EVALUATION OF OOCYTES RECOVERED FROM OVARIES OF BLACK BENGAL GOAT UNDER DIFFERENT RETRIVAL METHODS

MD. TARIKUL ISLAM

DEPARTMENT OF ANIMAL PRODUCTION AND MANAGEMENT
SHER-E-BANGLA AGRICULTURAL UNIVERSITY
DHAKA-1207

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BY

MD. TARIKUL ISLAM

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Approved by:

Dr. Md. Saiful Islam	**Prof. Dr. Md. Jahangir Alam**
Supervisor	Co-Supervisor

.

Dr. Md. Saiful Islam
Chairman
Examination Committee
Department of Animal Production and Management
Sher-e- Bangla Agricultural University
Dhaka, 1207

Department of Animal Production and Management
Sher-e- Bangla Agricultural University
Sher-e-Bangla Nagar, Dhaka-1207

CERTIFICATE

*This is to certify that thesis entitled, "**EVALUATION OF OOCYTES RECOVERED FROM OVARIES OF BLACK BENGAL GOAT UNDER DIFFERENT RETRIVAL METHODS**" submitted to the Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (MS) in ANIMAL SCIENCE**, embodies theresult of a piece of bona fide research work carried out by **MD. TARIKUL ISLAM**, Registration No. **12-05018** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed of during the course of this investigation, has duly been acknowledged.

Dated:
Dhaka, Bangladesh

_____
Dr. Md. Saiful Islam
Associate Prof. & Supervisor
Department of Animal Production & Management
Sher-e- Bangla Agricultural University
Dhaka, 1207

DEDICATED TO

MY BELOVED PARENTS

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ABSTRACT

Black Bengal goat is the national pride of Bangladesh. Ovary is the key female reproductive organ of all the vertebrates. With the aim to study the ovarian morphometry of Black Bengal goat, both right and left ovaries were collected from the slaughter houses. For each of the specimens, gross parameters such as right, left, corpus luteum (CL) present and absent group and evaluated on the basis of weight (g), length (cm), width (cm), follicles aspirated and number and state of cumulus-oocyte-complexes (COCs), normal COCs and abnormal COCs, the effect of collection techniques on cumulus oocyte complexes (COCs) of goat oocytes. Our study revealed that the right ovary (1.56□0.41 g) was heavier than the left (0.71□0.10 g). The length of the right ovary (1.33□0.24 cm) was lower than the left (1.91□0.25 cm) but the width of the right (1.25□0.18 cm) was greater than the left (0.97□0.25 cm). There is no significant differences were found in right and left ovaries. On the other hand significantly higher weight [(1.30±0.30) vs (0.98±0.17) g] and width [(1.08±0.24) vs (0.89±0.22) cm] were found in CL-present group than those of CL-absent group of ovaries. The recovery rates of grade A (31.11%) and B (22.41%) oocytes were higher than that of grade C (19.83%) and D (26.61%). It was observed that, the total number of COCs per ovary as well as the number of abnormal COCs/ovary were significantly higher (p<0.05) in slicing (3.28 and 1.44, respectively) and dissection (3.44 and 1.76, respectively) followed by aspiration (3.20 and 0.84, respectively) technique. In contrast, the number of normal COCs/ovary was significantly higher (p<0.01) in aspiration (2.36) followed by slicing (1.84) and puncture (1.68) techniques. Periods as 2, 4, 6 and 8 hours yield 78%, 69%, 60% and 53% oocytes counts of good, fair, poor and bad quality oocytes respectively. Yield of oocyte was highest in dissection technique compared with aspiration and slicing technique but maximum number of normal oocyte obtained from aspiration technique.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	= Percentage
>	= Greater than
<	= Less than
±	= Plus minus
AI	= Artificial insemination
BAU	= Bangladesh Agricultural University
BBG	= Black Bengal Goat
BLRI	= Bangladesh Livestock Research Institute
BW	= Birth weight
CC	=Cumulus cells
CL	=Corpus Luteum
COCs	=Cumulus Oocyte Complex
CV	= Coefficient of Variation.
DLS	= Department of Livestock Services
et al.	= Associate
FAO	= Food and Agricultural Organization
FF	=Follicular Fluid
GC	=Granulosa Cells
GDP	= Gross Domestic Product
GV	=Germinal Vesicle
gm	= Gram
IVC	=*In vitro* Culture
IVEP	=*In vitro* Embryo Production
IVF	= *In vitro* Fertilization
IVM	= *In vitro* Maturation
Ibs	=Pound
Kg	= Kilogram
MOET	= Multiple Ovulation and Embryo Trasfer
mL	= Milliliter

n	= Number of observation
NS	= Non-significant
PB	=Polar Body
PGCs	=Primordial Germ cells
SAS	= Statistical Analysis
SAU	= Sher-e- Bangla Agricultural University
SE	= Standard Error
WW	= Weaning Weight
ZP	=Zona Pellucida

CHAPTER I

INTRODUCTION

Bangladesh is an agricultural based country and livestock plays a vital role in national economy. Out of 920.60 million world goat population, Asia itself possesses about 551.23 million which is almost 59.8% of the total world population (FAO, 2010). Goats in Bangladesh are valued for their contribution for meat and skin. About 284 million pounds meat from goat is used for human consumption (FAO, 2010). Goat population in Bangladesh constitutes nearly 7.05% of the total population in Asia (FAOSTAT, 2009). Goat is numerically and economically very important and promising animal genetic resources in the developing countries like Bangladesh. Extensive research on *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of the resulting zygotes has so far been reported (Cognie *et al*., 2003) but limited information so far has been found on the evaluation of goat ovaries, the efficient collection and grading of oocytes. In Bangladesh, *in vitro* techniques in goat is a recent concept but a great deal of work has been still going on to standardize *in vitro* embryo production (IVEP) techniques followed by IVM and IVF (Ferdous, 2006; Islam *et al*., 2007; Mondal *et al*., 2008). Therefore, improvement of the traits is possible by individual selection and improving management because the goal of livestock department including goats is to produce a quantity of quality products with maximum efficiency. Therefore, collection and evaluation of goat ovaries will create vast opportunities to conduct the research work in the area of *in vitro* production (IVP) of goat embryos.

Goats are regarded as intimate and integral part of subsistence traditional rural farming system. These indigenous goats have an excellent ability to accommodate and adapt to fluctuation in environment. Although Black Bengal Goats are known as prolific breed, they often show some degree of reproductive failure. Infertility of does in many cases is manifested by abnormalities in the reproductive organs (tract and gonads). Ovaries are vital

organ that supplies the germ cells, oocytes and produce hormones for maintaining reproductive health. Primordial germ cells are formed and multiplied during fetal stage. Once these germ cells are differentiated and lose their motility while they are referred to as oogonia. The oogonia are proliferated by mitotic cell division. They enter meiotic cell division and are referred to as oocytes. Oocytes are enclosed by a single layer of squamous granulosa cells to form primordial follicles. Oocytes in primordial follicles grow accompanied by follicular development through primary, secondary and antral stages and are finally ovulated. It is found that remarkably lower numbers of ovarian follicles are considered as one of the major causes of infertility in Black Bengal goats (Amin *et al*., 2005). The level of reproductive performance depends on the interaction of genetic and environmental factors but this performance is particularly susceptible to latter, for example, the seasonal availability of nutrients can affect reproduction considerably (Riera 1982). Devendra and Burns (1983) stated that ability to accommodate to fluctuation in environment often involves some degree of reproductive failure. Several molecular factors are known to be responsible for the follicular development in mammals (Skinner 2005). The effect of environmental factors on the development of follicles is not understood well. Animals are exposed to adverse climatic conditions, e.g., acute sun shine, high temperature, humidity, rainfall and cool weather in tropical countries like Bangladesh. The effect of such environmental factors on morphology of follicles has not been understood.

The quality of the immature oocyte is determined by the quality of cumulus oocyte complexes (COCs) and oocyte diameter (Arlotto *et al*., 1996), but the result of aspiration of ovarian follicles are known have varying quality of cells (Lucas *et al*., 2002). The specific cause of the variation oocyte quality is unknown, but may be caused by a variety of follicle size and oocyte diameter were used, which relates to the process of folliculogenesis.

The conservation of indigenous and endangered species and their faster genetic improvement has been achieved by adopting modern biotechnological tools eg. Multiple ovulation and embryo transfer (MOET), *in vitro* fertilization (FVF), micromanipulation of gametes, cryopreservation of embryos and gene transfer etc. The refractoriness of the embryos of most domestic species by MOET to culture techniques, high costs and inconsistent outcome have impeded the progress in domestic species of economic interest.

Successful IVF in most species is dependant on availability of sufficiently large number of good quality oocytes. The fertile life span of a female can be extended by using immature as well as senile animals for the collection of oocytes. Several workers have conducted studies on producing embryos from oocytes recovered from the ovaries of slaughtered animals (Staigmiller and Moor. 1984; Wani *et al.*, 2000).

The method of in *vivo* collection of oocytes is expensive and the number of oocytes recovered per ovary is very small (Pawshe *et al.*, 1994); whereas ovaries of valuable slaughtered animals "a waste product" are the cheapest and the most abundant source of oocytes for large scale embryo production through *in vitro* maturation and *in vitro* fertilization (Agrawal *et al.*, 1995). *In vitro* matured and *in vitro* fertilized embryos are calculated to be five times cheaper than those obtained from superovulated donors (Wooliams and Wilmut, 1989). The abundant supply of oocytes and the resultant availability of pre-defined stage embryos provide opportunity to conduct experiments essential for understanding the regulatory mechanisms involved during preimplantation embryonic development (Le-Gal *et al.*, 1992).

Considering the above discussion, the present research study was undertaken with the following objectives:-

1. To evaluate oocyte recovery rates and their quality from ovine ovaries collected at local abattoir.
2. To characterize the ovary and oocyte physically in Black Bengal Goat.
3. To assess the quality of oocytes recovered from Black Bengal Goat under different retrieval methods.

CHAPTER II
REVIEW OF LITERATURE

2.1 Gametogenesis

Genetically and functionally competent gametes are a prerequisite for normal fertilization and early embryo development. The first phase in the sexual reproduction of an organism is gametogenesis, a process of formation of gametes from the germ cells in the testes and ovaries. This process is termed as spermatogenesis in the male and oogenesis in the female. It is the fundamental biological process in both the sexes and the key event of gametogenesis is the halving of the number of chromosomes to produce haploid germ cells (sperm and oocyte) through meiosis. Thus, in goat, where the chromosome number of somatic cells is 60, each sperm and each oocyte has only 30 chromosomes. However, until this point spermatogenesis and oogenesis resume their similarity. After this, in the male, each primary spermatocyte divides meiotically and produces four spermatids, each destined to become a functional sperm. In the female, on the other hand, out of the four cells produced from each primary oocyte only one finally becomes a functional oocyte.

2.2 Oogenesis

Oogenesis represents formation of female gametes, when oogonia formed from primordial germ cells (PGCs) reach the stage of primary oocytes. The process of oogenesis happens in the ovaries and starts three weeks after fertilization in the early fetal development with formation of PGCs (Edson *et al*., 2009), stops at birth and continues during puberty in the course of the reproductive life of the female (Rahman *et al*., 2008).

The maternal contribution to the development of the embryo is determined during formation and maturation of the female gamete, the oocyte. The ability of the oocyte to achieve sperm-oocyte fusion is acquired early in oogenesis or the process of oocyte formation. The oogenetic products synthesized during

oocyte growth must also be sufficient to support embryonic development from fertilization until the activation of the embryonic genome (Olszanska and Borgul, 1993). Ultimately, the nuclear and ooplasmic maturity of the oocyte influences the success of fertilization and embryo development. In mammals, oogenesis commences during early fetal development, stops at birth and continues during puberty throughout the reproductive life of the female. After continuation of meiosis, the oogenesis process until completion is very fast. Oogenesis in mammals includes seven steps: (a) generation of primordial germ cells (PGCs), (b) migration of PGCs to the prospective gonads, (c) colonization of the gonads by PGCs, (d) differentiation of PGCs to oogonia, (e) proliferation of oogonia, (f) initiation of meiosis and (g) arrest at the diplotene stage of first meiotic prophase or prophase 1 (Van den Hurk and Zhao, 2005).

Oogonia are the early germ cells in the ovary, which increase in number by mitosis. Oogonial multiplication begins during early fetal development and ends months to years later in the sexually mature adult (Picton *et al*., 1998). Once mitosis ceases, the oogonia then grow in size and enter the prophase of the first meiotic division at approximately day 55 of gestation in the ewe (McNatty *et al*., 1995) and are then referred to as primary oocytes (Wassarman and Albertini, 1994). Each oogonium or primary oocyte contains the diploid number of chromosome. The primary oocyte which is transformed from each oogonium is a cell which becomes enclosed in a follicle, known as primordial follicle. In goat, sheep and cow, large population (approximately 100,000) of primordial or pre-antral follicles with meiotically incompetent oocytes are present in the ovaries (Miyano, 2003; Miyano and Hirao, 2003; Zhou and Zhang, 2006). Most of them are lost at various stages of development owing to atresia and only a very minority of oocytes becomes available for ovulation. In contrast, Ariyaratna and Gunawardana (1997) found in their histological study that one pair of ovaries of Batu goat (a Sri Lankan goat breed) aged between 18 to 36 months contained 35,092 primordial follicles (which are 90% of total ovarian follicle population), 10.67 normal and 20.42 atretic vesicular (antral)

follicles (1-6 mm diameter). At birth, all oocytes from growing and dominant follicles are arrested at the diplotene stage of prophase 1 (Van den Hurk and Zhao, 2005). This dictyate stage is characterized by the enclosure of the chromosomes within the large nucleus also known as the Germinal Vesicle (GV) (Elder and Dale, 2000). The oocytes remain in the arrested state until a few hours before ovulation. Surprisingly, the oocytes may stay at this arrested stage for a longer period of time depending on the species, waiting for the signal to resume growth and subsequent development occurs at puberty. The age of puberty for female goat or doe is ranged between 5 and 7 months (Jainudeen *et al*., 2000). The reason for storing the oocytes in this remarkable frozen meiotic state is unknown (Johnson and Everitt, 1980).

2.2.1 Proliferation phase

In the first phase of oogenesis, primitive sex cords (medullary cords) were colonized by PGCs which undergo repeated mitotic divisions to form the primordial oocytes known as oogonia-stem cells. Oogonia, surrounded by somatic epithelial cells originating from genital ridge of mesenchymal cells, condense to form individual primordial follicles, which first develop in the inner areas of the ovarian cortex (Guraya, 2008). Then oogonia interrupt mitotic acitivity and enter meiosis (Goto *et al*., 1999). After meiosis has been started, the oogonial germ cell is called a primary oocyte. The primary oocyte is surrounded by a layer of follicular cells forming the primary follicle. Guraya (2008) reported that differentiation of oogonia into oocytes is closely followed up by their congregation with pregranulosa or follicular cells which get segregated from the somatic cells of the ovarian blastema.

In fetal ovary, the primary oocyte progress gradually, it enters meiosis I, over the different stages of prophase I and arrests at the diplotene stage of the first prophase with homologous chromosomes (Mandelbaum, 2000). At birth, all oocytes from growing follicles are arrested at the diplotene stage of prophase I. The prophase of the first meiotic division as long inactive phase lasts from

birth to reproductive age. The oocytes persist in the arrested stage until a few hours before ovulation. As generally known, oocytes of vertebrates are arrested in prophase of the first meiotic division for several weeks, months or years. The duration of this period depends on the species. The oocytes arrested in the diplotene stage of the first meiotic prophase are managed by a few determinants: follicle size, cumulus cell communication, gonadotropins, steroids, cAMP, cAMP-PDE, adenylate cyclase, PK-C, MAP-K, MPF and gene expression (Chaube, 2001). Cyclic AMP is the intracellular signaling molecule produced by granulosa cells and transported via gap junctions to the oocytes, which maintains the oocyte in the meiotic arrest (Desai *et al*., 2013).

Marteil *et al*. (2009) reported that the arrest in prophase of meiosis I correlates with the sensitivity to hormone activity which disenables early nuclear maturation of small oocytes. In the process of oogenesis, oocytes go thorugh different cytoplasmic changes. The first cytoplasmic alterations begins at the late diplotene stage of prophase I (Van Blerkom and Runner, 1984). In this protracted period, proportionally with increasing of their size, oocytes concentrate molecules of mRNA, increase a store of cytoplasmic enzymes, metabolic substrates, lipids and sugars necessary for their maturation and the starting of embryonic development (Marteil *et al*., 2009). In mammalian species, the developmental procedure commences when maternal RNA and protein are concentrated during growth and maturation of oocytes (Telford *et al*., 1990).

2.2.2 Oocyte growth and development

The growth and development of an oocyte occurs inside an ovarian follicle and oocyte undergoes a progressive series of morphological modifications as it grows and proceeds through the different stages of development (Eppig *et al*., 1994). Although data are lacking for doe, Ariyaratna and Gunawardana (1997) indicated from their study that follicular morphology and activity are similar in does and ewes. In ewe, primordial, primary and secondary follicles,

respectively, appear in the fetal ovary at days 75, 100 and 120 (McNatty *et al*., 1995). Once a primordial follicle oocyte is activated to grow, it embarks on a complex journey that involves numerous molecular and morphological changes to both the oocyte and the follicle. The modifications are carefully orchestrated and require sensitive communication between the oocyte and surrounding Granulosa Cells or GCs (Fair, 2003). These structural rearrangements facilitate the increasing energy and nucleic acid synthesis requirements of the developing oocyte and are a prerequisite to the oocyte achieving meiotic competence and embryo developmental potential. The first sign of morphological change when the oocyte begins to grow is turning of the flat GCs to cuboidal which is known as primary follicle. After completion of the morphological change, the GCs proliferate actively, which cause the follicles to develop and increase in size. Through a series of mitotic division of GCs, unilaminar primary follicles are converted to multilaminar secondary follicles, followed by the antral or tertiary follicles (Miyano and Hirao, 2003). The antral follicle is a highly complex unit consisting of several layers of GC surrounding a fluid-filled cavity or ‘antrum` in which the oocyte surrounded by somatic cells is bathed. In the doe, antrum formation began when the GCs are about six cell layers in thickness and the Zona Pellucida (ZP) is visible at this stage (Ariyaratna and Gunawardana, 1997). The fluid found in the ‘antrum` is known as Follicular Fluid (FF). During this growth phase there is a major increase in ooplasmic organelles. The follicle provides a microenvironment for oocyte growth, development and is responsible for the production of hormones. The walls of mature preovulatory follicles consist of membrana granulosa (mural granulosa), theca interna and theca externa. The GCs are cells of epithelial origin essential for the growth and survival of the oocyte. The GCs consist of the corona radiata; Cumulus Cells (CCs), membrana granulosa and antral granulosa cells. The CCs surround the oocyte, which nourish the oocyte, are involved in oocyte growth, maturation (Buccione *et al*., 1990) and participate in the formation of the ZP (made of a translucent acellular layer of glycoprotein). In addition, these cells have also been implicated in the modulation or generation of oocyte maturation

inhibitors (Tsafriri *et al*., 1982; Eppig and Downs, 1984). The CCs in close contact with the oocyte are known as corona radiata. They are in close contact with the oocyte through ooplasmic extensions or processes across the ZP (De Loos *et al*., 1991), which are known as gap junctions. The heterologous gap junctions provide the basis for extensive network of intracellular communication among GCs. Normally CCs or corona cells surrounding the goat oocyte shed ≥30 h after ovulation (Harper, 1982).

2.2.3 Oocyte maturation

As mentioned earlier that oocytes are arrested at the diplotene stage of the prophase 1 at birth, they resume meiosis after a long quiescent phase at puberty which involve sequential sub-cellular and molecular transformations by various components of the follicle. During postnatal life, starting from puberty, ovarian follicles continue to grow, mature and either ovulate or regress. Follicles are recruited continuously until the original store is exhausted. Reinitiation of meiosis in the fully-grown oocyte is the first sign of oocyte maturation, which involves condensation of interphase chromatin, breakdown of nuclear membrane (germinal vesicle breakdown: GVBD), spindle formation and chromosome segregation. *In vivo*, resumption of meiosis is initiated by a preovulatory LH surge and only occurs in fully grown, meiotically competent oocytes from dominant follicles. Small oocytes in primordial and primary follicles have no ability to resume meiosis. Oocytes acquire the competence to resume meiosis when their size exceeds 80% of their final diameter; they then become gradually competent to progress to metaphase 2 (MII) as the diameter increases to over 90% of the maximum (Miyano and Hirao, 2003). The diameter of mature goat oocytes excluding ZP (ooplasm) ranged from 119-146 μm (De Smedt *et al*., 1994; Crozet *et al*., 2000). Diameter of mature oocyte in different animals. During this follicle and oocyte growth phase, oocytes not only acquire competency to resume meiosis, but also acquire ooplasmic maturity, also known as oocyte capacitation, both of which are required to ensure normal fertilization and embryo development (Gosden *et al*., 1997;

Hyttel *et al*., 1997). From *in vitro* studies, it is found that goat oocytes acquired the ability to initiate meiotic resumption in early antral follicles of 0.5 to 0.8 mm in diameter and to reach MI in follicles of 1.0 to 1.8 mm in diameter (De Smedt *et al*., 1994). Although 86% of goat oocytes from follicles larger than 2 mm progress to MII (De Smedt *et al*., 1992), only a small proportion of them can support embryonic development (Crozet *et al*., 1993). In cattle, oocytes originating from follicles larger than 6 mm in diameter yield a significantly higher percentage of blastocyst than the smaller follicles (Lonergan *et al*., 1994). In an *in vitro* study, a significantly higher oocyte maturation, morula and blastocyst development rates were achieved with goat oocytes originating from larger than 5 mm follicles compared with medium (>3-5) and smaller (2-3) follicles (Crozet *et al*., 1995). To reach the maturity, goat oocytes (ooplasm) grow from 29.6 μm in primordial follicles to 119 to 146 μm (De Smedt *et al*., 1994; Crozet *et al*., 2000) in antral follicles (>2 mm in diameter). The ZP of a goat oocyte from antral follicle bigger than 2 mm is about 3.2 μm thick (Ariyaratna and Gunawardana, 1997). The growth of goat oocyte in relation to follicular growth in the ovary as described by Ariyaratna and Gunawardana (1997).

Before and at the time of LH surge, the oocyte is surrounded by a compact CCs investment. The oocyte undergoes a series of changes in its nucleus, ooplasm and organization of the plasma membrane (oolemma) during the period between the LH surge and ovulation, which is known as oocyte maturation. Completion of the meiosis-1 takes place when oocytes have undergone extensive growth in cellular interaction with GCs and theca cells. The oocyte undergoes asymmetric cytokinesis and extrudes the first polar body (PB-1) containing a haploid chromosome complement (Kupker *et al*., 1998). Immediately after the meiosis-1 is completed, the meiosis-2 is initiated and the oocytes are again arrested at MII stage until fertilization, when an activation stimulus provided by sperm penetration triggers the completion of the meiotic cycle and initiates embryonic development. Ooplasmic maturation is required to acquire to the conditions to block polyspermy in case of fertilization, to

decondense penetrated spermatozoon and to form pronucleus (PN). It includes redistribution of cell organelles, migration of mitochondria to perinuclear position and accumulation of granules along the oolemma (Van den Hurk and Zhao, 2005). The endpoint of this, *in vivo*, is the ovulation and release from the follicle of a MII oocyte with potential to support normal embryonic development (Elder and Dale, 2000). However, the doe ovulates two to three mature oocytes in each estrous cycle (Jainudeen *et al*., 2000). Compared with human or mouse, goat oocyte is dark in opacity (Betteridge, 2003) due to very dense ooplasm consisted of concentrated lipid materials (Keskintepe *et al*., 1997).

2.2.4 Nuclear maturation

The oocyte contains a large germinal vesicle (GV) with a large nucleolus, before maturation starts. Nuclear maturation means the meiotic process of chromosomal reduction to a haploid content, so as to produce a diploid organism upon fusion with sperm (Voronina and Wessel, 2003). Throughout nuclear maturation, redistribution of the cytoskeleton eventuate. This process includes actions correlated with the germinal vesicle breakdown (GVBD), condensation of chromosomes, metaphase I spindle formation, separation of the homologous chromosomes with extrusion of the first polar body and arrest at metaphase II (Josefsberg *et al*., 2000). With the second meiotic division, chromatids separate and the second polar body is formed. Finally, the chromatids remaining in the oocyte decondense and a pronucleus forms. Niimura *et al.* (2002) observed lipid droplets in porcine oocytes and reported that alteration in the size of lipid droplets is associated with nuclear maturation. For completion of nuclear maturation, bovine oocyte need 24 h (Sirard *et al*., 1989), and porcine 44 h (Kim *et al.* 2011). After successful nuclear maturation, an oocyte arrested in metaphase II is formed. In this phase the oocyte is physiologically relevant to finish the second meiotic division in the process of fertilization.

2.2. 5 Cytoplasmic maturation

For cellular functions during maturation, fertilization and early embryo development, the cytoplasm of oocytes ensures useful metabolic conditions for the production of energy indispensable in these processes (Cetica *et al*., 2002). Cytoplasmic maturation means both the ultrastructural changes occurring in the oocyte from the GV to the MII stage and the acquisition of developmental competence of the oocyte (Duranthon and Renard, 2001). Because of this characteristic, there is considerable interest in the cytoplasmic maturation of mammalian oocytes.

Cytoplasmic maturation is regulated and greatly affected by hormone fluctuations. During maturation, the alteration of hormonal concentration probably has a substantial pertinence for the secretory activity of cumulus cells, a subset of granulosa cells. Accordingly, meiotic or cytoplasmic maturation of oocytes may be affected indirectly by the grade and continuance of hormonal exposure of cumulus cells, surrounding maturing oocytes as cumulus oophorus inside follicle.

Cytoplasmic maturation includes organelle rearrangement, storage of mRNAs, proteins and transcription factors which participate in the process of maturation, fertilization and early embryogenesis (Ferreira *et al*., 2009). The same author classified cytoplasmic maturation into three phases: organelle distribution, cytoskeleton dynamics and molecular maturation.

2.2.6 Maturation in vivo and *in vitro*

The liberation of a mature oocyte from the follicle with the competence to support regular ermbryonic development is the final stage of maturation *in vivo* (Fulka *et al.*, 1998). Oocyte maturation *in vivo* is triggered by the preovulatory surge of gonadotropins via granulose cells (Eppig, 1991) and also cumulus expansion can be induced with FSH or LH hormones (Hillensjo and Channing, 1980). It is probable that spontaneous maturation *in vivo* lasts longer than *in vitro* maturation induced by hormones. Pincus and Enzmann (1935) first described meiotic maturation of mammalian oocytes *in vitro* in rabbits. A

number of studies suggest that oocytes isolated from large antral follicles of many species promptly advance to metaphase II in culture and undergo fertilization in a high percentage of instances. However, their developmental potential after fertilization is low. From these facts, it can be concluded that *in vivo* conditions are greater in comparison with *in vitro*. For the provision of energy during maturation, cumulus cells have an effect on the oocyte, because they manage the nutritive repository, across the control of fatty acid lipolysis and synthesis. During *in vitro* maturation, absence of cumulus cells influence metabolism of lipids and causes substandard maturation (Auclair *et al.,* 2013). Oocytes of different species required different intervals for *in vitro* maturation. The porcine oocytes achieved metaphase II after 44 h of maturation culture (Abeydeera *et al*., 1998; Gonzales-Figureueroa and Gonzales-Molfino, 2005), bovine after 24 h in maturation medium (Luna *et al*., 2001; Smiljakovic and Tomek, 2006), human from 24 h to 48 h (Roberts *et al.,* 2002) and equine between 24 and 32 h (Hinrichs *et al.,* 1993).

2.3 Meiotic competence

The meiotic competence represents the ability to resume and complete the first meiotic division and to arrest at the second meiotic metaphase. The ability to resume and complete the first meiotic division is connected to the process of oocyte growth, this is the reason for the complicated process of meiosis. After isolation and oocyte culture, the oocytes do not sustain spontaneous maturation until the final stage of growth, when oocyte acquire competence for spontaneous maturation *in vivo*. Evaluation of energy cytoplasmic markers during process of *in vitro* maturation and investigation of changes in this section during achievement of meiotic competence for development of oocytes is very important. Beyond meiotic competence, the oocyte has to complete its differentiation by acquiring the ability to support the cytoplasmic maturation and finally the ability to be successfully fertilized and develop into a viable embryo. The final differentiation of meiotically competent oocytes occurs at the end of folliculogenesis. Oocyte capacitation comes in the late period of oocyte differentiation.

Meiotic competence of porcine oocytes has been associated with oocyte size, nucleolar structure and function (Motlik *et al*., 1984). As generally known, the cells derived from large follicles are more competent compared to those obtained from smaller follicles. In early antral follicles, oocytes become able to resume meiosis but only in growing antral follicles they become able to complete meiosis up to metaphase I and progress to metaphase II (Marchal *et al.,* 2002). Porcine oocytes acquire meiotic competence gradually during growth of follicles (Motlik and Fulka, 1986). Marchal *et al*. (2002) reported that porcine oocytes acquired meiotic competence in ovarian follicles with a diameter of 3 mm and more. In growing porcine oocytes, acquisition of meiotic competence depends on their capacity to activate MPF and MAP kinase (Kanayama *et al.,* 2002). The acquisition of meiotic competence during follicular growth was also described in mouse (Eppig and Schroeder, 1989) and ruminant oocytes (Mermillod *et al.,* 1999).

2.4 Developmental competence

Developmental competence means the ability of matured oocytes to undergo fertilization, subsequent embryo cleavage, and embryonic development. Internal ability of development is reflected in the quality of oocytes. This is associated to biochemical and molecular processes which support maturation and fertilization of oocytes and enable subsequent embryo development (Gilchrist *et al.,* 2008). The developmental potential of an embryo is reliant on developmental potential of oocyte from which it derives. Mattioli *et al*. (1989) for the first time described *in vitro* developmental competence of porcine oocytes. Many immature oocytes are able to terminate meiosis *in vitro*, but only a small proportion of them is competent to continue development to the blastocyst stage (Krisher and Bavister, 1998).

According to Sirard *et al.* (2006), developmental competence of oocytes consists of a few important factors: meiosis resumption, cleavage following fertilization, capability to develop to the blastocyst stage and ability to induce

pregnancy and bring it to term in good health. The cumulus cells surrounding the oocyte have an impact on acquisition of oocyte developmental competence.

The quality and developmental competence of mammalian oocytes are characterized by specific cytoplasmic factors: lipids, microtubules, relocation of mitochondria and ATP production. The mature oocytes with high developmental competence have a different lipid structure in comparison with immature oocytes (Ami *et al*., 2011). The study of Kim *et al.* (2001) indicates that the fatty acids located in lipid droplets of bovine oocytes are important for oocyte competence. A low developmental competence is related to deficiency of microtubule network which prevents appropriate relocation of mitochondria (Brevini *et al.,* 2005). Several authors already confirmed differences among morphologically good and poor oocytes indicating that activity and relocation of mitochondria is in correlation with their different deveopmental ability after *in vitro* fertilization. Failure of mitochondrial function and insufficiency of ATP production has been associated to reduced developmental competence (Dalton *et al*., 2014). The nuclear transfer experiments indicated that decline of developmental competence was linked with cytoplasmic defects (Mermillod *et al.,* 1998).

The follicle ambience and maternal signals, transmitted via granulosa and cumulus cells, are important for the progressive gain of developmental competence and as a support for oocyte growth (Gilchrist *et al*., 2008). Khatir *et al.* (1997) reported important information that calf follicular fluid, irrespective of the size and quality of the follicle from which it originates, stimulates the acquisition of developmental competence by adult oocytes during maturation, as does adult follicular fluid, but is inactive on prepubertal oocytes. However they concluded that the follicular environment is not responsible for the low developmental competence of prepubertal oocytes, as these oocytes are unable to respond to the stimulatory components of follicular fluid. Koenig and Stormshak (1993) described differences between pubertal and third-estrous gilts, when embryos delivered during the first-estrus cycle are

not fully competent for *in vitro* development. The results of Menino *et al*. (1989) show that the reduced *in vitro* development of embryos collected from gilts mated at the first estrus may be due to an aberration in blastocoel formation and expansion.

The developmental competence of oocytes matured *in vitro* is lower in comparison with oocytes matured *in vivo*, reducing the general effectiveness of *in vitro* maturation process. According to Rodriguez and Farin (2004), oocytes matured in the presence of gonadotropic hormones have a better developmental competence. The acquisition of developmental competence of bovine oocytes increases with the age of the calves, and full developmental competence of oocytes is accomplished before puberty (Presicce *et al*., 1997). Volarcik *et al*. (1998) reported that the developmental competence of human oocyte decreases with age.

2.5 Folliculogenesis

The ovarian follicles are basic structural and functional unit of the ovary and they ensure micro-conditions essential for oocyte development and maturation (Abd-Allah, 2010). The process of ovarian follicle development is known as folliculogenesis. The process of folliculogenesis starts during foetal development in many mammalian species, involving porcine (Bielanska-Osuchowska, 2006). The same author reported that the creation of ovarian follicles starts on day 56 p.c., while follicles encircled by a single layer of somatic cells are present on day 106 p.c. During oestrus cycle, porcine females express only one wave of follicular activity (Ratky and Brussow, 1998). During the early phases of gestation, follicles are already present in the bovine fetus (Erickson *et al.*, 1966).

Process of folliculogenesis in different animals has been investigated *in vivo* and *in vitro* in many laboratories. Through this process, an ovarian follicle passes through several stages: primordial-resting, primary, secondary-preantral, tertiary-antral, and the preovulatory-Graafian follicle stage. Rüsse (1983) reported first appearance of primordial, primary, secondary and early antral

follicles in bovine fetus. Several million primordial follicles are present in the ovaries during the fetal period containing an oocyte surrounded by layers of somatic granulosa and theca cells (Knight and Glister, 2006; Krisher, 2013). The primordial follicles go through consequential growth and development forming preovulatory follicle containing a mature oocyte (Volarcik *et al*., 1998). The preantral phase is characterized by zona pellucida formation, granulosa cell proliferation, the recruitment of thecal cells to the follicular basal lamina and a dramatic increase in oocyte volume (Pedersen, 1969). Antral follicle is characterized by the existence of antrum in the granulosa and theca externa and a fibrous layer around theca interna. In the preovulatory follicle, the fully grown oocyte has collected nutrient stores, mRNA, proteins and organelles, also great number of mitochondria (Dunning *et al*., 2014). Immature mammalian oocytes liberated from ovarian follicles proceed in meiosis and complete *in vitro* maturation.

The beginning phase of folliculogenesis starts independently of gonadotrophic hormones (Roche, 1996). However, the oocytes resume meiosis throughout progress from preantral to antral stage and after stimulation by pituitary gonadotropins FSH and LH, the mature oocyte is removed from the follicle and transfer into the oviduct (Uhlenhaut and Treier, 2011). Findings of Tanaka *et al*. (2001) demonstrated that in bovine fetal ovary, the serum concentration of FSH may play an important role as an initiator of early follicular development. After formation of follicular antrum, which roughly corresponds to the final stage of oocyte growth, granulosa cells differentiate into the mural granulosa cells which have a steroidogenic role and cumulus cells which create a close relatedness with the oocyte (Gilchrist *et al.,* 2008).

In mammals, folliculogenesis is a greatly selective process that includes steps of differentiation and proliferation of somatic and germ cells (Hernandez-Medrano *et al.,* 2012). During prospering from the preantral to the antral stage, the follicle is structurally changed to three separate populations of somatic cells: theca cells, granulosa cells and cumulus oophorus (Piotrowska *et al*.,

2013). After activation of growth, the granulosa cells start to proliferate, at the same time the oocyte initiates the growth stage. After this processes of initiation, development of follicle consists of proliferation and differentiation of the granulosa layers and the oocyte development. Roche (1996) reported that inhibins, activin, insulin-like growth factor I (IGF-I) and their binding proteins have direct and indirect effects on granulosa and theca cells that can modulate follicular development and steroidogenesis. During oocyte growth and maturation, the cumulus cells are metabolically linked with the oocyte and these cells are involved in the process of ovulation and fertilization (Tanghe *et al.,* 2002).

The quality achievement of oocytes, their maturation, fertilization and development are complex processes and the size of follicles from which oocytes are obtained have a significant influence on their maintenance. Size of the follicle from which the oocyte is derived affects the oocytes ability to resume meiosis and reach optimum maturation during IVM (Hyttel *et al.,* 1997). The meiotic and developmental capability of oocytes is achieved gradually, during development of follicles (Eppig *et al.,* 1994; Schramm and Bavister, 1995). According to Marchal *et al.,* (2002), developmental competence rises simultaneously with the size of follicles. The porcine oocytes acquire capability to complete meiotic maturation when they accomplish their full size in antral follicles of approximately 2 mm of diameter and at the same time, the transcriptional activity of oocytes decreases (Motlik *et al.,* 1984).

In vitro embryo production (IVP) represents a method for enhancing the population of genetically valuable animals (Pfeifer *et al.,* 2008). The examination of folliculogenesis is significant for improving of IVP techniques. Machatkova *et al*. (2004) reported that interaction among follicle size and the phase of follicular wave has an impact on embryo production. The production of embryos was primarily affected by the number of oocytes collected from medium follicles and the lower developmental competence of oocytes from small follicles. The production of embryos *in vitro* is usually diminished, which

implies that all oocytes are not capable of successful *in vitro* maturation and fertilization. The quality of oocytes and *in vitro* conditions are the primary elements which characterize development of embryo and production of normal offspring.

2.6 Harvesting of oocytes

The number of good quality oocytes harvested from the ovary is an important consideration in the *in vitro* production of embryos. Oocytes for IVF are collected from one of the following sources; the oviducts soon after ovulation, mature follicles shortly before ovulation or immature and antral follicles usually from abattoir material (Wani, 2002).

2.6.1 Collection of ovaries

Ovaries of abattoir origin are generally used for the production of embryo in domestic animals. The ovaries of slaughtered animals are the cheapest and most abundant source of oocytes for large scale production of embryos through IVM- IVF (Agrawal *et al.*. 1995). The sheep ovaries obtained from slaughter house were brought to the laboratory in a normal saline solution or Dulbeccos phosphate buffered saline solution (Pugh *et al*, 1991). 20°C (Slavik *et al*, 1992), or at room temperature (Watson *et al*, 1994; Wani *et al.*, 2000). The time interval between collection of ovine ovaries and harvesting of oocytes also varied from 1-2 (Pugh *et al.,* 1991) to 3-4 h (Wani *et al,* 1999) without any deleterious effect on oocyte maturation. Similarly, Snyder, (1978) collected ovine ovaries after slaughter and transported them for about 3h at 30°C, 37°C and 21°C. The proportions of ova mattiring were 16.7, 50 and 37.8 per cent respectively, indicating 37°C to be the best temperature for transportation of ovine ovaries. In pigs, storage of ovaries for 2-3 h at 33-35°C was considered to be best (Sato *et al.*, 1977). Likewise, bovine ovaries can be stored for 11 h at 24-25°C (Yang *et al.,* 1990) and 8 h at 20=C (Gordon and Lu, 1990). 8.5 h at 20-35=C (Sato *et al, 2011)* without any significant effect on the maturation and fertilization of recovered oocyte. Many investigators have reported that the stage of estrous cycle (Leibfried and First, 1979: Fukui and Sakuma, 1980;

Leibfried-Rutledge *et al,* 1985: Tan and Lu, 1990) and pregnancy (Vazta *et al.,* 1992) of the donors did not influence the developmental potential of the isolated oocytes; the selection of ovaries was based on reproduction status, therefore, appears non-significant.

The age of animal had no effect on the number and quality of ovine oocytes harvested (Ward *et al.,* 1999) and maturation, fertilization or cleavage rates (0-Brien *et al.*. 1997). However, a greater number of IVM oocytes developed into blastocysts from adult sheep ovaries, compared to those from lambs. There was, however, no difference in pregnancy rates obtained (0-Brien *et ah,* 1997). Oocytes had also been harvested from the ovaries of prepubertal lambs for successful IVM - IVF (Salykbaev *et al.,* 1986; Armstrong *et al.,* 1994).

Presence/ Absence of corpus luteum (C.L.) on ovaries had marked effect on total number of oocytes recovered. Ward and co-workers (1999) reported higher oocyte recovery rates in ovaries without C.L. compared to ovaries with C.L. (10.5 ± 0.2 vs 4.7 ± 0. 4 oocytes / ovary, respectively). The cause of a lower number of oocytes with a C.L. might be attributed to the fact that C.L. inhibits the growth of follicles and increases their atresia (Hafez, 1993). The ftmctional activity of ovary also had an effect on IVM - IVF and development of follicular oocytes. The maturation rates of oocytes collected from fimctionally active and inactive ovaries were 76.9 and 7.7 per cent respectively (Im *et al.,* 1995). The size of the ovary also affects number and quality of oocytes obtained by aspiration of visible follicles. The large ovaries (>5x7x9mm") yielded 8.4 = 0.4 oocytes / ovary' compared to small ovaries (<5x7x9mm^) which yielded 6.1 i 0.5 oocytes / ovary following aspiration (Wani *et* al., 1999). It might be attributed to the low number of visible follicles present on the small ovaries, whereas the large sized ovaries had a good number of visible follicles for aspiration.

2.6.2 Isolation of oocytes

The recovey of sufficient number of good quality oocytes from sheep ovaries is of paramount importance. *In vivo* matured oocytes were obtained either by surgical or laparoscopic techniques (Baldassarre *et al.,* 1996). These methods were expensive and the number of oocytes recovered per ovary was small (Pawshe *et al.,* 1994). The procedures like laparoscopy (Lambert *et al.,* 1986) and transvaginal ultrasound guided techniques (Pieterse *et al.,* 1992) had also been employed to aspirate follicular oocytes in bovines.

Several techniques had been used for the collection of oocytes from ovaries of abattoh origin in cattle (Katska, 1984; Katska and Smorg, 1984: Iwasaki *et al,* 1987), goats (Mogas *et al,* 1992; Pawshe *et al,* 1994) and sheep (Wahid *et al,* 1992a b; Wani *et al,* 1999). The methods commonly employed to recover oocytes from abattoir ovaries were dissection of follicles, aspiration of visible antral follicles, puncturing of follicles, slicing and dissecting of ovaries. Follicular dissection was first used to recover ovine follicular oocytes (Crosby *et al,* 1981; Fukui *et al,* 1988). Currently, slicing (Wahid *et al,* 1992a b) and aspiration (Slavik *et al,* 1992; Watson *et al,* 1994; Wani *et al,* 2000) are being employed routinely for oocyte recovery- in sheep.

Wani and associates (1999) subjected 47, 61 and 51 ovaries to puncturing, slicing and aspiration technique, respectively, for recovery of oocytes. The total number of oocytes recovered per ovary by puncture (9.4 ± 0.45) and slicing (9.5 ± 0.4) were significantly higher than aspiration (6.8 ± 0.3). The percentage of good quality oocytes. however, was higher for aspiration (64.4%) compared to puncturing (54.7%) and slicing (54.3%). On the contrary, the oocyte recovery rates for puncturing and aspiration of ovine ovaries were 84.9 and 57.6 per cent, respectively, from sheep ovaries (lorenzo *et al.,* 1999), however, both the methods yielded similar quality oocytes. In goat, slicing yielded more oocytes per ovary (6.05) than dissection (1.71) or aspiration (1.25), however, *in vitro* fertilization capacity of oocyte obtained by slicing method was lower

(18.2 vs 29.1%) than those obtained by dissection (Martino *et al.,* 1994). The lower number of oocytes recovered by aspiration may be attributed to the presence of some follicles embedded deep within the cortex, which were released by puncture or slicing of the ovary. Some of the oocytes might even be lost during aspiration of follicles, whereas, slicing or puncturing reduces these chances (Wani *et al.,* 2000).

2.6.3 Selectiion of oocytes

The selection of oocytes is an important step which determines the success of *in vitro* development of immature oocytes. The presence of a compact and healthy population of cumulus cells surrounding the oocyte has been universally used to characterize the culturable quality oocytes and to assess their development potential *in vitro* (Lonergan *et ah,* 1992). Higher *in vitro* maturation, fertilization and cleavage rates had been achieved in compact cumulus enclosed bovine oocytes (Leibfried and First, 1979; Yang and Lu, 1990; Cox *et al.,* 1993). Similarly, selection of immature sheep oocytes had been based on compactness and number of enclosing cimiulus layers (Pawshe *et al,* 1994) as well as cytoplasmic characteristics (Wani *et al.,* 1999, 2000). The denuded bovine oocytes had lower frequencies of maturation and fertilization *in vitro* (Kim and Park, 1990; Lorenzo *et al.,* 1995) and none of them progressed to the blastocyst stage *(YangetaL* 1990).

Cetica and associates (1999) empliasized that class A oocytes in bovine were most likely to mature *in vitro* as they had a close association with their surrounding cumulus cells. Good and poor graded bovine oocytes showed maturation rate of 75 and 58.8 percent, respectively, indicating better maturation rates of good quality oocytes (Im *et ah,* 1995).

Size of the follicles / oocytes also influences the quality and developmental competence of recovered oocytes. The oocytes with a larger diameter produce morula and blastocyst at higher rates (Arlotto *et al.,* 1992). Ledda and

associates (1999) reported that the meiotic competence of the *in vitro* matured prepubertal and adult sheep oocytes were affected by the follicular size. Non-atretic follicles were dissected from ovaries of prepubertal and adult sheep and depending on follicle diameter were divided in three groups (<1, 1-2 and >2mm). On maturation, a lower percentage of adult and prepubertal oocytes of <lmm reached metaphase-II than those obtained from l-2mm and >2mm groups (70.4 vs 89.5 and 95.5% for adult ovine oocytes and 27.2 vs 79.8 and 81.8% for prepubertal ovine oocvies, respectively). The results indicated that oocytes with the same diameter derived from different follicles showed similar meiotic progression rates.

CHAPTER III

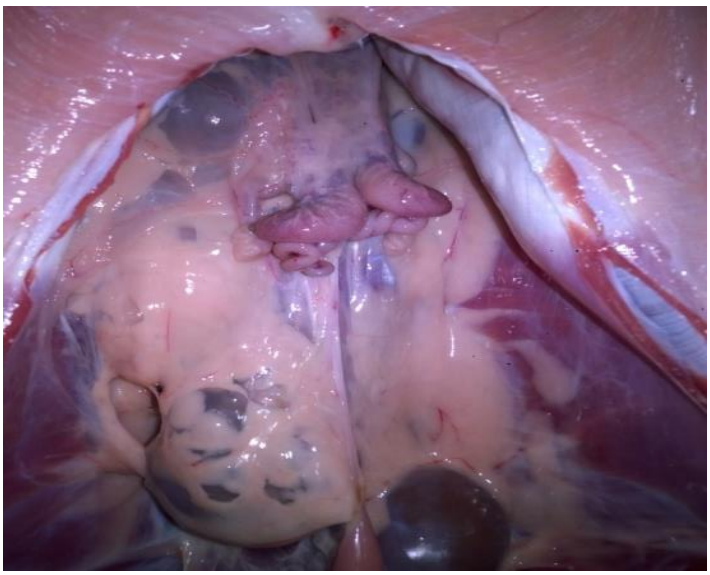
MATERIALS AND METHODS

3.1 Experimental site

The study was conducted in the laboratory of the Department of Animal Production and Management, Faculty of Animal Science and Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka.

3.2 Collection and transportation of ovaries

Both right and left ovaries of the female adult Black Bengal goats were collected from the slaughter houses and kept in collection vial containing 0.9 % physiological saline in a thermo flask at 25 °C to 30 °C and transported to the laboratory within 4 to 5 h of slaughter and transferred to sterilize Petri dishes with physiological saline solution before further processing. The ovaries were then transferred to sterilized petridishes and rinsed thoroughly by physiological saline solution at 25 °C before further processing (Haque *et al*. 2016).

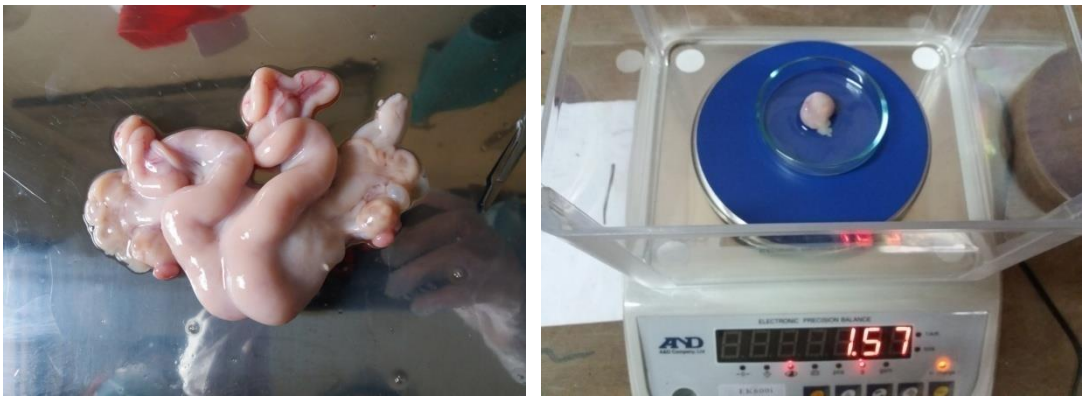


Palate 1. Collection of ovary at abattoir

3.3 Measurement of weight, length and width

After trimming individually right and left of ovaries, corpus luteum (CL)-present and absent ovaries were weighed (g) and recorded in tabular form. The length and width in (cm) of the right, left, CL-present and absent group ovaries were measured with the help of a measuring scale (Haque *et al*.2016).

All antral follicles for each ovary were counted and classified according to their diameter into small (<3mm), medium (3-5mm) and large follicles (>5mm) (Harris *et al*.2014).



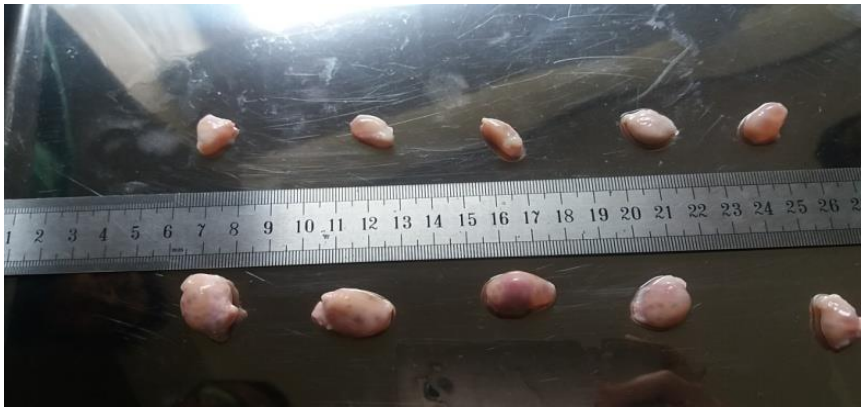
A B

C **D**

Palate 2. Measurement of ovary A) Ovary processing B) Measurement of weight C) Measurement of length D) Measurement of width

3.4 Physical evaluation of ovaries

Upon arrival at the laboratory the ovaries were washed three times in phosphate buffer saline (Gordon, 1994). By visual estimation, ovaries were divided into two groups depending on the presence or absence of corpora lutea (CL): CL bearing and non-CL bearing. The weight, length and width of the ovaries were measured.



Palate 3. Classification of the ovaries

3.5 Oocyte recovery

The occyte was collected aseptically from ovaries by three techniques (Ramsingh *et. al*.2013).

3.5.1 Aspiration technique

The visible follicles present on the surface of the ovary were aspirated with 22 G needle fixed to 5ml disposable syringe containing 1-2 ml of DPBS. The goat oocyte were aspirated from individuals ovary and placed in petridish containing 1 ml of Phosphate Buffer solution (PBS). Number of collected oocytes was recorded after grading; the only fair and good quality oocytes were subjected to the IVM and further IVF steps.

3.5.2 Dissection technique

The ovaries were placed in a sterile glass petridish containing 2 ml of DPBS. All the visible follicles were carefully subjected to blunt dissection with the

help of forceps. The follicles were ruptured and the follicular fluid will be allowed to flow into the DPBS.

3.5.3 Slicing technique

The ovaries were held firmly with the help of foreceps in a sterile glass petri desh containing 2 ml DPBS. The ovaries were sliced into possible thin sections. The oocytes containing DPBS media will be transferred to the petri desh and observed under microscope.

A

B

C

Palate 4. Different collection techniques A) Aspiration technique,

B) Disection technique, C) Slicing technique.

3.6 Microscopic study of ovaries

The numbers of visible follicles on the surface of different category of ovaries were counted and recorded. The ovaries were washed 2 to 3 times in saline solution at 30°C. Ten milliliters syringe was loaded with PBS (1~1.5 ml), and

the needle (19 G) was put in the ovary parenchyma near the vesicular follicles of more than 2 mm diameter and all follicles were aspirated near the point. After aspirating the follicles from one ovary, the aspirated follicular materials were transferred slowly into a 90-mm petridish, avoiding damage of the cumulus cells and the Cumulus Oocyte Complex (COCs) were searched and graded under microscope at low magnification. The COCs were classified according to the slight modification of the method of Khandoker *et al*.(2001) into 2 grades, normal: oocyte completely surrounded by cumulus cells; abnormal: oocyte partially surrounded by cumulus cells or completely denuded. The numbers of different grades of COCs in each category were recorded. In the meantime another petridish of Dulbeccos phosphate buffered saline (D-PBS) was prepared for pooling COCs and the COCs were picked up with an appropriate glass micropipette.

The tip diameter of the pipette was checked under the microscope to ensure COCs, which could be easily aspirated without damaging the cumulus cells. Basically the glass micropipettes were prepared slowly stretching the tip of Pasteur pipette above burners flame. Then the picked up COCs were washed 2 to 3 times into D-PBS.

3.7 Statistical Analysis

All values were expressed as Mean±SE. Statistical significance of differences between different parameters was evaluated by using student's t-test. The statistical analysis was done by SPSS program (Version 16.0; SPSS Inc., Chicago, IL, USA).

CHAPTER IV

RESULTS and DISCUSSION

From local slaughterhouses, goat ovaries were collected and recorded as right and left. On the presence or absence of corpus luteum (CL) they were also categorized as CL-present or absent group. Among 300 ovaries CL was found in 90 ovaries with the remaining 210 ovaries having no CL. The result of the different parameters is summarized.

4.1 Morphological evaluation of the ovaries

In different categories the mean weight, length and width in right and left ovaries are presented in Table 1.

Table 1: Quantitative parameters in right and left ovaries

	Right (Mean±SE)	Left (Mean±SE)	Level of significance
Weight (gm)	1.56±0.4	0.71±0.10	NS
Length (cm)	1.33±0.24	1.91±0.25	NS
Width (cm)	1.25±0.18	0.97±0.25	NS

NS=Non-significant

The mean weight, length and width in the present study were found numerically higher in right ovaries than those from left ovaries but no significant differences were found (p>0.05).

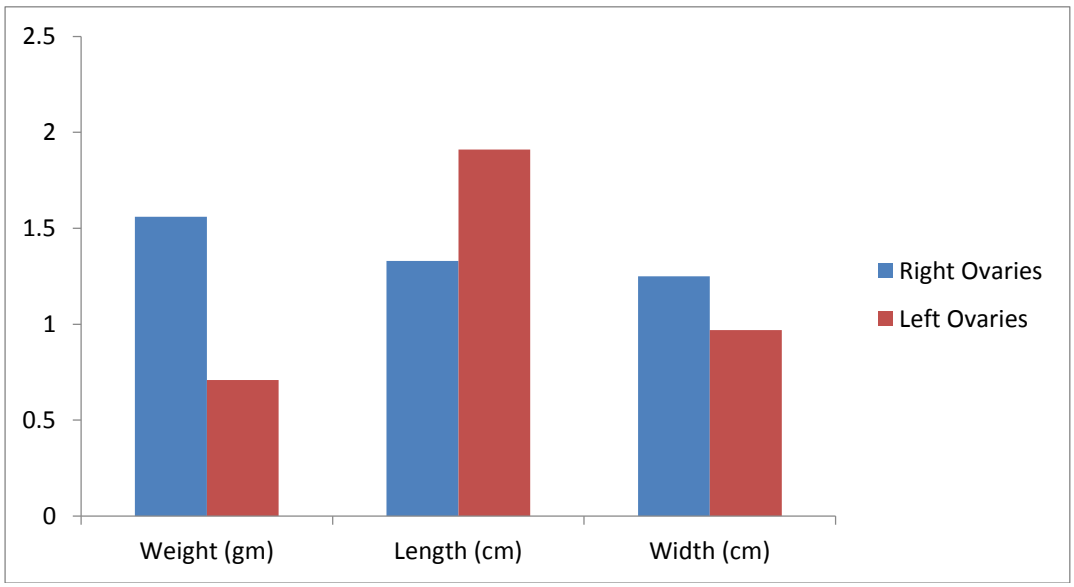


Figure 1: Quantitative parameters in right and left ovaries

The results of the different quantitative parameters of left and right ovaries in goat are summarized. In this study, it was observed that all the quantitative parameters of different follicular aspects of left ovary were not similar to the right ovary in goat (Table 1, Figure 1). The observation expressed that both left and right ovaries were equally active to normal physiological and/or ovarian activity.

The results of some previous study support that there was no significant difference in the parameters of left and right ovaries of goat (Singh *et. al*.,1974). In the present study, values that have not reached statistical significance may be ascribable to the species. Similar results were found in other report (Islam *et al*., 2007).

In accordance with the present study, (Islam *et. al*. 2007) worked on goat ovaries and expressed that the mean weight, length and width were found to be significantly higher in right ovaries than those of left ovaries and right ovaries were more active than left ones to show normal physiological and/or ovarian activity. Normal physiological explanation of ovarian activity is that right ovaries are more active than left ones (Singh *et al*., 1974; Rahman *et al*., 1977; Sarkar, 1993).

Table 2: Presence and absence of corpus luteum in ovaries

	CL-Present (Mean±SE)	CL-Absent (Mean±SE)	Level of significance
Weight (gm)	1.30 ± 0.30^{a}	0.98 ± 0.17^{b}	*
Length (cm)	1.42±0.35	1.45±0.18	NS
Width (cm)	1.08 ± 0.24^{a}	0.89 ± 0.22^{b}	*

NS=Non-significant at $p>0.05$

Means within the same row followed by different letters are significantly different at $p<0.05$

The weight and width were significantly higher in ovaries with CL than those of ovaries without CL (Table 2, Figure 2), ($p<0.05$).

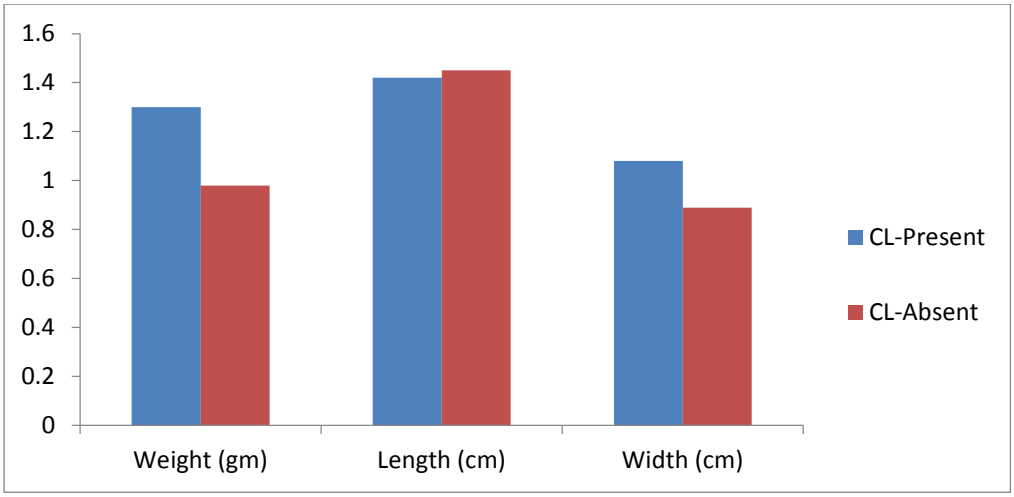


Figure 2: Quantitative parameters in corpus luteum-present and absent groups of ovaries

The CL is an extra cellular material within the ovary which made the differences of its width and weight. But no significant difference was found in the length of the ovaries with CL and without CL (Table 2, Figure 2).

The result is very usual as the hypertrophy of luteinized granulosa cells, hyperplasty of fibroblasts of the connective tissues and vascularity contribute to an increase in size of the CL (Jablonka-Shariff *et al*., 1993). The maximum diameter of CL is reached 6~9 d after ovulation and then regression starts between days 13 and 16 if maternal recognition does not occur (Jablonka-Shariff *et al*., 1993).

4.2 The effect of time elapsed from slaughtering to oocyte processing on oocyte quality

There is a direct effect of elapsed time from the period of slaughtering the donor animals toward time of specimens processing inside the Lab. The prolonged time storage affects the oocytes quality (Table 3).

Table 3: The effect of time elapsed from slaughtering to specimen processing

Time after slaughter (Hour)	Oocyte collection percentage (Ova %)[a]	Oocyte quality deterioration (Quality parameters)
2	78%	Good + to Good -
4	69%	Fair
6	60%	Poor
8	53%	Bad

[a]Ova collection percentage for the three collection methods.

The effect of elapsed time on the oocytes quality and that factor might interfere (impaired) with *in vitro* oocytes maturation (IVM) that yield low quality embryos. The present study also expressed that the good quality oocytes were gradually decreased with the increased processing time. It is better to transport the specimens from abattoir to the place of processing directly after slaughter if kept by cool box under 4-8°C. The samples yield a decreased ova numbers with the degree of ova damaged, aged and deteriorated if the time elapsed from period of slaughter to time of collection is increased.

4.3 Follicular development in right and left ovary

The number of follicles visible on the surface in relation to the right and left ovaries was counted as shown in Table 4. There were 1395 follicles recorded on the surface of the right ovaries.

Table 4: Follicular development in right and left ovary

Parameters	Right (150) (Mean±SE)	Left (150) (MEAN±SE)	Level of significance
Small follicle	2.71 ± 0.37	2.62 ± 0.43	NS
Medium follicle	1.56 ± 0.24	1.50 ± 0.34	NS
Large follicle	1.30 ± 0.21[a]	1.00 ± 0.27[b]	*

NS=Non-significant at $p>0.05$

Means within the same row followed by different letters are significantly different at $p<0.05$

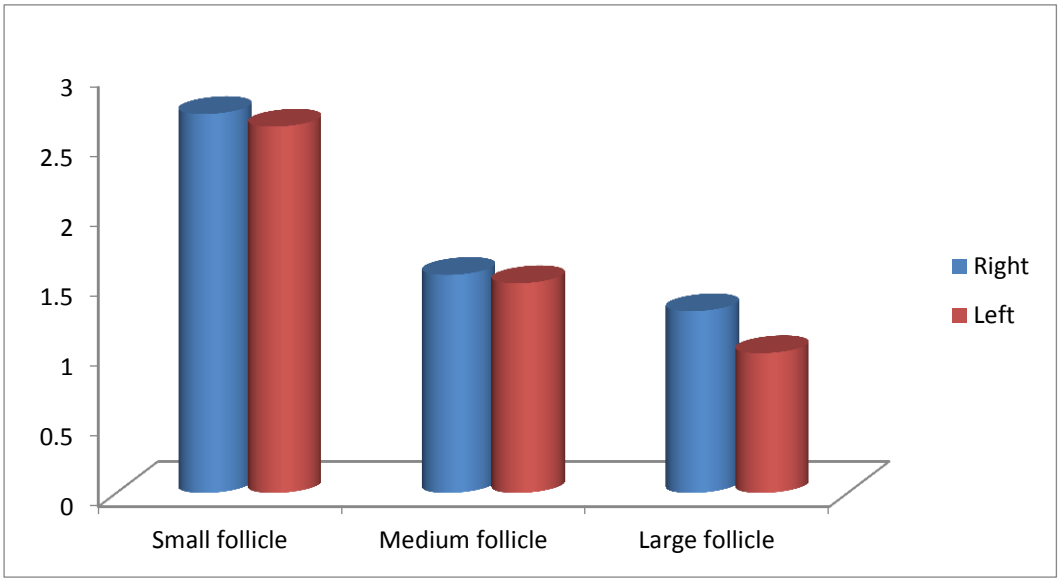


Figure 3: Follicular development in right and left ovary

The mean numbers of small and medium sized follicles were equivalent without a significant difference in right and left ovary. However, the average number of large follicles were significantly increased in the right ovary than that in the left ovary.

Crozet and co-workers (1995) dissected goat non-atretic follicles >2 mm in diameter and divided into three groups according to size (small = 2-3 mm; medium = 3.1-5 mm: large = >5 mm). Cumulus-oocyte complexes were isolated from the follicles and those with a compact multilayered cumulus were selected for *in vitro* maturation. After maturation, 70, 83 and 97 per cent of oocvles from small, medium and large follicles, respectively.

4.4 Grading of oocytes

The recovery rate of A, B, C and D grade of oocytes using different retrival methods was found to be 31.11%, 22.41%, 19.83% and 26.61% respectively (Table 5) and there was significant difference among the recovery rates of different grades of oocytes, (p<0.05) The results obtained in the present study showed that recovery percentage of grade A and B oocytes was higher than that of grade C and D.

Table 5: Rate of recovery of different Grades of Oocytes

Total number of ovary	Grade of oocyte	No of oocyte Recovered[a]	Rate (%) of oocyte recovered*
300	**Grade A**	289	31.11%
	Grade B	208	22.41%
	Normal (A+B)	497	53.52%
	Grade C	184	19.83%
	Grade D	247	26.61%
	Abnormal (C+D)	431	46.44%

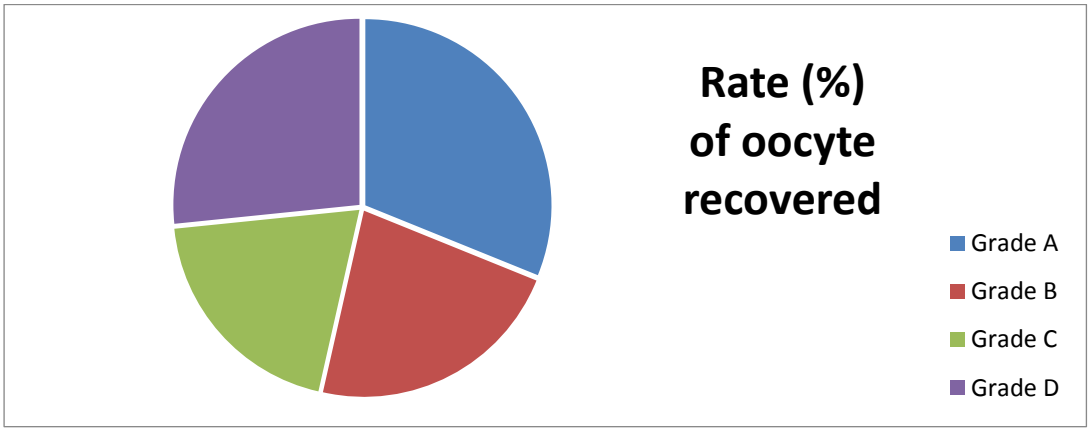


Figure 4: Rate of recovery of different Grades of Oocytes

This result was comparable with the findings of Ahmed *et al.* (2015) who recorded higher percentage of grade A and B oocyte than that of grade C and D in goat ovaries. In yak ovaries also recorded higher recovery rate of grade A and B oocytes using aspiration method, (Hussain 2011). On the other hand, Wang *et al.* (2007) recorded much lower rate of recovery of grade A oocytes in goat ovaries as compared to that observed in the present study. This discrepancy could be attributed to differences in the size of the ovary and follicle from which oocytes were recovered (Boni, 2012).

4.5 Effect of collection techniques on COCs recovery

The result of COCs recovery per ovary by three different techniques of aspiration, slicing and is dissection are summarized in Table 6. Total number of 320, 328 and 344 COCs were collected by aspiration, slicing and dissection techniques, respectively, from each of 100 ovaries. The results indicate that slicing and dissection yielded a significantly higher number of total COCs per ovary than that of aspiration technique.

Table 6: Number of collected and evaluated grade ova in regarding to collection methods

			Normal COCs Per ovary			Abnoral COCs per ovary		
Collection technique	**Total number of ovaries**	**Total number of COCs per ovary**	**Grade A**	**Grade B**	**Total**	**Grade C**	**Grade D**	**Total**
Aspiration	100	3.20^{b} ±0.22	1.52^{a} ±0.15	0.84^{a} ±0.13	2.36^{a} ±0.20	0.48^{b} ±0.12	0.36^{b} ±0.09	0.84^{b} ±0.14
Slicing	100	3.28^{a} ±0.30	1.12^{b} ±0.24	0.72^{b} ±0.13	1.84^{b} ±0.22	0.60^{a} ±1.2	0.84^{a} ±0.17	1.44^{a} ±0.20
Dissection	100	3.44^{a} ±0.27	1.16^{b} ±0.17	0.52^{b} ±0.11	1.68^{b} ±0.16	0.64^{a} ±0.14	1.12^{a} ±0.19	1.76^{a} ±0.20
Level of Significance		*	*	*	*	*	*	*

Values are shown in mean±SE
Means within the same column followed by different letters are significantly different at (p<0.05).

However, a significantly higher number of normal COCs per ovary was observed in aspiration (2.36) than those of slicing (1.84) and dissection (1.68) techniques (Table 6). The most commonly practiced methods of COCs recovery in goat are aspiration and dissection of visible follicles (Wang *et al*., 2007) .

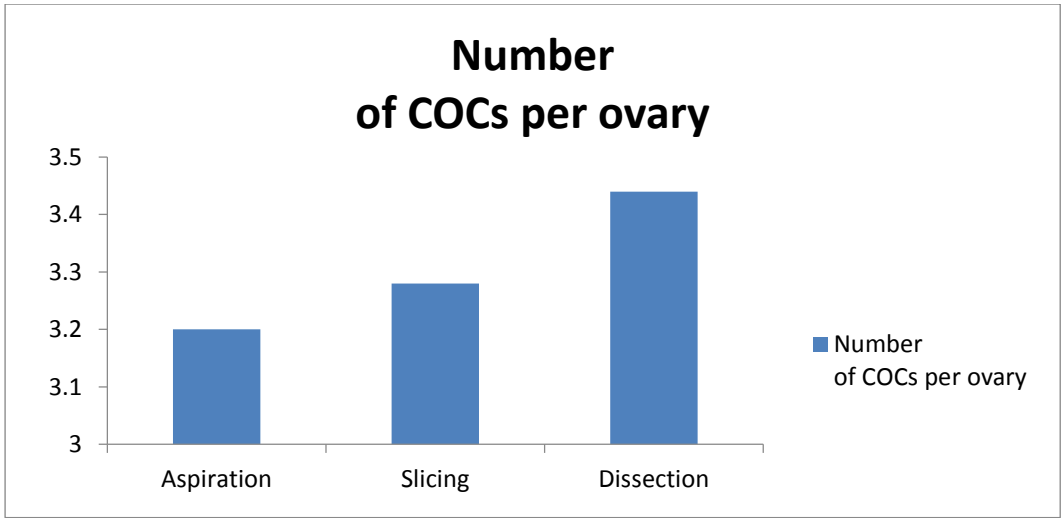


Figure 5: Different collection techniques

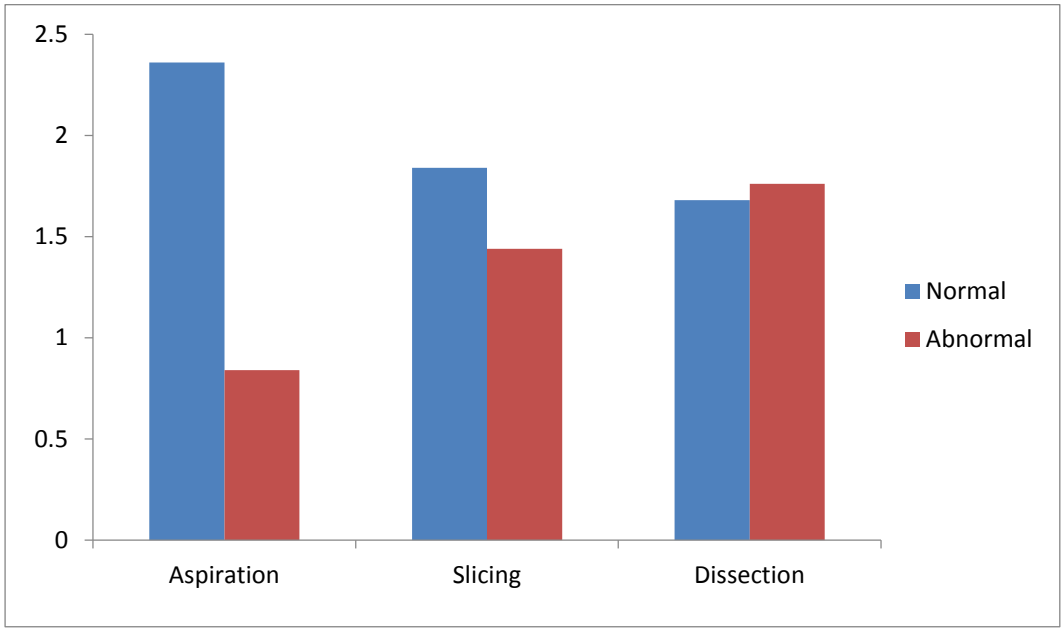
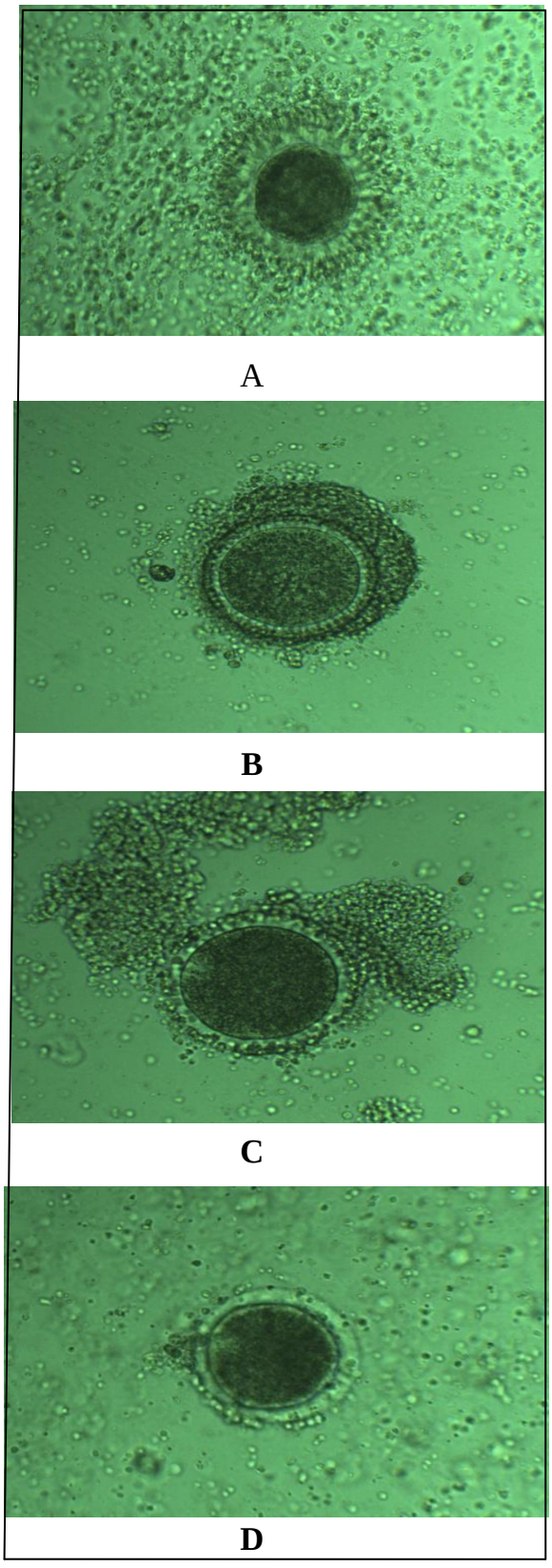


Figure 6: Grading of different techniques

In the aspiration technique, COCs were collected from 2 to 6 mm diameter of surface follicles using a hypodermic needle with 10 ml syringe. However, in the case of dissection, the whole ovarian surfaces were punctured by hypodermic needle and in the case of slicing, incisions were given along the whole ovarian surface using a scalpel blade that is all sizes of surface follicles were harvested.

Thus, the lower number of COCs recovered by the aspiration method in this experiment may be attributed to the presence of some follicles embedded deeply within the cortex, which can be released by dissection or slicing of the ovary. Higher yield of good quality oocytes obtained by aspiration technique as observed in the present study might be due to the collection of oocytes only from medium size follicles using wide lobe of 18 gauze needle which might had reduced the damage of cumulus cell layers during collection (Hashimoto *et al.*, 1999, Hosek *et al.*, 1986). Moreover, it might be due to the recovery of oocytes from the ovaries of animal with better reproductive status and containing higher number of medium and active follicles from which the oocytes were retrieved (Mehmood *et al.*, 2011). Ferdous (2006) reported that the numbers of normal COCs were found to be significantly higher ($p< 0.05$) in 2 to 6 mm diameter follicles than others. Besides, dissection and slicing techniques produce more debris which might interfere with the searching of oocytes under the microscope and also required more washing when compared to aspiration. As a result, a number of COCs were denuded from cumulus cells due to repeated washing.



Palate 5. Grading of oocyte. A) Grade A. B) Grade B. C) Grade C. D) Grade D

Hoque *et al* (2011) indicated that puncture and slicing yielded a significantly higher number of total COCs per ovary than that of aspiration technique, however, a significantly higher number of normal COCs per ovary was observed in aspiration technique than those of puncture and slicing techniques ($p<0.01$).

The result is comparable with the observation of oocytes from ovary of Boer goat by one of the three collection techniques (slicing, puncture, aspiration) Wang *et al*. (2007). They reported that, slicing and puncture technique of the ovaries yielded a higher *(*$p<0.05$) number of oocytes per ovary when compared to aspiration technique. Furthermore, Wani *et al*. (2000) reported that slicing and dissection technique yielded significantly ($p<0.05$) more COCs per ovary than aspiration technique in sheep but the percentage of good quality oocytes was higher in the aspiration method (64.4%), when compared with the puncture (54.7%) or slicing (54.3%) in ewe lamb which was also in accordance with the results of the present study. In contrast, Shirazi *et al*. (2005) reported that the number of COCs per ovary for slicing and aspiration did not differ significantly. This discrepancy might be due to species and size variation of Bengal goat and Iranian ewe. The final observation of this study is that, aspiration of 2 to 6 mm diameter vesicular follicles by an 18 gauge hypodermic needle is the best, simple and efficient way of recovering morphologically normal COCs from slaughterhouse goat ovaries.

CHAPTER V

SUMMARY AND CONCLUSION

Goats are regarded as intimate and integral part of subsistence traditional rural farming system. Collection and evaluation of goat ovaries will create vast opportunities to conduct the research work in the area of *in vitro* production (IVP) of goat embryos.

The present findings revealed that right and left ovaries both have a great potentiality to provide good number of oocytes for *in vitro* studies. Considering with the effects of CL on ovaries; highest number and normal grade oocyte would be collected from right ovaries. Moreover, this result creates a great opportunity of conducting further research on goat embryo production in Bangladesh

The oocytes remain firmly attached to the small and medium sized follicles before cumulus expansion and cannot be aspired, but can be easily recovered from the small follicles when the slicing method is employed. Slicing of ovaries is a simple and efficient tool for recovering good quality oocytes, but the aspiration technique is laborious and time consuming. The grade A and B oocytes were retrieved greatly from aspiration technique and dissection technique when compared to grade C and D oocytes; the latter were recovered to a greater extent by slicing method. From the results of the present study.The maximum per cent yield of grade A and B oocytes was observed by aspiration technique and dissection technique, while the grade C and D oocytes were retrieved by slicing technique. Overall yield of oocytes was highest with slicing technique when compared to the other techniques. It was the right thing not to discard the ovarian samples after aspiration in which they still gain more oocytes that can obtained by slicing those ovaries. Di ssection method for oocytes collection was better with less debris and cellular tissues remnant that

made the inspection for oocytes so easy. Time consuming for specimens transport as well as the temperature of transporting vessel affect the samples quality. We recommend using slaughter house specimens and slicing method for obtaining more numbers of oocytes.

Before any manipulation procedures and fertilization of oocytes, the quality of oocytes must be estimated exactly, because this has a high influence on embryo development. The suitability of oocytes for fertilization *in vitro* must be estimated most precisely using a complex evaluation of the characteristics of cumulus- oocyte complex structure, oocyte cytoplasm, polar body, perivitelline space, zona pellucida, and meiotic spindle at the same time. The less reproductively performing goats are usually slaughtered and most of them might be non-cyclic. So there is the possibility to get more non-cyclic ovaries from the slaughterhouse. The less number of CL group ovaries obtained in this experiment further supports the above statement.

Further studies are required for adopting and improving *in vitro* embryo production systems (maturation, fertilization and culture) of collected oocytes from local mammalian animals (cattle, buffalo, sheep and goat). Technological manipulation of the mammalian oocytes may increase the production of meat, milk and conserve species.

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