IN VITRO FERTILIZATION OF BOVINE OOCYTES USING FRESH, FROZEN AND EPIDIDYMAL SPERMATOZOA

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DECLARATION

I hereby declare that the thesis entitled "***IN VITRO* FERTILIZATION OF BOVINE OOCYTES USING FRESH, FROZEN AND EPIDIDYMAL SPERMATOZOA"** 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

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LIST OF ABBREVIATIONS USED

AR	Acrosome reaction
bFGF	Bovine fibroblast growth factor
BSA-V	Bovine serum albumin-fraction V
BMOC	Brinster medium for ova culture
BTS	Beltsville thawing solution
cAMP	Cyclic adenosine mono phosphate
CC	Chromosome condensation
CL	Corpus luteum
CO_2	Carbon dioxide
COCs	Cumulus oocytes complexes
Cox	Cycloxygenase
CSF	Cystostatic factor
DPBS	Dulbecco's phosphate buffered saline
ECS	Estrus cow serum
EGF	Epidermal growth factor
FCS	Foetal calf serum
FSH	Follicle stimulating hormone
FDA	Fluorescein diacetate
GalNAc	N-acetyl-galactosaminyl
GSH	Glutathione
GV	Germinal vesicle

GVBD	Germinal vesicle break down
h	Hour
hCG	Human chorionic gonadotropin
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
hpi	Hour post insemination
IGF	Insulin like growth factor
IP_3	Inositol triphosphate
IP_3R	Inositol triphospate receptor
IU	International units
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
kg	Kilogram
LH	Luteinizing hormone
MEM	Minimum essential medium
mg	Milligram
min	Minute
MI	Metaphase I
M II	Metaphase II
ml	Milliliter
mM	Millimole
MPF	Maturation promoting factor
mKRB	Modified krebs ringer bicarbonate
MPM	Modified parker medium

NBCS	New born calf serum
NIH	National institute of health
nm	Nanometer
No.	Number
OPU	Ovum pick up
PB	Polar body
PBS	Phosphate buffered saline
pH	Hydrogen ion concentration, minus log of
RER	Rough endoplasmic reticulum
mRNA	Messenger ribo nucleic acid
SER	Smooth endoplasmic reticulum
SFRE	Serum free
SOF	Synthetic oviduct fluid
TCM	Tissue culture medium
TGF	Transforming growth factor
TL-HEPES	Tyrode's lactate-(N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid])
TALP	Tyrode, albumine, lactate, pyruvate
°C	Degree centigrade
µg	Microgram
µm	Micrometer

Dedicated to.....

the farming community

Introduction

1. INTRODUCTION

Biotechnology has an important role to play in ensuring the reproductive efficiency of farm animals at the highest levels, compatible with animal welfare and protection of the environment (Morris and Sreenam, 2001). The success of application of technologies such as cloning by nuclear transfer, sperm and embryo sexing and production of transgenic animals is critically depended on the expertise in the basic reproduction techniques like *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of embryos (Kane, 2003). A major technical breakthrough was achieved during the last few decades in the world for large-scale production of bovine embryos through IVF of oocytes. Inspite of this considerable progress in the development of *in vitro* embryo production techniques, the success rates remain inconsistent.

In vitro reproduction technology has wide range applications with decisive advantages over *in vivo* methods. *In vivo* methods involve cumbersome procedures with high costs to recover the oocytes /early embryos, whereas the *in vitro* techniques are inexpensive and can provide a large number of oocytes /embryos in a short span of time to support various researches in the field of *in vitro* reproductive biotechnologies. In combination with ultrasound guided ovum pick up (OPU) from live cows, this technology can maximize the production of progenies from elite animals and thereby support the breeding bull production and genetic improvement through large-scale artificial insemination. Use of sexed sperm in conjunction with *in vitro* embryo production is a potentially efficient means of obtaining offspring of predetermined sex (Wheeler *et al.*, 2006). Progenies can be produced even from pre-pubertal and dead animals by IVF technique. Conservation of endangered species and rare livestock breeds are possible through IVF techniques (Neglia *et al.*, 2003). *In vitro* production (IVP) of embryos coupled with embryo transfer is a solution to

low fertility problems of lactating dairy cows (Rutledge, 2001). More over the IVF technology has relevance to assess the status of bull fertility (Gordon, 2003).

Embryo production through *in vitro* means involves a series of events such as oocyte recovery, *in vitro* maturation (IVM), IVF and IVC of embryos. IVM includes nuclear maturation and cytoplasmic maturation (Bevers *et al.*, 1997). During maturation process the meiotic arrest at diplotene is removed and meiosis resumes and progresses to metaphase II. Cytoplasm undergoes many changes in m-RNA, protein synthesis and its accumulation (Rodriguez and Farin, 2004).

Lot of scientific work on IVM and fertilization has been carried out in *Bos taurus*. In India, IVM and IVF studies have been carried out in buffalo (Totey *et al.*, 1996; Chauhan *et al.*, 1998 and Gupta *et al.*, 2001), goat (Vijayakumaran, 1995; Pawshe *et al.*, 1996 and Katiyar *et al.*, 1997) and sheep (Chauhan *et al.*, 1997). However, extensive studies on IVF of oocytes from Indian cattle are not many.

In Kerala Agricultural University, much research work in the field of multiple ovulations, *in vivo* embryo production, cryopreservation and its transfer have been carried out in the Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Mannuthy (Suresan Nair, 1992; Benjamin, 1994; Ramachandran, 2000; Joseph, 2003 and Ratheesh Babu, 2006). Some preliminary work has also been carried out in the field of IVP of embryos in this university. Priscilla (2001) carried out a study on retrieval and characterization of bovine oocytes collected from slaughterhouse ovaries and Magnus Paul (2005) conducted a study on IVM of bovine oocytes retrieved by various methods from slaughter house ovaries. The next process in IVP of embryos is IVF of oocytes, which forms the basis for the present study.

The key to a successful IVF is to promote sperm capacitation and acrosome reaction *in vitro* (Fukui *et al.*, 1983). Mammalian spermatozoa undergo a series of physiological events in the female reproductive tract prior to acquiring the fertilization capability (Chang, 1951). There are differences in the capacitation ability of ejaculated and epididymal spermatozoa. While capacitation of bovine epididymal spermatozoa can be achieved in normal saline (Ball *et al.*, 1983), ejaculated bovine spermatozoa do not undergo capacitation unless stimulated by capacitating agents (Parrish *et al.*, 1985, 1986). Decapacitation factors found in the seminal plasma (Chang, 1951) might be responsible for these differences. However, freshly ejaculated and frozen bull spermatozoa do not differ in their capacitation ability *in vitro* (Bondioli and Wright, 1983).

Improvement of *in vitro* embryo production system require carefully designed experiments to assess the specific effects of IVM, IVF and embryo culture procedures on the development of embryos and fetuses resulting from transfer of such embryos (Farin *et al.*, 2001). In India, most of these research works are at primitive stage and have a need for greater advancements to apply them effectively and economically. Studies comparing the *in vitro* fertilizing capacity of bovine spermatozoa from different sources of origin are very scanty. Perusal of literature did not reveal any work on this aspect from India. Hence the present study was intended to give a special stress on this aspect of IVF with the following as major objectives.

1. To assess the *in vitro* maturation rate of bovine oocytes
2. To compare the effect of the quality of oocytes on *in vitro* maturation and fertilization
3. To assess and compare the *in vitro* fertilizability of bovine oocytes using fresh, frozen and epididymal spermatozoa

Review of Literature

2. REVIEW OF LITERATURE

2.1. *IN VITRO* PRODUCTION OF BOVINE EMBRYOS

The first IVF calf (Virgil) was born in the USA (Brackett *et al.*, 1982). A wide range of factors influence the success of *in vitro* embryo production, varying from the bulls used for IVF to the technicians in the laboratory (Yang *et al.*, 1995). The IVP of embryos through IVF consists of a chain of four steps, namely harvesting of the oocytes, IVM of the recovered oocytes, IVF of the *in vitro* matured oocytes and IVC of the IVF zygote for development to the desired stage. The success of a particular step depends on the success of the previous step or steps (Gordon, 2003).

2.2. HARVESTING OF OOCYTES

Harvesting of oocyte is usually done by dissection, slicing or aspiration of follicles or oviductal flushing, depending upon whether pre or post ovulatory oocytes are required. Oocyte retrieval from live animal could be performed by ovariectomy, laparoscopy or ultrasound guided technique (Besenfelder *et al.*, 2001).

2.2.1. Recovery of Oocytes from Live Animals

The first calf from IVF was produced from an oocyte obtained by laparotomy procedures (Brackett *et al.*, 1982). This surgical procedure was later supplemented by the laparoscopic techniques and had been used successfully in several studies (Sirard and Lambert, 1985; Sirard *et al.*, 1985; Lambert *et al.*, 1986 and Stubbings *et al.*, 1988 a). Ovum pick up could be useful for collecting oocytes from genetically valuable cows that responded poorly to gonadotropin stimulation or in cows from which embryo recovery was no longer possible (Looney *et al.*, 1994). An eight year

old cow produced 176 embryos in a period of three years by weekly once OPU programme (Hasler, 1998). Vivanco and Mc Millan (1999) reported that, OPU could be valuable in breeding programmes that required many more births annually than was possible with conventional embryo transfer technology. Ultrasound guided OPU technique is widely used now a days for the collection of oocytes from live animals (Goodhand *et al*, 2000 and Hendriksen *et al.*, 2004). In India, Manik *et al.* (2002) demonstrated the OPU technique for recovering oocytes.

2.2.2. Recovery of Oocytes from Abattoir Ovaries

Many investigators confirmed that the stage of oestrus cycle and pregnancy status of the donor animal did not influence the developmental capacity of the isolated oocytes (Leibfried and First, 1979; Fukui and Sakuma, 1980 and Leibfried-Rutledge *et al.*, 1985). Therefore the selection of ovaries based on reproduction status appeared unnecessary. Ovaries from slaughter house are usually used for the retrieval of oocytes for IVP of embryos (Harada *et al.*, 1997 and Lequarre *et al.*, 2005). The recovery rate varied with collection procedures, selection of ovaries and procedural skill.

2.2.3. Collection of Ovaries

2.2.3.1. Time Interval from Slaughter to Isolation of Oocytes

Bovine ovaries from slaughter house collected in 0.9 per cent NaCl maintained at 35 to 37 ° C and brought to the laboratory within 3 h resulted in 82 per cent oocytes matured to M II (Chian *et al.*, 1995). The time interval preferred by researchers normally ranged from one hour (Ectors *et al.*, 1995 and Lechniak *et al.*,

1996), three to five hours (Wu *et al.*, 1997), one to three hours (Vassena *et al.*, 2003 and Matsushita *et al.*, 2004) and more than five hours (Ali *et al.*, 2004).

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.

2.2.3.2. Temperature and Media

Increased depolymerization of microtubules of oocytes were observed when the ovaries were kept below 20° C, resulting in abnormal meiosis (Aman and Parks, 1994). In various studies, ovaries were transported in normal saline with antibiotics at different temperatures like 20-30° C (Ectors *et al.*, 1995) with above 86 per cent maturation rate, 35-37° C (Chian *et al.*, 1995) with 82 per cent maturation rate, 30-37° C (Lechniak *et al.*, 1996) with approximately 75 per cent maturation rate and >20° C (Matsushita *et al.*, 2004) with 45.2 per cent maturation rate.

Khurana and Niemann (2000 a) used phosphate buffered saline (PBS) supplemented with one per cent new born calf serum (NBCS) at ambient temperature (~25°C) for the collection and transportation of ovaries and generated 30 to 35 per cent morulae /blastocysts of transferable quality. Dulbecco's phosphate buffered saline (DPBS) with antibiotics (Abdoon *et al.*, 2001 and de Wit and Kruip, 2001) or sterile normal saline supplemented with antibiotics (Ali and Sirard, 2002; Cetica *et al.*, 2002 and Bormann *et al.*, 2003) were the media usually used for the pre retrieval processing of ovaries.

2.2.4. Isolation of Oocytes from Ovaries

2.2.4.1. Isolation Media

Commonly used oocyte isolation media are TL-HEPES (TL-N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]), HEPES or Bicarbonate buffered TCM-199 (Tissue culture Medium-199), DPBS and synthetic oviduct fluid (SOF). Tyrode's medium (HEPES buffered) supplemented with 0.3 per cent Bovine Serum Albumin fraction V (BSA) and antibiotics was used for oocyte retrieval (Aman and Parks, 1994; Hasler *et al.*, 1995 and Azambuja *et al.*, 1998).

Tissue Culture Medium-199 buffered either with HEPES or bicarbonate, 10 per cent fetal calf serum (FCS) and antibiotics was used for oocyte ietrieval and washing by many workers (Lechniak *et al.*, 1996; Armstrong *et al.*, 1996; deMatos *et al.*, 1997 and Harada *et al.*, 1997). Cumulus Oocyte Complexes (COCs) were washed in Hepes-buffered M 199 medium supplemented with 10 per cent oestrus cow serum (ECS) by Guyader-Joly *et al.* (1999). Khurana and Niemann (2000 a) used fresh phosphate buffered saline (PBS) for collection and morphological evaluation of COC and obtained approximately 35 per cent morulae /blastocysts of transferable quality. Mizushima and Fukui (2001) washed COCs in TCM-199 supplemented with 0.3 per cent (w /v) BSA, 2mM sodium bicarbonate and 10 mM HEPES.

2.2.4.2. Isolation Methods

The collection of oocytes from ovaries was performed by dissection, slicing or aspiration (Carolan *et al.*, 1994). Aspiration of oocytes from 2-8 mm follicles using 18-21 gauge disposable needle and syringe or vacuum pump was a common

method of oocyte isolation from slaughter house ovaries (Dominguez, 1995; Furnus *et al.*, 1997; Wu *et al.*, 1997; Pugh *et al.*, 1998; Guyader-Joy *et al.*, 1999; Khurana and Niemann, 2000 a; Abdoon *et al.*, 2001; de Wit and Kruip, 2001 and Ali and Sirard, 2002). Aspiration of oocytes from 3-6 mm follicles with 18-21 gauge disposable needles with 10 ml disposable syringe was followed for oocyte retrieval from sow ovaries (Mori *et al.*, 2000; Algriany *et al.*, 2004 and Kim *et al.*, 2006).

Oocytes were collected by repeated slicing of ovaries with a sharp sterile surgical blade while it was held in a Petri dish containing the COC handling medium (Das *et al.*, 1996 a; Hochi *et al.*, 1996; Bols *et al.*, 1997; Hochi *et al.*, 1999 and Priscilla, 2001).

Repeated puncturing of slaughter house ovaries with 18 gauge needle was performed to recover oocytes into the medium kept in a Petri dish (Sharma *et al.*, 1996; Wani *et al.*, 1999 and Priscilla, 2001). To improve the efficiency of oocyte retrieval the follicular fluid was encouraged to ooze out by applying gentle pressure on the adjacent stroma of the punctured follicle (Das *et al.*, 1996 a and Priscilla, 2001). 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). Aspiration method of oocyte retrieval had the advantage of high speed oocyte recovery when compared to dissection or slicing (Gordon, 2003).

2.2.4.3. Oocyte Yield per Ovary

By employing aspiration technique, scientists could obtain oocyte yield, in various ranges like 3-10 (Katska and Smorag, 1984; Mermillod *et al.*, 1992; de

Oliveria *et al.*, 1994; Baruha *et al.*, 1998; Priscilla, 2001 and Magnus Paul, 2005) and 11-15 (Hamno and Kuwayama, 1993; Carolan *et al.*, 1994 and Arlotto *et al.*, 1996) from cattle ovaries.

Number of oocytes isolated from bovine ovaries by slicing method ranged between 15-25 (Sato *et al.*, 1990), 25-50 (Carolan *et al.*, 1994 and Takagi *et al.*, 1998), 10-15 (Rieger and Loskutoff, 1994 and Arlotto *et al.*, 1996), and 5-10 (Priscilla, 2001 and Magnus Paul, 2005).

Sato *et al.* (1990), Priscilla (2001) and Magnus Paul (2005) obtained 3-10 oocytes per ovary when puncture method was employed for oocyte retrieval. Das *et al.* (1996 a) and Das *et al.* (1996 b) obtained 2.6 and 11.13 oocyte per ovary in buffalo and sheep respectively by puncture method.

Hamno and Kuwayama (1993), Carolan *et al.* (1994), Takagi *et al.* (1998), Priscilla (2001) and Magnus Paul (2005) obtained maximum oocytes by slicing method than aspiration method, in cattle. Priscilla (2001) reported that, aspiration yielded more oocytes than puncture in cattle. Magnus Paul (2005) reported that, aspiration and puncture did not differ significantly in yield of COCs per ovary. Deepa (2007) found that slicing and puncturing yielded more oocytes per porcine ovary when compared to other methods and aspiration yielded significantly less oocyte than other methods.

2.2.5. Selection of Oocytes

Visual assessment of the compactness and the extent of cumulus investment has been the most widely used criterion to select bovine immature oocytes for IVM and further developmental potential (Leibfried and First 1979 and Madison *et al.*,

1992). Most predictive morphological criteria for selection of COCs continue to be intact cumulus cells and homogenous cytoplasm (Abdoon *et al.*, 2001). Most of the grading depended on a visual subjective appraisal by laboratory personnel and as such could vary with the individual and the laboratory (Gordon, 2003).

2.2.5.1. Cumulus Cell Character

Pawshe *et al.* (1994) graded oocytes with more than four layers of compact cumulus cells and uniform cytoplasm as good, less than four layers or with a partial cumulus mass with a uniform cytoplasm as fair and oocytes with out cumulus cells, but with uniform cytoplasm as poor quality oocytes. Das *et al.* (1996 a) classified oocytes with more than five layers of compact cumulus cells as good, two to five layers of cumulus cells as fair and fewer than two layers of cumulus cells and either completely or partially denuded as poor quality oocytes. Abdoon and Kandil (2001) classified oocytes as good, fair, poor and denuded. According to these authors, oocytes surrounded by more than six layers, three to five layers, one to two layers of cumulus cells and naked oocytes were good, fair, poor and denuded respectively. Gupta *et al.* (2006) classified the COCs from Murrah buffaloes with more than or equal to five layers of cumulus cells and uniform granulation of ooplasm as grade A, those with two to four layers of cumulus cells and uniform granulation of ooplasm as grade B, partially denuded and uniform granulation of ooplasm as grade C and completely denuded oocytes with uniform granulation as grade D.

2.2.5.2. Ooplasm Character

Leifried and First (1979) classified oocytes which had vacuolated or fragmented ooplasm and not evenly filling the zona pellucida or shrunken away from

zona pellucida as degenerated oocytes. Dominguez (1995) considered oocytes with condensed and vacuolated ooplasm as abnormal.

Oocytes with evenly granulated ooplasm and with dark scattered ooplasm were graded as good and poor quality respectively by Das *et al.* (1996 a). Majority of scientists selected oocytes with homogenous and evenly granulated cytoplasm for IVM and fertilization studies (Romero-Arredondo and Seidel, 1996; Tatemoto and Terada, 1996; Ikeda *et al.*, 2000 and Lequarre *et al.*, 2005). Good quality oocytes with homogenous evenly distributed cytoplasm were selected by Nashta *et al.* (1998) for IVM. Vassena *et al.* (2003) found that, oocytes with even, smooth and finely granulated ooplasm had the highest developmental competence.

2.2.5.3. Culture Grade Oocytes

Oocytes with multiple layers of cumulus cells (more than three layers) and uniform granulation of ooplasm were usually considered as culture grade oocytes. Hawk and Wall (1994) used limited number of quality grades in selecting oocytes for maturation to avoid delay in placing the oocytes into the maturation medium and found that highest developmental potential was obtained when oocytes with minimal cumulus cells were excluded. Cetica *et al.* (1999) studied the nuclear stage of four grades of immature oocytes selected on the basis of cumulus cell coverage and found that 88 per cent of the top grade oocytes were at the GV stage, which was significantly higher than in the other grades.

Khurana and Niemann (2000 b) examined the factors influencing the efficiency of embryo production and observed that the morphological quality of oocytes required for efficient embryo production was the compactness and number of layers of cumulus cells. Magnus Paul (2005) classified oocytes with more than

five complete layers of cumulus cells and uniform granulation of ooplasm as Class A, three to five complete layers of cumulus cells and uniform granulation of ooplasm as Class B, one to two layers of cumulus cells and uniform granulation of ooplasm as Class C and denuded oocytes with uniform granulation of ooplasm as Class D and found significantly higher maturation of oocytes in class A and B than C.

2.2.5.4. Yield of Culture Grade Oocytes

Culture grade oocyte recovery rate of 40-57 per cent was obtained in bovine (Katska, 1984 and Dominguez, 1995) and buffaloes (Naik *et al.*, 1999), by the aspiration method. Hamno and Kuwayama (1993) reported that more than 90 per cent of oocytes collected by aspiration method was of A and B class COCs. By employing aspiration technique Vassena *et al.* (2003) could collect only 62 per cent of culture grade COCs.

By aspiration method Katska and Smorag (1984) obtained four to five oocytes with multiple layers of cumulus cells per bovine ovary. A range of 3-37 multilayered COCs with an average of 17.8 oocytes per ovary were obtained by Takagi *et al.* (1992) by the same method in bovines.

Sato *et al.* (1990) obtained 12.3 multilayered COCs per ovary when slicing method was employed for oocyte recovery, whereas Takagi *et al.* (1992) obtained an average of 14.3 culture grade COC per ovary by same method.

Sato *et al.* (1990) obtained eight COCs per ovary with multiple layers of cumulus cells when puncture method of oocyte retrieval was employed. Studies by Magnus Paul (2005) revealed that there was no significant difference between these three methods in the yield of culture grade oocytes in cattle.

Dominguez (1995) opined that larger follicles were more in number in European breeds than Zebu and crossbred cattle. Factors like body condition, age, diseases, managemental practice and reproductive disorders could affect follicular development and oocyte yield.

2.3. *IN VITRO* MATURATION

2.3.1. Oocyte Maturation and Meiotic Resumption

The complex biological process that involved the meiotic resumption of oocyte, progress from prophase of first meiotic division to metaphase of the second meiotic division, that transform the primary oocyte into a mature ova is known as oocyte maturation. During this process the nucleus and cytoplasm of the oocyte undergo many changes, which make the oocyte competent to undergo fertilization and further embryonic development (Gordon, 1994). The oocyte from the dominant follicle that underwent ultra structural modifications at nuclear and cytoplasmic level and attained full developmental competence through a process that might be termed as oocyte capacitation (Hyttel *et al.*, 1997). The process by which oocytes obtain the factors that are essentially required for completing the developmental potential process is referred to as cytoplasmic maturation (Gordon, 2004).

2.3.1.1. Cytoplasmic Maturation

Cytoplasmic maturation was essential to acquire the ability to block polyspermy and to decondense penetrated spermatozoa to form pronuclei and after fertilization for redistribution of cell organelles, migration of mitochondria to a perinuclear position and accumulation of granules along the oolemma (Prather and Day, 1998 and Van den Hurk and Zhao, 2005).

The concentration of intracellular glutathione (GSH) was increased as cytoplasmic maturation completed and hence the estimation of GSH after IVM might be a valuable marker to assess the degree of cytoplasmic maturation in bovine and pig oocytes (Mizushima and Fukui, 2001).

Changes associated with maturation of oocyte, which occurred in one to eight hours of luteinizing hormone (LH) peak *in vivo* which included formation of periviteline space with loss of contact between the cumulus cell and the oocyte and roughening of nuclear membrane. These changes were followed by resumption of meiosis marked by germinal vesicle break down (GVBD), disappearance of rough endoplasmic reticulum (RER), and formation of clusters of mitochondria in association with lipid droplets and smooth endoplasmic reticulum (SER). The period from 8-19 h was characterized by intensive clustering of mitochondria in association with lipid droplets and elements of SER and appearance of ribosomes in cytoplasm. After 19 h, polar body (PB) extruded, mitochondria dispersed, and majority of organelles aggregated towards centre of the cell. Relatively organelle free cortical region contained cortical granules immediately adjacent to the plasma membrane, together with aggregates to tubular SER (Gasparrini, 2002).

2.3.1.2. Nuclear Maturation

Germinal vesicles break down occurred six to eight hours after beginning of IVC (Sirard *et al.*, 1989). Oocytes isolated from follicles and immediately fixed contained a GV in 100 per cent cases (Khatir *et al.*, 1998). It was further reported that, by 12 h of IVM most of the oocytes (84 per cent) were at metaphase I (M I), by 20 h of IVM 89 per cent of oocytes were at M II stage and at 24 h 86 per cent exhibited M II to extrusion of first PB. Diploid set of chromosomes were fully condensed and were arranged on equatorial plane during M I (Datta and Goswami,

1999). The authors observed darkly stained nucleus with very distinct nuclear membrane in GV stage. Transition from M I to M II stage of bovine oocyte maturation was triggered by reduction in Maturation Promoting Factor (MPF) (Anas *et al.*, 2000). Nuclear maturation was characterized by chromosome condensation (CC), nuclear envelope dissolution, spindle assembly and chromosome separation (Wehrend and Meinecke, 2001 and Krischek and Meinecke, 2002). According to Rodriguez and Farin (2004), nuclear maturation refers to acquisition of the ability to undergo dissolution of GV, GVBD, condensation of chromosomes, release of first PB and subsequent arrest at metaphase II (M II). Nuclear maturation process last about 24 h in cow and sheep, 44 h in pig and 36 h in horse (Van den Hurk and Zhao, 2005).

Conti *et al.* (2002) stated that a cytostatic factor (CSF) was an essential component and was responsible for the maintenance of M II arrest. Glucose metabolism, particularly pentose phosphate pathway, were involved in normal mechanism regulating the meiotic resumption and maintenance of developmental competence (Krisher, 2004). It was observed that Ca^{2+} was important for meiotic resumption in bovine oocytes and low Ca^{2+} affected progression of meiosis by inhibiting the activation of kinases.

2.3.2. Maturation Media

A variety of different media including, TALP medium (Ball *et al.*, 1983; Lenz *et al.*, 1983; Crister *et al.*, 1984; Leibfried-Rutledge *et al.*, 1986 and Hasler *et al.*, 1995), Minimum essential medium (MEM) (Hensleigh and Hunter, 1985 and Bavister *et al.*, 1992), Ham's F-12 medium (Fukushima and Fukui, 1985 and Bavister *et al.*, 1992), Ham's F-10 medium (Bavister *et al.*, 1992; Totey *et al.*, 1993 a; Totey *et al.*, 1993 b and Totey *et al.*, 1996), and TCM-199 (Bavister *et al.*, 1992;

Hochi *et al.*, 1996; Furnus *et al.*, 1997; Pugh *et al.*, 1998; Hochi *et al.*, 1999; Khurana and Niemann, 2000 a; Magnus Paul, 2005 and Deepa, 2007) have been used for IVM of bovine oocytes.

Tissue culture medium-199 was the most commonly used maturation medium with rates of maturation occasionally reaching 98-99 per cent (Utsumi *et al.*, 1988). Either bicarbonate buffered TCM (Bilodeau *et al.*, 1993; Furnus *et al.*, 1997 and Neglia *et al.*, 2003) or HEPES buffered TCM-199 (Hochi *et al.*, 1996; Hashimoto *et al.*, 1998; Pugh *et al.*, 1998; Hochi *et al.*, 1999; Liu *et al.*, 1999 and Khurana and Niemann, 2000 a) were also used for IVM of bovine oocytes. Tissue culture medium-199 is a complex medium and it contains vitamins, amino acids, purines and other substances, mainly in the same concentrations as it is found in the serum (Gordon, 2003).

2.3.3. Media Additives

Hormones like Follicle Stimulating Hormone (FSH), LH and Estrogen and growth factors like epidermal growth factor (EGF), insulin like growth factor (IGF-1) and Transforming growth factor-∞ (TGF-∞) can influence the IVM of bovine oocytes (Bevers *et al.*, 1997).

2.3.3.1. Hormones

Many scientists have investigated the effects of hormones on maturation of bovine oocytes *in vitro* at different sensitive stages such as nuclear maturation, fertilization, *in vitro* cleavage and development to morulae /blastocysts with disagreement in observations (Fukushima and Fukui, 1985; Hensleigh and Hunter, 1985; Stubbings *et al.*, 1988 b and Fukui and Ono, 1989).

Estrogens alone or along with FSH and /or LH had beneficial effect on maturation and further cleavage of the oocytes (Fukushima and Fukui, 1985). Similar to *in vivo* , where the oocyte is initially exposed to estradiol and then to FSH and LH, Stubbings *et al.* (1988 b) added estradiol at the beginning of culture and gonadotropins from the start or at six hour of culture and found that the sequential addition of hormones improved the frequencies of fertilization and subsequent embryonic development. But Fukui and Ono (1989) found no positive effects of hormonal supplementation. The authors attributed these contradictory results to the differences in doses of hormones and the choice of medium and the culture conditions.

2.3.3.1.1. Follicle Stimulating Hormone

Different sources of FSH like, recombinant bovine FSH (Barnes *et al.*, 1993), bovine FSH (Long *et al.*, 1994), Ovine FSH (Chian *et al.*, 1996; He *et al.*, 1997 and Pugh *et al.*, 1998) and porcine FSH (Armstrong *et al.*, 1996; Furnus *et al.*, 1997 and Calder *et al.*, 2001) were used for maturation of bovine oocytes *in vitro*.

Barnes *et al.* (1993) used recombinant bovine FSH at the rate of 45 µg /ml. Long *et al.* (1994) and Azambuja *et al.* (1998) used FSH at the rate of 0.01 NIH units per ml. The concentration of FSH used in most of the IVM studies was in the range of 0.5-1 µg /ml (Kobayashi *et al.*, 1994; Chian *et al.*, 1996; He *et al.*, 1997; Furnus *et al.*, 1997; Khurana and Niemann, 2000 a; Calder *et al.*, 2001 and Dode and Adona, 2001). Hasler *et al.* (1995) used 4 µg /ml FSH and Guyader-Joly *et al.* (1999) used 10 µg /ml FSH for IVM of oocytes.

Bevers *et al.* (1997) reviewed and stated that concentration of FSH and LH in most of the IVM studies ranged between 1-10 µg per ml.

Pawshe *et al.* (1996) reported that FSH enhanced early embryonic development rather than meiotic maturation. Addition of FSH alone in maturation medium resulted in higher rate of oocyte maturation *in vitro* (Sachan *et al.*, 1999). However, Gupta *et al.* (2001) reported that FSH was the most essential gonadotropin for cumulus expansion and IVM. According to Choi *et al.* (2001), FSH stimulated an increase of cyclic adenosine mono phosphate (cAMP) concentration and cumulus expansion. While FSH priming improved the maturation rate of IVM oocytes, FSH or human chorionic gonadotropin (hCG) priming did not improve development to the blastocyst stage (Stephen *et al.*, 2003).

2.3.3.1.2. Luteinizing Hormone (LH)

Various sources of LH like recombinant bovine LH (Barnes *et al.*, 1993), purified porcine LH (Armstrong *et al.*, 1996), bovine LH (Chian *et al.*, 1996 and He *et al.*, 1997), ovine LH (Pugh *et al.*, 1998 and Calder *et al.*, 2001) and recombinant human LH (Gordon, 2003) were used in IVM medium for bovine oocytes. Luteinizing hormone at a concentration of five microgram per ml was used for IVM of bovine oocytes (Long *et al.*, 1994; Kobayashi *et al.*, 1994; Gasparrini, 2002 and Neglia *et al.*, 2003). Leutinizing hormone at concentrations of 6 µg /ml (Hasler *et al.*, 1995) and 10 µg /ml (Furnus *et al.*, 1997 and Guyader-Joly *et al.*, 1999) were also used in maturation medium for bovine oocytes. It enhanced the quality of oocytes, which was reflected in increased embryo yield during IVF and further culture (Pawshe *et al.*, 1996).

2.3.3.1.3. Estradiol 17-β

Estradiol addition at a concentration of 1µg /ml was found to improve IVM of bovine oocytes (Harper and Brackett, 1993), buffalo oocytes (Sachan *et al.*, 1999)

and pig oocytes (Bing *et al.*, 2001). Estradiol was used at a concentration of one microgram per ml in maturation medium by many researchers for bovine oocytes (Barnes *et al.*, 1993; Furnus *et al.*, 1997; Pugh *et al.*, 1998; Lim *et al.*, 1999; Khurana and Niemann, 2000 a; Gasparrini, 2002 and Neglia *et al.*, 2003).

Bevers *et al.* (1997) reported that estradiol had a significant role in cytoplasmic maturation and that it was necessary for further fertilization and early post fertilization development.

2.3.3.2. Serum

Generally maturation of bovine oocytes *in vitro* had been accomplished in maturation media supplemented with FCS (Goto *et al.*, 1988; Sanbuissho and Threlfall, 1988; Hasler *et al.*, 1995; Furnus *et al.*, 1997; Pugh *et al.*, 1998; Hochi *et al.*, 1999; Guyader-Joly *et al.*, 1999; Magnus Paul, 2005 and Deepa, 2007), ECS (Fukui and Ono, 1989 and Khurana and Niemann, 2000 a), BSA (Bavister *et al.*, 1992) or Steer serum (Hochi *et al.*, 1996). Raghu *et al.* (2002) reported that, serum prevented sticking of oocytes to syringe surface and needle. Gasparrini (2002) opined that, serum prevented zona hardening and thereby improved the fertilizing ability of oocytes. Serum was known to contain a wide range of components like hormones, growth factors, amino acids and binding proteins (Gordon, 2003). Growth factors which were important for cytoplasmic maturation such as EGF and IGF-I were found in serum (Mikkelsen, 2004).

Some investigators observed no differences between FCS and ECS to support maturation of oocytes *in vitro* (Sanbuissho and Threlfall, 1988 and Fukui and Ono, 1989). Studies in Ireland showed that ECS had a significant and marked effect compared with FCS on subsequent developmental competence of oocytes. Some

studies suggested that pro ECS might be more effective in maturation of oocytes than ECS; analysis of the serum showed high levels of LH and prolactin (Gordon, 2003).

2.3.3.3. Granulosa cells

Several investigators (Critser *et al.*, 1986; Lu *et al.*, 1987, 1988; Fukui and Ono, 1989; Chian *et al.*, 1994; Konishi *et al.*, 1995 and Liu *et al.*, 1995) have supplemented the IVM media with granulosa cells, in order to mimic the intra follicular cellular interactions during COC maturation and to promote nuclear and cytoplasmic maturation of the bovine oocyte.

Critser *et al.* (1986) found that maturation of bovine oocytes with or without co-culture of granulosa cells (1 million /ml) did not influence the frequency of maturation or fertilization, but morulae and blastocysts were obtained only from oocytes co-cultured with granulosa cells. Lu *et al.* (1987), Xu *et al.* (1987) and Fukui and Ono (1989) have reported a favourable effect of granulosa cells when used at concentrations ranging from three to seven million /ml. Chian and Niwa (1994) in a study concluded that the presence of intact cumulus cells was necessary for at least 12 h of the 24 h culture period. Mingoti *et al.* (2002) observed considerable oestradiol accumulation in the maturation medium during the 24 h period of oocyte incubation. Granulosa cells were able to produce oestradiol (Gordon, 2003).

2.3.3.4. Growth Factors

Epidermal growth factor, IGF-I, TGF-∞ and Bovine fibroblast growth factor (bFGF) were the major growth factors used to enhance the oocyte maturation. Kobayashi *et al.* (1994) reported that EGF and TGF-∞ in maturation medium stimulated cumulus expansion and oocyte fertilizability. The authors added EGF (10

ng /ml), TGF-∞ (10 ng /ml) and bovine FGF (10 ng /ml), separately in maturation media and obtained cleavage rate of 70.3 per cent with EGF, 73.6 per cent with TGF-∞ and 40 per cent with bovine FGF. Bevers *et al.* (1997) reviewed the influence of EGF in the induction of cumulus expansion and promotion of nuclear maturation in several species including cow. Epidermal growth factor stimulated oocyte maturation by disrupting it's communication with cumulus cell and it created a positive maturation signal. Hochi *et al.* (1999) used 0.5 ng /ml EGF in maturation medium. Avery *et al.* (2000) obtained 71-79 per cent cleavage rate when added EGF in maturation medium at a concentration of 50 ng /ml. Gupta *et al.* (2002) reported positive effect of EGF on maturation of buffalo oocytes. When it was added at the rate of 20 ng /ml in TCM-199 medium supplemented with 10 per cent FCS, resulted in enhanced cumulus expansion (74 per cent) and maturation rate (80 per cent).

2.3.3.5. Pyruvate and Amino acids

Pyruvate and amino acids like cysteine, glutamine, taurine and glycerine had physiologically important role in the maturation of bovine oocytes *in vitro*. Hochi *et al.* (1996) and Khurana and Niemann (2000 a) used 2 mM sodium pyruvate and 22 µg /ml pyruvate respectively in maturation media. Sodium pyruvate was usually added to culture medium for IVM at the rate of 0.2 mM (Silva and Knight, 1998 and Avery *et al.*, 2000). Geshi *et al.* (2000) stated that, the cumulus cells metabolized glucose to pyruvate or Krebs cycle intermediates that could be passed to the oocyte and enhanced its quality. Without cumulus cells, cattle oocytes required sodium pyruvate for nuclear maturation in a protein free medium (Nagai, 2001).

Glutamine was added at the rate of 0.4 mM (Bols *et al.*, 1996), 1 mM (Hochi *et al.*, 1996) or 0.68 mM (Choi *et al.*, 2001) in the culture media for IVM. Abdoon *et al.* (2001) added glutamine, taurine and glycein in maturation medium

and proved the beneficial effect in developing bovine embryos. Ali *et al.* (2003) added cysteine and improved bovine embryo development *in vitro* in defined conditions containing low glucose. These amino acids could act as energy substrates, pH regulators or as a pool for *de novo* protein synthesis.

2.3.4. Culture conditions

2.3.4.1. Temperature

Bovine oocytes could undergo nuclear maturation *in vitro* at temperatures ranging from 35° C to 39° C (Lenz *et al.*, 1983). These authors observed that the oocytes matured at 39° C had significantly higher rates of fertilization than those matured at 35° C, 37° C or 41° C. Shi *et al.* (1998) reported that optimal embryo development was achieved between 38° C and 39° C. Most of the scientists used a temperature range of 38.5° C to 39° C for cattle IVM studies (Khurana and Niemann, 2000 a and Neglia *et al.*, 2003).

2.3.4.2. Gas Phase and Humidity

Since most of the media used for IVF studies were bicarbonate buffered, provision of an atmosphere of five per cent carbon dioxide (CO_2) in air has been a common practice. Most of the workers used five per cent CO_2 in atmospheric air and maximum humidity (around 95 per cent) for IVM and obtained good results (Chian *et al.*, 1996; de Matos *et al.*, 1997; Furnus *et al.*, 1997; Khatir *et al.*, 1998; Pugh *et al.*, 1998; Guyader-Joly *et al.*, 1999; Hochi *et al.*, 1999 and Khurana and Niemann, 2000 a). Optimal gas phase for IVM of oocytes were 2.5 to 5 per cent CO_2 and 20 per cent oxygen and maximum humidity is given during IVC of oocytes in order to prevent evaporation of medium (Gordon, 2003).

2.3.5. Assessment of Oocyte Maturation Status

2.3.5.1. Cumulus Cell Expansion

Hunter and Moore (1987) classified bovine oocytes after maturation based on the degree of cumulus cell expansion. According to them, grade 1 was with full cumulus cell expansion, grade 2 with moderate expansion and grade 3 with slight expansion. Cumulus cell mass and expansion of cumulus cells were found to be good predictors of blastocyst potential after IVF. In assessing oocyte maturation, due regard is paid to the degree of cumulus-cell expansion in the belief that this is a reflection of physiological normality. *In vitro* maturation of bovine oocyte involves well defined changes in the cumulus cells that surround it; the cumulus cells expand to form a spherical mass in three dimensions and the COCs appear to float in the culture dish (Gordon, 2003).

2.3.5.2. Nuclear Changes

Bilodeau *et al.* (1993) classified oocytes as GV stage or at intermediate stage (GVBD, chromosome condensation, M I) or mature (Anaphase I, Telophase I and Metaphase II). Nuclear maturation refers to acquisition of the ability to undergo dissolution of GV, condensation of chromosomes, release of first polar body and subsequent arrest at metaphase II (Rodriguez and Farin, 2004).

2.3.6. Maturation Rate

With 24 h *in vitro* culture of bovine oocytes in TCM-199 with gonadotropins, Kobayashi *et al.* (1994) obtained an overall cumulus expansion rate of 99.2±1.0 per cent. By 24 h culture of bovine oocytes retrieved by aspiration, Lorenzo *et al.*

(1994) obtained 58.8-71.4 per cent cumulus expansion in culture medium TCM-199, supplemented with gonadotropins. However, Quero *et al.* (1994) obtained a cumulus expansion rate of 78 per cent when aspirated oocytes with more than 3 layers of cumulus cells were cultured *in vitro*. Most of the workers used oocytes with multiple layers (≥3 layers) of cumulus investment for IVM and fertilization studies (Tornesi *et al.*, 1995). Calder *et al.* (2003) obtained an overall cumulus expansion rate of 90.3±2.5 per cent when bovine oocytes were cultured for 24 h in TCM-199 medium with gonadotropins, estrogen and 10 per cent serum. An overall cumulus expansion rate of 68.75 per cent was obtained by Magnus Paul (2005) when aspirated oocytes were matured *in vitro*. Cumulus expansion rate obtained for A grade, B grade and culture grade oocytes retrieved by aspiration method by the same author were 83.08 per cent, 68.29 per cent and 77.36 per cent respectively.

2.4. *IN VITRO* FERTILIZATION

2.4.1. Oocyte for *In Vitro* Fertilization

Many factors associated with oocytes influence the results of IVF in cattle. The major factors were source of oocytes, quality of oocytes and the steps that were taken in handling oocytes prior to the exposure to sperm. Mostly the matured oocytes were rinsed and transferred to the fertilization medium before the addition of prepared sperms (Gordon, 2003).

2.4.1.1. Processing Prior to Co-culture with Sperm

Oocytes were rinsed twice in TALP and placed in 0.5 ml of fertilization medium (Hasler *et al.*, 1995). The *in vitro* matured COCs were placed into IVF drops after washing three times with HEPES-buffered TALP supplemented with two

per cent BSA and an additional three times with the Tyrode's IVF medium (Hochi *et al.*, 1996).

Furnus *et al.* (1997) washed the expanded oocytes twice in Hepes-TALP, supplemented with 3 mg /ml BSA and placed into 50 µl drops of IVF medium. Following maturation, oocytes were washed in HEPES-buffered TALP medium, then in bicarbonate-buffered TALP containing 1 µM hypotaurine, 2 µM penicillamine and 10 µg /ml heparin (IVF medium) and then transferred to IVF drops (Pugh *et al.*, 1998).

Khurana and Niemann (2000 a) rinsed COCs twice in Fert-TALP medium and the oocytes were inseminated in groups of 10 in 100 µl droplets of fertilization medium under silicon oil.

2.4.1.2. Effect of Cumulus Cells on Sperm Penetration

Cumulus enclosed oocytes were routinely used for IVF studies in cattle. Saeki *et al.* (1994) examined the effects of cumulus cells on sperm capacitation, acrosome reaction (AR) and penetration of bovine oocytes in which denuded oocytes failed to be fertilized. These authors found that, cumulus cells did not affect the capacitation and AR, but the addition of cumulus cells was effective in facilitating sperm penetration of the denuded oocytes. It was further observed that, cumulus cells matured with gonadotropins and oestradiol enhanced the sperm penetration more than when immature cells were used. Chian *et al.* (1995) reported that cumulus cells participated in the mechanisms of sperm capacitation and the acrosome reaction and that polyspermy was directly affected by the number of capacitated and acrosome reacting sperm on the oocyte surface. Generally *in vitro* matured COCs without denuding the cumulus cells were rinsed and used for IVF (Hasler *et al.*,

1995; Furnus *et al.*, 1997; Pugh *et al.*, 1998; Guyader-Joly *et al.*, 1999 and Khurana and Niemann, 2000 a). Chian *et al.* (1996) concluded that, cumulus cells produced a chemical substance that either attracted spermatozoa or facilitated sperm penetration when very few sperms were around the oocyte. Fatehi *et al.* (2002) reported that, removal of cumulus cells prior to IVF significantly reduced cleavage rate (25 per cent for denuded oocytes vs. 56 per cent for COCs).

2.4.2. Sperm for *In Vitro* Fertilization

2.4.2.1. Source of Spermatozoa

Keskintepe *et al.* (2002) reported the production of karyotypically normal blastocysts after the injection of bull sperm that had been selected, freeze-dried (lyophilized) and stored at four degree centigrade until use. By the development of new techniques such as sperm micro injection in which spermatozoa may be employed in extremely small numbers and even when they are showing very poor motility, it is possible to achieve fertilization of oocytes. Sperm cells obtained from the epididymis or directly from the testis itself can be used to achieve fertilization of oocytes by sperm micro injection (Gordon, 2003). Many of the scientists have used both fresh and frozen bull semen for cattle IVF studies. But frozen semen is widely used for IVF studies in cattle. However, (Gordon, 2003) opined that when necessity arose, even sperm cells recovered from the epididymis could be used for IVF.

2.4.2.1.1. Fresh Ejaculated Spermatozoa

Katska *et al.* (1996) found that removal of seminal plasma from bull ejaculates immediately after collection could improve the outcome of *in vitro* embryo production. Verberckmoes *et al.* (2000) studied the influence of

centrifugation on the motility and membrane integrity of fresh bull sperm and reported that centrifugation might be required to obtain a sperm sample free of seminal or epididymal plasma. These authors concluded that, a centrifugation speed of 3214 x g was optimal for the separation of sperm and plasma without causing mechanical damage to the sperm membrane and motility. Washing of bull sperm to remove seminal plasma proteins rapidly and effectively prior to use in IVF is a recognized practice. This is usually done by washing the sperm by centrifugal sedimentation and resuspension in fresh medium (Gordon, 2003).

Goldman *et al.* (1998) examined the reaction of fresh and frozen bull sperm incubated for 5-24 h with bovine oviductal epithelial cells and found that the percentage of motile fresh sperm exceeded that of frozen sperm and more number of fresh spermatozoa attached to the epithelial cell monolayer. Krzyzosiak *et al.* (2001) found that the fertility of the sperm declined with storage of sperm at ambient temperature in citrate-based commercial diluents. De Pauw *et al.* (2002) found that, fresh sperm coated with Tris-egg-yolk diluents immediately after ejaculation, for five minutes, yielded significantly higher fertilization rates than uncoated sperm after four days of storage.

2.4.2.1.2. Frozen Spermatozoa

Frozen semen is widely used as major source of spermatozoa in IVF studies due to the ready availability of proven quality frozen semen. Hasler *et al.* (1995) prepared the percoll gradient with sperm-TALP (SP-TALP) and the centrifuged frozen thawed semen was overlaid on percoll gradient for 30 minutes at 700 x g. The recovered sperm pellet was resuspended in sperm-TALP and concentration determined with haemocytometer and adjusted the concentration. These authors obtained a cleavage rate of 77 per cent with these procedures.

Hochi *et al.*, (1996) washed frozen thawed semen twice with HEPES-buffered TALP solution by centrifugation at 200 x g and then resuspended in 0.6 to 0.8 ml HEPES buffered TALP solution. Then the sperm suspension was layered over glass wool column and allowed to filter by gravity and achieved a cleavage rate of 51.4 per cent.

Furnus *et al.* (1997) centrifuged the semen sample after it was applied on the top of the percoll gradient for 20 minutes at 500 x g and the pellet was resuspended in 300 µl Hepes TALP solution and again centrifuged at 300 x g for 10 minutes. After removal of the supernatant, spermatozoa were resuspended in IVF medium and adjusted the concentration. These authors obtained a fertilization rate of 80 per cent with these procedures.

2.4.2.1.3. Cauda Epididymal Spermatozoa

Ball *et al.* (1983) collected epididymal spermatozoa into a sterile centrifuge tube containing 10 ml of wash medium and washed in a modified TALP medium. After centrifugation (200 x g for 10 min), the spermatozoa were resuspended in fresh medium to a concentration of approximately 1 x 10^8 sperm /ml. Two micro liter of this suspension was added to droplets containing the oocytes and yielded a concentration of 4 x 10^6 sperm /ml. These authors obtained a sperm penetration rate up to 71 per cent.

Goto *et al.* (1989) washed epididymis with six per cent glucose solution and then several small incisions were made on the epididymis. The spermatozoa were squeezed out from the cauda epididymis into a dish containing one ml of yolk-Tris-citrate extender maintained at 35° C and then cryopreserved. The frozen plastic straws of epididymal semen were thawed in water at 32 to 35° C and washed three

times (centrifugation at 700 x g for five min) with BO medium without BSA but supplemented with five millimolar caffeine. The spermatozoa was then pre incubated for 2 to 3 h in five per cent CO_2 in air at 39° C in a 110 µl drop (12.5 to 18.7 x 10^6 sperm /ml) BO medium containing BSA (5 mg /ml) and caffeine (2.5 mM) and then inseminated into fertilization drops containing oocytes and obtained 55.2 to 64.3 per cent fertilization rate.

Frozen thawed cauda epididymal spermatozoa and ejaculated spermatozoa deprived of seminal plasma were used for IVF by Katska *et al.* (1996). These workers found that, absence of seminal plasma during capacitation had appeared favorable to embryo production efficiency. The results supported the reports in several species that seminal plasma contained factors that might adversely affect male fertility. These factors are believed to inhibit sperm capacitation, the acrosome reaction and the action of acrosomal enzymes (Gordon, 2003).

Herrick *et al.* (2004) collected and maintained testes from four species of free ranging African bovids at 5 to 10°C for 2–8 h before dissection and sperm recovery. The epididymis and vas deferens were dissected out from both testicles of individual males. The cauda epididymis and proximal vas deferens of each testicle was then flushed with two to three milliliter of isothermal Hepes-buffered SOF. Epididymal contents from both testicles of an individual male were pooled and cryopreserved for further studies. Straws were briefly (10 sec) thawed in air before being plunged into a 38°C water bath for approximately 2 min. Thawed samples were layered on to a 45 per cent: 90 per cent percoll gradient and centrifuged for 25 min. The resultant pellet of live, motile sperms was resuspended in five ml of an SOF based sperm washing medium and centrifuged for 5 to 10 min. Final sperm pellets were diluted to 20–30 x 10^6 sperm /ml in either SOF for fertilization or modified SOF. Both media contained 1.7 mM $CaCl_2$ and two non essential amino acids and were further supplemented

with penicillamine (10–20 mM), hypotaurine (5–10 mM), and either heparin (5 or 10 mg/ml) or caffeine (2.0 mM) and co-incubated with matured oocytes and obtained 33 per cent fertilization rate.

2.4.2.2. Separation of Highly Motile Spermatozoa

The most motile spermatozoa were quickly and effectively removed from seminal plasma, somatic cells and dead and morphologically abnormal spermatozoa by percoll density gradients.

Parrish *et al.* (1995) found that separation of sperm on a 45 and 90 per cent discontinuous percoll gradient required 15 min centrifugation (700 x g) to obtain optimal recovery of motile sperm, which were present almost exclusively in the bottom 0.5 ml of the gradient after centrifugation. The authors recorded a higher recovery of motile sperm with percoll than with swim-up technique and recommended the percoll method of sperm separation for routine use in cattle IVF.

Seidel *et al.* (1995) compared the centrifugation through percoll or BSA with swim-up technique and centrifugation method was found to be quicker and efficient.

Hochi *et al.*, (1996) layered sperm suspension over glass wool column and allowed to filter by gravity and achieved a cleavage rate of 51.4 per cent.

Guyader-Joly *et al.* (1999) and Khurana and Nieman (2000 a) separated motile spermatozoa by swim-up procedures. After a 45 minute swim-up period, the upper portion of the capacitation medium was collected and pelleted by centrifugation at 200 x g for 10 minutes and obtained good yield of embryos on IVF of bovine oocytes.

2.4.3. Co-culture of Oocyte and Spermatozoa

2.4.3.1. Sperm Concentration in Relation to Number of Oocytes

Although many workers have preferred a sperm concentration of 1 million per ml, concentrations ranging from 0.2 million /ml (Pavlok *et al.*, 1992) to 18 million /ml (Goto *et. al.*, 1988) have been employed successfully. Many scientists used a final sperm concentration of 1 x 10^6 sperms /ml for IVF studies, with good results (Pugh *et al.*, 1998; Guyader-Joly *et al.*, 1999 and Khurana and Niemann 2000 a). Tanghe *et al.* (2000) evaluated the effect on fertilization rates of increasing the sperm-oocyte ratio from 5000:1 to 50,000:1 and found that increasing the sperm-oocyte ratio above the normal 5000: 1 value did not significantly affect fertilization rate or the incidence of polyspermy. Rizos *et al.* (2002) used a fixed concentration of 1 x 10^6 spermatozoa /ml and a sperm oocyte ratio of 5000:1.

2.4.3.2. Activation of Sperm Motility

It is very important to have highly motile bull sperm available for IVF. This may be achieved by applying various procedures for isolating motile samples like swim-up techniques and percoll density gradients. There are also several pharmacological compounds that may be employed to stimulate and maintain the motility of bull sperm.

Merton *et al.* (2000) added PHE (Penicillamine, hypotaurine and epinephrine) and heparin to TALP fertilization medium at six hours prior to the insemination and at the time of insemination. A significant decrease in the yield of embryos recorded when the addition of these agents was six hours in advance rather than at the time of insemination. Peixer *et al.* (2002) used PHE and heparin in TALP

fertilization medium for IVF using Zebu cattle semen. Addition of penicillamine (20 μM), hypotaurine (10 μM) and epinephrine (1-2 μM) to the IVF medium enhanced the sperm motility and IVF results (Gordon, 2003).

2.4.3.3. Culture Medium

Simple chemically defined media such as modified TALP (Ball *et al.*, 1983; Parrish *et al.*, 1986, 1988 and Lu *et al.*, 1987) or modified BO medium (Goto *et al.*, 1988) have been used for IVF of bovine oocytes. Studies by Saeki *et al.* (1994) showed that protein supplementation was not essential when oocytes were enclosed by cumulus cells, but was essential when oocytes were cumulus free. A study by Holm *et al.* (1999) showed that exposure of oocytes matured and fertilized in defined conditions with serum for two minute prior to IVF enhanced cleavage rate and blastocyst yield. Schneider *et al.* (1999) exposed sperms to somatic cell co-culture and recorded a higher yield of fertilized and cleaved oocytes than somatic cell free controls. These authors observed that sperm cells co-cultured with buffalo-rat liver cells fertilized cattle oocytes that cleaved at higher rate and developed to the morula stage more readily than those co-cultured with bovine oviductal cells or cell-free controls. Pavlok (2000) showed that granulosa cells were capable of extending the fertile lifespan of bovine frozen thawed sperm. An alternative to TALP medium used for IVF in some laboratories has been a modified SOF formulation (Gordon, 2003).

2.4.3.4. Temperature and Gas Phase

Optimum fertilization results were obtained at a temperature of 38-39° C (Lenz *et al.*, 1983). Incubation for IVF was carried out at 39° C in five per cent CO_2 air with saturated humidity for 24 h by many workers (Furnus *et al.*, 1997; Pugh *et*

al., 1998 and Khurana and Niemann, 2000 a). A temperature of 39° C was optimal not only for the maturation of cattle oocytes but for sperm penetration as well (Gordon, 2003)

2.4.3.5. Humidity

Most of the scientists used maximum humidity (around 95 per cent) for IVF (Furnus *et al.*, 1997; Pugh *et al.*, 1998 and Khurana and Niemann, 2000 a). Maximum humidity is given in order to prevent evaporation of medium (Gordon, 2003).

2.4.3.6. Culture Duration

Ball *et al.* (1983) co-incubated bovine oocytes and spermatozoa for 40-48 h for IVF. Leibfried-Rutledge *et al.* (1985), Xu *et al.* (1987) and Fukui *et al.* (1991) co-incubated oocyte and spermatozoa for 21-24 h. Bovine oocytes and spermatozoa have been co-incubated for varying intervals such as 6 h (Goto *et al.*, 1988), 8 h (Iwasaki *et al.*, 1990) and 18-20 h (Madison *et al.*, 1992), for the purpose of IVF.

2.4.4. Events in Fertilization Process

2.4.4.1. Capacitation Process

Mammalian spermatozoa undergo a series of physiological events in the female reproductive tract prior to acquiring the fertilization capacity. An initial phase known as capacitation brings about membrane alteration and the removal of materials from surface of spermatozoa which are epididymal or seminal plasmatic in origin. The second phase referred to as the acrosome reaction involves fusion of the

plasma membrane and the outer acrosomal membrane, resulting in the release of enzymes that help the sperm in making their way towards the oocyte, first through the matrix of cumulus cells and then the zona pellucida. The capacitation stimulates hyper activation of spermatozoa, which may further facilitate penetration of the oocyte (Bedford, 1970). Fertilization in most animal species involves a transient increase in the intracellular free Ca^{2+} concentration of the oocytes, which takes the form of a Ca^{2+} wave starting from the site of sperm attachment. The origin of this calcium is believed to be the inositol triphosphate (IP_3) induced Ca^{2+} release from the endoplasmic reticulum (Gordon, 2003).

2.4.4.2. In vitro Capacitation

The key to a successful IVF is to promote sperm capacitation and induce the acrosome reaction. Various agents used to stimulate *in vitro* capacitation include, bovine follicular fluid, oviductal fluid, high ionic strength medium, calcium ionophore, heparin, caffeine and cAMP (Brackett *et al.*, 1980, 1982; Critser *et al.*, 1984; Parrish *et al.*, 1986, 1988, 1989 a, 1989 b and Goto *et al.*, 1988).

There are differences in the capacitation ability of ejaculated and epididymal spermatozoa. While capacitation of bovine epididymal spermatozoa could be achieved in normal saline (Ball *et al.*, 1983), ejaculated bovine spermatozoa did not undergo capacitation unless stimulated by capacitating agents (Parrish *et al.*, 1986). Decapacitation factors of seminal plasma might be responsible for these differences. However, freshly ejaculated and deep frozen bull spermatozoa did not differ in their capacitation ability *in vitro* (Bondioli and Wright, 1983).

Assumpcao *et al.* (2000) tested *in vitro* capacitation of thawed semen using heparin (100 µg /ml) for 6 h and calcium ionophore (5 µM) for one minute

incubation period, in which heparin treatment resulted in greater sperm capacitation rates than the calcium ionophore treatment.

Fresh semen required a longer capacitation and heparin treatment period than frozen. However, the quality of frozen- thawed semen deteriorates more rapidly than that of fresh semen. It is important to check the motility and other characteristics after the thawing of frozen semen. Whether semen is freshly collected from the buil or deep frozen, certain steps must be taken before the use in IVF to separate the highly motile and normal sperm from the rest of the population (Gordon, 2003).

2.4.4.3. Sperm and Oocyte Interaction

Fertilization in the cow and other farm animals is mainly controlled by interaction between the sperm and components of the oocyte. The oocyte and sperm surface adhesion molecules regulate the process of fertilization in mice and other mammals (Wassarman, 1994). Sun *et al.* (1994) recorded the changes in intracellular calcium concentrations in cattle oocytes, after penetration by sperm in the normal fertilization process. The mechanism involved in sperm oocyte binding is based on the interaction of proteins and carbohydrates present on the surface of the gametes.

Fucosyl and N-acetyl-galactosaminyl (GalNAc) binding sites on the bull sperm surface were involved in the induction of the acrosome reaction and zona pellucida recognition (Brandelli *et al.*, 1995). Nakada and Mizuno (1998) reported that, calcium oscillations occurred during meiotic maturation and at fertilization they triggered oocyte activation and continued until around the time when pronuclei were formed. Knott *et al.* (2002) noted that the inositol-triphosphate receptor (IP_3R) was

implicated in mediating the release of Ca^{2+} at the time of fertilization and played an important part in the activation of cattle oocytes.

2.4.4.4. Fertilization Process

Several research groups have reported on events at and after IVF in cattle in recent years (Sun *et al.*, 1994; Kochhar and King, 1998; Laurincik *et al.*, 1998 and Fair *et al.*, 2000). The time required for sperm penetration of IVM oocytes was less than four hour; sperm head decondensation occurred within one to two hour of oocyte penetration and the male pronucleus developed after a further three to five hour (Gordon,2003).

Chian *et al.* (1999) have reported on the morphological events that the sperm have to undergo during male pronucleus formation following IVF of cattle oocytes. The authors described that, the first evidence of sperm penetration was observed four hour post insemination (hpi) and reached its peak at nine hour post insemination. Male pronuclear formation was first recorded at nine hpi and reached its maximum rate at 14 hpi. These authors further described that, the morphological changes of the penetrated sperm during transformation into male pronucleus were (i) sperm nucleus decondensation (ii) recondensation of sperm nucleus (iii) redecondensation of sperm nucleus (iv) formation of prepronucleus and (v) male pronucleus. Protein phosphorylation might be related to the formation of the male pronucleus in cattle oocytes

2.4.4.5. Abnormal Fertilization

Galli and Lazzari (1996) reported that polyspermy was a major problem since polyspermic oocytes cleaved in most cases but did not develop to the blastocyst

stage. Sumantri *et al.* (1996) reported an increased polyspermy rate on prolonged co-culture of sperm and oocyte during IVF. Gomez and Diez (2000) reported that prolonged oocyte and sperm co-culture during IVF might have adverse effects on subsequent embryonic development.

2.4.4.6. Cleavage

One of the standard evaluation procedures employed in cattle IVF was the percentage of inseminated oocytes which have cleaved when examined two days later. The first cleavage occured somewhere around 30 hpi and by 48 hpi majority of embryos had reached the four cell stage (Gordon, 1994).

2.4.5. Assessment of Fertilization

Oocytes were separated from cumulus cells and sperm by bovine testicular 0.1 per cent hyluronidase treatment for three to five minutes, followed by repeated passage through a fine pipette (Chian *et al.*, 1996). Within a short period after penetration, the spermatozoa decondensed within the ooplasm, a nuclear envelope formed and swelling of the male pronucleus occurred. Simultaneously meiosis resumed, second PB extruded, a nuclear envelope formed around the decondensed female chromatin and female pronuclei were formed. Pronucleus was not visible unless special techniques such as differential interference contrast microscopy and staining were employed (Gordon, 2003).

2.4.5.1. Aceto Orcein Staining

Chian *et al.* (1995) mounted oocytes on glass slide and fixed in acetic alcohol (acetic acid: ethanol, 1:3 v/v) for 48-72 h at room temperature, stained with one per

cent orcein in 45 per cent acetic acid and examined for evidence of sperm penetration. Sperm penetration was identified with the presence of swollen sperm head, male pronuclei, oocytes with two pronuclei and a clear second PB. Aceto orcein stain used for staining oocytes in many of the studies but the concentration might be one per cent (Sawai *et al.*, 1997 and Wongsrikeao *et al.*, 2006) or two per cent (Torner *et al.*, 2004).

2.4.5.2. Lacmoid

Galli and Lazzari (1996) used lacmoid for staining oocytes after fertilization studies. The oocytes were fixed in 3:1 ethanol: acetic acid fixative solution for 24 h; stained with lacmoid and observed under phase contrast microscope for assessment of fertilization. Isobe *et al.* (1998) used one per cent lacmoid for staining of oocytes.

2.4.5.3. Mitbramycin or 4', 6-diamidino-2-phenylindole (DAPI)

Mori *et al.* (1988) employed fluorescence microscopic investigations of nuclear DNA stained with mitbramycin or 4',6-diamidino-2-phenylindole (DAPI) in oocytes and zygotes during IVF. Mitbramycin or DAPI bind stoic biometrically with G-C and A-T pairs of DNA respectively. These authors could visualize the GV, the nucleolus and chromosome in the oocyte and the nuclei, male and female pronuclei, polar bodies, nucleolus-associated chromatins and chromosomes in the zygote.

2.4.5.4. Hoechst 33342 (bis Benzimide trihydrochloride) Staining

Oocytes were incubated in bicarbonate buffered TCM-199 with 5μg /ml Hoechst 33342 stain for 15 minutes and viewed under fluorescent microscope with excitation filters of 340-380 nm and suppression filter of 425 nm (Smith,1993).

Simon (2005) used Hoechst 33342 (bis Benzimide) stock solution of 5 µg /ml for staining the nucleus of oocyte and obtained good results. Magnus Paul (2005) employed Hoechst 33342 stock solution of five microgram per milliliter for staining the nucleus of oocyte and obtained good results.

2.4.5.5 Fluorescein Diacetate (FDA) Staining

Katska and Smorag (1985) used 3'6'-Fluorescein diacetate (FDA) stain to assess the viability of bovine oocytes. The basic staining solution contained five microgram per ml of acetone. It was diluted to a final concentration of 1.7 µg FDA per ml of modified DPBS. Oocytes were stained in staining solution for three to five min. After staining oocytes were washed in Dulbecco's medium and observed under fluorescent microscope with excitation filters of 490 nm and suppression filter of 515 nm.

Magnus Paul (2005) stained oocytes with Fluorescein diacetate to assess the viability of oocytes and obtained good results. Fluorescein diacetate revealed both membrane integrity and cytoplasmic enzyme activity of cell by emitting apple green fluorescence.

2.4.6. Fertilizability in Relation to Spermatozoan Source

2.4.6.1. Fresh Ejaculated Spermatozoa

In experiments conducted with freshly ejaculated sperm from Jersey bull, five out of twelve preovulatory ova recovered from follicles and nine out of sixteen recently ovulated tubal ova were apparently fertilized *in vitro* (Brackett *et al.*, 1980).

Fresh BTS (Beltsville Thawing Solution) extended semen from two randomly selected boars was used for IVF studies by Kidson *et al.* (2003) and obtained 29-36 per cent total penetration rate and 15-18 per cent normal fertilization rate for sow oocytes. Fresh dog spermatozoa showed the highest average penetration rate of around 70 per cent with both immature and *in vitro* matured canine oocytes, in comparison to chilled or frozen sperm, while frozen sperm manifested the lowest, around 50 per cent of average penetration rate, with both oocytes types (De Los Reyes *et al.*, 2009). These authors reported around 70 per cent oocyte penetration rate with fresh ejaculated semen in canines.

2.4.6.2. Frozen Spermatozoa

Brackett *et al.* (1980) observed no signs of fertilization in two IVF experiments, in which frozen semen was employed. The most likely reason proposed was the inadequate condition of sperm cells used. Frozen semen was used as a major source of spermatozoa for IVF studies by many scientists. Chian *et al.* (1994) obtained a very high penetration rate of approximately 84 – 87 per cent when frozen semen was used for IVF. Up to 73.6 per cent of two pronuclei (2PN) and up to 95.5 per cent sperm penetration rate was achieved by Long *et al.* (1994) with frozen semen at different dose levels, source and incubation time. An oocyte penetration rate of 87 – 93 per cent was obtained by Chian *et al.* (1995) when bovine oocytes with cumulus cells were inseminated with frozen thawed spermatozoa at a final concentration ranging from $0.8 - 5.0 \times 10^6$ spermatozoa /ml.

Cryopreserved bull spermatozoa were used in an IVF study by Hazeleger *et al.* (1995) and obtained a fertilization rate up to 87 per cent, characterized by the presence of two pronuclei. Chian *et al.* (1996) obtained oocyte penetration rate up to 90 per cent with frozen semen in an experiment to study the effect of progesterone

and estradiol on sperm penetration *in vitro* of bovine oocytes. Azambuja *et al.* (1998) obtained a fertilization rate of 73.2 per cent for oocytes maintained at 39° C and fertilized *in vitro* using frozen semen. Oocyte penetration rate and fertilization rate achieved were up to 93±7 per cent and 71±6 per cent respectively, when frozen thawed semen was used in IVF studies by Hashimoto *et al.* (1998). Oocyte penetration rate up to 94.7 ±15.6 and fertilization rate up to 89.7±15.2 per cent were obtained by Mizushima and Fukui (2001) when frozen thawed semen was used in a study. Avery *et al.* (2003) obtained a fertilization rate of 56 per cent with frozen semen during IVF studies. Chohan and Hunter (2004) obtained 79.9 per cent fertilization rate when frozen semen was used for IVF of bovine oocytes.

Arlotto *et al.* (1996) obtained a fertilization rate up to 72±7 and cleavage rate up to 69±3 per cent when frozen semen was used as sperm source in IVF studies. Cleavage rate of 76 per cent was obtained 48 h post insemination using frozen semen in a study conducted by Keskintepe and Brackett (1996). Ali and Sirard (2002) used frozen semen in IVF studies and achieved a cleavage rate up to 76±5.7 per cent after 72 h post insemination. Percentage of cleaved zygote ranged from 70.5±6.88 to 81.1±2.08 when frozen semen was used as sperm source for IVF by Reis *et al.* (2002)

2.4.6.3. Epididymal Spermatozoa

Ball *et al.* (1983) reported to have obtained in an IVF study a penetration rate up to 71 per cent and fertilization rate up to 50 per cent by epididymal spermatozoa. Fertilization rate based on one pair of pronuclei and presence of one sperm tail ranged from 55.2 to 64.3 per cent in a study conducted by Goto *et al.* (1989). Herrick *et al.* (2004) reported fertilization rate of 33 per cent for *in vitro* matured springbok oocytes with cryopreserved epididymal spermatozoa.

Nagai *et al.* (1988) reported that, the percentage of penetrated porcine oocytes *in vitro* with frozen epididymal spermatozoa (0-40 per cent) was significantly higher when compared to the fertilization rate with frozen ejaculated spermatozoa (zero per cent).

Katska *et al.* (1996) studied the *in vitro* fertilizability of bull spermatozoa and reported that the *in vitro* embryo production efficiency with frozen thawed epididymal spermatozoa were 83.7 ± 10.3, 80.2 ± 11.8 and 30.6 ± 5.0 per cent respectively for fertilization, cleavage rate and blastocysts yield.

Pawshe *et al.* (1996) recorded a cleavage rate of 79.33 per cent for IVF studies in goats using epididymal spermatozoa. Choi *et al.* (1998) reported the highest fertilization rate of 78 per cent in IVF using epididymal spermatozoa in inbred BALB/c mice. Williams *et al.* (2001) reported a cleavage rate of 57 per cent with canine ova, using epididymal spermatozoa in IVF. Hishinuma and Sekine (2004) reported penetration rate of 39.3 per cent immature oocytes by epididymal spermatozoa in a sperm-oocyte penetration assay conducted in canines. Fan *et al.* (2008) obtained a fertilization percentage up to 41.4 per cent for epididymal spermatozoa in mouse.

2.4.7. Physiological and Morphological Characteristics of Cauda Epididymal Spermatozoa

Rapid forward progression was seen only in a few spermatozoa in the middle of the corpus whereas most of the spermatozoa in the cauda of the epididymis and in the vas deferens exhibited good motility. During transit through the epididymis no obvious morphological changes were observed in the tail of the normal spermatozoa, but a cytoplasmic droplet first observed at the neck of the spermatozoa slowly

migrated caudally down to the mid-piece. The cytoplasmic droplet was usually lost by the time of ejaculation and experiments have shown that migration of droplet was not essential for acquisition of motility. Changes in the sperm head during epididymal transit in many species included alterations in the shape and size of the acrosome (Harper, 1982).

Amann and Almquist (1962), Igboeli and Foote (1968), Deutscher *et al.* (1974) and Goovaerts *et al.* (2006) reported 32.1 per cent, 41 per cent, 66 per cent and 46.1 per cent motility respectively for epididymal sperm.

Deutscher *et al.* (1974) and Goovaerts *et al.* (2006) reported a sperm concentration of $3810 \times 10^6 \pm 460 \times 10^6$ per ml and $38487 \times 10^6 \pm 3888 \times 10^6$ per ml respectively in epididymal content.

Deutscher *et al.* (1974) and Goovaerts *et al.* (2006) reported 87 per cent and 84 per cent live spermatozoa respectively for epididymal sperm sample.

Goovaerts *et al.* (2006) reported 37 per cent, 3.4 per cent, 2.7 per cent, 13.7 per cent and 43.3 per cent normal spermatozoa, spermatozoa with abnormal heads, abnormal tails, proximal protoplasmic droplet and distal protoplasmic droplet respectively in sperm content from epididymis.

Materials and Methods

3. MATERIALS AND METHODS

3.1. SOURCE OF OVARY

Ovaries required for the study were collected directly from Corporation slaughter house, Kuriachira, Thrissur. South Indian breeds like Hallikar, Kangayam, Khillari and crossbred cattle of Kerala were mainly slaughtered at Corporation slaughter house.

3.2. COLLECTION AND TRANSPORTATION OF OVARY

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

3.3. PRE RETRIEVAL PROCESSING OF OVARIES

After trimming of the extraneous tissue, the ovaries were washed repeatedly in DPBS fortified with 100 IU /ml Benzyl penicillin and 100 µg /ml Streptomycin sulphate maintained at 36-38° C. The final washing was done with DPBS supplemented with 0.5 per cent BSA and maintained at 36-38° C.

3.4. RETRIEVAL OF OOCYTES

Oocytes were retrieved from ovaries by applying aspiration method. Surface follicles measuring 2-8 mm in size were aspirated with a sterile 18 gauge disposable needle connected to a 10 ml disposable syringe (Plate 1). The retrieval process was

carried out in COC handling media prepared with DPBS enriched with five percent day zero estrus cow serum and 0.5 per cent BSA and maintained at 39° C. A total of 81 ovaries were used for oocyte retrieval in the study.

3.5. POST RETRIEVAL PROCESSING OF OOCYTES

The medium containing retrieved oocytes were transferred into sterile 90 mm disposable Petridish having grid and examined under the zoom stereomicroscope at 10 x magnification for identification of oocytes (Plate 2). Identified oocytes were collected by means of unopette and transferred into 35 mm Petridish containing fresh COC handling media maintained at 39° C (Plate 3). Searching of oocytes was carried as fast as possible.

3.5.1. Assessment of Total Yield of Oocytes per Ovary

Oocytes were retrieved from a total of 81 ovaries by aspiration method and total and average yield of total oocytes per ovary were assessed.

3.6. MORPHOLOGICAL EVALUATION OF OOCYTES

The oocytes so collected were examined separately for quality under 40 x magnification of zoom stereomicroscope and based on the number of layers of cumulus cells and ooplasm character (Abdoon and Kandil, 2001 and Gupta *et al.*, 2006) the selected oocytes were graded into two categories as follows.

Grade I: More than five complete layers of cumulus cells and uniform granulation of ooplasm (Plate 4)

Grade II: Three to five complete layers of cumulus cells and uniform granulation of ooplasm (Plate 5)

Grade I and Grade II oocytes were placed separately in labeled maturation media and further processing for IVM and IVF study were done separately. However the pooled values of grade I and grade II oocytes were also analyzed together as a new class namely culture grade oocytes (Plate 6) for the purpose of interpretation of results. All other oocytes that were found to be inferior to grade II quality were discarded from further processing (Plate 7 and Plate 8)

3.6.1. Assessment of Yield of Culture Grade Oocytes

Oocytes obtained from 81 ovaries were examined separately under 40 x magnification of zoom stereomicroscope and the culture grade oocytes separated (Grade I and Grade II). The average and percentage yield of culture grade oocytes per ovary were assessed.

3.7. *IN VITRO* MATURATION OF OOCYTES

3.7.1. Media

Medium used for maturation of oocytes was freshly prepared Hepes modified TCM-199 (25 mM Hepes) enriched with 22 µg /ml sodium pyruvate, 2.2 mg /ml sodium bicarbonate and penicillin G sodium and streptomycin sulphate at the rate of 0.5 mg /ml each. This medium was further supplemented with hormones, 1µg /ml estradiol-17β, 0.5 µg /ml FSH, 0.06 IU hCG and 20% heat-inactivated estrus cow serum prior to employing it for IVM. Selected COCs were washed twice in maturation medium without hormones and transferred in groups of 5-10 into 100 µl drops of pre incubated (2 h) maturation medium under sterile mineral oil and allowed to complete incubation for a period of 24 h without any disturbance in the culture conditions (Khurana and Niemann, 2000 a).

3.7.2. Culture Conditions

Culture conditions set for this study were 39° C temperature, five per cent CO_2 tension and maximum humidity (Khurana and Niemann, 2000 a). Standard water jacketed CO_2 incubator was used to get this culture environment (Plate 9).

3.7.3. Assessment of Maturation Character

After 24 h culture in maturation medium, all the oocytes in the culture drops were evaluated under zoom stereomicroscope for maturation changes such as expansion and mucification of cumulus cells (Plate 10) and its correlation with oocyte quality. Oocytes with maximum degree of cumulus expansion only were selected for further IVF studies (Plate11). Oocytes with moderate to mild expansion of cumulus cells were considered as incompletely matured (Plate 12 and 13).

3.8. *IN VITRO* FERTILIZATION

3.8.1. Fertilization Media

The IVF medium consisted of Fert-TALP medium supplemented with 1 µM epinephrine, 10 µM hypotaurine, 20 µM pencillamine and 0.56 µg /ml heparin (Khurana and Niemann, 2000 a).

3.8.2. Pre Fertilization Processing of Oocyte

At the end of IVM, the selected COCs were rinsed twice in Fert-TALP medium (Table 1) and transferred in groups of 5-10 depending upon the availability of oocytes in 100 µl droplets of fertilization medium under mineral oil.

3.8.3. Pre Fertilization Processing of Spermatozoa

3.8.3.1. Extraction of Fresh Spermatozoa

Fresh ejaculated semen was obtained from healthy bulls maintained at the AI centre attached to the College of Veterinary and Animal Sciences, Mannuthy. Semen was maintained in a water bath at 35° C. Fifty microlitre of fresh semen was layered over percoll density gradients and the motile spermatozoa were separated as per Parrish *et al.* (1995).

3.8.3.2. Extraction of Frozen Spermatozoa

Frozen semen supplied by Kerala Livestock Development Board was used for the study. The semen in 0.25 ml straw was thawed at 37° C in water bath for 30 seconds. Then the semen sample was applied on the top of the percoll gradient and motile spermatozoa were separated as per Parrish *et al.* (1995).

3.8.3.3. Extraction of Epididymal Spermatozoa

Testes collected from six bulls that were recently slaughtered at the Department of Livestock Product Technology, College of Veterinary and Animal Sciences, Mannuthy formed the source of epididymal spermatozoa. Crossbred bulls after completion of their target production, brought from Kerala Livestock Development Board bull station for slaughter were selected for testis collection. The testis was transported to the laboratory in sperm-TALP medium maintained at 35° C. Epididymis was separated gently by blunt dissection after thorough washing of the testis. Sperms from cauda epididymis were collected by gentle slicing and squeezing of each cauda epididymis in five millilitre of sperm-TALP medium taken in a sterile

Petri dish as per the protocol of Goovaerts *et al.* (2006). Semen collected from both the epididymis was pooled together for further processing. Motile spermatozoa were separated by percoll density gradient separation as per Parrish *et al.* (1995).

3.8.3.3.1. Evaluation of Epididymal Sperm Character

The motility of the pooled sample was assessed subjectively (nearest five per cent) by phase contrast microscopy (200 x magnification). Concentration of sperm in the pooled sample was assessed with haemocytometer. Eosin/ nigrosin stained smears were used for the evaluation of the percentages of live and dead spermatozoa and for assessment of sperm morphology. At least 300 spermatozoa were counted in six samples, and classified as either: normal spermatozoa, spermatozoa with abnormal heads or tails, or spermatozoa with a proximal or distal protoplasmic droplet.

3.8.3.4. Standardization of Spermatozoan Concentration

Concentration of the sperms in IVF medium was determined with haemocytometer and adjusted to 20 x 10^6 /ml using IVF medium. The final concentration in fertilization droplet was adjusted to 20,000 sperms per oocytes (1 µl per oocyte).

3.8.4. *In Vitro* Co-Culture

Matured oocytes from grade I and grade II category of COCs after washing were loaded gently into separate pre incubated (2 h) IVF drops using unopette. Required volume of processed semen was inseminated gently into the fertilization droplet using micropipette and allowed to complete incubation for a period of 24 h

without any disturbance in the culture conditions. Culture conditions set for IVF were 39° C temperature, five per cent CO_2 tension and maximum humidity. Standard water jacketed CO_2 incubator was used to get this culture environment.

3.8.5. Assessment of Fertilization Character

Oocytes after 24 hour culture in fertilization medium were denuded by repeated pipetting (Plate 14) and examined under bright field of inverted phase contrast microscope at 200 x and 400 x magnification. The oocytes were then stained with aceto orcein after whole mount fixation of oocytes as per Chian *et al.* (1995) and examined for evidence of sperm penetration. Oocytes showing sperm penetration evidence like presence of swollen sperm head (Plate 15), male pronuclei, sperm tail in the cytoplasm, oocytes with two pronuclei (Plate 16), a clear second polar body and cleavage (Plate 17 and Plate 18) were considered as fertilized. Oocytes with more than two pronuclei were evaluated as polyspermic (Plate 19).

3.8.5.1. Assessment of In Vitro Fertilizability of Spermatozoa from Different Sources

Culture grade oocytes matured *in vitro* were subjected at random to IVF using fresh, frozen and epididymal spermatozoa in IVF medium as per the protocol of Khurana and Niemann (2000 a) and the IVF rates was assessed in relation to the three specified groups separately. The effect of quality of oocyte before IVM and effect of source of spermatozoa on IVF rate were assessed.

3.8.5.2 Assessment of Total Yield of In Vitro Fertilized Oocytes

The mean yield of *in vitro* fertilized oocytes per ovary was assessed by using the pooled value of IVF rate of fresh, frozen and epididymal semen.

3.9. RESEARCH MATERIALS USED

Specification details of equipment, media, hormones and chemicals used in this study are shown in Table 2 and Table 3.

3.10. DESIGN OF EXPERIMENT

The design of experiment in brief is shown in Fig. 1.

3.11. STATISTICAL ANALYSIS

Data were subjected to statistical analysis as per Snedecor and Cochran (1996). Data on maturation rate and fertilization rate were analyzed with students T-test/one way ANOVA. p-value of less than 0.05 was considered statistically significant.

Table 1. Composition of sperm-TALP and fert-TALP media

Ingredient	Sperm-TALP		Fert-TALP	
	mM	g /100 ml	mM	g /100 ml
NaCl	100 mM	0.5840 g	114 mM	0.6662 g
KCl	3.1 mM	0.0284 g	3.2 mM	0.0239 g
$NaHCO_3$	25 mM	0.2100 g	25 mM	0.2100 g
$NaH_2PO_4.H_2O$	0.3 mM	0.0041 g	0.3 mM	0.0041 g
$CaCl_2.2H_2O$	2 mM	0.0294 g	2 mM	0.0294 g
$MgCl_2$	0.4 mM	0.0038 g	0.5 mM	0.0048 g
HEPES	10 mM	0.2603 g	-	-
Sodium Lactate	21.6 mM	294 µl	10 mM	0.1121 g
Gentamicin	50 µg /ml	0.0050 g	50 µg /ml	0.0050 g
Phenol Red	0.01 µg /ml	1 µg	0.01 µg /ml	1 µg
Store at 4° C				
Sodium Pyruvate	1 mM	0.0110 g	0.2 mM	0.0022 g
BSA	6 mg /ml	0.6 g	6 mg /ml	0.6 g

Media prepared based on Parrish *et al.* (1988)

Table 2. Details of equipment

Sl.No	Name of Item	Patent Name	Manufacturer
1.	Zoom stereomicroscope	Leica MZ6	Leica micro systems, Germany
2.	Phase contrast microscope	Leica DMIL	Leica micro systems, Germany
3.	CO_2 Incubator	Labline	Labline Instruments Inc, USA
4.	Streamline Vertical Laminar Flow Cabinet	Esco	ESCO, India
5.	Millipore ultra water filtration unit	Milli Q UF Plus	Millipore Corporation, USA
6.	Digital pH Meter	Cyber Scan 2500	Cyber Scan Eutech Instrument, Singapore
7.	Electronic Analytical Precision Balance	CP-2245	Sartorius, Germany

Table 3. Details of media, hormones and chemicals

Sl.No	Name of Item	Patent Name	Manufacturer
1.	TCM-199	Medium 199	Sigma Chemicals, St. Louis, USA
2.	pFSH (FSH from Porcine Pituitary)	Folltropin-V	Vetrepharm Canada Inc.
3	Dulbecco's Phosphate Buffered Saline	Dulbecco's Phosphate Buffered Saline	Sigma Chemicals, St. Louis, USA
4.	17-β-Estradiol	β-Estradiol	Sigma Chemicals, St. Louis, USA
5.	Sodium Pyruvate	Sodium Pyruvate	Sigma Chemicals, St. Louis, USA
6.	Gentamicin Sulphate	Gentamicin	Hi media Laboratories Ltd. Mumbai
7.	Streptomycin Sulphate	Ambistryn	Alembic Ltd, Vadodara
8.	Benzyl Penicillin Sodium	Benzyl penicillin injection	Alembic Ltd, Vadodara
9.	Sodium Chloride	Sodium Chloride	Sigma Chemicals, St. Louis, USA
10.	Sodium Bicarbonate	Sodium bicarbonate	Sigma Chemicals, St. Louis, USA
11.	Penicillamine	DL-Penicillamine	Sigma Chemicals, St. Louis, USA
12.	Mineral Oil	Mineral Oil	Sigma Chemicals, St. Louis, USA
13.	Orcein	Natural Red	Himedia Laboratories Ltd. Mumbai
14.	Ethanol	Ethanol	Changshu Yangyuan chemicals, China
15.	Acetic Acid	Acetic Acid	BDH Laboratories, England
16.	Potassium Chloride	Potassium Chloride	Sigma Chemicals, St. Louis, USA
17.	Sodium Phosphate Monophosphate	Sodium Phosphate Monophosphate	Sigma Chemicals, St. Louis, USA
18.	Phenol Red	Phenol Red	Sigma Chemicals, St. Louis, USA
19.	Sodium Lactate	Sodium Lactate (60%)	Sigma Chemicals, St. Louis, USA
20.	Magnesium Chloride	Magnesium Chloride	Sigma Chemicals, St. Louis, USA

Table 3 continued

Sl.No	Name of Item	Patent Name	Manufacturer
21.	$CaCl_2$-$2H_2O$	Calcium Chloride	Sigma Chemicals, St. Louis, USA
22.	HEPES	Hepes	Sigma Aldrich, USA
23.	BSA fraction V	Albumin, Bovine Fraction V	Sigma Chemicals, St. Louis, USA
24.	Heparin Sodium Salt	Heparin	Sigma Chemicals, St. Louis, USA
25.	Epinephrine	Epinephrine	Sigma Chemicals, St. Louis, USA
26.	Hypotaurine	Hypotaurine	Sigma Chemicals, St. Louis, USA
27.	Percoll	Percoll	Sigma Chemicals, St. Louis, USA
28.	hCG	Chorulon	Intervet (India) pvt Ltd, Hyderabad

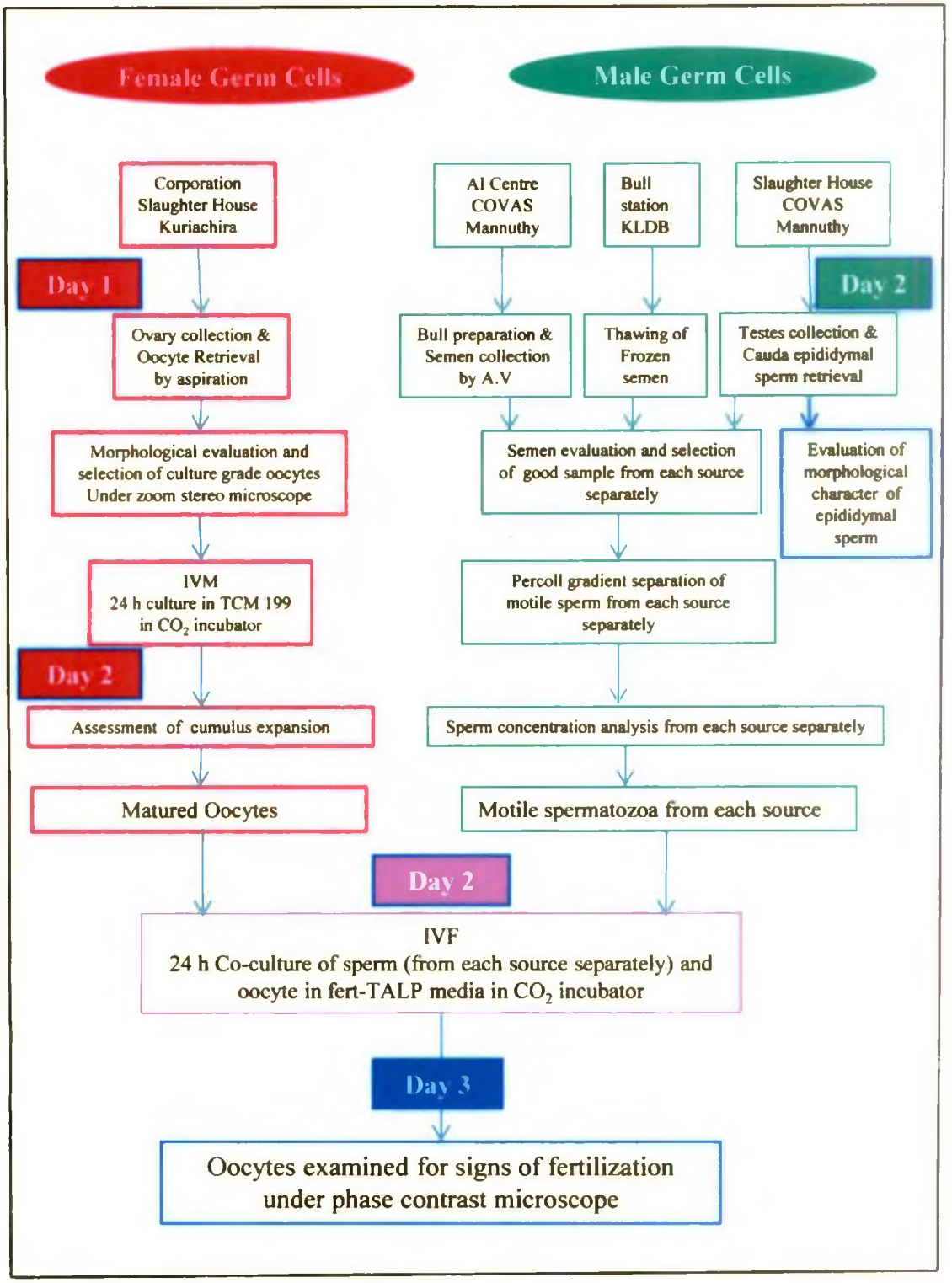


Fig.1. Design of experiment

Results

4. RESULTS

This study on IVF of follicular oocytes in cattle was carried out using oocytes collected by aspiration method from ovaries of recently slaughtered animals. Spermatozoa from three different sources viz., fresh, frozen and cauda epididymis were used for IVF.

4.1. YIELD OF TOTAL OOCYTES PER OVARY

A total of 81 ovaries were subjected to the study which on oocyte harvest by aspiration method yielded a total number of 939 oocytes. The mean number of total oocytes retrieved per ovary was found to be 11.59±0.10 (Table 4).

4.2. YIELD OF CULTURE GRADE OOCYTES

4.2.1. Yield of Culture Grade Oocytes per Ovary

All the 939 oocytes were subjected to quality grading. A total of 462 grade I, 138 grade II and 339 below grade II oocytes were obtained. Grade I and Grade II oocytes were considered together as culture grade oocytes (600 oocytes) and subjected to IVM. The remaining 339 oocytes were below grade II and hence discarded (Plate 4, Plate 5, Plate 6, Plate 7 and Plate 8).

Mean yield of grade I, grade II, culture grade and below grade II oocyte per ovary was found to be 5.70±0.06, 1.70±0.05, 7.41±0.07 and 4.19±0.04 respectively (Table 4& Fig.2). Yield of grade I oocyte was found to be significantly higher than that of grade II oocytes ($P<0.05$)

4.2.2. Percentage Yield of Culture Grade Oocytes

Out of 939 oocytes collected, the percentage yield of grade I, grade II, total culture grade and below grade II oocytes were 49.20±0.31, 14.70±0.41, 63.90±0.22 and 36.10±0.22 respectively (Table 4 & Fig. 3). The percentage yield of grade I oocytes was significantly higher than that of grade II oocytes ($P<0.05$).

4.3. *IN VITRO* MATURATION RATES OF OOCYTES BASED ON CUMULUS EXPANSION

After 24 hours of *in vitro* culture in standard culture conditions and media, the oocytes were examined for signs of maturation. Oocytes showing higher level of cumulus expansion, mucification and cumulus monolayer formation were evaluated as mature oocytes (Plate 10, Plate 11, Plate 12 and Plate 13).

4.3.1. Percentage and Mean Yield of *In vitro* Matured Oocytes

Among the 600 culture grade oocytes kept for maturation, 502 (83.67±0.35 per cent) oocytes exhibited good cumulus expansion. The mean yield of oocytes showing cumulus expansion per ovary was 6.22±0.06 (Table 5).

4.3.2. Effect of Quality of Oocytes on *In vitro* Maturation

4.3.2.1. Effect of Morphological Grade of Oocytes on In vitro Maturation Rate

Among the 462 grade I and 138 grade II oocytes, 407 (88.20±0.75 per cent) and 95 (69.21±1.97 per cent) oocytes respectively exhibited good cumulus expansion

(Table 5 & Fig. 4). On analysis it was found that grade I oocytes was having significantly higher cumulus expansion rate than grade II oocytes (P<0.01).

4.3.2.2. Effect of Morphological Grade on Mean Yield of In vitro Matured oocytes

Mean number of matured oocyte obtained per ovary was 5.04±0.06 and 1.18±0.03 from grade I and grade II oocytes respectively (Table 5 & Fig.5). The mean number of matured oocytes obtained per ovary from grade I oocytes was found to be significantly higher than that of grade II oocytes (P<0.01).

4.4. *IN VITRO* FERTILIZABILITY OF *IN VITRO* MATURED OOCYTES

A total of 502 matured oocytes were subjected to IVF studies under standard procedures and culture conditions using fresh, frozen and epididymal spermatozoa. Oocytes showing fertilization signs like presence of decondensed or swollen sperm head (Plate 15), male pronuclei, oocytes with two pronuclei (Plate 16), a clear second polar body and cleavage (Plate 17 and Plate 18) were considered as fertilized.

4.4.1. *In vitro* Fertilizability of Fresh Spermatozoa

The fertilization rate obtained with fresh semen was 36.52±1.68, 45.00±5.63 and 37.86±0.47 per cent for grade I, grade II and total culturable grade oocytes respectively (Table 6).

4.4.2. *In vitro* Fertilizability of Frozen Spermatozoa

The fertilization rate obtained with frozen semen was 28.65±0.76, 23.89±3.03 and 27.72±0.89 per cent for grade I, grade II and total culture grade oocytes respectively (Table 6).

4.4.3. *In vitro* Fertilizability of Cauda Epididymal Spermatozoa

The fertilization rate obtained with cauda epididymal spermatozoa was 46.53±1.32, 35.20±4.62 and 44.51±0.57 per cent for grade I, gradeII and total culture grade oocytes respectively (Table 6).

4.4.4. Total Yield of Fertilized Oocyte per ovary and Overall Fertilization Rate

The overall yield of *in vitro* fertilized oocyte per ovary was 2.28±0.10 and the overall fertilization rate was 36.70±1.71 per cent (Table 6 and 7).

4.4.5. Correlation between the Source of Spermatozoa and Oocyte Quality on *In Vitro* Fertilization

4.4.5.1. Effect of Source of Spermatozoa on Fertilization Rate

The fertilization percentage obtained with grade I oocytes was 36.52±1.68 (50/138), 28.65±0.76 (37/129) and 46.53±1.32 (65/140) per cent when fresh, frozen and cauda epididymal spermatozoa was used for IVF (Table 6 & Fig.6). *In vitro* fertilization using cauda epididymal spermatozoa yielded significantly higher fertilization rate and IVF using frozen semen yielded lowest among the group when grade I oocytes alone considered for IVF ($P<0.01$).

Grade II oocytes yielded 45.00±5.63 per cent (14/31), 23.89±3.03 per cent (8/33) and 35.20±4.62 per cent (11/31) when fresh, frozen and cauda epididymal spermatozoa was used for IVF (Table 6 & Fig.6). There was significantly higher results observed with fresh semen than with frozen semen in the yield of fertilized oocytes ($P<0.01$). But no significant difference was observed between epididymal

source of spermatozoa and fresh semen when grade II oocytes were considered (P>0.05).

Fertilization percentage obtained with total culture grade oocytes were 37.86±0.47 (64/169), 27.72±0.89 (45/162) and 44.51±0.57 (76/171) with fresh, frozen and cauda epididymal spermatozoa respectively (Table 6 & Fig.6). Significant difference was observed between these three treatment groups (P<0.01). *In vitro* fertilization rate using epididymal spermatozoa was significantly higher followed by fresh ejaculated semen and then frozen semen (P<0.01).

4.4.5.2. Effect of Source of Spermatozoa on the Mean Yield of Fertilized Oocytes per Ovary

The mean number of oocytes fertilized per ovary from total culture grade oocytes were 2.38±0.05 (64/169), 1.73±0.07 (45/162) and 2.72±0.03 (76/171) respectively for fresh ejaculated, frozen thawed and epididymal spermatozoa (Table 7 & Fig.7). There was significant difference between these three groups (P<0.01). Mean yield of fertilized oocytes per ovary was significantly higher for epididymal spermatozoa than for fresh ejaculated semen. Similarly fresh ejaculated semen yielded significantly higher number of fertilized oocytes per ovary than frozen semen.

The mean number of fertilized oocytes per ovary obtained from grade I oocyte were 1.87±0.06, 1.43±0.04 and 2.33± 0.05 respectively when fresh, frozen and epididymal spermatozoa were used for IVF (Table 7 & Fig.7). From grade II oocytes the yield of fertilized oocytes per ovary obtained were 0.51±0.07, 0.31±0.05 and 0.39±0.06 when fresh, frozen and epididymal spermatozoa were used for IVF (Table 7 & Fig.7). A significant difference was observed in the yield of fertilized

oocytes between the source of spermatozoa with grade I oocytes (P<0.01). Significantly higher results were obtained with epididymal source than with fresh semen, followed by frozen semen. There was significant difference noticed between fresh and frozen semen with grade II oocytes (p<0.01). Significant difference was observed between epididymal and frozen semen also with grade II oocytes (p<0.01). But no significant difference was observed between fresh and epididymal spermatozoa with grade II oocytes (P>0.05).

4.4.5.3. Effect of Quality of Oocytes on Fertilization Rate

Fertilization rate of 36.52±1.68 and 45.00±5.63 per cent; 28.65±0.76 and 23.89±3.03 per cent and 46.53±1.32 and 35.20±4.62 per cent were obtained for grade I and grade II oocytes when fresh, frozen and epididymal spermatozoa were used for IVF (Table 8 & Fig.8). Over all fertilization rate obtained with fresh, frozen and epididymal spermatozoa for grade I and grade II oocytes were 37.24± 1.91 and 34.70±3.24 per cent respectively (Table 8 & Fig.8). There was no significant difference noticed between grade I and grade II oocytes on fertilization rate in each category (P>0.05).

4.4.5.4. Effect of Quality of Oocytes on the Mean Yield of Fertilized Oocyte per Ovary

The mean number of fertilized oocytes per ovary obtained from grade I and grade II oocyte was 1.87±0.06 and 0.51±0.07; 1.43±0.04 and 0.31±0.05 and 2.33± 0.05 and 0.39±0.06 when fresh, frozen and epididymal spermatozoa was used for IVF (Table 8 & Fig.9). The overall mean number of fertilized oocytes per ovary obtained from grade I and grade II oocytes was 1.87±0.09 and 0.40±0.04

respectively. A significant difference was observed between grade I and grade II oocytes in each category ($P<0.01$).

4.5. PHYSIOLOGICAL AND MORPHOLOGICAL CHARACTERISTICS OF CAUDA EPIDIDYMAL SPERMATOZOA

Testes from six recently slaughtered bulls collected from slaughter house were used for the study. Epididymal sperm content retrieved from each testis by slicing and squeezing into five ml of sperm-TALP medium was pooled together for the assessment of motility, concentration and morphological evaluation.

4.5.1. Motility of Epididymal Spermatozoa

Epididymal spermatozoa were examined under phase contrast microscope at 200 x magnification and assessed the motility subjectively (nearest five per cent). The mean motility obtained for epididymal sperm content was 49.17±9.26 per cent (Table 9).

4.5.2. Concentration of Sperms in Epididymal Content

Concentration of sperm in the pooled sample was assessed with haemocytometer. The mean concentration obtained was $37,175x10^6 \pm 7612x10^6$ per ml (Table 9).

4.5.3. Morphological Evaluation of Epididymal Spermatozoa

The morphology of epididymal spermatozoa was assessed by examining slides prepared by Eosin-nigrosin staining technique. The mean percentage of live

and dead sperms were 84.5±8.02 per cent and 15.5±8.02 per cent respectively (Table 9). The mean percentage of normal spermatozoa, spermatozoa with abnormal heads, abnormal tails, spermatozoa with a proximal protoplasmic droplet and distal protoplasmic droplet were 35.67±2.30 per cent, 3.17±1.58 per cent, 2.33±0.61 per cent, 11.67±4.01 per cent and 47.17±3.17 per cent respectively (Table 9).

Table 4. Morphological grades of retrieved follicular oocytes

Parameter	Morphological grade oocytes				Total
	Culture grade oocytes			Grade <II	
	Grade I	Grade II	Total		
Total Yield of oocytes	462	138	600	339	939
Percentage yield (Mean±S.E)	49.20±0.31*	14.70±0.41*	63.90±0.22	36.10±0.22	100.00
Oocyte yield per ovary (Mean±S.E)	5.70±0.06*	1.70±0.05*	7.41±0.07	4.19±0.04	11.59±0.10

* denotes significance between grade I and grade II at 5% level

Table 5. Effect of quality of cumulus oocyte complexes on *in vitro* maturation

Variable	Grade I	Grade II	Culture Grade
No. of oocytes kept for maturation	462	138	600
No. of oocytes showed cumulus expansion	407	95	502
Expanded COCs (%) (Mean±S.E)	88.20±0.75**	69.21±1.97**	83.67±0.35
Matured oocyte yield per ovary (Mean±S.E)	5.04±0.06**	1.18±0.03**	6.22±0.06

** denotes significance between grade I and grade II at 1% level

Table 6. Effect of source of spermatozoa on the rate of *in vitro* fertilization

Sl. No	Source of Spermatozoa	Morphological grade of oocytes								
		Grade I			Grade II			Total Culture grade oocytes		
		No. of oocytes subjected to IVF	Oocytes showed signs of fertilization		No. of oocytes subjected to IVF	Oocytes showed signs of fertilization		No. of oocytes subjected to IVF	Oocytes showed signs of fertilization	
			No.	(% Mean±S.E)		No.	(%Mean±S.E)		No.	(%Mean±S.E)
1	Fresh	138	50	36.52±1.68[a]	31	14	45.00±5.63[a]	169	64	37.86±0.47[a]
2	Frozen	129	37	28.65±0.76[b]	33	8	23.89±3.03[b]	162	45	27.72±0.89[b]
3	Cauda Epididymal	140	65	46.53±1.32[c]	31	11	35.20±4.62[a]	171	76	44.51±0.57[c]
4	Total	407	152	37.24±1.91	95	33	34.70±3.24	502	185	36.70±1.71

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

Table 7. Effect of source of spermatozoa on the yield of fertilized oocytes per ovary

Sl. No	Source of Spermatozoa	No. of ovaries used	Morphological grade of oocytes								
			Grade I			Grade II			Total Culture grade oocytes		
			Oocytes showed signs of fertilization			Oocytes showed signs of fertilization			Oocytes showed signs of fertilization		
			No. of oocytes used	No. of oocytes fertilized	Mean number of fertilized oocyte per ovary (Mean±S.E)	No. of oocytes used	No. of oocytes fertilized	Mean number of fertilized oocyte per ovary (Mean±S.E)	No. of oocytes used	No. of oocytes fertilized	Mean number of fertilized oocyte per ovary (Mean±S.E)
1	Fresh	27	138	50	1.87±0.06[a]	31	14	0.51±0.07[a]	169	64	2.38±0.05[a]
2	Frozen	26	129	37	1.43±0.04[b]	33	8	0.31±0.05[b]	162	45	1.73±0.07[b]
3	Cauda Epididymal	28	140	65	2.33±0.05[c]	31	11	0.39±0.06[a]	171	76	2.72±0.03[c]
4	Total	81	407	152	1.87±0.09	95	33	0.40±0.04	502	185	2.28±0.10

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

Table 8. Effect of quality of oocytes on *in vitro* fertilization

Variable	Source of spermatozoa	Grade I (Mean±S.E)	Grade II (Mean±S.E)
Percentage of oocytes which showed fertilization signs	Fresh	36.52±1.68^{NS}	45.00±5.63^{NS}
	Frozen	28.65±0.76^{NS}	23.89±3.03^{NS}
	Cauda epididymal	46.53±1.32^{NS}	35.20±4.62^{NS}
	Overall	37.24±1.91^{NS}	34.70±3.24^{NS}
Mean number of oocytes per ovary which showed fertilization signs	Fresh	1.87±0.06**	0.51±0.07**
	Frozen	1.43± 0.04**	0.31±0.05**
	Cauda epididymal	2.33± 0.05**	0.39±0.06**
	Overall	1.87±0.09**	0.40±0.04**

NS denotes non significant; ** denotes significant at 1% level

Table 9. Physiological and morphological characteristics of epididymal spermatozoa

Parameter	Source of cauda epididymal semen						
	Bull 1	Bull 2	Bull 3	Bull 4	Bull 5	Bull 6	Overall (Mean±S.E)
Motility (%)	55	10	50	65	40	75	49.17±9.26
Concentration (per ml)	40200×10^6	4200×10^6	39450×10^6	42300×10^6	35100×10^6	61800×10^6	$37,175 \times 10^6 \pm 7612 \times 10^6$
Live Sperms (%)	91	45	92	96	87	96	84.5±8.02
Dead Sperms (%)	9	55	8	4	13	4	15.5±8.02
Normal Spermatozoa (%)	36	24	36	37	34	47	35.67±2.30
Spermatozoa with Abnormal Heads (%)	1	7	2	0	9	0	3.17±1.58
Spermatozoa with Abnormal Tails (%)	3	3	3	1	4	0	2.33±0.61
Spermatozoa with a Proximal Protoplasmic Droplet (%)	6	31	11	6	11	5	11.67±4.01
Spermatozoa with a Distal Protoplasmic Droplet (%)	54	35	48	56	42	48	47.17±3.17

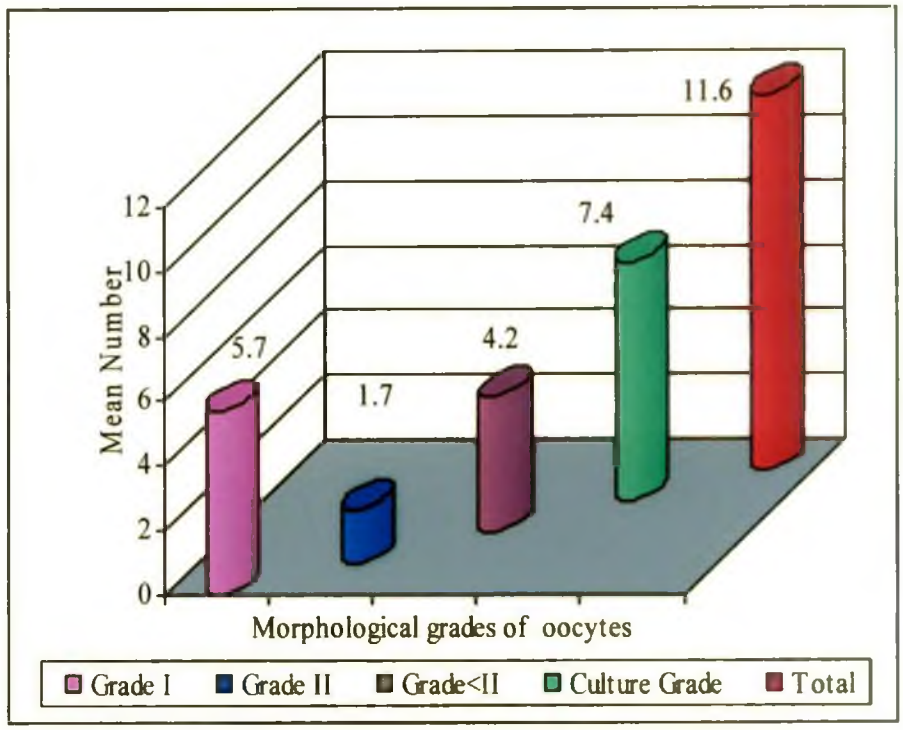


Fig. 2. Mean yield of various morphological grade oocyte per ovary

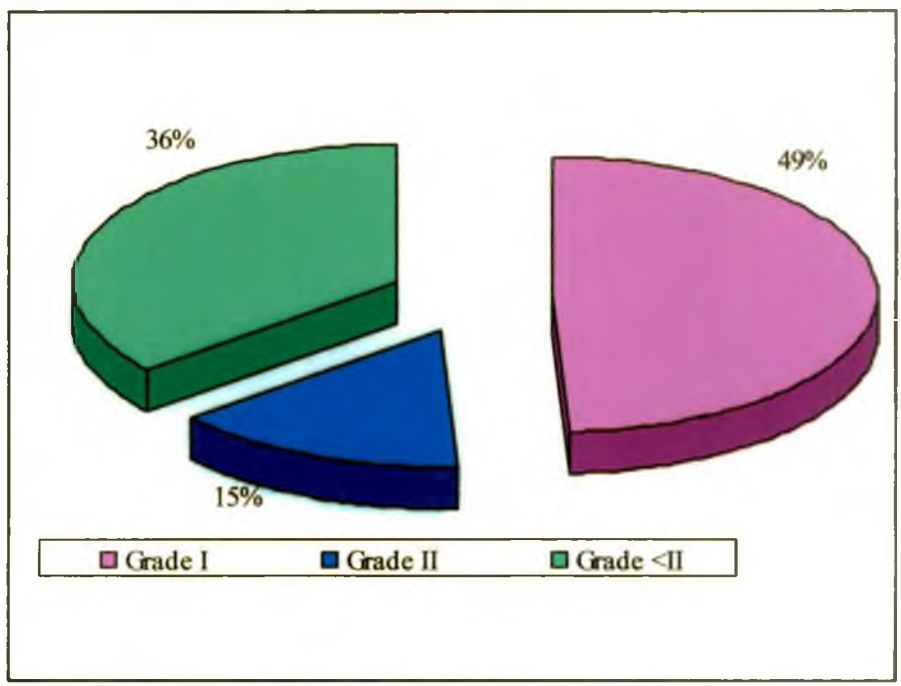


Fig. 3. Percentage yield of various morphological grade oocytes

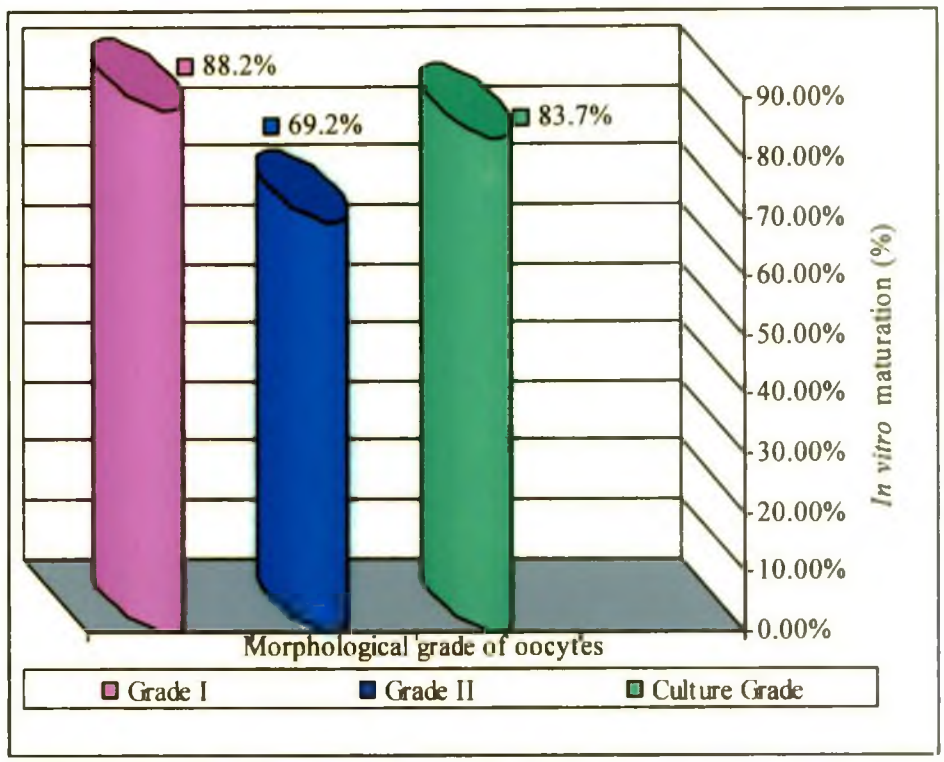


Fig. 4. Effect of quality of oocytes on *in vitro* maturation rate

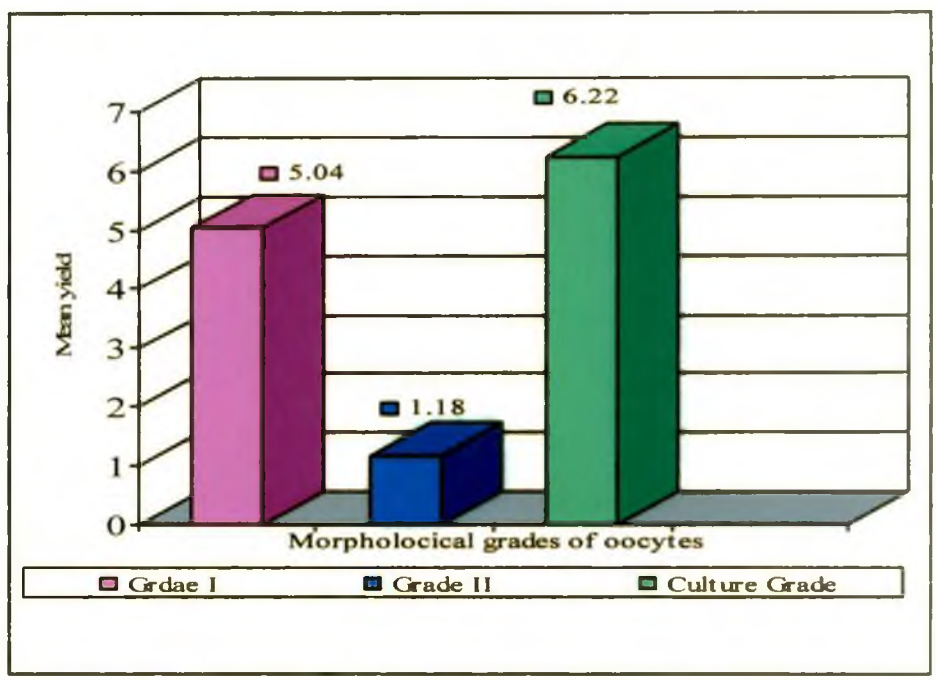


Fig. 5. Effect of quality of oocytes on mean yield of *in vitro* matured oocytes per ovary

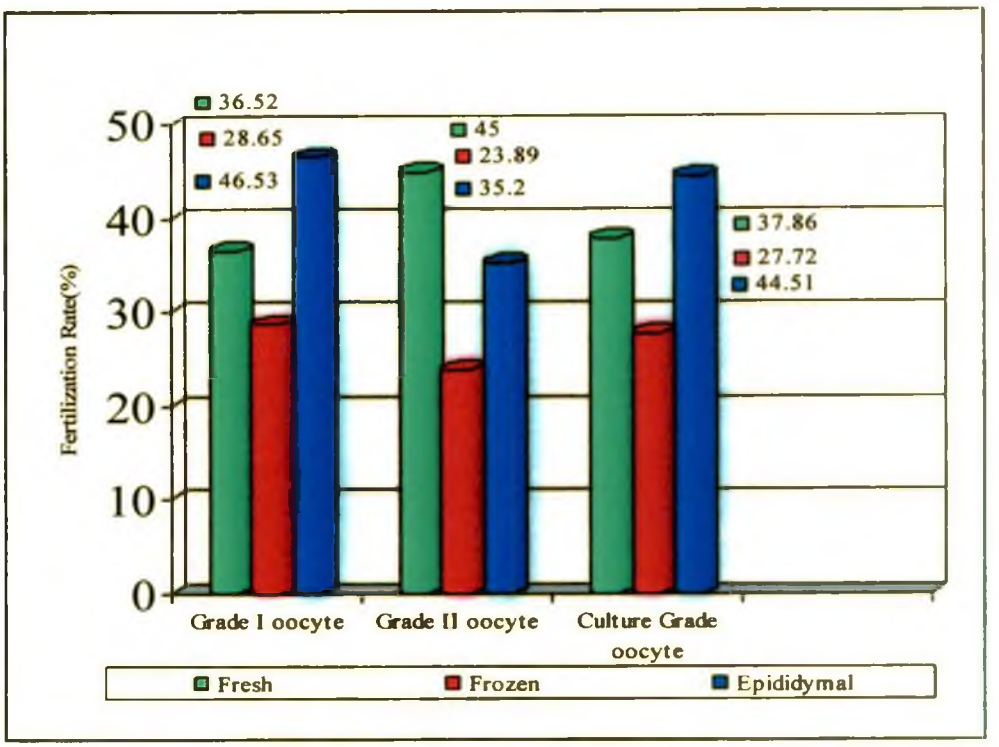


Fig. 6. Effect of source of spermatozoa on *in vitro* fertilization rate

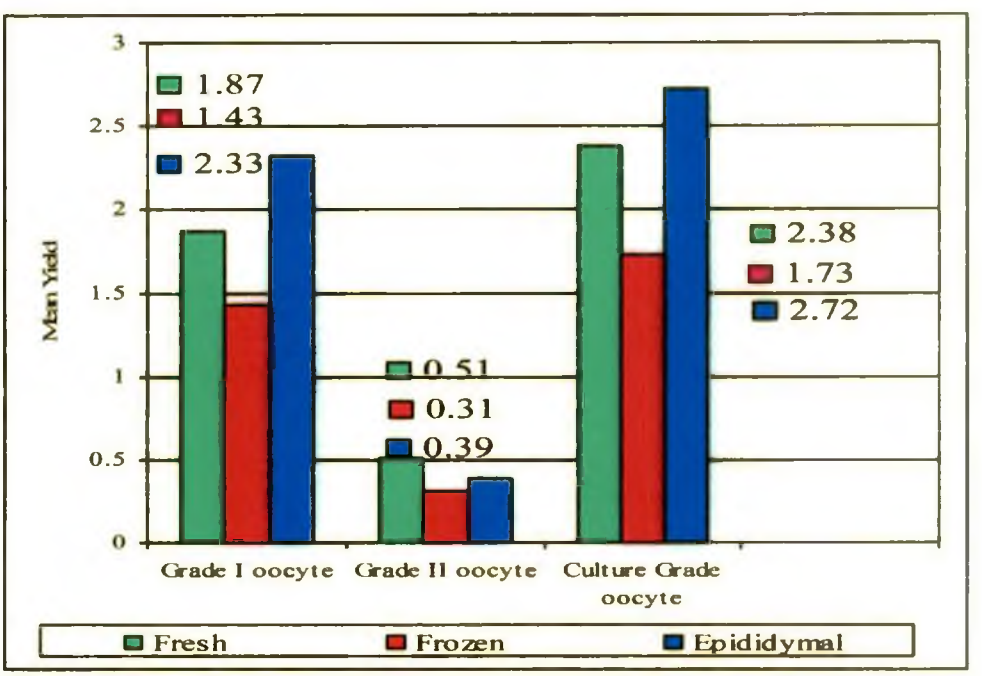


Fig. 7. Effect of source of spermatozoa on yield of *in vitro* fertilized oocyte per ovary

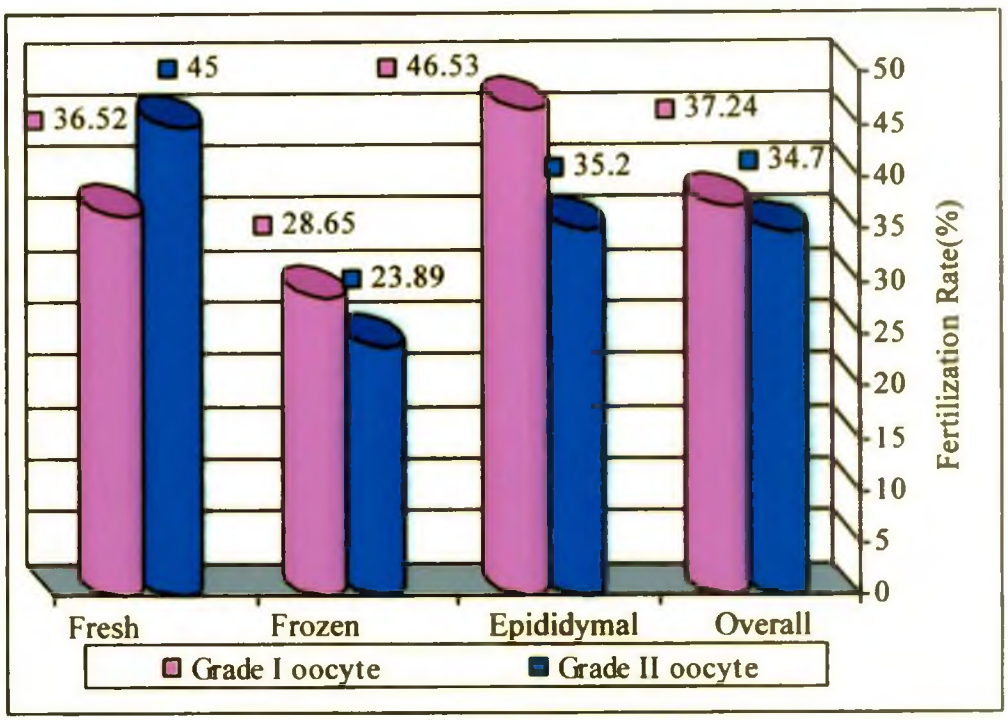


Fig. 8. Effect of quality of oocyte on *in vitro* fertilization rate

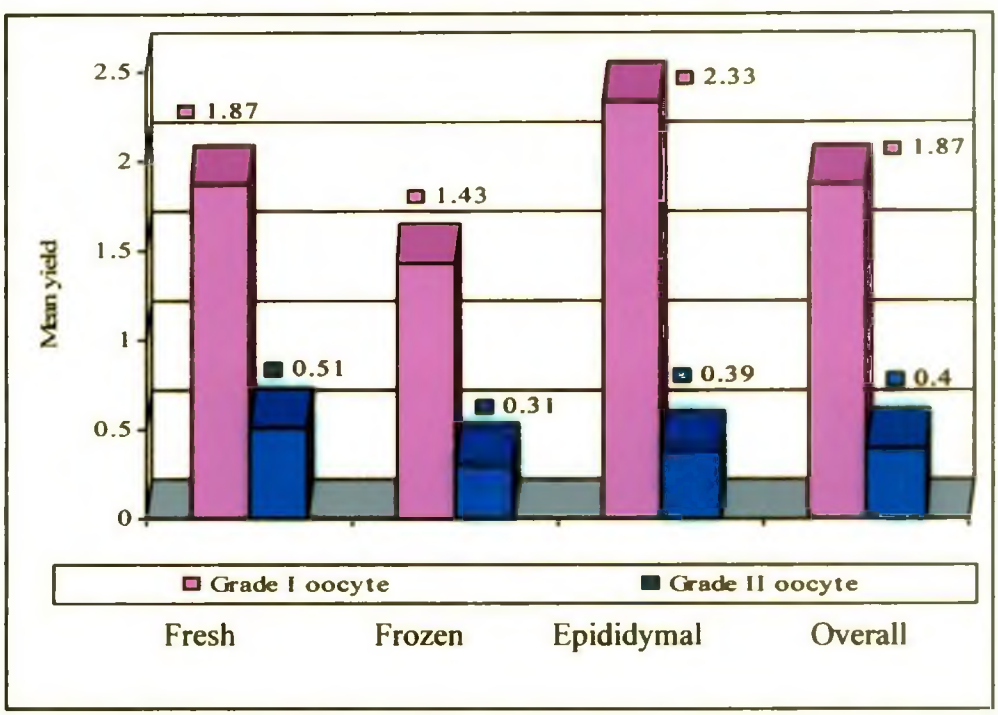
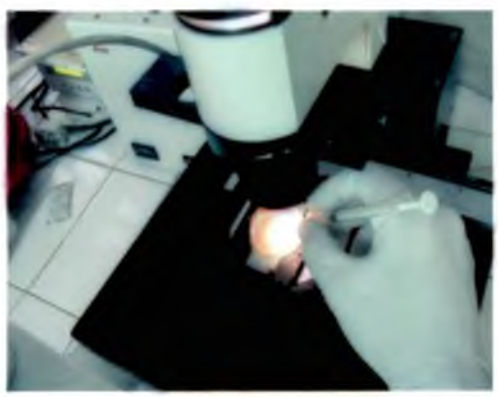


Fig. 9. Effect of quality of oocytes on the mean yield of in vitro fertilized oocyte per ovary

Oocyte Retrieval

Plate 1. Oocyte retrieval by aspiration method

Isolation of Oocytes



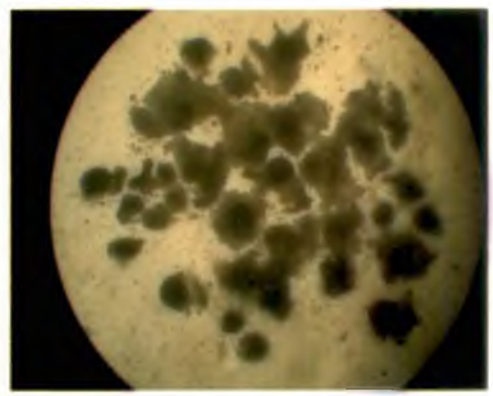


Plate 2. Isolation of oocytes under zoom stereo microscope

Plate 3. Zoom stereo microscopic view of isolated oocytes (20 x)

Morphological Classification of Cumulus Oocyte Complexes

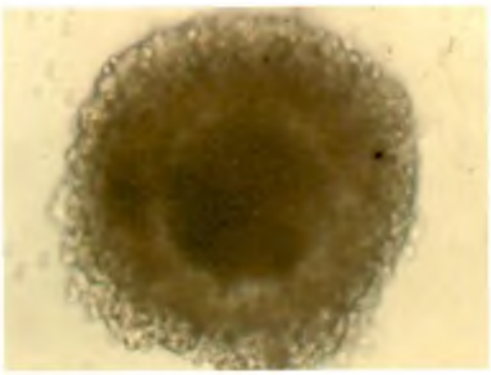


Plate 4. Grade I oocyte (400 x)

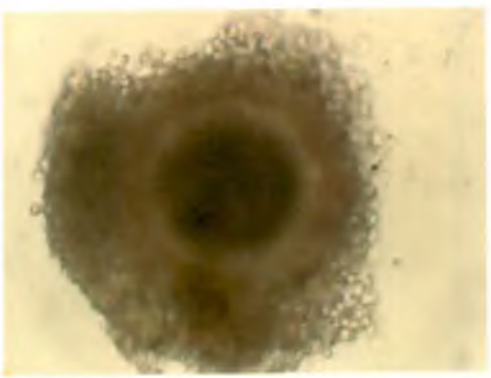


Plate 5. Grade II oocyte (400 x)

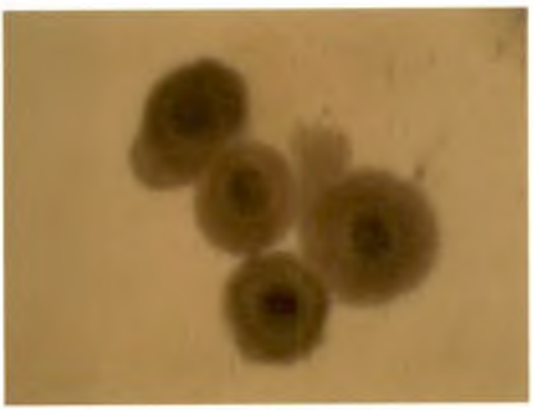


Plate 6. Culture grade oocytes (100 x)

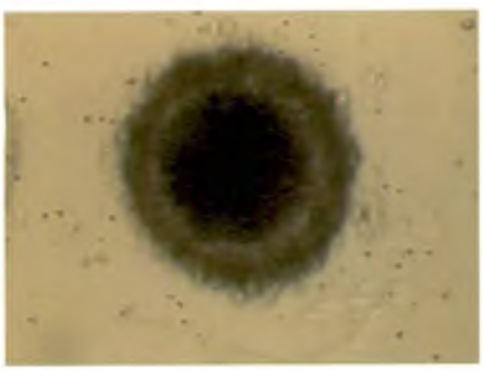


Plate 7. Grade < II oocyte (400 x)

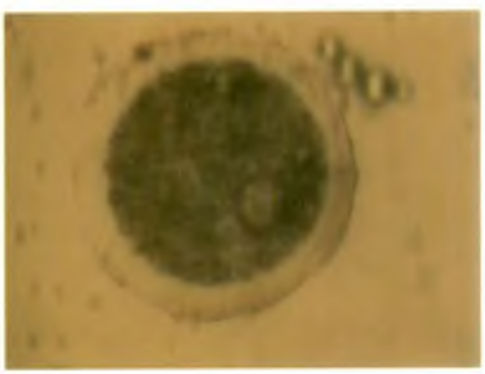


Plate 8. Denuded oocyte (400 x)

In Vitro Culture of Oocytes

Plate 9. *In vitro* culture of oocytes in CO_2 incubator

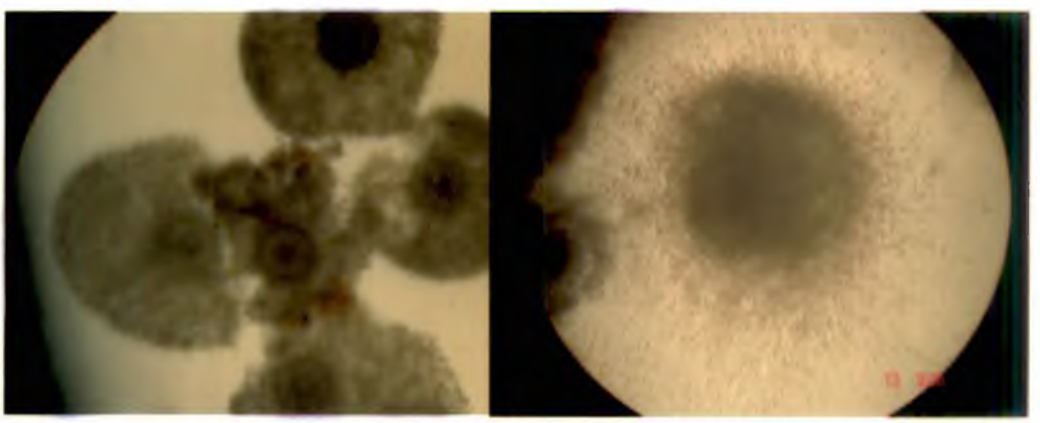


Plate 10. Zoom stereomicroscopic appearance of *in vitro* matured oocytes (200 x)

In vitro Matured Oocytes- Classification based on Cumulus Expansion

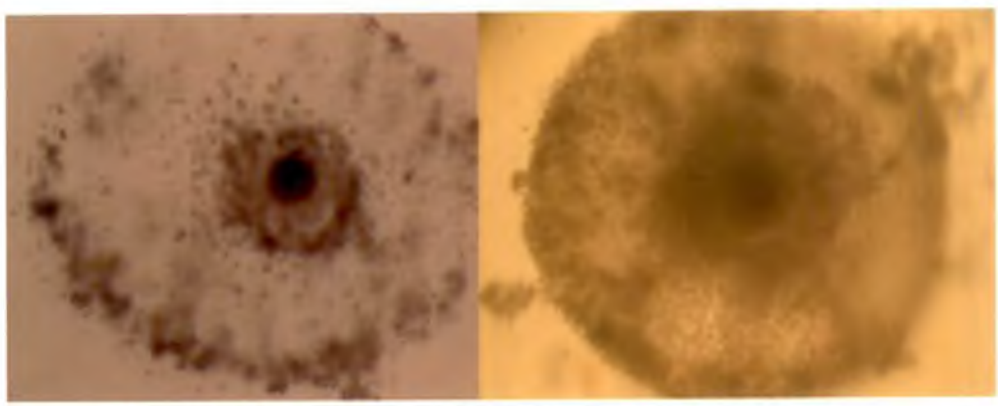


Plate 11. Maximum degree cumulus expansion (400 x)

Plate 12. Moderate degree cumulus expansion (400 x)

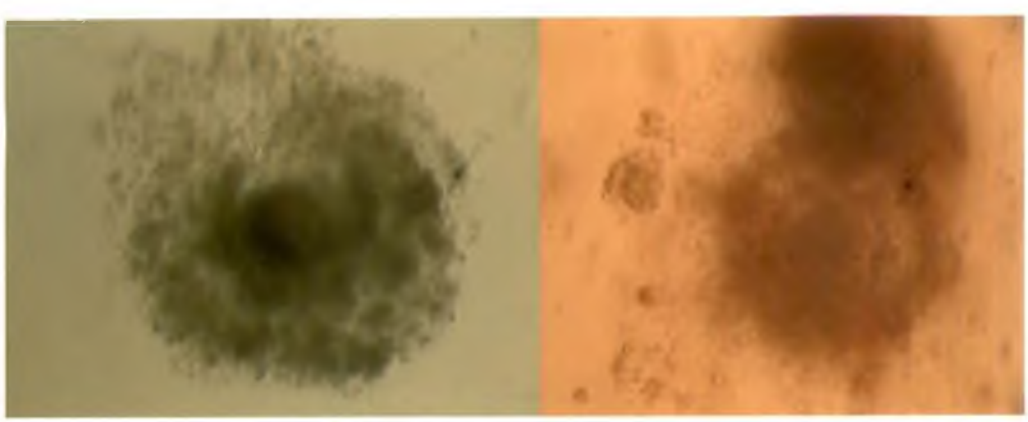


Plate 13. Mild degree cumulus expansion (400 x)

Characteristic signs of *In Vitro* Fertilization

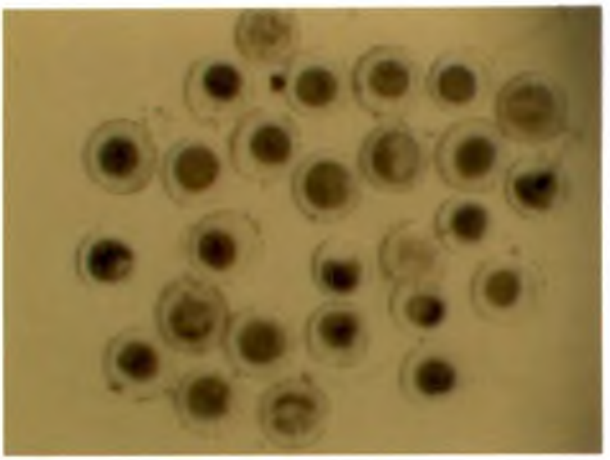


Plate 14. Denuded oocytes after IVF (100 x)

Plate 15. Sperm head undergoing decondensation in the ooplasm (Aceto orcein staining - 400 x)

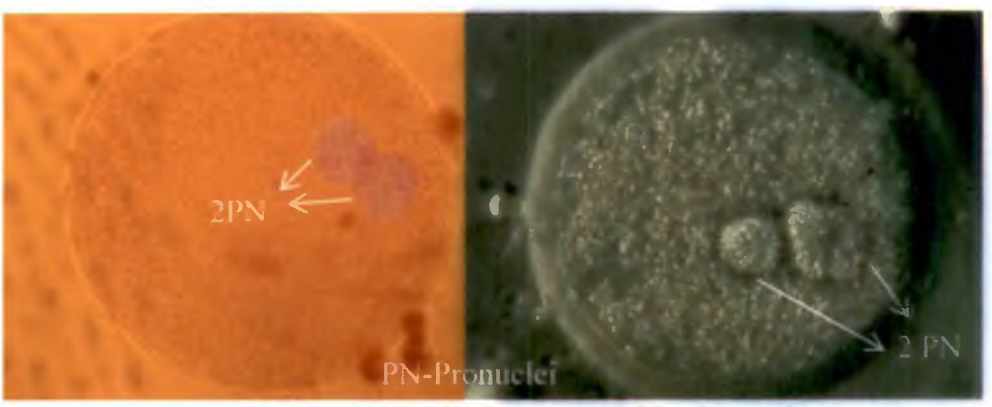


Plate 16. Oocytes with two pronuclei in the ooplasm (Aceto orcein staining- 400 x)

Characteristic signs of In vitro Fertilization (contd)

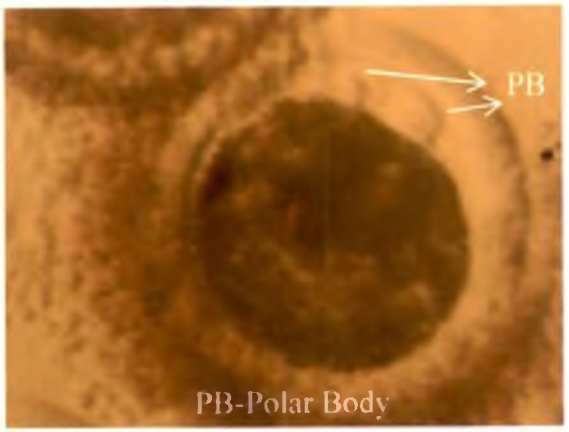


Plate 17. Extruded second polar body (400 x)

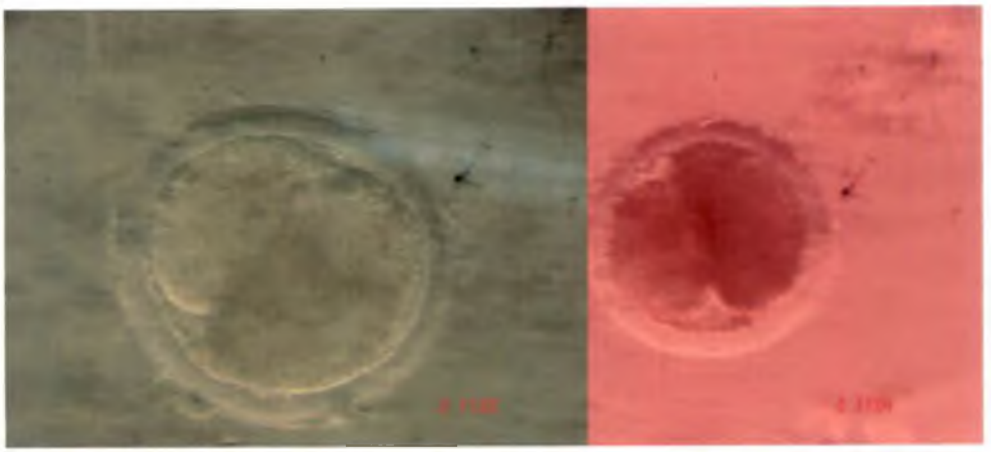


Plate 18. Fertilized oocyte undergoing cleavage - 2 cell stage (400 x)

Plate 19. Polyspermy - presence of more than two pronuclei
(Aceto orcein staining – 400 x)

Discussion

5. DISCUSSION

This study was designed to assess the fertilizability of *in vitro* matured bovine follicular oocytes on co-culture with fresh, frozen and epididymal spermatozoa. It was also envisaged to assess the effect of oocyte quality on the rate of maturation and fertilization *in vitro*. A total of 939 oocytes retrieved by aspiration technique from 81 ovaries of recently slaughtered cows were used for the study.

5.1. TOTAL OOCYTE YIELD PER OVARY

The mean number of total oocytes obtained as per the present study was 11.59±0.10 per ovary. This is in agreement with the results obtained for Hamno and Kuwayama (1993) and Arlotto *et al.* (1996). Carolan *et al.* (1994) obtained a slightly higher oocyte yield of 13.9±3.2 per ovary by aspiration technique. However, Mermillod *et al.* (1992), de Oliveria *et al.* (1994), Baruha *et al.* (1998), Priscilla (2001) and Magnus Paul (2005) obtained a lower yield of total oocyte per ovary than the results of the present work.

Reports on the mean yield of total oocytes per ovary by aspiration technique are highly variable. Vijayakumaran (1995) opined that, the laboratory environment, procedure adopted, expertise of the person concerned, breed of the animals used and the quality of the ovaries used might be the probable reasons for variation in the result of oocyte retrieval. Ovaries from South Indian breeds like Hallikar, Kangayam, Khillari and crossbred cattle of Kerala were used for the study. The breed, age and physiological status of the donor animal used, the retrieval procedure adopted, expertise of the person concerned and variation in the laboratory environment might be the reasons for variation of the result in the present study when compared to others.

5.2. YIELD OF CULTURE GRADE OOCYTE PER OVARY

5.2.1. Grade I Oocytes per Ovary

Percentage yield of grade I oocyte in the present study was 49.20±0.31 per cent. This is in agreement with the results obtained for Katska (1984), Katska and Smorag (1984) and Magnus Paul (2005). These authors obtained 45, 50 and 45 per cent respectively for grade I oocytes. Slightly lower percentage of A class oocytes (41.3 per cent) was obtained by Hamno and Kuwayama (1993), whereas Madison *et al.* (1992) and Carolan *et al.* (1994) obtained much lower percentage of grade I oocytes in comparison to the present results. As per the reports of above authors the percentage of grade I oocyte was 22 and 30.9 respectively.

Average yield of grade I oocytes obtained by aspiration method was 5.70±0.06 per ovary. This is comparable to the result obtained by Katska and Smorag (1984) and Carolan *et al.* (1994). The mean number of grade I oocytes reported by the above authors were 4.63±3.98 and 4.3±0.9 respectively per ovary. Priscilla (2001) and Magnus Paul (2005) obtained a slightly lower yield of grade I oocyte (2-4 oocyte per ovary), when compared to the results of present study. The highest quality oocyte obtained in the present study was grade I, which is in agreement with the results of Katska and Smorag (1984), Carolan *et al.* (1994) and Magnus Paul (2005).

5.2.2. Grade II Oocytes per Ovary

Percentage yield of grade II oocytes from bovine ovaries obtained in the present study was 14.70±0.41. This is comparable with the results of Carolan *et al.* (1994) and Priscilla (2001) who recorded grade II oocyte yield of 18 per cent and

13.77 per cent respectively. Magnus Paul (2005) obtained higher yield (32.79 per cent) of B class oocytes than the present result.

Mean number of Grade II oocytes obtained by aspiration method in the present study was 1.70±0.05 per ovary. This was higher than the result (1.22±0.12) obtained by Magnus Paul (2005), whereas the result (2.5±0.6) recorded by Carolan *et al.* (1994) was slightly higher than the present values.

5.2.3. Culture grade Oocytes per Ovary

Percentage of total culture grade oocytes obtained in the present study was 63.90 ±0.22. This is comparable with results of Vassena *et al.* (2003) and Katska (1984). Dominguez (1995) obtained slightly lower value (54.9 per cent) than the present result. But Hamno and Kuwayama (1993) and Magnus Paul (2005) obtained 76 per cent of culture grade oocytes, which is higher than the present result. Dominguez (1995) used ovaries from Europian, Zebu and cross bred cows and classified the oocytes with homogenous ooplasm, intact zona pellucida and a complete compact layer of cumulus cells as normal and remaining oocytes as abnormal. The breed of the donor animal used, the retrieval procedure adopted and the criteria of oocyte selection might be the reasons for variation in the result.

The mean number of culture grade oocyte per ovary obtained by aspiration method in the present study was 7.41±0.07 per ovary. Katska and Smorag (1984) and Magnus Paul (2005) obtained a lower values (5.24±4.03 and 2.88±0.19 respectively) than the result of the present study, whereas, Takagi *et al.* (1992) obtained 17.8±1.9 culture grade oocytes per ovary, which was very high in comparison. Variation in the mean yield of total oocytes per ovary in various studies might be due to difference in the procedure and the criteria of oocyte selection

adopted, which in turn caused the proportionate variation in the yield of culture grade oocyte per ovary.

5.3. *IN VITRO* MATURATION OF OOCYTES

5.3.1. Maturation of grade I Oocytes

Cumulus expansion rate obtained for grade I oocytes was 88.20±0.75 per cent in the present study, which is in agreement with the results of Rabahi *et al.* (1993) and Magnus Paul (2005). Higher cumulus expansion rate (98±3.3 per cent) was reported by Romero-Arredondo and Seidel (1996) for class A oocytes in cattle. Mean number of matured oocytes obtained per ovary was 5.04±0.06 from grade I oocytes. Perusal of literature failed to show any report on comparison of mean yield of matured oocyte per ovary.

5.3.2. Maturation of grade II Oocytes

Cumulus expansion rate obtained for grade II oocytes in the present study was 69.21±1.97 per cent, which is in agreement with the results of Magnus Paul (2005). Konishi *et al.* (1995) obtained a higher maturation rate for class B oocytes in bovine (76 per cent), whereas Chauhan *et al.* (1998) obtained a comparable result in buffaloe. Mean number of matured oocytes obtained per ovary was 1.18±0.03 from grade II oocytes.

5.3.3. Maturation of Culture Grade Oocytes

Overall cumulus expansion rate obtained for total culture grade oocytes was 83.67±0.35 per cent in the present study, which is comparable to the result of Calder *et al.* (2003) and Quero *et al.* (1994). The above authors have obtained overall

cumulus expansion rate of 90 and 79 per cent respectively. The overall cumulus expansion rate obtained by Magnus Paul (2005) was 68.75 per cent. The mean number of oocytes showing cumulus expansion per ovary was 6.22±0.06 in the present study.

5.3.4. Effect of Oocyte Quality on Maturation Rate

On overall analysis, the cumulus expansion percentage of matured oocytes per ovary obtained from grade I oocyte showed a significantly higher result than that of grade II oocytes in the present study, which is comparable to the result of Magnus Paul (2005). Magnus Paul (2005) obtained higher cumulus expansion rate of 83.08 per cent for class A oocyte when compared to 68.29 per cent with class B oocytes. The mean number of matured oocytes per ovary obtained from grade I oocytes was significantly higher than that of grade II oocytes. Perusal of literature failed to show any reports on comparison of mean number of cumulus expanded oocytes per ovary. The proportionate yield of grade II oocyte per ovary was significantly lower than that of grade I oocytes in the present study. Moreover, the cumulus expansion percentage of grade II oocytes was significantly lower than that of the grade I oocytes and this might be the probable reason for the low yield of cumulus expanded oocytes per ovary from grade II oocytes in the present study.

Raghu *et al.* (2002) opined that removal of cumulus cells perturbed the cytoplasmic maturation and hence developmental competence was reduced. In the absence of cumulus cells, oocytes exhibited no maturation or exhibited only reduced maturation (Kobayashi *et al.*, 1994). Cumulus cells communicate to the oocyte through corona radiata cells, which penetrate the zona pellucida and form gap junctions with oolemma. These gap junctions allow metabolic transfer and help in nutrition of oocytes, which play a vital role in oocyte maturation (Buccione *et al.*,

1990; Armstrong *et al.*, 1996; Bols *et al.*, 1996; de Matos *et al.*, 1997; Datta and Goswami, 1999 and Geshi *et al.*, 2000). Cumulus cells metabolized glucose to pyruvate or Kreb's cycle intermediates, that could be passed to the oocyte and enhance its quality (Geshi *et al.*, 2000). Cumulus oocyte complex quality have definite role in the IVM of bovine oocytes. Oocytes with multiple layers of cumulus cells matured better than oocytes with reduced cumulus cells. Oocytes with more than three complete layers of cumulus cells were found to be ideal for IVM.

5.4. *IN VITRO* FERTILIZATION OF OOCYTES

5.4.1. Fertilizability of Fresh Spermatozoa

The fertilization rate obtained was 36.52±1.68, 45.00±5.63 and 37.86±0.47 per cent respectively for grade I, grade II and culture grade oocytes, when fresh ejaculated semen was used for IVF. This result is comparable to the results obtained by Brackett *et al.* (1980) in bovines. Kidson *et al.* (2003) obtained comparable fertilization rate (29 to 36 per cent) for sow oocytes when fresh semen was used for IVF. De Los Reyes *et al.* (2009) used fresh dog spermatozoa for IVF and obtained average penetration rate of around 70 per cent with *in vitro* matured canine oocytes, which is well above the result of the present study. Laboratory environment, species of the animal used and the level of expertise might be the reason for variation in the results.

5.4.2. Fertilizability of Frozen Spermatozoa

The fertilization rate obtained with frozen semen was 28.65±0.76, 23.89±3.03 and 27.72±0.89 per cent for grade I, grade II and total culture grade oocytes respectively. This result is lower than the results obtained by Long *et al.*,

1994; Hazeleger *et al.*, 1995; Chian *et al.*, 1996; Azambuja *et al.*, 1998; Hashimoto *et al.*, 1998; Mizushima and Fukui, 2001; Avery *et al.*, 2003 and Chohan and Hunter, 2004). Brackett *et al.* (1980) reported no signs of fertilization in two IVF experiments in which frozen semen was employed. The most likely reason proposed by the authors was the inadequacy in the processing procedure of sperm cells. Frozen semen stored in ampoules was thawed according to direction of the supplier and the sperm cells were immediately treated in a way known to allow *in vitro* capacitation of rabbit sperm and used for their IVF study in cattle.

5.4.3. Fertilizability of Cauda Epididymal Spermatozoa

The fertilization rate obtained with cauda epididymal spermatozoa were 46.53±1.32, 35.20±4.62 and 44.51±0.57 per cent for grade I, grade II and total culture grade oocytes respectively. Ball *et al.* (1983) and Goto *et al.* (1989) obtained comparable results when epididymal spermatozoa were used for IVF in bovine oocytes, whereas, Katska *et al.* (1996) achieved a higher fertilization rate (83 per cent) of oocytes when epididymal spermatozoa were used for IVF of bovine oocytes. Semen samples which showed comparable survival rates only were selected for the above IVF experiment and that might be the probable reason for higher fertilization rate when compared to the result of the present study.

Hishinuma and Sekine (2004) reported a similar fertilization rate when epididymal spermatozoa were used in a sperm-oocyte penetration assay conducted in canine. Nagai *et al.* (1988) and Fan *et al.* (2008) also recorded similar values for epididymal spermatozoa in porcine and mouse oocytes respectively.

Herrick *et al.* (2004) reported a much lower fertilization rate (Three per cent) in comparison to the result of the present study when springbok oocytes were

fertilized *in vitro* using cryopreserved epididymal spermatozoa. When Goat epididymal spermatozoa were used for IVF studies, Pawshe *et al.* (1996) obtained a cleavage rate up to 79.33 per cent. Williams *et al.* (2001) reported a cleavage rate of 57 per cent for canine ova when epididymal spermatozoa were used for IVF. Choi *et al.* (1998) recorded a higher fertilization rate with epididymal spermatozoa for inbred BALB/c mice.

5.4.4. Overall Fertilization Rate

The fertilization rate based on the pooled data of fresh, frozen and epididymal spermatozoa was 36.70±1.71 per cent and the yield of *in vitro* fertilized oocyte per ovary was 2.28±0.10 in the present study. Perusal of literature failed to show any reports on comparative assessment of overall fertilization rate of oocytes using fresh, frozen and epididymal spermatozoa.

5.4.5. Correlation between the Source of Spermatozoa and Oocyte Quality on *in vitro* Fertilization

The fertilization percentage obtained with grade I oocytes were 36.52±1.68, 28.65±0.76 and 46.53±1.32 per cent respectively for fresh, frozen and cauda epididymal spermatozoa. Grade II oocytes yielded 45.00±5.63, 23.89±3.03 and 35.20±4.62 per cent fertilization rate respectively when fresh, frozen and cauda epididymal spermatozoa were used for IVF. Total culture grade oocytes fertilized was 37.86±0.47, 27.72±0.89 and 44.51±0.57 per cent respectively with fresh, frozen and cauda epididymal spermatozoa.

The mean number of fertilized oocytes per ovary obtained from grade I oocyte were 1.87±0.06, 1.43±0.04 and 2.33± 0.05 respectively when fresh, frozen

and epididymal spermatozoa were used for IVF. From grade II oocytes the yield of fertilized oocytes per ovary reported were 0.51±0.07, 0.31±0.05 and 0.39±0.06 respectively for fresh, frozen and epididymal spermatozoa. The mean yield of fertilized oocytes per ovary from culture grade oocytes were 2.38±0.05, 1.73±0.07 and 2.72±0.03 respectively for fresh, frozen and cauda epididymal spermatozoa.

5.4.5.1. Differences Due to Sources of Spermatozoa

In vitro fertilization rate and mean yield of fertilized oocyte per ovary using cauda epididymal spermatozoa for grade I and culture grade oocytes were significantly higher than that of fresh and frozen semen ($p<0.01$). Similarly the difference in fertilization rate and mean yield of fertilized oocyte per ovary between fresh and frozen semen were found significantly different ($p<0.01$). The lowest fertilization rate and mean yield per ovary was obtained for frozen semen.

With respect to the grade II oocytes, no significant difference in fertilization rate and mean yield of fertilized oocyte per ovary were observed between fresh and epididymal spermatozoa, though the rate and mean yield for fresh semen was slightly higher. The fertilization rate and mean yield of fertilized oocyte per ovary for frozen semen was significantly lower than other two sources of spermatozoa.

5.4.5.2. Differences Due to Oocyte Quality

No significant difference was observed between grade I and grade II oocytes on fertilization rate, when fresh, frozen and epididymal spermatozoa were used for IVF. Whatever be the quality of oocytes before maturation, only those oocytes that showed maximum cumulus expansion were used for the IVF in the present study and this might be the reason for non significant difference between grade I and II

oocytes. But a significant difference was observed between grade I and grade II oocytes in the mean yield of fertilized oocytes per ovary with fresh, frozen and epididymal spermatozoa. The yield of grade I oocytes per ovary in the present study was significantly higher than the yield of grade II oocytes and this might be the reason for the significantly higher yield of fertilized oocytes per ovary from grade I than grade II oocytes.

5.4.5.3. Overall Effect Due to Source of Spermatozoa and Oocyte Quality

Fertilization rate obtained with fresh, frozen and epididymal spermatozoa was comparable to the individual results of many scientists (Brackett *et al.*, 1980; Ball *et al.*, 1983; Nagai *et al.*, 1988; Goto *et al.*, 1989; Hishinuma and Sekine, 2004 and Fan *et al.*, 2008). But the results obtained in the present study was lower than the results of certain other workers (Long *et al.*, 1994; Hazeleger *et al.*, 1995; Chian *et al.*, 1996; Azambuja *et al.*, 1998; Hashimoto *et al.*, 1998; Mizushima and Fukui, 2001; Avery *et al.*, 2003; Chohan and Hunter, 2004 and De Los Reyes *et al.*, 2009), which might be due to multiple reasons like laboratory environment, breed and nutritional status of donor animal and level of expertise. Multiple factors were proved critical to the outcome of IVF and embryo culture. Boone and Shapiro (1990) reported that, factors like cytotoxic materials, water quality, gas composition, temperature, hydrogen ion concentration and osmolality of media and light exposure might be critical to the outcome of IVF and embryo culture. The reduced IVF rate obtained in this study might be due to the cumulative effect of the above factors.

Perusal of literature failed to show any reports on comparison of *in vitro* fertilizability of fresh, frozen and epididymal spermatozoa and comparative efficiency of IVF of grade I and grade II oocytes using fresh, frozen and epididymal spermatozoa in bovines.

Fresh bull semen showed significantly higher fertilization rate with all the quality grades of oocytes when compared to frozen semen in the present study, which is in agreement with the results of De Los Reyes *et al.* (2009) in canines. The authors reported that, fresh dog spermatozoa showed significantly higher penetration rate with both immature and in *vitro* matured canine oocytes in comparison to frozen sperm. Nagai *et al.* (1988) obtained significantly higher percentage of penetrated porcine oocytes ***in vitro*** with frozen epididymal spermatozoa when compared to the fertilization rate, with frozen ejaculated spermatozoa, which is also in agreement with the results of the present study.

Katska *et al.* (1996) studied the ***in vitro*** fertilizability of bull spermatozoa and obtained higher fertilization with frozen-thawed epididymal spermatozoa when compared to the results with fresh semen which is in agreement with the results of the present study. It was further reported that, the seminal plasma deprived ejaculated sperm showed significantly more oocytes fertilized, cleaved and developed to the blastocyst stage when compared to ejaculate semen. It was opined that, the absence of seminal plasma during capacitation had a favorable effect on embryo production efficiency ***in vitro***.

Bedford (1970) reported that, capacitated sperm loose the ability to undergo the acrosome reaction when sperms were treated with seminal plasma and proved the presence of a factor which prevented the acrosome reaction. Reports on several species including cattle suggested that seminal plasma contained factors that might influence male fertility (Miller *et al.*, 1990 and Killian *et al.*, 1993). Generally, these factors are believed to inhibit sperm capacitation, the acrosome reaction, or acrosomal enzymes and ultimately interfere with fertilization. On the other hand, in many studies the positive role of seminal plasma in capacitation of bull sperm,

especially increasing the number of binding sites for heparin, has been emphasized (Miller *et al.*, 1990).

Spermatozoa from cauda epididymis, which have not been exposed to seminal plasma proteins are fertile (Holtz and Smidth, 1976). Ejaculated and epididymal spermatozoa differ in their ability to capacitate. Bovine epididymal spermatozoa can be capacitated in simple salt solution (Ball *et al.*, 1983), but ejaculated bovine spermatozoa do not capacitate well unless capacitating agents are used. Killian *et al.* (1993) have shown in bull seminal plasma, the existence of four fertility associated proteins that appeared to be of value in predicting small differences in the *in vivo* relative fertility among bulls. Watson (1995) reported that, cryopreservation induced modifications to sperm membranes, making them more reactive to their environment after thawing such that cryopreserved sperm were in partially capacitated state (cryocapacitation). This cryocapacitation was partially responsible for reduced fertility of frozen thawed bull semen (Cormier and Bailey, 2003). It was further suggested that, exposure of fresh spermatozoa in the egg yolk-tris-glycerol extender used for semen cryopreservation and cooling to 4° C for four hours promoted capacitation (premature capacitation) which might be responsible for the compromised *in vivo* fertility of frozen thawed spermatozoa. Once capacitated, spermatozoa exhibit elevated metabolic rates, increased membrane fluidity and permeability and if they do not achieve fertilization, they undergo spontaneous acrosome reactions due to an uncontrolled influx of Ca^{2+}. Hence the fertilizing life span of capacitated spermatozoa is limited.

Difference in fertilizing ability of fresh, frozen and cauda epididymal spermatozoa observed in the present study might be due to the factors described above. Katska *et al.* (1996) recommended the use of frozen cauda epididymal spermatozoa for mass embryo production of unknown genetic value.

5.5. PHYSIOLOGICAL AND MORPHOLOGICAL CHARACTERISTICS OF CAUDA EPIDIDYMAL SPERMATOZOA

5.5.1. Motility

Epididymal sperm extract was examined under phase contrast microscope at 200 x magnification and assessed the motility subjectively (nearest five per cent). The mean motility percentage obtained for epididymal sperm was 49.17±9.26 per cent. This was in agreement with the results by Amann and Almquist (1962), Igboeli and Foote (1968) and Goovaerts *et al.* (2006), which were 32.1 per cent, 41 per cent and 46.1 per cent respectively. Deutscher *et al.* (1974) reported a higher motility (66±4.7 per cent) of epididymal spermatozoa collected by cannulation technique from four angus bulls aged two to three years old, when compared to the motility observed in the present study. Singh *et al.* (2007) and Hiron (2007) also reported a higher motility of epididymal spermatozoa in buffaloe (71.7±1.14 and 78.83±0.64 per cent respectively) and Filliers *et al.* (2008) in cats (69.9 per cent), when compared to the results of the present study. Filliers *et al.* (2008) collected epididymal spermatozoa in Hepes-TALP medium and placed in a conventional incubator at 39° C for 20 minutes to allow for movement of the passively liberated spermatozoa into the surrounding medium. Singh *et al.* (2007) and Hiron (2007) obtained epididymal spermatozoa by puncturing the epididymis at multiple sites and collecting in Tris diluter for evaluation of semen. Deutscher *et al.* (1974) reported that, the rate of motility of the epididymal spermatozoa showed a trend for being lower than in the ejaculate. They opined that, the dilution of the epididymal sperm with seminal plasma during ejaculation stimulated more vigorous movement and a combination of dilution, oxygenation and increased temperature was observed to increase the rate of motility. The species, breed, age and the procedures adopted might be the probable reasons for the variation in the results.

5.5.2. Concentration

Concentration of sperm in the pooled epididymial sperm content from both sides was assessed with haemocytometer. The mean concentration obtained for epididymal sperm was $37,175x10^6$ $\pm 7612x10^6$ per ml. This was in agreement with the results ($38487 \times 10^6 \pm 3888 \times 10^6$ per ml) of Goovaerts *et al.* (2006). Deutscher *et al.* (1974) reported a much lower concentration ($3810 \times 10^6 \pm 460 \times 10^6$ per ml) of spermatozoa in epididymal extract, when compared to the concentration obtained in the present study. Epididymal sperm collected by cannulation technique from four Angus bulls aged two to three years with no breeding experience was used in their study. Moreover, each bull was electro ejaculated twice daily for two alternate days before cannulation surgery. These factors might be the probable reasons for obtaining low concentration of epididymal spermatozoa when compared to the result of present study.

5.5.3. Viability

The mean percentage of live and dead sperms was 84.5±8.02 per cent and 15.5±8.02 per cent respectively in the present study, which is in agreement with the results of Deutscher *et al.* (1974) and Goovaerts *et al.* (2006) in bulls; Singh *et al.*(2007) and Hiron (2007) in buffaloe and Filliers *et al.* (2008) in cats.

5.5.4. Morphological Characteristics

The mean percentage of normal spermatozoa, spermatozoa with abnormal heads, abnormal tails, proximal protoplasmic droplet and distal protoplasmic droplet were 35.67±2.30, 3.17±1.58, 2.33±0.61, 11.67±4.01 and 47.17±3.17 per cent respectively. This is in agreement with the results of Goovaerts *et al.* (2006) in bulls.

Deutscher *et al.* (1974) reported a slightly higher percentage of head and tail abnormalities (4.3±0.64 and 2.8±0.33) in bull epididymal sperm collected by cannulation technique from four Angus bulls aged two to three years with no breeding experience, when compared to the results of the present study. The breed, age, and breeding status of the animals used might be the probable reasons for variation of results. Amann and Almquist (1962) and Deutscher *et al.* (1974) reported that, the types of abnormal sperm and their percentages were very comparable between the epididymal and ejaculate samples.

Igboeli and Foote(1968) recovered cauda epididymal sperm of four bulls, stored it at 5° C and used it within 60 h of slaughter to inseminate 100 cows and the mean non-return rate (NRR) obtained was 69 per cent and the mean NRR of ejaculated semen from these same bulls was 75 per cent. Spermatozoa from cauda epididymis, which have not been exposed to seminal plasma proteins were fertile (Holtz and Smidth, 1976). Harper (1982) reported that, the cytoplasmic droplet was usually lost by the time of ejaculation and migration of the droplet was not essential for acquisition of motility. The high sperm concentration and the presence of more number of distal protoplasmic droplets might be due to the maturation and storage function of epididymis (Goovaerts *et al.*, 2006). It was further reported that the progressive motility of ejaculated semen were significantly higher than that of epididymal sperm. This might be due to higher percentage of static spermatozoa in epididymal sample. The presence of a high percentage of non-moving spermatozoa in the epididymal sperm extract can be attributed to metabolic inactivity of the spermatozoa while in the cauda epididymis. In some cases, epididymal sperm is the only available source of male gametes for use in assisted reproduction programmes. This can be the only source when spermatozoa have to be urgently retrieved from a severely injured or suddenly diseased donor and can be vitally significant with a donor belonging to an endangered species. In some situations, superior males have

to be handled or captured under anesthesia and this might impair normal ejaculation. Moreover, when an ejaculate is devoid of any spermatozoa due to some block in the vas deference or in case of testicular biopsy, there may be necessity for aspiration of epididymal spermatozoa for IVF. Collection of epididymal sperm further offers the possibility to acquire and use genetic material from elite males even after their death.

The use of fresh or cryopreserved epididymal spermatozoa in assisted reproduction has already led to production of offspring in domestic and wild species including cattle, goat, eland and mouflon (Igboeli and Foote, 1968; Garde *et al.*, 1995; Blash *et al.*, 2000 and Bartels *et al.*, 2001). Epididymal sperm can either be used fresh, or be frozen and stored in genetic resource bank projects (Gilmore *et al.*, 1998).

CONCLUSION

The yield of grade I oocyte from slaughter house ovary was significantly higher than grade II oocyte (5.70±0.06 and 1.70±0.05). Morphological appearance of cumulus oocyte complexes had definite role in the success rate of IVM and IVF of bovine oocytes. Grade I oocytes with more than five layers of cumulus cells matured better than the grade II oocytes having three to four layers of cumulus cells (88.20±0.75 and 69.21±1.97 per cent). However, grade I and grade II oocytes did not differ significantly in their fertilizing ability, provided, they have comparable level of *in vitro* maturation status. It is clearly observed from this study that the source of spermatozoa has definite influence on the *in vitro* fertilization rate of bovine oocytes. *In vitro* fertilization using epididymal spermatozoa showed significantly more fertilization rate than fresh semen and this was closely followed by frozen semen (44.51±0.57, 37.86±0.47 and 27.72±0.89 per cent respectively). Hence it is suggested that, epididymal spermatozoa retrieved from slaughtered bulls

could be efficiently used for IVF of *in vitro* matured bovine oocytes equally or even better than the fresh ejaculated or frozen semen. *In vitro* fertilization of oocytes using sexed spermatozoa and IVC of IVF oocytes are the major areas coming in line with the present study requiring attention in future.

Summary

6. SUMMARY

A study was carried out on *in vitro* maturation and fertilization of follicular oocytes retrieved by aspiration method from ovaries of recently slaughtered cows. The major objective of this study was to assess the fertilizabilty of bovine oocytes matured *in vitro* on co-culture with fresh, frozen or epididymal spermatozoa. The main observations made in this study were (1) total yield of oocytes per ovary (2) yield of culture grade oocytes per ovary (3) yield of oocytes matured *in vitro* (4) yield of *in vitro* fertilized oocytes when inseminated with fresh, frozen or epididymal spermatozoa (5) total yield of *in vitro* fertilized oocytes and (6) morphological characteristics of epididymal semen.

A total of eighty one ovaries of abattoir origin from South Indian breeds like Hallikar, Kangayam, Khillari and crossbred cattle of Kerala were subjected to oocyte retrieval by aspiration method. Grade I (462) and grade II (138) COCs so obtained were subjected to maturation in separate groups for 24 h in Hepes modified TCM-199 enriched with sodium pyruvate, sodium bicarbonate, antibiotics, estradiol-17β, FSH, hCG and 20 per cent heat-inactivated day zero estrus cow serum. Culture environment was set as 39° C temperature, five per cent CO_2 tension and maximum humidity in a standard CO_2 incubator. Maturation status of COCs was assessed by observing cumulus expansion and mucification.

Oocytes with maximum degree of cumulus expansion from each group (407 and 95) were subjected to IVF using fresh, frozen and epididymal spermatozoa in Fert-TALP medium supplemented with epinephrine, hypotaurine, pencillamine and heparin (5-10 oocytes in 100 µl droplet). Fifty micro liters of fresh semen obtained from healthy cross bred bulls was layered over percoll density gradients and separated the motile spermatozoa. With respect to frozen semen, motile spermatozoa

and culture grade COCs were 5.04±0.06, 1.18±0.03 and 6.22±0.06 respectively. Grade I oocytes showed significantly higher maturation rate and mean yield per ovary than grade II oocytes.

The fertilization rate obtained with grade I oocytes were 36.52±1.68, 28.65±0.76 and 46.53±1.32 per cent respectively for fresh, frozen and cauda epididymal spermatozoa. Percentage yield of Grade II oocytes were 45.00±5.63, 23.89±3.03 and 35.20±4.62 respectively when fresh, frozen and cauda epididymal spermatozoa were used for IVF. Fertilization rate obtained with total culture grade oocytes were 37.86±0.47, 27.72±0.89 and 44.51±0.57 per cent with fresh, frozen and cauda epididymal spermatozoa respectively.

The mean number of fertilized oocytes per ovary obtained from grade I oocyte were 1.87±0.06, 1.43±0.04 and 2.33± 0.05 respectively when fresh, frozen and epididymal spermatozoa were used for IVF. From grade II oocytes the yield of fertilized oocytes per ovary reported were 0.51±0.07, 0.31±0.05 and 0.39±0.06 respectively for fresh, frozen and epididymal spermatozoa. The mean yield of fertilized oocytes per ovary from culture grade oocyte were 2.38±0.05, 1.73±0.07 and 2.72±0.03 respectively for fresh, frozen and cauda epididymal spermatozoa.

In vitro fertilization rate and mean yield of fertilized oocyte per ovary of cauda epididymal spermatozoa for grade I and total culture grade oocytes were significantly higher than that of fresh and frozen semen ($p<0.01$). Similarly, the difference in fertilization rate and mean yield per ovary between fresh and frozen semen were also found to be significantly different ($p<0.01$). The lowest fertilization rate and mean yield per ovary were obtained when frozen semen was used.

With respect to the grade II oocytes, no significant difference in fertilization rate and mean yield per ovary were observed between fresh and epididymal spermatozoa, though the fertilization rate and mean yield for fresh semen were slightly higher. The fertilization rate and mean yield per ovary for frozen semen was significantly lower than other two sources of spermatozoa.

No significant difference was observed between grade I and grade II oocytes on fertilization rate, when fresh, frozen and epididymal spermatozoa were used for IVF. But a significant difference was observed between grade I and grade II oocytes in the mean yield of fertilized oocytes per ovary with fresh, frozen and epididymal spermatozoa.

The mean motility (per cent), concentration (x 10^6 /ml), percentage of live sperms, dead sperms, normal spermatozoa, abnormal heads, abnormal tails, proximal protoplasmic droplet and distal protoplasmic droplet of epididymal semen were 49.17±9.26, 37175±7612 , 84.5±8.02, 15.5±8.02, 35.67±2.30, 3.17±1.58, 2.33±0.61, 11.67±4.01 and 47.17±3.17 respectively.

The present study revealed that more number of grade I oocytes could be obtained by aspiration method from cow's ovaries than grade II oocytes (5.70±0.06 and 1.70±0.05). Grade I oocytes were matured better than grade II oocytes (88.20±0.75 and 69.21±1.97 per cent). But grade I and grade II oocytes did not differ significantly in their fertilizing ability after proper maturation (37.34±1.91 and 34.70±3.24 per cent). Epididymal spermatozoa retrieved from bulls after slaughter could be efficiently used for IVF of *in vitro* matured bovine oocytes equally or even better than the freshly ejaculated or frozen semen. Epididymal spermatozoa showed significantly more fertilization rate than fresh semen and this was closely followed by frozen semen (44.51±0.57, 37.86±0.47 and 27.72±0.89 per cent respectively).

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IN VITRO FERTILIZATION OF BOVINE OOCYTES USING FRESH, FROZEN AND EPIDIDYMAL SPERMATOZOA

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ABSTRACT

The study was designed to assess the fertilizability of bovine oocytes matured *in vitro* on co-culture with fresh, frozen or epididymal spermatozoa. The yield of oocytes and effect of cumulus oocyte complex (COC) morphology on *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) was also studied. A total of eighty one ovaries of abattoir origin from South Indian breeds like Hallikar, Kangayam, Khillari and crossbred cattle of Kerala were subjected to oocyte retrieval by aspiration method. Grade I (462) and grade II (138) COCs so obtained were subjected to maturation in separate groups for 24 h in Hepes modified TCM-199 enriched with sodium pyruvate, sodium bicarbonate, antibiotics, estradiol-17β, FSH, hCG and 20 per cent heat inactivated day zero estrus cow serum. Culture environment was set as 39° C temperature, five per cent CO_2 tension and maximum humidity in a standard CO_2 incubator. Maturation status of COCs was assessed by observing cumulus expansion and mucification.

Oocytes with maximum degree of cumulus expansion from each group (407 and 95) were subjected to IVF using fresh (n=169), frozen (n=162) and epididymal (n=171) spermatozoa in Fert-TALP medium supplemented with epinephrine, hypotaurine, pencillamine and heparin (5-10 oocytes in 100 µl droplet). Good quality spermatozoa isolated by percoll density gradient separation technique were used for IVF. Culture conditions set for IVF were 39° C temperature, five per cent CO_2 tension with maximum humidity in a standard water jacketed CO_2 incubator.

After 24 h co-culture in fertilization medium, the oocytes were evaluated for evidence of sperm penetration like presence of swollen decondensed sperm head, male pronuclei, two pronuclei, a clear second polar body and cleavage.

The total yield of oocytes by aspiration method per ovary was 11.59±0.10 (939 /81) and percentage yield of grade I, grade II and total culture grade oocytes were 49.20±0.31 (462), 14.70±0.41 (138) and 63.90±0.22 (600) respectively. Mean number of grade I, grade II and culture grade oocyte per ovary were 5.70±0.06, 1.70±0.05 and 7.41±0.07 respectively. The percentage and yield of grade I oocytes were significantly higher than grade II oocytes.

Cumulus expansion rates of grade I, grade II and total culture grade oocytes were 88.20±0.75 (407), 69.21±1.97 (95) and 83.67±0.35 (502) per cent respectively. The mean number of oocytes showing cumulus expansion per ovary from grade I, grade II and culture grade COCs were respectively 5.04±0.06, 1.18±0.03 and 6.22±0.06. Grade I oocytes showed significantly higher maturation rate and mean yield of matured oocyte per ovary than grade II oocytes.

The fertilization rates obtained with fresh, frozen and epididymal spermatozoa were respectively 36.52±1.68, 28.65±0.76, 46.53±1.32 for grade I; 45.00±5.63, 23.89±3.03, 35.20±4.62 for grade II and 37.86±0.47, 27.72±0.89, 44.51±0.57 per cent for culture grade oocytes. The mean number of oocytes fertilized per ovary by fresh, frozen and epididymal spermatozoa were respectively 1.87±0.06, 1.43±0.04, 2.33± 0.05 for grade I; 0.51±0.07, 0.31±0.05, 0.39±0.06 for grade II and 2.38±0.05, 1.73±0.07, 2.72±0.03 for culture grade oocytes. Significant difference was observed between three sources of spermatozoa for grade I and culture grade oocytes on fertilization rate and mean yield of fertilized oocytes per ovary. No significant difference could be observed between three sources of spermatozoa with respect to fertilizability when grade II oocytes were used.

There was no significant difference between grade I and grade II oocytes on fertilization rate of fresh, frozen and cauda epididymal sources of

spermatozoa. But the mean number of fertilized oocytes per ovary obtained from grade I oocytes was significantly higher than that from grade II oocytes for fresh, frozen and cauda epididymal sources of spermatozoa.

The overall fertilization rate obtained was 36.70±1.71 per cent and *in vitro* fertilized oocyte per ovary was 2.28±0.10 in the present study.

The mean motility (per cent), concentration (x 10^6 /ml), percentage of live sperms, dead sperms, normal spermatozoa, abnormal heads, abnormal tails, proximal protoplasmic droplet and distal protoplasmic droplet of epididymal semen were 49.17±9.26, 37175±7612 , 84.5±8.02, 15.5±8.02, 35.67±2.30, 3.17±1.58, 2.33±0.61, 11.67±4.01 and 47.17±3.17 respectively.

The present study revealed that more number of grade I oocytes could be obtained by aspiration method from cow's ovaries than grade II oocytes. Even though COC morphology has a significant role in maturation rate of oocytes, the fertilizing ability of grade I and grade II oocytes did not differ significantly after proper maturation. Epididymal spermatozoa retrieved from bulls after slaughter could be efficiently used for IVF of *in vitro* matured bovine oocytes equally or even better than the freshly ejaculated or frozen semen. Epididymal spermatozoa showed significantly more fertilization rate than fresh semen and this was closely followed by frozen semen (44.51±0.57, 37.86±0.47 and 27.72±0.89 per cent respectively).