COMPARATIVE SEASONAL ASSESSMENT ON THE QUALITY OF BLACK BENGAL GOAT OOCYTES IN VIEW OF *IN VITRO* EMBRYO PRODUCTION

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CERTIFICATE

This is to certify that the thesis entitled **COMPARATIVE SEASONAL ASSESSMENT ON THE QUALITY OF BLACK BENGAL GOAT OOCYTES IN VIEW OF *IN VITRO* EMBRYO PRODUCTION** submitted to the Department of Animal Production and Management, Sher-e-Bangla Agricultural University, Dhaka in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (MS) in ANIMAL SCIENCE,** embodies the results of a piece of bona fide research work carried out by **SHIKHA SARKAR,** bearing Registration No. **13-05318** under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma in any other institution.

I further certify that any help or sources of information received during the course of this investigation has duly been acknowledged.

Dated:

Dhaka, Bangladesh

_____

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DEDICATED TO

My beloved parents

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ABSTRACT

In vitro maturation (IVM) of oocytes provide an excellent opportunity for cheap and abundant embryos for carrying out animal improvement. With the aim to study the oocyte quality in different seasons 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 season on cumulus oocyte complexes (COCs) of goat oocytes. Our study revealed that the average number of follicles in both left and right ovaries were higher in summer (12.50; 10.48) than in winter season (8.08; 8.21). Both right and left ovaries are great source for large number of oocyte and without-CL ovaries are considered as suitable source to supply quality oocyte for *in vitro* embryo production of goat. In different corpus luteum-present ovaries total number of follicles were distinctly higher in summer season (9.00±1.00) compared to that of winter season (6.50±1.84). In different corpus luteum-absent ovaries total number of follicles were distinctly higher in summer and winter season (13.52; 11.84) compared to that of CL present ovary in summer and winter season (9.00; 6.50). The oocyte recovery rate (2.14) was higher in summer than in (1.97) winter season. The results obtained in the present study showed that recovery rate of grade A and B oocytes were higher in summer than that of in winter and recovery rate of grade C and D were higher in winter than that of in summer. In the present study a significantly greater number of oocytes per ovary were recovered from ovaries without a corpus luteum (2.23; 2.02) than from ovaries with a corpus luteum(1.50; 1.50). Greater number of oocytes per ovary were recovered from ovaries with and without a corpus luteum (1.50; 2.23) in summer than from ovaries with and without a corpus luteum (1.50; 2.02) in winter. So the summer was the best season for recovery of COCs which have a worthy competence to be matured *in vitro*.

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LIST OF ABBREVIATIONS AND SYMBOLS

>	= Greater than
<	= Less than
±	= Plus minus
AETE	=European Embryo Transfer Association
AI	=Artificial Insemination
AR	=Acrosome Reaction
BCB	=Brilliant Cresyl Blue
CAK	=Cdc Activating Kinase
cAMP	=Cyclic Adenosine Monophosphate
CC	=Cumulus Cells
CL	=Corpus Luteum
COCs	=Cumulus-oocyte-complexes
CR	=Cortical Reaction
eCG	=Equine chorionic gonadotrophin
EGF	=Epidermal Growth Factor
ESS	=Estrous Sheep Serum
et al.	= Associate
FCS	=Foetal Calf Serum
FF	=Follicular Fluid
FPN	=Female PN
GCs	=Granulosa Cells
GH	=Growth Hormone

G_6PDH	=Glucose6-phosphate dehydrogenase
GV	=Germinal Vesicle Stage
GVBD	=Germinal Vesicle Breakdown
hCG	=Human chorionic gonadotrophin
ICM	=Inner Cell Mass
ICSI	=Intracytoplasmic Sperm Injection
IETS	=International Embryo Transfer Society
IVD	=in vivo derived
IVEP	=In vitro Embryo Production
IVF	=In Vitro Fertilization
IVM	=In Vitro Maturation
IVP	=In vitro production
JIVET	=Juvenile in vitro Embryo Transfer
LOS	=Large Offspring Syndrome
MEM	=Minimal Essential Medium
MI	=Metaphase I
MII	=Metaphase II
MOET	=Multiple Ovulation and Embryo Transfer
MPN	=Male PN
MPF	=Maturation Promoting Factor
OPU	=Ovum Pick Up
OSS	=Oestrous Sheep Serum

PB-1	=Polar Body
PBS	=Phosphate Buffer Solution
PUFA	=Poly Unsaturated Fatty Acids
PVS	=Perivitelline space
SCNT	=Somatic cell nuclear transfer
SOF	=Synthetic Oviductal Fluid
SS	=Steer Serum
TALP	=Tyrode’s Albumin Lactate Pyruvate
ZP	=Zona Pellucida

CHAPTER 1

INTRODUCTION

Goat is a multi-functional animal and contributes a great role to the rural economy of Bangladesh and it plays an important role in the livelihood of a large proportion of small farmers particularly women, landless and marginal farmers, who seldom have other means of survival (Choudhury *et al*., 2012). Bangladesh has the second highest population of goat among the Asiatic countries which accounts for about 60.60 million heads representing 57% of total ruminant livestock (FAOSTAT, 2009). Goats significantly contribute to the national GDP through the production of 130000 MT of meat (25% total meat), 1312000 MT milk and 391000 MT Skin each year (FAO, 2003). Goat population has increased at the rate of 10% per annum with a simultaneous reduction of cattle (0.21% per annum) from 1970 to 2003 (FAO, 1970 and 2003).

As goat rearing is the main means of survival for many women in remote villages, there is a need to develop a scientific method of goat rearing without causing adverse impact on the environment (Choudhury *et al*., 2012) and it is also noted that heritability for body weight and average daily gain of kids is low and variable (Petrovic *et al*., 2012). So, *in vitro* embryo production from slaughterhouse ovaries might be considered as a low cost and sustainable technique in Bangladesh condition (Rahman, 2003). In Bangladesh, *in vitro* technique in goat is a recent concept but a great deal of work has been still going on to standardize IVEP techniques (Ferdous, 2006; Islam *et al*., 2007; Mondal *et al*., 2008; Hoque, 2009). Season appears to influence the fertilization and viability rates of sheep embryos (Va´ zquez *et al*., 2008). Season also can affect some of the characteristics of the embryos (Mitchell *et al*., 2002). Mitchell *et al*. (2002) observed that the number of fertilized ova and embryos that had <16 cells impacted embryo quality, which was lower during anoestrus than during the reproductive season (RS). In addition, Stenbak *et al*. (2001) found the *in vitro* fertilization (IVF) rates of oocytes recovered from superovulated ewes were higher in the reproductive season than they were during anoestrus. Melatonin

implants can be an effective method of inducing oestrous cycles, increasing lambing rates and prolificacy during anoestrus (Haresign *et al*., 1990; Robinson *et al*., 1991; Haresign, 1992; Abecia *et al*., 2007). Abecia *et al*. (2008) summarized the effects of exogenous melatonin on the ovary as well as early embryos in ewes. Though melatonin treatments during anoestrus did not improve fertilization rates in superovulated ewes (McEvoy *et al*., 1998; Forcada *et al*., 2006), this pineal hormone can improve the viability of embryos (Forcada *et al*., 2006). Nutrition can have a significant effect on numerous aspects of reproduction, including hormone release, fertilization, and early embryo development (Boland *et al*., 2001). Nutritional condition can be correlated with embryo survival and the efficiency of assisted reproductive technologies (Webb *et al*., 2004). Borowczyk *et al*. (2006) found that the oocytes derived from underfed ewes yielded fewer blastocysts and had lower rates of cleavage and blastocyst formation than control ewes. Lozano *et al*. (2003) observed lower cleavage rates in underfed ewes than overfed ewes and undernutrition can impair embryo viability (Va´ zquez *et al*., 2008). As melatonin can increase embryo survival and IVF rates (Valasi *et al*., 2006), the author hypothesized that this hormone might override the effects of undernutrition and season on oocyte competence, which is the ability of an oocyte to be fertilized and develop to the blastocyst stage.

In vitro maturation (IVM) of oocytes provide an excellent opportunity for cheap and abundant embryos for carrying out research and for the application of emerging biotechnologies like cloning and transgenic (Li *et al*., 2006). Several workers have studied different aspects of IVM in mammalian oocytes (Roa *et al*., 2002 and Kharche *et al*., 2005) and the maturation media with the selection of protein supplements and hormones play an important role for IVM in and subsequent for IVF and in vitro development (Motlagh *et al*., 2008). In different studies on IVM of animal oocytes, the basic medium is supplemented with different kinds of sera (Motlagh *et al*., 2008 and Hegab *et al*., 2009) and the importance of sera may due to its contents of hormones, trace nutrients and

proteins such as globulin and futuin (Hsu *et al*., 1987). The addition of serum to the culture medium provides a source of albumin and it balances the osmolarity and acts as a free radical scavenger (Thompson, 2000). Also, in some experiments, the protein additives of the same species were utilized for *in vitro* maturation of their occytes and examples of such protein additives were estrous goat serum for caprine occytes (Keskintepe *et al*., 1994), water buffalo follicular fluid for buffalo oocytes (Tajik *et al*., 2000), porcine follicular fluid for porcine oocytes (Funahashi *et al*., 1997), and equine follicular fluid for equine oocytes (Hinrichs *et al*., 1995). Sheep oocytes have also been studied for different aspects of maturation (Roa *et al*., 2002) and in all experiments, maturation media was supplemented with FCS (Ghasemzadeh- Nava and Tajik, 2000), estrous sheep serum (ESS) (Ghasemzadeh- Nava and Tajk, 2000), human serum (Thomson *et al.*, 1998) and mare serum (Motlagh *et al*., 2008).

Seasonal effect was found to be an important consideration in this respect, especially in buffalo (Kadoom, 1995 and Das *et al*., 1996), mare (Bruck *et al*., 1996 and Colleoni *et al.*, 2004) and cattle (Silva *et al.*, 2006). In buffalo, the incubation temperature during the *in vitro* maturation (IVM) influenced the fertilization rate but had no significant effect on maturation and subsequent embryo development (Ravindranatha *et al*., 2003). Al-Katanani *et al*. (2002) concluded that summer depression in oocyte quality in Holstein cow was evident, but cooling cow for 42 days did not alleviate that seasonal effect. Singla *et al.* (1999) reported that buffaloes subjected to heat stress yielding fewer good quality oocytes than their unstressed counterparts the developmental competence of the oocytes under *in vitro* conditions has been investigated. Rutledge *et al*. (1999) emphasized that the production of cattle blastocyst was reduced in mid summer to late summer. In contrast, Rocha *et al*. (1997) in *Bos indicus* (Brahman) cows and Rivera *et al*. (2000) in bovine failed to indicate an effect of season on *in vitro* embryo production in a subtropical environment. Seasonality is less pronounced between males of domestic species, but there are

differences in behavior and sperm characteristics depending on the time of the year (Gerlach *et al*., 2000, Ciereszko *et al*., 2000 and Chacon *et al*., 2002).

So, the present study was undertaken with a view to collection and evaluation of goat slaughter house ovaries, follicles and COCs (Cumulus oocyte complexes) in different seasons to create opportunities to conduct the research work in the area of IVP (*In vitro* production) of goat embryos.

The present study was, therefore, designed with the following objectives:-

- To evaluate oocyte recovery rates and their quality from goat ovaries collected at local abattoir in two different seasons.
- To characterize the ovary and oocyte physically in Black Bengal goat in two different seasons.
- To assess the quality of oocytes recovered from Black Bengal goat in two different seasons.

CHAPTER 2

REVIEW OF LITERATURE

Genetically and functionally competent gametes are a prerequisite for normal fertilization and embryo development and the first phase in the sexual reproduction of an organism is gametogenesis. It is 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 and 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. So, in goat, where the chromosome number of somatic cells is 60, each sperm and each oocyte has only 30 chromosomes, until this point spermatogenesis and oogenesis resume their similarity.

2.1 Oogenesis

During oogenesis, mammalian oocytes initiate meiotic division and progress to the diplotene stage of prophaseI, defined as the germinal vesicle stage (GV), where they remain arrested until meiotic maturation and after a period of growth, mammalian fully-grown oocytes are able to resume meiosis spontaneously when released from their surrounding follicular environment (Pincus and Enzman, 1935; Edwards, 1965). Meiotic maturation is characterized by germinal vesicle breakdown (GVBD), chromosome condensation, metaphaseI (MI) spindle formation, extrusion of the first polar body and arrest at metaphaseII (MII) and resumption of meiosis and progression up to MII is orchestrated by the maturation promoting factor (MPF), the central component in this process, whose activity was first demonstrated by Masui and Markert (1971).

The MPF is a heterodimeric protein kinase composed of a regulatory subunit, cyclinB, and a catalytic subunit p34cdc2 (Dunphy *et al*., 1988; Lohka *et al*., 1988; Labbe´*et al*., 1989) and MPF activity, that mainly regulates events of

oocyte maturation, has been described in several mammalian species: it appears shortly before GVBD reaches a high level at MI, then decreases and again reaches a high level at MII (mouse: Hashimoto and Kishimoto, 1988; Choi *et al.*, 1991; Fulka *et al*., 1992; Verlhac *et al*., 1994; pig: Naito and Toyoda, 1991; cattle: Collas *et al*., 1993; Kalous *et al.*, 1993; rabbit: Jelink ova *et al*.,1994; goat: Dedieu *et al.*, 1996). The protein kinase activity of p34cdc2 is strictly dependent on its association with cyclin B and on its own phosphorylation status and to be active, p34cdc2 must be phosphorylated on threonine by a protein kinase called CAK (Cdc activating kinase) and dephosphorylated on threonine 14 and tyrosine15 (Dunphy and Kumagai, 1991; Gautier and Maller, 1991; Solomon *et al*., 1992; Fesquet *et al*., 1993; Poon *et al.*, 1993).

These dephosphorylations are controlled by a protein phosphatase, whose activity is in part regulated by the protein phosphatase 2A (Dunphy and Kumagai, 1991; Gautier and Maller, 1991). 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) and 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 and after continuation of meiosis, the oogenesis process until completion is very fast. Oogenesis in mammals includes seven steps: (1) generation of primordial germ cells (PGCs), (2) migration of PGCs to the prospective gonads, (3) colonization of the gonads by PGCs, (4) differentiation of PGCs to oogonia, (5) proliferation of oogonia, (6) initiation of meiosis and (7) 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 and 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) and 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 and 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 and 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) and 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 and 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) and the reason for storing the oocytes in this remarkable frozen meiotic state is unknown (Johnson and Everitt, 1980).

2.2 Oocyte growth and development

The development of mammalian oocytes is generally correlated with the development of follicular somatic cells and the initiation of oocyte growth usually coincides with a thickening of the granulosa cell layer, and continued oocyte growth occurs concurrently with proliferation of granulosa cells in preantral follicles (Brambell, 1928). Follicular antrum formation occurs about the time that oocytes near completion of their growth and acquire the capacity to resume the first meiotic division (Brambell, 1928; Szybec, 1972; Erickson & Sorensen, 1974; Sorensen & Wassarman, 1976) and oocytes can achieve advanced phases of maturation sequentially, and this development is also correlated, at least temporally, with the progression of follicular development (Sorensen & Wassarman, 1976; Eppig & Schroeder, 1989). Oocytes of many mammalian species undergo gonadotrophin-induced maturation just before ovulation and development of ovarian follicles in mice begins shortly after birth.

A large group of follicles develops almost synchronously in neonatal and juvenile mice (Peters, 1969), so oocytes and follicles at increasing stages of development can be isolated from the ovaries of mice until they are about 1 month old (Szybec, 1972; Mangia & Epstein, 1975; Sorensen & Wassarman, 1976) and follicular antrum formation occurs in mice when they are approximately 15 days old and this coincides with the acquisition of the capacity of oocytes to undergo germinal vesicle breakdown upon liberation from the follicles and culture in a supportive medium (Szybec, 1972; Sorensen & Wassarman, 1976; Eppig & Schroeder, 1989). Many oocytes isolated at this stage, however, failed to progress through metaphase I and produce a polar body and an increased frequency of polar body production was observed when the oocytes were isolated from mice that were a few days older (Sorensen & Wassarman, 1976; Eppig & Schroeder, 1989). Acquisition of capacities to undergo germinal vesicle breakdown and subsequently complete meiosis I are, therefore, experimentally separable and sequential events in oocytes. Oocytes from unprimed immature mice that underwent spontaneous maturation *in vitro*

were fertilized and underwent embryonic development with frequencies that depended on the age of the mice (Eppig & Schroeder, 1989) and there was no difference in the frequency of germinal vesicle breakdown or polar body production when oocytes were cultured after isolation from 20-26-dayold mice, nor was there a difference in the frequency of cleavage to the two-cell stage after insemination. However, there was a difference in the frequency at which the embryos completed the two cell stage to blastocyst transition; only 30% of the two-cell stage embryos derived from oocytes isolated from 22-day-old mice developed to blastocysts in contrast with 55% of the two-cell stage embryos derived from oocytes isolated from 26-day-old mice (Eppig & Schroeder, 1989) and the capacity of oocytes to complete pre-implantation development after maturation and insemination in vitro is, therefore, acquired after they develop the capacity to complete meiosis I and to cleave to the two-cell stage.

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) and 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 the ewe, primordial, primary and secondary follicles, respectively, appear in the fetal ovary at days 75, 100 and 120 (McNatty *et al*., 1995) and 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) and 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 and 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) and 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) and the fluid found in the ‘antrum` is known as Follicular Fluid (FF). During this growth phase there is a major increase in ooplasmic organelles and the follicle provides a microenvironment for oocyte growth, development and is responsible for the production of hormones.

The GCs consist of the corona radiata; Cumulus Cells (CCs), membrana granulosa and antral granulosa cells and the CCs surround the oocyte, that 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) and 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), that are known as gap junctions. The heterologous gap junctions provide the basis for extensive network of intracellular communication among GCs and normally CCs or corona cells surrounding the goat oocyte shed ≥30 h after ovulation (Harper, 1982).

2.2.1 Oocyte maturation

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) and the diameter of mature goat oocytes excluding ZP (ooplasm) ranged from 119-146 μm (De Smedt *et al*., 1994; Crozet *et al*., 2000). 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) and 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) and 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) and 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 goat oocyte from antral follicle bigger than 2 mm is about 3.2 μm thick (Ariyaratna and Gunawardana, 1997). Completion of the meiosis 1 takes place when oocytes have undergone extensive growth in cellular interaction with GCs and theca cells and the oocyte undergoes asymmetric cytokinesis and extrudes the first polar body (PB-1) containing a haploid chromosome complement (Kupker *et al*., 1998). 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) and 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) and 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).

2.3 Activation of sperm and sperm-oocyte interaction

In mammals, fertilization process is internal and the male gametes (spermatozoa) must be introduced into the female reproductive tract at coitus or artificially and as already mentioned, when the ejaculated spermatozoa are deposited into the female reproductive tract at that time they have no fertilizing capability. Therefore, the spermatozoa need to gain this ability, or in other word, need to be activated or made in ready state first before they can interact with oocyte to ensure fertilization success and the known physiological events occurring during sperm activation and sperm-oocyte interaction have been reviewed mainly based on mice models (Bedford, 1982; Yanagimachi, 1994; Tulsiani *et al*., 1997; Bedford, 1998).

2.3.1 Activation of spermatozoa

In mammalian species, activation of spermatozoa follows several physiological and structural changes upon exposure to environmental signals in the female reproductive tract before they interact with the oocytes towards a successful fertilization and these include various alterations in the plasma membrane and intracellular components and changes in motility pattern and metabolism of the spermatozoa. Two main processes, capacitation and acrosome reaction, must occur to activate the spermatozoa before the sperm interact with the oocyte.

2.3.2 Capacitation

The spermatozoa must reside a minimum period in the female reproductive tract before gaining the ability to fertilize oocytes (Austin, 1951; Chang, 1951) and it is speculated that during this time, glycoproteins from the sperm surface are removed, thus exposing receptor sites that can respond to oocyte signals and lead to acrosome reaction. This process allows the spermatozoa to bind to the ZP, which subsequently leads to the Acrosome Reaction (AR), penetration of the ZP and fertilization of the oocyte and in another word, the spermatozoa gains the capacity to fertilize oocyte and, therefore, is termed as capacitation (Markert, 1983; Elder and Dale, 2000). This phenomenon is first noted by Chang (1951) and Austin (1951) and the term 'capacitation` was referred by Austin (1951) and the time required for sperm capacitation varies among different species of animals and ranges from less than one hour in male mouse to 6 h in man (Elder and Dale, 2000). The changes that occur during capacitation in the sperm plasma membrane include modification of ion channels, increased adenylate cyclase and cyclic adenosine monophosphate (cAMP), changes in surface glycoprotein moieties that lead to changes in lectin binding patterns and also enzymatic modification of surface proteins such as sugar transferases and metabolic changes include increased glycolytic activity and oxygen consumption, hyperactivation associated with activation of adenylate cyclase system and loss of Zn^{+2} ions that lead to increased nuclear stability (Yanagimachi, 1994; Tulsiani *et al*., 1997).

A number of enzymes and factors that present in the female reproductive tract have been implicated to affect sperm capacitation, such as arylsulphatase, fucosidase and taurine and however, till to date, the exact mechanism on how these enzymes and factors capacitate sperm remains unknown (Elder and Dale, 2000). During capacitation, sperm motility is apparently regulated by changes in the intracellular concentration of calcium ions through a calcium channel (named CatSper) which is only expressed in the tail of mature sperm (Ren *et al*., 2001). It is thought that the capacitation phenomenon is necessary for

spermatozoon to fuse with the oolemma, but not for subsequent steps of fertilization such as DNA decondensation and PN formation and this is supported by in vitro experiments in Xenopus laevis, an amphibian species (Brun, 1974), mouse (Kimura and Yanagimachi, 1995) and human (Palermo *et al.*, 1992) in which injection of intact sperm into the ooplasm resulted in development of normal offspring. However, discrepancies also exists, for example, the capacitation step appears to be critical in cattle (Goto *et al*., 1990; Sutovsky *et al.*, 1997), goat (Keskintepe *et al*., 1997; Wang *et al*., 2003) and in other farm animals where ICSI is problematic (Catt and Rhodes, 1995) and the failure of ICSI in some farm animals may be due to the failure of removal of the perinuclear theca, a cytoskeletal capsule present between the sperm membrane and the nuclear envelope (Sutovsky *et al*., 1997). This structure could block the access of cytoplasmic factors involved in decondensation of the sperm nucleus and this is supported by results where artificially capacitated bull spermatozoa resulted in PN formation and production of a normal calf after ICSI (Goto *et al*., 1990). Capacitation may be attained *in vivo* or *in vitro and d*ifferent *in vitro* conditions are used to capacitate spermatozoa from various species (Yanagimachi, 1994). Capacitation is temperature dependant and occurs at 37° to 39°C.

2.3.3 Acrosome reaction (AR)

The first interaction between the spermatozoon and the oocyte is at the ZP, an extracellular coat that surrounds all mammalian oocytes (Wassarman *et al*., 2001) and the sperm nucleus is covered by membrane bound secretory vesicle known as the acrosome which is a large, Golgi-derived lysosome-like organelle that overlies the nucleus in the apical region of the sperm head (Kaji and Kudo, 2004). The acrosome or its surrounding membranes contains a large array of hydrolytic enzymes including hyaluronidase, acrosin, serine protease, proacroson, phosphatase, arylsulphatase, colagenase, phospholipase C and β-galactosidase (Elder and Dale, 2000) and the spermatozoon interacts with the ZP by the plasma membrane overlying the acrosome. The specific binding of

the spermatozoon to the ZP induces in the acrosome a calcium-mediated signal transduction process that leads to AR. The AR brings exposure of the inner acrosomal membrane to the outside by breaking the organelle and involves fusion of the outer acrosomal membrane with the overlying plasma membrane allowing the acrosomal content to be released (Wassarman *et al.,* 2001) and this process is necessary for the spermatozoon to acquire fusibility with the oolemma (Kaji and Kudo, 2004). The AR is accompanied by modifications in the sperm plasma membrane, that exposes receptors for ZP binding and possibly factors exposed on the equatorial segment in preparation for sperm-oocyte fusion (Kupker *et al*., 1998).

In mammals, Austin and Bishop (Austin and Bishop, 1958) first described the AR and although, AR is now considered as a separate process (Bavister, 2002), that triggers after capacitation, however, discrepancies exists as some authors believe that it was a part of the capacitation process (Austin and Bishop, 1958) and others considered AR as the final phase of capacitation (Yanagimachi, 1969). The AR is considered as the final prerequisite step in the sperm activation process before it gets the ability to fuse with the oocyte and it is obligatory that this reaction only occur in the presence of a rise in the intracellular calcium (Ca^{2+}) level (Florman and Babcock, 1991; Yanagimachi, 1994; Ben-Yosef and Shalgi, 1998; Wassarman, 1999). This increase of intracellular calcium can be achieved *in vitro* by exposing sperm to Ca^{2+} ionophores or phosphodiesterase inhibitors (Kupker *et al*., 1998) and an artificially high pH of 9.0-9.5 could also induce AR (Elder and Dale, 2000). Although, it is not clear whether the AR is initiated whilst the sperm is interacting with the cumulus mass, however, it is speculated that the reaction may be started in the CCs as a major component of the cumulus matrix is hyaluronic acid and the acrosome contains hyaluronidase and the AR is relatively rapid once the correct trigger signals have been received and may take 2 to 15 min *in vitro* (Elder and Dale, 2000).

2.3.4 Sperm-oocyte interaction

The ZP of the oocyte is made up of protein and carbohydrates in the form of glycoprotein units which are probably stabilized by disulphide bonds. The principal types of carbohydrate found are fucose and glycoprotein, which are synthesized by the oocyte itself (Elder and Dale, 2000). After completion of AR, the sperm penetrate the ZP. Following penetration, the acrosome-reacted sperm passes through PVS and interacts with the sperm plasma membrane of the oocyte (oolemma) (Schultz and Kopf, 1995) and this initial interaction involves specific recognition, binding and then fusion between the sperm plasma membrane in the region of the equatorial segment and the oolemma (Yanagimachi, 1994). The sperm head fuses with microvilli on the oolemma (Kupker *et al*., 1998) and the attachment of the sperm head to the ZP inevitably alters the permeability of the sperm plasma membrane, causing a transient change in the concentration of several intracellular ions (Tulsiani *et al*., 1997).

Subsequently the entire spermatozoon including the tail is drawn into the ooplasm (Kupker *et al*., 1998) and this initiates syngamy and further development of the zygote (Tulsiani *et al*., 1997). The exact mechanism of sperm penetration of the ZP and the complementary molecules which initiate penetration of the ZP by the sperm are not yet known. However, it is generally believed that the process involves receptor-ligand interaction between sperm-surface proteins and ZP glycoproteins and in rodents, these consist of sperm membrane galactosyltransferase and N-acetylglucosamine residues of the ZP. The receptor-ligand interaction induces the AR leading to exocytosis and release of acrosomal enzymes from the acrosomal cap and the specific sperm protein(s) responsible for sperm-oocyte interaction are yet to be fully characterized.

However, four ZP proteins with variable roles in sperm-oocyte interaction have been isolated in mammals and in the mouse, ZP3 plays the primary role, while ZP1 is the primary sperm receptor in pig, rabbit and non-human primates (Dunbar *et al*., 1998). It is not known yet whether such a characterization is

available for the goat and it is thought that the penetration of the ZP by the sperm is mediated by lytic acrosomal enzymes (Tulsiani *et al*., 1997; Bedford, 1998) or by the mechanical action of the sperm itself (Tulsiani *et al*., 1997; Bedford, 1998) and the latter author argues that available evidence is in favor of mechanical instead of lytic penetration.

2.4 Fertilization and early embryogenesis

Fertilization can be defined as the process of union of two germ cells, namely spermatozoon and oocyte, whereby the somatic chromosome number is restored and the development of a new and unique individual exhibiting characteristic of the same species is initiated (Wassarman, 1999). Transferring genetic information from one generation to the next, it ensures the immortality of an individual and by creating variation it allows evolutionary forces to operate (Elder and Dale, 2000). Following ZP penetration, a series of events take place leading to syngamy and the production of the zygote and these steps have been summarized previously (Bedford, 1982; Yanagimachi, 1994).

2.4.1 Activation of oocyte

Oocyte activation is the process of releasing the oocyte from the second meiotic arrest when the spermatozoon fertilizes it and it is a cell signaling event that results in events including the cortical granule reaction or Cortical Reaction (CR), decondensation of the sperm nucleus, maternal RNA recruitment, resumption of meiosis as evidenced by the extrusion of second polar body (PB-2) and later events such as PN formation and initiation of DNA synthesis and 23+999 rise in Ca^{2+} concentration. In human, this increase occurs within 1 to 3 min of sperm-oocyte fusion and takes the form of a wave originating at the point of spermatozoon entry (Ben-Yosef and Shalgi, 2001) and the site of Ca^{2+} release and sequestration is thought to be the endoplasmic reticulum, where inositol 1, 4, 5-triphosphate (IP_3) receptors are present (Kline and Kline, 1992). The first Ca^{2+} transient is followed by a series of shorter Ca^{2+} transients of high amplitude

(Ca^{2+} oscillations). In IVF studies, Ca^{2+} oscillations were observed in all mammalian species including goat (Jellerette *et al*., 2006), although their frequency is species specific (Ben-Yosef and Shalgi, 2001) and as fertilization progresses, the amplitude and frequency of the Ca^{2+} transients decrease, while the duration increases until an absolute cessation of Ca^{2+} oscillations during entry into interphase and PN formation, several hours after sperm entry (Jones *et al*., 1995). Calcium oscillations require a continuous Ca^{2+} influx to refill endoplasmic reticulum stores (Miyazaki, 1995) and it was suggested that although a single rise is sufficient to produce activation, oscillations might be required for additional developmental events (Ozil, 1990). There are two postulated hypotheses of oocyte activation mechanism, namely 'receptor hypothesis` and sperm factor hypothesis, which describe the contribution of the spermatozoa to successful oocyte activation (Kimura *et al*., 1998; Fissore *et al*., 1998) and in the receptor hypothesis, it is the spermatozoon interacting with the oolemma that results in oocyte activation. In this model, an oocyte surface receptor is coupled to a G-protein (Miyazaki *et al*., 1993) or a tyrosine-kinase-mediated (Ben-Yosef and Shalgi, 1998) signaling pathway and when activated by a sperm, activates phospholipase C that results in the formation of IP_3 and hence intracellular Ca^{2+} is released and the second hypothesis, which is called the sperm factor hypothesis, suggests that the release of intracellular Ca^{2+} is triggered by diffusible messenger(s) in sperm cytoplasm which enters the ooplasm following sperm-oocyte fusion. This hypothesis gained support with the development of ICSI technique where initial Ca^{2+} rise, Ca^{2+} oscillations and full oocyte activation occur by injecting sperm or sperm extract into the ooplasm (Fissore *et al*., 1998).

2.4.2 Formation of pronuclei

Soon after oocyte activation through sperm entry the PB-2 extrudes (Payne *et al*., 1997), the PB-2 is generally extrudes immediately adjacent to the PB-1. The mammalian sperm nucleus is packed with distinct protamine and when sperm enters the ooplasm, the sperm nuclear envelope breaks down, the protamines are

lost, the sperm head enlarges in the ooplasm and chromosome or nuclear decondensation takes place a few hours by reduction of disulphide bonds between protamines by the action of glutathione (Kupker *et al*., 1998). This coincides with decondensation of maternal chromatin and formation of the female PN (FPN) and usually, but not always, the MPN appears near the site of sperm entry whereas the FPN forms close to the PB-2 (Payne *et al*., 1997).

2.4.3 Cleavage and early embryogenesis

DNA replication and cell divisions occur without an increase in cell mass to attain a ratio similar to somatic cells and this process is referred to as cleavage (Hafez and Hafez, 2000b). The cleavage furrow often goes through the region where the PN resided at the initiation of syngamy and the resulting daughter cells are known as blastomeres. The plane of second division is also vertical and passes through the main axis but at a right angle to the initial plane of cleavage, resulting in 4 blastomeres and the third cleavage division occurs approximately at a right angle to the second, resulting in 8 blastomeres. This doubling sequence is followed on through the remainder of early cleavage (Hafez and Hafez, 2000b) and cleavage divisions are always mitotic and each blastomere receives full assortment of chromosomes. Slight variations in blastomere sizes within the same embryo are probably unimportant, but major differences may indicate defects in underlying cellular processes (Hardarson *et al*., 2001) and although oocyte size (diameter of ooplasm) was measured in most of the species, there was paucity of information on blastomeres size.

A search of available literature revealed one paper where morphometric measurements of human ICSI-derived cleaved embryos were performed up to 4-cell stage and using computer-controlled morphometric analysis of blastomere size, it was found that average diameter of blastomeres of 2-, 3-and 4-cell stage embryos were 80.1, 68.7 and 64.9 μm, respectively (Hnida *et al*., 2004). The diameter of ooplasm of a fully grown human oocyte or 1-cell embryo is 135 to 160 μm (Oppenheimer and Lefevre, 1989) that is about the size of a mature goat

oocyte (119-146 μm) (Crozet *et al*., 2000). It was found from their study that blastomere size significantly affected by degree of fragmentation and multinuclearity and that computer-assisted, multilevel analysis of blastomere size may function as a biomarker for embryo quality (Hnida *et al*., 2004) and in goat, initial and principal activations of embryonic genome (cell stage) are reported to be occurring at 2-cell and 8-cell stage, respectively (Kelk *et al*., 1994). In goat, compaction of the embryo begins at 8-cell stage (Sakkas *et al*., 1989). In goat, the blastocoel appears at 120 h post-fertilization (Sakkas *et al*., 1989) and the embryo is confined within the ZP (analogous to eggshell in bird, reptiles or monotremes) throughout the early stages of development. The goat embryo stays in the oviduct for 48 to 72 h from the time of ovulation (Moore, 1977) and the trophectoderm cells of the embryo secrete proteolytic enzymes that digest a passage through the ZP and allow the blastocyst to hatch. The hatching of goat blastocyst occurs around 168 h (7 days) after fertilization (Sakkas *et al*., 1989) and the exposed cells of the goat blastocyst then make firm physical contact with the uterine wall, implantation stage begins, embryo becomes elongated and the final implantation in the uterine endometrium occurs at day 16 post-fertilization (Gurdon *et al*., 1995).

2.5 *In vitro* embryo production procedures

The influence of season on oocyte quality has been considered, as a factor of high impact. In Bangladesh, *in vitro* production of goat embryos is a very recent work but a great deal of work has been done regarding evaluation and grading of bovine ovaries, collection of COCs from slaughterhouse ovaries and grading of bovine oocytes followed by IVM, IVF of the oocytes and IVC of the resulting zygotes (Rahman *et al*., 2003; Goswami *et al*., 2004; Pervage *et al*., 2007) and *in vitro* culture of mouse embryo (Khandoker *et al*., 2005). Preliminary research on goat embryo culture (Islam *et al*., 2007; Ferdous, 2006) have been done, but, there exists vast opportunity to conduct the research work on *in vitro* goat embryo production. Multiovulation and Embryo Transfer (MOET) and *In vitro* Embryo Production (IVEP) are assisted reproductive technologies aimed at

increasing the genetic diffusion of females and both *in vivo* derived (IVD) embryos and *in vitro* produced embryos together with cryoconservation techniques will be essential to increase sheep and goat productivity (Paramio, 2010).

Thus, 3-month-old females in a JIVET scheme reduces this interval to 7 months and using oocytes obtained from 3- to 4-week-old females to 6 months with an increases in the rate of genetic gain of approximately 5% (Morton, 2008). The mean embryo production was 1.5 embryos per OPU session (AETE Newsletter 40, December 2013) and in goats and sheep, the numbers of transferable embryos were 406 and 265, respectively, France being the country with the highest number recorded. Only six countries reported embryo transfer in goats, with 1805 IVD embryos collected and 1013 embryos transferred (IETS Newsletter, December 2013) and there is less research on assisted reproductive technologies in small ruminants compared to other livestock species such as cattle and pigs.

However, in the last few years, there has been significant research in small ruminant embryo studies due to the rising importance of these animals in economically fast developing countries such as China and India and also because of the growing interest in small ruminants, mainly goats, as animals to express recombinant proteins in milk (Paramio and Izquierdo, 2014). Spanish Agriculture Ministry data (www.magrama.gob.es, 2013) show 16.6 million sheep and 2.8 million goats with an increasing interest in milk production. The economic values of this products are 537 and 75 USD millions for meat and 569 and 315 for milk in sheep and goats respectively. According to FAO (www.faostat.fao.org, 2013), the number of sheep and goats in the world was 1169 and 996 million respectively and the number of sheep in Africa, America, Asia, Europe and Oceania was 322, 87, 526, 129 and 106 million, respectively, and goats, 334, 35, 595, 17 and 4 million respectively.

2.5.1 Grading of oocytes

Goat ovaries from the slaughter house were found to be an economical source of oocytes for IVM, IVF and IVC research (Martino *et al*., 1995). The average number of good quality oocytes recovered from ovaries without corpora lutea was more as compared to the ovaries with corpora lutea, which can be effectively used for IVF (Kumar *et al*., 2004) and in addition it has been shown that a comparable higher maturation rate could be reached within 24 hours of culture if the oocytes had a compact cumulus structural cell.

Oocytes with intact cumulus or at least four layers of cumulus cells gave good result for IVM and IVF (Yang *et al*., 1993) and whereas, denuded oocytes or oocytes with few cumulus cells are usually rejected because of their low capacity of fertilization and/or in vitro development (Crister *et al*., 1986). Beside this, culture condition, hormone and protein supplementation also plays an important role for IVM, IVF and IVC (Bavister and Rose-Hellenkant, 1992; Fukui and Ono, 1989; Moor *et al*., 1980; Tsafri and Thibault, 1975) and though, a lot of experiments in this context have been performed such as IVM, IVF and IVC of embryos in cattle (Fukuda et al., 1990; Kotsuji *et al.*, 1996; Khandoker *et al.*, 2001; Chanson *et al*., 2001), in buffalo (Totey *et al*., 1993) in sheep and goat (Gardner *et al*., 1994; Cognie *et al*., 2003), horses (Cognie *et al*., 1992) and in rat and mouse embryos (Khandoker and Tsujii, 1999). Similar experiments in Bangladesh have not been done in the past and previously experiment on collection and evaluation of cumulus-oocyte-complexes (COCs) from slaughterhouse goat ovaries conducted and reported that the higher number of follicles was found in the ovaries without corpus luteum (Islam *et al*., 2007). The number of follicles measuring 2-6 mm diameter was found to be higher in ovaries without CL than ovaries with functional and regressed CL (Ferdous, 2006) and it was also suggested that ovaries having no CL might be the source of quality COCs (Islam *et al*., 2007).

2.5.2 *In vitro* maturation

Due to the heterogeneous nature of immature oocytes (oocytes from a wide range of follicle stages) used for IVEP, their response to the same IVM condition is different and unpredictable (Cognie *et al.,* 2004; Tibary *et al.,* 2005) and thus, the percentage of oocytes that resume nuclear maturation and reach the metaphase II stage can be more than 80% both in sheep (Guler *et al.,* 2000; Bai *et al.,* 2008; Shirazi *et al.,* 2010; Shabankareh *et al*., 2011) and goat (Izquierdo *et al.,* 2002; Rodriguez Gonzalez *et al*., 2003a,b; Urdaneta *et al*., 2003; Souza Fabjan *et al.,* 2014a).

Conventionally, oocytes of sheep and goat are *in vitro* matured in groups (25–50 oocytes with a ratio of 1 oocyte/2–5 ll medium) and incubated at 38–39°C in humidified atmosphere of 5% CO2 in air for 24–27 h and the culture systems used for *in vitro* embryo production (IVEP) can be classified according to their formulation in- (1) undefined media, where serum and/or a somatic cells co-culture is used; (2) semi-defined, without somatic cells and serum which is replaced albumin; (3) defined, where albumin is replaced by macromolecules, such as polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP) (Farin *et al*., 2001; Vanroose *et al.*, 2001). Several IVM media have been proposed for small ruminant oocytes (MEM, Waymouth, Ham-F10, etc.), but however, the most widely used is the TCM199 medium, bicarbonate buffered and containing minerals, carbon and energy sources (glucose, glutamine) as well as vitamins and amino acids (Mermillod *et al*., 2006), supplemented with L-glutamine, pyruvate, hormones (FSH, LH, 17b-E2,) plus complex fluids such as heat-treated serum and follicular fluid recovered from healthy non-atretic follicles (Cognie *et al*., 2003; Tibary *et al*., 2005; Paramio, 2010). Nevertheless, there are some research teams using SOF medium instead of TCM1999 in sheep (Shabankareh *et al*., 2012) and goat (Ongeri *et al.,* 2001; Bormann *et al.,* 2003; Herrick *et al.,* 2004) oocytes.

The atmospheric conditions of the IVM are air with 5% CO2 in air and it means 3–4 times more O2 than in the oviduct (Mastroianni and Jone, 1965) resulting

in increased reactive oxygen species (ROS) production (Luvoni *et al*., 1996; Agarwal *et al*., 2006) in the *in vitro* culture harmful for oocytes. Compound thiols such as cysteamine, 2-mercaptoethanol, cysteine, and cystin and glutathione (GSH) added to IVM media protect the oocytes from ROS (de Matos *et al*., 2002) and hese thiols also increase Intracytoplasmic GSH concentration which has a positive effect on fertilization and male pronucleus formation in oocytes from adult (de Matos *et al*., 2002) and prepubertal (Rodriguez-Gonzalez *et al*., 2003a,b; Bai *et al*., 2008) females. Addition of 100 lM cysteamine to the IVM medium improves blastocyst yield in goats (Rodriguez-Gonzalez *et al*., 2003a,b; De *et al*., 2011). Moreover, Wani *et al*. (2012) adding 200 lM cysteamine enhances *in vitro* embryo development rates in sheep and on the other hand, lamb oocytes matured in presence of 100 lM 2-mercaptoethanol and 600 lM cysteine produce embryos that reach the blastocyst stage earlier (Bai *et al*., 2008).

Vitamins are one of the components of many defined media and vitamins have been shown to increase glucose metabolism and to act as an antioxidant for developing embryos (Gardner *et al*., 1994). The effects of vitamins have been tested on oocyte maturation and embryo culture and have indicated some benefits for oocyte maturation and embryonic development in goat (Bormann *et al*., 2003) and sheep (Shabankareh *et al*., 2012) and the minimal essential medium (MEM) contain six water-soluble vitamins (thiamine hydrochloride, riboflavin, pyridoxal hydrochloride, folic acid, d-calcium pantothenate and nicotinamide) and two quasi-vitamins (myoinositol and choline chloride). Also, TCM-199 contains the same vitamins that MEM and using semi-defined media, the addition of vitamins improves embryo development. Thus, the addition of MEM vitamins to a SOF maturation medium plus BSA increases the percentage of blastocysts in goat (Bormann *et al*., 2003) and sheep (Shabankareh *et al*., 2012) and gonadotrophin hormones and 17b-estradiol are generally used in *in vitro* maturation protocols to improve nuclear and cytoplasmic oocyte

maturation as well as expansion of the surrounding cumulus cells in ovine (Guler *et al*., 2000) and caprine (Pawshe *et al.,* 1996) oocytes.

The concentration of FSH, LH and 17bestradiol vary among laboratories and gonadotrophin concentration commonly added to IVM medium are 10 lg/ml LH, 10 lg/ml FSH and 1 lg/ml 17b-E2 (goat: Cognie *et al*., 2004; Hammami *et al*., 2013; sheep: Leoni *et al*., 2007; Wang *et al.,* 2013) and 5 lg/ml FSH, 5 lg/ml LH and 1 lg/ml 17b – estradiol (sheep: Loi *et al.,* 2008; Catala *et al*., 2012; goat: Kharche *et al.,* 2009). In prepubertal goats, supplementation with high concentrations of 17b-estradiol (10 and 100 lg/ml) was found to be inhibitory on the meiotic progression up to metaphase II compared with 1 lg/ml (Lv *et al*., 2010) and human chorionic gonadotrophin (hCG) and equine chorionic gonadotrophin (eCG) also are added to IVM, in goats (Rahman *et al*., 2011) and sheep (Shabankareh *et al.,* 2012).

Conventionally, IVM media are supplemented with Serum and Follicular Fluid (FF), both of them with unknown composition and their actions are not fully understood, but it is believed that they provide proteins and/or some growth factors that contribute to the success of in vitro maturation and subsequent development. Despite the undefined and variable nature of serum composition, this supplementation is widely generalized in a conventional concentration of 10–20% v/v and the 4 sera mainly used are- (1) Foetal Calf Serum (FCS) (goat: Cognie *et al.,* 2004; Zhang *et al.,* 2013; sheep: Garcia-Garcia *et al.,* 2007; Wani *et al*., 2012), (2) Steer Serum (SS) (goat: Urdaneta *et al.,* 2003; JimenezMacedo *et al*., 2006; Romaguera *et al*., 2010), (3) Oestrus goat serum (OGS) (goat: De *et al*., 2011; sheep: Shabankareh *et al*., 2011) and (4) Oestrous Sheep Serum (OSS) (goat: Tajik and Esfandabadi, 2003; sheep: Shabankareh *et al*., 2011). Follicular fluid is also used as a supplement in the IVM media in goat and sheep (Cognie *et al.,* 2004) and the FF constitutes the microenvironment of the oocyte during follicular maturation and contains molecules involved in nuclear and cytoplasmic maturation, ovulation and fertilization (Yoshida *et al*., 1992). The supplementation of IVM media with FF from nonatretic healthy or

gonadotrophin stimulated large follicles (>4 mm) resulted in beneficial effects in both sheep and goat oocytes (Tibary *et al*., 2005) and however, Shabankareh *et al.* (2011) have compared the use of IVM media supplemented with human menopausal serum (HMS), OGS, OSS, bovine follicular fluid (BFF) and ovine follicular fluid (OFF) on *in vitro* maturation of sheep oocytes and have observed that HMS, OSS and OGS in the presence of FCS and hormones increased cleavage and blastocyst rates (90 and 18%, 87 and 43%, 86 and 38% respectively) compared with media containing FCS only or OFF and BFF (56 and 18.4%, 53 and 14%, 54 and 15% respectively).

Growth factors also have been added to IVM to improve embryo results and epidermal growth factor (EGF) is the most of the used. Thus, EGF is routinely added to IVM medium plus serum in goat (Zhang *et al.,* 2013) and sheep (Shabankareh *et al*., 2012; Wani *et al*., 2012) and EGF is added to IVM without serum supplementation in goat (Bormann *et al.,* 2003; Souza *et al*., 2013; Souza-Fabjan *et al.,* 2014a) and sheep (de Matos *et al.,* 2002). A used IVM defined medium of TCM199, EGF and cysteamine is used with good results in goat (Cognie *et al.,* 2004; Locatelli *et al.,* 2008; Souza *et al*., 2013; Souza-Fabjan *et al*., 2014b) and sheep (Cocero *et al*., 2011) and Insulin-like growth factor-I (IGF-I) has also been used to stimulate oocyte maturation and promoting blastocyst development in sheep (Guler *et al.,* 2000) and goat (Magalh~aes-Padilha *et al*., 2012). In sheep, Shabankareh and Zandi (2010) have studied the addition of EGF plus IGF-I plus cysteamine in a defined-IVM, semi-defined-IVM, and undefined-IVM medium on the cleavage and embryo rates concluding that these supplements added to undefined-IVM produced a higher percentage of morula and blastocysts than in a defined and semi-defined media and also, the positive influence of growth hormone (GH) on oocyte maturation has been reported.

In ovine (Shirazi *et al*., 2010), addition of 300 ng/ml GH in serum-containing maturation medium during IVM has significantly increased embryo development in terms of cleavage, blastocyst and hatching rates compared to the

control and in contrast, GH supplementation in serum-free oocyte maturation medium had no effect on subsequent cleavage, blastocyst and hatching rate. Finally, some laboratories have studied other molecules to try to find a defined medium for IVM and thus, Herrick *et al.* (2004) have obtained good results in embryonic development using for IVM goat oocytes hyaluronate and citrate in SOF medium (22% blastocysts) instead of SOF supplemented with BSA (19% blastocysts) and TCM199 with 10% OGS (5% blastocysts).

Thus, Souza-Fabjan *et al.* (2014a) testing three different IVMs showed no differences in blastocyst production between (1) defined medium (TCM199 plus cysteamine plus EGF), (2) TCM199 with FCS and (3) complex medium TCM199 with serum, hormones, EGF, Insulin Like Growth Factor, vitamins, thiol compounds and ITS with 46% and 45% and 45% of blastocysts respectively. Oocyte quality is the key factor to produce blastocysts and however, the quality of these blastocysts is affected mainly by the embryo culture conditions (Rizos *et al.,* 2002).

2.5.3 *In vitro* fertilization

The first step is to select the most motile and viable spermatozoa from the whole fresh ejaculate or the frozen-thawed sperm and the ejaculate comprises of a mixture of seminal plasma, mature and immature spermatozoa, non-reproductive cells, various microorganisms and non-specific debris. The most common method used for separating the sample into motile and non-motile fractions from fresh ejaculate is the swim-up technique (sheep: Shabankareh and Akhondi, 2012; Wani *et al.,* 2012; Shirazi and Motaghi, 2013; goat: Jimenez-Macedo *et al.,* 2007; Anguita *et al*., 2007a,b; Romaguera *et al*., 2010; De *et al.,* 2011; Pradeep *et al.,* 2011; Hammami *et al*., 2014) and thus, an aliquot of sperm suspension (50–100 ll) is layered under 2 ml of capacitation medium in several tubes at 38.5°C and after 0.5–1 h the top medium (0.4–1 ml) is collected from each tube. The pooled medium containing highly motile spermatozoa is centrifugated (200 9 g) for 10 min and for frozen-thawed semen, conventionally, motile spermatozoa are obtained by centrifugation on a discontinuous Percoll

gradient (sheep: Li *et al*., 2006; Garcia-Garcia *et al.,* 2007; Wan *et al*., 2009; Heidari *et al*., 2013; Wang *et al.,* 2013; goat: Bormann *et al.,* 2003; Herrick *et al.,* 2006; Souza *et al.,* 2013) and Percoll consists of colloidal silica particles of 15–30 nm diameter (23% w/w in water) that have been coated with polyvinyl pyrrolidone (PVP).

Rho et al. (2001) have compared selection of frozenthawed buck spermatozoa by swim-up, glass-wool filtration and Percoll density gradient centrifugation and the percentages of both cleavage and blastocyst formation in the Percoll density-gradient group (62% and 18% respectively) were significantly higher ($p < 0.05$) than in the swim-up (50% and 11% respectively) and glass-wool filtration (44% and 8% respectively) groups. Once the most viable and motile spermatozoa are selected, sperm capacitation is carried out *in vitro and s*everal capacitating agents have been used to capacitate spermatozoa and to yield good fertilization and cleavage rates, such as (1) Oestrous sheep serum (OSS) (ovine: Berlinguer, 2012; Catala *et al.,* 2011, 2012; Forcada *et al.,* 2013; Lahoz, 2013; Shirazi and Motaghi, 2013; Wang *et al.,* 2013), (2) oestrous goat serum (OGS) (goat: Koeman *et al*., 2003; Katska-Ksiazkiewicz *et al.,* 2007; Kharche *et al*., 2009), (3) heparin (goat: Izquierdo *et al.,* 1999; Hammami *et al*., 2014; ovine: Wani *et al.,* 2012; Heidari *et al.,* 2013; Sreenivas *et al*., 2013), (4) heparin and ionomycin (Wang *et al*., 2002; Urdaneta *et al.,* 2004), (5) heparin and PHE (penicillamine, hypotaurin and epinephrine) (ovine: Shabankareh and Akhondi, 2012), (6) heparin and serum (ovine: Cox and Alfaro, 2007; goat: Bormann *et al.,* 2003; Cox and Alfaro, 2007) and (7) heparin, PHE and serum (ovine: Wan *et al.,* 2009; goat: Katska-Ksiazkiewicz *et al*., 2007).

These capacitating agents can be used previously to the IVF, incubating the spermatozoa with them 15 min to 1 h, or to be presents in the fertilization medium and the use of oestrous serum instead of BSA in the IVF medium significantly increases the cleavage rate of sheep (78% and 0% respectively; Li et al., 2006) and goat (35% and 12% respectively; Kharche *et al*., 2009) oocytes. Moreover, the addition of heparin in the IVF medium supplemented with 5–10%

OSS does not seem to enhance the cleavage rate by improving the efficiency of ram sperm capacitation (Li *et al.,* 2006) and regarding the fertilization media used for IVF in small ruminants, the Synthetic Oviductal Fluid (SOF) medium is the most used in ovine (Leoni *et al*., 2007; Bai *et al.,* 2008; Wan *et al.,* 2009; Catala *et al*., 2012; Wang *et al*., 2013), whereas the Tyrode's Albumin Lactate Pyruvate (TALP) medium is widely used as IVF medium in goat (Katska-Ksiazkiewicz *et al*., 2007; Hammami *et al*., 2013).

For IVF, groups of 15–30 oocytes are transferred to drops of 50–100 ll of IVF medium where are inseminated and the final sperm concentration used in the IVF drop is 19106 spermatozoa/ml but it can vary from 0.5 9 106 cells/ml (Bai *et al.,* 2008) to 12–15 9 106 cells/ ml (Herrick *et al.,* 2004, 2006) depending on the male and IVF system used. Sperm and oocytes are co-incubated for 16–24 h at 38–39°C and concerning the atmosphere used during IVF, most laboratories use a humidified atmosphere of 5% CO2 in air (Cognie *et al.,* 2003). However, Leoni *et al.* (2007) suggest that low oxygen atmosphere during the IVF period improves the number of high quality sheep blastocysts as evaluated through kinetic development to blastocyst stage and cryotolerance to vitrification procedures and semen can be sex-sorted before IVF. But, Beilby *et al*. (2011) did not find differences between X- or Y-chromosome enriched ram sperm and non-sorted ram sperm.

2.5.4 *In vitro* embryo culture

The last step of *in vitro* embryo production is the culture of the presumptive zygotes to reach the stage of blastocysts at 6–8 days after *in vitro* fertilization in ruminant (Gardner *et al.,* 1994) and the post-fertilization culture is the period having the greatest impact on the blastocyst quality (Rizos *et al*., 2002). Embryonic genomic activation, which occurs at the 8- to 16-cell stage in ruminants, coincides with an increase in metabolic activity (Rieger *et al*., 1992), oxygen consumption and uptake of carbohydrates (Thompson *et al*., 1996) to the blastocyst stage and thus, early embryos cultured in vitro fail to develop past the 8to 16-cell stages in traditional culture media. This block occurred around

time of activation of the embryonic genome and serum and cells have been added to the culture to avoid this block and thus, since Gandolfi and Moor (1987) showed that co-culture with epithelial oviduct cells could support sheep embryos past the 8- to 16-cell stage, a large number of teams have used this undefined system, especially in laboratories with incubator atmosphere of 20% of O_2. Co-culture cells allow reduce O_2 tension to less toxic levels and moreover, somatic cells used in co-culture may produce unknown embryo growth promoting factors or delete embryo toxic factor from culture medium (Bavister, 1995). Although a wide variety of different cell types have been used, the most widely employed are the oviductal epithelium cells (Rho *et al.*, 2001; Katska-Ksiazkiewicz *et al.*, 2007). In the laboratory, with prepubertal goat oocytes, the author compared embryo development in 4 co-culture systems, oviductal epithelial cells (OEC) and cumulus cells (CC), both caprine and bovine, and the best results in blastocyst yield was obtained with OEC co-culture regardless of the species from which the cells are taken (Izquierdo *et al.*, 1999). Co-culture with somatic cells presents several disadvantages such as contamination risks, dubious quality control and long preparation time and moreover, the results are not predictable because of the unknown physiological status of the cells. To avoid the inconveniences of co-culture in atmospheres 20% O_2 a strategy for decreasing oxidative stress and oxidative damage is to add antioxidants to the media and thus, supplementation of IVC medium with 200 lM cysteamine (De *et al.*, 2011) has improved the blastocyst yield (33% and 49%, without and with cysteamine respectively) in goats.

A smaller concentration (100 lM cysteamine) has not been sufficient to alleviate the oxidative stress (Urdaneta *et al.*, 2003; De *et al.*, 2011) and in sheep, a-tocopherol (Natarajan *et al.*, 2010a) or L-ascorbic acid (Natarajan *et al.*, 2010b) added to SOF medium has enhanced embryo development at 20% O_2 (18% and 8% blastocysts, with and without a-tocopherol respectively; and 14% and 9% blastocysts, with and without L-ascorbic acid respectively). However, at 5% oxygen concentrations, exogenous antioxidants do not influence bovine

blastocyst formation rate or quality (Amiridis and Cseh, 2012) and several studies have carried out to test biologically derived components, such as serum and albumin, added to the IVC medium in order to avoid co culture cells media.

However, as mentioned previously, the composition of serum is also undefined and variable and it has been shown that exposure to serum can greatly alter embryo morphology, developmental rates, newborn weight and gestation length (Thompson *et al.,* 1995) and the developmental abnormalities associated with IVC, collectively termed 'large offspring syndrome' (LOS), include a range of features and phenotypes, for example, increased early embryo losses, large foetuses, disproportionate foetal organ growth and abnormalities of placental development. Thus, LOS and several other developmental abnormalities have been reported also in lambs produced *in vitro* (Tibary *et al*., 2005) and Rooke *et al*. (2007) concluded that the presence of serum during the first 2 days of IVC resulted in increased weights of gravid uterus, placenta, foetus, foetal heart and liver of lambs. The cell cycles during this sensitive window in the sheep cover the period from which the embryo is dependent on maternal RNA and proteins through to the onset of major genome activation and in goats, we have not found studies about LOS after IVEP. But, Wilmut *et al*. (2002) concluded that in cloning goats, placenta-abnormalities, LOS, respiratory or cardiovascular dysfunction, organ dysplasia, high perinatal mortality or abnormal postnatal development have not been observed as it was observed in calves and sheep and different culture media have been successfully used for small ruminant embryo development such as TCM 199 (Wani *et al.,* 2012), B2 (Katska-Ksiazkiewicz *et al*., 2007) and 'Sydney IVF Blastocyst' medium (Beilby *et al*., 2011).

However, the most widely used medium is the synthetic oviduct fluid (SOF) and Tervit *et al*. (1972, 1974) were the first to report successful culture of ruminant zygotes to the blastocyst stage in vitro using SOF medium, which was based on the composition of ovine oviduct fluid. Subsequently, changes to the original composition have been made. Some laboratories routinely supplement SOF medium with 5– 10% FCS (goat: Hammami *et al.,* 2013; sheep: Catal a *et al*.,

2012) but others only add BSA (sheep: Leoni *et al.*, 2007; Shabankareh and Akhondi, 2012; Wang *et al*., 2013) and with sheep and goat oocytes recovered by LOPU, Cox and Alfaro (2007) have obtained a high blastocyst rate (50% and 61.5%, goat and sheep respectively) culturing the embryos in SOF medium plus BSA 5 days and then 2 days more in TCM199 plus BSA.

Recently, it has published some works testing the effect of some molecules until now no proved in culture media of embryos of small ruminants ghrelin is a widespread hormone that several studies have linked with reproductive physiology (Garcia *et al.*, 2007; Tena-Sempere, 2008). In sheep (Wang *et al.*, 2013), the blastocyst rate, total cell number of blastocysts and the expression levels of the GLUT1 and IFNT genes were increased when 50 ng/ml ghrelin was added during IVC to the SOF medium and on the other hand, activin is an important member of the transforming growth factor b (TGFb) superfamily. Expression of protein and mRNA for activin-A and activin receptors has been localized in both oocyte and granulosa cells of follicles at various developmental stages of ovine (Thomas *et al*., 2003); and caprine (Silva *et al.,* 2004) and supplementation of SOF medium with 10 ng/ml activin-A has enhanced embryo development of prepubertal goat oocytes (Hammami *et al*., 2014). In goat, Rodrıguez-Dorta *et al.* (2007) have compared the effect of two culture conditions, SOF in a humidified atmosphere of 5% O2, 5% CO 2 and 90% N2 vs goat OEC (GOEC) co-culture in a 5% CO2 in air, on the pregnancy rate, embryo survival rate and offspring development after direct transfer of vitrified/thawed caprine IVP embryos and the percentage of blastocyst in SOF (28%) was higher than in GOEC (20%).

The progress in understanding of the requirements of the developing embryo resulted in the development of sequential media where components change according to the needs of the embryo (Thompson, 2000) and these sequential media would mimic the change in environment experienced by the developing embryo *in vivo*, enabling the biochemical and morphological changes of embryos. Thus, physiological sequential media are formulated to reflect the

carbohydrate levels of the reproductive tract and reduce cellular stress on the embryo (Lane *et al.*, 2003) and in goat, the sequential G1.2/G2.2 media supplemented with BSA have been shown to support embryo development until blastocyst stage (Ongeri *et al.*, 2001; Bormann *et al.*, 2003; Koeman *et al.*, 2003).

In sheep, Garcia-Garcia *et al.* (2007) have tested sequential G1.3/G2.3 media supplemented with BSA compared to SOF plus 5% FCS and they have obtained a similar blastocyst rate (21.5% and 24.1% respectively) in both IVC systems and however, blastocysts developed in G1.3/G2.3 serum-free were of lower quality in terms of hatching (44.3% and 86.6% respectively). Finally, embryos are routinely cultured in groups and in fact, most researchers report higher blastocyst rates and improvement embryo quality following group, compared to single culture, because oocytes and embryos stimulate reciprocally during their *in vitro* development (Gardner *et al.*, 1994). From a practical point of view, an individual oocyte culture system would be very useful when working with endangered species or other animals of high genetic value, and/or when LOPU-sessions, or other retrieval methods yield only one or a few cumulus oocyte complexes and methods of culturing embryos individually, or in groups, but in an individually identifiable manner using microfluidics (Krisher and Wheeler, 2010), wells-in-wells (Vajta *et al*., 2008) or on adhesive substances (Matoba *et al.*, 2010) are being studied. More recently, innovative ways of bar-coding individual embryos have even been reported (Novo *et al*., 2013) and in conclusion, embryo culture media are divers and also according with the experimental conditions of the laboratory.

In goat and sheep routinely presumptive zygotes are cultured for 8 days after insemination in an atmosphere of 5% O2 using SOF medium plus serum and addition of serum increases blastocyst yield because its effect in the mitosis activation but also serum have a significant effect on the incidence of chromosomal abnormalities in the resulting blastocysts (Lonergan *et al*., 2004). In our laboratory assessing chromosomal anomalies in blastocysts obtained of

oocytes from prepubertal and adult females an cultured in SOF plus 10% FCS we observed that 90% of blastocyst showed mixoploidy with an average of 23.68% of abnormal blastomere per embryo and without differences between prepubertal and adult blastocysts (Romaguera *et al*., 2011) and a chemical defined serum-free media is also an important aim to reach in IVEP of small ruminants.

2.5.5 Relationship between follicle size, oocyte diameter and embryo development

The incorporation of Juvenile *in vitro* Embryo Technology (JIVET) into breeding programmes is advantageous because it can reduce the generation interval and increase the rate of genetic gain and another advantage of oocyte collection from prepubertal ovaries is the early availability of large numbers of oocytes per female (Koeman *et al.*, 2003). Thus, this technology would allow a fast multiplication of unique and expensive animals such as endangered species (Tervit, 1996) and nevertheless, the greatest limitation of JIVET is that the production of embryos and their development to term after transferring to recipient females is lower in comparison to their adult counterparts. This fact has been shown in cattle 1.2 vs 2.2 blastocysts per animal in calf and cow (Palma, 1993), in sheep 20% vs 40% blastocysts in lamb and ewe (Ledda *et al.*, 1997), in pig 21% vs 34% blastocysts in prepubertal and adult females (Marchal *et al.*, 2001) and in goats stimulated hormonally 24% vs 34% blastocysts in prepubertal and adult females (Leoni *et al*., 2009) and this low embryo development of oocytes obtained from prepubertal females might be caused because these ovaries present a low number of follicles larger than 3 mm.

In goats, the author have observed 1.1 follicles larger than 3 mm per ovary in prepubertal females (Martino *et al*., 1994) and in adult goats, Crozet *et al.* (1995) obtained a percentage of blastocysts of 6%, 12%, 26% and 41% of oocytes recovered from small (2–3 mm), medium (3.1–5 mm), large (5 mm) follicles and ovulated oocytes, respectively. The positive and direct relationship between follicle size, oocyte diameter and embryo development is well-known (Gandolfi

et al., 2005) and studies in laboratory have shown ultrastructural and functional deficiencies in prepubertal goat oocytes such as altered distribution of cortical granules (Velilla *et al*., 2004) and mitochondria (Velilla *et al.*, 2006), disorganization of microtubules and microfilaments (Velilla *et al.*, 2005) and alteration in total RNA content, p34 (cdc2) and cyclin B1 expression and maturation promoting factor (MPF) activity (Anguita *et al*., 2007a,b, 2008).

However, when selecting prepubertal oocytes from follicles larger than 3 mm, the percentage of blastocysts obtained after IVF was similar to those obtained from adult females by LOPU (Laparoscopy Ovum Pick Up) (18 vs 20% respectively) (Romaguera *et al*., 2011) and ovaries from prepubertal animals have a high percentage of antral follicles with a diameter smaller than 3 mm (Martino *et al*., 1994), making it difficult to release oocytes by follicular aspiration or select oocytes by their follicular diameter as is conventionally done in adult ovaries. Prepubertal female oocytes are routinely obtained by slicing the ovary surface recovering a pool of oocytes with a heterogeneous degree of growth and atresia and coming from unknown follicles and in this case, oocytes are selected by diameter and the morphological appearance of cumulus cells and oocyte cytoplasm. Thus, Anguita *et al.* (2008) classified prepubertal goat oocytes diameters in 4 categories: <110 lm; 110–125 lm; 125– 135 lm and larger than 135 lm observed a blastocyst development of 0%, 0%, 7% and 10%, respectively, after IVF. Using Intracytoplasmic Sperm Injection (ICSI), Jimenez-Macedo *et al*. (2006) found significant differences between oocytes 125–135 lm diameter and larger than 135 lm in cleavage (67% and 75% respectively) but blastocyst yield was not different (16% and 11% respectively) and assessment of oocyte diameter is a time-consuming procedure which affects IVEP output because the long exposure of oocytes to light and harmful atmosphere.

The absence of enzymatic activity of G6PD can be an indirect measure of fully-grown oocytes that have finished their intraovarian growth phase and Rodriguez Gonzalez *et al*. (2002) using BCB showed that the mean diameter of BCB+

oocytes (136.6 ± 6.3 lm) was larger than BCB oocytes (125.5 ± 10.2 lm) in prepubertal goats. In prepubertal sheep oocytes, BCB+ oocytes were also larger (123.66 ± 2.72 lm) than BCB- (106.82± 2.82 lm) with a blastocyst ±development after IVF of (21% and 8% respectively) (Catala *et al.,* 2011). and however, these significant differences in blastocyst yield were not observed after BCB selected oocytes fertilized by ICSI (14% and 11% for BCB+ and BCB- oocytes) (Catala *et al.,* 2012).

The results of JIVET using oocytes from 30- to 45-day-old goats and 90- to 120-day-old lamb show yields of 18% and 21% of blastocysts/total oocytes respectively and in a recent review by Souza-Fabjan *et al.* (2014b) summarized a production of blastocysts in goats from 7% to 55% and in sheep from 10 to 42% with oocytes from adult females.

Katska-Ksiazkiewicz *et al.* (2007) tested the BCB stain to select goat oocytes for blastocyst production concluding that the percentage of blastocysts was significantly higher in BCB+ (13%) and control (19%) than BCB- oocytes (0%) and in ovine oocytes, Wang *et al*. (2012) observed significantly higher percentages of blastocyst (34%) of BCB+ oocytes than BCB- (6%) and Mohammadi-Sangcheshmeh *et al.* (2012) described 34%, 4% and 20% of blastocyst rate for BCB+, BCB- and control oocytes respectively.

Variability in IVEP output has also been observed according to season and in laboratory with prepubertal goat oocytes and fresh semen capacitated with heparin we have observed a different blastocyst production in summer, autumn, winter and spring with 13%, 2%, 17% and 25% respectively (Catala *et al*., 2013). In adult females, Souza-Fabjan *et al*. (2014a) also found higher blastocyst production of goat oocytes recovered at the slaughterhouse during the anoestrus season and in contrast, Mara *et al.* (2013) in sheep testing during 3 years the output of IVEP programme concluding that the percentage of blastocysts was higher during the breeding season, but there were no differences in pregnancy and lambing rates among blastocysts produced throughout the year.

2.5.6 Seasonal effect on the quality of oocytes

Seasonal effect was found to be an important consideration in this respect, especially in buffalo (Kadoom, 1995 and Das *et al*., 1996), mare (Bruck *et al*., 1996 and Colleoni *et al.*, 2004) and cattle (Silva *et al.*, 2006). In buffalo, the incubation temperature during the *in vitro* maturation (IVM) influenced the fertilization rate but had no significant effect on maturation and subsequent embryo development (Ravindranatha *et al*., 2003). Al-Katanani *et al*. (2002) concluded that summer depression in oocyte quality in Holstein cow was evident, but cooling cow for 42 days did not alleviate that seasonal effect. Singla *et al.* (1999) reported that buffaloes subjected to heat stress yielding fewer good quality oocytes than their unstressed counterparts the developmental competence of the oocytes under *in vitro* conditions has been investigated. Rutledge *et al*. (1999) emphasized that the production of cattle blastocyst was reduced in mid summer to late summer. In contrast, Rocha *et al*. (1997) in *Bos indicus* (Brahman) cows and Rivera *et al*. (2000) in bovine failed to indicate an effect of season on *in vitro* embryo production in a subtropical environment. Seasonality is less pronounced between males of domestic species, but there are differences in behavior and sperm characteristics depending on the time of the year (Gerlach *et al*., 2000, Ciereszko *et al*., 2000 and Chacon *et al*., 2002).

2.6 Conclusion

Genetic improvement of goat in Bangladesh could be made by planned artificial insemination (AI) with frozen semen, multiple ovulation and embryo transfer (MOET) and *in vitro* production (IVP) of embryos. But to produce good embryos, quality oocyte is obligatory. Though lot of ovaries are waste in slaughter house but it may be a good source of quality livestock production that can full fill the existing scarcity of meat, milk and skin and ovary collection, evaluation and grading technique results in rapid genetic gain of outstanding females. So, *in vitro* embryo production from slaughterhouse ovaries might be considered as a low cost and sustainable technique in Bangladesh condition.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental site

The experiment was conducted in the laboratory of the Dept. of Animal Production & Management and Dept. of Animal Nutrition, Genetics & Breeding, Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University.

3.2 Materials

Sl No.	Name of the Item
1	Petridesh 90mm
2	Goat ovary
3	Slide
4	Beaker 250 ml
5	Gloves
6	Syringe 10mm
7	Mask
8	Scalpel
9	Micro pippete
10	Microscope
11	Digital Balance
12	Test Tube
13	Tube Rack
14	Sodium chloride
15	Cotton
16	Alcohol
17	PBS (Phosphate buffer solution)
18	Slide Calipers

3.3 Methods

3.3.1 Collection and processing of ovaries

3.3.1.1 PBS (Phosphate Buffer Solution) formulation

Before collection of ovaries PBS (Phosphate Buffer Solution) was formulated. For this 500 ml distilled water was required and kept in a beaker. Then 4 g NaCl (Sodium Chloride), 0.1 g KCl (Pottassium Chloride), 0.71 g $Na_2HPO_4.2H_2O$ (Sodium Hydrogen Phosphate) and 0.12 g KH_2PO_4 (Pottassium Hydrogen Phosphate) were weighed in a digital balance and put one by one in the 500 ml distilled water containing beaker and mixed them by jerking.Then PBS **(Phosphate Buffer Solution)** was ready for use.

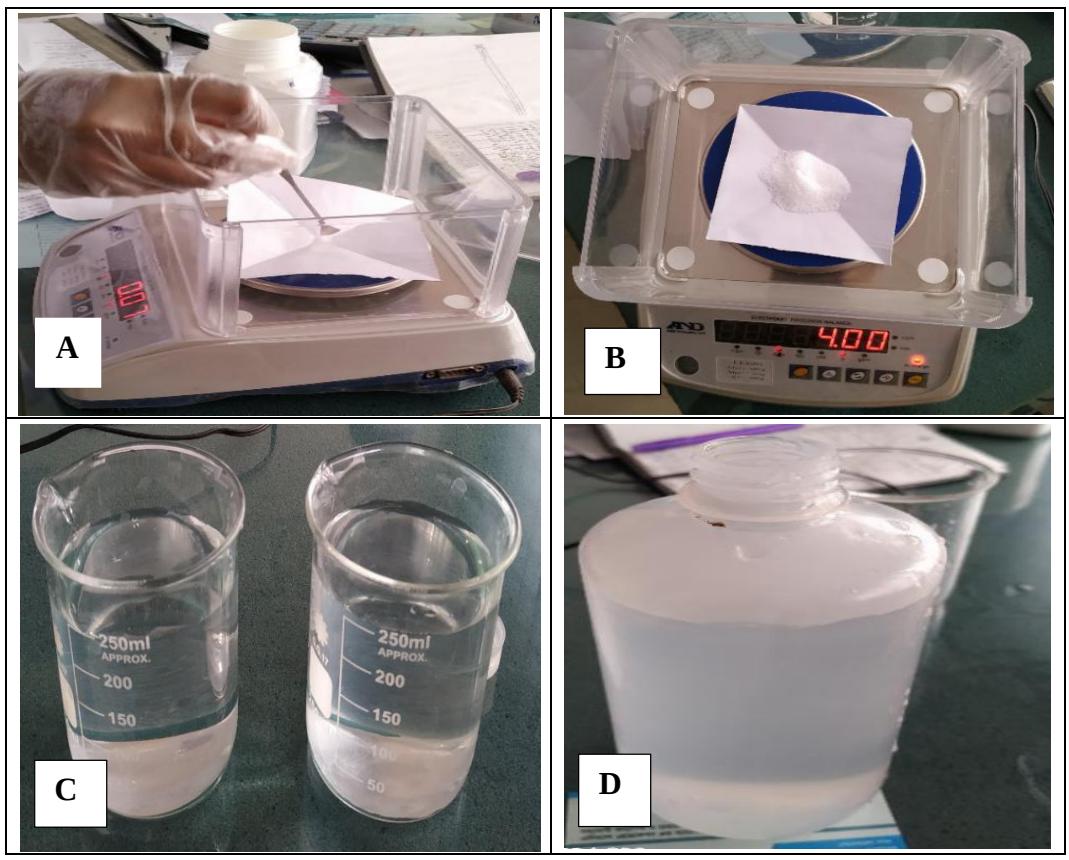

Plate 1. **PBS (Phosphate Buffer Solution) formulation,** A.Taking powder from bottle in a digital balance, B. Powder weighed in a digital balance, C. 500 ml distilled water containing beaker, D. PBS (Phosphate Buffer Solution)

3.3.1.2 Collection of ovaries

Collection of ovaries and processing have been performed according to the slight modification as described previously (Jamil *et al.,* 2008). Briefly ovaries from sexually mature goats were collected within 30 min of slaughter from the slaughter house. During collection, ovary was marked whether it is right or left. They were then transported within 2 h of slaughter to the laboratory in a vacuum flask containing sterilized phosphate buffered saline (PBS) at 25-30 °C. Ovary collection times were classified into 2 breeding seasons, i.e. Summer season (July, August and September) and Winter season (December, January and February). Among 101 ovaries CL was found in 6 ovaries with the remaining 95 ovaries having no CL.

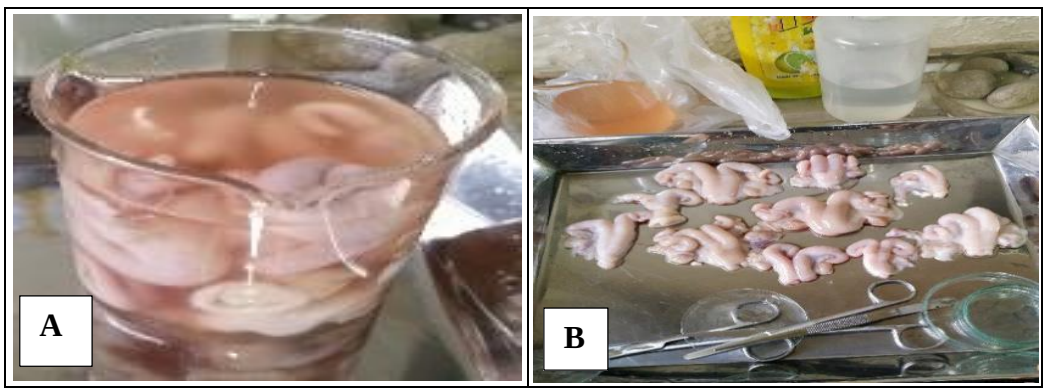

Plate 2. **Collection of ovaries,** A.Female reproductive tract at flask, B. Female reproductive tract at steel tray

3.3.1.3 Processing of ovaries

Upon arrival at the laboratory the ovaries were then transferred to sterilized petridishes and rinsed thoroughly by physiological saline at 25 °C before further processing. The adipose tissues and surrounding bursa were removed from ovaries surface. After collection and trimming, ovaries were evaluated on the basis of presence and absence of CL.

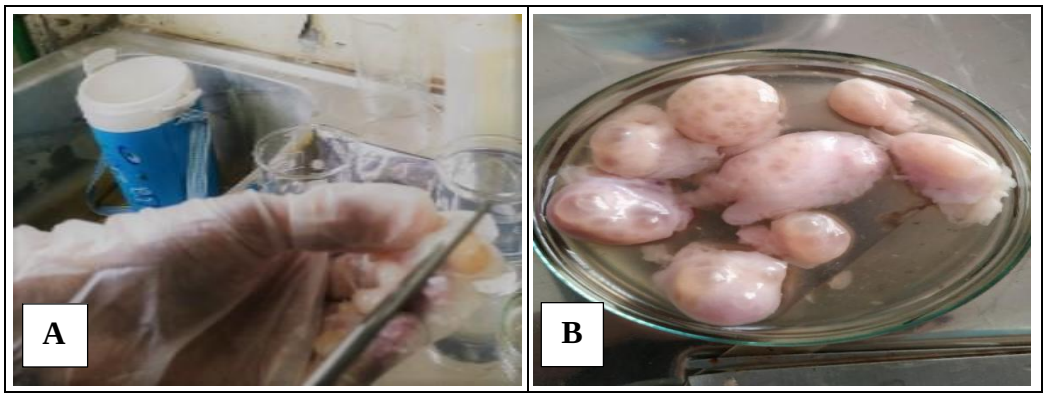

Plate 3. **Processing of ovaries,** A. Trimming of ovaries, B. Processed ovaries at PBS containing petridish

3.3.2 Grouping of ovary

To investigate the influence of the corpus luteum on the quantity and the quality of cumulus-oocyte complexes (COCs) recovered per ovary, the ovaries were divided into 2 groups, i.e. the ovaries with and without a corpus luteum. The total as well as usable COCs recovered from each ovary of the 2 ovary groups were recorded.

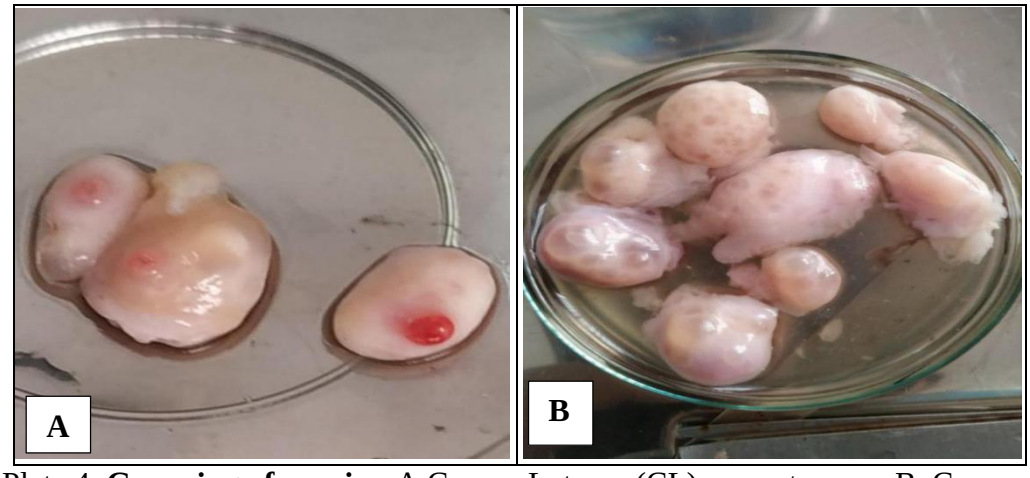

Plate 4. **Grouping of ovaries,** A.Corpus Luteum (CL) present ovary, B. Corpus Luteum (CL) absent ovary

3.3.3 Evaluation of ovary

After collection and trimming ovaries were evaluated on the basis of the following parameters

3.3.3.1 Measurement of weight, length and width of ovaries

Individually right, left, CL-present and CL-absent ovaries were weighed in gm in a digital balance and the weight was recorded in tabular form. The length and width in cm of right, left, CL- present and CL-absent ovaries measured with the help of a digital slide calipers.

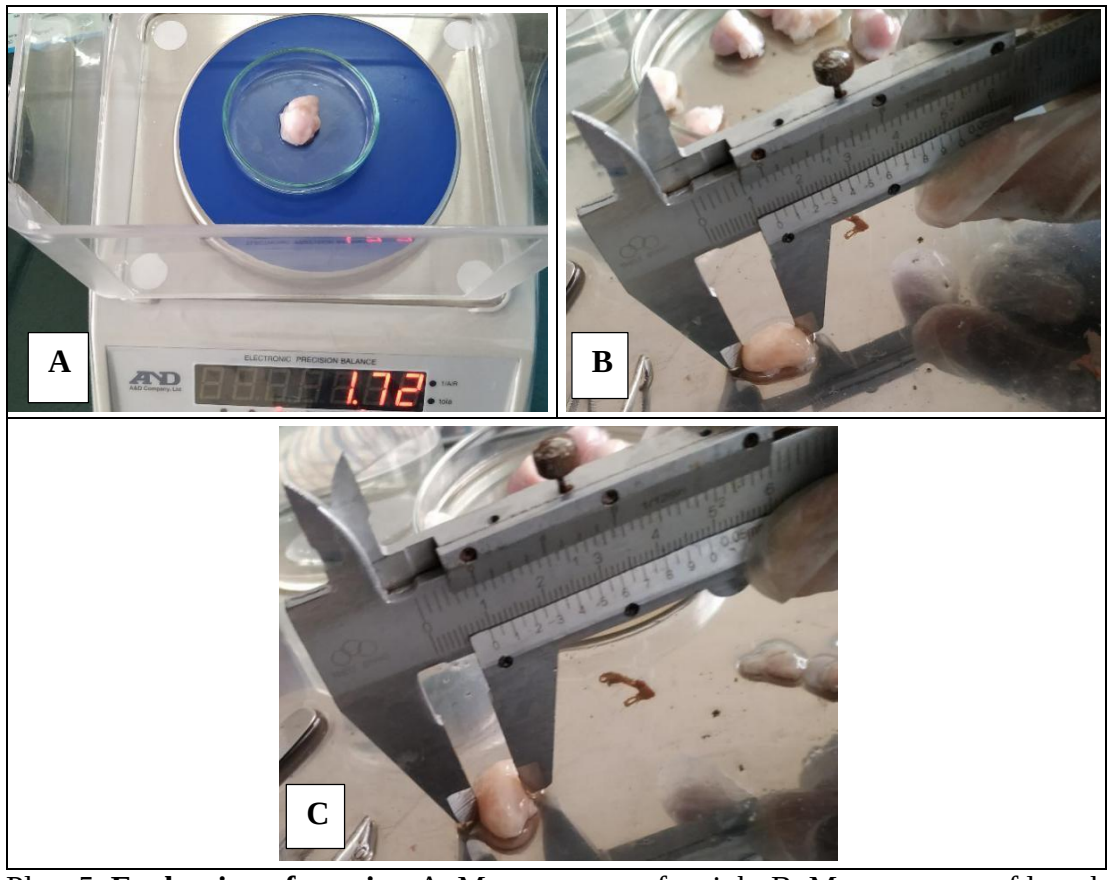

Plate 5. **Evaluation of ovaries**, A. Measurement of weight B. Measurement of length C. Measurement of width

3.3.3.2 Counting of follicle on the surface of the ovary

Different size of follicles on the surface of both ovaries are present. The number of visible follicles on the surface of different category (with or without CL) of ovaries were counted and recorded for further analysis.

Plate 6. **Counting of follicle on the surface of the ovary**

3.3.4 Oocyte collection and COCs evaluation

The ovaries were washed 2-3 times in saline solution at 30°C. They were then placed in a beaker and kept in a water bath at 30°C. Each ovary was individually handled, and oocytes were recovered by the following way:

3.3.4.1 Aspiration technique

In this technique, 18-gauge hypodermic needles were used to puncture the ovarian surface. The 10 ml syringe was loaded with D-PBS (1.0 -1.5ml), and the needle (18G) was put in the ovary parenchyma near the vesicular follicles and all follicles were aspirated near the point at the same time. After aspirating the follicles from one ovary, the aspirated follicular materials were transferred slowly into a 35-mm Petridis, avoiding damage to the cumulus cells. Then the

petridishes were kept undisturbed for 5 minutes, allowing the COCs to settle down. Then the COCs were searched and graded under an inverted microscope (Olympus, Tokyo, Japan) at low magnification.

Plate 7. **Oocyte collection,** Aspiration technique

3.3.4.2 Catagories of COCS

The COCs were classified according to the slight modification of the method of Khandoker *et al*. (2001). Briefly quality of cumulus oocyte complexes were analyzed based on layers of granulose cells. There are four categories of cumulus oocyte complexes: category A (good quality), oocytes surrounded by cumulus cells with large quantities (more than 3 layers) and compact with a homogeneous ooplasm; category B (medium quality) cumulus cells surrounding the oocytes or oocytes partially surrounded by less than 3 layers of cumulus cells with homogeneous ooplasm; category C (poor quality) the oocytes not covered by cumulus cells or oocytes surrounded by cumulus cells slightly. And grade D: degeneration observed both in oocytes and cumulus cells. Here grade A and B will be considered as normal COCs and grade C and D as abnormal COCs.

3.3.5 Statistical analysis

All values were expressed as Mean±SE. The *p* value less than 0.05 were considered as significant. Data were analyzed with the SAS (Statistical Analysis System) software using one-way Analysis of Variance (ANOVA).

CHAPTER 4
RESULTS & DISCUSSION

4.1 Effect of season on morphology of ovaries

The ovaries were found almond-shaped, pale colored structures situated in the edge of the mesovarium near the lateral margin of the pelvic inlet (Plate 8). This report corresponds to the report of May (1970). Each ovary had an irregular surface by follicles of various sizes projecting from the surface. The uterine extremity of the ovaries was connected with the extremity of the horn of uterus by a proper ligament of the ovary. There was no demarcation between the horn of the uterus and the flexuous uterine tubes (Plate 8).

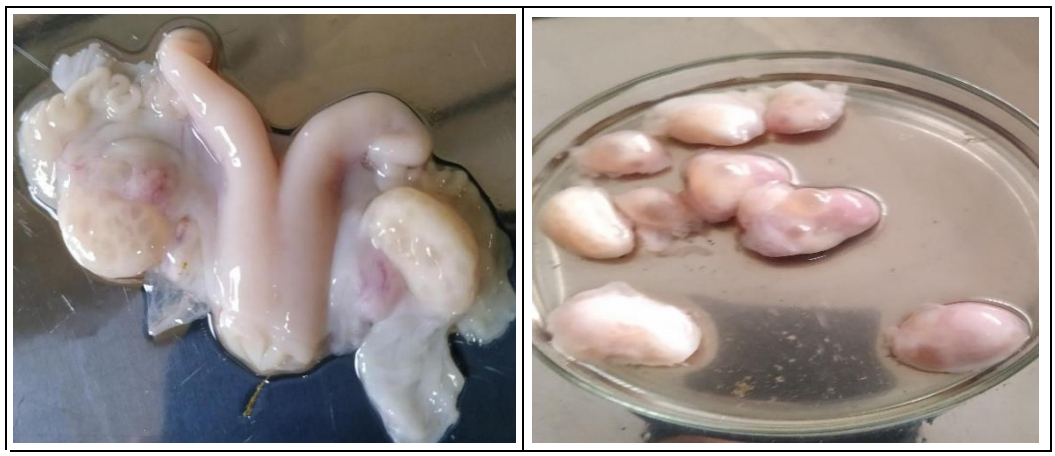

Plate 8. **Morphological evaluation of ovaries,** Ovary and associated structures

In left ovaries the mean weight and length were distinctly higher (Table 1, Figure 1) in summer season [(1.00±0.11) g and (1.32±0.06) cm respectively] compared to that of winter season [(0.74±0.07) g and (1.22±0.05) cm respectively]. On the other hand the length of the left ovary was 1.71±0.27 cm respectively in Nigerian goats (Adigwe and Fayemi, 2005). This differs with the present study. Species difference may cause this. But width was distinctly higher in left ovaries (Table 1, Figure 1) in winter season [(0.83±0.06) cm respectively] compared to that of summer season [(0.82±0.05) cm respectively].

In right ovaries the mean weight and width were distinctly higher (Table 1, Figure 2) in summer season [(1.08±0.15) g and (0.91±0.06) cm respectively] compared to that of winter season [(0.92±0.07) g and (0.85±0.04) cm respectively], but length was distinctly higher (Table 1, Figure 2) in winter season [(1.32±0.04) cm respectively] compared to that of summer season [(1.29±0.08) cm respectively].

Table 1: Effect of season on qualitative and quantitative parameters in left and right ovary

ovary	Parameter	summer season	winter season	Level of significance
Left ovary	Weight (g)	1.00±0.11	0.74±0.07	NS
	Length (cm)	1.32±0.06	1.22±0.05	NS
	Width (cm)	0.82±0.05	0.83±0.06	NS
Right ovary	Weight (g)	1.08±0.15	0.92±0.07	NS
	Length (cm)	1.29±0.08	1.32±0.04	NS
	Width (cm)	0.91±0.06	0.85±0.04	NS

Values are shown in mean±SE, (Standard Error)
NS=Non-significant at $p>0.05$

The observation expressed that in both seasons ovaries were equally active to normal physiological and/or ovarian activity. The observation also revealed that there was no significant difference (P>0.05) in the parameters of left ovary in summer and winter seasons of goat. The mean weight and width in the present study were numerically found higher in right ovaries in summer than those of winter season but no significant ($p>0.05$) differences were found. The mean weight (g) and width (cm) of right ovaries in summer and the weight (g), length (cm) and width (cm) of right ovaries in winter were found higher than left ovaries. The length of the right ovary was 1.73±0.27 cm respectively in Nigerian goats (Adigwe and Fayemi, 2005). This doesnot match with the present study. This may cause due to species difference.

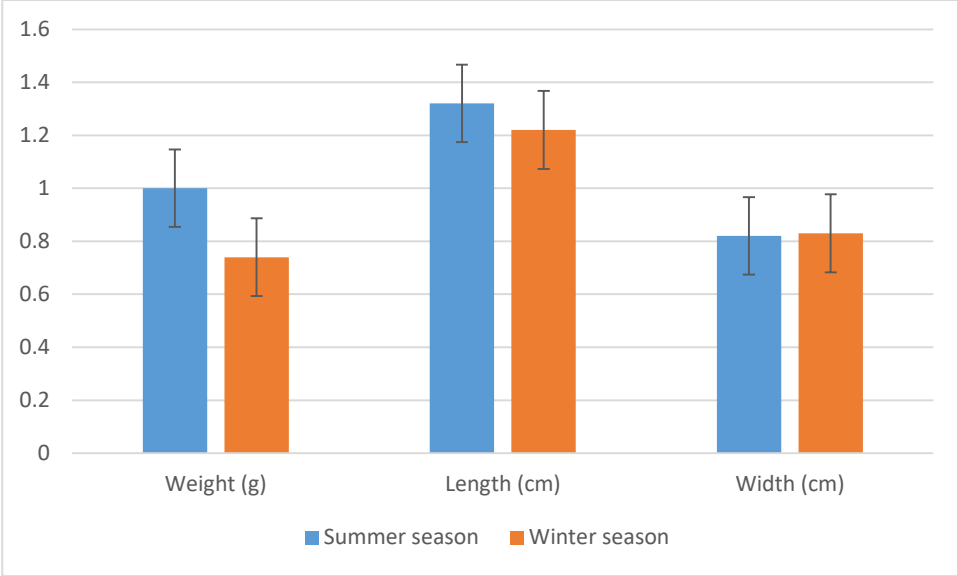

Figure 1: Effect of season on qualitative and quantitative parameters in left ovary

The results of the different qualitative, quantitative parameters of ovaries in different seasons in goat are summarized in (Figure 1 and 2). In this study, it was observed that all the qualitative and quantitative parameters of different follicular aspects of left ovary were not similar to the right ovary in goat in different seasons were not similar and non-significant (P>0.05) in goat. 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 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.

Regarding to normal physiological explanation of ovarian activity is that right ovaries are more active than left ones, according to previous reports (Singh *et al*., 1974; Rahman *et al*., 1977; Sarkar, 1993). In the present study, values that have not reached statistical significance (P>0.05) may be ascribable to the species. Similar results were found in goat (Islam *et al*., 2007). But the results of a previous study, Singh *et al*. (1974) supports that there was no significant difference (P>0.05) in the parameters of left and right ovaries of goat.

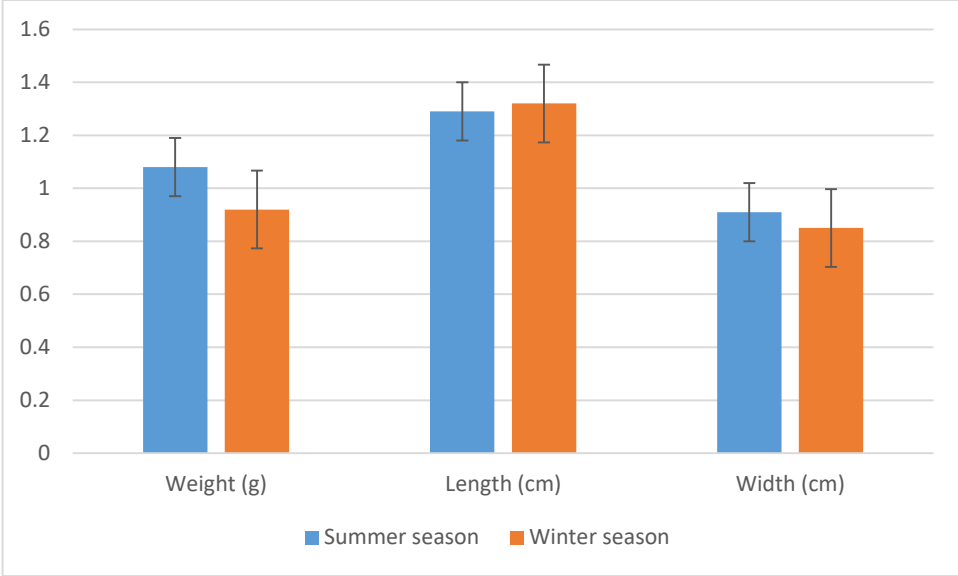

Figure 2: Effect of season on qualitative and quantitative parameters in right ovary

Table 2: Effect of season on qualitative and quantitative parameters in corpus luteum-present ovary

Seasons	Weight (g) (Mean±SE)	Length (cm) (Mean±SE)	Width (cm) (Mean±SE)
Summer season	0.78±0.32	1.32±0.25	0.86±0.20
Winter season	0.58±0.15	1.21±0.14	0.89±0.05
Level of significance	NS	NS	NS

Values are shown in mean±SE, (Standard Error)
NS=Non-significant at $p>0.05$

In corpus luteum-present ovaries the mean weight and length were numerically higher (Table 2, Figure 3) in summer season [(0.78±0.32) g and (1.32±0.25) cm respectively] compared to that of winter season [(0.58±0.15) g and (1.21±0.14) cm respectively]. But width was distinctly higher (Table 2, Figure 3) in winter season [(0.89±0.05) cm respectively] compared to that of summer season [(0.86±0.20) cm respectively]. No significant ($p>0.05$) differences were found in the weight (g), length (cm) and width (cm) of corpus luteum-present ovary in summer and winter seasons of goat.

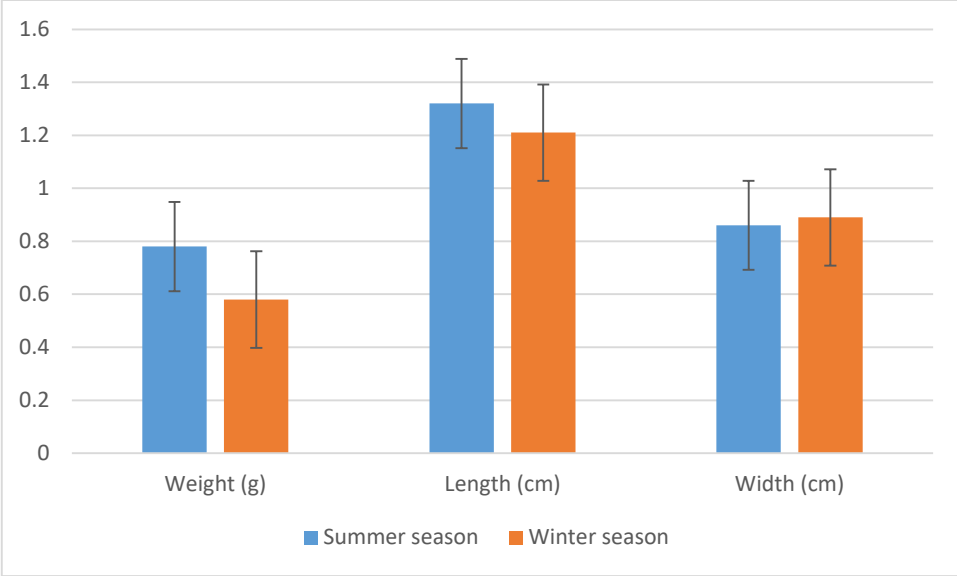

Figure 3: Effect of season on qualitative and quantitative parameters in corpus luteum-present ovary

The CL is an extra cellular material within the ovary which made the differences of its width and weight. 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 Effect of season on follicular development in ovary

The number of follicles visible on the surface in relation to the right and left ovaries were counted and presented in (Table 3, Figure 4). The average number of follicles in both left and right ovaries were higher (Table 3, Figure 4) in summer (12.50; 10.48) than in winter season (8.08; 8.21). No significant ($p>0.05$) differences were found in the total number of follicles in left and right ovary in summer and winter seasons of goat.

Table 3: Seasonal effect on number of follicles of ovary

No. of follicle (Mean±SE)			
Ovary	Winter season	Summer season	Level of significance
Left ovary	8.08±0.77	12.50±2.56	NS
Right ovary	8.21±0.95	10.48±1.98	NS

Values are shown in mean±SE, (Standard Error)
NS=Non-significant at $p>0.05$

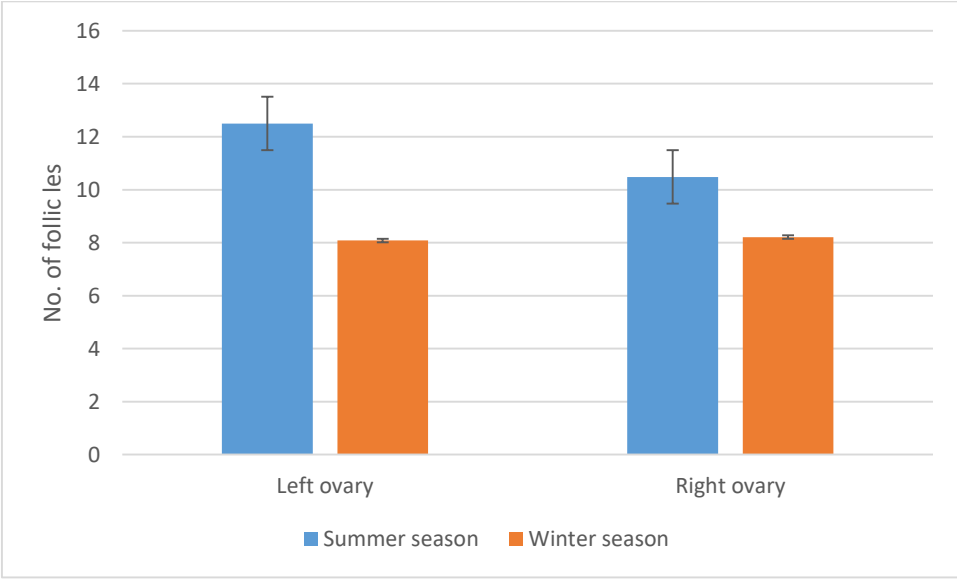

Figure 4: Seasonal effect on number of follicles of ovary

According to (Farag *et al.*, 2010) the results revealed that more categories COCs and the total number of recovered oocytes per ovary were aspirated during spring than those collected during winter, summer and autumn seasons. This may attributed to the presence of more follicles during the breeding season (spring). These findings are similar to those reported on sheep by Attia (2001), who found that the reduction in the proportion of good oocytes (class A and B) was evident among those recovered during summer and autumn (16.46%, 24.19% and 15.76%, 23.57%, respectively) than those harvested during winter and spring (50.83%, 57.77% and 28.90%, 28.88%, respectively). It could be suggest that oocytes were compromised during development and differentiation when climatic factor are not ideal for reproduction. The present findings were

also supported by Datta and Goswami (1998) who observed that, the average number, as well as, the proportion of good quality oocytes that were retrieved from buffalo ovaries during cool months (<25°C) was significantly higher than the corresponding values obtained during moderately hot (25-30°C) and hot (>30°C) periods. They suggested that oocytes are compromised during development and differentiation when climatic factor are not ideal for reproduction. Also, Zohier *et al* (2007) found that the proportion of collected number of good buffalo oocytes significantly increased during spring and winter than those collected during summer and autumn (71 and 74.6 vs. 50 and 56.9%, respectively). In addition, Brück *et al.* (1996) revealed that the rate of oocytes recovery in mare was significantly higher in May/June (57.3%) than in August / September (44%). Also, more category (I) and total oocytes per ovary in camel were recovered during the breeding season than non breeding season (Abdoon, 2001). According to (Va´ zquez *et al.*, 2014.) This author attributed this difference to the presence of more follicles during the breeding season (from February to April). But all this results donot support my observation. More follicles were found during summer season than winter season from my observation. It may happen due to nutritional aspect, temperature or species difference. In summer season grass is more available than winter season. This may be a cause of this findings.

Table 4: Effect of season on total number of follicles in corpus luteum-present and corpus luteum-absent ovary

	Total No. of follicle (Mean±SE)		
Ovary	Summer season	Winter season	Level of significance
CL present ovary	9.00±1.00	6.50±1.84	NS
CL absent ovary	13.52±0.50	11.84±1.71	NS

Values are shown in mean±SE, (Standard Error)
NS=Non-significant at $p>0.05$

In corpus luteum-present ovaries total number of follicles were distinctly higher (Table 4, Figure 5) in summer season [(9.00±1.00) respectively] compared to that of winter season [(6.50±1.84) respectively]. In corpus luteum-absent ovaries total number of follicles were distinctly higher (Table 4, Figure 5) in summer and winter season (13.52; 11.84) compared to that of CL present ovary in summer and winter season (9.00; 6.50). No significant ($p>0.05$) differences were found in the total number of follicles in corpus luteum-present and absent ovary in summer and winter seasons of goat.

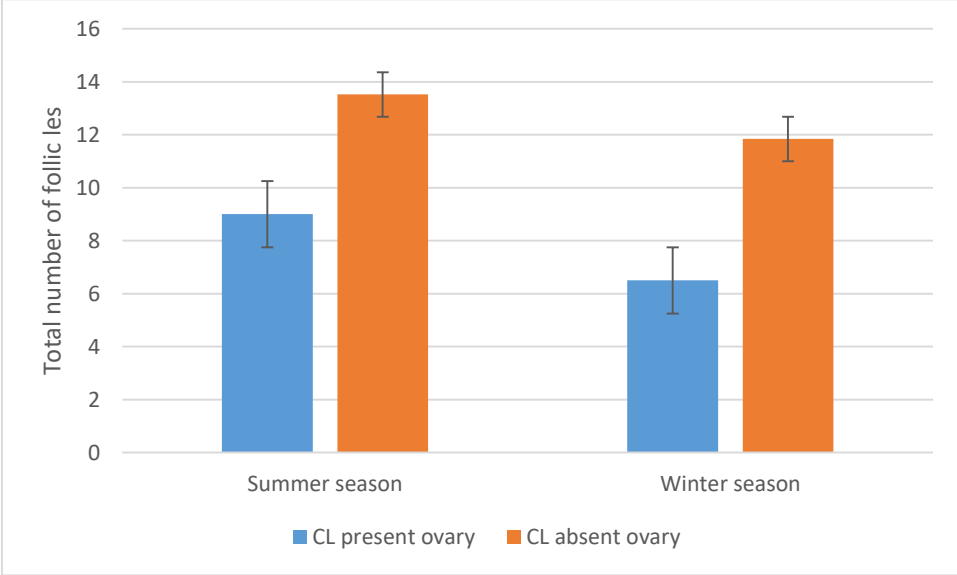

Figure 5: Effect of season on total number of follicles in corpus luteum-present and corpus luteum-absent ovary

As follicle bears oocyte, so we can say that more follicle contains more oocyte and less follicle contains less oocyte. According to Nandi *et al*. (2000) the oocyte recovery rate decreased when ovaries had a corpus luteum and this supports this result.

4.3 Grading of oocytes

The oocyte recovery rate (Table 5) (2.14) is higher in summer than in (1.97) winter season. The recovery rate of A, B, C and D grade of oocytes were found to be 2.20, 2.42, 2.07 and 1.71 (table 5) in summer and 2.00, 1.25, 2.42 and 2.25 in winter. The results obtained in the present study showed that recovery rate of

grade A and B oocytes were higher in summer than that of in winter and recovery rate of grade C and D were higher in winter than that of in summer. The results also obtained in the present study showed that recovery rate of total grade A and B oocytes (2.24) was higher in summer than that of in winter (1.79) and recovery rate of total grade C and D was higher in winter (2.33) than that of in summer (1.95).

Table 5: Grading of oocytes in different seasons

Parameters		Summer season	Winter season	Level of significance
Total No. of ovaries		57	44	--
Total No. of oocytes		122	87	--
Oocytes per ovary (mean±SE)		2.14±0.11	1.97±0.11	NS
Normal oocyte	Grade A	2.20±0.16	2.00±0.13	NS
	Grade B	2.42^{a}±0.36	1.25^{b}±0.16	*
	Total	2.24^{a}±0.15	1.79^{b}±0.12	*
Abnormal oocyte	Grade C	2.07±0.23	2.42±0.36	NS
	Grade D	1.71±0.18	2.25±0.31	NS
	Total	1.95±0.16	2.33±0.23	NS

Values are shown in mean±SE, Standard Error
Mean values in the same column with different superscripts (a, b) differ significantly at $p<0.05$
NS=Non-significant at $p>0.05$
*Significant at $p<0.05$

Ovary collection times in goat were classified into 2 breeding seasons, i.e. peak breeding season (September, October, and November) and low breeding season (March, April, and May) (Jamil *et al.*, 2008). The recovery rate of B oocytes and total of A and B oocytes was significantly higher ($P < 0.05$) during the spring season than the winter season. The recovery of oocytes was significantly higher ($P < 0.05$) during the peak breeding season than the low breeding season (Huma *et al.*, 2006). This result supports this findings partially. A similar trend was observed with the recovery of usable oocytes (Jamil *et al.*, 2008). Bovine reproduction has a complex dependency on soil, plant, and climatic factors

(Predojevic *et al.,* 1988), particularly in tropical and subtropical parts of the world. Although buffaloes are polyestrous, they exhibit a distinct seasonal variation in breeding activity. Results of other studies performed in Pakistan are consistent with those performed in India; about 64%-75% of buffalo exhibited estrus between September and December (Singh *et al., 1993*). It has been observed that the maximum number of services occurred during the fall season (September, October and November), followed by the winter season (December, January and February) (Shah *et al.,* 1988). The seasonal effect on the incidence of estrus was very significant ($P < 0.05$). The poor recovery of oocytes during the low breeding season was probably due to the relatively inactive status of ovaries (Perera *et al.,* 1984) that often renders buffalo as seasonal breeders (Pandey *et al.,* 1979). The results of the present study revealed that a significantly higher ($P < 0.05$) oocyte recovery rate per ovary was obtained from the ovaries collected during the peak breeding season.

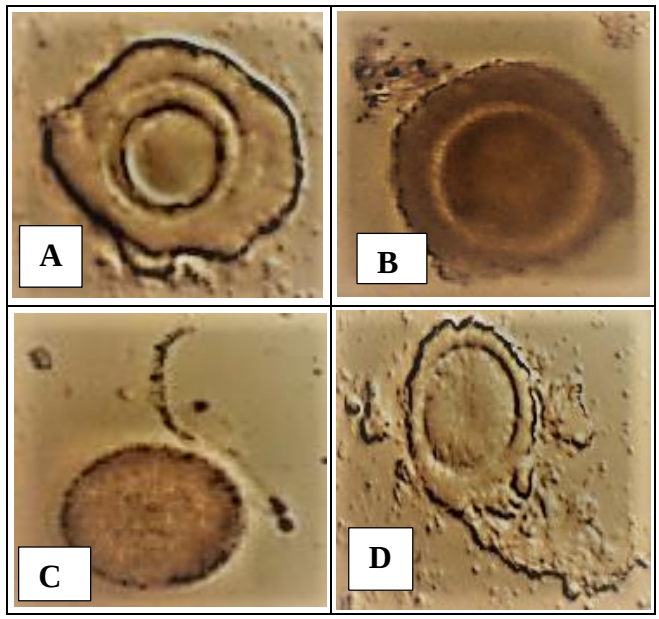

Plate 9. **Grading of oocytes,** A. Grade A, B. Grade B, C. Grade C, D. Grade D

According to (Farag *et al.,* 2010) during the study, the average number of COCs categories and total oocytes per ovary were slightly raised during summer than

those collected during winter (1.31; 3.68 vs. 1.26; 3.45, respectively). These findings are similar to those reported by Gou *et al*. (2009) who observed that proportion of sheep COCs was significantly (P<0.05) decreased in winter compared to summer and autumn (51.4 vs. 84.9 and 83.6%, respectively). Also these findings are in agreement with those reported by Kadoom *et al.* (1995) who found that, the total recovery rate of oocytes from buffalo ovaries was higher in summer season than in other seasons. The same findings were also obtained by Seydou *et al*. (1999) in goat. This findings support this observation.

According to Nagwa Hassan *et al.* (January 2010) the spring was the best season for recovery of COCs which have a worthy competence to be matured *in vitro*. Stenbak *et al*. (2001) reported that IVF of oocytes recovered from superovulated ewes was higher in the breeding season than during the anoestrus. In addition, season influences *in vivo* fertilization rate, and some studies have shown a greater number of fertilized oocytes recovered from super-ovulated ewes in the breeding season than during the anoestrus (Mitchell *et al*., 2002; Gonza´ lez-Bulnes *et al*., 2003). Changes in semen quality might contribute to the seasonal differences in IVF, but low seasonal variations in the volume and quality of the ejaculates from Rasa Aragonesa rams (Martı´*et al*., 2007) suggest that the lower cleavage rates in the anoestrous season might be mainly caused by lower oocyte quality.

4.4 Effect of season on oocytes recovery in corpus luteum-present and corpus luteum-absent ovary

In the present study a significantly greater number of oocytes (Table 6, Figure 6) per ovary were recovered from ovaries without a corpus luteum (2.23; 2.02) than from ovaries with a corpus luteum (1.50; 1.50). Greater number of oocytes (Table 6, Figure 6) per ovary were recovered from ovaries with and without a corpus luteum (1.50;2.23) in summer than from ovaries with and without a corpus luteum (1.50; 2.02) in winter.

Table 6: Effect of season on total number of oocytes in corpus luteum-present and corpus luteum-absent ovary

No. of oocyte (Mean±SE)			
Ovary	Summer season	Winter season	Level of significance
CL present ovary	1.50±0.50	1.50±0.28	NS
CL absent ovary	2.23±0.14	2.02±0.12	NS

Values are shown in mean±SE, (Standard Error)
NS=Non-significant at *p*>0.05

No significant (p>0.05) differences were found in the total number of oocytes in corpus luteum-present and absent ovary in summer and winter seasons of goat.

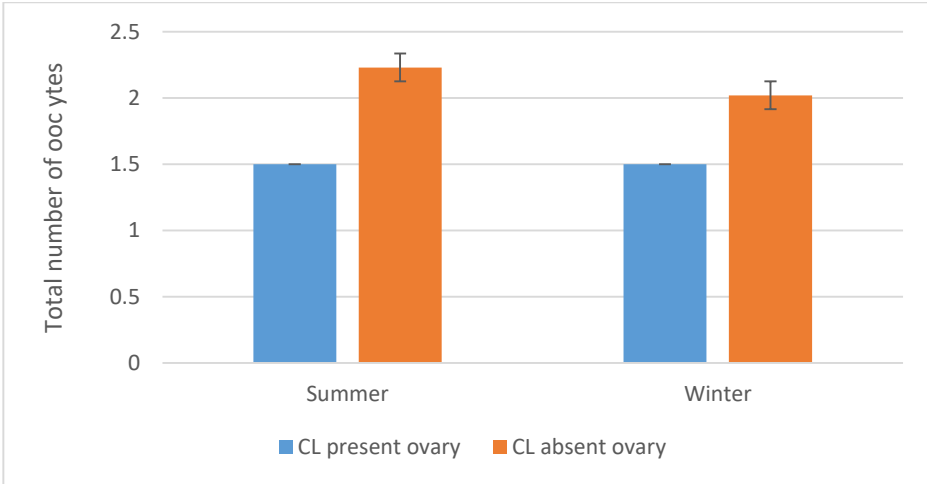

Figure 6: Effect of season on oocytes recovery in corpus luteum-present and corpus luteum-absent ovary

According to Nandi *et al*. (2000) the oocyte recovery rate decreased when ovaries had a corpus luteum. This is because follicular development is restricted, as lutein cells occupy most of the ovary (Kumar *et al.*, 1997). The dominant follicle is usually observed in the corpus luteum-bearing ovary, and the other follicles are very small and remain mostly inaccessible (Gasparrini *et al.*, 2000). Cow (Moreno *et al.*, 1993) and goat (Agrawal *et al.*, 1992) ovaries containing a corpus luteum yielded a lower number of oocytes than ovaries without a corpus

luteum. Several researchers have reported that the presence of a corpus luteum yielded a lower number of oocytes per ovary and a lower proportion of usable oocytes (Moreno *et al.*, 1993). In contrast, Boediono *et al.* (1995) and Das *et al*. (1996) found no difference in the mean number of oocytes per ovary between corpus luteum-bearing and non-bearing ovaries. In conclusion it was determined that the method of oocyte collection, season, and ovarian status at the time of oocyte collection significantly affects the recovery of usable oocytes in buffalo for use in IVF programs.

CHAPTER 5
SUMMARY AND CONCLUSION

In vitro maturation (IVM) of oocytes provide an excellent opportunity for cheap and abundant embryos for carrying out animal improvement. 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 present study was, therefore, designed with the objectives of evaluating oocyte recovery rates and their quality from goat ovaries collected at local abattoir in different seasons, characterizing the ovary and oocyte physically in Black Bengal goat in different seasons and assessing the quality of oocytes recovered from Black Bengal goat in different seasons.

The experiment was conducted in the laboratory of the Department of Animal Production and Management and the Department of Animal Nutrition, Genetics and Breeding, Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University. From local slaughterhouses, goat ovaries were collected and recorded as right and left. At the presence or absence of corpus luteum (CL) they were also categorized as CL-present or -absent group. Ovary collection times were classified into 2 breeding seasons, i.e. summer season (July to September) and winter season (December to February). Among 101 ovaries CL was found in 6 ovaries with the remaining 95 ovaries having no CL. The result of the different parameters is summarized.

Our study revealed that the left ovary (1.00±0.11) g was heavier in the summer than the ovary (0.74±0.07) g in the winter. The length of the ovary (1.32±0.06 cm) was higher in summer than that (1.22±0.05) cm in the winter but the width of the ovary distinctly higher in winter season [(0.83±0.06) cm respectively]

compared to that of summer season [(0.82±0.05) cm respectively]. On the other hand higher weight [(1.08±0.15) vs (0.92±0.07) g] and width [(0.91±0.06) vs (0.85±0.04) cm] were found in right ovaries in summer than winter season. The average number of follicles in both left and right ovaries were higher in summer (12.50; 10.48) than in winter season (8.08; 8.21). In different corpus luteum-present ovaries the mean weight and length were distinctly higher in summer season [(0.78±0.32) g and (1.32±0.25) cm respectively] compared to that of winter season [(0.58±0.15) g and (1.21±0.14) cm respectively]. But width was distinctly higher in winter season [(0.89±0.05) cm respectively] compared to that of summer season [(0.86±0.20) cm respectively].

Both right and left ovaries were great source for large number of oocytes and without-CL ovaries were considered as a suitable source to supply quality oocytes for *in vitro* embryo production of goat. In different corpus luteum-present ovaries total number of follicles were distinctly higher in summer season [(9.00±1.00) respectively] compared to that of winter season [(6.50±1.84) respectively]. In different corpus luteum-absent ovaries total number of follicles were distinctly higher in summer and winter season (13.52; 11.84) compared to that of CL present ovary in Summer and Winter season (9.00; 6.50).

The oocyte recovery rate (2.14) was higher in summer than in (1.97) winter season. The recovery rate of A, B, C and D grade of oocytes were found to be 2.20, 2.42, 2.07 and 1.71 in summer and 2.00, 1.25, 2.42 and 2.25 in winter. The results obtained in the present study showed that recovery rate of grade A and B oocytes were higher in summer than that of in winter and recovery rate of grade C and D were higher in winter than that of in summer. The results also obtained in the present study showed that recovery rate of total grade A and B oocytes (2.24) was higher in summer than that of in winter (1.79) and recovery rate of total grade C and D was higher in winter (2.33) than that of in summer (1.95).

In the present study a significantly greater number of oocytes per ovary were recovered from ovaries without a corpus luteum (2.23; 2.02) than from ovaries with a corpus luteum (1.50; 1.50). Greater number of oocytes per ovary were

recovered from ovaries with and without a corpus luteum (1.50; 2.23) in summer than from ovaries with and without a corpus luteum (1.50; 2.02) in winter. So the summer was the best season for recovery of COCs which have a worthy competence to be matured *in vitro*.

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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