IN VITRO MATURATION OF CAPRINE FOLLICULAR OOCYTES

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DECLARATION

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

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CERTIFICATE

Certified that the thesis entitled "*IN VITRO* **MATURATION OF CAPRINE FOLLICULAR OOCYTES**" is a record of research work done independently by **Ambili John,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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LIST OF ABBREVIATIONS USED

BSA	Bovine serum albumin
COC	Cumulus oocyte complex
DPBS	Dulbeco's phosphate buffered saline
EGF	Epidermal growth factor
EGS	Estrous goat serum
FBS	Fetal bovine serum
FF	Follicular fluid
FSH	Follicle stimulating hormone
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
hCG	Human chorionic gonadotropin
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
IGF-I	Insulin-like growth factor-I
IU	International Units
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
LAP	Laparoscopic aspiration procedure

LH	Luteinizing hormone
LOPU	Laparoscopic ovum pick up
mg	Milligram
MI	Metaphase I
MII	Metaphase II
MOET	Multiple ovulation and embryo transfer
NGF	Nerve growth factor
oFSH	Ovine Follicle stimulating hormone
PBS	Phosphate buffered saline
SOF	Synthetic oviduct fluid
TALP	Tyrode, albumine, lactate, pyruate
TCM-199	Tissue culture medium-199
TUGA	Trans vaginal ultrasound guided aspiration

Introduction

1. INTRODUCTION

Vast and diverse genetic resources of goats are found in India and among this, Malabari breed, a native of Kerala, is well known for its milk production and adaptability. This small ruminant plays a major role in the rural economy of the state. Recently, there is a stimulated interest for goat rearing in Kerala as evidenced by the increase in goat population as against a decline in cattle and buffalo (AHD, 2004). But we are facing the challenges of massive fragmentation of land holdings and diversion of land for non-agricultural purposes, making a drastic reduction in grazing facilities available for livestock. Further, there is progressive decline in the number of farmers due to diversion to other profitable enterprises. Hence, adoption of advanced techniques of production and reproduction to enhance profitability of goat rearing is the need of the hour.

The success of application of reproductive technologies such as cloning by nuclear transfer, sperm and embryo sexing and production of transgenic animals is critically dependent on the expertise in the basic reproduction techniques such as *in vitro* maturation (IVM) of oocytes, *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of embryos (Kane, 2003). The major applications of these techniques include, the conservation of endangered species and rare livestock breeds, commercial supply of oocytes and embryos of different stages of development and production of progenies even from pre-pubertal and dead animals (Holtz, 2005). The potential benefits of *in vitro* embryo production techniques include greater genetic gain obtained, reduced chances of disease transmission and easiness in transportation of embryos, compared to animals.

The future trends in goat production are expected to be dictated by the emerging technologies of reproduction such as Multiple Ovulation and Embryo Transfer (MOET), *in vitro* embryo production, cryopreservation and

micromanipulation. Economical means for *in vitro* embryo production can be expected to play a prominent role in future goat breeding strategies (Ishwar and Menon, 1996). *In vitro* production (IVP) of embryos is currently the central focus in livestock industry including goats. For the successful IVP programme in goat and other livestock, *in vitro* maturation (IVM) of cumulus oocyte complex (COC) is a primary requirement.

Maturation of oocytes means the process of acquisition of competence by oocytes to achieve fertilization and to develop into an embryo. Like other mammals, the primary oocytes of goat become arrested at the diplotene stage of meiosis at birth. They are capable of resuming meiosis spontaneously when removed from their follicles and cultured *in vitro* .(Gilchrist and Thompson, 2007). Goat oocytes must undergo both nuclear and ooplasmic maturation for normal fertilization and embryonic development. During the maturation process 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 the laboratory, under suitable conditions, maturation of immature oocytes can be carried out successfully. In India, IVM studies were carried out in buffalo, cattle, pig, sheep and goat. In Kerala Agricultural University, much research work in the field of multiple ovulations, *in vivo* embryo production, cryopreservation and its transfer in different species have been carried out in the Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Mannuthy (Nair, 1992; Benjamin, 1994; Ramachandran, 2000; Joseph, 2003 and Babu, 2006). In the field of *in vitro* embryo production, Priscilla (2001) carried out a study on retrieval and characterization of bovine oocytes collected from slaughter house ovaries, Paul (2005) conducted a study on IVM of bovine oocytes retrieved by various methods from the slaughter house ovaries and Binoy (2009) studied the *in vitro* fertilization of bovine oocytes using fresh, frozen and epididymal

spermatozoa. Regarding the other species of animals, Deepa (2007) carried out a study on *in vitro* maturation of porcine follicular oocytes, but no research work has been conducted so far in the area of *in vitro* embryo production of goats, the first step of which is *in vitro* maturation of oocytes, which forms the basis for the present study.

The success rate of *in vitro* production of embryos is less than that of *in vivo* production techniques. Improvement in the efficiency of *in* vitro maturation procedures can improve the quality and quantity of IVF/IVP embryos. Various factors such as follicle size, follicular fluid or cells, composition of IVM medium, age of the donor goat and the culture conditions are involved in the successful IVM of goat oocytes (Rahman *et al.*, 2008a). It is in this context that the present study on IVM of caprine follicular oocytes was undertaken with the following major objectives.

1. To analyse the effect of oocyte retrieval methods and cumulus mass on *in vitro* maturation of caprine oocytes.
2. To identify the best oocyte retrieval method for *in vitro* maturation.
3. To assess the *in vitro* maturation rate of caprine oocytes.

Review of Literature

2. REVIEW OF LITERATURE

2:1 *IN VITRO* MATURATION OF FOLLICULAR OOCYTES

In most mammalian species, some hours before rupture of the follicle and ovulation, the fully grown oocyte resumes meiosis, progressing from prophase of the first meiotic division to metaphase of the second division. This complex biological process, which transforms the primary oocyte into an egg which is ready to undergo fertilization, is known as oocyte maturation. Oocyte maturation involves both cytoplasmic and nuclear maturation.

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. In live animal, the oocyte that has been arrested in the dictyate stage of the first meiotic division, resumes meiosis in response to the signals associated with preovulatory luteinizing hormone (LH) surge (Gordon, 2003).

In vitro embryo production in small ruminants provides an excellent source of low-cost embryos for basic research on developmental biology and physiology and for commercial application of emerging biotechnologies. *In vitro* maturation is an important step involved in the *in vitro* production of embryos. The process of harvesting oocytes from ovaries of recently slaughtered animals, assessing their quality and subjecting the oocytes to IVM process are the preliminary steps involved in the *in vitro* production of embryos (Zhou and Zang, 2006).

2.2 HARVEST OF OOCYTES

2.2.1 Harvest from Live Animals

Oocytes from live goats could be retrieved following superovulation or without hormonal stimulation using various procedures like follicle aspiration or slicing of ovariectomized ovary, follicle aspiration through laparotomy, laparoscopic ovum pick-up (LOPU), or transvaginal ultrasound guided aspiration (TUGA) (Graff *et al.*, 1999; Samake *et al.*, 2000; Alberio *et al.*, 2002 and Rahman *et al.*, 2008b).

Graff *et al.* (1999) reported that, the number of follicles detected and oocytes harvested by TUGA (9.5 and 4.3, respectively) was less than that obtained by laparoscopic aspiration procedure (LAP) (17.4 and 14.4, respectively) and unlike donor does subjected to a repeated LAP, there was no evidence of adhesions in donor does subjected to repeated TUGA.

In an experiment using superovulated goats, Samake *et al.* (2000) retrieved 18 oocytes per goat through aspiration of follicles after ovariectomy and 8.3 oocytes per goat through aspiration of exteriorized ovaries by laparotomy and reported a maturation rate of 100 per cent.

In superovulated goats, oocyte recovery using LOPU technique ranged from 33% to 80% with an average yield that ranged between 5.6 and 13.4 oocytes per animal (Alberio *et al.*, 2002; Baldassare *et al.*, 2002; Baldassare *et al.*, 2003; Baldassare and Karatzas, 2004). The LOPU procedure was recognized as less traumatic than laparotomy (Koeman *et al.*, 2003). It has been found that LOPU can be repeated up to five times in the goats at different intervals and in different seasons with little or no important change in overall response (Pierson *et al.*, 2004).

2.2.2 Harvest from Abbatoir Ovaries

Goat ovaries from slaughtered animals are the cheapest and the most abundant source of primary oocytes and this was widely used for *in vitro* maturation (Agrawal *et al.*, 1995; Katiyar *et al.*, 1997; Ongeri *et al.*, 2001; Bormann *et al.*, 2003; Rodriguez-Gonzalez *et al.*, 2003 and Tajik and Esfandabadi, 2003). The use of ovaries collected from animals at the abattoir as a source of oocytes for IVM-IVF allows for the large scale production of embryos that can be used in the development of new biotechnologies such as cloning and genetic engineering (Gordon, 2003).

2.2.2.1 Collection of Ovaries

2.2.2.1.1 Media for Ovary Transportation

To maintain the healthiness of caprine ovaries and its follicle during the transport from slaughterhouse to laboratory Pawshe *et al.* (1993), Pawshe, *et al.* (1994), Sharma *et al.* (1996), Katiyar *et al.* (1997), Malik, *et al.* (1999), Tajik and Esfandabadi (2003) and Wang *et al.* (2007a) used normal saline with or without antibiotic supplementation like Penicillin, Streptomycin or Gentamycin for keeping it free of contamination. Ovaries were transported in PBS supplemented with 0.1 µg polyvinyl alcohol (Keskintepe *et al.*, 1996). PBS supplemented with sodium pyruate, glucose and antibiotics was used for transportation of ovaries (Le Gal, 1996). Various others used PBS supplemented with antibiotics (Izquierdo *et al.*, 1998; Izquierdo *et al.*, 1999; Rodriguez-Gonzales *et al.*, 2003 and Kharche *et al.*, 2005) for transportation of caprine ovary. Rodriguez-Gonzales *et al.* (2003) made use of DPBS containing 50µg/ml gentamycin for transporting prepubertal goat ovaries from slaughterhouse to laboratory.

2.2.2.1.2 Temperature of transport media

In various studies caprine ovaries were transported from abattoir to laboratory within the temperature range of 25-30°C (Pawshe *et al.*, 1993 and

Bormann *et al.,* 2003) or 30-33°C (Keskintepe *et al.,* 1996) or 30-35°C (Sharma *et al.,* 1996; Rho *et al.,* 2001 and Tajik and Esfandabadi, 2003) or 32-37°C (Katiyar *et al.,* 1997) or 35-37°C (Mogas *et al.,* 1997; Izquierdo *et al.,* 1998; Izquierdo *et al.,* 1999; Rodriguez-Gonzalez *et al.,* 2003 and Kharche *et al.,* 2009). Evecen *et al.* 2010 reported that oocytes obtained from bitch ovaries transported at 4° C had higher maturation rates than those transported at 35-38° C irrespective of the stage of the estrous cycle at the time the oocytes were harvested.

2.2.2.1.3 Time Interval from Slaughter to Harvest of Oocytes

The time interval preferred by researchers normally ranged from one hour, two hours, two to three hours, four hours and six hours (Martino *et al.,* 1994; Pawshe *et al.,* 1994; Keskintepe *et al.,* 1996; Tajik and Esfandabadi, 2003; Wang *et al.* 2007a and Kharche *et al.,* 2009)

Time interval from slaughter to retrieval of oocyte influenced the success rate of IVM, fertilization and subsequent development of embryos (Ali *et al.,* 2004). It was further reported that ovaries maintained at 35° C for 4 h after slaughter showed higher rate 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 IVM. Wani and Nowshari (2005) reported that there was no difference in the proportion of oocytes reaching M II stage from the ovaries collected and stored in normal saline solution at room temperature for 12 h and those collected in warm normal saline solution and processed immediately after arrival in laboratory.

2.3 OOCYTE RETRIEVAL

2.3.1 Pre-retrieval Processing of Ovaries

The pre retrieval processing like removal of extraneous tissue, repeated washing etc. of ovaries reduced the chance of contamination in the oocyte culture (Martino *et al.,* 1994 and Sachan *et al.,* 1999). Researchers used different media for

washing of ovaries prior to oocyte retrieval. Ovaries were washed thoroughly in normal saline before oocyte retrieval by Pawshe *et al.* (1994). Sharma *et al.* (1996) and Izquierdo *et al.* (2002) washed ovaries in PBS supplemented with antibiotics prior to processing.

2.3.2 Retrieval Methods

Several methods were used for oocyte retrieval from goat ovaries. Collection of oocytes from individual follicles after dissection and isolation of follicles from ovaries was performed by some workers (Katska, 1984; Le Gal, 1996 and Crozet *et al.*, 2000). Aspiration, puncturing, slicing and post aspiration slicing were different techniques commonly used for oocyte retrieval from slaughter house ovaries (Pawshe *et al.*, 1994; Vijayakumaran, 1995; Katiyar *et al.*, 1997; Tajik and Esfandabadi, 2003 and Wang, *et al.*, 2007a).

2.3.2.1 Aspiration

Oocyte aspiration from ovarian follicles was performed by simple aspiration of follicular contents using a syringe and a needle of 18-22g or aspiration with a constant vacuum source (Gordon and Lu, 1990; Martino *et al.*, 1994; Keskintepe *et al.*, 1996; Keskintepe *et al.*, 1998; Yadav *et al.*, 1998; Velilla *et al.*, 2002; Tajik and Esfandabadi, 2003 and Wang *et al.* 2007b). The number of oocytes harvested per ovary was lower after aspiration than puncture method because oocytes remained firmly attached to the small and medium size follicles before cumulus expansion and could not be aspirated (Das *et al.*, 1996b). Wani *et al.* (2000) recovered a higher percentage of good quality oocytes by aspiration (64.4 per cent) method than by puncture (54.7 per cent) or slicing (54.3 per cent). Aspiration had the advantage of high speed of oocyte recovery, when compared to dissection or slicing (Gordon, 2003).

2.3.2.2 Slicing

Slicing or dissection of individual follicles was used for collection of goat oocytes by various workers (Younis *et al.,* 1991; Mogas *et al.*, 1993). Cutting the surface of the ovary and releasing the oocyte from the follicle with a razor blade or slicing of the ovaries followed by simple rinsing of the slices (Martino *et al.,* 1994; Pawshe *et al.,* 1994; Pawshe *et al.,* 1996; Ongeri *et al.,* 2001; Priscilla, 2001 and Rho *et al.,* 2001) were done. Martino *et al.* (1994) reported that slicing of goat ovary was a simple and efficient tool for recovering a high number of cumulus-enclosed, good quality oocytes that were competent to mature, fertilize and develop *in vitro.*

Pawshe *et al.* (1994) opined that time required for processing each ovary by the slicing method was comparatively less than that required for aspiration or puncturing and significantly more good quality usable oocytes enclosed with compact cumulus mass were obtained by slicing (0.9±0.06) than by aspiration(0.5±0.07) or by puncturing (0.5±0.06). By slicing method oocytes from surface follicles as well as follicles of deeper cortical stroma were released, whereas by puncture and aspiration, only oocytes from surface follicles were released (Das *et al., 1996*a). Slicing resulted in the production of more tissue debris that interfered adversely with recovery of oocytes (Wani *et al.,* 1999).

2.3.2.3 Puncture

Repeated puncturing of slaughterhouse ovaries with 18 gauge needle was performed to recover oocytes into the medium kept in a petri dish (Vijayakumaran, 1995; Sharma *et al.,* 1996; Wani *et al.,* 1999; Priscilla, 2001 and Kharche *et al.,* 2006). To improve the efficiency of oocyte retrieval the follicular fluid was allowed to ooze out by applying gentle pressure on the adjacent stroma of the punctured follicle (Das *et al.,* 1996a and Priscilla, 2001). Sharma *et al.* (2006) recovered more number of excellent to good quality cumulus enclosed oocytes by puncture of ovarian surface follicles. Kharche *et al.* (2008a) recovered an average of 2.5

culturable oocytes per ovary by puncture method and he recorded a maturation rate of 68.42 per cent and the birth of a normal healthy female kid after transfer of *in vitro* produced embryos. An average of two oocytes per ovary were recovered by puncture method Kharche *et al.* (2008b)

2.3.3 Retrieval Media

Commonly used oocyte isolation media are HEPES or bicarbonate buffered TCM-199, TL-HEPES (TL-N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]), DPBS and synthetic oviduct fluid (SOF).

Pawshe *et al.*, (1993), Pawshe *et al.*, (1994) and Ongeri *et al.*, (2001) dissected ovaries in TL-HEPES media supplemented with 2-4 mg/ml BSA. TCM-199 with serum and antibiotic supplementation was used for oocyte retrieval and washing by many workers (Le Gal, 1996; Katiyar *et al.*, 1997; Mogas *et al.*, 1997; Izquierdo *et al.*, 1998 and Izquierdo *et al.*, 1999). HEPES buffered TCM-199 was used by Le Gal (1996) and Izquierdo *et al.*, (2002). Das *et al.* (1996b) and Sharma *et al.* (1996) used PBS supplemented with 10 per cent heat inactivated goat serum for retrieval of oocyte of caprine origin. To avoid the coagulation of retrieval medium with the follicular fluid, heparin was added @ of 1IU/ml or 11.1µg/ml to TCM (Malik *et al.*, 1999 and Rodriguez-Gonzalez *et al.*, 2003). Rho *et al.* (2001) used Ham's F-10 medium for the collection of oocytes. Bormann *et al.* (2003) collected oocytes in HEPES buffered SOF medium supplemented with 14.24 mg/ml heparin.

2.4 QUALITY GRADING OF OOCYTES

COCs are usually classified under zoom stereomicroscope with 40x – 60x magnification. Most predictive morphological criteria for selection of COCs continued to be an intact cumulus cells and homogenous cytoplasm (Vijayakumaran, 1995; Wang *et al.* 2007b and Kharche *et al.*, 2008b).

2.4.1 Based on Cumulus Cell Layer

The COCs were graded as good, fair and poor according to the character of cumulus cells (Pawshe *et al.,* 1994); oocytes with many complete layers of cumulus cells and uniform ooplasm were graded as good, oocytes with thin or incomplete layers of cumulus cells and uniform ooplasm as fair, and oocytes with few or no cumulus cells as poor. Vijayakumaran, (1995) classified oocytes into five grades according to the character of cumulus cells and ooplasm. Morphologically normal oocytes with more than three layers of compact, cumulus cells and uniform ooplasm were classified as Grade A, morphologically normal oocytes with three or less than three layers or with a partial cumulus mass and uniform ooplasm as Grade B, morphologically normal oocytes with corona radiata only and uniform ooplasm as Grade C, morphologically normal oocytes without any cumulus or corona radiata and uniform ooplasm as Grade D and morphologically abnormal oocytes with ooplasm showing signs of degeneration as Grade E.

Koemon *et al.* (2003) classified the COCs into Grade A ($\geq$ 3 cumulus layers), Grade B(1-2 cumulus layers), Grade C (denuded) and Grade D (expanded cumulus). Recovered oocytes were graded depending on their cumulus investment and cytoplasmic distribution as excellent or A grade (oocytes with more than four layers of compact cumulus masses with an evenly granulated cytoplasm), good or B grade (oocytes with at least 2-4 layers of cumulus cell masses with an evenly granulated cytoplasm), fair or C grade (oocytes with at least one complete layer of cumulus cell mass with an evenly granulated cytoplasm) and poor or D grade (oocytes with no cumulus cell or an incomplete layer of cumulus cells or expanded cumulus mass with or without a dark or unevenly granulated cytoplasm) Kharche *et al.* (2008b) and Kharche *et al.* (2009).

2.4.2 Based on Ooplasm Character

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). A good goat oocyte had ooplasm which filled the entire part of vitelline space (Rajikin *et al.,* 1994). Oocytes with condensed and vacuolated cytoplasm were considered abnormal (Dominguez, 1995)

In IVM/IVF studies, majority of the scientists selected oocytes with homogenous and evenly granulated cytoplasm (Vijayakumaran, 1995; Pawshe *et al.,* 1996; Kharche *et al.,* 2008b). Oocytes with ooplasm showing signs of degeneration were considered morphologically abnormal (Vijayakumaran, 1995). Das *et al.* (1996a) graded oocytes with evenly granulated cytoplasm as culturable grade and dark scattered cytoplasm as poor.

2.5 YIELD OF OOCYTE

2.5.1 Oocyte Yield per Ovary

The average number of oocytes recovered per ovary by Martino *et al.* (1994) was 1.71, 1.27 and 6.05 for dissection, aspiration and slicing, respectively. Pawshe *et al.* (1994) recovered an average of 2.7±0.15, 2.4±0.12 and 2.2±0.13 oocytes per ovary by aspiration, puncturing and slicing respectively. Vijayakumaran, (1995) recovered an average of 4.99 ± 0.87, 2.95 ± 0.79, 3.25 ± 0.51 and 2.40 ± 0.43 oocytes per ovary respectively by slicing, puncturing, aspiration and post aspiration slicing. Katiyar *et al.* (1997) recovered an average of four oocytes per ovary from 2-6 mm follicles by slicing method.

Wani *et al.* (1999) indicated that slicing (9.5 ± 0.4) and puncture (9.5 ± 0.4) yielded significantly more oocyte per ovary than follicle aspiration (6.8 ± 0.3) in sheep. Wani *et al.* (2000) recovered an average total number of 9.5 ± 0.45, 9.5 ± 0.40 and 6.8 ± 0.30 oocytes per ovary by puncture, slicing and aspiration respectively.

Shirazi *et al.* (2005) reported that the number of oocytes per ovary for slicing (4.0) and aspiration (3.7) did not differ significantly in ewe lambs. Wang *et al,* (2007b) reported that slicing (6.3) and puncture (5.8) of Boer goat ovaries yielded a significantly higher number of oocytes per ovary compared to aspiration (3.1).

2.5.2 Effect of Retrieval Technique on the yield of Different Quality Grades of Oocytes

2.5.2.1 Grade A

Oocytes with multiple layers of cumulus cells (>3 layers) and uniform granulation of ooplasm were considered as A class oocytes. Retrieval methods like aspiration, slicing and puncture were adopted for COC recovery (Pawshe *et al.,* 1994 and Vijayakumaran, 1995)

Pawshe *et al.* (1994) obtained 0.56 ± 0.07, 0.57 ± 0.06 and 0.91 ± 0.06 A class oocytes per ovary by aspiration, puncturing and slicing method respectively and significantly high number of A class ooctytes per ovary was retrieved by slicing technique compared to aspiration and puncture. Vijayakumaran (1995) recovered an average of 31.78, 33.67, 26.15 and 27.08 per cent A class oocytes respectively by aspiration, slicing, puncture and post aspiration slicing technique and reported maximum yield of A class oocytes by slicing method, followed by aspiration, post aspiration slicing and puncture. By resorting to aspiration technique, Gogoi *et al.* (2001) recovered 30-40 per cent of A class oocytes. Wang *et al.* (2007a) reported that 58.1, 61.9 and 55.2 per cent good quality oocytes were recovered by aspiration, slicing and puncture technique respectively.

64.4, 54.3 and 54.7 per cent of A class oocytes were recovered per ovine ovary by aspiration, slicing and puncture techniques respectively (Wani *et al.* 2000). Number of A class oocytes yielded per bovine ovary by practicing aspiration technique ranged between 2-4 (Priscilla, 2001). Percentage of A class oocytes

recovered by aspiration technique was 44.86 per cent and 49.20 per cent ((Paul, 2005 and Binoy, 2009) for bovine ovaries and 27 per cent for porcine ovaries (Deepa, 2007). Wang *et al.* (2007a) reported that the percentage of A class oocytes recovered was highest by slicing technique followed by aspiration and puncture.

2.5.2.2 Grade B

Pawshe *et al.* (1994) obtained 0.88 ± 0.12, 0.75 ± 0.09 and 0.80 ± 0.07 B class oocytes per ovary respectively by aspiration, puncturing and slicing of caprine ovaries and reported no significant difference in the yield of B class oocytes between aspiration, slicing and puncture. Vijayakumaran (1995) recovered an average of 24.49, 16.73, 30.62 and 17.29 per cent B class oocytes respectively by aspiration, slicing, puncture and post aspiration slicing technique and no significant difference in the yield of B class oocytes between aspiration, slicing and puncture was reported, but the author reported a significantly low number of B class oocytes recovered by post aspiration slicing. Wang *et al.* (2007a) reported that 22.6, 20.6 and 25.8 per cent B class oocytes were recovered by aspiration, slicing and puncture technique respectively.

Wani *et al.* (2000) recovered 22.4, 22.8 and 21.5 per cent of B class oocytes per ovine ovary by aspiration, slicing and puncture techniques respectively. Percentage of B class oocytes recovered from bovine ovary by aspiration, slicing and puncture technique was 32.97, 29.89 and 31.69 per cent respectively (Paul, 2005). Deepa (2007) recovered 30.89, 29.32, 25.91 and 17.06 per cent B class oocytes per porcine ovary by aspiration, slicing, puncture and post aspiration slicing respectively. Wang *et al.* (2007a) reported that the percentage of B class oocytes recovered was highest by puncture technique followed by slicing and aspiration.

2.5.2.3 Grade C

An average of 1.33 ± 0.14, 0.96 ± 0.12 and 0.69 ± 0.04 C class oocytes were obtained by aspiration, puncture and slicing method respectively and it was recorded that the highest number of C class oocytes was recovered by aspiration technique compared to slicing and puncture. (Pawshe *et al.,* 1994). Vijayakumaran (1995) recovered 16.74, 11.80, 18.77 and 14.33 per cent C class oocytes from caprine ovaries by aspiration, slicing, puncture and post aspiration slicing methods respectively and reported highest number of C class oocytes by slicing method and recorded that there is significant difference between aspiration and puncture. 19.3, 17.5 and 19.0 per cent of C class oocytes were recovered by Wang *et al.* (2007a) by aspiration, slicing and puncture method respectively.

Wani *et al.* (2000) recovered 13.2, 22.9 and 23.8 per cent of C class oocytes per ovine ovary by aspiration, slicing and puncture techniques respectively. Percentage of C class oocytes recovered from bovine ovary by aspiration, slicing and puncture techniques were 32.97, 29.89 and 31.69 per cent respectively (Paul, 2005). Deepa (2007) recovered 30.89, 29.32, 25.91 and 17.06 per cent C class oocytes per porcine ovary by aspiration, slicing, puncture and post aspiration slicing respectively. No significant difference was observed by Wang *et al.* (2007a) in the yield of C class oocytes between aspiration, slicing and puncture.

2.6 OOCYTE MATURATION *IN VITRO*

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.

2.6.1 Maturation Media

The culture medium used in IVM not only affected the proportion of oocytes that reach metaphase II (M II) and become capable of fertilization, but also

influenced subsequent embryonic development (Bavister, *et al.,* 1992). Maturation is carried out in complex tissue culture media with addition of gonadotropins, steroid hormones, pyruate, serum of various sources, growth factors and antibiotics.

Complex tissue culture media like TCM-199 (Totey *et al.,* 1993; Pawshe *et al.,* 1996; Katiyar *et al.,* 1997 and Wang *et al.,* 2007b), Ham's F-10 (Totey *et al.,* 1993; Vijayakumaran, 1995; Le Gal, 1996), Ham's F-12 (Pawshe *et al.,*1996), Minimum Essential Medium-α (MEM-α), RPMI-1640, Waymouth medium and synthetic oviductal fluid (Bormann *et al.,* 2003 and Evecen *et al.,*2010) were used for maturation of caprine oocytes. These media originally designed for cell culture were not capable of supporting high level of oocyte maturation and hence were supplemented with sera (Gasparrini, 2002).

2.6.1.1 TCM 199

The basic culture medium most commonly used for the maturation of caprine oocytes was TCM-199 (tissue culture medium- 199: HEPES modification, with Earl's salta, L-Glutamine, and 25 mM HEPES without sodium bicarbonate) supplemented with hormones, serum and other substances at different concentrations (Pawshe *et al.,* 1994; Keskintepe *et al.,* 1996; Pawshe *et al.,* 1996; Katiyar *et al.,* 1997; Izquierdo *et al.,* 2002; Baldassare *et al.,* 2003; Baldassare and Karatzas, 2004; Zhou and Zhang, 2006; Wang *et al.,* 2007a and Kharche *et al.,* 2009). From a comparative study of M-199 and Ham's F-12 medium supplemented with or without serum and hormones for supporting *in vitro* maturation of goat oocytes, Pawshe *et al.* (1996) concluded that the combination of M-199 with FSH, LH and Estradiol 17β, supplemented with 10 per cent FCS was the most efficacious medium for *in vitro* maturation and subsequent embryonic development of goat oocytes. TCM-199 without any additives could support maturation of oocytes, fertilization and culture *in vitro* which resulted in formation of hatched blastocyst (Bevers *et al.,* 1997).

2.6.1.2 Ham's F-10 Medium

Deb and Goswami (1990) cultured cumulus enclosed goat oocytes in Ham's F-10 medium and obtained a maturation rate of 0.00 per cent after 24 h, 18.2 per cent after 30 h and 14.8 per cent after 34 h of culture *in vitro*. Vijayakumaran (1995) employed Ham's F-10 medium for caprine oocyte maturation and obtained satisfactory maturation rates.

2.6.1.3 Other Media

Zhiming *et al.* (1990) observed that goat oocytes matured to 43 per cent in TALP enriched with newborn calf serum, BSA and gonadotropins. Chauhan and Anand (1991) observed a maturation rate of 69.1 per cent in Hams's F-12 supplemented with BSA and FCS in the presence of FSH. Vijayakumaran (1995) used Ham's F-10 medium for IVM of caprine oocyte and obtained satisfactory maturation levels. Pawshe *et al.* (1996) compared two different culture media namely Ham's F-12 and M-199 for supporting IVM of goat oocytes and reported that neither medium, when supplemented with EGS and hormones showed any notable changes in the maturation rate. It was further reported that Ham's F-12 medium supplemented with FCS and hormones showed a considerable increase in the maturation rate.

2.6.2 Culture Conditions

2.6.2.1 Temperature

De Smedt *et al.* (1992) indicated that immature goat oocytes were particularly sensitive to temperature. Though most of the *in vitro* maturation works in mammals could be performed at 37° C, best results could be obtained only if the normal basal body temperature of the respective species could be provided (Eppig, 1996).

In case of goat, the most widely practiced temperature levels were 38° C (Younis *et al.*, 1991; Pawshe *et al.*, 1993 and Pawshe *et al.*, 1994), 38.5° C (Katiyar

et al., 1997; Keskintepe *et al.*, 1996; Le Gal, 1996; Zhou and Zhang, 2006 and Kharche *et al.*, 2009) and 39° C (Le Gal, 1996; Sharma *et al.*, 1996 and Baldassare and Karatzas, 2004).

2.6.2.2 Gas Phase

Two most common gas mixtures used during *in vitro* maturation of gametes were 5 per cent CO_2, 5 per cent O_2 and 90 per cent N_2 or 5 per cent CO_2 in 95 per cent air with the later being more common (Eppig, 1996).

In goats majority of the investigators used 5 per cent CO_2 in air (Pawshe *et al.*, 1993; Sharma *et al.*, 1996; Katiyar *et al.*, 1997; Baldassare and Karatzas, 2004; Wang *et al.* 2007a and Kharche *et al.*, 2009) and obtained good results. Keskintepe *et al.*, (1996) employed 5 per cent CO_2, 5 per cent O_2 and 90 per cent N_2. Zhou and Zhang (2006) used 5 per cent CO_2 and 95 per cent O_2.

2.6.2.3 Humidity

Many of the workers used maximum humidity (around 95-100 per cent) for IVM of goat oocytes and obtained good maturation rates (Pawshe *et al.*, 1993; Sharma *et al.*, 1996; Rho *et al.*, 2001 and Zhou and Zhang, 2006) Gilchrist and Thompson (2007) opined that high humidity of 90-100 per cent was suitable for *in vitro* maturation of oocytes, high humidity helped to maintain osmolality by preventing evaporation. The highest rate of oocyte maturation was obtained with 95 per cent humidity.

2.6.2.4 Culture Duration

Many reports indicated that, a wide range of time was required for completion of meiosis, ranging from 24 to 32 h in goats (Chauhan and Anand 1991 and Crozet *et al.*, 1995). It has been demonstrated that an extensively long or short IVM period can cause an increase in polyspermy, due to an alteration in the content or a malfunction

of the cortical granules (Niwa *et al.,* 1991). Martino *et al.* (1994) reported that the percentage of maturation increased significantly on increasing the time from 24 h to 25.5 or 27 h. Pawshe *et al.* (1994), Le Gal (1996), Izquierdo *et al.* (2002) and Baldassare and Karatzas (2004) cultured oocytes *in vitro* for 24-27 h with good results. Sharma *et al.* (1996) reported that the percentage of oocytes reaching MII was less at 30, 31 and 36 h of culture compared to 32 h of culture and the author opined that 32 h of culture was the most suitable time for maturation of goat oocytes *in vitro.*

On the basis of sequential configuration of chromosomes, it was found that the optimal duration of *in vitro* maturation of oocytes is 32, 30, and 24 h for sheep, goats and buffalo, respectively (Yadav *et al.,* 1997). Katiyar *et al.* (1997) reported that the percentage of oocytes reaching metaphase II after 30 h of culture was higher than that obtained after 32 h of culture. It was reported that incubation time necessary for maturation of goat oocytes seemed to be longer than that needed for sheep or cattle and a higher proportion of goat oocytes reached metaphase II after 27 h than after 24 h of culture (Rho *et al.,* 2001). Zhou and Zhang (2006) cultured oocytes from preantral follicles for 48 hours with 54.08 per cent maturation rate. Kharche *et al.* (2009) obtained a maturation rate of 80 per cent after 27 hours of IVM.

2.7 ASSESSMANT OF OOCYTE MATURATION

2.7.1 Based on Cumulus Cell Expansion

The cumulus cells were known to supply nutrients, energy substrates and messenger molecules for the development of oocyte and to mediate the effect of hormones on the cumulus oocyte complex (Buccione *et al.,* 1990). Cumulus expansion has been postulated to be important in achieving complete oocyte maturation, as the expansion of COCs has been reported to be related to fertilization rate and developmental potential in oocytes (Cox *et al.,* 1993). Chauhan *et al.* 1997 assessed the cumulus expansion as degree zero when there was no expansion, degree

one when cumulus cells were homogenously spread and clustered cells was still observed and degree two when cumulus cells were homogenously spread and clustered cells were no longer present. Gupta *et al.* (2005) graded the degree of cumulus expansion after IVM as zero, one, two, and three. When the cumulus expansion was not observed it was graded as zero, slight cumulus expansion involving only outer most layer was scored one, partial cumulus expansion involving all layers except corona radiata as two and full cumulus expansion involving all layers including the corona radiata as three. A first polar body was found in all the oocytes in which grade two or three cumulus expansion was observed and hence all the grade two and grade three oocytes were considered matured. Optimal expansion of cumulus mass appeared to be essential for ooplasmic maturation (Chen *et al.*, 2007).

2.7.2 Based on Cytoplasmic and Nuclear Changes

Crozet *et al.* (1995) suggested that cytoplasmic maturation conferred the oocyte, the ability to support early embryonic development. Eppig, (1996) defined cytoplasmic maturation as a variety of changes in the intracellular organization and physiology of oocytes that allow normal embryo development after fertilization and it was further reported that ooplasmic maturation involved accumulation of protein and mRNA, development of calcium regulatory mechanism, changes in the activity of MPF and MAPK and redistribution of cellular organelles.

Gasparrini (2002) reported that changes associated with cytoplasmic maturation happening in 1-8 h of LH peak in oocyte *in vivo* and *in vitro* included formation of perivitelline space with loss of contact between the cumulus cell and the oocyte, and roughening of nuclear membrane, 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 the cytoplasm. After 19 h, polar body got extruded; mitochondria dispersed and majority of organelles were located towards centre of the cell. Relatively organelle free cortical region contained cortical granules immediately adjacent to the plasma membrane together with aggregates of tubular SER.

Rahman *et al.* (2008a) suggested a number of criteria to assess ooplasmic maturation viz. cytoskeletal organization of oocytes such as migration of cortical granules to the oolemma, increased number of mitochondria and lipid droplets, changes in the arrangement of golgi apparatus and the presence of only granular endoplasmic reticulum; MPF activity and oocyte metabolism.

De Smedt *et al.* (1992) found that the acquisition of meiotic competence was accompanied by nucleolar compaction and a dramatic decrease in RNA synthesis. The chromosomal configurations of the oocyte at different hours of incubation were classified into germinal vesicle (GV- if a prominent nucleus with dispersed chromatin or round shaped nucleus was found), germinal vesicle break down (GVBD- the nucleolus and nuclear membrane disappeared and chromosomes appeared as condensed and coiled up filaments), metaphase I (recognized by the appearance of paired chromosomes and bivalents), anaphase I (identified by chromosomes pulling apart from each other and moving to opposite ends of the spindle), telophase I (when two equally spread groups of chromosomes were found), metaphase II (the chromosomes of the first polar body were clustered and the oocyte chromosomes were spread out) and degenerated or abnormal and non defined (Agrawal *et al.*, 1995)

The sequential nuclear changes during *in vitro* oocyte maturation were reported to be germinal vesicle stage, germinal vesicle break down stage, metaphase I stage and metaphase II stage (Sharma *et al.*, 1996 and Yadav *et al.*, 1996). Weherend and Meinecke (2001) reported that nuclear maturation was characterized by

chromosome condensation (CC), nuclear envelope dissolution (GVBD), spindle assembly and chromosome separation.

Nuclear maturation refers to the acquisition of the ability to undergo dissolution of germinal vesicle (GV) (nuclear membrane), condensation of chromosomes, release of first polar body and subsequent arrest at metaphase II (Rodriguez and Farin, 2004). van den Hurk and Zhao (2005) reported that nuclear maturation last about 24 h in sheep and cattle, about 44 h in pig and about 36 h in horse and comprises several steps, that include two 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.

The follicular oocytes which were usually arrested at the prophase I of the first meiotic division until the onset of puberty, under the influence of gonadotropins resumed meiosis just before ovulation which resulted in the disappearance of the nuclear membrane and germinal vesicle break down, followed by chromosome condensation with the occurrence of the M I stage and subsequently, upon extrusion of the first polar body, the oocytes reached the M II stage and remained in this stage until penetration by the spermatozoa (Kharche *et al.*, 2006).

2.7.2.1 Sequential Changes of Nucleus Associated with Maturation

At zero hour of culture, 55.55 per cent of GV stage oocytes were observed which gradually decreased to 37.38, 11.34, 3.77, 8.90 and 4.38 per cent at 6, 12, 18, 24 and 30 h of culture (De Smedt *et al.*, 1992). GVBD was the dominant configuration at 6 h of incubation. Anaphase I and telophase I stage first observed at six and 12 h of incubation respectively and were the stages of short duration. Metaphase I of the meiosis was found at 12 and 18 h of incubation. Meiotically mature oocytes (Metaphase II) first observed at 12 h reached the highest level at 24 h of incubation. A decline in metaphase II was obtained at 30 h of incubation.

Oocytes were matured and fixed at different time intervals to establish the chronology of meiotic events occurring in goat oocytes during *in vitro* maturation (Sharma *et al.,* 1996), and it was revealed that at 0 h of culture 78.3 per cent of oocytes were at the Germinal vesicle (GV) stage, 57.4 per cent of oocytes were found in germinal vesicle break down (GVBD) stage at 6 h culture, peak (51.1 per cent) metaphase I was noted at 12 h, MII was first observed after 18 h of culture, and at 32 h the maximum (71.6 per cent) oocytes were in MII stage.

Yadav *et al.* (1996) reported that at 0 h most of the oocytes were at the GV stage, GVBD started within 6 h of culture, and most of the oocytes completed this stage by 16 h of culture. Further condensation of chromosomes lead to the MI stage at 12 to 20 h followed by the MII stage at 22 to 36 h, with the highest number of oocytes reaching the MII stage at 30 h of culture.

Kharche *et al.* (2006) reported that oocytes remained in the GV stage from the onset to 6-8 h of culture, the GVBD occurred between 7 and 9 h and the metaphase I became established within 12-18 h and finally most oocytes reached the M II stage after 27 h.

2.7.2.1.1 Germinal Vessicle Stage

The germinal vesicle stage was identified as containing a single large nucleus with uniformly distributed filamentous chromatin subsequently condensing to form a ring of condensed chromatin around the compact nucleolus (Yadav *et al.,* 1996). A chromosome configuration was designated as GV, when having a single large nucleus with uniformly distributed filamentous chromatin subsequently condensing to form a ring of condensed chromatin around the compact nucleus (Kharche *et al.,* 2006 and Kharche *et al.,* 2008a)

2.7.2.1.2 Germinal Vesicle Breakdown Stage

Germinal vesicle breakdown (GVBD) was identified by the disappearance of a compact nucleolus and nuclear membrane and by gradual condensation of chromatin material into distinguishable chromosome identities (Yadav *et al.*, 1996). According to Kharche *et al.* (2006) and Kharche *et al.* (2008a), in the GVBD category of oocytes, the nucleolus and nuclear membrane had disappeared and chromosomes appeared as condensed and coiled up filaments.

2.7.2.1.3 Metaphase I stage

Metaphase I was described as characterized by the formation of individual bivalents with chiasmata , subsequent terminalization of chiasmata, appearance of tetrads, separation of two chromosome sets towards opposite poles (anaphase-I) and completion of separation of two chromosome sets (telophase-I) (Yadav *et al.*, 1996). The metaphase I stage was recognized by the appearance of paired chromosomes and bivalents (Kharche *et al.*, 2006 and Kharche *et al.*, 2008a)

2.7.2.1.4 Metaphase II Stage

The percentage of oocytes reaching M II stage *in vitro* was approximately 90 per cent (Younis *et al.*, 1991). Martino *et al.* (1995) reported that the proportion of oocytes in M II increased significantly in culture time from 53 per cent at 24 h to 73 per cent at 27 h. Metaphase II was marked by the emission of first polar body resulting in haploid set of chromosomes (Yadav *et al.*, 1997) and the M II stage of meiosis was considered ideal for fertilization and the *in vitro* maturation process was supposed to be completed when the highest percentage of M II was observed. Wani and Nowshari (2005) opined that dromedary oocytes required 32-44 h of *in vitro* culture to have an optimum number of oocytes in M II stage. In the M II stage emission of first polar body, resulting in the formation of haploid set of

chromosomes in the oocytes was observed (Kharche *et al.,* 2006 and Kharche *et al.,* 2008a)

2.7.2.2 Staining of Oocytes

To evaluate the nuclear stage after maturation, oocytes after IVM are denuded of cumulus cells, fixed on a slide and stained and examined. A number of stains were used; one per cent aceto orcein (Pawshe *et al.,* 1994; Vijayakumaran, 1995; Tajik and Esfandabadi, 2003; Paul, 2005; Kharche *et al.,* 2006). Fixing of oocytes with methanol: acetic acid (3:1) and staining with 2 per cent Giemsa solution have made it possible to accurately identify the sequential stages of chromatin formation (Katiyar *et al.,* 1997 and Yadav *et al.,* 1997). One per cent lacmoid was used by Rodriguez-Gonzalez *et al.* (2002) and Wang *et al.* (2007b), whereas Kharche *et al.* (2008b) used 0.1 µg/ml 4,6-diamino-2-phenylindol for the evaluation of nuclear status after maturation of oocytes *in vitro.*

2.7.2.2.1 Aceto Orcein

Oocytes were denuded by vortexing (Paul, 2005) or repeated pipetting (Pawshe *et al.,* 1994; Sharma *et al.,* 1996; Kharche *et al.,* 2004) and cumulus free oocytes were than mounted on glass slides. A cover slip supported by four droplets of a mixture of Vaseline and paraffin wax (3:1 v/v) was placed on to the oocytes. Oocytes were then fixed in a fixation solution (acetic acid: methanol 1:3 v/v) for 12-24 h and stained with one per cent (w/v) orcein in 40 per cent (v/v) acetic acid (Vijayakumaran, 1995)

2.7.2.2.2 bis Benzimide (Hoechst 33342)

Bormann *et al.* (2003) stained blastocysts mounted on siliconized slides and squashed under cover slip with 0.1mg/ml Hoechst 33342 DNA stain diluted in SOF HEPES in 30µl drops for 30 min for studying the nuclear changes.

2.7.2.2.3 FDA

Katska (1984) used 3'6'-Fluorescein diacetate (FDA) for viability assessment of bovine oocytes. The basic staining solution contained 5 µg FDA/ml of acetone. Immediately before use, it was diluted, giving a final concentration of 1.7 µg FDA/ml of modified Dulbeco'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 Dulbeco's medium and observed under fluorescent microscope. It was reported that the degree of fluorescein accumulation was a reflection of quality of oocyte cytoplasm. FDA revealed both membrane integrity and cytoplasmic enzyme activity of cell by emitting apple green fluorescence. 1.7 µg FDA/ml of Dulbeco's phosphate buffered saline was used for staining (Paul *et al.*, 2006).

2.8 FACTORS AFFECTING *IN VITRO* MATURATION

2.8.1 Follicle Diameter

De-Smedt *et al.* (1992) showed that 80 per cent of goat oocytes recovered from follicles 2-6 mm in diameter progressed to M II, whereas only 24 per cent of oocytes from follicles 1-1.8 mm attained that stage. Martino *et al.* (1994) opined that most follicles smaller than 2.5 or 3 mm contained oocytes which were not competent in achieving meiosis. Crozet *et al.* (1995) found a direct positive relationship between follicle diameter and meiotic competence and reported that 56 and 96 per cent oocytes were able to reach the MII stage after IVM, when oocytes were recovered from follicles of 2-3 mm and larger than 3 mm diameter, respectively. Gall *et al.* (1996) reported that oocytes from follicles less than 0.5 mm in diameter failed to undergo GVBD and the author opined that meiotic competence of goat oocytes was acquired progressively during folliculogenesis and was achieved in follicles of 3 mm in diameter when the oocytes had completed their growth phase. Teotia *et al.*

(2001) collected oocytes from > 4 mm size follicles and reported an average maturation rate of 80 per cent.

2.8.2 Oocyte Quality

Oocyte quality was found to influence the maturational potentiality of follicular oocytes (Vijayakumaran, 1995). While A and B grade oocytes having compact cumulus mass and evenly granulated ooplasm exhibited a higher maturation rate (75.13 and 69.94 per cent), it was significantly low for C, D and E grade oocytes (35.53, 18.79 and 1.70 per cent respectively). Bovine oocytes with more than three layers of cumulus cells and evenly granulated ooplasm exhibited better polar body extrusion rate (Paul, 2005)

2.8.3 Components of Maturation Media

2.8.3.1 Hormones

Gonadotropins are the primary regulators of *in vitro* nuclear maturation in mammalian oocytes. Gonadotropins alter the metabolism of the cumulus cells and induce resumption of meiosis in the oocytes by interrupting the mode of inhibitory substances through the gap junctions (Ball *et al.*, 1983 and Salustri and Siracusa, 1983).

Oocytes matured *in vitro* in the presence of gonadotropins and estradiol showed high maturation and fertilization rates compared with those matured without hormones. (Totey *et al.*, 1993). It was observed that gonadotropins and estradiol usually cause synergestic enhancement of nuclear maturation, depending on the type of serum supplement used in the maturation medium (Pawshe *et al.*, 1993). The beneficial effect of gonadotropins in the medium was more pronounced for oocytes from juvenile or prepubertal females (Mogas *et al.*, 1995). The addition of gonadotropins (FSH and LH) and estradiol-17β to the medium significantly improved oocyte maturation rates (Izquierdo *et al.*, 1998 and Izquierdo *et al.*, 1999).

Estradiol may be involved in cytoplasmic maturation by stimulating DNA polymerase β and enhancing the synthesis of presumed male pronucleus growth factors. Blastocyst production was significantly increased for oocytes matured in the presence of estradiol-17β (Pawshe and Totey, 2003).

2.8.3.1.1 Follicle Stimulating Hormone (FSH)

Follicle stimulating hormone was found to enhance early embryonic development rather than meiotic resumption (Eyestone *et al.*, 1993). Pawshe *et al.* (1993) observed a higher maturation rate when oocytes were incubated in TCM-199 in the presence of FSH in comparison to incubation without FSH. The optimum concentration of FSH was found to be 10 µg/ml, which gave a maturation rate of 91.8 per cent. Follicle stimulating hormone at both low and high concentrations (1µg and 20 µg/ml respectively) was less effective. Pawshe *et al.* (1996) opined that FSH enhanced early embryonic development rather than meiotic resumption. Supplementation of FSH-P (5 µg/ml) in TCM-199 containing 10 per cent FBS resulted in significantly higher maturation rates (81 against 56 %) of goat oocytes after 30 h of culture (Katiyar *et al.*, 1997).

When FSH was added to *in vitro* maturation medium, it appeared to stimulate cumulus cells of COC to secrete a positive factor that could override arrest due to hypoxanthine which could trigger meiotic resumption. In addition, FSH stimulated an increase of cAMP concentrations and cumulus expansion (Choi *et al.*, 2001).

2.8.3.1.2 Luteinizing Hormone (LH)

Addition of LH for bovine *in vitro* maturation enhanced the quality of oocytes, which was reflected in an increased embryo yield after IVF and embryo culture (Bavister *et al.*, 1992). Luteinizing hormone 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). Final concentration of LH ranged from 3 µg/ml to 100 µg/ml (Keskintepe *et al.*, 1994 and Ongeri *et al.*, 2001)

van den Hurk and Zhao (2005) reported that LH surge activated adenylcyclase, promoting a rapid rise in cAMP and stimulated rapid increase in intracellular calcium in granulosa cells through activation of phospholipase-C, evoking a rapid turnover in phosphoinositol hydrolysis and thus the production of inositoltriphosphate. Intracellular calcium mobilization was followed by a calcium influx from extracellular environment and this increase in calcium triggered a series of changes resulting in germinal vesicle breakdown.

2.8.3.1.3 Estradiol 17-β

Estradiol improved the completion of maturational changes including the synthesis of presumed male pronucleus growth factor (Thibault *et al.*, 1975 and Moor, 1978). Estradiol had a significant role in cytoplasmic maturational changes that was necessary for fertilization and post fertilization development (Bevers *et al.*, 1997). Supplementation of maturation medium with estradiol 17β (100 ng/ml) led to a significant increase in blastocyst yield (Guler *et al.*, 2000). Gasparrini (2002) obtained a maturation rate of 94 per cent when estradiol was used at a concentration of 1 µg/ml.

2.8.3.2 Growth Factors

In sheep, nerve growth factor (NGF) was produced *in vitro* by granulosa cells in response to gonadotropin stimulation and might be involved in the control of oocyte maturation (Barboni *et al.*, 2000). Completion of meiosis was stimulated, and cleavage rates after IVF were significantly increased in sheep oocytes by the presence of 10 ng/ml of EGF in a defined culture medium (Guler *et al.*, 2000), whereas 100 ng/ml of IGF-I did not appear to have an effect on oocyte maturation.

The addition of NGF to the maturation medium resulted in resumption of meiosis in more than 70 per cent of the oocytes (Barboni, 2000). Oocytes matured *in vitro* in the presence of EGF had greater cumulus cell expansion and higher fertilization rates in sheep (Grazul-Bilska *et al.*, 2003). Epidermal growth factor (EGF) influenced oocyte maturation and blastocyst production in various mammalian species and may be involved in the regulation of follicular growth and oocyte maturation in goats and sheep (Gall *et al.*, 2005).

Zhou and Zhang (2005) reported that the growth factors, EGF and IGF-I can individually or synergistically regulate the survival and growth rate of caprine preantral follicle oocytes. Goat oocytes were found to express EGF receptors, one of the regulators of oocyte maturation and it was found that EGF triggered signaling through the Mitogen-Activated Protein Kinase (MAPK) pathway during IVM in goat oocyte (Gall *et al.*, 2005). Wang *et al.*, 2007b cultured oocytes in TCM-199 with 10 ng/ml of epidermal growth factor (EGF).

2.8.3.3 Serum

In some experiments, serum of the same species was utilized for IVM of oocytes; estrus goat serum for caprine oocytes (Keskintepe *et al.*, 1994), equine follicular fluid for equine oocytes (Hinrichs *et al.*, 1995) and porcine follicular fluid for porcine oocytes (Funahashi *et al.*, 1997).

Maturation media for caprine oocytes contained calf serum (Crozet *et al.*, 1993), estrous goat serum (EGS) (Keskintepe *et al.*, 1994), bovine serum albumin along with EGS (Rajikin *et al.*, 1994) and fetal bovine serum (FBS) (Martin-Lunas *et al.*, 1996) as protein supplement. Rodriguez-Gonzalez *et al.* (2003) supplemented IVM medium with 10 per cent steer serum and obtained an average maturation rate of 64 per cent.

Tajik and Esfandabadi, (2003) used different protein supplements like FBS, EGS and estrous sheep serum (ESS) at 10, 15 and 20 per cent levels in the caprine oocyte maturation media and obtained a maturation rate of 83, 79 and 84 per cent for FBS, 86, 77 and 85 per cent for EGS and 94, 82 and 74 per cent for ESS respectively. For all the three sera used, there was a significant difference between serum supplementation and non supplementation and no significant difference was observed among different concentrations of the serum.

In most of the studies, fetal calf serum has been widely used for oocyte maturation *in vitro* in many species such as goat (Crozet *et al.*, 2000) and sheep (Ghasemzadeh-Nava and Tajik, 2000). It was found that serum provided nutrition to the cells in the COCs and prevented zona pellucida hardening in sheep oocytes (Wani, 2002).

Semi-defined protein preparation such as serum was usually included in IVM media because it contains undefined growth factors, hormones and peptides that may support growth and development of oocytes. Maturation media were usually supplemented with 10-20 per cent heat-treated serum (56° c for 30 min) (Tibary *et al.*, 2005).

The maturation rate of the caprine oocytes in media without protein supplementation was 28.57 per cent whereas, the protein supplemented in the form of 10 per cent EGS significantly increased the maturation rate to 61.9 per cent and the maturation rate was further increased with 15 and 20 per cent EGS supplementation to 72.7 and 78.6 per cent respectively (Kharche *et al.*, 2006). A 68.42 per cent maturation rate with 20 per cent EGS was reported in caprine oocytes without hormone supplementation (Kharche *et al.*, 2008b).

2.8.4 Oocyte Retrieval Technique

2.8.4.1 Aspiration

Oocytes collected by follicle aspiration exhibited a maturation percentage of 87.1 per cent which was lower than that obtained for oocytes collected by slicing or puncture (Pawshe *et al.,* 1994). Vijayakumaran (1995) opined that retrieval systems were having only negligible influence on *in vitro* maturation capability of goat oocytes. Maturation rates obtained for the oocytes collected by aspiration (71.85 per cent), slicing (73.65 per cent) and puncture (74.59 per cent) techniques did not differ significantly. A maturation rate of 100 per cent was reported for goat oocytes aspirated from 2-6 mm follicles and incubated for 24 h in TCM 199 medium supplemented with 10 per cent fetal calf serum (FCS), 100 mg/ml LH, 0.5 mg/ml FSH and 1 mg/ ml estradiol 17β at 38.5° C in humid five per cent CO_2 in air (Samake *et al.,* 2000).

2.8.4.2 Slicing

Martino *et al.* (1994) reported that the percentages of maturation obtained for slicing (56.9 per cent) were lower than those obtained for dissection and aspiration (69.3 and 72 per cent respectively). Pawshe *et al.* (1994) obtained a maturation rate of 90.4 per cent for oocytes recovered by slicing and the author observed significant differences in the maturation rates following the three methods of oocyte collection. Wang *et al.* (2007a) reported that there was a statistically lower proportion of metaphase II stage after *in vitro* maturation of the oocytes harvested by slicing method compared to aspiration and puncture. The author suggested that this lower maturation rate may be due to more preantral follicles collected by slicing techniques than by other techniques. It was observed that, the good quality oocytes recovered by three collection methods had the similar cleavage rate and blastocyst yield of the embryos after IVF of the M II stage oocytes.

2.8.4.3 Puncture

Retrieval method was found to have no significant effect on *in vitro* maturation potential of bovine COCs (Paul, 2005). A maturation rate of 68.42 per cent with addition of 20 per cent EGS and without hormones was recorded in caprine oocytes recovered by puncture of ovarian follicles (Kharche *et al.*, 2008a).The percentage of GV, GVBD, M I and M II stages of development after IVM of oocytes retrieved by puncture of ovarian follicles was reported to be 7.24, 4.82, 14.82 and 61.37 per cent respectively (Kharche *et al.*, 2008b).

2.8.5 Other Factors

For IVM of goat oocytes, follicular fluid (FF) recovered from large follicles (>4 mm) could be used as a supplement in maturation medium containing TCM 199 and 100 ng of oFSH, and this positive effect was enhanced when FF was recovered from non-atretic or gonadotropin stimulated follicles (Cognie *et al.*, 1995 and Cognie and Poulin, 2000). Factors contained in FF, such as growth hormone, activin, or inhibin could stimulate oocyte maturation and that the inhibin α- subunit could decrease the developmental potential of oocytes (Stock *et al.*, 1997).

Tyagi *et al.* (1997) reported that granulosa cell monolayer delayed maturation of goat oocytes for a few hours, but increased the maturation percentage significantly. A study was conducted to compare the two oocyte maturation systems, a granulosa cell monolayer and granulosa cell co-culture for their effects on IVM, IVF and developmental competence of caprine oocytes and it was concluded that granulose cell monolayer better supported cytoplasmic maturation of growing caprine oocytes, which was evident by a better maturation rate, active fertilization and improved cleavage rates and subsequently a higher rate of morula formation (Teotia *et al.*, 2001).

Bormann *et al.* (2003) suggested that addition of vitamins during caprine oocyte maturation was beneficial for subsequent blastocyst development and viability. It was reported that addition of vitamins significantly increased overall blastocyst development and tended to increase the percentage of cleaved embryos. The possible functions of vitamins during maturation of caprine oocytes were suggested to be due to the significant role played by vitamins in the metabolism of carbohydrates and amino acids and to act as an antioxidant.

Gupta *et al.* (2005) matured ovine oocytes in TCM-199 with different concentrations of a novel peptide (Mr 26.6 kDa), isolated and partly purified from buffalo ovarian follicular fluid. The author observed a dose dependent increment in the cumulus expansion as well as *in vitro* maturation rates of oocytes.

2.9 FERTILITY STATUS OF IVM OOCYTE

Caprine oocytes matured and fertilized *in vitro* and transferred to a recipient have resulted in the birth of live offspring (Crozet *et al.*, 1993). It was reported that blastocyst development from *in vitro* matured and fertilized caprine oocytes remained inefficient because less than a third of the embryos resulting from *in vitro* maturation and *in vitro* fertilization developed to morula stage (Crozet *et al.*, 1993; Martino *et al.*, 1995; Rho *et al.*, 2001 and Teotia *et al.*, 2001). Kids born after complete *in vitro* maturation, fertilization and culture were reported by Keskintepe *et al.* (1994). Kharche *et al.* (2005) studied the effect of vitrification of oocyte on *in vitro* maturation and fertilization capacity and obtained a maturation and fertilization rate of 27.5 and 17.8 percent and 74.1 and 54.4 percent for vitrified and non vitrified oocytes respectively.

Materials and Methods

3. MATERIALS AND METHODS

3.1 SOURCE OF OVARY

Ovaries required for the study were collected from the corporation slaughter house, Kuriachira, Thrissur. Malabari goats and its crossbreds are mainly slaughtered here.

3.2 COLLECTION AND TRANSPORTATION OF OVARY

Ovaries dissected out from freshly slaughtered animals were transported to the laboratory within 1-2 h of slaughter at 35-37°C in a thermo flask containing freshly prepared normal saline solution fortified with 100 IU/ml Benzyl penicillin and 100 µg/ml Streptomycin sulphate.

3.3 PRE RETRIEVAL PROCESSING OF OVARIES

The ovaries were washed in normal saline solution to remove excess blood and tissue debris. After trimming of the extraneous tissue, the ovaries were washed several times in sterile normal saline solution containing penicillin and streptomycin at 37°C. The final washing was done with Dulbeco's Phosphate Buffered Saline (DPBS) supplemented with 0.5 per cent Bovine Serum Albumin (BSA) and maintained at 36-38°C.

3.4 RETRIEVAL OF OOCYTES

Oocytes were retrieved from the ovaries by applying any one of the three retrieval methods namely aspiration, slicing and puncture, in Cumulus Oocyte Complex (COC) handling media prepared with DPBS enriched with five per cent day zero oestrus goat serum and 0.5 per cent BSA and maintained at 38°C. A total of 138 ovaries were collected, and 42, 48 and 48 ovaries were processed under retrieval

methods namely aspiration, slicing and puncture respectively and yield of different quality grades of oocytes under each retrieval method was assessed.

3.4.1 Aspiration

Surface follicles measuring 2-8 mm in size were aspirated with a sterile 20 gauge disposable needle connected to a 10 ml disposable plastic syringe.

3.4.2 Slicing

All the visible surface follicles and the whole ovary were gently sliced with a Bard-Parker blade No: 22. Ovaries were held with an artery forceps in a petri dish containing 5 ml COC handling medium during the slicing process. Large sized ovarian tissue debris was removed from the medium prior to further processing.

3.4.3 Puncture

All the visible follicles and ovarian surface were repeatedly punctured with a 20 gauge needle until antrum from all surface follicles were exteriorized. During this process, the ovaries were kept in a petri dish containing 5 ml COC handling medium and held in position with an artery forceps.

3.5 POST RETRIEVAL PROCESSING OF OOCYTES

The medium containing oocytes from all retrieval methods were transferred into separate sterile 90 mm petri dishes having grid and examined under zoom stereomicroscope at 10x magnification for identification of oocytes. Identified oocytes were collected by means of unopette and transferred into 35 mm petri dish containing fresh COC handling media maintained at 38° C. The total yield of oocytes from different retrieval systems and their morphological grades were recorded separately.

3.6. CHARACTERIZATION AND CLASSIFICATION OF OOCYTES

All the oocytes obtained from different retrieval systems were examined separately under 40x magnification of zoom stereomicroscope for their morphological character. Based on the number of layers of cumulus cells and ooplasm character (Vijayakumaran, 1995), the oocytes were graded into three categories as follows.

Grade A: Morphologically normal oocytes with more than three complete layers of compact cumulus cells and uniform granulation of ooplasm.

Grade B: Morphologically normal oocytes with two to three complete layers of cumulus mass and uniform granulation of ooplasm.

Grade C: Morphologically normal oocytes with single complete layer of cumulus cells and uniform granulation of ooplasm.

All other oocytes that were found to be inferior to grade C quality were considered as poor quality and were discarded from further processing. Each class of selected oocytes under different retrieval methods were put separately in labelled drops of COC handling medium until further processing for *in vitro* maturation.

3.7 *IN VITRO* MATURATION OF OOCYTES

A total of 129, 162 and 140 oocytes collected respectively by aspiration, slicing and puncture were subjected to *in vitro* maturation. The number of oocytes under each quality grade, retrieved by each retrieval method was noted separately.

3.7.1 Media

Medium used for *in vitro* maturation of oocytes was freshly prepared Hepes modified TCM- 199 (25 mM Hepes) enriched with 22 µg /ml sodium pyruate, 2.2 mg /ml sodium bicarbonate and penicillin G sodium and streptomycin sulphate at the rate

of 500 µg /ml each. This medium was further supplemented with hormones, 1 µg /ml estradiol- 17β, 0.5 µg /ml FSH, 0.06 IU hCG and 20 per cent heat- inactivated oestrus goat serum prior to employing it for IVM. Sufficient numbers of 50 µl maturation drops were prepared with this media in 35 mm sterile petri dishes to accommodate each category of oocytes. Sterile mineral oil was layered over these drops and was equilibrated in CO_2 incubator for at least 2 h prior to introducing the oocyte. Two small dishes of maturation media were also maintained in the incubator for pre and post maturation washing of oocytes.

3.7.2 Culture Conditions

Culture conditions set for this study were 38.5 ° C temperature, five per cent CO_2 tension and maximum humidity in a standard water jacketed CO_2 incubator.

3.7.3 Pre Culture Washing of Oocytes

Each category of oocyte was washed repeatedly in COC handling media and then subjected to final washing with pre-equilibrated maturation media.

3.7.4 *In vitro* Culture of Oocytes

On completion of final washing, each category of oocyte was loaded gently into separate maturation drops at five to seven oocytes per drop using unopette and allowed to complete incubation for 24 h without any disturbance in the culture environment.

3.8 ASSESSMENT OF MATURATION

Maturation rates of different quality grades of oocytes obtained from three retrieval methods were assessed based on the cumulus expansion and for the purpose of confirmation, a minimum of 30 oocytes from each retrieval method were examined for nuclear changes.

3.8.1 Cumulus Cell Expansion

After 24 h of incubation, oocytes in the culture drops were examined under zoom stereomicroscope for the signs of maturation such as cumulus expansion and mucification of cumulus cells.

3.8.2 Assessment of Nuclear Maturation of Oocytes

A minimum of 30 oocytes showing cumulus expansion from each retrieval method were taken out of the culture drops and washed with pre-equilibrated maturation media to free it off from the traces of residual mineral oil, denuded by repeated pipetting, fixed in acetic acid and methanol (1:3) stained with one per cent aceto-orcein, and then examined for changes associated with nuclear maturation.

3.8.2.1 Denudation of Oocytes

Oocytes after washing in pre-equilibrated maturation media were stripped off from their cumulus investment by repeated pipetting for five minutes with a small bore capillary tube connected to a tuberculin syringe. Denuded oocytes were transferred to another fresh drop to free them from the liberated cumulus cells.

3.8.2.2 Whole Mount Fixation

Denuded oocytes were then placed on a labeled grease free clean glass slide in a small drop of maturation medium. Small quantity of Vaseline paraffin jelly was applied on the four corners of a clean cover glass. It was then placed gently over the denuded oocytes already kept on the glass slide. Cover glass was pressed down carefully over to the oocytes by applying gentle pressure alternately on all the four corners of the cover glass with the tip of a forceps until it touched the fluid medium. There after extra care was taken while pressing down to get a good squash on the oocytes, without actually breaking it. The processed slide was gently dipped into

acetic acid : methanol (1:3 ratio) fixative. It was allowed to remain in the fixative for about 12 to 24h.

3.8.2.3 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 30 oocytes from each retrieval method was stained with 1 per cent orcein in 45 per cent 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.

3.8.2.4 Maturation Changes of Nucleus

In aceto-orcein staining, the germinal vesicle appeared like a circular ring with a small dark spot at the centre representing nucleolus. Germinal vesicle break down (GVBD) stage was characterized by nuclear membrane expanding, fragmenting and at the same time chromosomes becoming more condensed. Metaphase I appeared like a single set of chromosome in the equatorial plane. Metaphase II appeared like two sets of chromosomes in two different planes of focus, one for polar body and the other for MII chromosomes.

3.9 STATISTICAL ANALYSIS

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

Table 1. Details of media, hormones, chemicals, glasswares and equipments

Sl. No.	Name of Item	Product Name	Manufacturer
(1)	**Media, hormones and chemicals**		
1.	TCM-199	Medium 199	Sigma Chemicals St. Louis, USA
2.	Dulbecco's Phosphate Buffered Saline	Dulbecco's Phosphate Buffered Saline	Sigma Chemicals, St. Louis, USA
3.	p FSH (FSH from Porcine Pituitary)	Folltropin – V	Vetrepharm Canada Inc.
4.	hCG	Chorulon	Intervet (India) pvt Ltd, Hyderabad.
5.	17-β-Estradiol	β-Estradiol	Sigma Chemicals St. Louis, USA
6.	Sodium pyruvate	Sodium Pyruvate	Sigma Chemicals St. Louis, USA
7.	Streptomycin sulphate	Ambistrin	Alembic Ltd. Vadodra
8.	Benzyl penicillin sodium	Benzyl penicillin injection	Alembic Ltd., Vadodra
9.	Sodium Chloride	Sodium Chloride	Sigma Chemicals St. Louis, USA
10.	Sodium bicarbonate	Sodium bicarbonate	Sigma Chemicals St. Louis, USA
11.	Mineral oil	Mineral oil	Sigma Chemicals St. Louis, USA
12.	Orcein	Natural red	Hi media Laboratories Ltd. Mumbai
13.	Methanol	Methanol	Merck, Germany
14.	Acetic acid	Acetic acid	BDH Laboratories England
15.	BSA fraction V	Albumin, Bovine Faction V	Sigma Chemicals St. Louis, USA

Sl.No	Name of Item	Product Name	Manufacturer
(2)	**Disposable / Glass Wares**		
1.	Petri dish (35 mm)	Falcon	Becton Dickinson Labware,NewJersy, USA
2.	Petri dish (60 mm)	Falcon	Tarson Products, India
3.	Petri dish (90 mm)	Falcon	Tarson Products, India
4.	Syringe filter units (0.22 µm) 25 mm	Millex – GS	Millipore Corporation USA
5.	Disposable pipette tips 10 µl, 200 µl, 1000 µl	Brand	BRAND Germany
6.	Plastic syringe (2 ml, 5 ml, 10ml)	Dispovan	Hindustan Syringes and Medical Devices Ltd., India
7.	20 g needle 20 x 1 ½ 1.20 x 38 mm	Dispovan	Hindustan Syringes and Medical Devices Ltd., India
8.	Conical centrifuge tubes	Falcon	Becton, Dickinson, Labware NEW Jersy USA
9.	Capillary pipettes	Unopette	Becton Dickinson vacutainer systems USA
10.	Microcentrifuge tubes (1.5 ml)	Tarson	Tarson Products, India
11.	Serum vials (2 ml)	Tarson	Tarson Products, India

Sl.No	Name of Item	Product Name	Manufacturer
(3)	**Equipments**		
1.	CO_2 Incubator	Lab Line	Lab Line instruments Inc. USA
2.	Zoom stereomicroscope	Leica MZ6	Leica micro system, Germany
3.	Phase contrast microscope	Leica DMIL	Leica micro system, Germany
4.	Stage warmer	Linkam MC 60	Linkam, England
5.	Streamline vertical laminar flow cabinet	Esco	ESCO, India
6.	Millipore ultra water filtration unit	Milli Q UF Plus	Millipore corporation, USA
7.	Micropipettes 0.5-10 µl, 100-1000 µl	Labopette	Hirschman Laborgerate, Germany
8.	Micropipette 10-200 µl	Transferpetter	Brand, Germany
9.	Micropipette helper	Brand	Brand, Germany
10.	Digital pH meter	Cyber scan 2500	Cyber scan Eutech Instrument, Singapore
11.	Electronic analytical precision balance	CP-2245	Sartorius, Germany
12.	Thermal flask	Eagle	Eagle, India

OOCYTE RETRIEVAL METHODS

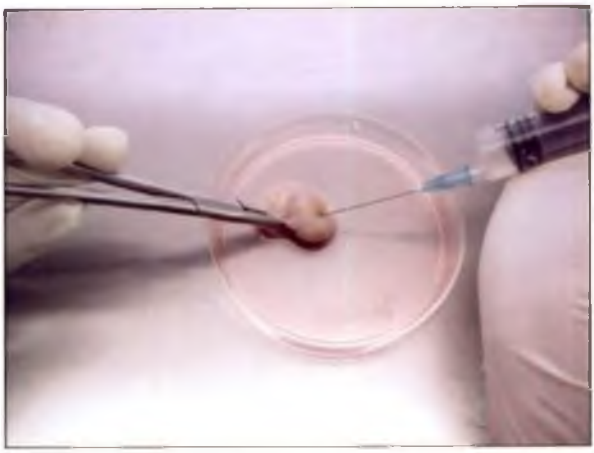


Plate 1 : Aspiration

Plate 2 : Slicing

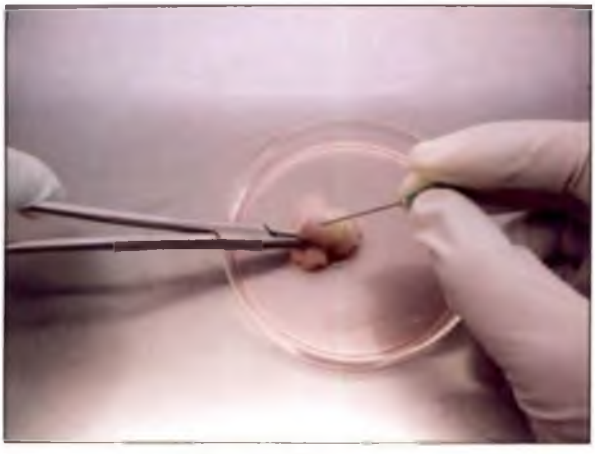


Plate 3 : Puncture

CLASSIFICATION OF CUMULUS - OOCYTE COMPLEXES

Plate 4 : A grade Oocyte (200x)

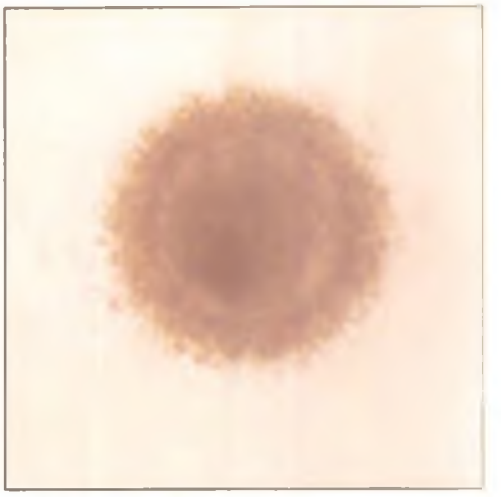


Plate 5 : B grade Oocyte (200x)

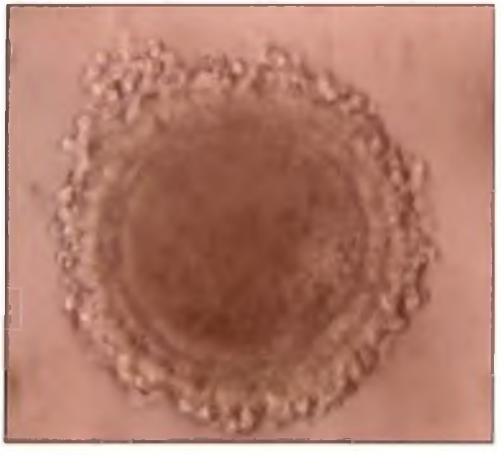


Plate 6 : C grade Oocyte (200x)

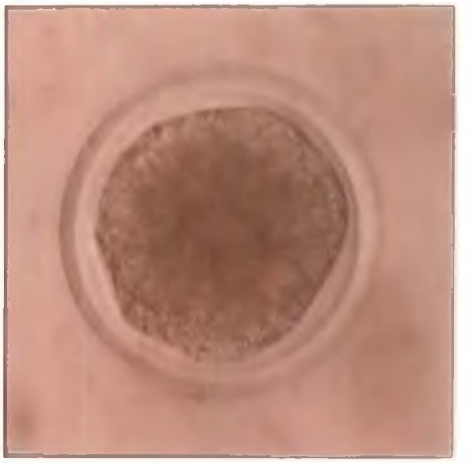


Plate 7 : Poor Quality Oocyte (200x)

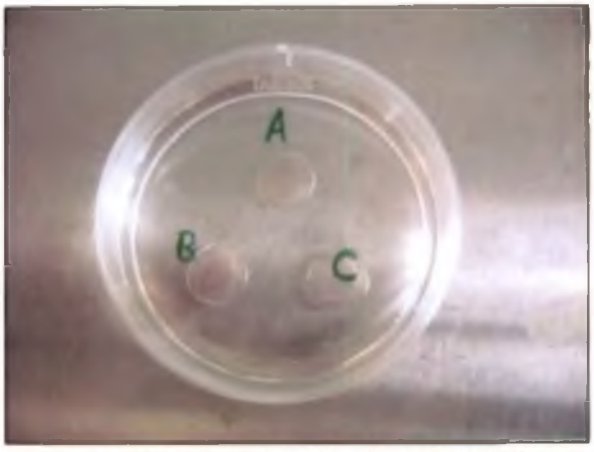


Plate 8 : Maturation Media TCM-199 as 50 µl drops under mineral oil overlay

Plate 9 : *In vitro* maturation of oocytes in CO_2 incubator

Plate 10 : Assessment of maturation under phase contrast microscope.

Results

4. RESULTS

This study on *in vitro* maturation of caprine follicular oocytes was carried out using oocytes collected by three retrieval techniques from ovaries of freshly slaughtered animals. A total of 550 oocytes from 138 ovaries collected by three retrieval methods were used in the study.

4.1 EFFECT OF RETRIEVAL TECHNIQUES ON YIELD OF OOCYTES PER OVARY

A total of 138 ovaries were subjected to the study which on harvest by three retrieval methods viz. aspiration, slicing and puncture yielded a total of 550 oocytes. Forty two ovaries were subjected to aspiration and 48 each were subjected to slicing and puncture. The yield of oocytes under each retrieval method was 165 by aspiration, 213 by slicing and 172 by puncture. The mean yield of oocytes per ovary was found to be 3.93 ± 0.11, 4.44 ± 0.06 and 3.59 ± 0.07 by aspiration, slicing and puncture respectively (Table 2 and Fig. 1). It was found that aspiration slicing and puncture differ significantly ($P< 0.01$) in the mean yield of oocytes.

4.2 EFFECT OF RETRIEVAL TECHNIQUES ON THE QUALITY OF COCs

4.2.1 Comparative Efficiency between Retrieval Techniques

4.2.1.1 A Class Oocytes

The yield of Class A oocytes by aspiration, slicing and puncture were 26.74, 23.08 and 29.01 per cent respectively (Table 3 and Fig. 4). On statistical analysis, it was found that there was significant difference ($P< 0.01$) in the percentage yield of A class oocytes between aspiration and slicing or puncture and slicing. But aspiration and puncture did not differed significantly in the yield of A class oocytes.

Maximum number of A class oocytes per ovary was obtained by aspiration followed by slicing and puncture as 1.05 ± 0.05, 1.04 ± 0.04 and 1.04 ± 0.04 respectively (Table 3 and Fig. 2)

4.2.1.2 B Class Oocytes

Class B oocytes obtained by aspiration, slicing and puncture were 26.62, 28.15 and 30.26 per cent respectively (Table 3 and Fig. 4). From statistical analysis it was found that aspiration differed significantly ($P< 0.01$) from puncture in the yield of B class oocytes. There was no significant difference between aspiration and slicing or puncture and slicing. However, maximum percentage of B class oocytes was obtained by puncture followed by slicing and aspiration

Mean yield of B class oocytes per ovary was found to be 1.05 ± 0.08, 1.25.± 0.05 and 1.08 ± 0.03 respectively by aspiration, slicing and puncture (Table 3 and Fig. 2). Maximum B class oocytes per ovary were obtained by slicing followed by puncture and aspiration.

4.2.1.3 C Class Oocytes

Aspiration, slicing and puncture yielded 24.77, 24.44 and 22.07 per cent C class oocytes respectively (Table 3 and Fig. 4). On statistical analysis it was found that aspiration, slicing and puncture did not differ significantly in the percentage yield of C class oocytes. But it was found that a maximum percentage of C class oocytes were obtained by slicing, followed by aspiration and puncture.

The mean number of C class oocytes retrieved per ovary was found to be 0.98 ± 0.07, 1.08 ± 0.07 and 0.79 ± 0.04 by aspiration, slicing and puncture respectively (Table 3 and Fig. 2). Maximum number of C class oocytes per ovary was obtained by slicing followed by aspiration and puncture.

4.2.1.4 Poor Quality Oocytes

The yield of poor quality oocytes by aspiration, slicing and puncture were 21.87, 23.96 and 18.66 per cent respectively (Table 3 and Fig. 4). It was found that there was significant difference ($P< 0.01$) in the percentage yield of poor quality oocytes between slicing and puncture. But aspiration and puncture did not differ significantly and similarly aspiration and slicing did not differ significantly in the percentage yield of poor quality oocytes.

The maximum number of poor quality oocytes per ovary was obtained in slicing followed by aspiration and puncture as 1.06 ± 0.06, 0.86 ± 0.04 and 0.67 ± 0.03 respectively (Table 3 and Fig. 2).

4.2.2 Comparative Efficiency within each Retrieval Technique

4.2.2.1 Aspiration

A total of 165 oocytes were obtained from 42 ovaries subjected to aspiration. Of this, 44 (26.74 per cent) were A class oocytes, 44 (26.62 per cent) were B class, 41 (24.77 per cent) were C class and 36 (21.87 per cent) were poor quality oocytes (Table 4 and Fig. 3). Yield of A class oocytes was maximum followed by B and C class even though they did not differ significantly. It was also observed that yield of A and B class oocytes were significantly ($P<0.05$) higher than that of poor quality oocytes and yield of C class oocytes did not differed significantly from the yield of A, B or poor quality grade oocytes.

The mean yield of A, B, C and poor quality grades of oocytes per ovary were found to be 1.05 ± 0.05, 1.05 ± 0.08, 0.98 ± 0.07 and 0.86 ± 0.04 respectively (Table 4 and Fig. 2).

4.2.2.2 Slicing

On slicing of 48 ovaries, 213 oocytes were obtained. Among this, A, B, C and poor quality grades were 50 (23.08 per cent), 60 (28.15 per cent), 52 (24.44 per cent) and 51 (23.96 per cent) respectively (Table 4 and Fig. 3). It was found that yield of A class oocyte differed significantly ($P<0.05$) from that of B class oocytes. Yield of B and C class oocytes was significantly higher than that of poor quality oocytes.

Oocyte yield per ovary on slicing was 1.04 ± 0.04, 1.25 ± 0.05, 1.08 ± 0.07 and 1.06 ± 0.06 for A, B, C and poor quality grades of oocytes respectively (Table 4 and Fig. 2).

4.2.2.3 Puncture

When puncture was performed in 48 ovaries, 172 oocytes were obtained. Of this, 50 (29.01 per cent) were A class, 52 (30.26 per cent) were B class, 38 (22.07 per cent) C class and 32 (18.66 per cent) were poor quality oocytes respectively (Table 4 and Fig. 3). The yield of A and B class oocytes did not differ significantly but significant difference ($P<0.05$) was observed between C class and poor quality oocytes.

Mean yield of A, B, C and other quality grades of oocytes per ovary by puncture method was 1.04 ± 0.04, 1.08 ± 0.03, 0.79 ± 0.04 and 0.67 ± 0.03 respectively (Table 4 and Fig. 2).

4.3 EFFECT OF RETRIEVAL METHOD AND COC MORPHOLOGY ON CUMULUS EXPANSION

After 24 h of culture in standard culture conditions and media, oocytes were examined for cumulus expansion. Oocytes showing cumulus expansion were evaluated as mature oocytes.

4.3.1 Comparison between Methods of Retrieval under Same Class

4.3.1.1 A Class COCs

The cumulus expansion rate of A class oocytes obtained by aspiration, slicing and puncture was 77.75 per cent, 69.70 per cent and 71.49 per cent respectively (Table 5 and Fig. 5). Even though there was no significant difference in the cumulus expansion rates of A class oocytes retrieved by aspiration, slicing and puncture, maximum cumulus expansion rate was obtained for oocytes retrieved by aspiration followed by puncture and slicing.

4.3.1.2 B Class COCs

Cumulus expansion rate of 63.48 per cent, 51.37 per cent and 63.39 per cent was obtained for B class oocytes retrieved by aspiration, slicing and puncture respectively (Table 5 and Fig. 5). It was found that the cumulus expansion rate of B class oocytes retrieved by slicing was significantly ($P<0.01$) low when compared to other methods.

4.3.1.3 C Class COCs

Class C oocytes obtained by aspiration, slicing and puncture when subjected to *in vitro* maturation exhibited a cumulus expansion rate of 39.17, 32.57 and 37.29 per cent respectively (Table 5 and Fig. 5). No significant difference in the cumulus expansion rate between different retrieval methods was observed but maximum cumulus expansion rate was obtained for oocytes retrieved by aspiration followed by puncture and then slicing.

4.3.2 Comparison between Morphological Grades under each Retrieval Method

4.3.2.1 Aspiration

When 129 oocytes retrieved by aspiration were kept for maturation, 79 (68.99 per cent) exhibited well to moderate cumulus expansion. Among the 44 A class, 44 B class and 41 C class oocytes kept for maturation, 35 (77.75 per cent), 28 (63.48 per cent) and 16 (39.17 per cent) exhibited cumulus expansion respectively (Table 6 and Fig. 6). It was found that there was a significant difference ($P<0.05$) between the cumulus expansion rates of A, B and C class oocytes.

4.3.2.2 Slicing

One hundred and sixty two oocytes retrieved by slicing were kept for maturation and 86 (53.09 per cent) exhibited cumulus expansion. Among 50 A class, 60 B class and 52 C class oocytes, 35 (69.70 per cent), 31 (51.37 per cent) and 20 (32.57 per cent) exhibited cumulus expansion respectively (Table 6 and Fig. 6). Significant difference ($P<0.05$) was found in the cumulus expansion rate among different grades of oocytes.

4.3.2.3 Puncture

When 140 oocytes retrieved by puncture were kept for maturation, 83 (59.29 per cent) exhibited cumulus expansion. Out of 50 A class, 52 B class and 38 C class oocytes, 36 A class (71.49 per cent), 33 B class (63.39 per cent) and 14 C class oocytes (37.29 per cent) exhibited cumulus expansion (Table 6 and Fig. 6). No significant difference was observed between the cumulus expansion rate of A and B class oocytes but cumulus expansion rate of C class oocytes was significantly ($P<0.05$) lower than that of A and B class.

4.4 EFFECT OF RETRIEVAL METHOD AND COC MORPHOLOGY ON NUCLEAR MATURATION

4.4.1 Comparison between Methods of Retrieval under Same Class

4.4.1.1 A class Oocytes

Fourty, 30 and 50 per cent of A class oocytes retrieved by aspiration, slicing and puncture respectively exhibited nuclear maturation (Table 7 and Fig. 7). It was found that there was no significant difference between aspiration and slicing or aspiration and puncture but there was significant difference ($P<0.01$) between slicing and puncture in the nuclear maturation rate of A class oocytes.

Polar body extrusion rate in aspiration, slicing and puncture was 30, 20 and 30 per cent respectively (Table 8). Statistical analysis revealed no significant difference between these methods on polar body extrusion rate of A class oocytes.

4.4.1.2 B Class Oocytes

Class B oocytes retrieved by aspiration, slicing and puncture exhibited a nuclear maturation rate of 20, 10 and 30 per cent respectively (Table 7 and Fig. 7). There was no significant difference between aspiration and slicing or aspiration and puncture but there was significant difference ($P<0.01$) between slicing and puncture in the nuclear maturation rate.

Class B oocytes retrieved by aspiration, slicing and puncture exhibited a polar body extrusion rate of 10, 10 and 20 per cent respectively (Table 8). No significant difference between aspiration, slicing and puncture on polar body extrusion rate of B class oocytes was there.

4.4.1.3 C Class Oocytes

Percentage of C class oocytes exhibiting nuclear maturation when retrieved by aspiration, slicing and puncture were 10, 10 and 20 per cent respectively (Table 7 and Fig. 7). No significant difference was noticed between retrieval techniques in nuclear maturation rate of C class oocytes. Only one C class oocyte (10 per cent) retrieved by puncture exhibited polar body extrusion (Table 8).

4.4.2 Comparison between Morphological Grades under each Retrieval Method

4.4.2.1 Aspiration

In aspiration method 30 oocytes (10 oocytes each of A, B and C grade) were examined for nuclear maturation. Among the 10 A class oocytes examined, four (40 per cent) exhibited nuclear maturation with presence of M II stage chromosomes. Among these, three (30 per cent) exhibited polar body extrusion. Out of the 10 B class oocytes examined, two (20 per cent) exhibited nuclear maturation and one (10 per cent) exhibited polar body extrusion. Among the 10 C class oocytes only one (10 per cent) exhibited nuclear maturation and none of the oocytes exhibited polar body extrusion (Table 9 and Fig. 8). It was found that there was significant ($P<0.01$) difference in the nuclear maturation rate and polar body extrusion rate of each class of oocytes. The overall nuclear maturation and polar body extrusion rate in aspiration was found to be 23.33 and 13.33 per cent respectively.

4.4.2.2 Slicing

Among 30 oocytes denuded and examined for nuclear changes, three (30 per cent) out of 10 A class oocytes revealed M II stage and two (20 per cent) revealed extruded polar body. Out of 10 B class oocytes only one (10 per cent) exhibited both nuclear maturation and polar body extrusion. With respect to the C class oocytes retrieved by slicing, out of the 10 oocytes examined, only one (10 per cent) exhibited M II stage and none exhibited polar body extrusion (Table 9 and Fig. 8). There was

significantly higher nuclear maturation rate of A class oocytes than B or C class oocytes ($P< 0.01$). No significant difference was noticed between A and B class oocytes in the polar body extrusion rate. An overall nuclear maturation and polar body extrusion rate of 16.67 and 10 per cent respectively were recorded.

4.4.2.3 Puncture

A total of 30 oocytes were examined for nuclear changes. Out of the 10 A class oocytes, five (50 per cent) exhibited M II stage and three (30 per cent) exhibited polar body extrusion. Among the 10 B class oocytes, three (30 per cent) and two (20 per cent) exhibited nuclear maturation and polar body extrusion respectively. Out of the 10 C class oocytes, two (20 per cent) exhibited M II stage and one (10 per cent) exhibited extruded polar body (Table 9 and Fig. 8). There was significant difference ($P<0.01$) between A, B and C class oocytes in the nuclear maturation rate. But polar body extrusion rate of A and B class oocytes or B and C class oocytes did not differ significantly. The overall nuclear maturation rate was 33.33 per cent and polar body extrusion rate was 20 per cent.

Table 2. Effect of three harvesting techniques on oocyte yield and Mean(±S.E) recovery rate of oocytes per ovary

Sl. No.	Retrieval methods	Number of ovaries	Total yield of oocytes	Mean number of oocytes (Mean ± S.E)
1	Aspiration	42	165	3.93 ± 0.11^{a}
2	Slicing	48	213	4.44 ± 0.06^{b}
3	Puncture	48	172	3.59 ± 0.07^{c}
4	Total	138	550	$3.99 \pm 0.09^{*}$

Values with different superscripts (a,b,c) in the same column differ significantly ($P<0.01$)

*Indicate average

Table 3. Percentage grades of oocyte and oocyte yield per ovary recovered by the different retrieval techniques

Sl. No.	Morphological grade of COCs	Oocyte retrieval systems								
		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	44	26.74[a]	1.05 ± 0.05	50	23.08[b]	1.04 ± 0.04	50	29.01[a]	1.04 ± 0.04
2	B	44	26.62[a]	1.05 ± 0.08	60	28.15[ab]	1.25 ± 0.05	52	30.26[b]	1.08 ± 0.03
3	C	41	24.77[a]	0.98 ± 0.07	52	24.44[a]	1.08 ± 0.07	38	22.07[a]	0.79 ± 0.04
4	Poor	36	21.87[ab]	0.86 ± 0.04	51	23.96[b]	1.06 ± 0.06	32	18.66[a]	0.67 ± 0.03

Values with different superscripts (a,b,c) in the same row differ significantly ($P<0.01$)

Table 4. Effect of retrieval technique on oocyte quality - Comparative efficiency within retrieval techniques

Sl.No.	Oocyte retrieval system	Number of ovaries studied	Total yield of oocytes	Morphological grade of COCs	Number of oocytes in each grade	Percentage of total yield	Mean number per ovary
1	Aspiration	42	165	A	44	26.74[a]	1.05 ± 0.05
				B	44	26.62[a]	1.05 ± 0.08
				C	41	24.77[ab]	0.98 ± 0.07
				Poor	36	21.87[b]	0.86 ± 0.04
2	Slicing	48	213	A	50	23.08[a]	1.04 ± 0.04
				B	60	28.15[b]	1.25 ± 0.05
				C	52	24.44[ab]	1.08 ± 0.07
				Poor	51	23.96[a]	1.06 ± 0.06
3	Puncture	48	172	A	50	29.01[a]	1.04 ± 0.04
				B	52	30.26[a]	1.08 ± 0.03
				C	38	22.07[b]	0.79 ± 0.04
				Poor	32	18.66[c]	0.67 ± 0.03

Values with different superscripts (a,b,c) in the same column differ significantly (P<0.05)

Table 5. Percentage cumulus expansion rate of each grade of oocytes from different retrieval systems

Sl. No.	Grade of COCs	Oocyte retrieval systems								
		Aspiration			Slicing			Puncture		
		Oocytes kept for maturation	Oocytes showing cumulus expansion	Expanded COCs (%)	Oocytes kept for maturation	Oocytes showing cumulus expansion	Expanded COCs (%)	Oocytes kept for maturation	Oocytes showing cumulus expansion	Expanded COCs (%)
1	A	44	35	77.75[a]	50	35	69.70[a]	50	36	71.49[a]
2	B	44	28	63.48[a]	60	31	51.37[b]	52	33	63.39[a]
3	C	41	16	39.17[a]	52	20	32.57[a]	38	14	37.29[a]
4	Total	129	79	68.99*	162	86	53.09*	140	83	59.29*

Values with different superscripts (a,b,c) in the same row differ significantly ($P<0.01$)

*Indicate average

Table 6. Percentage cumulus expansion rate of different grade oocytes within each retrieval method.

Sl. No	Oocyte retrieval system	Morphological grade of COCs	Oocytes kept for maturation	Oocytes showing cumulus expansion	Expanded COCs (%)
1	Aspiration	A	44	35	77.75[a]
		B	44	28	63.48[b]
		C	41	16	39.17[c]
		Total	129	79	68.99[*]
2	Slicing	A	50	35	69.70[a]
		B	60	31	51.37[b]
		C	52	20	32.57[c]
		Total	162	86	53.09[*]
3	Puncture	A	50	36	71.49[a]
		B	52	33	63.39[a]
		C	38	14	37.29[b]
		Total	140	83	59.29[*]

Values with different superscripts (a,b,c) in the same column differ significantly (P<0.05)

Table 7.Percentage nuclear maturation rate of each grade of oocytes obtained by different retrieval systems.

Sl. No.	Grade of COCs	Oocyte retrieval systems								
		Aspiration			Slicing			Puncture		
		No. of oocytes examined	No. of M II stage oocytes	Percentage of nuclear maturation	No. of oocytes examined	No. of M II stage oocytes	Percentage of nuclear maturation	No. of oocytes examined	No. of M II stage oocytes	Percentage of nuclear maturation
1	A	10	4	40^{ab}	10	3	30^{a}	10	5	50^{b}
2	B	10	2	20^{ab}	10	1	10^{a}	10	3	30^{b}
3	C	10	1	10^{a}	10	1	10^{a}	10	2	20^{a}
	Total	30	7	23.33^{*}	30	5	16.67^{*}	30	10	33.33^{*}

Values with different superscripts (a,b,c) in the same raw differ significantly ($P<0.01$)

*Indicate average

Table 8. Percentage of polar body extrusion rate of each grade of oocytes obtained by different retrieval systems.

Sl. No.	Grade of COCs	Oocyte retrieval systems								
		Aspiration			Slicing			Puncture		
		No. of oocytes examined	No. of Polar body extruded	Percentage of polar body extrusion	No. of oocytes examined	No. of Polar body extruded	Percentage of polar body extrusion	No. of oocytes examined	No. of Polar body extruded	Percentage of polar body extrusion
1	A	10	3	30[a]	10	2	20[a]	10	3	30[a]
2	B	10	1	10[a]	10	1	10[a]	10	2	20[a]
3	C	10	0	0	10	0	0	10	1	10
4	Total	30	4	13.33*	30	3	10*	30	6	20*

Values with different superscripts (a,b,c) in the same raw differ significantly ($P<0.01$).

*Indicate average

Table 9. Nuclear maturation and polar body extrusion rate of different grade oocytes within each retrieval method

Sl. No	Oocyte retrieval system	Morphological grade of COCs	Number of oocytes examined	M II	PB	Percentage of nuclear maturation	Percentage of polar body extrusion
1	Aspiration	A	10	4	3	40^{a}	30^{a}
		B	10	2	1	20^{b}	10^{b}
		C	10	1	0	10^{c}	0
		Overall	30	7	4	23.33	13.33
2	Slicing	A	10	3	2	30^{a}	20^{a}
		B	10	1	1	10^{b}	10^{a}
		C	10	1	0	10^{b}	0
		Overall	30	5	3	16.67	10
3	Puncture	A	10	5	3	50^{a}	30^{a}
		B	10	3	2	30^{b}	20^{ab}
		C	10	2	1	20^{c}	10^{b}
		Overall	30	10	6	33.33	20

Values with different superscripts (a,b,c) in the same column differ significantly ($P<0.01$)

MATURED OOCYTES

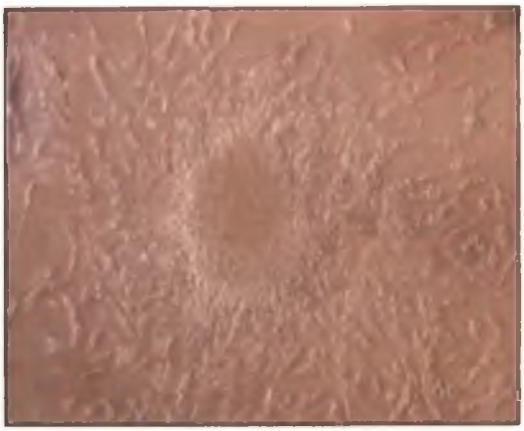


Plate 11 : Matured Oocyte showing Mucification (200x)

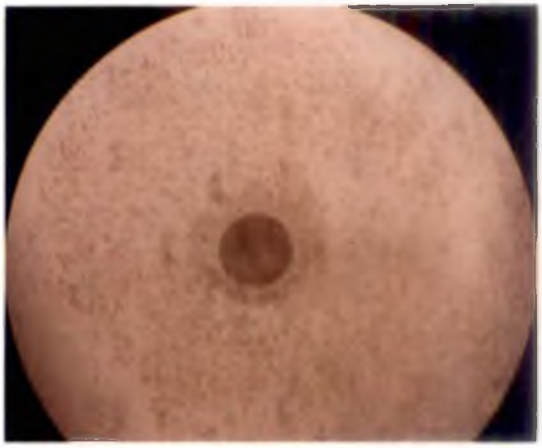


Plate 12 : Matured Oocyte showing Cumulus Expansion (200x)

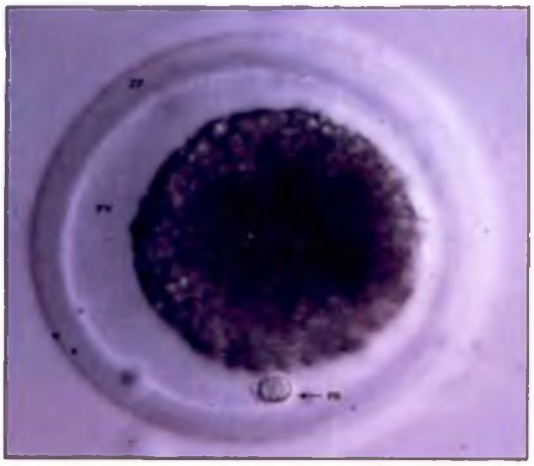


Plate 13 : Matured Oocyte with extruded first polar body (400x)

PB - Polar Body
ZP - Zona Pellucida
PV - Perivitelline Space

NUCLEAR MATURATION - SEQUENTIAL CHANGES

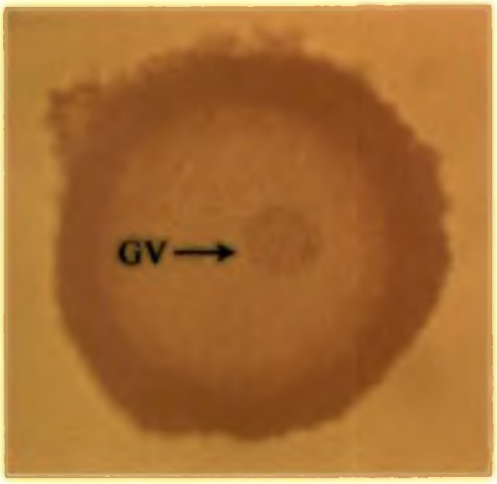


Plate 14 : Oocyte showing Germinal Vesicle Stage (400x)

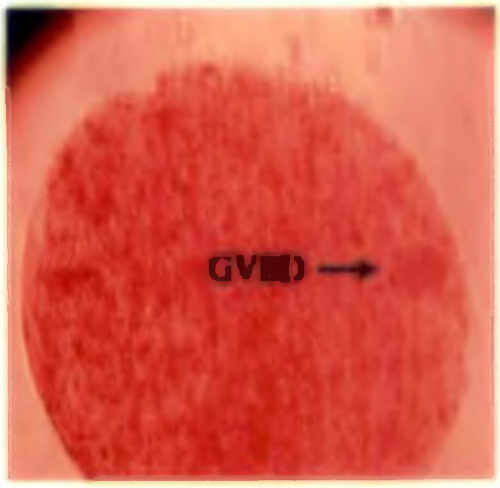


Plate 15 : Oocyte showing Germinal Vesicle Break Down Stage (400x)

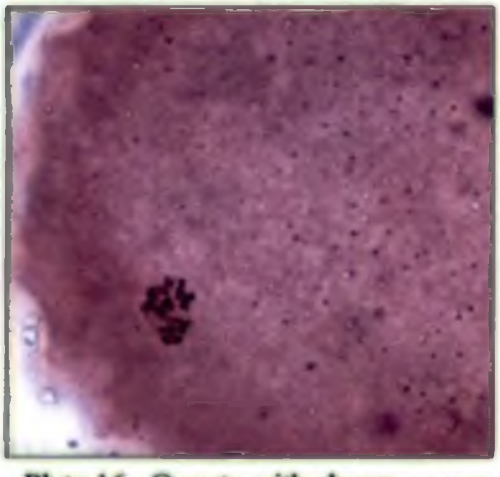


Plate 16 : Oocyte with chromosomes at Metaphase I (M I) Stage (400x)

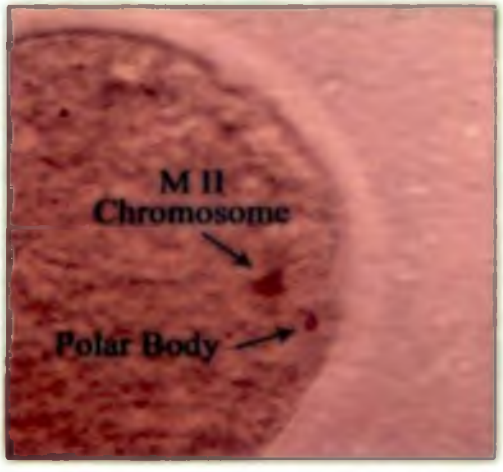


Plate 17 : Oocyte showing Metaphase II plates and polar body

Fig. 1. Effect of three harvesting techniques on oocyte yield

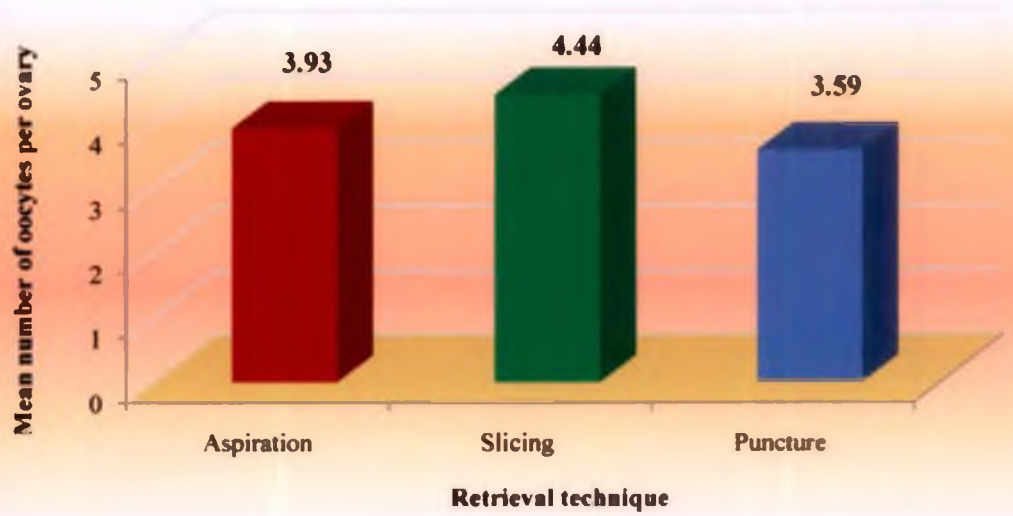


Fig. 2 Effect of retrieval technique on oocyte quality - Comparative efficiency within retrieval technique

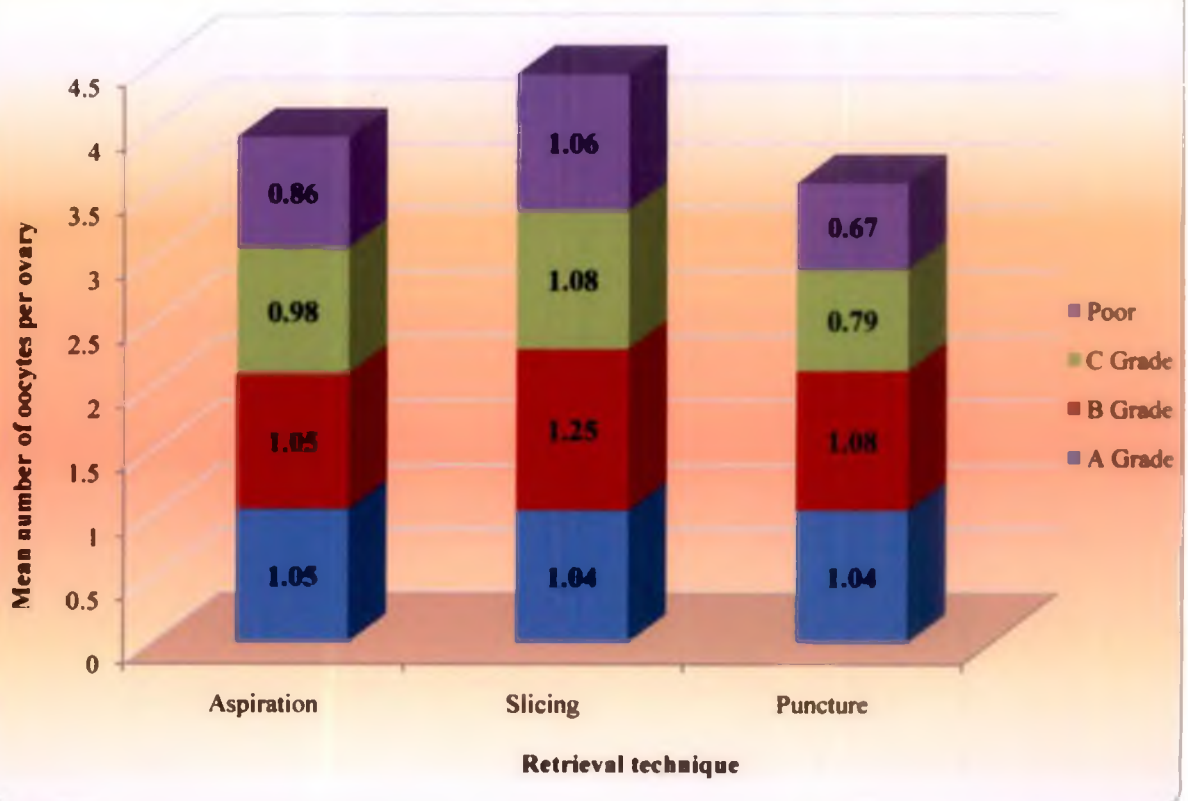


Fig. 3 Effect of retrieval technique on the percentage yield of each quality grades of oocytes

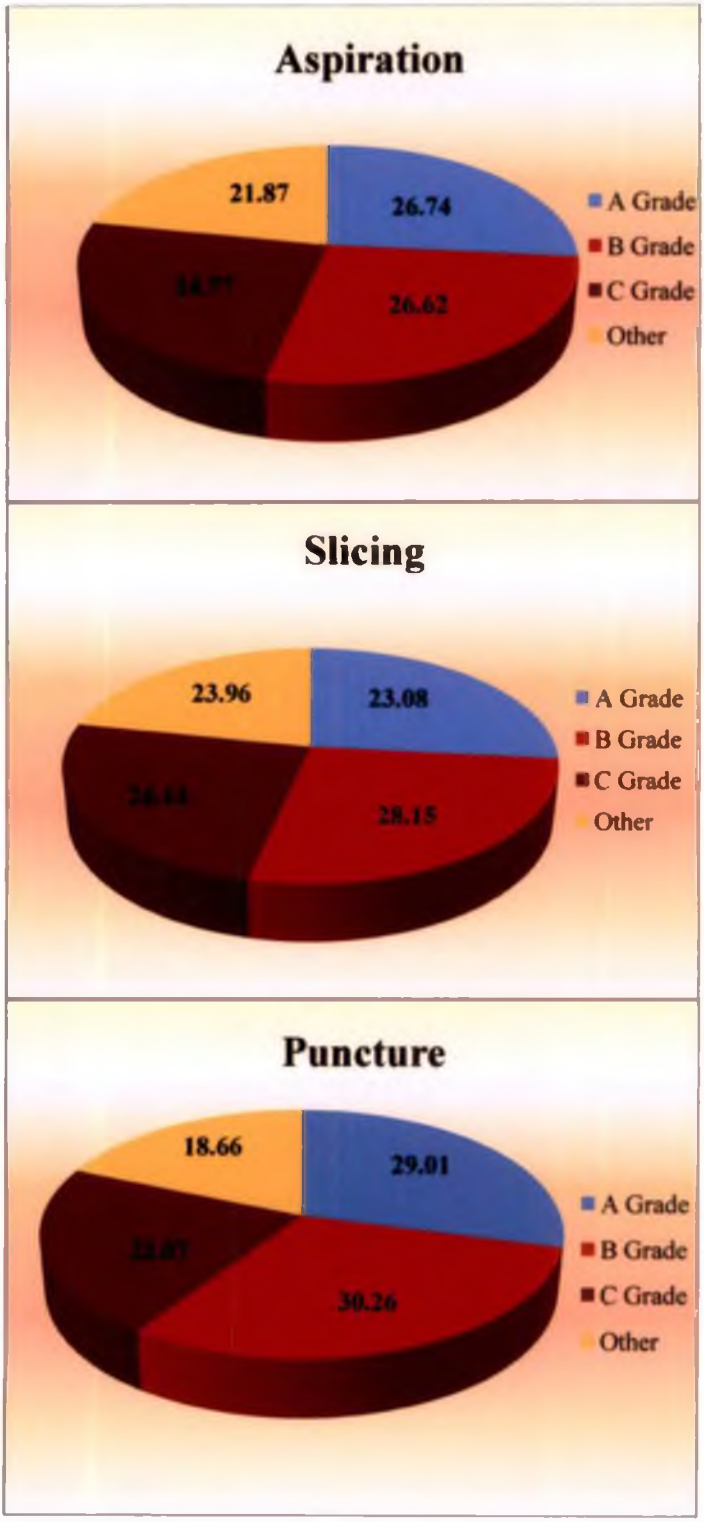


Fig. 4 Effect of retrieval technique on oocyte quality - Comparative efficiency between retrieval techniques

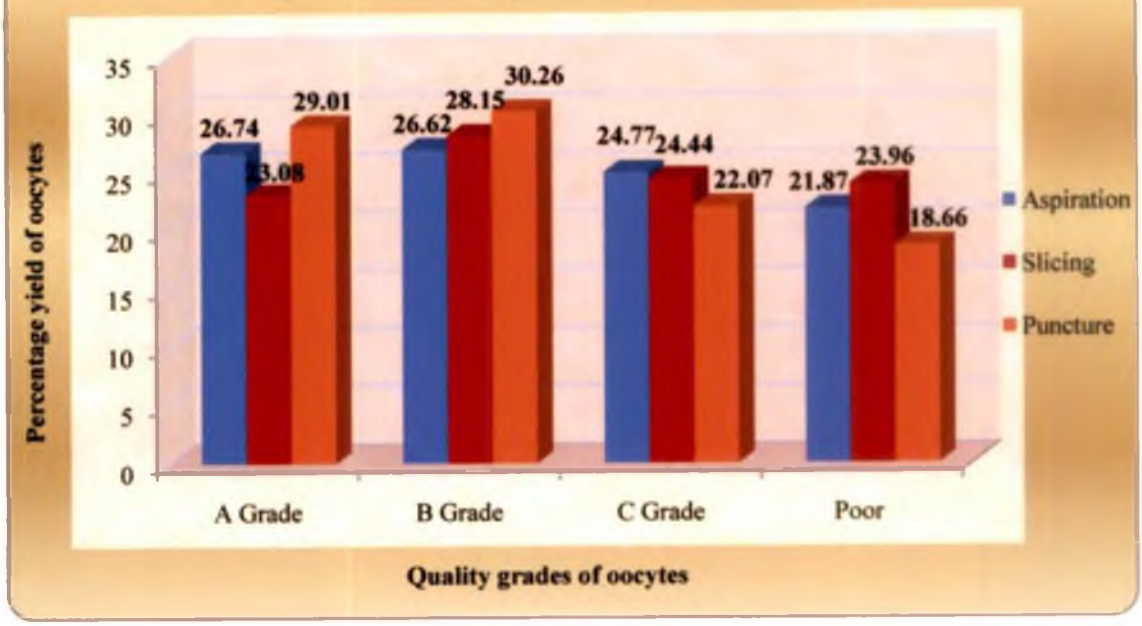


Fig. 5 Cumulus expansion rate of each quality grade of oocytes from different retrieval systems.

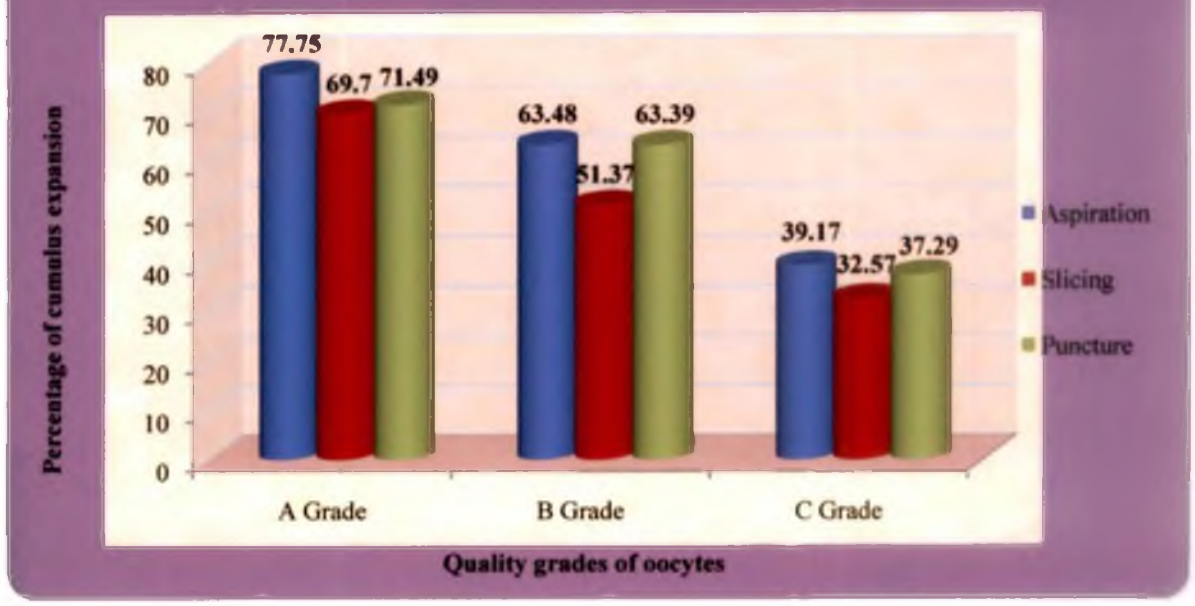


Fig. 6 Cumulus expansion rate of different quality grades of oocytes within each retrieval method

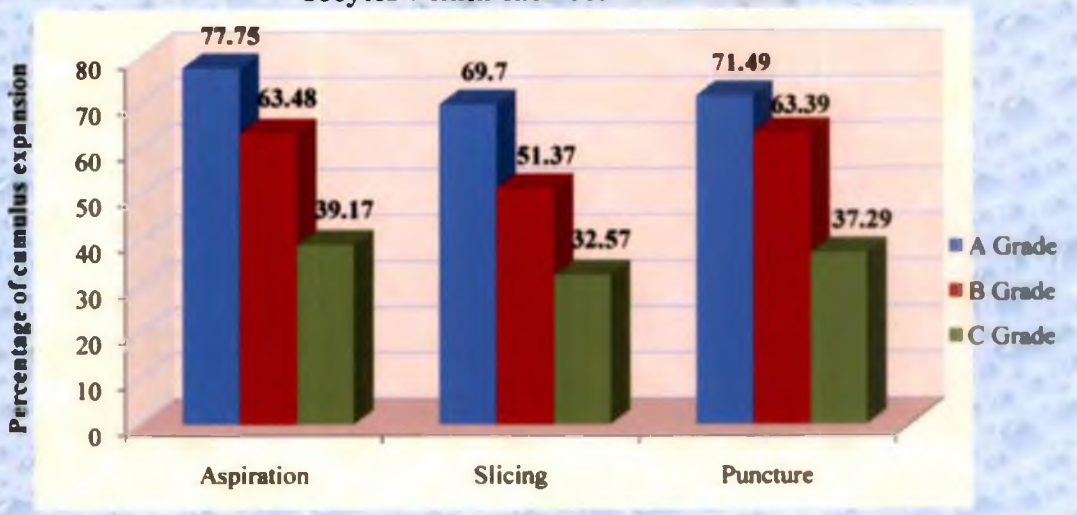


Fig. 7 Nuclear maturation rate of each quality grade of oocytes obtained by different retrieval systems

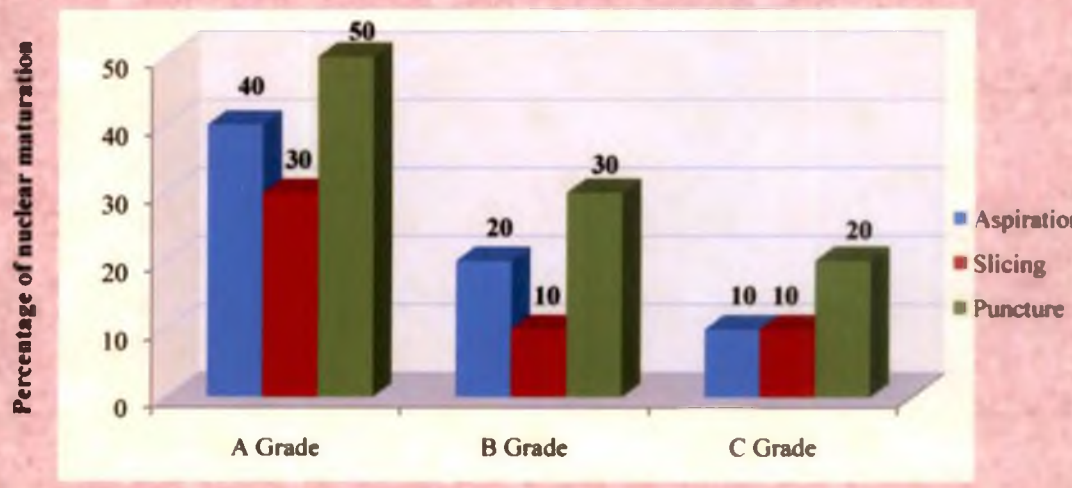
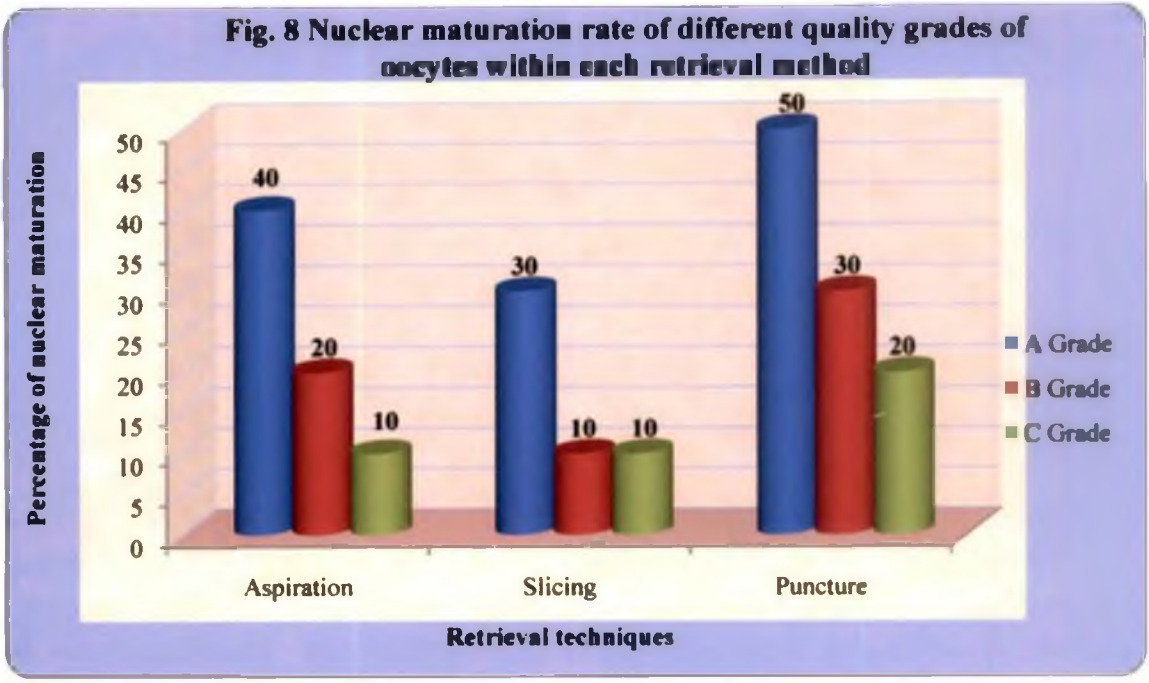


Fig. 8 Nuclear maturation rate of different quality grades of oocytes within each retrieval method



Discussion

5. DISCUSSION

The present study was undertaken to assess the effect of three oocyte retrieval methods viz. aspiration, slicing and puncture on the yield of different quality grades of oocytes and to evaluate the *in vitro* maturation rate of different grades of caprine oocytes.

5.1 EFFECT OF RETRIEVAL TECHNIQUES ON YIELD OF OOCYTES PER OVARY

The mean yield of oocytes per ovary was found to be 3.93 ± 0.11, 4.44 ± 0.06 and 3.59 ± 0.07 for aspiration, slicing and puncture respectively (Table 2 and Fig. 1). Aspiration, slicing and puncture differed significantly in the yield of COCs per ovary. Slicing yielded significantly higher number of COCs per ovary.

The yield of oocyte per ovary by aspiration method is in agreement with Vijayakumaran (1995) and Tajik and Esfandabadi (2003). Martino *et al.* (1994), Pawshe *et al.* (1994) and Wang *et al.* (2007a) recovered a lower number of oocytes by aspiration. Various other workers recovered higher number of oocytes than the present study (Keskintepe *et al.*, 1998; Yadav *et al.*, 1998 and Velilla *et al.*, 2002).

Result of slicing method, is in agreement with Martino *et al.* (1994), Vijayakumaran (1995) and Katiyar *et al.* (1997). Higher number of oocytes were recovered by Martino *et al.* (1994); Ongeri *et al.* (2001) and Wang *et al.* (2007a). Pawshe *et al.* (1994) recovered comparatively low number of oocytes by slicing method.

Results obtained for puncture are in agreement with Wang *et al.* (2007a). The yield was low compared to the present study as reported by Martino *et al.* (1994); Pawshe *et al.* (1994) and Vijayakumaran (1995).

In the present experiment significantly higher COC per ovary was obtained in slicing method, when compared to aspiration or puncture. This is in agreement with the observations of Martino *et al.* (1994), Vijayakumaran (1995) and Wang *et al.* (2007a) in goats.

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

5.2 EFFECT OF RETRIEVAL TECHNIQUES ON THE QUALITY OF COCs

5.2.1 Comparative Efficiency between Retrieval Techniques

5.2.1.1 A Class Oocytes

The yield of Class A oocytes by aspiration, slicing and puncture methods are given in table 3 and Fig. 4. The yield of Class A oocytes by aspiration was 26.74 per cent. This is in agreement with the results of Pawshe *et al.* (1994) and Vijayakumaran (1995). Wang *et al.* (2007a) obtained much higher percentage of A class oocytes per ovary by aspiration. Average yield of A class oocytes by aspiration was 1.05 ± 0.05 per ovary which is comparable to the results obtained by Martino *et al.* (1994), Vijayakumaran (1995) and Wang *et al* (2007a). However, Pawshe *et al.* (1994) obtained a lower number of A class oocytes by aspiration, compared to the present study.

When slicing method was employed, 23.08 per cent A class oocytes were obtained which is in agreement with the results obtained by Mogas *et al.* (1993) and Pawshe *et al.* (1994). Vijayakumaran (1995) and Ongeri *et al.* (2001) obtained higher percentage of A class oocytes by slicing. Mean number of A class oocytes per

ovary obtained by slicing was 1.04 ± 0.04 and this is in accordance with the results of Martino *et al.* (1994), Pawshe *et al.* (1994) and Vijayakumaran (1995).

Puncture yielded 29.01 per cent A class oocytes which is in agreement with the results of Vijayakumaran (1995) and Wani *et al.* (1999). Pawshe *et al.* (1994) obtained a comparatively lower percentage than the present study. However, Kharche *et al.* (2006) and Wang *et al.* (2007a) recovered obtained a higher percentage than the present study. The mean number of A class oocytes obtained by puncture was 1.04 ± 0.04. This result is in agreement with that of Wani *et al.* (1999). Vijayakumaran (1995) and Pawshe *et al.* (1994) obtained lower yield per ovary. In contrast, Wang *et al.* (2007a) recorded comparatively high values than the present study.

5.2.1.2 B Class Oocytes

The yield of B class oocytes by aspiration was 23.08 per cent. The result is in agreement with that of Vijayakumaran (1995). The yield obtained by Pawshe *et al.* (1994) and Wang *et al.* (2007a) was higher than that obtained in the present study. The mean number of B class oocytes retrieved by aspiration was 1.05 ± 0.08, which agrees with the reports of Pawshe *et al.* (1994) but slightly lower number was recorded by Vijayakumaran (1995) and Wang *et al.* (2007a).

Slicing yielded 28.15 per cent B class oocytes. Pawshe *et al.* (1994) obtained similar results. However Vijayakumaran (1995) and Wang *et al.* (2007a) obtained a lower percentage of B class oocytes by slicing. The mean yield of B class oocytes was 1.25 ± 0.05 by slicing. This result is in agreement with that of Wang *et al.* (2007a). The yield was slightly lower than the values reported by Pawshe *et al.* (1994) and Vijayakumaran (1995).

By puncture 30.26 per cent B class oocytes were obtained. Pawshe *et al.* (1994), Vijayakumaran (1995) and Wang *et al.* (2007a) obtained similar results. The mean yield of 1.08 ± 0.03 per ovary is in accordance with the values obtained by

Wang *et al.* (2007a); but slightly lower values were reported by Pawshe *et al.* (1994) and Vijayakumaran (1995).

5.2.1.3 C Class Oocytes

The percentage yield of C class oocytes by aspiration was 24.77. Similar result was recorded by Keskintepe *et al.* (1996). The percentage yield reported by Pawshe *et al.* (1994) was considerably higher than that of the present study. Vijayakumaran (1995) and Wang *et al.* (2007a) recovered lower percentage of C class oocytes than the present study. The mean number of C class oocytes recovered by aspiration was 0.98 ± 0.07. This result is in agreement with that of Wang *et al.* (2007a). While Vijayakumaran (1995) recovered a lower number Pawshe *et al.* (1994) recovered higher number of C class oocytes by aspiration.

Slicing yielded 24.44 per cent C class oocyte which is in accordance with the results of Pawshe *et al.* (1994). Vijayakumaran (1995) and Wang *et al.* (2007a) recovered a lower percentage of C class oocytes by slicing. The mean yield of C class oocytes by slicing was 1.08 ± 0.07. Wang *et al.* (2007a) obtained similar results. However, Pawshe *et al.* (1994) and Vijayakumaran (1995) obtained lower number of C class oocytes by slicing.

Percentage yield of C class oocytes recovered by puncture was 22.07. The yield obtained by Vijayakumaran (1995) and Wang *et al.* (2007a) is lower than the present study. Pawshe *et al.* (1994) obtained a higher percentage of C class oocytes than the present study. The mean yield of C class oocytes by puncture was 0.79 ± 0.04 per ovary. The results of Pawshe *et al.* (1994) are comparable with the present study. While Wang *et al.* (2007a) reported a higher yield; Vijayakumaran (1995) recovered a lower number compared to the present results.

5.2.1.4 Poor Quality Oocytes

The yield of poor quality oocytes by aspiration, slicing and puncture were 21.87, 23.96 and 18.66 per cent respectively. The result is in agreement with that of Vijayakumaran (1995).

5.2.2 Comparative Efficiency within each Retrieval Technique

5.2.2.1 Aspiration

Yield of different quality grades of oocytes retrieved by aspiration is shown in Table 4 and Fig. 3. Percentage yields of A, B, C and other quality grades of oocytes retrieved by aspiration were 26.74, 26.62, 24.77 and 21.87 respectively.

Yield of A class oocytes were comparable with the results of Pawshe *et al.* (1994) and Vijayakumaran (1995). Wang *et al.* (2007a) obtained much higher percentage of A class oocytes per ovary by aspiration. Mean yield of A class oocytes by aspiration was 1.05 ± 0.05 per ovary which was in accordance with the results obtained for Martino *et al.* (1994), Vijayakumaran (1995) and Wang *et al* (2007a). However, Pawshe *et al.* (1994) obtained a lower yield of A class oocytes by aspiration compared to the present study.

The yield of B class oocytes by aspiration was in agreement with that of Vijayakumaran (1995). The yield obtained by Pawshe *et al.* (1994) and Wang *et al.* (2007a) was higher than the values obtained in the present study. The mean number of B class oocytes by aspiration was 1.05 ± 0.08, which agrees with that of Pawshe *et al.* (1994) but slightly lower values were recorded by Vijayakumaran (1995) and Wang *et al.* (2007a).

The percentage yield of C class oocytes by aspiration was 24.77. Similar result was recorded by for Keskintepe *et al.* (1996). The percentage yield reported by

Pawshe *et al.* (1994) was considerably higher than that of the present study. Vijayakumaran (1995) and Wang *et al.* (2007a) recovered lower percentage of C class oocytes than the present study. The mean number of C class oocytes recovered by aspiration was 0.98 ± 0.07. This result is in agreement with that of Wang *et al.* (2007a). While Vijayakumaran (1995) recovered a lower number, Pawshe *et al.* (1994) obtained a higher yield of C class oocytes by aspiration.

The yield of other quality grade oocytes by aspiration was 21.87 per cent, which was in agreement with that of Vijayakumaran (1995). Perusal of literature did not reveal many reports on the yield of poor quality oocytes.

5.2.2.2 Slicing

Yield of different quality grades of oocytes retrieved by slicing is shown in Table 4 and Fig. 3. Percentage yields of A, B, C and other quality grades of oocytes retrieved by slicing were 23.08, 28.15, 24.44 and 23.96 respectively.

The percentage yield of A class oocytes in the present study was in agreement with the results obtained by Mogas *et al.* (1993) and Pawshe *et al.* (1994). Vijayakumaran (1995) and Ongeri *et al.* (2001) obtained a higher percentage of A class oocytes by slicing. Mean yield of A class oocytes per ovary recovered by slicing was 1.04 ± 0.04 and this was in agreement with the results of Martino *et al.* (1994), Pawshe *et al.* (1994) and Vijayakumaran (1995).

Slicing yielded 28.15 per cent B class oocytes. Pawshe *et al.* (1994) also recorded similar results. However, Vijayakumaran (1995) and Wang *et al.* (2007a) recorded a lower percentage of B class oocytes by slicing. The mean yield of B class oocytes was 1.25 ± 0.05 by slicing. This result is in agreement with that of Wang *et al.* (2007a). The yield was slightly higher than the reports of Pawshe *et al.* (1994) and Vijayakumaran (1995).

Slicing yielded 24.44 per cent C class oocytes which is in accordance with the results obtained by Pawshe *et al.* (1994). Wang *et al.* (2007a) recovered a lower percentage of C class oocytes by slicing. The mean yield of C class oocytes recovered by slicing was 1.08 ± 0.07. Wang *et al.* (2007a) obtained similar results. However, Pawshe *et al.* (1994) and Vijayakumaran (1995) recovered lower number of C class oocytes by slicing.

The yield of other quality grade oocytes by slicing was 23.96 per cent which is in agreement with that of Vijayakumaran (1995).

5.2.2.3 Puncture

Yield of different quality grades of oocytes retrieved by puncture is shown in Table 4 and Fig. 3. Percentage yields of A, B, C and other quality grades of oocytes retrieved by puncture were 29.01, 22.07, 24.44 and 23.96 respectively.

Puncture yielded 29.01 per cent A class oocytes which was in agreement with the results of Vijayakumaran (1995) and Wani *et al.* (1999). Pawshe *et al.* (1994) obtained a comparatively lower percentage than the present study. Kharche *et al.* (2006) and Wang *et al.* (2007a) recorded higher values than the present study. The mean number of A class oocyte obtained by puncture was 1.04 ± 0.04. This result agrees with that of Wani *et al.* (1999). Vijayakumaran (1995) and Pawshe *et al.* (1994) recorded lower number per ovary. Wang *et al.* (2007a) obtained comparatively high number than the present study.

By puncture 22.07 per cent B class oocytes were obtained. Pawshe *et al.* (1994), Vijayakumaran (1995) and Wang *et al.* (2007a) obtained similar results. The mean yield was 1.08 ± 0.03 per ovary which is in agreement with the values recorded by Wang *et al.* (2007). However, Pawshe *et al.* (1994) and Vijayakumaran (1995) reported lower values.

Percentage yield of C class oocytes by puncture was 22.07. The results recorded by Vijayakumaran (1995) and Wang *et al.* (2007a) were lower than the present study. Pawshe *et al.* (1994) obtained a higher percentage of C class oocytes than the present study. The mean yield of C class oocytes obtained by puncture was 0.79 ± 0.04 per ovary. The results of Pawshe *et al.* (1994) agree with the present study. In contrast, Wang *et al.* (2007a) recovered higher number and Vijayakumaran (1995) recorded lower values.

The yield of other quality grade oocytes by puncture was 18.66 per cent and this was in agreement with that of Vijayakumaran (1995).

Various workers have reported many factors that affect the yield of oocytes viz. breed, season, time interval from collection of ovaries to oocyte harvest, temperature of media for transport of ovaries, retrieval technique and the criteria used for classification of culture grade oocytes (Rahman *et al.*, 2008). The difference in the yield of oocytes obtained by various workers may be attributed to various factors such as difference in breed, nutritional status, stage of estrus cycle, agro-climatic conditions, methodology and skill in the technique etc.

5.3 EFFECT OF RETRIEVAL METHOD AND COC MORPHOLOGY ON MATURATION RATE

5.3.1 Maturation Rate of COCs

Maturation status of caprine oocytes was assessed by cumulus expansion and nuclear maturation. Nuclear maturation was assessed by staining denuded oocytes with one per cent aceto-orcein. Oocytes with M II chromosomes were considered as matured. Polar body extrusion rate was also assessed. On perusal of literature, it was noted that cumulus expansion and polar body extrusion were considered as signs of maturation but maturation rate was assessed based on the appearance of M II stage chromosomes.

Cumulus expansion rate of different quality oocytes obtained by various retrieval systems are shown in Table 5 and 6 and Fig. 5 and 6. Nuclear maturation and polar body extrusion rate of different quality oocytes obtained by various retrieval systems are shown in Table 9 and Fig. 7 and 8.

Cumulus expansion rate of A, B, and C class oocytes obtained by aspiration, slicing and puncture were 77.75, 63.48 and 39.17 per cent, 69.70, 51.37 and 32.57 per cent and 71.49, 63.39 and 37.29 per cent respectively.

Maturation rate of A class oocytes obtained by aspiration was 40 per cent. Pawshe *et al.* (1994), Vijayakumaran (1995) and Sharma *et al.* (1996) obtained a higher maturation rate for A class oocytes retrieved by aspiration. Maturation rate of B class oocytes recovered by aspiration was 20 per cent. Pawshe *et al.* (1994), Vijayakumaran (1995), Sharma *et al.* (1996) and Wang *et al.* (2007a) reported a higher maturation rate than the present study. Class C oocytes exhibited a maturation rate of 10 per cent when recovered by aspiration. The results recorded by Pawshe *et al.* (1994), Mogas *et al.* (1995) and Sharma *et al.* (1996) is higher than that of the present study.

Class A oocytes recovered by slicing exhibited a maturation rate of 30 per cent which was lower than the reports of Pawshe *et al.* (1994), Vijayakumaran (1995) and Sharma *et al.* (1996). Maturation rate of B class oocytes recovered by slicing was 10 per cent. Pawshe *et al.* (1994), Vijayakumaran (1995), and Wang *et al.* (2007a) reported a higher maturation rate than the present study. When recovered by slicing, C class oocytes exhibited a maturation rate of 10 per cent. A higher maturation rate was obtained for Pawshe *et al.* (1994), Mogas *et al.* (1995) and Sharma *et al.* (1996).

Percentage of A class oocytes exhibiting maturation when retrieved by puncture is 50 per cent. The results of Pawshe *et al.* (1994), Vijayakumaran (1995) and Sharma *et al.* (1996) revealed higher maturation rate of A class oocytes. Class B oocytes retrieved by puncture exhibited a maturation rate of 30 per cent, which was

lower than that reported by Pawshe *et al.* (1994), Vijayakumaran (1995), and Wang *et al.* (2007a). When recovered by puncture, C class oocytes exhibited a maturation rate of 20 per cent. A higher maturation rate was reported by Pawshe *et al.* (1994), Mogas *et al.* (1995) and Sharma *et al.* (1996).

Various factors that affect the *in vitro* maturation of oocytes include, stage of the estrus cycle, age of the animals, technique of oocyte retrieval, culture media, culture conditions, assessment of maturation etc. (Wang *et al.*, 2008). The lower maturation rate of oocytes obtained in the present study might be due to the lower duration of culture provided when compared to the other studies. The variation may also be due to the difference in the concentration of components in the medium like serum and hormones. Vijayakumaran (1995) used a different medium than the present study; Ham's F-10, while others used the same medium with slight changes in the concentration of various components like serum and hormones. Many workers followed culture duration of 27-32 h. The duration selected in the present study was 24 h. Other factors like breed and nutritional status of animals selected for the study should also be taken into account for the variation in results from other authors.

Class A oocytes obtained by aspiration, slicing and puncture exhibited a polar body extrusion rate of 30, 20 and 30 per cent respectively. Class B oocytes recovered by aspiration, slicing and puncture exhibited a polar body extrusion rate of 10, 10 and 20 per cent respectively. None of the C class oocytes recovered by aspiration or slicing exhibited polar body extrusion, 10 per cent of C class oocytes obtained by puncture exhibited a polar body extrusion.

CONCLUSION

It was clear from this study that the retrieval method had definite role in the yield of COCs per ovary. Yield of COCs by aspiration, slicing and puncture differed significantly and slicing method yielded more number of oocytes. Slicing was a better method than aspiration or puncture for retrieval of oocytes from caprine

ovaries as far as the total yield of oocytes per ovary is concerned. Percentage yield of each quality grade of oocyte did not differ significantly between retrieval methods. But the yield of different grades of oocytes within each retrieval method differed significantly. Retrieval method was found to have no significant effect, whereas COC morphology was found to have significant effect on cumulus expansion, nuclear maturation and polar body extrusion rates of different grades of oocytes. It is also clear from this study that, A class oocytes with more than three compact layers of cumulus cells and uniform ooplasm exhibits the highest cumulus expansion rate, nuclear maturation rate and polar body extrusion rate.

Summary

6. SUMMARY

A study was carried out on *in vitro* maturation of caprine follicular oocytes using oocytes obtained by three retrieval techniques from ovaries of recently slaughtered animals. The major objectives of this study were (1) to analyse the effect of oocyte retrieval methods and cumulus mass on *in vitro* maturation of caprine oocytes, (2) to identify the best oocyte retrieval method for *in vitro* maturation and (3) to assess the *in vitro* maturation rate of oocytes.

Ovaries used for the study were of recently slaughtered goats belonging to Malabari breeds and its crosses. Ovaries dissected out from freshly slaughtered animals were transported to the laboratory within 1-2 h in a thermo flask containing freshly prepared normal saline solution fortified with antibiotics; Benzyl penicillin and Streptomycin sulphate. A total of 138 ovaries were processed in the study. After repeated washing of ovaries in normal saline and final washing in DPBS, oocytes were recovered by aspiration, slicing or puncture into COC handling medium (DPBS enriched with five per cent day zero oestrus goat serum and 0.5 per cent BSA) maintained at 38°C.

Recovered oocytes were rinsed several times in COC handling media. These oocytes were classified into different quality grades based on number of cumulus cell layers and ooplasm character as A, B, C and poor quality grades. Grade A COCs were morphologically normal with more than three complete layers of compact cumulus cells and uniform granulation of ooplasm. Grade B oocytes had normal morphology with two to three complete layers of cumulus cells and uniform granulation of ooplasm. Oocytes in the C grade were morphologically normal with single complete layer of cumulus cells and uniform granulation of ooplasm. All other oocytes that were found to be inferior to grade C quality were graded as poor quality oocytes.

Each class of oocytes obtained by different retrieval methods were cultured separately in 50 µl drops of maturation media. Medium used for *in vitro* maturation of oocytes was freshly prepared Hepes modified TCM- 199 (25 mM Hepes) enriched with 22 µg /ml sodium pyruate, 2.2 mg /ml sodium bicarbonate and penicillin G sodium and streptomycin sulphate at the rate of 500 µg /ml each. This medium was further supplemented with hormones, 1 µg /ml estradiol- 17β, 0.5 µg /ml FSH, 0.06 IU hCG and 20 per cent heat- inactivated oestrus goat serum prior to employing it for IVM. Culture conditions were set in a standard water-jacketed type of CO_2 incubator with a temperature of 38.5° C, five per cent CO_2 tension and maximum humidity. After 24 h of culture, oocytes were examined for maturational changes like cumulus expansion. A total of 90 oocytes, 30 each from each retrieval method showing cumulus expansion were stained with one per cent aceto orcein and examined for nuclear maturational changes.

Average yield of COC per ovary by aspiration, slicing and puncture was 3.93 ± 0.11, 4.44 ± 0.06 and 3.59 ± 0.07 respectively. Yield was significantly higher in slicing method than aspiration or puncture. Higher yield by slicing method might be due to recovery of oocytes from surface follicles as well as follicles of deeper cortical stroma.

The percentage yield of A, B, C and poor quality grades of oocytes by aspiration method was 26.74 per cent, 26.62 per cent, 24.77 per cent and 21.87 per cent respectively. Mean yield of oocytes in each quality grade by aspiration was 1.05 ± 0.05, 1.05 ± 0.08, 0.98 ± 0.07 and 0.86 ± 0.04 respectively. Slicing yielded 23.08 per cent A class, 28.15 per cent B class, 24.44 per cent C class and 23.96 per cent poor quality oocytes. Mean yield of oocytes in these classes obtained by slicing method was 1.04 ± 0.04, 1.25 ± 0.05, 1.08 ± 0.07 and 1.06 ± 0.06 respectively. Percentage yield of A, B, C and poor quality grades of oocytes by puncture was 29.01 per cent, 30.26 per cent, 22.07 per cent and 18.66 per cent respectively. Mean

yield of oocytes per ovary by puncture method was 1.04 ± 0.04, 1.08 ± 0.03, 0.79 ± 0.04 and 0.67 ± 0.03 for A, B, C and poor quality grades of oocytes respectively.

No significant difference was observed in the yield of A, B and C class oocytes between aspiration, slicing and puncture. Poor quality oocytes were significantly more in slicing method. This may be due to recovery of oocytes from all kinds of follicles irrespective of the follicle diameter.

The cumulus expansion rate of A class oocytes obtained by aspiration, slicing and puncture was 77.75 per cent, 69.70 per cent and 71.49 per cent respectively. Class B oocytes exhibited a cumulus expansion rate of 63.48 per cent, 51.37 per cent and 63.39 per cent respectively when collected by aspiration, slicing and puncture methods. Class C oocytes obtained by aspiration, slicing and puncture when subjected to *in vitro* maturation exhibited a maturation rate of 39.17, 32.57 and 37.29 per cent respectively. Retrieval method was found to have no significant effect on cumulus expansion potential of caprine oocytes, whereas, the COC morphology had significant effect on cumulus expansion potential. Oocytes with three or more layers of cumulus cells exhibited better cumulus expansion rate than oocytes with less than three layers of cumulus cells.

Nuclear maturation rate of A class oocytes collected by aspiration, slicing and puncture method was respectively 40, 30 and 50 per cent and polar body extrusion rate was 30, 20 and 30 per cent respectively. Class B oocytes exhibited nuclear maturation rate of 20, 10 and 30 percent and polar body extrusion rate of 10, 10 and 20 per cent respectively by aspiration, slicing and puncture. Nuclear maturation rate of 10, 10 and 20 per cent was noticed in C class oocytes retrieved by aspiration, slicing and puncture respectively and 10 per cent polar body extrusion was observed in C class oocyte by puncture. None of the C class oocyte exhibited polar body extrusion in aspiration or slicing.

Analysis of results revealed that the method of retrieval is having effect on neither nuclear maturation nor polar body extrusion rate of caprine oocytes. But the COC morphology is having significant effect on nuclear maturation and polar body extrusion rates. Oocytes with three or more layers of cumulus cells exhibited better nuclear maturation and polar body extrusion rate.

This study proved that slicing is a better method than aspiration or puncture for retrieval of oocytes from caprine ovaries as far as the total yield of oocytes per ovary is concerned. Retrieval method was found to have no significant effect, whereas COC morphology was found to have significant effect on cumulus expansion, nuclear maturation and polar body extrusion rates of different grades of oocytes.

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IN VITRO MATURATION OF CAPRINE FOLLICULAR OOCYTES

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ABSTRACT

This study was designed to analyse the effect of three oocyte retrieval methods, aspiration, slicing and puncture on the yield of different quality grades of oocytes and to evaluate the *in vitro* maturation rate of different grades of caprine oocytes. One hundred and thirty eight ovaries of Malabari goats and its crossbreds collected from the slaughter house were subjected to the oocyte retrieval methods. The oocytes harvested were graded based on the number of cumulus cell layers and ooplasm character into A, B, C and poor quality grades. Oocytes of A, B and C grades were subjected to maturation for 24 h in TCM-199 medium under standard culture conditions.

Average yield of COC per ovary by aspiration, slicing and puncture was 3.93 ± 0.11, 4.44 ± 0.06 and 3.59 ± 0.07 respectively. Yield was significantly higher in slicing method than aspiration and puncture.

The percentage yield of A, B, C and poor quality grades of oocytes by aspiration method was 26.74 per cent, 26.62 per cent, 24.77 per cent and 21.87 per cent respectively. Mean yield of oocytes of each quality grade by the same method were 1.05 ± 0.05, 1.05 ± 0.08, 0.98 ± 0.07 and 0.86 ± 0.04 respectively. Slicing yielded 23.08 per cent A class, 28.15 per cent B class, 24.44 per cent C class and 23.96 per cent poor quality oocytes. Mean yield of oocytes per ovary in these classes by slicing method were 1.04 ± 0.04, 1.25 ± 0.05, 1.08 ± 0.07 and 1.06 ± 0.06 respectively. Percentage yield of A, B, C and poor quality oocytes by puncture was 29.01 per cent, 30.26 per cent, 22.07 per cent and 18.66 per cent respectively. Mean yield per ovary by puncture method was 1.04 ± 0.04, 1.08 ± 0.03, 0.79 ± 0.04 and 0.67 ± 0.03 for A, B, C and poor quality oocytes respectively.

No significant difference was observed in the yield of A, B and C class oocytes between aspiration, slicing and puncture. Yield of poor quality oocytes were significantly more in slicing method.

The cumulus expansion rate of A class oocytes obtained by aspiration, slicing and puncture was 77.75 per cent, 69.70 per cent and 71.49 per cent respectively. Class C oocytes exhibited a cumulus expansion rate of 63.48 per cent, 51.37 per cent and 63.39 per cent respectively when collected by aspiration, slicing and puncture method. Class C oocytes obtained by aspiration, slicing and puncture when subjected to *in vitro* maturation exhibited a cumulus expansion rate of 39.17, 32.57 and 37.29 per cent respectively. Retrieval method was found to have no significant effect on cumulus expansion potential of caprine oocytes, whereas the COC morphology had significant effect on cumulus expansion potential.

Nuclear maturation rate of A class oocytes collected by aspiration, slicing and puncture method were respectively 40, 30 and 50 per cent and polar body extrusion rate was 30, 20 and 30 per cent respectively. Class B oocytes exhibited nuclear maturation rate of 20, 10 and 30 per cent and polar body extrusion rate of 10, 10 and 20 per cent respectively by aspiration, slicing and puncture. Ten, 10 and 20 per cent of C class oocytes retrieved by aspiration, slicing and puncture exhibited nuclear maturation and 10 per cent polar body extrusion was observed in C class oocytes retrieved by puncture. None of the C class oocytes collected by aspiration or slicing exhibited polar body extrusion.

This study proved that slicing is a better method than aspiration or puncture for retrieval of oocytes from caprine ovaries as it yielded more number of oocytes per ovary. Retrieval methods had no significant effect, whereas COC morphology was found to have significant effect on cumulus expansion, nuclear maturation and polar body extrusion rates of different grades of oocytes