EFFECT OF BOVINE SERUM ALBUMIN ON *IN-VITRO* MATURATION OF BOVINE OOCYTE

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CERTIFICATE

*This is to certify that the thesis entitled "**EFFECT OF BOVINE SERUM ALBUMIN ON IN-VITRO MATURATION OF BOVINE OOCYTE**" submitted to the Department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (MS) in ANIMAL BREEDING AND GENETICS**, embodies the results of a piece of bona fide research work carried out by **MOUMITA SAHA**, RegistrationNo.**18-09076**, 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:

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DEDICATED TO

Professor

Dr. Lam Yea Asad

and my beloved parents

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EFFECT OF BOVINE SERUM ALBUMIN ON *IN-VITRO* MATURATION OF BOVINE OOCYTE

ABSTRACT

The present experiment was to investigate the effect of different concentration of Bovine Serum Albumin on *in vitro* maturation of bovine oocyte. Cumulus oocytes complexes (COCs) were collected from bovine ovaries by aspiration method and matured for 48 hours in TCM-199 basic medium supplemented with different concentration of BSA (0%, 5%, 10% and 15%). The Significantly higher ($p<0.01$) number of follicles were aspirated in ovaries without CL (Corpus Luteum) (4.60±0.28) than with CL containing ovaries (3.00±0.37). Higher numbers of COCs were found in ovaries without CL (4.42±0.30) than ovaries with CL (2.40±0.40). Number of follicle aspirated in left ovaries (4.76±0.20) and in right ovaries (4.74 ±0.20).The collected number of COCs higher in left ovaries (4.47±0.19) compared to right ovaries (4.34±0.18).Three levels of cumulus cell expansion after 48 hours of *in vitro* maturation (at 38.5 °C and 5% CO_2 in an incubator) observed under 10x magnification of microscope and different stages of nuclear maturation was observed based on chromosomal configuration. Oocytes reached to Level-1, Level-2 and Level-3 cumulus cell expansion level with 0%, 5%, 10% and 15% of BSA concentration were 0.32%, 0.26%, 0.29% and 0.50%; 0.38%, 0.43%, 0.86% and 0.31%; 0.13%, 0.96%, 0.71% and 0.06% respectively, where, 5% BSA supplementation was reached 0.96% at cumulus cell expansion level-3. The percentage of COCs reached to Metaphase-I, Metaphase-II, GVBD (germinal vesicle break down) and GV(Germinal vesicle) stages with 0%, 5%, 10% and 15% of BSA supplementation were 0.00%, 0.92%, 0.47% and 0.28%; 0.00%, 0.00%, 0.12% and 0.00%; 0.64%, 0.62%, 0.82% and 0.61%; 0.29%, 0.19%, 0.24%,and 0.44% respectively. These result further indicate that the maturation and subsequent development rate could be significantly increased ($p<0.01$) by supplementing 5% level of BSA than that of control. This trend maintained up to 10% of BSA, but no further improvement was found by increasing level of BSA up to 15%. It is conducted that the left ovary without CL is a good source of normal grade oocytes and 10% level of BSA supplementation has a positive effect of *in vitro* maturation of bovine oocytes.

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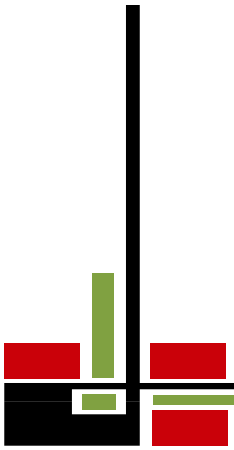
LIST OF ABBREVIATIONS AND SYMBOLS

%	= Percentage
>	= Greater than
<	= Less than
±	= Plus minus
AI	= Artificial Insemination
ANOVA	= Analysis of Variance
BAU	= Bangladesh Agricultural University
B.C	= Before Christ
BLRI	= Bangladesh Livestock Research Institute
BSA	= Bovine Serum Albumin
CL	= Corpus Luteum
COCs	= Cumulus-Oocyte-Complexes
DF	= Degree of Freedom
DLS	= Department of Livestock Services
et al.	= Associate
FAO	= Food and Agricultural Organization
FAOSTAT	= Food and Agricultural Organization Statistics
GDP	= Gross Domestic Product
Gm	= Gram
GVBD	= Germinal Vesicle Break Down
IVC	=*In Vitro* Culture

LIST OF ABBREVIATIONS AND SYMBOLS (cont'd)

IVF	= *In Vitro* Fertilization
IVM	= *In Vitro* Maturation
IVP	= *In Vitro* Production
Kg	= Kilogram
MOET	= Multiple Ovulation and Embryo Transfer
MS	= Mean Square
No.	= Number
NS	= Not significant
PBS	= Phosphate Buffered Saline
SAARC	= South Asian Association for Regional Co-operation
SAS	= Statistical Analysis System
SAU	= Sher-e-Bangla Agricultural University
SAURES	= Sher-e-bangla Agricultural University Research System
SE	= Standard Error
SS	= Sum of Squares
Viz	= Namely



Chapter 1
Introduction

CHAPTER-1

INTRODUCTION

Latest developments in gametes and embryo cellular biology, the field of molecular embryology on farm animals has been explored due to the limited availability of the suitable experimental materials at a acceptable cost. After a dramatic development of cellular biology over the last decades, lots of research efforts have been moved towards the implementation of assisted reproductive technologies (ARTs) such as multiple ovulation and embryo transfer (MOET*), In-vitro* production (IVP) of embryos, cloning and transgenesis to transfer a targeted number of embryos produced from animal having desired genetic make-up. In MOET, embryos are collected *in-vivo* from super ovulated donars at the required developmental stage and transferred to a number of synchronized recipients. Cattles are numerically and economically very important and promising animal genetic resources in developing country like Bangladesh and accounted for about 24.3 millons head (DLS-2020). Cattle significantly tribute to the GDP in Bangladesh through production of milk, meat and skin.

Bangladesh is an agriculture-based developing country and livestock plays a vital role as a major component of agricultural sector, in the national economy of Bangladesh. Although livestock plays a major role (16%) in agricultural sector (economic statistics, 2005). Due to lower genetic potentiality, indigenous livestock can not fulfil the demand of milk and meat. This is being considered as an important problem for livestock development and this can be overcome through genetic improvement of indigenous stock by an appropriate breeding technology such as reproductive and molecular genetic technologies. Assisted reproductive technologies include artificial insemination(AI), multiple ovulation and embryo transfer (MOET), *in vitro* production (IVP) of embroyo etc. By enhancing selection intensity and reducing generation interval these technologies could bring tremendous change in animal production system.

In vitro maturation of immature oocytes from ovaries at slaughter, followed by IVF and IVC the resulting zygotes has allowed extensive research on modern reproduction techniques in farm animals. Mukherjee (1972) reported that mouse oocyte could be matured and fertilized *in vitro* and developed to final term, there have been intensive attempts in cattle and pigs with the

technique. Hanada *et al*., (1986) finally succeeded in getting calves from IVM oocytes that were fertilized *in vitro*. In pigs, Mattioli *et al*., (1989) succeeded in getting piglets from IVM-IVF oocytes in 1989. Fulka and Okolski first performed the IVM of mare oocytes in 1981.

Conventional embryo transfer technology is based on the superovulation of high quality donor animal and subsequent recovery of embryo by flushing the uterus a week after breeding. In cattle, this technology is well established, but in goat, considering the poor ovulatory response, the application of this technology is difficult. An alternative to conventional superovulation procedures is *in vitro* production (IVP) of embryos. This technology allows the predictable supply of embryos from ovaries of slaughtered females or from lives selected animals, via repeated recovery of primary oocytes.

For *in vitro* production of bovine embryos, the efficient collection method and grading of cumulus-oocytes-complexes (COCs) are the primary steps to be done. To obtain immature oocyte from ovaries several methods such as aspiration of total follicular material, collection of intact follicles with subsequent isolation of the COCs and slicing of the ovaries are employed. Slicing of the ovaries, flushing of the follicles or rupturing the isolated follicles may increase the number of recovered oocyte as compared with that of aspiration of follicular materials (Alm and Torner, 1994).

Oocyte maturation is traditionally defined as those events associated with the initiation of germinal vesical breakdown (GVBD) and completion of the first meiotic division, reffered to here as nuclear maturation. The ability of the mammalian oocytes to resume meiosis and complete nuclear maturation is acquired in stepwise fashion, which has been shown to increase with age of female (Sorensen and wassarman,1976) and size of follicle (Tsafriri and Channing,1975), though this ability differs among species studies (Motlik and Fulka,1986). Mouse oocytes recovered from young female showed increasing frequencies of GVBD. The ability of the oocyte to complete the first meiotic division was not evident until oocytes had nearly completed their growth phase (77-78µm oocyte diameter) and was associated with antrum formation (Linder *el al*.,1980). The process of oocyte maturation, however, also includes changes within the cytoplasm, of which the production of male pronuclear growth factor is a notable example (Thibault *et al*.,1975). More correctly, oocyte maturation should be defined as

those events that render the oocyte capable of fertilization and able to initiate the program that directs pre implantation embryonic development.

oocyte maturation is crucial for subsequent embryo development; however, oocyte mitochondrial and lipid-droplet behaviour are still poorly understood. Although excessive lipid accumulation during *in vitro* production (IVP) of bovine embryos has been linked with impaired cryotolerance, lipid oxidation is essential for adequate energy supply. Fetal bovine serum (FBS) and bovine serum albumin (BSA) are supplements used during IVM, containing high and low lipid content, respectively. IVM media containing FBS increased total lipid content 18-fold and resulted in higher lipid accumulation in oocytes when compared with media with BSA. IVM using a lower FBS concentration combined with BSA resulted in satisfactory maturation and embryo development and also reduced lipid accumulation in blastocysts. In conclusion, IVM causes changes in mitochondrial and lipid dynamics, which may have negative effects on oocyte development rates and embryo lipid accumulation. Moreover, decreasing FBS concentrations during IVM may reduce embryo lipid accumulation without affecting production rates.

Bovine serum albumin (BSA) is a globular protein that is often used as a protein concentration standard in lab experiments as well as in numerous other biochemical applications. Derived from cows, BSA is extracted from cow blood using one of three different purification methods: cold-organic solvent fractionation, heat shock, and ion exchange chromatography.

BSA improves maturation, fertilization, blastocyst formation and hatching rates *in vitro* (Visconti *et al*., 1995; Bhattacharyya,1992) and it has been widely used in medium for the capacitation of sperm (Rajikin *et al.*,1994; Dow and Bavister, 1989) and the acrosome reaction (Yoshida *et al*.,1993;Andrews and Bavister,1989). It is generally believed that the beneficial effects of serum are due to cyclic adenosine monophosphate, catecolamines, vitamins, putative growth factors, lipids and albumin. It has been also demonstrated that the beneficial effect of BSA supplement is due to the presence of a relatively high molecular weight protein which contributes to maturation of oocytes (Kane, 1985; Kane and Headon,1980). It may also have a nutritional role to play by supplementing amino acids after hydrolysis (Thompson and Peterson, 2000; Biggers *et al.*,1997), thereby maintaining the intracellular amino acid pools (Thompon *et al.*,1998). BSA also provide yet underfined embryotrophic (e.g.citrate, steroids) compounds (Biggers *et al*., 1997; Bavister, 1995; Gray *et al*.,1992), functioning as a heavy metal ion

chelator/free radical scavenger, protecting cellular constituents against the effect of toxins and regulating redox potential, p^H and osmolarity (Bigger *et al*., 1997; Alveraz and Storey,1995; Flood and Shirley, 1991).

However, a great deal of work has been done regarding collection of cumulus-oocyte-complexes (COCs) from slaughter house ovaries, grading of collected oocytes, IVM, IVF of the oocytes and IVC of the zygotes throughout the world. But in Bangladesh, no such work has no far been undertaken. Slaughter house ovaries can be an economic source of oocytes for IVM, IVF and IVC experiment. Embryos can be produced from ovaries of the cows that are usually being slaughteded in slaughter houses for meat purpose and the embryos thus produced can be transferred to the recipient cows. Moreover, recent advances in biotechnology have enables the researchers to produce cloned and genetically modified animals by manipulating *in vitro* produced embryos. The present research work has been undertaken for the first time in Bangladesh as a very preliminary approach to embryos produced. The further approach to conduct a trial for *in vitro* maturation experiment could not be undertaken because of unavailability of some necessary instrument and apparatus at our disposal. But then it is hoped that the work would be a base line for the future researchers who will attempt to make further contribution in this field of animal biotechnology.

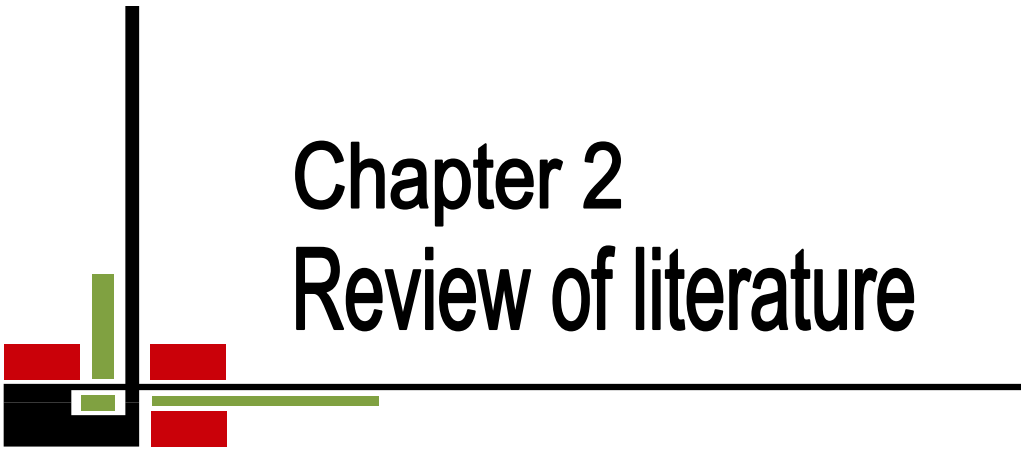
In Bangladesh, many female cattle are slaughtered year round in the slaughter house and at the home. Ovaries from slaughter house female can be an economic source of oocytes for IVM, IVF and IVC experiment. Embryos produced from oocytes collected from ovaries can be transferred to the recipient cows. Moreover, recent advances in biotechnology have enabled the researchers to produced cloned and genetically modified animal by manipulating *in vitro* produced embryos. The present research work has been undertaken in Bangladesh to embryo production from oocytes collected from slaughter house ovaries. Under these circumstances the main objectives of the present study are as follows:

1. To investigate oocyte recovery rate and grading procedures of cumulus-oocyte-complexes

   (COCs) obtained from slaughter house bovine ovaries.

2. To establish the relationship between ovarian condition and morphological quality of COCs.

3. To know the effect of bovine serum albumin on *in vitro* maturation of bovine oocytes.



Chapter 2
Review of literature

CHAPTER-2

REVIEW OF LITERATURE

Substantial research works have been carried out in different countries of the world related to *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of recorded COCs in different ruminant species of cattle, buffalo, sheep and goat. In Bangladesh, reports with this kind of researches have been done to very limited extend. However, relevant information related finding research work carried out in different countries of the world are reviewed in this chapter.

2.1 Collection and evaluation of bovine ovary, follicles and cumulus-oocytes-complexes (COCs)

Rajesh *et al*. (2018) collected the cumulus oocyte complexes (COCs) were aspirated aseptically from the follicles of ≥ 6 mm diameter present on the surface of the ovary by using 18 G needle attached to 5 ml disposable syringe containing 2 ml of collection media. The oocytes were graded based on the presence of the cumulus cells complex around the oocytes. The mean A grade (3-5 cumulus cell layer) oocyte recovery rate per ovary was 1.35 from the ovaries with CL, while those without CL were 1.97, and the mean oocyte recovery rate per ovary was 1.78.

Asad *et al*. (2016) collected from goat ovaries by three methods aspiration, puncture and slicing. The normal grade oocyte per ovary was significantly ($P<0.05$) higher in aspiraton (54.78%) and in puncture (54.70%) method than slicing (30.71%). The oocyte recovery rate was significantly lower ($p<0.05$) in CL containing ovaries (2.03±0.23) than that of ovaries without CL (4.92±0.27). Higher number of normal quality COCs per ovary were obtained from without CL ovaries (2.47±0.12) compared to ovaries (0.98±0.14) having CL.

Khandoker *et al*. (2011) collected ovaries for evaluating length, width and weight, number of follicles, aspirated follicles and state of COCs. Number of follicles were higher ($p<0.05$) in the left (7.25±0.31) compare to the right (6.22±0.32) ovaries. The length, width and weight of ovaries with CL were higher ($p<0.05$) whereas, number of observed follicles, aspirated follicles,

number of COCs and number of normal COCs were significantly ($p<0.05$) higher in ovaries without CL.

Ferdous (2006) was collected COCs by aspiration method and reported that the average number of normal COCs was 1.77 and 2.04 for CL-present and CL-absent group of ovaries respectively. Significantly higher number of COCs and follicles of 2-6 mm diameter as well was obtained from CL-absent group of ovaries while no significant variation was found in the number of follicles measuring <2 mm and >6 mm diameter in CL-present and absent group of ovaries. Normal COCs were found to be significantly higher in number of 2-6 mm diameter.

Rizos *et al.* (2004) isolated bovine COCs from abattoir ovaries by follicular aspiration using an 18 guage needle connected to a vacuum system. Serum TCM-199, buffered with an mM HEPES and 26 Mm bicarbonate and containing 50 IU/ml heparin was used for washing COCs. Pools of 50-60 COCs from 28 ovaries were matured *in vitro* in 0.5 ml TCM-199 containing 26 mM bicarbonate and 2.5mM pyruvate in 4-well plates at 38.5 ºC in a 5% CO_2 in a humidified air atmosphere.

Grondahl *et al*. (1998) recovered the bovine oocytes by mechanical puncture of the follicle and found that the average number of oocytes recovered per ovary was 2.6±0.17. The ovaries were stored in isolated thermoflask contain normal saline supplement with 50 mg/ml gentamycin at 25 to 30 ºC . Cumulus-oocyte-complexes (COCs) were collected from 2 to 6 mm diameter follicles with an 18-gauge needle attached to a 10-ml disposable syringe.

Echert and Niemann (1995) collected bovine ovaries from the local slaughter house and transported to the laboratory within 2 to 3 hours in phosphate buffered saline (PBS) at 25 ºC where they were washed twice in fresh PBS. The surface of the ovaries was sliced with a surgical blade and flushed with fresh PBS containing 2 IU heparin and 1 mg/ml polyvinyl-alcohol (PVA). Cumulus-oocytes -complexes (COCs) were collected into fresh PBS.

Dell' Aquila *et al.* (1995) collected ovaries from mares of unknown reproductive history during the breeding season (April to July) at local slaughter house in southern Italy (41 degree North latitude). The ovaries were put in 0.9% saline and were transported (at 25 to 30 ºC)to the laboratory within 1 to 3 hours in a thermoflask. Follicular fluid was aspirated from follicle measuring less than 3 cm in a diameter through an 18-gauge needle.

Rath *et al.* (1995) collected ovaries from prepuberal gilt from local abattoir and transported to the laboratory in prewarmed (30 ºC) thermo flask within 1 hour. All ovaries were washed twice in dulbeccos phosphate buffered saline (d-PBS; Sigma) supplemented with 15 % new born calf serum. Follicles between 2 to 5 mm in diameter were punctured with the help of an 18-gauge needle connected to a 5-ml syringe at room temperature.

Brinsko *et al.* (1995) reported that there was no difference between age groups in the proportion of follicles available for examination or the proportions of normal, abnormal oocytes.

Crozet *et al.* (1995) studied on oocytes from follicles of three different sizes(small: 2-3mm; medium: 3.1-5mm; large:>5mm) and reported that oocytes from small and medium follicles yielded a significantly lower proportion of hatched blastocysts (0% and 3% respectively than did those from large follicle ovaries and from ovulated oocytes (15% and 34% respectively).

Pawshe *et al.* (1994) conducted a series of experiments on the recovery methods of goat oocytes by using 3 methods: aspiration, puncturing and slicing and they concluded that the average number of oocytes recovered per ovary was significantly higher by aspiration(2.7±0.15) than by puncturing (2.2±0.13) or by slicing (2.4±0.12).They also reported that significantly more good-quality usable oocytes covered with compact cumulus cells were obtained by slicing (0.9±0.06) than by aspiration (0.5±0.06) and the percentages of oocytes maturing, fertilizing and developing *in vitro* differed significantly among recovery methods.

Totey *et al.* (1993) obtained ovaries from adult buffalo at local abattoir and the ovaries were transported to the laboratory within 2 hours after slaughter. The ovaries were stored in isolated thermoflask contain normal saline supplement with 50 mg/ml gentamycin at 25 to 30 ºC. Cumulus-oocyte-complexes (COCs) were collected from 2 to 6 mm diameter follicles with an 18-gauge needle attached to a 10-ml disposable syringe.

Palma *et al.*(1993) conducted an experiment to compare the efficiency of using either calf or cow ovaries in an IVF-programme. They observed that the recovery rate of follicle (follicles/ovary) was 25,27 and 62% for small, middles and large ovaries, respectively.

2.2 Grading of cumulus-oocytes-complexes (COCs)

Das *et al*. (2018) the oocytes collected from the groups were classified into 3 categories separately as Type A, B and C in respect of the morphology of cumulus cell layers tightly adhered with the zonapellucida of oocytes and cytoplasmic appearance of oocyte. The rate of recovery in aspiration technique was found to be the highest for grade A (62.27±1.60) and the least in grade C (13.98±1.41) type of oocytes; while for slicing technique, highest in grade B (51.36±2.01) and the least in grade C (18.23±1.31) type of oocytes. The rate of recovery of culturable oocytes (grade A+B) was 86.01±1.41 and 81.76±1.31, by aspiration and slicing technique, respectively.

Žilaitis *et al*. (2018) selected follicles which diameter, were medium (6-9 mm). A total of 125 COCs were aspirated from 124 ovaries. Among 72 COCs were aspirated of ovaries presence of CL, and 53 COCs absence of CL. Only Grade A and B oocytes COCs were used for maturation. A higher percentage of oocytes was collected from medium size follicles of ovaries with presence of corpus luteum (34.15 percent) than absence of corpus luteum ($p<0.05$). In ovaries group with presence of CL, COCs matured 84.72 percent (Grade A and B). In ovaries group with absence of CL, COCs matured 64.15 percent (Grade A and B). A significant difference was detected between the presence and absence of CL group ($p<0.05$).

Saleh (2017) collected oocytes were 55, 68, 87 and 106 oocytes respectively; slicing methods yield more oocytes count. Period of time between slaughtering and samples processing significantly affect oocytes collected percentage and quality, periods as 2, 6, 12 and 24 hours yield 75%, 68%, 61% and 55% oocytes counts of good, fair, poor to aged and bad quality oocytes respectively. Two hours period yield an elevated oocytes count with good quality. Maturation index of oocytes according to the type of collected methods showed 44, 37, 39 and 42 with 12, 8, 6 and 6 good oocyte quality for the four methods respectively.

Khandoker *et al*. (2016) the follicular materials collected from both techniques were observed under microscope to categorize the COCs as A (oocyte surrounded with cumulus cells homogenously), B (surrounded with cumulous cells partially), C (oocyte not surrounded at all by cumulous cells) and D (degeneration observed both in oocyte and cumulous cells). Grade A and

grade B were classified as normal and grade C and grade D were considered as abnormal COCs. Ovaries having no CL contributing more total number of COCs per ovary (6.8±1.0) and also contributing higher normal COCs (5.7±0.9) than that of ovaries with CL (6.0±2.0 and 4.5±1.5, respectively). But same trend of result was not found in aspiration technique. Similarly, higher percentage of COCs recovery rate was also recorded in blunt dissection (61.6±4.6% vs 16.5±4.9%, on total basis) than aspiration (48.6±2.9% vs 11.7±4.1% on normal basis) technique.

Hoque *et al*. (2011) COCs were collected by three techniques viz. puncture, slicing and aspiration of goat ovaries obtained at slaughter house. The total number of COCs /ovary as well as the number of abnormal COCs/ ovary were significantly higher ($p<0.01$) in puncture (4.22 and 2.38, respectively) and slicing (4.14 and 2.22, respectively) followed by aspiration (3.28 and 0.80, respectively) technique. In contrast, the number of normal COCs/ovary was significantly higher ($p<0.01$) in aspiration (2.48) followed by slicing (1.91) and puncture (1.85) techniques.

Mondal *et al*. (2008) Cumulus-oocyte-complexes (COCs) were collected from 2 to 6 mm diameter follicles with an 18-gauge needle attached to a 10-ml disposable syringe and obtained higher maturation rate in grade A COCs (71.70%) than that of grade B (51.52%) when cultured in TCM-199 supplemented with FCS and bovine serum albumin (BSA) at 38.5% with 5% CO_2. Only Grade A and B oocytes COCs were used for maturation.

Salim *et al.* (2005) studied on reproductive tract of four- category (category 1 = acyclic; category 2=cyclic but not conceived; category 3= post partum anestrus and category 4= normal breeder and kidder). Black Bengal does to investigate the causes of infertility and reported that the average number of normal follicles were significantly higher in category 4 compared to other category and degenerated follicles were reverse to that of the result of the normal follicles.

Goswami *et al.* (2004) classified the ovaries in three catagories on the basis of the state of the CL and reported that the average number of follicles harvested per ovary was 4.37, 5.28, 6.48 in type- I,type- II and type- III, respectively. Higher number grade A and B COCs was obtained from type- III ovaries.

Rahman *et al.* (2003) classified the ovaries in three catagories on the basis of the state of the CL and reported that the average number of follicles harvested per ovary was 4.37, 5.28 and 6.48 in

type- I, type- II and type- III respectively. Higher number grade A and B COCs was obtained from type- III ovaries.

Echert and Niemann (1995) divided the collected COCs into 2 morphological categories COCs with a homogeneous evenly granulated cytoplasm possessing least three layers of compact cumulus-cells designated as category 1. COCs with less than three layers of cumulus-cells and were partially denuded possessing a homogeneous evenly granulated cytoplasm as category 2. All other morphological types of COCs were discarded and comparatively better result was found with the former catagory of COCs.

Leibfried and First (1986) classified cumulus-oocytes-complexes based on their cumulus morphology into 5 categories- 1) Compact of COCs with a sheet of compact cumulus cells completely surrounding the oocytes like a hood, 2) In homogeneous COCs covering the oocytes partially, 3)Partially expanded COCs with an expanding cumulus but still appearing cellular, 4) Totally expanded COCs with a completely expanded cumulus having sparsely cellular gelatinous cloud around the oocyte and 5)Degenerated COCs with clumped, dark cumulus cells. They observed better results in IVM study from first 3 categories of COCs.

2.3 Effect of bovine serum albumin (BSA) supplementation in macroscopic observation of cumulus cell expansion

Khandoker *et al*. (2017) collected cumulus oocytes complexes (COCs) from slaughter house goat ovaries by aspiration method. COCs were matured for 24 hours in TCM-199 basic medium and supplemented with different levels of BSA (2mg/ml,4mg/ml and 6 mg/ml) where 0mg/ml was considered as control. Three levels of cumulus cell expansion after 24 h of *in vitro* culture (at 38.5ºC and 5% CO_2 in an incubator) observed under 10x magnification of microscope and the different stages of nuclar maturation was observed based on chromosomal configuration. Metaphase-II stages were 40.78±3.84, 67.52±0.85, 68.95±1.88 and 57.74±2.39%.Normal fertilization (formation of 2 pronuclei) were 23.28±3.00, 35.52±1.21, 37.74±1.24 and 29.30±3.73 for 0 mg (control), 2 mg, 4 mg and 6mg level of BSA respectively.

Dey *et al*. (2016) bovine ovaries were collected from local abattoir; cumulus-oocyte-complexes (COCs) were aspirated from 3 to 8 mm diameter follicles using a 10 ml disposable syringe attached with a 21G needle. The COCs were selected based on morphological characteristics

and selected COCs were transferred into *in vitro* maturation (IVM) medium for 22 to 24 hrs. The maturation rates of COC were examined through detection of first polar body and cumulus cell expansion. Results showed that 74.16± 5.49% of the total immature COCs were matured as detected by the presence of first polar body. The diameter of matured COC was 2.21 times higher than that of the immature COC. Moreover, about 64.30 ± 6.71% COC showed full expansion of their cumulus cell.

Asad *et al*. (2012) collected goat ovaries using the aspiration method. Media were prepared using TCM-199 supplemented with 0.5% bovine serum albumin for 27 h, fertilized with capacitated fresh semen in Brackett and Oliphant (BO) medium for 6 h and then cultured up to 7 days, at 38.5ºC with 5% CO_2 under humidified air. It was observed with 0% to 15% of gFF that 53.8-75.0% of the oocytes reached the cumulus cell expansion level-3; 41.5-67.8% reached metaphase- II; 28.6-38.4% exhibited normal fertilization (formation of 2-pronuclei); 12.3-33.7% were 2 cell embryos.

Hoque *et al*. (2011) Only normal quality COCs were cultured in TCM-199 supplemented with 2.5% bovine serum albumin (BSA) plus 10% goat follicular fluid (gFF). The matured COCs were then fertilized in BO medium with fresh buck semen. The rates of COCs that reached the maximum cumulus cell expansion (level-3) were 64.14, 65.93 and 65.73% respectively.

Luciano *et al.*(2005) studied on cumulus oophorus cells in regulation of female gamete development, meiotic maturation and oocyte-sprerm interaction. T heir results suggested that direct interaction between oocyte and cumulus cell (CCs) is not essential during IVM and IVF of denuded oocyte. They found that putative diffusible factor (s), produced by CCs and /or by the cross talk between oocyte and CCs in the intact complex play key role in the acquisition of developmental competence of the denuded female gamete.

Cumulus oocytes complexes (COCs) collected from local slaughter house were matured in synthetic oviductal fluid (SOF) medium supplemented with BSA, luteinizing hormone(LH), follicle stimulating hormone (FSH) and epidermal growth factor (EGF) in the presence or absence of minimum essential medium (MEM) vitamins for 24 h. The maturation rates of COC were examined through detection of first polar body and cumulus cell expansion and after

fertilization the overall blastocyst development were increased significantly in the presence of MEM vitamins (Bormann *et al*. 2003, Schmitt and Nebrad, 2002 and Choi *et al.*2001).

Rejane *et al.* (2003) investigated the role of growth hormone (GH) on *in vitro* cumulus expansion in bovine oocyte and to approach its way of action. Bovine COCs were cultured in a control medium (TCM 199, 5mg/ml BSA, 1 µg/ml estradiol and antibiotics). After culture cumulus expansion was high and nuclear level maturation was noticeable.

The inclusion of different concentrations of BSA-V, fetal calf serum (FCS) or PVA during IVM had no positive effect on the developmental capacity of the bovine oocytes compared with the use of SOF alone with no supplement but significantly decreased the percentage of embryos reaching the morula and blastocyst stages. However, when BSA-V was replaced with purified BSA, BSA that was essentially free of fatty acids, or chicken egg albumin, embryonic development rates were restored. These studied showed that the effect of FSH on nuclear maturation and cumulus cell expansion is dependent on substrates present in IVM medium (Ali and Sirard, 2002).

Calder *et al.* (2001) revealed that concentrations of PGE_2 above 50 ng/ml resulted in moderate cumulus expansion of bovine COCs, but expansion did not oocur in the absence of serum. Although the PGE_2 pathway is involved in cumulus expansion, serum factors are required to mediate PGE_2 induced expansion.

Geshi *et al.* (2000) concluded that without cumulus cells bovine oocytes required supplementation of sodium pyruvate for nuclear maturation protein free medium. Presence of cumulus cells during maturation *in vitro* seems *in vitro* in a not to be an absolute requirement in cattle, since blastocysts obtained developmental competence to be normal calves after embryo transfer at a low rate.

Beker *et al.* (2000) reported that GHRH and VIP no effect on nuclear maturation or cumulus expansion of bovine COCs but retard cytoplasmic maturation, as reflected by delayed cortical granule migration.

Cetica *et al*. (1999) collected ovaries at an abattoir and the oocytes harvested. As regards seletion criteria, immature oocytes were classified as class A, B, C and D according to the character of

the cumulus cells. As regards maturation criteria, there was no cumulus expansion when oocytes were matured in TCM-199 without supplementation, partial expansion with the addition of fetal calf serum and full expansion when supplemented with steer serum or bovine follicular fluid.

Hashimoto *et al.* (1998) stated that the addition of cumulus cell (1.6×10(6) cells/ml) improved the development of bovine corona-enclosed oocytes, however, addition of a similar number of cumulus cells as cumulus-oocytes-complexes (COCs, cumulus cell density:4.2× 10 (6) cells/ml) had no effect on the development oocytes denuded from their somatic cells. Cumulus cells benefit bovine oocytes development either by secreting soluble factors, which induce developmental competence, or by removing an embryo development suppressive component from the medium.

Furnus *et al.* (1997) addition of cysteine and cysteamine to IVM medium increased GSH levels in COCs and Dos. N-Acetylcysteine increased GSH levels only in Dos. Cumulus cells during IVM play an important role in oocytes GSH synthesis, allowing the oocytes to use cystine and contributing to the stimulatory effect exerted by cysteine and cysteamine. IVM medium supplemented with cysteine of cysteamine increased GSH content in oocytes without cumulus mass (DOs) and in the absence of a cumulus cell monolayer. These may be useful to increase the efficacy of IVM of those oocytes having few cumulus cell layers, in a system without co-culture.

Lorenzo *et al.* (1995) studied on the chronological changes in the meiotic progress of *in vitro* maturation of bovine oocytes. The results demonstrated that the immature oocytes at the time of collection (0 h) were in the germinal vesical stage (GV), and that the highest maturation rate was at 24 h of culture in all treatments. Sperm treatments enhanced the maturation rates obtained (52.1 and 55.7%) compared to the control (serum-free) medium (42.7%) in cumulus-cell-enclosed oocytes. In denuded oocytes, the maturation rates were lower compared to cumulus-cell-enclosed oocytes in all treatments. In conclusion, meiotic progression of bovine oocytes can be influenced by the inclusion of sera in the maturation media and by the presence of the cumulus cells.

Yang *et al.* (1996) collected oocytes from antral follicles (2-7 mm in diameter) from cows and heifers, within three hours after slaughter. Oocytes with intact cumulus or with at least 4 layers of cumulus cells were selected for *in vitro* maturation and *in vitro* fertilization and showed good result.

Lonergan *et al.*(1994) demonstrated that both tissue culture medium 199 (M199) and synthetic oviduct fluid (SOF) are capable of supporting the IVM of bovine oocytes at the high rates in the absence of macromolecular supplements, as evidenced by subsequent development to the cumulus cell expansion and blastocyst stage. Inclusion of bovine serum albumin (3 mg/ml) was not beneficial and in fact significantly depressed development when added to SOF.

Totey *et al.*(1993) observed that oocytes cultured for 24 hours in TCM-199 or Ham's medium containing 10% FCS or BES had a significantly higher maturation rare than those the medium alone. The maturation rate was higher in both medium supplemented with 10% FCS than with 10% BES. Addition of hormones alone or in combination with sera further improved the cumulus cell expansion level and maturation rate.

Shabpareh *et al.*(1993) conducted an experiment in which oocytes were collected from ovaries after slaughter. Follicles were aspirated with PBS 5 to 6, 6 to 7 and 7 to 8 hours after slaughter. The oocytes were cultured for 30 hours in medium 199 with or without 100 IU/ml horse chorionic gonadotropin. Horse chorionic gonadotropin and the time from slaughter to oocyte collection did not affect cumulus expansion or oocytes maturation.

Cognie *et al.* (1992) reported that mammalian oocytes removed from the follicles are able to undergo spontaneous nuclear maturation *in vitro*. Culture of cumulus-oocyte-complexes with granulosa cells in a medium supplemented with FSH, LH and ostradiol increased the development capacity of *in vitro* fertilized oocytes. The time to complete maturation *in vitro* differed among species: 24 hours in sheep, 27 hours in goats and 36 hours in mares.

Zuelke *et al*. (1990) showed that luteinizing hormone enhance cell expansion and maturation of immature ooctytes obtained from slaughtered cattle as reflected by elevated proportions of oocytes that fertilized and reached blastocyst stages in vitro after in vitro fertizilation (IVF).

Chen *et al.* (1990) explained that the down-regulation of cumulus gap junctions is required for the initial phase of cumulus cell disaggregation and confirm earlier reports that hyaluronic acid systhesis plays a major role in additional expansion of the cumulus. The failure of cultured complexes to expand maximally can be overcome by the addition of substrates acid synthesis to the culture medium.

Crister *et al.* (1986) demonstrated that addition of serum to oocyte treated with heparin decreased cumulus cell expansion. Heparin and mixed glycosaminoglycans stimulate events related to oocyte maturation but were not capable of preparing oocytes for fertilization. The addition of serum or desulfation of heparin inhibited heparin effects on *in vitro* oocytes maturation.

Bovine serum albumin (BSA) and fetal calf serum (FCS) were evaluated as protein supplements for *in vitro* maturation and fertilization of oocytes from cows and hamsters. BSA and low does of FCS (0.1 or 1.0%) did not support viability or maturation of cumulus-oocyte complex as well as higher doses of FCS (5, 10 or 20%) for either species. BSA failed to support cumulus expansion for bovine or hamster cumulus-oocyte complexes. All doses of FCS examined supported cumulus expansion in bovine cumulus-oocyte complexes, whereas the hamster complexes required at least 1.0% FCS to induce cumulus expansion. The addition of a serum with BSA improved viability of the cumulus in the bovine but did not support cumulus expansion or completion of M- I in bovine complex (Rultedye *et al*.,1986).

2.4 Effect of bovine serum albumin (BSA) supplementation *in vitro* nuclear maturation

Saha *et al*. (2018) collected cumulus oocytes (COCs) from goat ovaries by aspiration method and matured for 24 hours in TCM-199 basic medium supplemented with bovine serum albumin and different level of FF (5%, 10% and 15%). The percentage of COCs reached to Metaphase- II stages were 43.33±3.33, 51.67±0.83, 66.66±0.00 and 67.79±1.92 respectively (0%, 5%,10% and 15% of FF supplementation).

Garcia *et al*. (2017) studied that TCM-199 supplemented with either 7 mg/ml of Bovine Serum Albumin (TCM+BSA) or 10% Fetal Bovine Serum (v/v; TCM+FBS) was used. Bovine oocytes were matured in vitro and placed in the previously mentioned media for further 18 hours, in the absence of added sperm (sham fertilization) and their chromatin conformation was evaluated. After IVM, 78.9% of the initial oocytes had reached the M-II stage.

Bhuiyan *et al*. (2014) the ovaries of cows were collected from local slaughter house followed by aspiration of follicular fluid. The cumulus-oocyte-complexes (COCs) with more than 3 compact cumulus cell layers were cultured in tissue culture medium (TCM) 199 for maturation. The rate of maturation of occytes ranged from 51.9 ± 9.4 % (18 hours) to 59.0 ±17.1 % (27 hours) and the difference in maturation rate among different culture durations was not significant ($P>0.05$). To determine an effective protein supplementation, 63 oocytes from 19 ovaries were cultured separately in TCM 199 supplemented with either fetal bovine serum (FBS) or bovine serum albumin (BSA).The rate of maturation was significantly ($P<0.01$) higher in medium supplemented with FBS (55.63 ±6.19 %) than that of BSA (14.82 ± 9.36 %).

Asad *et al*. (2012) collected goat ovaries using the aspiration method and media were prepared using TCM-199 supplemented with 0.5% bovine serum albumin plus four levels of gFF at concentrations of 0%, 5%, 10% and 15% and oocytes were matured for 27 h, fertilized with capacitated fresh semen in Brackett and Oliphant (BO) medium for 6 h and then cultured up to 7 days, at 38.5ºC with 5% CO_2 under humidified air. Metaphase- II stages were 44.8±1.01, 53.8±0.89, 66.8±0.87, 67.8±0.99 respectively.

Reza *et al*. (2012) cumulus oocytes complexes (COCs) were matured in TCM-199 supplemented with 10% BFF, 5% BSA or without supplementation (control). The percentage of COCs reached to Metaphase- II stages were 40.78±3.64, 65.74±2.39 and 67.52±0.85; normal fertilization (formation of 2 pronuclei) were 23.28±3.00, 29.30±0.73 and 30.52±1.21 for control,10% BFF and 5% BSA supplementation, respectively.

Tareq *et al*. (2011) COCs were cultured in TCM-199 supplemented with 2.5% bovine serum albumin (BSA) plus 10% goat follicular fluid (gFF). The COCs reached to metaphase-II (M-II) stage were 57.75, 58.23 and 58.57%; normal fertilization (formation of male and female pronuclei) were 34.43, 35.03 and 34.65% in puncture, slicing and aspiration techniques, respectively.

Asad *et al*. (2010) (COCs) were collected by aspiration of 2-6 mm diameter follicles. Upon grading, only normal quality COCs were matured in TCM-199 for 48 hours. The percentage of COCs reached to the M- II stag was 61.41±1.97%.

Anguita *et al.* (2007) matured oocytes in TCM 199 medium supplemented with 275 mg/ml sodium pyruvate, 146 mg/ml L- glutamine, 10% (v/v) steer serum, 10 mg/ml 0-LH, 10 mg/ml FSH, 1mg/ml 17-b estradiol, 400 mM cysteamine and 50 mg/ml gentamycin with 5% CO_2 ,5% O_2 and 90% N_2 at 38.5ºC for 27 h and the percentage of oocytes that reached to GVBD, M- I and M- II stages were found be 11.1%, 18.68% and 78.02% respectively.

Wang *et al*. (2007) studied the effects of oocytes harvesting techniques of slicing, puncture, aspiration- I and aspiration- II. They cultured the COCs in TCM-199 supplemented with 10µg/ml of epidermal growth factor (EGF) or 10% fetal calf serum (FCS), either alone or with 1 IU/ml FSH for 24 h, fertilized matured oocytes in TALP medium 10 µg/ml of heparin with frozen semen (10^6 sperm /ml),then cultured the zygotes in B2 medium containing 10% fetal bovine serum (FCS) with 5% CO_2 in humid air at 38.5ºC.They found that the percentages of oocytes reached to M- II stages were 48-63 with 58.7-62.5% cleavage rates and 13.3-15.0% rate of blastocyst development.

Barretto *et al.* (2006) reported that oocytes were matured *in vitro* in a 10% knockout (SR) supplemented TCM-199 medium (control) with either 0.5mM IBMX or 25 muM rescovitine (ROSC).They also concluded that maturation rate varies with the culture time and treatment groups.

Gilchrist *et al.*(2005) explained that nuclear maturation was 1.3- fold lower Bovine *in Vitro* Mat cultures containing 2.3mM glucose compared with 5.6mM glucose and this effect was independent of glucosamine supplementation. They also demonstrated that glucose concentrations and the timing of the introduction of gonadotrophin during IVM have variable effects on nuclear maturation.

Sun *et al.* (2004) studied on charges in germinal vesicle (GV) chromatin configurations during growth and maturation of bovine oocytes using a new method that allows a clearer visualization of both nucleolus and chromatin after Hoechst staining. The GV chromatin of bovine oocytes was classified info five configurations, based on the degree of chromatin condensation, and on nucleolus and nuclear membrane disappearance. *In vitro* maturing oocytes showed a large proportion of GV3 and GV4 configurations. There was no significant difference in distribution

of chromatin configurations between oocytes with more than two layers of cumulus cells and those with less than one layer or on cumulus cells.

Rodriguez *et al*. (2004) cultured COCs for 20 h in tissue culture medium (TCM-199) supplemented with 10% estrus cow serum (ECS), 5 micro/ml FSH and 1 micro/ml estradiol in the presence of 120 micro M DRB. COCs were then released from meiotic arrest and cultured for 20 h in DRB-free medium, with culture initiated concomitant to the release of DRB-treated COCs from meiotic arrest. Nuclear maturation was assessed after 0,5,10,15, and 20 h of culture in DRB-free medium. The proportion of DRB-arrested oocytes reaching metaphase II (M- II) following 20 h culture in DRB-free medium was not significantly different from controls (96+/-4% versus 99+/-4%).

Payton *et al.* (2004) stated culture of GV-stage COCs at 41ºC increased the proportion that had type III cortical granules and reduced the proportion that progressed to metaphase-II after *in vitro* maturation.

Imai *et al.*(2002) reported that CR1aa was superior to TCM-199 for the potential development of *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of bovine oocytes.

Mayes *et al.* (2001) aspirated bovine COCs and classified according to the state of their cumulus cells and cytoplasm(classes 1 to 3). Groups of 15 to 20 COCs were fixed at 0 h or after an incubation period of 4 h and evaluated for state of nuclear maturation. Result show that at 0 h, COCs from class 3 have fewer oocytes at the GV stage than COCs from class 1 and class 2 (respectively 69.3+/-3.2 vs 88.8+/-3.4% and 86.9%GV+/-4.3%SEM; $P<0.05$). After 4 h of incubation, all COCs classes show a significant decrease in the number of COCs at the GV stage.

Khurana *et al.* (2000) reported that morphological grading of immature oocytes is an appropriate selection criterion for their developmental ability. Essential medium supplemental with energy substrates according to the findings of metabolic studies was less effective in supporting *in vitro* maturation and subsequent development than TCM-199. The presence of serum improved the rate of *in vitro* development of one-cell embryos.

Geshi *et al.* (2000) reported that without cumulus cells bovine oocytes required supplementation of sodium pyruvate for nuclear maturation *in vitro* in a protein free medium.

Ikeda *et al.* (2000) found that the presence of midkine (MK) during IVM of bovine granulose-enclosed oocytes can enhance their developmental competence to the blastocyte stage after IVF and suggest that the enhancing effects might be mainly mediated by GCs.

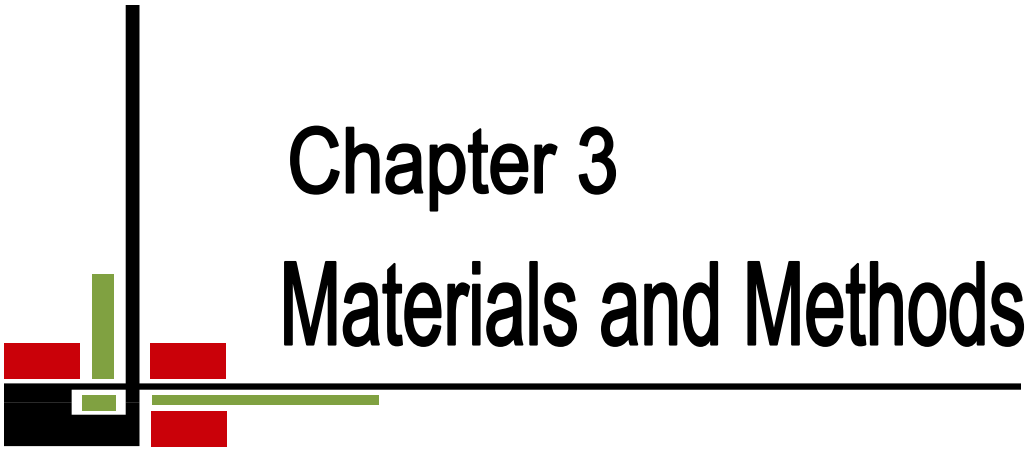
Khatir *et al.*(1998) compared the kinetics of nuclear maturation between calf and cow oocytes in order to determine if there are differences between the 2 groups which could explain their disparate developmental capacity. They concluded that the difference in developmental capacity between cow and calf oocytes may be explained by a difference in the kinetics of nuclear maturation, which was significant at 20 h of culture (with 89% of cow oocytes at metaphase II and 71% of calf oocytes).

Izadyar *et al.* (1996) showed that at 4 and 8 h the percentage of oocytes in GV stage after GH treatment (54% and 19%) was significantly lower than the control (64% and 41%). Similarly at 16 and 22 h the percentage of oocytes in M-II stage was significantly higher in the GH- treated group; (58% and 77%) and (46% and 62%) for GH and control respectively. The number of oocytes in M-II beyond 22 hr of culture did not differ; 100 and 1000ng/ml GH induced significant cumulus expansion ($p<0.05$), which was not observed in the absence of GH. Growth hormone (GH) present during IVM has a beneficial effect on subsequent development.

Izadyar *et al.*(1996) found that activin present during maturation in the absence of serum and gonadotrophic hormones also did not alter the proportion of fertilization. IVM in the presence of bovine activin A has no effect on subsequent embryonic development.

Brinsko *et al. (*1995) observed that the overall maturation rate to the second metaphase (M-II) was 52.7% after 24 hours of culture. Maturation rates for oocytes obtained from young and middle-aged mares were similar.

Nagai (1993) reported that cumulus cells during maturation period supported IVM of the porcine oocytes to the metaphase II- 68.9% and were involved in the cytoplasmic maturation needed for developmental competence of post-fertilization such as male pronucleus formation in porcine oocytes.



Chapter 3

Materials and Methods

CHAPTER-3

MATERIALS AND METHODS

The experiment was conducted at the Animal Nutrition, Breeding and Genetics Laboratory under the department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka from January 2019 to December 2019.

3.1 Preparation of the laboratory

Before starting the experiment, all the necessary equipment were properly installed and checked. If needed, these things repaired and/or reinstalled. Finally all equipment cleaned and sterilised with 70% alcohol. All the reusable, equipment were properly washed, dried, covered with aluminium foil, sterilised and finally kept in a cleaned and sterilised chamber until use. All the essential disposal equipment as well as media, chemicals and reagents were made readily available before starting the experiment.

The list of recruitments are mentioned below-

a) Microscope
b) CO2 incubator
c) Laminar Air Flow Cabinet
d) Ph meter
e) Weighing balance
f) Disposal 10 ml syringes
g) Disposal 18 G needles
h) Sterilised rubber gloves
i) Sterilised beaker
j) Sterilised measuring 100 ml cylinder
k) Culture dishes of 35mm
l) Pasture pipette
m) Petre dishes (90mm)
n) Water bath

o) Sterilized test tube 10 ml
p) Glass microscope
q) Distilled water
r) Digital pipette
s) Bovine serum albumin (BSA)
t) Paraffin oil
u) Tissure culture media 199 (TCM-199) for maturation
v) Measuring scale
w) Scissors
x) Watch glass
y) Forceps

3.2 Collection and processing of ovaries

3.2.1 Preparation for ovary collection

Physiological saline (0.9% Nacl) was prepared and sterilized in autoclave and stored in refrigerator for further use. On the day of collection, 1000 mg of zentamycin were added per litre of saline. The solution was warmed at 25 to 30 ºC and kept in a thermos box to maintain this temperature during transporting the ovaries from slaughter house to laboratory. Dulbecco's phosphate buffered saline (D-PBS) solution was also prepared by adding one pack of PBS salt in one litre of distilled water. Then it was sterilized in autoclave and stored in refrigerator for further use.

3.2.2 Collection of ovaries and trimming

The representative photograph of the ovaries is shown in plate 3.1 (A). The ovaries were kept in collection vial containing 0.9% Physiological saline in a thermoflask at 25 to 30 ºC and transported to the laboratory within 2 to 3 hours of slaughter. The ovaries were then transferred to the sterilised petridishes containing same saline at 25 ºC. In the laboratory each ovary was trimmed to remove the surrounding tissue and overlying bursa (Plate 3.1) (B). Each ovary was treated to three washing in D-PBS.

3.2.3 Evaluation of ovary

Ovaries were evaluated on the basis of following measures.

3.2.3.1. Measurement of weight, length and width

Individually right and left ovary and ovaries with CL and without CL was weighed in a digital balance as shown in plate 3.1 (E) and the weight was recorded in tabular form. The length and width in cm of the right and left ovary and ovaries with CL and without CL were measured with the help measuring scale (plate 3.1) (D).

3.2.3.2. Counting of follicles on the surface of the ovary

There are numerous follicles on the surface of the both ovaries. The number of visible follicles on the surface of different category of ovaries were counted and recorded.

3.2.3.3. Collection of follicles by intact follicle collection method

Each ovary was incised with scissors to collect all the visible follicle within it. Then follicles were stored in a saline watch glass at room temperature. From each follicle the follicular material were harvested with the help of forceps and needle by blunt dissection on a sterilised culture dish (35 mm). The numbers of COCs were counted (Plate 3.1) (G). The COCs were classified into 4 grades (Plate 3.2) Grade-A= COCs completely surrounded by cumulus cells; Grade-B= COCs partially surrounded by cumulus cells; Grade-C=Oocytes not surrounded by cumulus cell; Grade-D=Degeneration observed both cumulus cells and oocytes. Grade A and Grade B considered as normal COCs. Grade C and Grade D considered as abnormal COCs. The number of different grades of COCs in each category were recorded.

3.2.4. COCs aspiration and grading

The ovaries were brought to the laboratory and washed 2-3 times in saline solution at 30ºC. ovary was picked up in left hand. The 10 ml syringe was loaded with PBS (1-1.5 ml), and needle (19G) was put in the ovary parenchyma near the vesicular follicles (2 to 6 mm diameter) by right hand and all 2 to 6 mm diameter 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 90 mm petridish, avoiding damaged to the cumulus cells and the COCs were searched and

graded under microscope at low magnification (4X). The COCs were classified into 4 grades as described previously. The numbers of different grades of COCs in each categories were recorded. The representative photograph of the COCs is shown in Plate 3.2. In the meantime another petridish of D-PBS was prepared for pooling COCs and the COCs were picked up with an appropriate glass micropipette.

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

3.3. *In vitro* maturation (IVM) of bovine COCs

3.3.1. Preparation of medium and droplet culture dish

The maturation medium, TCM-199 supplemented with 0% (control), 5%, 10% and 15% bovine serum albumin (BSA) was prepared (Plate 3.3) (A) and its P^H was fixed at 7.4 on the day of aspiration and sterilized by filtration. About 2.5 to 3.5 ml of the medium was poured into each of two 35 mm culture dishes. In another culture dish 4 drops of each about 100 µl of maturation medium were poured and covered with paraffin oil photograph represent as 3.3 (F).The above two culture dishes with evenly distributed medium and dish with droplet were kept in an incubator at 38.5 ºC with 5% CO_2 in air. The first two dishes could be used for maturation of the oocytes.

3.3.2. Macroscopic observation of cumulus cell expansion

Normal graded (grade A and B) oocytes were washed to 3 times separately in PBS and then transferred into the maturation medium (TCM-199+ 0%, 5%,10% and 15% BSA) and washed 2 to 3 times. Droplets containing normal graded oocytes were kept in a CO_2 incubator at 38.5 ºC with 5% CO_2 in air for 48 hours (Plate 3.3) (G). The numbers of oocytes according to grade used for maturation and the times of initiation of maturation were recorded. After 48 hours of IVM, cumulus expansion was determined by three level same magnificent under microscope as 1:

indicating no expansion of COCs; 2: indicating moderate expansion and 3: indicating marked expansion cells with a compact layer of choronaradiata (Plate 4.1).The number of oocytes classified on the basis of expansion rate COCs was recorded.

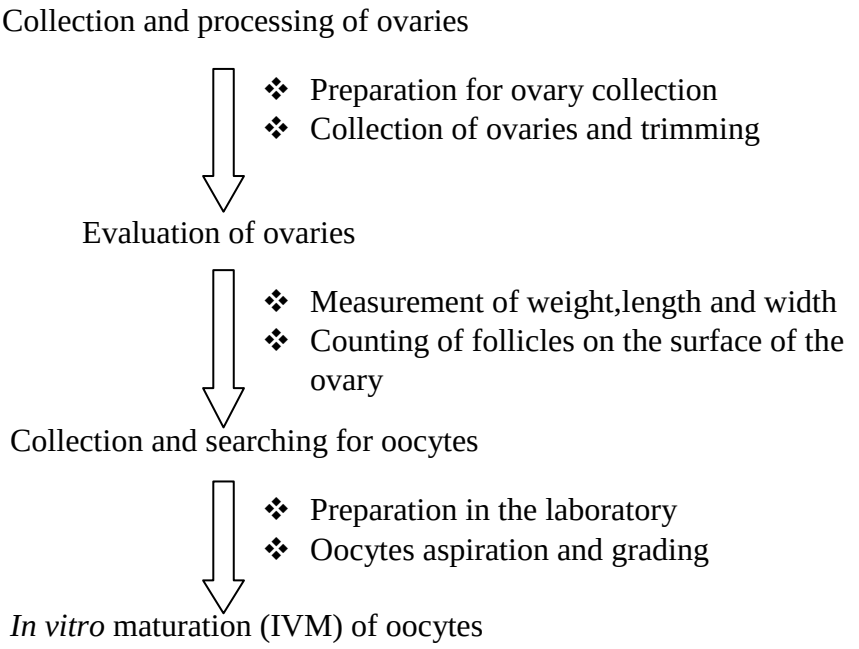


Figure 1. Protocol of collection, evaluation and *in vitro* maturation of bovine oocyte

3.3.3. Nuclear maturation

The nuclear level maturation was checked in representation sample. For the purpose, after *in vitro* maturation for 48 hours. COCs were denuded from cumulus cells by repeated pipetting. Then oocytes were washed in PBS three times. After that the oocytes were transferred to Na-Citrate solution for 5-10 minutes. Oocytes were mounted on a glass slide and washed with aceto-alcohol up to complete removal of cytoplasm from the oocytes. Then it was stained with 1% aceto-orcein for 30 minutes (Plate 3.3 H). After drying, the slides were examined under inverted microscope at high magnification(100X) with immersion oil through USB 2.0 camera for germinal vesicle break down (GVBD), Metaphase-I (M-I) and Metaphase-II (M-II) stage. Finally percentage of maturation was calculated (Plate 4.2).

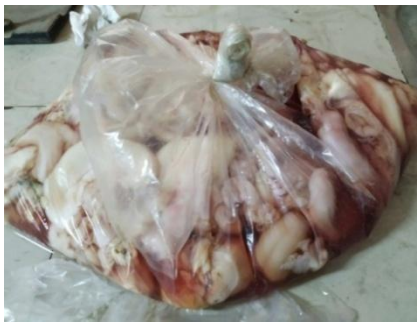


Plate-A

Plate-B

Plate-C

Plate-D

Plate-E

Plate-F

Plate-G

Plate3.1 Representative photograph showing

A) Collection of ovaries

B) Cutting of ovary

C) Placing left and right ovary on tray

D) Measuring length and width (cm) of the ovary

E) Measuring weight of ovary on weighing balance

F) Aspiration technique

G) Microscopic examination of COCs

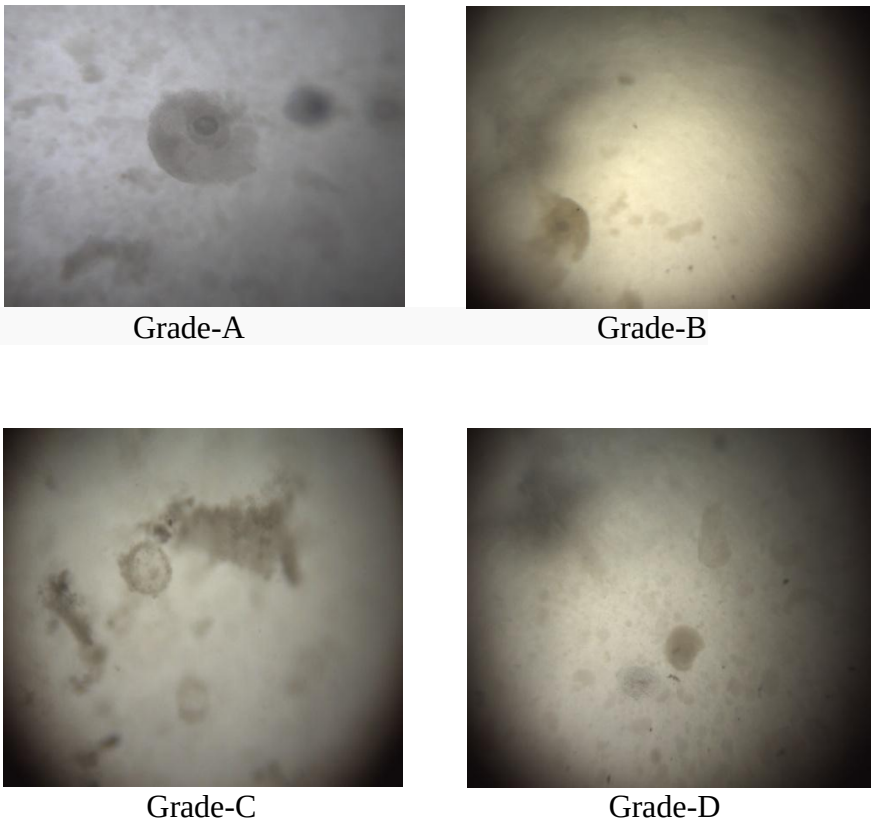


Plate 3.2 Representative photograph showing different grades of cumulus-oocyte-complexes

Grade-A= COCs completely surrounded by cumulus cells

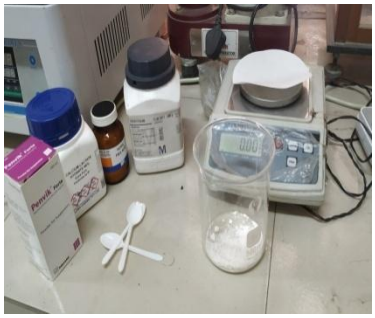
Grade-B= COCs partially surrounded by cumulus cells

Grade-C= Oocytes not surrounded by cumulus cell

Grade-D= Degeneration observed both cumulus cells and oocytes

Grade A and Grade B considered as normal COCs

Grade C and Grade D considered as abnormal COCs



(A)

(B)

(C)

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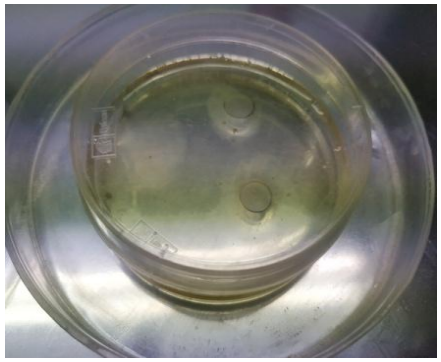
(E)



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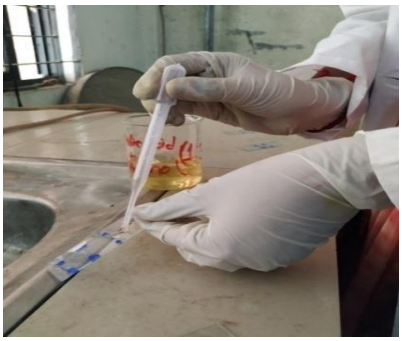

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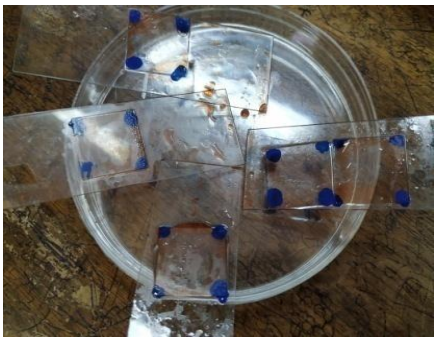


(I)

Plate. 3.3 Representative photograph showing

A,B,C) Measuring and preparation of TCM and BSA

D) Preparation of water bath

E) COCs aspiration

F) Preparation the droplet of maturation medium

G) Droplet in CO2 incubator

H,I) Macro-droplet for COCs washing and staining

3.4 Preparation of maturation medium (TCM-199)

Table 1: Maturation medium (TCM-199)

Material	Amount
Medium 199 (sigma)	1 vial
Hepes	2.383 g/liter
Penicillin	1 vial
Streptomycin	100mg/liter
Sodium pyruvate	0.1000 g/liter
Calcium lactate	0.9002 g/liter
Sodium bicarbonate	2.2 g/liter
D glucose	0.549g/liter

The medium (TCM-199) was supplemented with 0% (control), 5% 10% and 15% bovine serum albumin (BSA).Finally P^H was adjusted to 7.2-7.3 by adding 1N NaOH and the medium was sterilized by filtration through disposable 20-22 µ milipore filter.

3.5. Statistical model and methods of data analysis

All values were expressed as Mean±SE. Comparison of means Duncan's multiple range test (DMRT) was applied with the help of statistical analysis system (SAS,1998).

Chapter 4
Results and Discussion

CHAPTER -4

RESULTS AND DISCUSSION

4.1 Ovarian classification (left and right) and other parameters per ovary

From local slaughter house cow ovaries were collected and were recorded as right and left. On the basis of presence and absence of corpus luteum (CL) again they were grouped as with CL and without CL. Among 70 ovaries CL found in 25 ovaries and remaining 45 ovaries having no CL. The result of the different parameters is summarized in table 4.1, 4.2, 4.3 and appendices of 1-20.

Table: 4.1 Ovarian Classification (Left and Right) and other parameters per ovary

Ovarian type	Weight (g) (Mean ±SE)	Length (cm) (Mean ±SE)	Width (cm) (Mean ±SE)	Total no of visible follicle (Mean ±SE)	No of follicle aspirated (Mean ±SE)	Collected COCs per ovary (Mean±SE)				
						Total	Normal		Abnormal	
							A	B	C	D
Right (70)	1.14 ±0.26	1.18^{b} ±0.04	0.86 ±0.03	6.84 ±0.26 (176)	4.74 ±0.20 (113)	4.34 ±0.18 (76)	2.11^{a} ±0.09 (23)	1.52^{a} ±0.08 (24)	0.33^{b} ±0.09 (6)	0.37^{b} ±0.09 (23)
Left (68)	1.13 ±0.26	1.31^{a} ±0.04	0.90 ±0.03	6.51 ±0.27 (198)	4.76 ±0.20 (121)	4.47 ±0.19 (86)	0.37^{b} ±0.09 (17)	0.24^{b} ±0.08 (10)	1.68^{a} ±0.09 (21)	2.19^{a} ±0.09 (38)
Significant level	NS	**	NS	NS	NS	NS	**	**	**	**

Means with different superscripts differ significantly from each other within the same column ** ($p<0.01$)
NS= Not significant
Parenthesis indicates the total number.

Among 138 ovaries (consisting right ovary 70 and left ovary 68) a number of 45 belonged without CL and 25 with CL. The result of different parameters are summarized inTable-4.1 and Table-4.2. The length (cm) of left (1.31±0.04) was significantly ($p<0.01$) higher than the right ovary (1.18±0.06) but no significant differences were found in the width (cm) and weight (g) of right ovaries (0.86±0.03 and 1.14±0.26) and left ovaries (0.90±0.03 and 1.13±0.26) respectively (Table-4.1) and Appendix (1-3). The number of 374 follicles were recorded on the surface of the ovaries (Appendix-4) and 234 follicles were aspirated from the surface of both (right and left) and (Appendix-5). Ovaries among them 113 were obtained with a mean of (4.74±0.20) per ovary from right and 121 from left ovaries with a mean of (4.76±0.20) per ovary (Table-4.1) and (Figure-4.1). The collected number of COCs higher in left ovaries (4.47±0.19) compared to right ovaries (4.34±0.18) (Appendix-6). When the COCs were classified as normal and abnormal groups, the highest number of normal COCs were found in right (Grade-A; 2.11±0.09 and Grade-B; 1.52±0.08) than that of left (Grade-A; 0.37±0.09 and Grade-B; 0.24±0.08) ovary (Figure-4.2) and (Appendix 7-10).

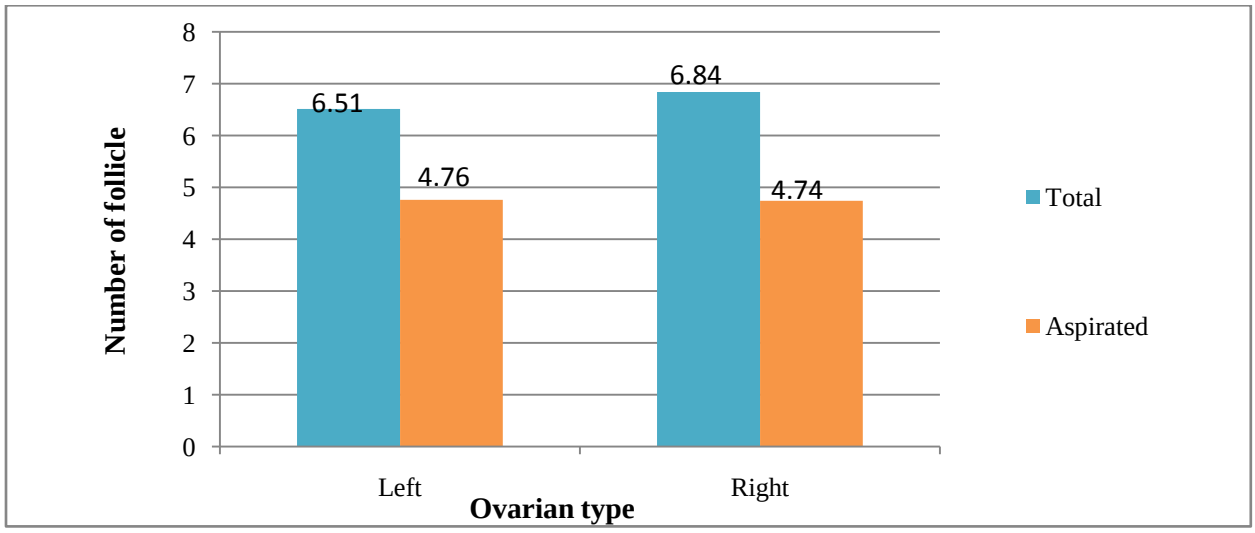


Figure 4.1. Total number of follicles and number of follicles aspirated per ovary

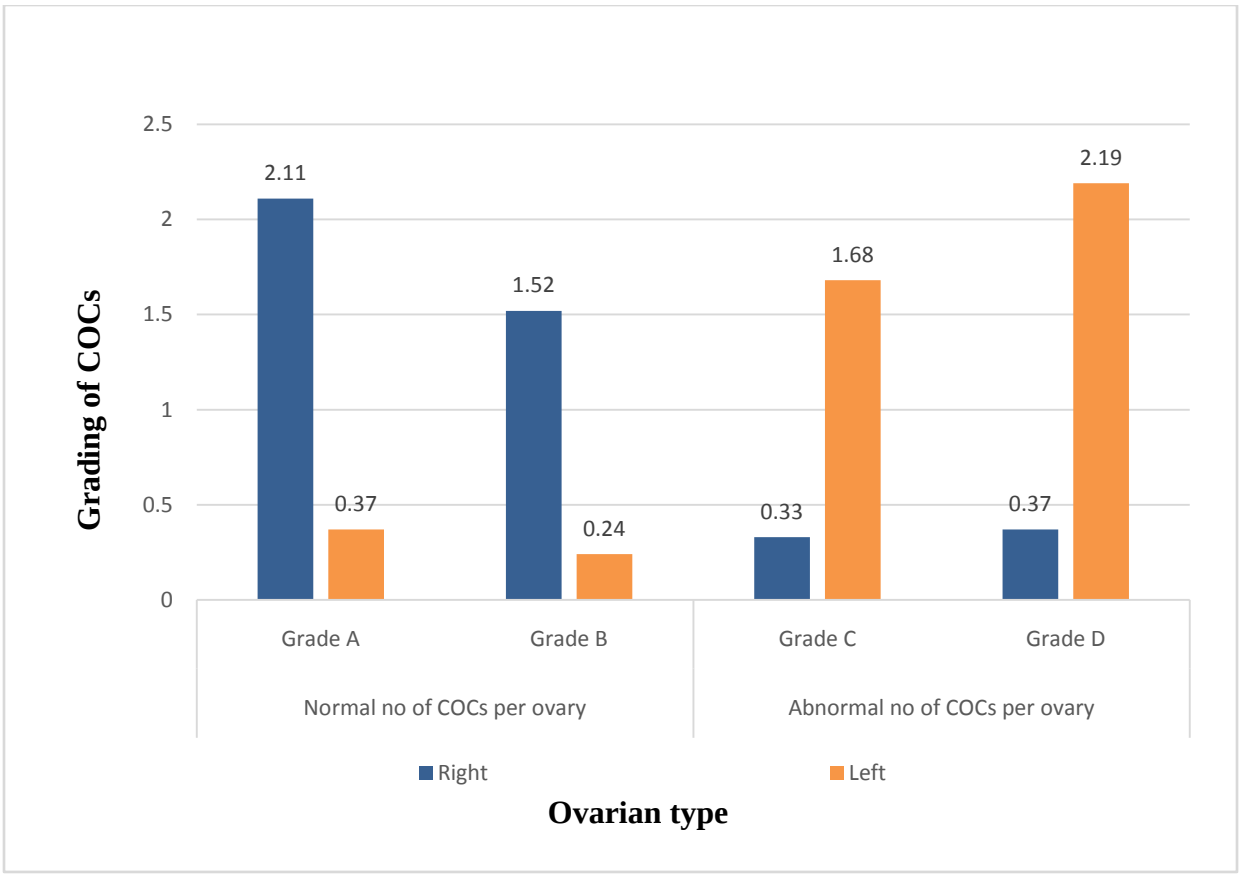


Figure 4.2. Normal and abnormal number of COCs per ovary

Several method have been used for harvesting of oocytes from slaughter house ovaries. Age, season, nutritional status (body condition) and cyclicity of animals at the time of slaughter , size and functional status of follicles, methods of oocyte retrieval are some of the factors that might contribute to recorded variation in oocyte quality (Nandi *et al*., 2001; Zoheir *et al*., 2007 ; Amer *et al*., 2008). A number of research works have been conducted to compare the efficiency of the oocyte collection techniques in cattle (Katska, 1984; Lonergan *et al*., 1991), sheep (Wahid *et al*., 1992; Wani *et al*., 2000) and goat (Mogas *et al*., 1992; Wang *et al*., 2007). In Bangladesh, few researches have performed in IVP of goat embryos, where COCs were collected only by aspiration of 2 to 6 mm diameter follicles (Asad *et al*., 2016; Islam *et al*., 2007; Mondal *et al*., 2008; Ferdous, 2006). Moreover, puncture, slicing techniques produce more debris which might interfare with the searching of oocytes under microscope and also required more washing when compared to aspiration. As a result, a number of COCs were denuded from cumulus cell due to repeated washing and ultimately resulted in a lower number of normal COCs when compared to aspiration at the final observation.

4.2 Ovarian classification (with CL and without CL) and other parameters per ovary

Table: 4.2 Ovarian Classification (with CL and without CL) and other parameters per ovary

Ovarian type	Weight (g) (Mean± SE)	Length (cm) (Mean± SE)	Width (cm) (Mean± SE)	Total no of follicle (Mean± SE)	No of follicle Visible aspirated (Mean ±SE)	Collected COCs per ovary (Mean±SE)				
						Total	Normal		Abnormal	
							A	B	C	D
With CL (25)	5.14^{a} ±0.21	2.75^{a} ±0.08	2.26^{a} ±0.06	4.32^{b} ±0.43 (108)	3.00^{b} ±0.37 (75)	2.40^{b} ±0.40 (60)	0.40^{b} ±0.21 (10)	0.24^{b} ±0.14 (6)	0.24 ±0.12 (6)	1.52^{a} ±0.15 (38)
Without CL (45)	3.28^{b} ±0.15	2.27^{b} ±0.06	1.68^{b} ±0.05	5.93^{a} ±0.32 (267)	4.60^{a} ±0.28 (207)	4.42^{a} ±0.30 (199)	2.26^{a} ±0.16 (102)	0.75^{a} ±0.11 (34)	0.48 ±0.09 (22)	0.91^{b} ±0.11 (41)
Significant level	**	**	**	**	**	**	**	**	NS	**

Means with different superscripts differ significantly from each other within the same column** ($p<0.01$), NS= Not significant
Parenthesis indicates the total number

In other case 282 follicles were aspirated out of 375 follicles on the surface of both ovaries from CL group (Luteal phase) and without CL (Follicular phase) Appendix (14-15). The width, length and weight were significantly ($p<0.01$) higher in ovaries with CL(5.14^{a}±0.21, 2.75^{a}± 0.08 and 2.26^{a} ±0.06) than those of ovaries without CL(3.28^{b}±0.15, 2.27^{b}±0.06 and 1.68^{b}± 0.05) (Table-4.2) and (Appendix 11-13). The CL is an extra cellular material within the ovary which made differences of its width and weight (Jablonka-Sharif *et al.*1993). This result contradicts with the previous result of Singh *et al*. (1994), Rahman *et al*. (1977) and Sanker *et al*.(1993) and it might be due to less number of ovaries were processed. The female cow destined slaughtering were usually less reproductive performer and most of them might be non-cyclic. So, there had

been the possibility to get more non-cyclic ovaries from the slaughterhouse during random sampling.

The Significantly higher ($p<0.01$) number of follicles were aspirated per ovary in ovaries without CL (4.60±0.28) than in CL containing ovaries (3.00±0.37) (Table-4.2) and (Figure-4.3). The causes of higher number of follicles found in ovaries without CL than those of with CL containing group were understood well as it fits the endocrinological explanation. It is known that all female mammals are born with a large number of follicles which rapidly decline as puberty approaches; but whether the early losses represent a mechanism of physiological wastage or not, is not definitely known. Follicle growth initiation is one of the most important and least understood aspects of ovarian biology and represents a major challenge for experimental study. Changes in the local micro environment (i.e, pH, hormonal concentration, etc) might occur during evolving of primary follicular stage may affect follicular dynamics and quality in the ovaries.

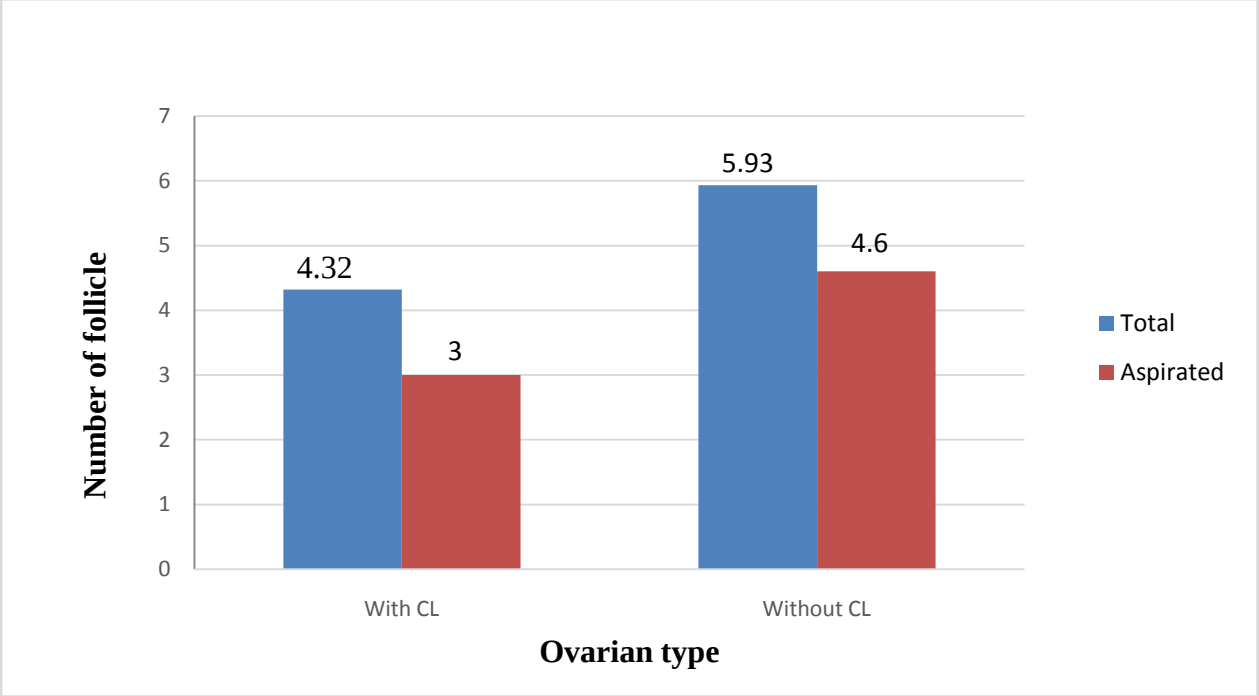


Figure 4.3.Total number of follicles and number of follicles aspirated per ovary

Higher numbers of COCs were found in ovaries without CL (4.42±0.30) than ovaries with CL (2.40±0.40) (Appendix-16). Furthermore, COCs were classified in normal and abnormal groups,

the significantly higher ($p<0.01$) number of normal COCs was found in ovaries without CL (Grade-A;2.26±0.16 and Grade-B; 0.75±0.11) than those ovaries with CL(Grade-A;0.40±0.21 and Grade-B; 0.24±0.14) and the reverse trend was found in abnormal group (Grade-C;0.48±0.09 and Grade-D; 0.91±0.11) and (Grade-C;0.24±0.12 and Grade-D; 1.52±0.15) follicles per ovary respectively (Figure 4.4) and (Appendix 17-20). When compared the ovaries in between with CL and without CL group, significantly ($p<0.01$) higher number of normal COCs were found in without CL group than that of with CL group. The result strongly supported by the previous finding of Asad (2015) who reported that higher number of follicles aspirated per ovary without CL group (2.92±0.08) than those of the with CL group (2.52±0.11) in goat. Similarly, finding also found in buffalo ovaries by Khandoker *et al.* (2011) where significantly higher number of follicles were collected in ovaries without CL (6.78±0.18) than in CL containing ovaries (4.09±0.26). Similar results also reported in goat (Saha *et al*., 2014 ; Mondal *et al*., 2008 and Islam *et al*., 2007). In case of cows, ovaries having without CL contributing more total number of COCs per ovary (6.8±1.0) and also contributing higher normal COCs (5.7±0.9) than that of ovaries with CL (6.0±2.0 and 4.5±1.5 respectively) (Khandoker *et al.* 2016). These findings of further supports the statement that for economic reason, typically less reproductive performing cows are slaughtered in slaughter house and most of them might be non cyclic. Commonly, the older or finisher non cyclic animals were brought for slaughter in the slaughter house.

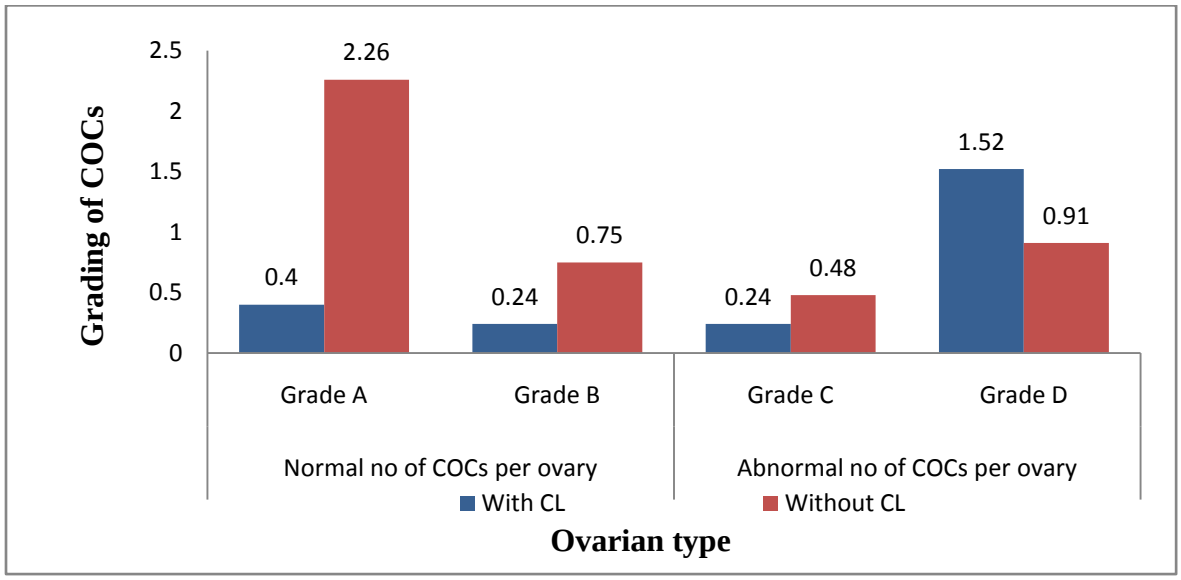


Figure 4.4. Normal and abnormal number of COCs per ovary

The follicular growth is inhibited while atresia is increased in presence of CL in the ovary (Hafez, 1993). These might be the physiological explanation for lower numbers of COCs in the ovaries having CL, the negative effect of progesterone on anterior pituitary might not be functional in this ovary. So, the highest number of normal COCs in this category other than CL functional group explains the role of hormonal balance on bovine folliculogenesis. Similarly, the higher number of normal COCs in without CL ovaries may be due to the hormonal effect of CL. This result supports the previous report of Hafez results (1993) on the role of progesterone on bovine follicular degeneration

Follicular growth initiation is one of the most important and least understood aspects of ovarian biology and represent a major challenge for experimental study. The presence of CL in cyclic female's ovary produces a higher level of progesterone hormones that signals a negative response to anterior pituitary gland for the restriction of gonadotrophin secretion and ultimately follicular degeneration oocurs (Webb *et al.*1999). In this study, the average number of collected COCs per ovary were significantly higher ($p<0.01$) in CL-absent ovaries due to the absence of corpus luteum in non-cyclic female. Nandi *et al*. (2000) stated that when ovaries has corpus luteum, the oocytes recovery rate decreases. This is because there will be restriction of follicular development as lutein cells occupy most of the ovary (Kumar *et al*., 2004). So the highest number of normal COCs in this category other than CL functional group explains the role of hormonal balance (FSH and LH) on cow folliculogenesis. The negative effect of progesterone might not be functional and estrogen-progesterone remains in balanced level which allows follicular growth and oocytes maturation. Within the category, the highest number of normal COCs than that of abnormal COCs further supports the above statement(Khandoker *et al*., 2011; Asad, 2015) who found that presence of a CL significantly reduced the recovery rate as well as the quality of the oocytes. These statement can be the physiological explanation for lower number of COCs in the CL ovaries compared to without CL ovaries. Our finding further supported by other researchers, they have done their research in goats (Asad, 2015; Khandoker *et al* ., 2011; Mondal *et al*., 2008 and Islam *et al*., 2007)

From the observation of this study it was shown that among the 375 ovaries collected from slaughter house , 282 follicle were aspirated where,207 ovaries were observed as without corpus luteum, 75 ovaries obtain with corpus luteum , The number of ovaries having no corpus luteum

usually obtaining from non cyclic cows were slaughtered for economic reason. Commonly less reproductive performing cows were slaughtered and cause high possibility to get more CL-ovaries from the slaughter house during sample collection.

4.3 *In vitro* maturation (IVM) of COCs

Bovine Serum Albumin (BSA) has a nutritional role to play by supplementing amino acids after hydrolysis, thereby maintaining the intracellular amino acid pools. BSA also provide underline embryo tropics (e.g citrate, steroids) compounds functioning as a heavy metal ion cheltor free radical scanverger, protecting cellular constituents against the effect of toxins, which enhance the fertilization of oocytes.

In vitro maturation of COCs can be divided into nuclear and cytoplasmic processes. Nuclear maturation involves resumption of meiosis and progression to metaphase II. Cytoplasmic maturation encompasses a variety of cellular processes that must be completed in order for the embryo to be fertilized and develop into a normal embryo and offspring (Eppig,1996). The maturation and fertilization rates depends on oocytes quality, sufficiency and efficacy of the media and optimization of incubation condition. In this experiment, only normal quality of COCs (Grade-A and Grade-B) collected by aspiration technique were taken and similar media and condition were used for aspiration technique for maturation and further processed to fertilization. The maturation of COCs was initially measured by macroscopic observation of cumulus cell expansion level and then confirmed by nuclear maturation.

a) Cumulus cell expansion of COCs after 48 h culture

The result of the cumulus cell expansion of COCs cultured in TCM-199 supplemented with different levels of Bovine Serum Albumin (BSA) are presented in Table-4.3 and Figure-4.5. No significant difference ($p>0.05$) was found in level-1 but highest value was found when TCM was supplemented with 15%BSA ($0.50^{a}\pm0.13$). In level-2, highest value was found when TCM is supplemented with 10% BSA ($0.86^{a}\pm0.15$) and significant difference ($p<0.05$) were observed when TCM was with 0%, 5%,10% and 15% BSA ($0.38^{ab}\pm0.17$, $0.43^{ab}\pm0.14$ and $0.31^{b}\pm0.17$). Incase of Level-3, highest value was observed ($0.96^{a}\pm0.13$) when TCM was supplemented with 5% BSA but no improvement was observed when TCM was increased at 15% level ($0.06^{b}\pm0.16$) BSA.

Table:4.3 *In-Vitro*-Maturation of COCs after 48 hours of culture in different category of ovaries:

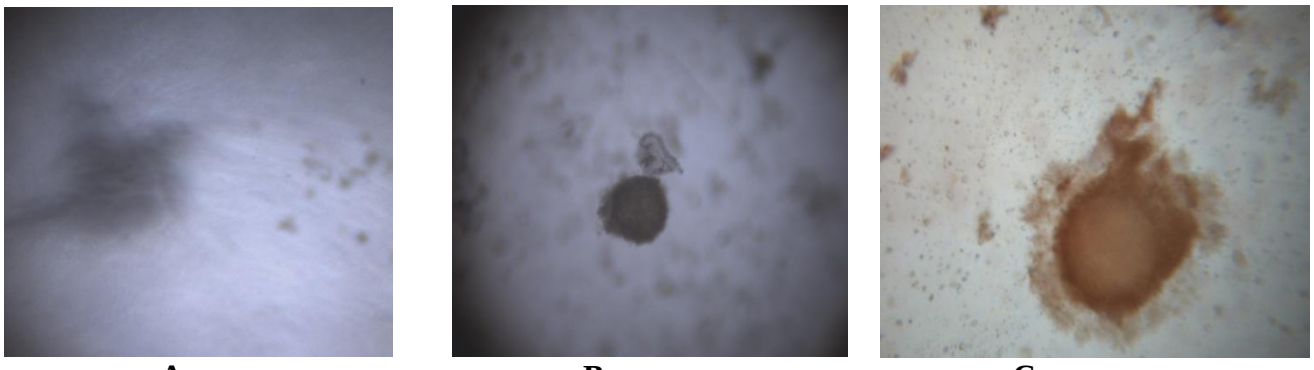
Level of Bovine serum albumin (BSA)	Total no of normal oocytes (COCs)	Rate of Expansion level % (mean±SE)		
		Level-1	Level-2	Level-3
TCM+0%BSA (without supplementation)	16	0.32±0.13	0.38^{ab}±0.17	0.13^{b}±0.16
TCM+5%BSA	23	0.26±0.11	0.43^{ab}±0.14	0.96^{a}±0.13
TCM+10%BSA	21	0.29±0.11	0.86^{a}±0.15	0.71^{a}±0.14
TCM+15%BSA	16	0.50±0.13	0.31^{b}±0.17	0.06^{b}±0.16
Significant level		NS	*	*

Values are shown in Mean±SE

Means with different superscripts within the column* differ significantly ($p<0.05$)

NS=Not significant

The result of cumulus cell expansion are also demonstrated in Figure 4.1, which clearly indicated the differences of expansion level of COCs under different BSA supplementation.



A **B** **C**

Plate 4.1. Photograph of different level of cumulus cell expansion

Where; A. Cumulus cell expansion level-1 (less expansion)
B. Cumulus cell expansion level-2 (moderate expansion)
C. Cumulus cell expansion level-3 (marked expansion)

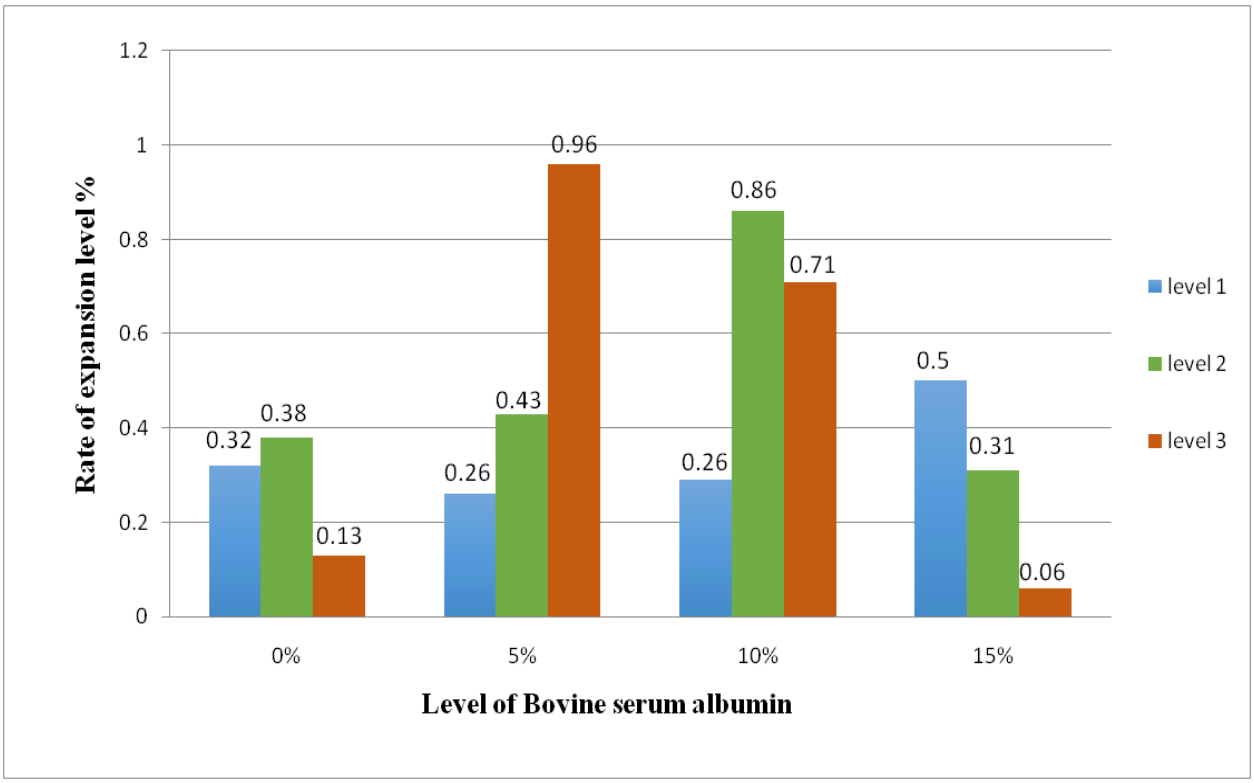


Figure 4.5. *In-vitro* cumulus cell expansion of COCs after 48 hours of culture

Result of BSA supplementation in IVM media at 5% level reported in the experiment (0.96% maturation rate) was almost similar with the maturation rate of oocytes using TCM-199 supplemented with follicular fluid (Asad *et al*., 2012); with fetal calf serum (Mondal *et al.*, 2008) and estrus goat serum (Kharache *et al*., 2006). This similarly further supports the above findings that 5% BSA supplementation in maturation media was optimum for bovine IVM and the level not increase further.

b) *In-vitro* nuclear maturation

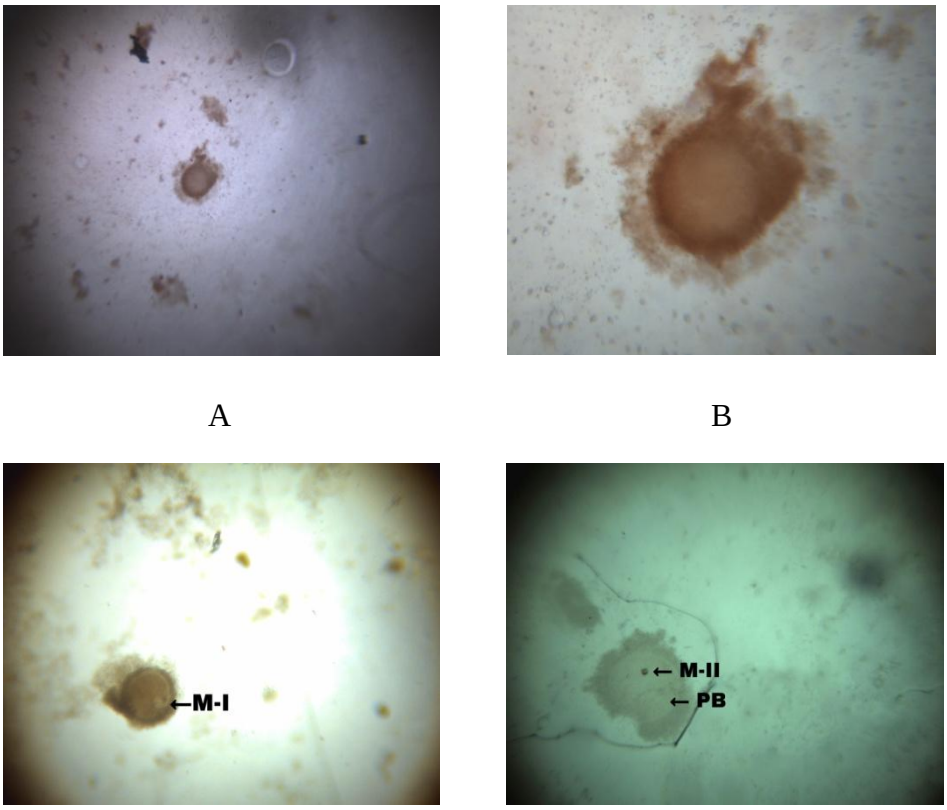
The result of nuclear maturation of COCs after 48 hours cultured in different levels of BSA is presented in Table-4.4. The percentage of COCs matured up to metaphase II stage were 0.00%, 0.00%, 0.12% and 0.00%; metaphase- I were 0.00%, 0.92%,0.47%, and 0.28% ; GVBD were 0.64%, 0.62%,0.82% and 0.61% and GV 0.29%, 0.19%, 0.24% and 0.44% for without (control), 5%, 10%,15% level of BSA respectively (Table-4.4).

Table: 4.4 Effect of different levels of BSA on *in vitro* nuclear maturation of COCs

Level of Bovine serum albumin (BSA)	Total no of normal oocytes (COCs)	Rate of nuclear maturation % (Mean± SE)			
		M-I	M- I I	GVBD	GV
TCM+0%BSA (without supplementation)	14	0.00^{c}±0.17	0.00±0.04	0.64±0.20	0.29±0.14
TCM+5%BSA	21	0.92^{a}±0.14	0.00±0.03	0.62±0.16	0.19±0.11
TCM+10%BSA	17	0.47^{b}±0.15	0.12±0.04	0.82±0.18	0.24±0.13
TCM+15%BSA	18	0.28^{bc}±0.15	0.00±0.03	0.61±0.17	0.44±0.12
Significant level		*	NS	NS	NS

Values are shown in Mean±SE
Means with different superscripts within the column*differ significantly($p<0.05$)
NS=Not significant
M-I= Metaphase- I, M-II= Metaphase- II
GVBD= Germinal vesical break down .
GV=Germinal vesicle

In this study, significant difference ($P<0.05$) were found in the oocytes classified as M-II stages between Bovine Serum Albumin supplemented 5% and 10% and between 0%(control) and 5% level of Bovine Serum Albumin but no significant difference ($p>0.05$) found and the level of Bovine Serum Albumin decresed from 10% to 15%. No difference was found in metaphase-II but the highest M-II was found in 10% BSA (0.12%) supplementation. Significant difference ($p<0.05$) was found in metaphase-I and highest value was found in 5% BSA (0.92^{a}±0.14). No significant difference was found in GVBD and GV but highest was in 10% BSA (0.82^{a}±0.18) and 15% BSA (0.44^{a}±0.12) respectively (Table-4.4 and Figure-4.6).



A B

C D

Plate 4.2 Photograph showing different stages of nuclear maturation of COCs based on choromosomal configuration

Where,

A. Germinal vesicle(GV)

B. Germinal vesicle breakdown (GVBD)

C. Metaphase- (M-I) indicated by arrow and

D. Metaphase- (M-II) indicated by arrow and PB indicates polar body.

In the present study, protein supplementation in culture medium influenced the *in vitro* maturation rate of oocytes as indicated by presence of significant difference in maturation rate between oocytes cultured in medium supplemented with BSA. As protein supplementation, in culture medium for *in vitro* maturation of oocytes, usually fetal bovine serum (FBS) or fetal calf serum (FCS) or oestrus cow serum (OCS) or BSA is used. Holm *et al*. (1999) started that BSA is essential during *in vitro* maturation of bovine oocytes. Moreover, BSA was used with

culture media as a source of protein by Rose and Bavister (1992). However, serum may act as a source of disease transmission in culture medium. Nevertheless, it has been documented that BSA may probably be chemically impure and contaminated with some low molecular weight compound (Kane, 1987; Asad *et al*. (2017) who obtained that three level of cumulus cell expansion after 24 h of *in vitro* maturation (at 38.5ºC and 5%CO_2 in an incubatior) observed under 10x magnification of microscope and metaphase-II stages were 40.78±3.84, 67.52±0.85, 68.95±1.88 and 57.74±2.39% at 0, 2, 4 and 6 mg/ml of BSA supplementation on goat embryo.

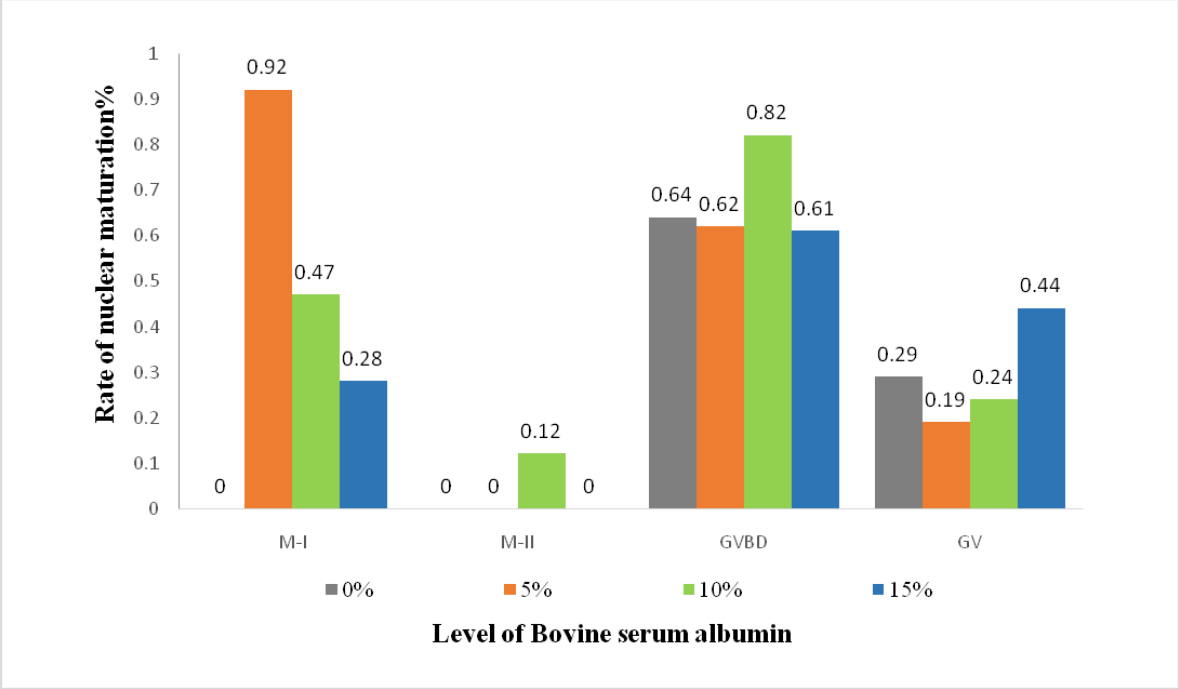


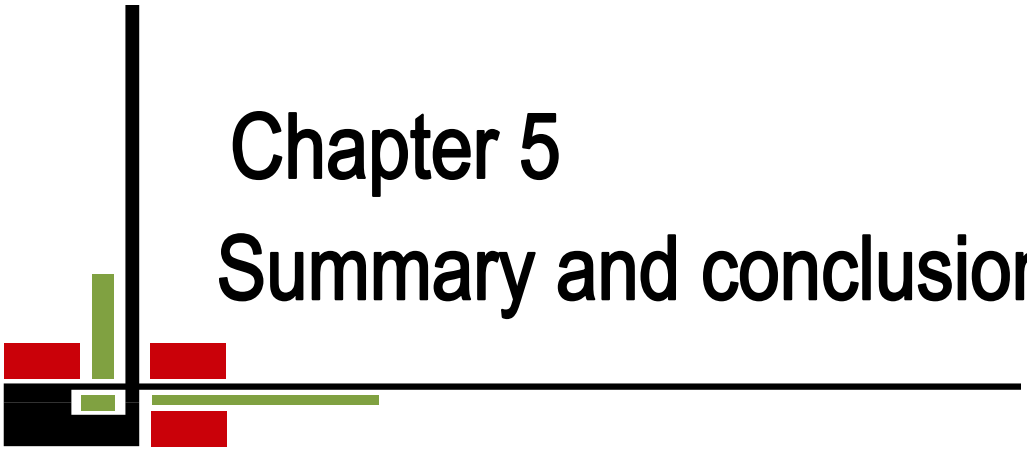
Figure 4.6 *In-vitro* nuclear maturation of COCs cultured in media with different levels of bovine serum albumin

The nuclear maturation rate at M-II (0.12%) of oocytes using 10% BSA supplementation in maturation media was comparable with the maturation rates obtained with 10% fetal bovine serum supplemented (63.7%; Kharche *et al.*,2006), 10-20% estrus goat serum (58-71%; Kharche *et al.*,2009) and with 10% fetal calf serum (58.8-60.4%; Wang *et al.*, 2007). The maturation rate was also comparable to the results of Asad *et al*. (2018) who obtained the COCs reached to M-II at 10% level of FF(Follicular Fluid) supplementation was 66.66 ±0.00% in goat embryo and Wang *et al*. (2007) who obtained 48-63% maturation rate in Boer goat. The maturation rate was

also comparable to the result of Hoque (2009) who obtained 58.7% maturation rate in Black Bengal Goat. The findings of the present study were comparable with those of cattle (Choi *et al*. 1997 and Caralon *et al*.1992); buffalo (Asad *et al*. 2012 and Chauhan *et al*. 1997); sheep (Wani *et al*. 2000) and also with goats (Pawshe *et al*.1994). It showed that the maturation rate does not depend on the collection techniques. Cumulus cell expansion level might be considered as the tool of oocytes maturation. Cumulus cells expansion during *in vitro* oocytes maturation was beneficial for completion of maturation process. The role of the cumulus cells might revolve around their ability to produce pyruvate to provide energy substrate during this period (Ball *et al*. 1984). Moreover, the culture condition condition of the present experiment might be optimum for *in vitro* maturation (IVM) of bovine oocytes in the context of Bangladesh.

In the present study, the rate of overall maturation is 0.12% contrasting to the present finding, the *in vitro* maturation rate of oocytes of zebu cows was higher in Bangladesh (Das *et al*., 2006; Talukder *et al*., 2008). Moreover, grades of oocytes may influence the *in vitro* maturation rates of oocytes as variation in rate of maturation *in vitro* was demonstrated between good and poor grade oocytes (Goswani 2002). However, all retrieved oocytes were cultured for maturation irrespection of grading which may contribute for obtaining lower maturation rate in the present study than earlier.

The present study focused on the influence of different level of BSA supplementation on IVM of bovine oocytes. After the above discussion, we could conclude that, considering the effects of BSA, on the *in vitro* maturation of bovine, 10 % BSA level can be advantageous as a supplementation of maturation. Moreover, this result creates a great opportunity of conducting further research on bovine embryo production.



Chapter 5
Summary and conclusion

Chapter-5

SUMMARY AND CONCLUSION

The research work was conducted at the Animal Nutrition, Genetics and Breeding Laboratory Department of Animal Nutrition, Genetics and Breeding, Sher-E-Bangla Agricultural University, DHAKA-1207 with a view to establish the suitable method of oocyte collection, evaluation of slaughterhouse bovine ovaries and COCs depending on some parameters to establish the procedure of *in vitro* nuclear maturation of bovine oocyte and also the culture condition of IVM. The objective of research work was to find out the effect of bovine serum albumin on *in-vitro* maturation of bovine oocytes.

In this research, 282 follicles were aspirated out of 375 follicles on the surface of both ovaries from CL group (Luteal phase) and without CL (Follicular phase). The Significantly higher ($p<0.01$) number of follicles were aspirated per ovary in ovaries without CL (4.60±0.28) than in CL containing ovaries (3.00±0.37). Higher numbers of COCs were found in ovaries without CL (4.42±0.30) than ovaries with CL (2.40±0.40), the significantly higher ($p<0.01$) number of normal COCs was found in ovaries without CL than those ovaries with CL with the mean of (Grade-A; 2.26±0.16 and Grade-B; 0.75±0.11) and (Grade-A; 0.40±0.21 and Grade-B; 0.24±0.14) follicles per ovary respectively. Number of follicle aspirated in left ovaries (4.76±0.20) and in right ovaries (4.74 ±0.20). The collected number of COCs higher in left ovaries (4.47±0.19 per ovary) compared to right ovaries (4.34±0.18 per ovary). So, left ovary without CL is a good source of normal grade oocytes for *in vitro* maturation of bovine oocytes

In case of bovine serum albumin (BSA) supplementation in IVM media, it was found that the percentage of COCs reaching the cumulus cell expansion level-3 were 0.13±0.16, 0.96± 0.13, 0.71± 0.14, 0.06±0.16; M-II stages were 0.00±0.04, 0.00±0.03, 0.12±0.04, 0.00±0.03 with 0%(control), 5%, 10% and 15% of BSA supplementation. Where, 5% BSA supplementation was reached 0.96% at cumulus cell expansion level-3 and nuclear maturation at 10% BSA reached 0.12% M-II. The result of this experiment indicate that 10% BSA level could be used as a supplement in TCM-199 maturation media.

Finally, It can be conducted that, left ovaries contain more COCs and higher number of follicles than right ovaries; without CL group ovaries with 10% level of bovine serum albumin supplementation are suitable for *in vitro* nuclear maturation of bovine oocytes.

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Appendices

APPENDICES

Appendix 1: Analysis of variance (ANOVA) for weight (g) in different right and left ovary

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	0.002	0.002	0.00	0.982
Within Catagory	136	643.087	4.728		
Total	137	643.089			

NS=Not significant

Appendix 2: Analysis of variance (ANOVA) for length (cm) in different right and left ovary

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	0.549	0.549	6.08	0.01**
Within Catagory	136	12.294	0.090		
Total	137	12.843			

Significant ($p<0.01$)

Appendix 3: Analysis of variance (ANOVA) for width (cm) in different right and left ovary

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	0.065	0.065	1.20	0.275
Within Catagory	136	7.344	0.054		
Total	137	7.409			

NS= Not significant

Appendix 4: Analysis of variance (ANOVA) for total number of follicle in different right and left ovary

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	3.714	3.714	0.77	0.381
Within Catagory	136	654.256	4.811		
Total	137	657.971			

NS= Not significant

Appendix 5: Analysis of variance (ANOVA) for number of follicle aspirated and collected in different right and left ovary

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	0.016	0.016	0.01	0.940
Within Catagory	136	399.606	2.938		
Total	137	399.623			

NS= Not significant

Appendix 6: Analysis of variance (ANOVA) for total number of COCs in different right and left ovary

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	0.563	0.563	0.24	0.628
Within Catagory	136	324.712	2.387		
Total	137	325.275			

NS= Not significant

Appendix 7: Analysis of variance (ANOVA) for normal number (Grade-A) of COCs in different right and left ovary

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	105.228	105.228	207.72	0.0001
Within Catagory	136	68.894	0.506		
Total	137	174.123			

Significant ($p<0.01$)

Appendix 8: Analysis of variance (ANOVA) for normal number (Grade-B) of COCs in different right and left ovary

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	57.691	57.691	127.21	0.0001
Within Catagory	136	61.678	0.453		
Total	137	119.369			

Significant ($p<0.01$)

Appendix 9: Analysis of variance (ANOVA) for normal number (Grade-C) of COCs in different right and left ovary

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	62.667	62.667	114.67	0.0001
Within Catagory	136	74.325	0.546		
Total	137	136.992			

Significant ($p<0.01$)

Appendix 10: Analysis of variance (ANOVA) for normal number (Grade-D) of COCs in different right and left ovary

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	114.222	114.222	219.23	0.0001
Within Catagory	136	70.857	0.521		
Total	137	185.079			

Significant ($p<0.01$)

Appendix 11: Analysis of variance (ANOVA) for weight (g) in CL present and CL absent group of ovaries

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	55.685	55.685	52.19	0.0001
Within Catagory	68	72.553	1.066		
Total	69	128.239			

Significant ($p<0.01$)

Appendix 12: Analysis of variance (ANOVA) for length (cm) in CL present and CL absent group of ovaries

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	3.655	3.655	21.77	0.0001
Within Catagory	68	11.414	0.167		
Total	69	15.069			

Significant ($p<0.01$)

Appendix 13: Analysis of variance (ANOVA) for width (cm) in CL present and CL absent group of ovaries

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	5.323	5.323	54.20	0.0001
Within Catagory	68	6.679	0.098		
Total	69	12.003			

Significant ($p<0.01$)

Appendix 14: Analysis of variance (ANOVA) for total number of follicle in CL present and CL absent group of ovaries

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	41.831	41.831	8.94	0.0039
Within Catagory	68	318.240	4.680		
Total	69	360.071			

Significant ($p<0.01$)

Appendix 15: Analysis of variance (ANOVA) for number of follicle aspirated and collected in CL present and CL absent group of ovaries

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	41.142	41.142	12.02	0.0009
Within Catagory	68	232.800	3.423		
Total	69	273.943			

Significant ($p<0.01$)

Appendix 16: Analysis of variance (ANOVA) for total number of COCs in CL present and CL absent group of ovaries

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	65.722	65.722	16.14	0.0001
Within Catagory	68	276.977	4.073		
Total	69	342.700			

Significant ($p<0.01$)

Appendix 17: Analysis of variance (ANOVA) for normal number (Grade-A) COCs in CL present and CL absent group of ovaries

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	56.000	56.000	50.91	0.0001
Within Catagory	68	74.800	1.100		
Total	69	130.800			

Significant ($p<0.01$)

Appendix 18: Analysis of variance (ANOVA) for normal number (Grade-B) COCs in CL present and CL absent group of ovaries

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	4.278	4.278	8.33	0.0052
Within Catagory	68	34.871	0.512		
Total	69	39.142			

Significant ($p<0.01$)

Appendix 19: Analysis of variance (ANOVA) for normal number (Grade-C) COCs in CL present and CL absent group of ovaries

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	0.995	0.995	2.62	0.1099
Within Catagory	68	25.804	0.379		
Total	69	26.800			

NS= Not significant

Appendix 20: Analysis of variance (ANOVA) for normal number (Grade-D) COCs in CL present and CL absent group of ovaries

Sources of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significant level
Between Catagory	1	5.958	5.958	10.16	0.0022
Within Catagory	68	39.884	0.586		
Total	69	45.843			

Significant ($p<0.01$)