparable result was obtained for Katiyar *et al.* (1997) in A class caprine oocytes obtained by slicing method, but there the maturation time was 30 h. Pawshe *et al.* (1996) reported a higher metaphase II rate in A class caprine oocytes obtained by slicing method. Slicing method exhibited a polar body extrusion rate of 42.1% in A class COC's.

When puncture method was the method of oocytes retrieval, A class oocytes exhibited a metaphase II rate of 80 per cent. A comparable result was obtained for Schellander *et al.* (1990) when bovine A class oocytes were subjected for maturation treatment. Sharma et al. (1996) observed a lower maturation rate in A class goat oocytes when puncture method was employed. A polar body extrusion rate of 44% was obtained in A class oocytes by puncture method.

Maximum maturation rate was observed in A class oocytes obtained by aspiration, followed by puncture and slicing. But statistical analysis revealed no significant difference between methods of retrieval in A class oocytes. This agreed with the results of Pawshe *et al.* (1996) in goats who observed no significant difference in maturation rate of A class oocytes obtained by different methods of retrieval. Polar body extrusion rate of aspiration, slicing and puncture method did not differed significantly on statistical analysis.

5.3.1.2 B Class

Cumulus expansion rate obtained in B class oocytes by aspiration, slicing and puncture was 68.29%, 53.0% and 62.07% respectively. Statistical analysis revealed no significant difference between methods of retrieval in cumulus expansion rate. Nuclear maturation rate of B class oocytes was 57.1%, 41.67% and 52.63% for aspiration, slicing and puncture respectively. By aspiration method in bovines Konishi *et al.* (1996) obtained a higher maturation rate in B class COCs whereas Chauhan *et al.* (1998a) obtained a comparable result in buffalo COC's when same retrieval method was used. Statistical analysis of nuclear maturation rate revealed no significant difference between methods of retrieval. B class oocytes exhibited a polar body extrusion rate of 28.6%, 25.0% and 26.3% by aspiration, slicing and puncture methods. Statistically there was no significant difference between these methods in polar body extrusion rate.

5.3.1.3 C Class

C class oocytes harvested by aspiration, slicing and puncture revealed a cumulus expansion rate of 44.74%, 35.29% and 38.46% respectively. By aspiration method maximum maturation rate was obtained in B class oocytes followed by puncture and slicing. Statistical analysis revealed no significant difference between the methods of retrieval in cumulus expansion rate. Nuclear maturation rate of C class oocytes obtained by aspiration, slicing and puncture was 38.46, 28.57 and 30.77 per cent respectively. Maximum maturation rate was obtained by aspiration method, and least in slicing method. Results of nuclear maturation obtained by aspiration method was comparable to that of Leibfried and First (1979). Higher rate of maturation was obtained for de Loose *et al.* (1989), Konishi *et al.* (1996) and Warriach and Chohan (2004) in cattle and Datta and Goswami (1999) in buffaloe. C class oocytes obtained by slicing of caprine ovaries by Martino *et al.* (1992) exhibited a higher maturation rate than the present result. Statistical analysis of nuclear maturation rate of C class oocytes revealed no significant difference between methods of retrieval. C class oocytes exhibited a polar body extrusion rate of 15.4%, 14.3% and 15.4% respectively, which did not differ significantly on statistical analysis.

5.3.1.4 D Class

In the present study, most of the denuded oocytes failed to exhibit nuclear maturation. Only one oocyte in aspiration method exhibited nuclear maturation (12.5%). Most of the studies revealed such low maturation rate in D class oocytes (Leibfried and First, 1979; de loose *et al.*, 1989;Geshi *et al.*, 2000). However some scientists obtained exceptionally high values in nuclear maturation of denuded oocytes (Lorenzo *et al.* (1994); Tatemoto and Terada (1995) Konishi *et al.* (1996)). D class oocytes failed to reveal extruded polar body after *in vitro* maturation in this study.

5.3.1.5 Culture Grade

Culture grade oocytes obtained by aspiration slicing and puncture revealed a cumulus expansion rate of 77.36%, 62.36% and 66.91% respectively (Table 6 and Fig.5). Cumulus expansion rate obtained by aspiration method was similar to the results of Quero *et al.* (1994). A slightly lower cumulus expansion rate was obtained for Olson *et al.* (1990) and Lorenzo *et al.* (1994). Comparable result was obtained in buffaloe for Nandi *et al.* (2002) and Raghu *et al.* (2002) when aspiration method was employed. Statistical analysis revealed no significant difference between methods of retrieval in cumulus expansion rate of A and B class oocytes.

Nuclear maturation rate of culture grade oocytes obtained by aspiration, slicing and puncture was 72.22%, 64.52% and 68.20% respectively (Table 8 and Fig 7).

Results obtained in aspirated bovine oocytes agreed with the results of Arlotto *et al.* (1990), Quero *et al.* (1994) and Geshi *et al.* (2002) in bovines. Results of Tornesi *et al.* (1995), Leibfried-Rutledge *et al.* (1986b) and Ali and Sirard (2002) was slightly lower than these results. A lower result was also obtained for Leibfried and First (1979); Lorenzo *et al.* (1994); and Nakagawa *et al.* (1994) when aspiration was used for recovery of culture grade oocytes. However a higher nuclear maturation rate was obtained for Jiang *et al* (1991), Wiemer *et al.* (1991), Tatemoto and Terada (1995), Tatemoto and Terada (1996) and Gandolfi *et al.* (1997). A comparable nuclear maturation rate was also obtained for Toety *et al.* (1993b) and Nandi *et al.* (2002) in buffaloes when aspiration was employed for harvesting culture grade oocytes. In aspiration method when culture grade oocytes were taken together, a polar body extrusion rate of 38.9% was obtained.

Result of Arlotto *et al.* (1990) agreed with the maturation rate of culture grade oocytes obtained by slicing method. For Rieger and Loskutoff (1994) a higher maturation rate was obtained in culture grade oocytes collected by slicing.

Kobayashi *et al.* (1994) employed slicing method and reported still higher maturation rate in culture grade oocytes. Wahid *et al.* (1992) in sheep and Katiyar *et al.* (1997) in goat also obtained a higher maturation rate in culture grade oocytes on slicing. In culture grade oocytes a polar body extrusion rate of 35.5% was obtained.

Sato *et al.* (1990) obtained a higher value for culture grade oocyte maturation when collection method was ovarian puncture. Polar body extrusion rate in puncture method was 38.6%, when culture grade oocytes were taken together.

Statistical analysis revealed no significant difference between collection methods of oocytes on the nuclear maturation rate and polar body extrusion rate of culture grade oocytes. These results clearly indicate that the collection methods like aspiration, slicing and puncture are equally good for collection of COC's for *in vitro* maturation purpose.

These methods did not differ significantly in polar body extrusion rate. Maximum polar body extrusion rate was exhibited in aspiration (45.5%) followed by puncture (44%) and slicing (42.1%).

5.3.1.6 Overall maturation rate of COCs

Overall cumulus expansion rate was 68.75, 56.33 and 60.67% by aspiration, slicing and puncture (Table 6 and Fig 5). Calder *et al.* (2003) obtained a higher overall cumulus expansion rate when COCs were collected by aspiration method. Kobayashi *et al.* (1994) also obtained a higher cumulus expansion rate than present study when slicing was employed for COC collection. Statistical analysis of overall cumulus expansion rate exhibited no significant difference between methods of retrieval.

When aspiration, slicing and puncture of ovaries where performed for COC retrieval an overall nuclear maturation rate of 63.27%, 53.66% and 59.65% were obtained (Table 8 and Fig 7). Result obtained for Sanbuissho and Threlfall

(1985),Sanbuissho and Threlfall (1990), de Oliveira *et al.* (1994) and Palasz *et al.* (2000) when aspiration was used for COC collection revealed slightly lower values than the present study. Values reported by Arlotto *et al.* (1990), Arlotto *et al.* (1996) and Lechniak *et al.* (2002) agreed with the results obtained in aspiration method. Baruha *et al.* (1998) on aspiration of oocytes, a lower maturation rate was obtained when compared to present result. For Monaghan *et al.* (1993), Laroca *et al.* (1993), Chian (1996), Chian and Niwa (1994), Khatir *et al.* (1998), Dode *et al.* (2001), Ikeda *et al.* (2003) and Calder *et al.* (2003) obtained very high overall maturation rate by aspiration method. Tajik and Esfandabadi (2003) also obtained a higher, overall maturation rate in goat oocytes when aspiration was used for oocytes collection. Overall polar body extrusion rate in aspiration method was 32.7%.

Results of overall maturation rate obtained by slicing method agreed with the results of Arlotto *et al.* (1990). Carolan *et al.* (1994) obtained a higher maturation rate than the present study when oocytes were collected by surface dissection of bovine ovaries. Results of Martino *et al.* (1992) in goat agreed with the present values. Slicing method exhibited an overall polar body extrusion rate of 29.3%.

By puncture method, Iritani and Niwa (1977) obtained a comparable result with the present study. Schellander *et al.* (1990) by puncture method obtained a higher overall maturation rate when compared to this study. A polar body extrusion rate of 33.3% was obtained when all classes were combined.

There was no significant difference between the methods of retrieval in overall maturation and polar body extrusion rate. So aspiration, slicing and puncture methods were equally efficient in producing nuclear maturation in bovine oocytes. These findings agreed with the results obtained for Carolan *et al.* (1992) in cattle, Wahid *et al.* (1992) in sheep, and Pawshe *et al.* (1994) in goat. But this disagreed with the results of Iwasaki *et al.* (1987), Arlotto *et al.* (1990), Sato *et al.* (1990) and Carolan *et al.* (1994) who recorded a lower maturation rate

in slicing than puncture and aspiration method. A similar observation in goat oocytes with less than 3 layers of cumulus cells was noticed by Martino *et al.* (1992). One of the reasons suggested for this reduction was that slicing yielded smaller and immature oocytes located deep inside the ovary, which were having less developmental potential than larger oocytes obtained by puncturing and aspiration of larger follicles (Arlotto *et al.*, 1990; Carolan *et al.*, 1994; Arlotto *et al.*, 1996).

Das *et al.* (1996a) in buffaloes and Pawshe *et al.* (1994) in goat reported that by slicing oocytes located in deep and surface follicles, small and large follicles are released, so a heterogeneous population of follicles were involved. Hence this resulted in poor quality and immature oocytes. This can be attributed to reduction in maturation rate in sliced COCs, observed by some authors. In present study maturation rate in all class of COCs were lowest in slicing method. But this was not a significant reduction when compared to aspiration and puncture method.

As such results of this experiment clearly proved that aspiration, slicing and puncture method for COC collection is equally competent to produce good maturation rate in bovine COCs. Hence, any of these methods can be used for collection of COCs for *in vitro* maturation purpose. But their effect upon *in vitro* fertilization and embryo development are to be evaluated before selecting them for *in vitro* production of bovine embryos.

5.3.2 Maturation Rate in Different Retrieval Technique – A Comparison

The effect of number of cumulus cell layers on the maturation rate of bovine oocytes was analyzed. Each retrieval system was considered separately in the process. Parameters used for identification of maturation of oocytes were cumulus expansion, nuclear maturation and polar body extrusion.

5.3.2.1. Aspiration

In aspirated bovine oocytes A, B and C class oocytes exhibited a cumulus expansion rate of 83.08%, 68.29% and 44.74% respectively (Table 7 and Fig 6). Maturation rate observed in A class bovine oocytes obtained through follicular aspiration was comparable to the result of Rabahi *et al.* (1993) when employed similar procedures. Statistical analysis revealed that A class and B class oocytes did not differ significantly in cumulus expansion rate when aspiration was the method of retrieval. But C class oocytes were having significantly low cumulus expansion/maturation rate when compared to A class and B class oocytes.

Nuclear maturation rate of A, B, C and D class oocytes were 81.8%, 57.1%, 38.46% and 12.5% respectively. Maturation rate of A class oocytes obtained by aspiration method was slightly lower than the results of Tatemoto and Terada (1995) and Konishi *et al.* (1996). Leibfried and First (1979) when employed same method and same class of oocytes for maturation studies a lower maturation rate was obtained in bovines. Maturation rate of Choi *et al.* (1998) and Rodriguez *et al.* (2004) was much higher than the present study when bovine oocytes were used for the experiments. A comparable result was obtained in buffaloes when aspirated A class oocytes were matured *in vitro* (Chauhan *et al.*, 1998, Datta and Goswami, 1999). However Pawshe *et al.* (1994) obtained a higher maturation rate in aspirated caprine oocytes.

Chauhan *et al.* (1998) obtained a comparable result in aspirated B class buffaloe oocytes after *in vitro* maturation studies. Result of Konishi *et al.* (1996) was much higher than the present values reported in B class oocytes.

Slightly higher rate of C class oocyte maturation was observed by Leibfried and First (1979) in aspirated bovine oocytes. Higher maturation rates were also observed in cattle by de Loose *et al.* (1989) and Warriach and Chohan (2004). Datta and Goswami (1999) obtained higher maturation rate in buffaloes. Konishi *et al.* (1996) obtained a very high maturation rate in grade III bovine oocytes on 24 h of maturation treatment.

Denuded oocytes exhibited very poor maturational ability in aspirated bovine oocytes. A comparable result was observed by Leibfried and First (1979), de Loose *et al.* (1989) and Geshi *et al.* (2000). But some scientists obtained high maturation rate in denuded oocytes (Lorenzo *et al.*, 1994 and Konishi *et al.*, 1996). Tatemoto and Terada, (1995) observed very high maturation rate in D class oocytes.

Statistical analysis revealed that oocytes with ≥3 layers of cumulus cell layers (A and B) exhibited significantly higher maturation rate than oocyte with less than 3 layers of cumulus cells. Majority of denuded oocytes failed to mature. This agreed with the results of Leibfried and First (1979), Konishi *et al.* (1996) in cattle. Chauhan *et al.* (1998) reported a similar observation in buffaloe. Therefore, it is evident that morphology of COCs has a definite role in maturation of aspirated bovine oocytes. Oocytes with ≥3 layers of cumulus cells mature better than denuded or oocytes with less than 3 layers of cumulus cells. A, B and C class oocytes exhibited a polar body extrusion rate of 45.5%, 28.6% and 15.4% respectively in aspiration method. A and B class oocytes did not differ significantly in polar body extrusion rate. But polar body extrusion rate of C class oocytes was significantly lower than A and B class oocytes.

5.3.2.2. Slicing

A, B and C class oocytes obtained by slicing method exhibited a cumulus expansion rate of 69.70, 53.01 and 35.29% respectively (Table 7 and Fig 6). A class oocytes exhibited significantly higher cumulus expansion rate than B class and C class oocytes. B class oocytes exhibited significantly higher cumulus expansion rate than C class oocytes.

Nuclear maturation rate of A, B and C class oocytes in slicing method was 78.94%, 41.67% and 28.57% (Table 9 and Fig 8). Out of nine D class oocytes examined none exhibited nuclear maturation. Result of Carolan *et al.* (1992) was slightly higher than the maturation rate of A class oocytes, obtained by slicing in the present study. Much lower maturation rate was obtained for

Arlotto *et al.* (1996) in A class oocytes obtained by slicing. A higher maturation rate in A class caprine oocytes was also reported by Pawshe *et al.* (1994). Results obtained for Martino *et al.* (1994) in caprine oocytes with more than 3 layers of cumulus cells was higher than the present results in B class oocytes obtained by slicing. Martino *et al.* (1994) obtained higher maturation rate in sliced oocytes with 1-2 layers of cumulus cells when compared to C class oocytes of present study.

In slicing method when the number of layers of cumulus cells were more maturation rate was high. Highest maturation rate was observed in A class oocytes. This was similar to the observations of Martino *et al.* (1994) in sliced caprine oocytes. Oocytes with more than five layers of cumulus cells exhibited greatest maturation rate in slicing method. Polar body extrusion rate in slicing method was 42.1%, 25% and 14.3 % respectively in A, B and C class oocytes. Polar body extrusion rate was significantly higher in A class oocytes than B class and C class oocytes but B and C class oocytes did not differ significantly.

5.3.2.3. Puncture

Cumulus expansion rate of A, B and C class of oocytes in puncture method was 70.37%, 62.07% and 38.46% respectively. A class oocytes were having highest cumulus expansion rate and C class was having the least. Statistical analysis revealed that A class and B class did not differ significantly in cumulus expansion rate but the cumulus expansion rate of C class was significantly lower than both A and B classes.

Nuclear maturation rate in puncture method was 80%, 52.63%, 30.77% in A, B and C class oocytes. D grade oocytes exhibited no nuclear maturation. Maturation rate of A class oocytes in puncture method was comparable to the results of Schellander *et al.* (1990) in cattle. Sharma *et al.* (1996) obtained a slightly lower maturation rate in compact multiple layered oocytes obtained by puncture method. Statistical analysis revealed that highest maturation rate was observed in A class oocytes, followed by B. But there was no significant

difference between them. Maturation rate of C class oocytes were significantly lower than A class oocytes. D class oocytes failed to mature. A, B and C class oocytes obtained by puncture method revealed a polar body extrusion rate of 44%, 26.3% and 15.4% respectively. A and B class oocytes exhibited significantly higher polar body extrusion rate than C class oocytes. A and B class did not differ significantly in polar body extrusion rate.

Crosschecking of nuclear maturation with Hoechst 33342 stain revealed a nuclear maturation rate of 66.67% when 18 oocytes were stained. Aceto orcein stain revealed chromosome structures in much detail (Plate.26 and 27) than Hoechst 33342. FDA staining of 10 oocytes revealed 80% viability in matured oocytes. FDA stain reveals both membrane integrity and cytoplasmic esterase activity of oocytes (Chruch and Raines1980; Mohr and Trounson 1980; Overstorm 1996).

Results of all these studies clearly indicate that the morphology of COCs have significant effect on maturation of bovine oocytes. Greater the number of cumulus layers greater was the maturation rate. Oocytes with more than 3 layers of cumulus cells exhibited significantly higher maturation rate than the denuded oocytes or oocytes with partial cumulus layers. These results agreed with the results of Leibfried and First (1979), Martino *et al.* (1992), Konishi *et al.* (1996) and Chauhan *et al.* (1998). Raghu *et al.* (2002) opined that removal of cumulus cells perturbed the cytoplasmic maturation and hence developmental competence is reduced.

Cumulus cells are potentially important in the acquisition of full developmental competence. In absence of cumulus granulosa cells oocytes exhibited no maturation or reduced maturation (Hoshi *et al.*, 2001; Kobayashi *et al.*, 1994). Bols *et al.* (1996) reported that presence of intact cumulus cells for 12 h was necessary for normal cytoplasmic maturation. A cumulus cell density of 1.6-3.2 x 10^6 cells/ml during maturation was essential for continuous development of corona enclosed oocytes (Hashimoto *et al.*, 1998). Cellular

process involved in GVBD and late diakinesis were fully dependent on cumulus cells (Rieger and Loskutoff, 1994).

Cumulus cells communicate to the oocyte across zona pellucida through corona radiata cells, which penetrate the zona pellucida and form gap junctions with oolemma. These intercellular communications allow metabolic transfer as molecules of small molecular weight and help in nutrition of oocytes, which ultimately plays a vital role in oocyte growth and maturation (Geshi *et al.*, 2000; de Matos *et al.*, 1997; Buccione *et al.*, 1990; Armstrong *et al.*, 1996; Datta and Goswami 1999; Bols *et al.*, 1998). Staigmiller and Moor (1984) reported that granulosa cells provide energy substrate, some amino acids, nucleotides and phospholipids precursors to the oocyte, that generate some interactional signals which influence the nucleus and direct the synthesis of certain structural proteins and maturation specific proteins.

Cumulus cells metabolized glucose to pyruvate or Kreb's cycle intermediates, that could be passed to the oocyte and enhance its quality (Geshi *et al.*, 2000). Nagai (2001) reported that in absence of cumulus cells cattle oocyte required sodium pyruvate directly rather than glucose, in a protein free medium.

Connexine 43 (Cx43) plays an important role in intercellular communication mediated through gap junctions. Gap junctions are made up of proteins belonging to connexine family. Gap junctional network is essential for the oocyte meiotic maturation (Gordon, 2003).

Among gonadotrophins, FSH was most essential for cumulus expansion and maturation *in vitro* (Gupta *et al.*, 2001, Choi *et al.*, 2001). Action of FSH is mediated through gap junctions of cumulus oophorus (Atef *et al.*, 2005). One of the LH induced process culminating in ovulation is cumulus expansion, whereby cumulus secrete hyaluronan to form a muco-elastic extracellular matrix with proteins derived from the serum and follicle. This matrix structure was of importance for oocyte extrusion from the follicle and for pick up by the fimbria. In addition, a function of selective barrier for sperm had also been reported for

this matrix structure. Apart from LH, oocyte derived GDF-9 played an important role in the induction of cumulus expansion. LH activated GDF-9 by induction of proteases that loosen it from its inactivating proteoglycan or by stimulating the formation of a specific receptor for GDF-9. Activated GDF-9 induced the secretion of hyaluronan by the cumulus cells that, together with protein derived from the serum and follicle, form the muco-elastic matrix characteristic for cumulus expansion (van den Hurk and Zhao, 2005).

Combined action of FSH and LH on cumulus cells to synthesize pyruvate, stimulated TCA cycle to produce ATP for the energy requirement of oocyte. FSH stimulated increase of cAMP concentration (Choi *et al.*, 2001) in cumulus cells. Bilodeau *et al.* (1993) reviewed that when cAMP was high in COC, cAMP content was also high in oocytes derived from these complexes. They reported increase of cAMP in cumulus cells could activate a stimulatory signal for maturation and that this signal could overcome the inhibitory effect of high content of cAMP in oocyte itself. They argued that increasing cyclic AMP in cumulus cells induces maturation, by promoting oocyte cAMP hydrolysis. Choi *et al.* (2001) reported that FSH stimulated cumulus cells to secrete a positive factor, which could override the meiotic arrest due to hypoxanthine and trigger resumption of meiosis.

LH activates adenyl cyclase (AC) promoting a rapid rise in cAMP which results in activation of phospholipase C, evoking a rapid turnover in phosphoinositide hydrolysis and leading to production of inocitol triphosphate. This stimulates mobilization of intracellular calcium, followed by influx from the extracellular environment in cumulus cells. Increase in intra oocyte calcium level following LH stimulation of surrounding somatic cell occur either through direct transport from the cumulus or through inocitol triphosphate transport into the oocyte, followed by calcium release in the oocyte. This increase in calcium triggers a series of changes resulting ultimately in germinal vesicle breakdown (GVBD) by overriding the negative effect of cAMP inside the oocyte (van den Hurk and Zhao, 2005).

Lorenzo *et al.* (1994) reported both EGF and IGF-I alone or together stimulated nuclear maturation in immature bovine oocytes and opined that their beneficial effect was mediated through cumulus cells. Receptors for EGF have been demonstrated in bovine cumulus and small antral granulosa cells and the number of EGF binding site has been reported to be influenced by gonadotrophins. EGF exert its stimulatory effect by binding to granulosa cells since this phenomenon was seen only in cumulus enclosed oocyte, not in denuded oocyte (Chauhan *et al.*, 1999b). IGF II with FSH synergistically enhanced DNA synthesis, protein synthesis and steroidogenesis in the presence of granulosa cells. The synergistic effect was mainly caused by increase of IGF-II receptors in granulosa cells by FSH (Pawshe *et al.*, 1998).

Guoliang *et al.* (1994) reported that oocyte cumulus connections are crucial as far as initiating production of a meiosis inducing substance was concerned. Glutathione is another important substance needed for maturation and transported to oocyte through cumulus cells. Byskov *et al.* (2002) reported that cumulus cells were needed for production of maturation promoting factor (MPF). MPF is responsible for onset of oocyte maturation (Van den Hurk and Zhao, 2005).

Bols *et al.* (1996) reported that the cumulus improved fertilization by providing a capacitation inducing mechanism, hence the removal of cumulus cells even after maturation but before fertilization decreased developmental potential substantially. The rate and degree of polyspermy was significantly increased in denuded oocytes.

Maturation of oocytes in TCM – 199 with 10% foetal calf serum and 0.5 µg FSH / ml, 5 µg LH / ml and 1 µg estrogen / ml and 0.2 mM sodium pyruvate in 5% CO_2 at 38.5°C in maximum humidity gave good maturation results. This media is suited for basic maturation studies of Indian cattle but further experiments using different media have to be performed to determine most suited one.

So this experiment proved that the cumulus oocyte complex morphology have definite role in the *in vitro* maturation of bovine oocytes. Oocytes with multiple layers of cumulus cells (Culture grade) matured better than the denuded oocytes or oocytes with reduced cumulus number of cells (C and D class). Best quality oocytes for *in vitro* maturation purpose are oocytes with more than three complete layers of cumulus cells (A class and B class) irrespective of their retrieval methods.

CONCLUSION

It is clear from this study that the retrieval method has definite role in the mean yield of COCs per ovary. Slicing yielded significantly more number of COCs per ovary, whereas yield of COCs by aspiration and puncture did not differ significantly. Percentage yield of each grade of oocyte did not differ significantly between methods of retrieval. But mean yield of oocyte in each grade was maximum in slicing method. The yield of A & B class oocytes were significantly more in all retrieval methods. Yield of D class oocytes were least in all methods. Retrieval method was found to have no significant effect on the cumulus expansion, nuclear maturation and polar body extrusion. So oocytes collected by any method of retrieval can be used for *in vitro* maturation purpose. The COC morphology had definite role in the *in vitro* maturation of bovine oocytes. Oocytes with more than three layers of cumulus cells with uniform granulation of ooplasm are (Culture grade) best suited for *in vitro* maturation purpose.

Summary

6. SUMMARY

A study was carried out on *in vitro* maturation of follicular oocytes in cattle using oocytes obtained by various harvesting techniques from ovaries of recently slaughtered animals. The major objectives of this study were (1) to compare the different recovery methods on total yield of oocytes, (2) to compare the effect of recovery methods on quality grades of oocytes, (3) to compare the effect of retrieval method and quality of oocytes on IVM and (4) to identify a suitable protocol for IVM of follicular oocytes from cattle.

Ovaries used for the study were of recently slaughtered cattle belonging to South Indian breeds like Kangayam, Khillari, Hallikar and crossbred cattle of Kerala. Ovaries were dissected out and transported to laboratory within 2-4 h in freshly prepared normal saline fortified with antibiotics such as Penicillin and Streptomycine. A total of 150 ovaries were processed in the study. After repeated washing of ovaries in normal saline and TL-HEPES media kept at 36-38°C, oocytes recovery was performed by aspiration, slicing and puncture method. 50 ovaries were processed under each method of retrieval. Recovery of oocytes was performed while keeping ovaries in TL-HEPES medium enriched with BSA @ 0.6% 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. Class A COCs were characterised by more than five complete layers of cumulus cells and uniform granulation of ooplasm (Plate 8). Class B had 3-5 complete layers of cumulus cells and uniform granulation of ooplasm (Plate 9) and class C with 1-2 complete layers of cumulus cells and uniform granulation of ooplasm (Plate 10). Class D were denuded oocytes with uniform granulation of ooplasm (Plate 11

and 12). Pooled data of A and B class oocytes were analysed as a new class namely Culture grade oocytes for the purpose of drawing conclusion.

Each class of oocytes obtained by different recovery method were cultured separately in 100 µl maturation drops. Maturation media used for this study was TCM-199 enriched with 0.5 µg/ml FSH, 5 µg/ml LH, 1 µg/ml estrogen, 0.2 mM sodium pyruvate and 10% foetal calf serum (FCS). 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% and maximum humidity. After 24 h culture, oocytes were assessed for maturational changes like cumulus expansion and metaphase II plates. All oocytes showing maturation changes were observed for extruded polar body before and after staining. Staining was done mainly with 1% aceto-orcein. Few oocytes were stained with fluorescent stain -Hoechst 33342- as a method for cross checking. Viability status of few matured oocytes were also tested with vital stain like Fluorescein Diacetate (FDA).

The total yield of COC by aspiration, slicing and puncture was 3.7 ± 0.43, 5.42 ± 0.56 and 3.66 ± 0.43 respectively. Yield was significantly higher in slicing method than aspiration and puncture. Higher yield by slicing method might be due to recovery of oocytes from surface follicles as well as follicles of deeper cortical stroma.

Proportionate yield of A, B, C, D and culture grade oocytes by aspiration method was 44.86%, 32.97%, 18.30%, 3.7% and 76.22% respectively. Mean yield of oocytes in each of these class by same method was 1.66 ± 0.25, 1.22 ± 0.12, 0.68 ± 0.20, 0.14 ± 0.06 and 2.88 ± 0.19 respectively. Slicing yielded A, B, C, D and culture grade oocytes at the proportion of 37.27%, 29.89%, 20.66%, 12.10% and 67.16% respectively. Mean yield in these class in slicing method was 2.02 ± 0.25, 1.62 ± 0.20, 1.12 ± 0.15, 0.66 ± 0.09 and 3.64 ± 0.23 respectively. Percentage yield of A, B, C, D and culture grade oocytes in puncture method was 43.1%, 31.69%, 19.13%, 6.01% and 74.86% respectively.

Mean yield per ovary in puncture method was 1.58 ± 0.25, 1.16 ± 0.16, 0.70 ± 0.07, 0.22 ± 0.03 and 2.74 ± 0.21 for A, B, C, D and culture grade oocytes.

Aspiration puncture and slicing did not differ significantly in yield of A, B, C and culture grade oocytes. D grade oocytes were significantly more in slicing method. This might be due to recovery oocytes from all kinds of follicles. The percentage yield of culture grade oocytes was significantly more in aspiration (76.22) than slicing (67.16) and puncture(74.86). Yield of D Class oocytes was minimum in all methods.

Cumulus expansion rate of A class oocytes were 83.08 per cent, 69.70 per cent and 70.37 per cent in aspiration, slicing and puncture method respectively. B class oocytes exhibited cumulus expansion rate of 68.29 per cent, 53.0 per cent and 62.07 per cent in aspiration, slicing and puncture method respectively. In C class oocytes, the expansion rate was 44.74, 35.29 and 38.46 per cent respectively for the same methods. Retrieval method was found to have no significant effect on cumulus expansion potential of bovine COCs, whereas the COC morphology has significant effect on cumulus expansion potential. Oocytes with more than three layers of cumulus cells exhibited better cumulus expansion rate than oocytes with less number of cumulus cell layers.

Nuclear maturation rate for A class oocytes in aspiration, slicing and puncture method was respectively 81.8, 78.94 and 80.0 per cent and polar body extrusion rate was respectively 45.5, 42.1 and 44.0 per cent. B class oocytes exhibited nuclear maturation and polar body extrusion rates of 57.10 per cent, 41.67 per cent, 52.63 per cent and 28.6 per cent, 25.0 per cent and 26.3 per cent in aspiration, slicing and puncture respectively. 38.46, 28.57 and 30.77 per cent nuclear maturation and 15.4, 14.3 and 15.4 polar body extrusion rate was observed in C class oocytes by aspiration, slicing and puncture respectively. Most of D class oocytes failed to exhibit neither nuclear maturation nor polar body extrusion in all the methods. Analysis of results revealed that the method of oocytes retrieval is having neither effect on nuclear maturation nor polar body

extrusion of bovine oocytes. But the COC morphology is having significant effect on nuclear maturation and polar body extrusion. Oocytes with more than three layers of cumulus cells were exhibited better nuclear maturation and polar body extrusion rate.

Out of 18 oocytes crosschecked for nuclear maturation with Hoechst 33342, 12 (66.67%) exhibited metaphase II plates. Eight (80%) out of 10 oocytes tested for viability with FDA was found to be viable after IVM. Protocol and culture environment used for nuclear maturation in this study was found ideal for *in vitro* maturation studies of bovine oocytes. TCM-199 medium with 5 µg FSH/ml, 0.5 µg LH/ml, 1 µl Estradiol/ml, 0.2 mM pyruvate and 10 per cent serum was found good for *in vitro* maturation of bovine oocytes.

This study proved that slicing is a better method than aspiration and puncture for retrieval of oocytes from bovine ovaries as far as total yield of oocytes were concerned. The yield of A class and B class oocytes was more than other classes in all retrieval methods. Retrieval method was found to have no significant effect on the yield of each morphologic grade, cumulus expansion rate, Metaphase II rate and polar body extrusion rate. This experiment proved that oocytes with more than three layers of cumulus cells are better suited for maturation purpose irrespective of their retrieval method.

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EFFECT OF OVUM RETRIEVAL METHODS AND CUMULUS-OOCYTE COMPLEX MORPHOLOGY ON *In vitro* MATURATION OF BOVINE OOCYTES

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ABSTRACT

This study was designed to evaluate the effect of different retrieval methods like aspiration, slicing and puncture on yield of different grades of oocytes and their *in vitro* maturation potential. The effect of cumulus oocyte complex morphology on *in vitro* maturation of bovine oocyte was also studied. Slaughterhouse derived bovine ovaries from South Indian breeds like Kangayam, Khillari, Hallikar and crossbred cattle of Kerala were subjected to three retrieval methods to yield different quality grades of oocytes. In this study, a total of one hundred and fifty ovaries were processed. Fifty ovaries were processed under each method of retrieval. Each quality grade of oocyte obtained through three retrieval method were subjected to maturation for 24 h in TCM-199 medium supplemented with LH, FSH, Estrogen, Pyruvate and Foetal calf serum. Culture environment was set as 38.5°C temperature, 5% carbondioxide tension and maximum humidity in standard CO_2 incubator. Maturation changes were assessed by cumulus expansion, formation of M II plates and polar body extrusion.

Total yield of COCs in slicing was 5.42 ± 0.56, which was significantly higher than aspiration and puncture which yielded 3.7 ± 0.43 and 3.66 ± 0.43 COCs respectively. This could be due to recovery of oocytes from a heterogeneous population of follicles in slicing method. Aspiration, slicing and puncture yielded A class oocytes at the rate of 44.86%, 37.2% and 43.17% respectively, wherein mean yield per ovary in same methods were 1.66 ± 0.25, 2.02 ± 0.25 and 1.58 ± 0.25 respectively. Yield of B class oocytes by aspiration, slicing and puncture were 32.97%, 29.89% and 31.69% respectively. Mean yield of B class oocytes per ovary was 1.22 ± 0.12, 1.62 ± 0.20 and 1.16 ± 0.16 respectively. Proportionate yield of D class oocytes by aspiration, slicing and puncture was 3.7%, 12.10% and 6.01% respectively. Aspiration, slicing and puncture did not differ significantly in yield of A, B and C class oocytes. But D

class oocytes were proportionately more in slicing method. This finding could be attributed to the fact that the slicing recovered oocytes from a heterogeneous population of follicles, which included all kinds of growing follicles. When percentage yield of different classes of oocytes in same retrieval method was compared aspiration yielded A and B class oocytes in significantly higher proportion than C and D class oocytes. The same trend was observed in slicing and puncturing. In all the methods, the yield of D class oocytes was least.

Cumulus expansion rate of A class oocytes were 83.08 per cent, 69.70 per cent and 70.37 per cent in aspiration, slicing and puncture method respectively. B class oocytes exhibited cumulus expansion rate of 68.29 per cent, 53.0 per cent and 62.07 per cent in aspiration, slicing and puncture method respectively. Retrieval method was found to have no significant effect on cumulus expansion potential of bovine COCs. Oocytes with more than three layers of cumulus cells exhibited better cumulus expansion rate than oocytes with less cumulus cell layers.

Nuclear maturation rate of A class oocytes in aspiration, slicing and puncture was 81.08, 78.84 and 80.0% respectively, whereas in B class oocytes under same methods the percentage of nuclear maturation was 57.10, 41.67 and 52.63% respectively. D class oocytes failed to mature in all methods of retrieval except one oocytes in aspiration method. Aspiration, slicing and puncture did not differ significantly in the maturation percentage of oocytes. Polar body extrusion rate of A class oocytes in aspiration, slicing and puncture method was 45.5 per cent, 42.1 per cent and 44.0 per cent respectively. B class oocytes exhibited polar body extrusion rates of 28.6 per cent, 25.0 per cent and 26.3 per cent respectively in aspiration, slicing and puncture method respectively. Oocytes with more than three layers of cumulus cells exhibited better polar body extrusion rate irrespective of their retrieval method. Staining of 18 oocytes with fluorescent dye Hoechst 33342 as a method of cross checking, revealed 66.67 per cent Metaphase II rate. Ten oocytes tested for viability with FDA stain revealed 80 per cent viability after maturation. TCM-199 medium with 5 µg FSH/ml, 0.5 µg LH/ml,

1 µl Estradiol/ml, 0.2 mM pyruvate and 10 per cent serum was found to be ideal medium for maturation of bovine oocytes *in vitro*.. Oocytes with more than three layers of cumulus cells matured better than oocytes with less number of cumulus cell layers or denuded oocytes. These experiments proved that cumulus oocytes complex morphology have a very significant role in maturation of oocytes rather than retrieval methods.