SUPPLEMENTATION OF BOVINE SERUM ALBUMIN AND OVURELIN HORMONE TO DETERMINE AN EFFECTIVE MEDIA ON *IN VITRO* MATURATION AND FERTILIZATION OF CATTLE OOCYTES

A Thesis

By

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*This is to certify that the thesis entitled on "**SUPPLEMENTATION OF PROTEIN AND HORMONE TO DETERMINE AN EFFECTIVE MEDIA ON IN VITRO MATURATION AND FERTILIZATION OF CATTLE OOCYTES**" submitted to the Department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka-1207, 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 NASRIN AKHTER , Registration No 13-05511, 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 have duly been acknowledged.

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The Author

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATION	FULL WORD
%	= 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

LIST OF ABBREVIATIONS AND SYMBOLS (CONT'D.)

ABBREVIATION	FULL WORD
Gm	= Gram
IVF	= *In Vitro* Fertilization
IVM	= *In Vitro* Maturation
IVP	= *In Vitro* Production
IVEP	= *In Vitro* Embryo Production
IVEP-ET	= *In Vitro* Embryo Production-Embryo Transfer
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

LIST OF ABBREVIATIONS AND SYMBOLS (CONT'D.)

ABBREVIATION	FULL WORD
SS	= Sum of Squares
Viz	= Namely

SUPPLEMENTATION OF BOVINE SERUM ALBUMIN AND OVURELIN HORMONE TO DETERMINE AN EFFECTIVE MEDIA ON *IN VITRO* MATURATION AND FERTILIZATION OF CATTLE OOCYTES

ABSTRACT

The present experiment has been undertaken to know the effect of hormone and protein supplementation on *in vitro* maturation and fertilization of cattle oocytes. Ovaries from slaughter were brought to the laboratory and washed in a saline solution at 30℃ for 2 to 3 times. Cumulus oocytes complexes (COCs) were collected by aspiration method and 475 follicles were aspirated out of 631 visible follicles on the surface of both ovaries from CL group (Luteal phase) and without CL (Follicular phase),Where the COCs were classified as normal (Grade A and Grade B) and abnormal (Grade C and Grade D) groups, the highest numbers of normal COCs were found in the ovary without CL (3.11±0.21%). The COCs were matured for 48 hours in TCM-199 basic medium supplemented 10% Bovine Serum Albumin (BSA) and Gonadotropin Hormone (Ovurelin). Cumulus Cell expansion of COCs were studied in TCM-199 cultured media with Ovurelin and 10% BSA. COCs were fertilized with frozen semen after maturation in TCM-199 supplemented with 10% BSA and 10.15µg/ml Ovurelin. The maturation rate is significantly higher at (66.25±2.67%) when 10% BSA and 10.15µg/ml Ovurelin used combined. Similarly, the fertilization rate was significantly higher ($P<0.05$) and high number of normal pronuclei formation (2PN) (66.25±2.67%) was found when supplemented with 10% BSA and 10.15µg/ml Ovurelin. Thus, it can be concluded that the combined media of 10% BSA and Ovurelin might be used as a good supplement for *in vitro* maturation and fertilization of this study.

CHAPTER-I

INTRODUCTION

1.1 General Background

Research on *in vitro* maturation (IVM) and fertilization (IVF) in various cattle breed has been carried out in exotic breed such as in Fries Holland, Limousin and Ongole etc. For the past two years, *in vitro* production research has been undertaken at our lab. This is based on suitable infrastructure and facilities as well as the findings of this field's research. When compared to *in-vivo* embryo production technique, laboratory *in vitro* embryo production (IVEP) technology offers a number of benefits. First, problem cows that don't respond to super ovulatory therapy can be treated with IVEP. Second, IVEP can be utilized to preserve a sick cow's genetic potential even when they are not expected to react to traditional embryo production. Third, oocytes taken from a female cow's ovary, which generates a large number of oocytes, can be fertilized with semen from different bulls to create embryos. Fourth, oocytes for IVEP can be extracted from the ovaries from slaughter house or from live donor ovaries utilizing ovum pick up (OPU) (Suthar and Shah, 2009).

In vitro culture, *in vitro* fertilization, and *in vitro* maturation make up this IVEP process. In the past ten years, research into the technique of *in vitro* embryo generation using cattle oocytes has been conducted as an effective alternative to *in vitro* systems to create embryos for propagation faster than germplasm, for study in developmental biology, and for developing biotechnology.

The implementation of assisted reproductive technologies (ARTs), such as multiple ovulation and embryo transfer (MOET), *in vitro* production of embryos (IVEP), cloning, and transgenesis to transfer a targeted number of embryos produced from animals having desired genetic make-up, has attracted a lot of research attention in recent years. MOET in volves the *in-vivo* collection of embryos from super-ovulated donors at the necessary developmental stage and the transfer of those embryos to a number of synchronized recipients. In underdeveloped nations like Bangladesh, where there are 25.7 million head of cattle are very important and commercially promising animal genetic resources (DLS, 2021). Through the production of milk, meat, and skin, cattle considerably contribute to Nations GDP of Bangladesh.

1.2 State of the problems

The first and most crucial phase in any IVEP-ET program in cattle is *in vitro* maturation (IVM) of the oocytes. However, despite the fact that various exploratory investigations on IVM of zebu cow oocytes in Bangladesh have been undertaken (Goswami, 2002). The IVM rate still needs to be raised (Rahman, 2004; Das *et al*., 2006; Islam *et a*l., 2007; Morshed *et al*., 2014).

The most important procedures to complete for the *in vitro* development of bovine embryos are the effective collecting method and grading of cumulus-oocytes-complexes (COCs). Several techniques, including aspiration of all follicular material, harvest of intact follicles with subsequent isolation of the COCs, and ovarian slicing, are used to retrieve immature oocyte from ovaries. When compared to aspiration of follicular materials, slicing the ovaries, flushing the follicles, or rupturing the isolated follicles may result in more retrieved oocytes (Amer *et al*., 2008).

1.3 Justification of the study

In light of this, the current study was conducted with the intention of collecting and evaluating bovine ovaries, follicles, and COCs obtained from the slaughterhouse. It also aimed to examine the effects of BSA supplementation in the media used for *in vitro* maturation and the fertilization rate.

Oocytes are fertilized by sperm outside of the womb through a technique called *in vitro* fertilization (IVF). In the *in vitro* procedure, the ova from the female ovaries are removed, and the sperm fertilizes them in an appropriate culture medium. The recipient uterus is subsequently given the fertilized oocytes with the goal of establishing a viable pregnancy. Efficiency of IVP is influenced by a number of variables, including transport time, temperature change from abbatoir to laboratory, follicle size, oocyte development stage, media composition (Lonergan *et al*., 1994), hormones (Zuelke and Brackett,1990), and addition of serum and protein to the basic culture medium (Avery *et al*.,2003).

Bovine oocytes are often cultured *in vitro* using Tissue Culture Medium-199 (TCM-199), which is typically buffered (Singha *et al*., 2015). Scientists supplemented this basic medium with various additives from various sources while maintaining varied levels in order to create a well-

defined medium (Saha and Asad, 2022). For instance, gonadotropin (FSH and LH) and estradiol-17β (Rahman *et al*., 2008), as well as human chorionic gonadotropin (hCG) combined with FSH and estradiol-17β, have been introduced (Urdaneta *et al*., 2003). Additional growth-promoting elements, such as vitamins and other chemicals (Silva *et al.*, 2006).

In Bangladesh, a lot of female cattle are slaughtered year-round in slaughter houses. Oocytes for IVM, IVF, and IVC experiments can be economically obtained from female slaughter house ovaries. Oocytes extracted from ovaries can be used to create embryos, which can then be given to recipient cows. Additionally, recent developments in biotechnology have allowed researchers to manipulate *in vitro* created embryos to create cloned and genetically altered animals.

1.4 Objectives

The current study project has been carried out in Bangladesh to produce embryos using oocytes obtained from slaughter house. Given these circumstances, the following are the primary goals of the current study:

1. To explore at cumulus-oocyte-complexes (COCs) obtained from slaughter house cow ovaries in terms of oocyte recovery rate and grading practices;
2. To determine the connection between ovarian vitality and COCs morphological quality;
3. To know the effect of hormone and protein supplementation used on *in vitro* maturation and in vitro fertilization of cattle oocytes.

CHAPTER-II
REVIEW OF LITERATURE

Significant research has been conducted in various countries around the world on the *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of recorded COCs in various ruminant species such as cattle, buffalo, sheep, and goat. This type of research has been reported in Bangladesh have been carried out to a very limited extent. However, relevant information related to research discovery. This chapter examines work done in various countries around the world.

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

Rajesh *et al.* (2018) used an 18 G needle attached to a 5 ml disposable syringe containing 2 ml of collection media to aspirate cumulus oocyte complexes (COCs) from 6 mm diameter follicles on the surface of the ovary. The presence of a cumulus cell complex around the oocytes was used to grade them. The mean of A grade (3 to 5 cumulus cell layer) oocyte recovery rate per ovary was 1.35 in CL ovaries, 1.97 in CL ovaries, and 1.78 in CL ovaries.

Saleh (2017) was removed from the ovaries surrounding tissues and cleaned extensively with dish water, normal saline, and finally MEM medium containing antibiotics and nystatin to remove contaminants. Aspiration, slashing, slicing following aspiration, and slicing were used to collect oocytes. The collected oocytes were 55, 68, 87, and 106 oocytes, respectively; slicing approaches result in a higher oocyte count. The amount of time between slaughtering and sample processing has a significant impact on the percentage and quality of oocytes collected; for example, periods of 2, 6, 12, and 24 hours yield 75 percent, 68 percent, 61 percent, and 55 percent oocyte counts of good, fair, poor, aged, and bad quality, respectively oocytes and oocytes A two-hour timeframe results in a high-quality oocyte count. Oocyte maturation index based on the type of data collected.

Rahman *et al*. (2016) used three ways to harvest DNA from goat ovaries: aspiration, puncture, and slicing. The suction (54.78%) and pierce (54.70%) procedures produced substantially more normal grade oocytes per ovary than the slicing method ($P<0.05$) (30.71%). CL-containing ovaries had a poorer oocyte recovery rate ($p<0.05$) than CL-free ovaries (4.92±0.27). The

number of normal quality COCs per ovary in ovaries without CL (2.47±0.12%) was higher than in ovaries with CL (2.47±0.13%).

Khandoker *et al.* (2011) measured the length, breadth, and weight of ovaries, as well as the number of follicles, aspirated follicles, and COCs. The left (7.25±0.31) ovary had more follicles than the right ($p<0.05$) (6.22±0.32). The number of detected follicles, aspirated follicles, 7 COCs, and normal COCs were substantially ($p<0.05$) higher in ovaries without CL, as were the length, width, and weight of ovaries with CL.

Ferdous (2006) collected COCs using the suction method and discovered that the average number of normal COCs for the CL-present and CL-absent groups of ovaries was 1.77 and 2.04, respectively. The CL-missing group of ovaries had a substantially larger number of COCs and follicles measuring 2-6 mm diameter, while the CL-present and absent groups of ovaries exhibited no significant difference in the number of follicles measuring 2 mm and >6 mm diameter. Normal COCs had a much higher number of COCs with a diameter of 2-6 mm.

Rizos *et al.* (2008) isolated bovine COCs from abattoir ovaries via follicular aspiration with an 18-gauge needle attached to a vacuum system. COCs were washed with serum TCM-199, which included 50 IU/ml heparin and was buffered with mM HEPES and 26 Mm bicarbonate. Pools of 50-60 COCs from 28 ovaries were cultivated in vitro in 4-well plates containing 0.5 ml TCM-199 containing 26 mM bicarbonate and 2.5 mM pyruvate at 38.5℃ in a humidified air environment with 5% CO_2.

2.2 Grading of cumulus-oocytes-complexes (COCs)

Das and Arur (2018) shown that oocytes 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 and 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%) than absence of corpus luteum ($p<0.05$).

Saleh (2017) found oocytes with slicing approaches yielding higher oocyte counts. The time between slaughter and sample processing has a significant impact on the percentage and quality of oocytes collected; for example, periods of 2, 6, 12 and 24 hours yield 75 percent, 68 percent, 61 percent, and 55 percent oocyte counts of good, fair, poor to aged, and bad quality oocytes, respectively. A two-hour period results in a high oocyte count with good quality. The maturation index of oocytes was 44, 37, 39, and 42, with 12, 8, 6, and 6 good oocyte quality for the four methods, respectively.

Khandoker *et al.* (2016) used a microscope to classify COCs as A (oocyte completely surrounded by cumulus cells), B (oocyte partially surrounded by cumulus cells), C (oocyte not surrounded at all by cumulus cells), and D (oocyte not surrounded at all by cumulus cells) (degeneration observed both in oocyte and cumulous cells). COCs in grades A and B were regarded normal, but COCs in grades C and D were considered abnormal. Ovaries with no CL provide a larger overall number of COCs per ovary (6.8±1.0) and also a higher number of normal COCs (5.7±0.9) than ovaries with CL (6.0±2.0 and 4.5±1.5, respectively). However, the aspiration approach did not produce the same results. Similarly, blunt dissection (61.6±4.6 percent versus 16.5±4.9 percent on a total basis) had a larger percentage of COCs recovered than aspiration (48.6±2.9 percent vs 11.7±4.1 percent) on a normal basis.

Asad *et al.* (2015) 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% CO2 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.

Kim *et al.* (1996) 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.

2.3 *In vitro* maturation (IVM) and fertilization (IVF) of oocytes

During *in vitro* development, three primary processes can be distinguished: oocyte maturation, oocyte maturation and fertilization, and zygote development into a viable embryo. A lot of things influence the preceding procedure. Several oocyte IVM and IVF trials have been undertaken around the world, and the results are presented below.

Kharche *et al.* (2008) Oocytes were cultured in TCM -199 containing FSH (5g/ml), LH (5g/ml), and estradiol -17ß (1g/ml), supplemented with 20% fetal bovine serum, at 38.50°C and 5% CO2 in a humidified incubator for 27 hours. The average rate of maturation was 63.72 %. Oocytes were divided into four groups after maturation (IVM) and co-incubated with sperm in TALP medium containing 10g/ml heparin plus 6mg/ml crystalline bovine serum albumin (BSA) 3mg/ml crystalline BSA, 10% estrous goat serum (Group 2); 6mg/ml fatty acid free BSA (Group 3) and 20% estrous goat serum (Group 4). The average rate of formation of pronuclei was 31.56-37.39 %.

Mondol *et al.* (2008) cultured goat COCs for 22 hours in TCM-199 medium supplemented with 5% fetal calf serum (FCS) in air at 38.50°C in a setting of 5% CO2 and observed a maturation rate of 51.52-71.70 percent. Fertilized zygotes were grown in TCM -199 medium supplemented with 5% fetal calf serum (FCS) at the same conditions for 6-7 days after being fertilized in BO media. In grade A COCs, the fertilization rate was significantly greater ($p<0.05$) (38.23 %). The rate of development to compact morula was significantly greater ($p <0.05$) in grade A COCs (25.64%) than in grade B COCs (6.89%), and the rate of blastocyst was significantly higher ($P<0.05$) in grade A COCs (12.82%) than in grade B COCs (6.89%).

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, mg/ml o-LH, 10 mg/ml FSH, 1 mg/ml 17-b estradiol, 400 mM cysteamine, and 50 mg/ml gentamycin, with The fertilization

rate was reported to be 44.07-56.99 percent when oocyters were co-cultured with capacitated spermatozoa in modified Tyrode's medium (mTALP) supplemented with 1mg/ml hypotaurine.

Garg and Purohit (2007) developed oocytes in TCM -199 (5-10 oocytes/drop) supplemented with 5µg/ml FSH, 5µg/ml LH, 1ng/ml oestradiol, 25 mM hepes, 0.25 mM pyruvate, and antibiotics (streptomycin 50µg/ml and penicillin 500IU/ml) in an incubator with 5% CO_2 and The matured oocytes were fertilized in TALP medium supplemented with 30µg penicillamine/ml and 15 mM hypotaurine/ml after the sperm concentration was adjusted to 3×10^{10} ml-^{1}, and the rate was measured as 40.86 percent.

Cox and Alfaro (2007) incubated goat oocytes for 24-26 hours at 39ºC and 5% CO2 in air in maturation media (TCM 199+10% FCS), LH and estradiol, insulin, and growth factors. The mature oocyetes were fertilized with 106 sperm/ml in TALP media supplemented with heparin (1-10g/ml) and 10% heat treated oerstrus-sheep serum. The maturation and fertilization rates were found to be 88.3% and 50.0%, respectively.

Kharche *et al.* (2006) investigated the optimal concentration of estrous goat serum for goat oocyte maturation in vitro. COCs from slaughterhouse ovary were randomly separated into four groups and cultivated in TCM 199 medium without serum addition (Group-1), with 10% EGS supplementation (Group-2), 15% EGS supplementation (Group 3), and 20% EGS supplementation (Group 4). It was seen in all amounts of estrous goat serum after 24-27 hours of IVM as compared to individuals who did not receive serum supplementation. However, there was no statistically significant change in maturation percentage ($P<0.01$) between 10 and 20% serum supplementation in culture media. To summarize, supplementing TCM-199 medium with 20% EGS may be employed to attain appropriate maturation rates.

Tajik and Esfandabadi (2003) aspirated oocytes from caprine ovaries, cleaned them, and cultured them in TCM-199, which contained penicillin, streptomycin, and 10, 15, or 20% fetal bovine serum (FBS), as well as estrous goat serum (EGS). High maturation rates (74-94%) were seen in concentrations of the three distinct sera studied after 24-26 hours of culture. There was no significant difference between different concentrations or different sera. In the absence of protein supplement, almost complete maturation (4%) was seen.

Amer *et. al* (2008) grouped ovarian pairs into 3 types : Type I (with functional corpus luteum), Type II (with regressed corpus luteum), or Type III (without corpus luteum). In Type III, there were more vesicular follicles and aspirated oocytes than in Type II or Type I. In Type III ovaries, the number of Grade A and Grade B oocytes was substantially higher ($P< 0.01$) than in other kinds. Oocytes with >3 layers of cumulus cells matured faster than those with partial remnants or no cumulus cells, but they matured slower than those with 1 to 3 layers of cumulus cells.

Fargay *et al.* (2015) cultivated bovine immature oocytes for 72 or 120 hours in M-199 with 10 IM of each inhibitor (roscovitine, cilostamide, or forskolin), followed by up to 48 hours in maturation media supplemented with 7.5 IU FSH/luteinizing hormone (LH). The eggs were subsequently inseminated with frozen-thawed bull sperm after they had matured in vitro. Fertilization was measured as two-cell division 48 hours after insemination, as well as blastocyst development. For the 72-hour group, total maturation rates were 73 percent, 70 percent, 66 percent, and 69 percent for roscovitine, forskolin, cilostamide, and FSH/LH control, respectively, with no significant differences, demonstrating that inhibitors have no deleterious influence on oocyte maturation rate. Cilostamide (47%) and roscovitine (35%) fertilization rates were considerably higher in the 72-hour group than in the FSH/LH control group (20 percent). The total blastocyst formation rates per inseminated oocytes demonstrated that roscovitine (20%) had a considerably greater rate than forskolin (10%) among treatment groups (9%).

Morohaku *et al*.(2016) aspirated oocytes from mice and collected COCs were induced to resume meiosis, using gonadotropins and epidermal growth factor. Female fetal gonads without mesonephros were cultured in Transwell-COL membranes (Corning) for 17 d. α-MEM (Gibco, Thermo Fisher Scientific) supplemented with 1.5 mM 2-O-α-D glucopyranosyl-L-ascorbic acid (Tokyo Chemical Industry), 10 units/mL penicillin, and 10 μg/mL streptomycin (Sigma-Aldrich) was used as a basal medium (referred to here simply as α-MEM). FBS (Gibco, Thermo Fisher Scientific), SPS (SAGE In-Vitro Fertilization), β-estradiol (Santa Cruz Biotechnology), and the estrogen receptor antagonist ICI 182,780 (Tocris Bioscience) were added at the indicated concentrations for each experiment. Gonads were cultured for 17 d at 37 °C under 5% CO2 and 95% air. Approximately half of the medium in each well was replaced with fresh medium every other day (3, 11, 15, 38).A crucial factor underlying the successful establishment of in vitro

system stemmed from an observation of hypoplastic follicle formation in the ovaries cultured in α-MEM + FBS.

Rahman *et al.* (2004) cultured oocytes for maturation in medium for 24 hours at 38.5oC with 5% CO2 in humidified air. Under an inverted microscope, the presence of initial polar body extrusion in denuded oocytes was used to assess oocyte maturation. The oocytes were grown in fetal bovine serum (FBS) supplemented tissue culture medium (TCM), modified synthetic oviduct fluid (mSOF), and Tyrodes albumin lactate pyruvate (TALP) media to determine an effective basic medium. TCM medium had a much greater maturation rate (74±4.2) than TALP medium (58.2±6.2). The oocytes were cultivated in either follicle stimulating hormone (FSH) or gonadotrophin supplemented TCM to establish an effective hormone supplementation for maturation medium. In FSH augmented oocytes, the maturation rate was considerably (73.3±4.0) higher ($p>0.05$). The oocytes were grown in FBS, oestrus cow serum (OCS), and bovine serum albumin (BSA) supplemented TCM 199 to evaluate an efficient protein supplementation. In medium supplemented with FBS, OCS, and BSA, oocyte maturation rates were (73.0±5.7), (71.1±2.8), and (62.5±9.4), respectively ($p>0.05$).

Singha *et al.* (2015) used an efficient basic medium was determined after culturing COCs in either TCM 199 or SOF medium in Experiment 1 and an efficient hormone supplementation was determined after culturing COCs in either FSH or gonadotrophin supplemented TCM 199 in Experiment 2. An efficient protein supplementation was determined after culturing COCs in either FBS or Oestrous cow serum (OCS) supplemented TCM 199 in Experiment 3. The oocyte recovery rate per ovary was 3.35. The overall rate of IVM was 74.6%. The maturation rate was 75.5±3.9 and 62.2±20.2% in TCM and SOF medium, respectively ($P>0.05$). The maturation rate of oocytes was significantly higher (76.6±13.2%) in FSH supplemented medium than gonadotrphin supplemented counterpart (69.7±10.8%) ($P<0.05$).

Asad *et al.* (2018) collected Cumulus oocytes complexes (COCs) were from goat ovaries by aspiration method and matured for 24 hours in TCM-199 basic medium supplemented with different levels of FF (5%, 10% and 15%). The percentages of COCs reached to Metaphase-II stages with 0%, 5%, 10% and 15% of FF supplementation were 43.33±3.33%, 51.67± 0.83%, 66.66±0.00% and 67.79±1.92% respectively. The normal fertilization (formation of two pronuclei) was recorded as 27.47±0.55%, 31.69±1.09%, 36.54±1.73% and 38.69±2.95%,

respectively. After fertilization the rate of development to compact morula was found as 7.95±1.35%, 10.17±0.17%, 13.34±3.33% and 12.63±1.33%, respectively.

Wang *et al.* (2007) matured oocytes in TCM-199 supplemented with 10 ng/ml epidermal growth factor (EGF) or 10% FCS, either alone or with 1 IU/ml FSH, or oocytes were matured in TCM-199 supplemented with 1 IU/ml FSH. The proportion of blastocysts in oocytes matured in TCM-199 medium supplemented with EGF or FCS with FSH was considerably greater ($P < 0.05$).

CHAPTER-III

MATERIALS AND METHODS

From June 2020 to July 2021, the present experiment was carried out at the Animal Nutrition, Genetics and Breeding Laboratory under the Department of Animal Nutrition, Genetics and Breeding at Sher-e-Bangla Agricultural University, Dhaka.

3.1 Preparation of the laboratory

All of the necessary equipment was properly installed and tested prior to the start of the experiment. These items should be repaired and/or reinstalled if necessary. Finally, all equipment was cleaned and sterilized with 70% alcohol. All reusable equipment was washed, dried, wrapped in aluminum foil, sterilized, and finally stored in a clean and sterile chamber until use. Before beginning the experiment, all necessary disposal equipment, as well as media, chemicals, and reagents, were made readily available.

The list of recruitments are mentioned below-

A. Permanent equipment

a) Phase contrast microscope with USB 2.0 Camera
b) CO_2 incubator at 38.5ºC with 5% CO_2 in humidified air
c) Personal computer
d) Laminar Air Flow Cabinet
e) pH meter
f) Weighing balance
g) Magnetic Stirrer
h) Autoclave machine
i) Digital pH meter
j) Digital micro pipette
k) Water bath

B. Reusable equipment

1. Glassware

a) Beaker

b) Measuring Cylinder

c) Petri dishes (90mm)

d) Pasteur pipette

e) Test tube (10 ml)

f) Conical flask

g) Bottles for media

h) Glass micropipette

2. Collection vial (for ovary collection)

3. Thermo Flask at 25℃ to 30℃

4. Necessary surgical toolkits

C. Disposable equipment

1. Centrifuge tube
2. 10 ml syringes
3. 18G and 19 G needles
4. Sterilized rubber gloves
5. Culture dishes

D. Chemicals, reagents and media

1. Media

a. 0.9% Physiological saline solution

b. Dulbecco's phosphate buffered saline (D-PBS) solution

c. Bovine serum albumen (10% BSA)

d. Tissue culture medium 199 (TCM-199)

e. Gonadotropin Hormone (Ovurelin)

f. Brackett and Oliphant (BO) medium only for fertilization

2. Chemicals and Reagents

Chemicals and reagents are listed in the composition tables.

Preparation of different media for IVP of cattle embryos COCs collection medium

Table 1: Composition of physiological saline

Material	Amount (g)	Preparation
Nacl	9	Dissolved in 100 ml distilled water

[Note: The saline was autoclave before use on the day of collection, 1000 mg of zentamycin were added per litter of saline solution**]**

Table 2: Composition of Dulbecco's phosphate buffered saline (D-PBS) solution

A-Solution

Material	Amount (g)	Preparation
NaCl	4.00	Dissolved in 400 ml distilled water
KCl	0.1	
Na_2HPO_4	0.575	
KH_2PO_4	0.1	

B- Solution

Material	Amount(g)	Preparation
$CaCl_2$	0.05	Dissolved in 50 ml distilled water

C-Solution

Material	Amount (g)	Preparation
$Mgcl_2.6H_2O$	0.05	Dissolved in 50 ml distilled water

[Note: These 3 solutions were autoclave separately and then mixed to prepare the final D-PBS]

Preparation of 10% BSA

To make a 10% (100 mg/mL) stock BSA, dissolve 1g powder fraction V or molecular biology grade BSA in 10 mL distilled H_2O, layering the powder on the surface of the liquid to avoid clumping. Without stirring, gently rock the capped tube until the BSA has completely dissolved.

Preparation BO medium

Table 3: Composition of BO stock solution

A-Solution

Material	Amount	Preparation
Nacl	4.3092g	
Kcl	0.1974g	
$Cacl_2.2H_2O$	0.2171g	Dissolved in 500 ml distilled water
$NaH_2PO_4.2H_2O$	0.0840g	
$Mgcl_2.6H_2O$	0.0697g	
Phenol Red (0.5%)	0.1ml	

[Note: Each component of the A-solution was dissolved in the order written above. Ten times the weight of the $NaH_2PO_4.2H_2O$ and $MgCl_2.6H_2O$ were dissolved in 10 ml distilled water and 1ml of this solution was used.**]**

B-Solution

Material	Amount	Preparation
NaHCO	2.5873g	Dissolved in 200 ml distilled water
0.5% Phenol red solution	0.04 ml	

Table 4: Composition of BO medium

Material	Amount	Preparation
A-solution	380 ml	
Sodium pyruvate	0.01375g	
Penicillin	10,000 IU	Total of 500 ml solution was properly stirred
Streptomycin	10mg	
B-solution	120 ml	

[Note: The medium was prepared on the day of use and sodium pyruvate and antibiotics were added to A-solution and then diluted with B-solution.Finally it was sterilized by filtration**]**

Washing Solution and dilution solution

Table 5: Composition of semen washing solution

Material	Amount
BO Medium	50 ml
Caffeine	0.1942g

Table 6: Composition of COCs washing solution

Material	Amount
BO Solution	40 ml
BSA	400 mg

Table 7: Composition of semen dilution solution

Material	Amount
BO solution	10 ml
BSA	200 mg
Gonadotropin hormone (Ovurelin)	10.15µg/ml

3.2 Processing of ovaries

3.2.1 Ovary collection

Physiological saline (0.9% NaCl) was prepared and sterilized in an autoclave before being stored in the refrigerator. On the day of collection, 1000 mg of zentamycin per litre of saline was added. The solution was warmed to 25ºC to 30ºC and kept in a thermos box to maintain this temperature while the ovaries were transported from the slaughterhouse to the laboratory. Dulbecco's phosphate buffered saline (D-PBS) solution was made by dissolving one pack of PBS salt in one litre of distilled water. It was then sterilized in an autoclave and refrigerated for future use.

3.2.2 Trimming of ovaries

Plate 1 depicts a representative photograph of the ovaries. The ovaries were stored in a thermo-flask containing 0.9% Physiological saline at 25 to 30℃ and transported to the laboratory within 2 to 3 hours of slaughter. At 25℃, the ovaries were transferred to sterile petridishes containing

the same saline. Each ovary was trimmed in the lab to remove the surrounding tissue and overlying bursa (Plate 1). Each ovary was washed three times in D-PBS.

3.2.3 Evaluation of ovary

Ovaries were evaluated on the basis of following measures.

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.

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 sterilized culture dish (35 mm). The numbers of COCs were counted (Plate 4). The COCs were classified into 4 grades (Plate 6) 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 was recorded.

3.2.4 COCs aspiration and grading

The ovaries were brought to the laboratory and washed in a saline solution at 30℃ 2-3 times. The ovary was grasped in the left hand. The needle (19G) was placed 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 slowly transferred into a 90 mm petridish to avoid damaging the cumulus cells, and the COCs were searched and counted and graded under a low magnification microscope (4X).

COCs were classified into four grades, as previously described. In each category, the number of different grades of COCs was recorded. Plate 2 depicts a representative photograph of the COCs. Meanwhile, another petridish of D-PBS was prepared for pooling COCs, and the COCs were

collected using a glass micropipette.

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

3.2.5 Media and droplet preparation for *in vitro* Maturation

Tissue Culture Medium (TCM-199) (Hyclone, USA) supplemented with 10% bovine serum albumin (BSA) and 10.15µg/ml Gonadotropin (Ovurelin) (Bayer, Germany) was prepared as the maturation medium (Plate 2) (A), and its pH was fixed at 7.4 on the day of aspiration and sterilized by filtration. In each of two 35 mm culture dishes, 2.5 to 3.5 ml of medium was poured. In another culture dish, 4 drops of each about 100 µl of maturation medium were poured and covered with paraffin oil. The two culture dishes with evenly distributed medium and the droplet dish were kept in an incubator (NuAir, UK) at 38.5ºC with 5% CO_2 in the air. The first two dishes could be used to help the oocyte mature.

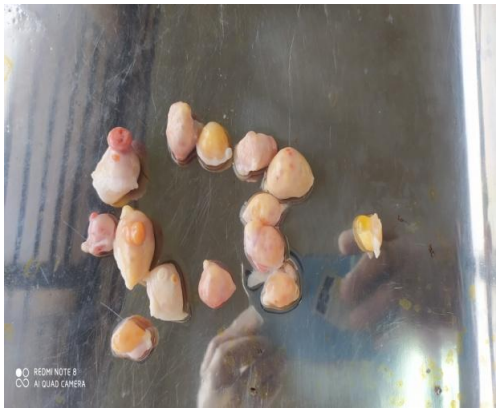
3.2.6 Microscopic observation of cumulus cell expansion

Normal graded (grade A and B) oocytes were washed three times in PBS before being transferred to the maturation medium (TCM-199+ 10% BSA and 10.15µg/ml Ovurelin) and washed two to three times more. Each droplet containing 8 to 10 normal graded oocytes were kept in a CO_2 incubator for 48 hours at 38.5ºC with 5% CO_2 in air (Plate 4). The number of oocytes per grade used for maturation, as well as the times of maturation initiation, were recorded. After 48 hours of IVM, cumulus expansion was determined under a microscope in three levels (Plate 6):

Level 1: No COCs expansion,
Level 2: Moderate expansion and
Level 3: Marked expansion cells with a compact layer of choronaradiata.

The number of oocytes classified according to the basis of expansion rate COCS was recorded.

A



B

C

D

Plate 1: Representative photograph showing

A. **Collection of ovaries**
B. **Cutting of ovaries**
C. **Ovary with CL**
D. **Ovary without CL**

A

B

C

Plate 2: Representative photograph showing

A. **Oocyte aspiration**

B. **Petridishes with different supplementation**

C. **Droplet preparation**

A

B

C

D

Plate 3: Representative photograph showing

A, B,C&D- Different chemicals, reagents and supplementation

A

B

C

Plate 4: Representative photograph showing

A. Incubator with 38.5^{0}C temperature and 5% CO_2

B. Microscopic observation

C. Capture of image

3.3 *In vitro* fertilization (IVF)

3.3.1 Medium Preparation

Brackett and Oliphant (BO) fertilization medium was prepared (Table1), its pH was adjusted to 7.8 on the day of use, and it was finally sterilized.

3.3.2 Semen Collection

Frozen semen of bull was collected from Central Artificial Insemination Laboratory, Savar and brought to the laboratory in icebox (at 4-5℃) within a short period.

3.3.3 Semen Preparation (Sperm Capacitation)

Each frozen straw was removed and immersed in a 37℃ water bath for 30 to 40 seconds. The straw was removed from the water bath and wiped dry, laboratory ends of the straw were cut, only the sperm suspension was expelled into the center of a flattened 100-µL droplet of semen dilution. The sperm concentration was finally adjusted to 10^6 per ml by adding semen dilution (BO+10% BSA and BO+10.15µg/ml Ovurelin, (Table 11). Then, depending on the number of matured COCs, 1-4 insemination droplets (100 µl) of BO medium were prepared in a 35 mm culture dish, covered with paraffin oil, and kept in the incubator for 3-4 hours for pre-incubation.

3.3.4 Incubation of oocyte with sperm

Following 48 hours of maturation, the remaining half of the matured COCs (the other half was used for nuclear maturation) were fertilized. Two 35-mm culture dishes were filled with COCs washing solution (BO+10% BSA and BO+ 10.15µg/ml Ovurelin) and washed three times. Approximately 15-20 COCs with a minimum volume of medium were transferred to each of the previously prepared sperm drops and then incubated for 5 hours in an incubator at 38.5ºC with 5% CO_2 in humidified air.

A

B

C

D

Plate 5: Representing photograph showing

A&B. **Frozen semen collection**

C. **Slide preparation**

D. **Cutting of frozen semen straw**

3.3.5 Checking the fertilization rate

After 5 hours of incubation, all COCs from each drop were pipetted away from cumulus cells. These oocytes were then fixed in a glass slide with aceto-ethanol (1:3, v:v) and stained with 1 percent aceto-orcein. After drying, the slides were examined under high magnification (100X) with emersion oil to observe the formation of pronuclei (PN) as-

i.Oocyte with two PN – Normal fertilization

ii.Oocyte with one PN- asynchronous PN development/ parthenogenetic activation or one PN was obscured by lipid droplets

iii. Oocyte with more than two PN- Polyspermia

iv. Oocye with no pronuclei

Finally the rate of fertilization was calculated. The representative photographs of pronuclei formation is shown in (Plate 9).

3.3.6 Statistical analysis

The difference between groups was assessed using Student's t-test, and all findings were presented as Mean±SE. When the P-value was less than 0.05, the difference between the groups was regarded as significant. With the use of a statistical analysis system, the Duncan's multiple range test (DMRT) was used to compare means (SAS, 1998).

CHAPTER-IV

RESULTS AND DISCUSSION

4.1 Ovarian categories and number of follicle and oocytes collected per ovary

In this study, total number of ovary with corpus luteum and ovary without corpus luteum were 49 and 69. About 475 follicles were aspirated out of 631 follicles on the surface of both ovaries from CL group (Luteal phase) and without CL (Follicular phase) which is showed in table 8. Total number of visible follicle from ovary with CL and without CL were 4.40±0.13 and 6.01±0.29. The number of follicle aspirated from with CL and without CL, these were 3.06±0.13 and 4.71±0.28. Both from ovary with CL and without CL collected COCs per ovary of normal, abnormal and total were (0.71±0.09 & 3.11±0.21), (1.79±0.07 & 1.42±0.08) and (2.51±0.13 & 4.53±0.25) . Data analysis reveals that each case has a considerable difference.

When the COCs were classified as normal (Grade A and Grade B) and abnormal (Grade C and Grade D) groups, the highest numbers of normal COCs were found in the ovary without CL (3.11 ±0.21**)**, which supports the previous result of Saha and Asad (2022), Rahman *et. al,* (2016) and Islam *et al.,* (2007) in goat and bovine.

Oocytes from slaughterhouse ovaries have been harvested using a variety of techniques. Age, the time of year, the animals' nutritional health (body condition) and cyclicity at the time of slaughter, the size and functional status of the follicles, and the methods used to retrieve the oocytes are a few of the variables that could affect the documented difference in oocyte quality (Nandi *et al.,* 2004; Zoheir *et al*., 2007).

Numerous studies have been done to examine the effectiveness of oocyte collecting methods in sheep, cattle, and goats (Amer *et al.*, 2008, Lonergan *et al.*, 1994 and Wang *et al.*, 2007). IVP of goat embryos has only been the subject of a few studies in Bangladesh, where COCs have only been collected. Few studies have been conducted in Bangladesh on IVP of goat embryos, where COCs were only obtained by aspirating follicles with a diameter of 2 to 6 mm (Asad *et al.,* 2018; Islam *et al.*, 2007)In addition, compared to aspiration, piercing and slicing techniques produce more material that could interfere with the examination of oocytes under a microscope and necessitate more washing (Mondal *et al*.,2008 and Ferdous, 2006). As a result of the repeated

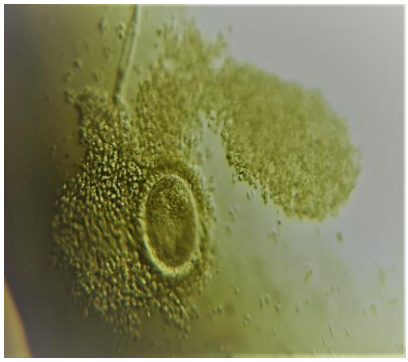
washing, a number of COCs were denuded from the cumulus cell, resulting in a decreased number of normal COCs when compared to aspiration at the final observation.

Table 8: Ovarian categories and number of follicle and oocytes collected per ovary

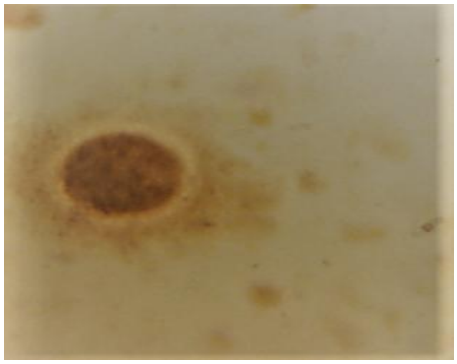
Ovarian Type	Total	Total No. of visible follicle (Mean±SE)	Number of follicle aspirated (Mean±SE)	Collected COCs per ovary (Mean± SE)		
				Normal (Grade A &Grade B)	Abnormal (Grade C &Grade D)	Total
Ovary with Corpus Luteum	49	4.40^{b}±0.13 (216)	3.06^{b}±0.13 (150)	0.71^{b}±0.09 (35)	1.79^{b}±0.07 (88)	2.51^{b}± 0.13 (123)
Ovary without Corpus Luteum	69	6.01^{a}± 0.29 (415)	4.71^{a}±0.28 (325)	3.11^{a}±0.21 (215)	1.42^{a}±0.08 (98)	4.53^{a}±0.25 (313)

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

Figure in the parenthesis indicates the total number.



A



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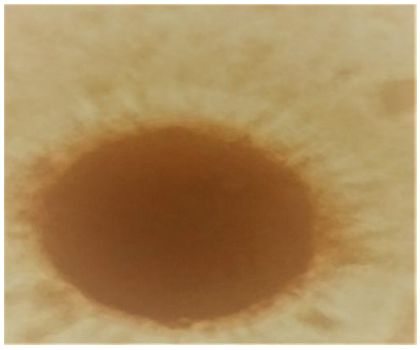

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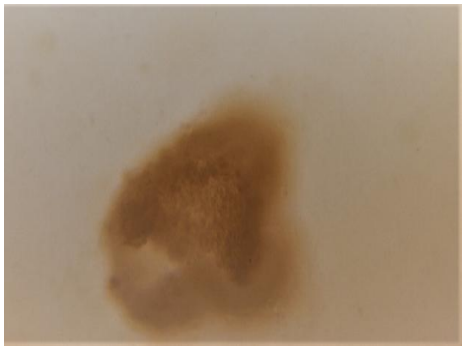


D

Plate 6: Photograph of Different Grading of Cumulus Oocyte Complexes (COCs)

Where, A= Grade A (oocytes completely surrounded by COCs)

B= Grade B (oocytes partially surrounded by COCs)

C= Grade C (oocytes with no COCs)

D= Grade D (degeneration occurred both in nucleous and COCs)

Significantly higher (P<0.05) number of follicles were aspirated per ovary in ovaries without CL(4.71±0.28**)** than in CL containing ovaries (3.06±0.13**)** which is similar to the findings of Saha and Asad,*(*2022).

The findings of Asad (2015), who found that in goats, more follicles were aspirated per ovary when the CL group was absent (2.92±0.08) than when it was present (2.52±0.11), substantially support the current data. Similar findings were made in buffalo ovaries by Khandoker *et al*., (2011), who discovered that ovaries without CL had considerably more follicles (6.78±0.18) than

ovaries with CL (4.09±0.26). Similar outcomes in goats were also documented (Saha *et al*., 2014; Mondal *et al*., 2008 and Islam *et al*., 2007). In the case of cows, ovaries without CL contributed more COCs overall (6.8±1.0) and higher normal COCs (5.7±0.9) than ovaries with CL (6.0±2.0) and (4.5±1.5), respectively (Khandoker *et al*. 2016).

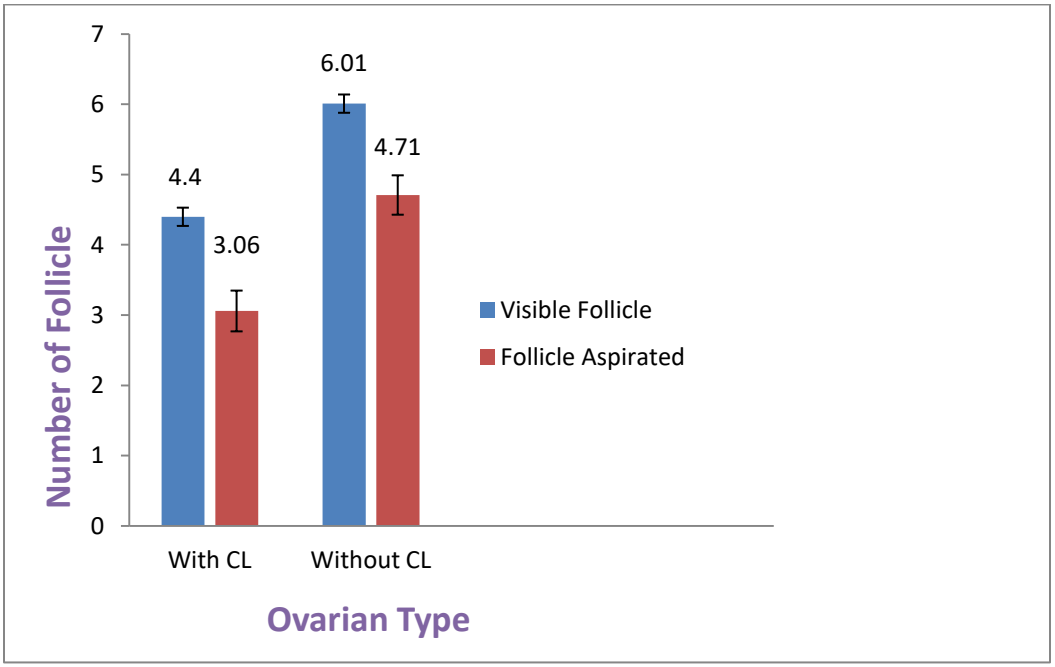


Figure 1: Total number of visible follicle and number of follicles aspirated

These results provide more evidence in support of the claim that, for economic reasons, slaughterhouses usually kill less reproductively active cows, many of whom may not be cyclic. The elder or finisher non-cyclic animals were typically transported to the slaughterhouse for execution.

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).

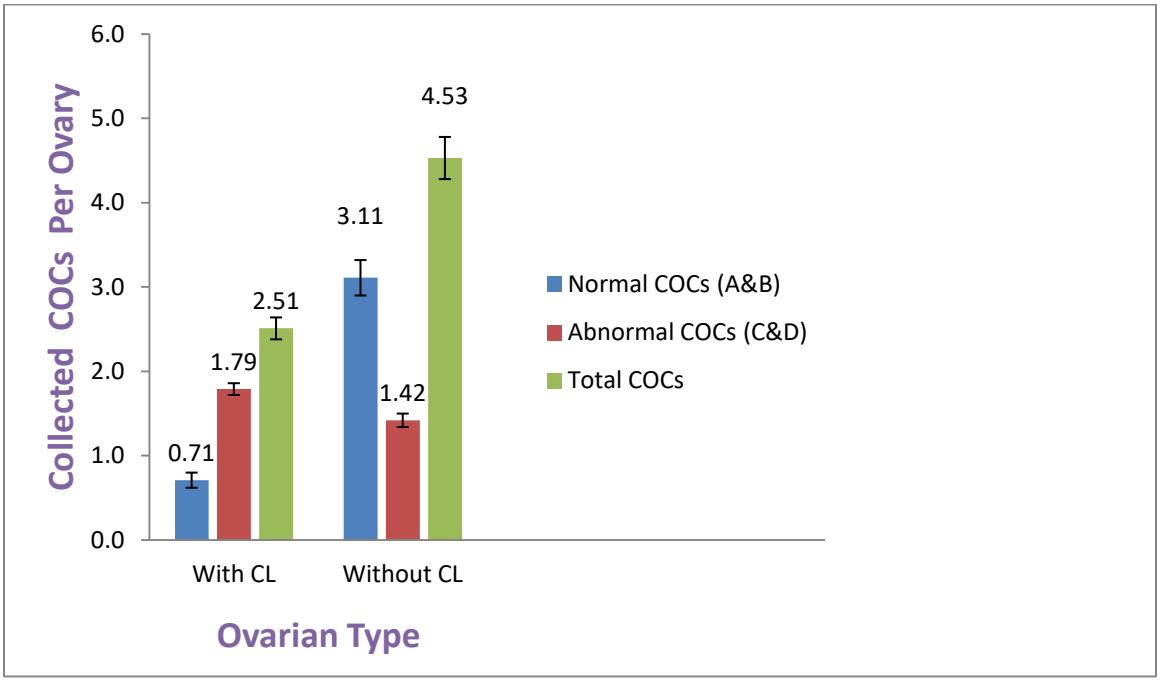


Figure 2: Collected COCs (normal and abnormal) per ovary

4.2 *In vitro* maturation of COCs

4.2.1 Determination of an Effective Hormone and Protein Medium for Oocytes Maturation

To find out the effectiveness of hormone (ovurelin) dose for oocyte maturation, different doses were applied (10.15µg/ml, 5.07µg/ml and 1.02µg/ml). Under this study, we can see that gonadotropin hormone (ovurelin) has a dosage-dependent influence on ovarian oocyte maturation, with the maximum maturation rate (50%) reported at a dose of 10.15µg/ml (Table 9). Throughout this experiment, 10.15µg/ml Ovurelin (Gonadotropin Hormone) was employed.

Table 9: Dose Dependent effect of ovurelin *in vitro* maturation rate of cattle oocyte

Doses of Ovurelin (µg/ml)	No. of oocyte culture	No. of oocyte mature	Maturation rate	Comment
10.15	**20**	**10**	**50%**	**Good**
5.07	**15**	**5**	**33%**	**Fair**
1.02	**15**	**2**	**10%**	**Poor**

Gonadotropins are the primary stimulators of oocyte development in IVM, and FSH is required for oocytes to be qualified for *in vitro* fertilization (Wang *et a*l., 2007). In contrast to the current investigation, the embryo development rate after IVF did not differ between bovine oocytes cultivated in FSH or gonadotrophin supplemented medium, despite the fact that the IVM rate was not compared. Furthermore, the rate of IVM in buffalo oocytes developed on FSH or PMSG supplemented medium did not differ (Ruhil and Purohit, 2015).

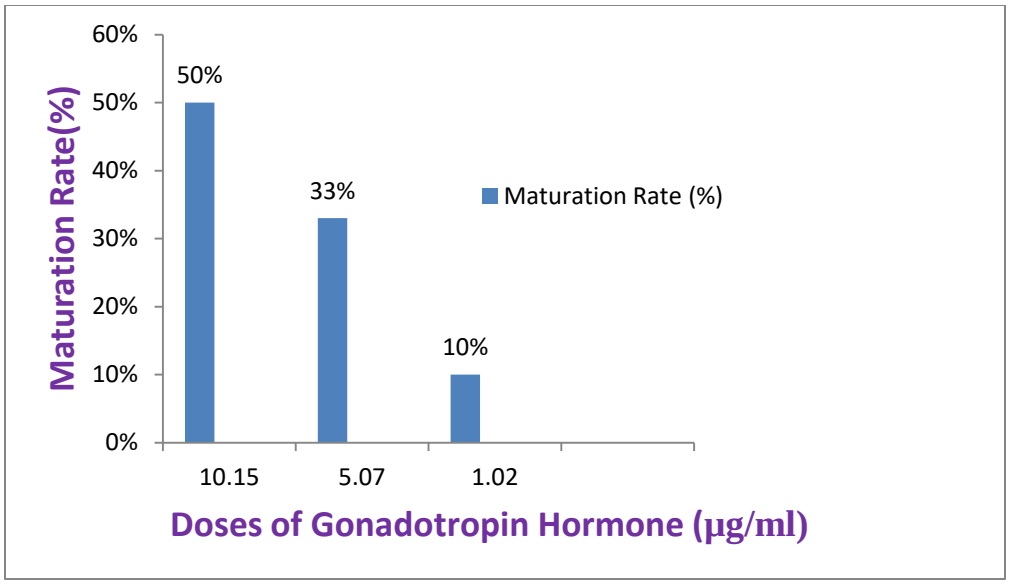


Figure 3: Dose dependent effect of ovurelin *in vitro* maturation of cattle oocyte

Because a prior investigation in our lab had shown that 10% BSA delivers the maximum maturation rate when it comes to *in vitro* maturation, 10% BSA was employed in this experiment to supplement the protein during the *in vitro* maturation of cattle oocytes.

4.2.2 Determination of an Effective Basic Medium for Oocytes maturation

The maturation rate of oocytes in basic media is presented in Table 10. In table 10, Protein supplement (Bovine Serum Albumin) has been provided in two ways. One way was providing 10% BSA with TCM-199 and another is providing 10% BSA with 10.15µg/ml ovurelin (Gonadotropin Hormone).

The maturation rate of oocytes was (44.52±3.28%) in TCM 199 (Control Group). To observe the cumulus cell expansion, oocytes were cultured in TCM-199 supplemented with Gonadotropin (10.15µg/ml Ovurelin) hormone, 10% Bovine Serum Albumin (BSA) and 10% BSA+ 10.15µg/ml ovurelin. The maturation rates of cattle COCs cultured in 10.15µg/ml ovurelin+ TCM-199, 10% BSA+ TCM-199 and 10% BSA+ 10.15µg/ml+ TCM-199 were (54.69±3.95%), (63.13±2.83%) and (66.25±2.67%).

The most often used culture medium for this purpose is TCM 199 (Kumar *et al*., 2004). The inclusion of some elements in TCM-199 medium's composition, such as essential amino acids and glutamine, may accelerate DNA and RNA synthesis and enhance cell division, which may

explain its positive effect on IVM of animal oocytes (Pawshe *et al.*, 1994; Gardner *et al.*, 1994). Furthermore, TCM 199 was found to boost the rate of IVM of oocytes in buffaloes better (Rajesh *et al*, 2018). For many years, it has been recognized that glucose and glutamine are insufficient energy sources for cumulus cell-free rodent oocytes. TCM 199 was utilized as a control in this study when just TCM 199 was used. The control group has lower oocyte maturation (44.5±23.28%) than the Hormone and Protein supplementation group. When the maturity rate of buffalo oocytes was compared between TCM 199 and SOF, Singha *et al*., (2015) found no difference in IVM rates (P>0.05) as Pawshe *et al.,* (1994) found no difference in IVM rates when the maturation rate of buffalo oocytes was compared between TCM 199 and SOF. In contrast to these results, Tajik *et al.,* (2003) found that TCM had a higher percentage of mature oocytes than SOF in buffaloes.

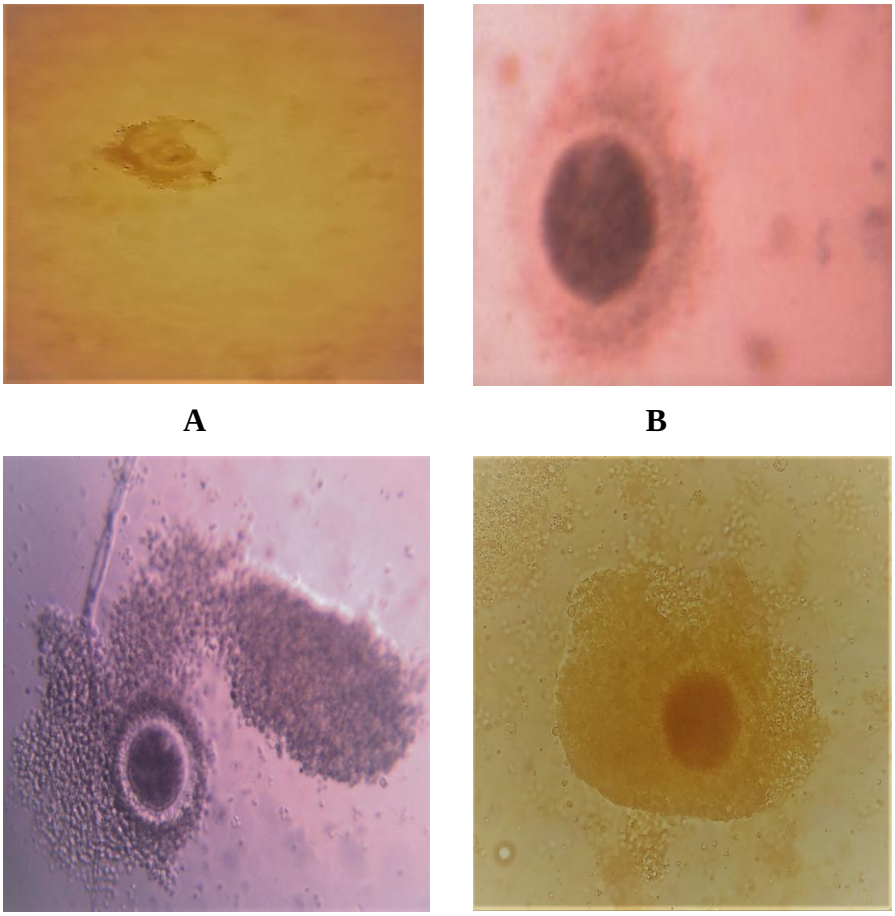
The oocyte maturation rate is higher (66.25±2.67%) when 10% BSA was supplemented with gonadotropin (ovurelin) and TCM-199 compared to administered just with TCM-199 (63.13±2.83 %). Simillarly, when gonadotropin hormone (Ovurelin) supplemented with 10% BSA and TCM-199 ,the maturation rate is higher (66.25±2.67%) compared with TCM-199 (54.69±3.95%) only.

The present maturation rate is similar to the earlier study reported by Morshed *et al*., (2014) in indigenous zebu cows. However, contrasting to the present findings, higher maturation rate was reported by Singha *et al.,* (2015) (74.5%) and Das *et al.,* (2006) (65.4%) in indigenous zebu cows. The differences in maturation rates between studies could be attributable to differences in basic media and the percentage of serum supplementation employed for oocyte maturation. Furthermore, oocyte grades may influence oocyte maturation rates *in vitro*, as differences in maturation rates *in vitro* have been seen between good and bad grade oocytes (Goswami, 2002). However, regardless of grading, all recovered oocytes were cultured for maturity, which may have contributed to Singha *et al.,* (2015) attaining a greater maturation rate than the current study. Oocytes with at least three compact cumulus cell layers were employed for maturation in this study, which may help to achieve a suitable rate of oocyte maturation for *in vitro*.

Table 10: *In Vitro* maturation of cattle COCs cultured in media supplemented with BSA and ovurelin

Supplementation	No. of oocytes cultured	No. of oocytes matured	Maturation rate (%) (mean±SE)
TCM-199 (Control)	54	22	44.52^{d}±3.28
Gonadotropin (ovurelin10.15 µg/ml)+TCM-199	56	31	54.69^{c} ±3.95
10% BSA+TCM-199	60	38	63.13^{b}±2.83
10%BSA+Gonadotropin (Ovurelin 10.15 µg/ml)+ TCM-199	60	40	66.25^{a}±2.67

Proportion values are Mean ± SE. Maturation rate are significantly different from each other ($P<0.05$).



A **B**

C **D**

Plate 7: Photograph of Different level of Cumulus Cell Expansion during Maturation

Where, A**. Cumulus cell expansion level-1 (less expansion)**

B**. Cumulus cell expansion level-2 (moderate expansion)**

C & D. **Cumulus cell expansion level-3 (marked expansion)**

The maturation of COCs was first determined by observing the level of cumulus cell expansion under a microscope**.** Cumulus cell expansion after 24 hours of culture was observed when TCM was supplemented with 0%, 5%, 10%, and 15% BSA, demonstrating that 10% BSA level can be effective as a supplementation of maturation for bovines *in vitro* (Saha and Asad, 2022).

The COCs were grown in TCM 199 supplemented with 10% FBS, 10% OCS (Oestrous Cow Serum), or 3% BSA (Bovine serum albumin) with 5g/ml FSH and 1g/ml OE2 to identify an effective protein supplementation in maturation medium (Rahman *et al.*, 2016) and Oocyte maturation rates in medium supplemented with FBS, OCS, and BSA were (73.0±5.7), (71.1±2.8), and (62.5±9.4), respectively ($p<0.05$), which is similar to current study.

The oocytes were cultivated in TCM-199 supplemented with 5g/ml FSH or 10 IU/ml Gonadotrophin, 10% FBS, and 1g/ml OE2 to test an effective hormone supplementation in maturation medium (Rahman *et al.*, 2016).

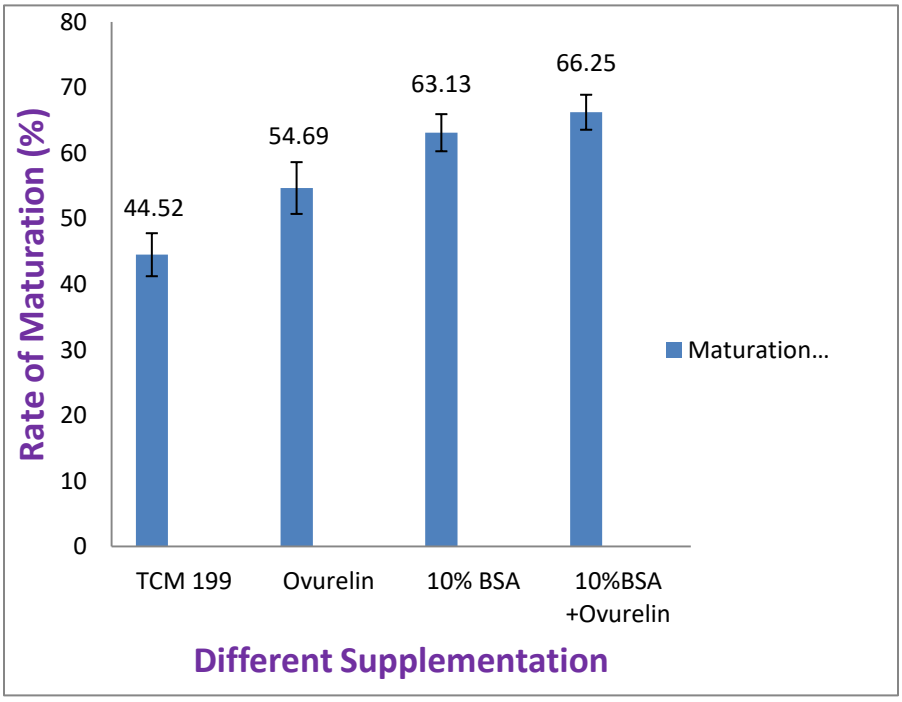


Figure 4: *In vitro* maturation of cayttle COCs cultured in media supplemented with BSA and ovurelin

The maturation rate of oocytes in Protein (FSH) supplemented TCM-199 was greater (73.3±4.0%) than in gonadotrphin supplemented TCM-199 (60.2±6.6%) (Rahman *et al*., 2016), which is similar to this investigation.

4.3 *In vitro* Fertilization of cattle oocytes with frozen semen

After maturation, COCs were cultured in BO medium with frozen semen for 5 hours, with pronuclei development used to determine the fertilization rate. (Table 11).

COCs were fertilized with frozen semen after maturation in TCM-199 supplemented with protein supplementation 10% Bovine serum albumen (10% BSA) and hormone supplementation (10.15µg/ml Ovurelin). Plate 4 shows the pronuclei production, with the results reported in Table 11. The combined supplementation of 10% BSA and 10.15µg/ml Ovurelin resulted in a significantly higher ($P<0.05$) trend (Table 11). When 10% BSA and ovurelin was administered the percentage of normal fertilization was significantly higher ($p<0.05$) and that were (37.04^{a}±5.1%) and (33.37^{b}±5.3%), respectively. The normal fertilization rate increased and abnormal fertilization (creation of 1 and more than 2 pronuclei, as well as no fertilization) reduced with the combination therapy of 10% BSA and Gonadotropin. When protein and hormone supplements were coupled in this study, the maximum 2PN was generated.

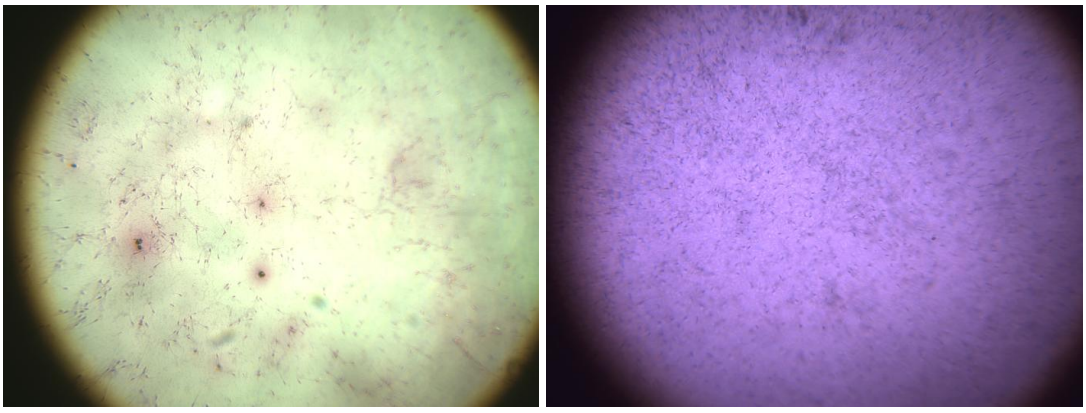


Plate 8: Frozen semen under microscope

Table 11: *In vitro* fertilization of cattle COCs by using frozen semen with BSA and ovurelin

Supplementaion	Total no. of oocytes	Fertilization rate (%) based on pronuclei (mean± SE)			
		2PN	1PN	>2PN	NPN
TCM-199	20	20.00^{d}±86 (5)	20.67^{a}±5.59 (4)	25.67^{a}±2.14 (5)	30.67^{c}±5.21 (6)
Gonadotropin (ovurelin 10.15µg/ml) +TCM-199	29	24.00^{c}±4.9 (9)	13.61^{c}±4.43 (4)	21.11^{b}±5.33 (6)	36.11^{b}±5.92 (10)
10%BSA+ TCM-199	36	33.37^{b}±5.35 (13)	21.35^{a}±7.61 (7)	15.10^{c}±4.85 (5)	31.67^{c}±4.20 (11)
10%BSA+ (Ovurelin 10.15µg/ml) TCM-199	38	37.04^{a}±5.19 (16)	15.00^{b}±5.28 (6)	9.37^{d}±4.83 (3)	39.52^{a}±4.33 (13)

Proportion values are Mean ± SD. Fertilization rate are significantly different from each other ($P<0.05$).

Here, PN= Pronuclei and NPN= No Pronuclei

Asad *et al*., (2018) found that supplementation boosted normal fertilization ($p<0.01$) and decreased fertilization (creation of 1 and more than 2 pronuclei) as well as no fertilization ($p<0.05$) compared to control, which is similar to present study.

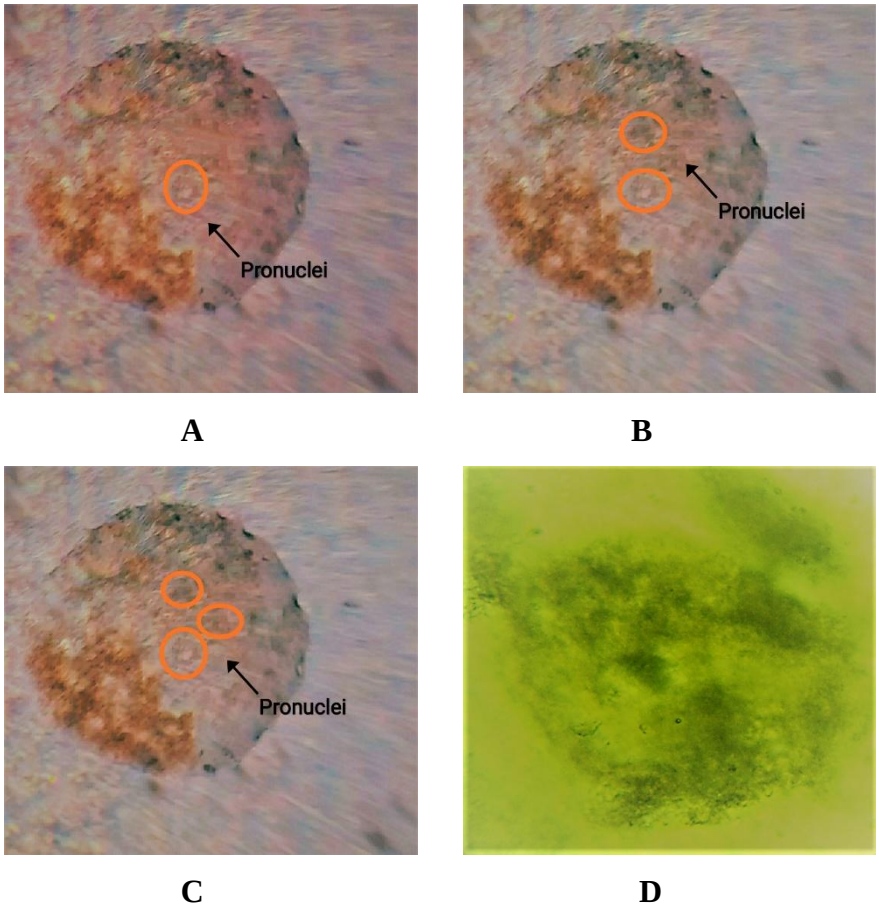


Plate 9: Photograph of Oocyte Fertilization

Where, A= **Oocyte with one pronuclei (1PN)**

B=**Oocyte with two pronuclei (2PN- Normal Fertilization)**

C= **Oocyte with three pronuclei (3PN- Polyspermi)**

D= **Oocyte with no pronuclei**

Here, Circle indicates pronuclei.

The rate of fertilization is directly proportional to oocyte maturation (Larocca *et al*., 1993) found that the inclusion of FF in culture medium during IVM-IVF boosted the fertilization rate and percentage of morula/blastocysts when compared to oestrus cow serum. Kim *et al*., (1996) discovered that adding follicular fluid to the maturation medium improved the maturation and development capabilities of bovine oocytes.

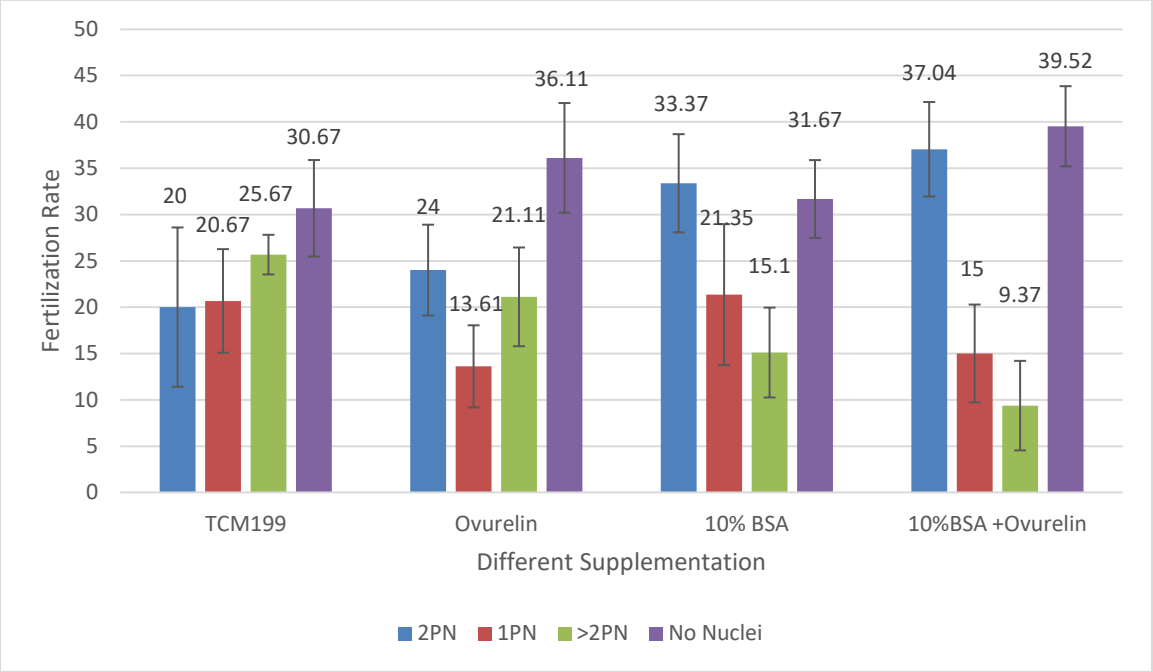


Figure 5: *In vitro* fertilization of cattle COCs by using frozen semen

The purpose of this study was to see how protein and hormone supplementation affected IVM and IVF in cow oocytes. After analyzing the effects of BSA and Gonadotropin on cattle in vitro maturation and fertilization, we can infer that combining the effects of 10% BSA and 10.15µg/ml Gonadotropin as a supplementation of maturation and fertilization can be beneficial.

CHAPTER-V

SUMMARY AND CONCLUSION

The research work was conducted at the Animal Nutrition, Genetics and Breeding Laboratory under the Department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka-1207 with a view to establish determination of effective media depending on some parameters to establish the procedure of *in vitro* maturation and fertilization of cattle oocytes and also the culture . The objective of research work was to find out the effect of Protein (10% BSA) and hormone (Ovurelin) supplementation on *in vitro* maturation and fertilization of cattle oocytes. In this research, 475 follicles were aspirated from the 118 ovaries removed from the abattoir, and it was discovered that 69 of them lacked the corpus luteum. The corpus luteum was discovered to be present in the remaining 49 ovaries. Protein supplement (Bovine Serum Albumen) has been supplied in two methods. One technique was supplying 10% BSA with TCM-199 and another is providing 10% BSA with 10.15µg/ml ovurelin hormone (gonadotropin hormone). The oocyte maturation rate is higher (66.25±2.67 percent) when 10 percent BSA was supplemented with gonadotropin (ovurelin) and when TCM 199 administered solely with 10% BSA then maturation rate was (63.13±2.83 percent) and maturation rate of control group (TCM-199) and (10.15µg/ml ovurelin+ TCM-199) were (44.52±3.28%) and(54.69±3.95%).

According to this study, we found that gonadotropin effects ovarian oocyte maturation in a dose dependent manner, with the greatest maturation rate being reported at a dose of 10.15µg/ml. So, 10.15µg/ml ovurelin (gonadotropin hormone) was used during this experiment. After maturing in TCM-199 supplemented with protein (10% Bovine Serum Albumen) and hormone (10.15µg/ml Ovurelin) supplementation, COCs were fertilized with frozen semen. 10.15µg/ml Ovurelin and 10% BSA supplemented together led to a considerably higher ($P<0.05$) trend (37.04±5.19%). The percentage of normal fertilization (the formation of two pronuclei) was considerably greater ($p<0.05$) as (33.37±5.35%) and (24.00±4.9%), when provided with 10% BSA and gonadotropin respectively. With the 10% BSA and Gonadotropin combination therapy, the normal fertilization rate increased (37.04±5.19%) and aberrant fertilization that was production of 1 pronuclei (15.00±5.28%) and more than 2 pronuclei (9.37±4.83%), as well as no pronuclei (39.52±4.33%) were decreased. In this study, the highest 2PN was produced (37.04±5.19%) when protein and hormone supplements were combined. Additionally, this discovery provides up a wide range of

opportunities for additional study into the production of bovine embryos. However, more research employing varying doses of the Gonadotropin hormone is needed in this area.

CHAPTER- VI

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CHAPTER-VII

APPENDICES

Appendix 1: Analysis of variance (ANOVA) for total number of follicle in ovary with CL and without CL

Sources of Variation	Degree of Freedom	Total Sum of Squares	Mean Square	F- Value	Significant Level
Between Category	1.41	119.76	1.01	6.45	0.0001
Within Category	116	464.82	3.93		
Total	117.41	584.59			

Significant ($p<0.05$)

Appendix 2: Analysis of variance (ANOVA) for number of visible follicle aspirated in ovary with CL and without CL

Sources of Variation	Degree of Freedom	Total Sum of Squares	Mean Square	F- Value	Significant Level
Between Category	1.41	77.94	0.66	5.86	0.000001
Within Category	116	417.01	3.53		
Total	117.41	494.92			

Significant ($p<0.05$)

Appendix 3: Analysis of variance (ANOVA) for normal (Grade A and Grade B) COCs in ovary with CL and without CL

Sources of Variation	Degree of Freedom	Total Sum of Squares	Mean Square	F- Value	Significant Level
Between Category	1.41	165.26	1.40	7.15	0.000001
Within Category	116	245.07	2.07		
Total	117.41	410.33			

Significant ($p<0.05$)

Appendix 4: Analysis of variance (ANOVA) for abnormal (Grade C and Grade D) COCs in ovary with CL and without CL

Sources of Variation	Degree of Freedom	Total Sum of Squares	Mean Square	F- Value	Significant Level
Between Category	1.41	4.04	0.03	1.65	0.06
Within Category	116	46.77	0.39		
Total	117.41	50.81			

NS= Not significant

Appendix 5: Analysis of variance (ANOVA) for total number of COCs in ovary with CL and without CL

Sources of Variation	Degree of Freedom	Total Sum of Squares	Mean Square	F- Value	Significant Level
Between Category	1.41	117.61	0.99	5.13	0.0001
Within Category	116	349.40	2.96		
Total	117.41	467.01			

Significant ($p<0.05$)