DETERMINATION OF EFFECTIVE MEDIA AND ITS HORMONE SUPPLEMENTATION ON IN VITRO MATURATION AND FERTILIZATION OF COW OOCYTES

A THESIS

BY

SOMA RANI ROY

DEPARTMENT OF ANIMAL NUTRITION, GENETICS AND BREEDING

SHER-E-BANGLA AGRICULTURAL UNIVERSITY, DHAKA -1207

BANGLADESH

DECEMBER, 2021

DETERMINATION OF EFFECTIVE MEDIA AND ITS HORMONE SUPPLEMENTATION ON IN VITRO MATURATION AND FERTILIZATION OF COW OOCYTES

By

Soma Rani Roy

Reg. No. 14-05829

A Thesis

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
SEMESTER: July-December, 2021

Approved By:

Dr. Lam Yea Asad
Professor and Supervisor
Department of Animal Nutrition, Genetics and Breeding
Sher-e-Bangla Agricultural University
Dhaka-1207

Dr. Al-Nur Md. Iftekhar Rahman
Associate Professor and Co-Supervisor
Department of Animal Nutrition, Genetics and Breeding
Sher-e-Bangla Agricultural University
Dhaka-1207

Dr. Mofassara Akter
Associate Professor and Chairman
Examination committee
Department of Animal Nutrition, Genetics and Breeding
Sher-e-Bangla Agricultural University
Dhaka- 1207

DEPARTMENT OF ANIMAL NUTRITION, GENETICS AND BREEDING

Sher-e-Bangla Agricultural University
Sher-e-Bangla Nagar, Dhaka –1207

CERTIFICATE

This is to certify that the thesis entitled "DETERMINATION OF EFFECTIVE MEDIA AND ITS HORMONE SUPPLEMENTATION ON IN VITRO MATURATION AND FERTILIZATION OF COW OOCYTES" 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 Soma Rani Roy, Registration No.14-05829 , 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 such help or source of information as has been availed of during the course of this investigation has duly been acknowledged.*

Date:

Dhaka, Bangladesh

Dr. Lam Yea Asad
Professor & Supervisor
Department of Animal Nutrition, Genetics and Breeding
Sher-e-Bangla Agricultural University
Dhaka-1207

DEDICATED

TO

Professor Dr.

Lam Yea Asad

Acknowledgement

By the name of almighty God, for His greatest, gracious, kindness and merciful of the universe who gave me chance to collaborate myself during this aid period with such a laborious job which definitely showed vision towards long cherished hope.

The work was done in the Department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka. I really feel emotional to express my heartfelt gratitude and honor to my ***supervisor professor, Dr. Lam Yea Asad****, Department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka whose consistent eye, inspiration, immense help, constructive criticism, valuable time, guidance and instruction paved the way to walk with this research work. My lifelong salute and respect to her. I wish to pay my deep respect and gratitude to my* ***co-supervisor Dr. Al-Nur Md. Iftekhar Rahman****, Associate Professor, Department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka, for his constructive criticism, necessary correction, instruction and advice during my research work and presentation.*

The author wishes to special thanks to Bishrat Farhana Amy, Nasrin Akhter Shyanti and all of my friends and well-wishers for their kind help, encouragement, co-operative and extended support in experimental work and data collection.

It is my privilege to pay respect and honor in this regard to my parents, brother and loving son Cassiopeian Roy Bissoy. Whose continuous good wish, support and unconditional love inspired me to go further ahead in the field of education and study. Finally, everlasting respect to almighty God a gain for his kind grace to finish this research work successfully.

The Author

DETERMINATION OF EFFECTIVE MEDIA AND ITS HORMONE SUPPLEMENTATION ON INVITRO MATURATION AND FERTILIZATION OF COW OOCYTE

ABSTRACT

The present experiment was conducted to determine an effective basic medium and its hormone supplementation for *in vitro* maturation and fertilization of cow oocytes. To determine an effective basic medium and hormone the effect of Tissue Culture Media 199 (TCM 199), Tissue Culture Media 199 (TCM 199) supplemented with different doses of Gonadotropin (0.5, 1,5,10 and 15 µg/ml) hormone on *in vitro* maturation and fertilization (of cow oocyte were evaluated throughout the study. Synthetic ovurulin used as substitute of gonadotropin. Cumulus Oocytes Complexes (COCs) were collected from cow ovaries by aspiration method and matured for 48 hours at 37.5ºC with 5% CO_2 in humidified air in basic and supplemented media. Significantly higher ($p<0.05$) number of follicles were aspirated in ovaries without CL (Corpus Luteum) (4.59±0.27) compared with ovaries containing CL (3.05). Consequently higher numbers of COCs were found in ovaries without CL (4.61) than ovaries with CL (1.98). The maturation rate was significantly higher (65.71) in (TCM 199 with Gonadotropin10 µg/ml) medium than those of other treatment. Higher maturation rate was found up to the 10 µg/ml Gonadotropin supplementation but when the dose exceeds 10 µg/ml the maturation rate began to decline. *In vitro* fertilization rate depends on, the condition of the used semen whether it is fresh or frozen, oocyte quality and maturation rate. Comparatively higher fertilization rate based on pronucli formation was found in Tissue Culture Media 199 (TCM 199), supplemented with Gonadotropin 10 µg/ml (40.14) than other media. Therefore, ovaries without CL could be used in Tissue Culture Media 199 (TCM 199), basic medium supplemented with Gonadotropin (10 µg/ml) level may be used as a basic medium for optimum *in vitro* maturation rate of cow's oocytes in Bangladesh.

LIST OF CONTENTS

CHAPTER		TITLE	PAGE NO
		ACKNOWLEDGEMENT	**i**
		ABSTRACT	**ii**
		LIST OF CONTENTS	**iii-iv**
		LIST OF TABLES	**v**
		LIST OF FIGURES	**vi**
		LIST OF PLATES	**vii**
		LIST OF APPENDICES	**viii**
		LIST OF ABBREVIATIONS AND SYMBOLS	**ix-x**
I		**INTRODUCTION**	**1-3**
	1.1	Background	**1**
	1.2	State of the problems	**1**
	1.3	Justification of the study	**2**
	1.4	objectives	**3**
II		**REVIEW OF LITERATURE**	**4-16**
	2.1	Collection and evaluation of bovine ovary, follicles and cumulus-oocytes-complexes (COCs)	4-7
	2.2	Use of different culture media in *in vitro* maturation (IVM)	7-13
	2.3	Effect of hormone supplementation in *in vitro* maturation	13-16
III		**MATERIALS AND METHODS**	**17-27**
	3	Preparation of the laboratory	17-18
	3.1	Preparation for ovary collection	18
	3.1.1	Collection and processing of ovaries	18
	3.1.2	Trimming of ovaries	19
	3.2	Evaluation of ovary	19
	3.3	Collection of oocyte and evaluation of COCs	20
	3.3.1	Counting of follicles on the surface of the ovary	20

LIST OF CONTENTS (cont'd)

CHAPTER		TITLE	PAGE NO.
	3.3.2	Aspiration	20
	3.3.3	COCs grading	21
	3.4	*In vitro* maturation(IVM) of COCs	22
	3.5	*In vitro* fertilization(IVF) cow oocyte	24
	3.5.1	Culture medium preparation	24
	3.5.2	Semen collection	24
	3.5.3	Semen preparation (sperm capacitation)	25
	3.5.4	Insemination (incubation with sperm)	25
	3.6	Evaluation the fertilization rate	26
	3.7	Preparation of maturation medium (TCM-199)	28
	3.8	Statistical model and methods of data analysis	28
IV		**RERULTS AND DISCUSSION**	**29-36**
	4.1	Ovarian Categories of collected samples	29-31
	4.2	Determination of an effective basic medium for oocytes maturation	32-34
	4.3	In vitro fertilization of cow COCs by using frozen semen	34-36
V		**SUMMARY AND CONCLUSION**	**37-38**
		REFERENCES	**39-48**

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
1	Preparation of maturation medium (TCM-199)	28
2	Ovarian Categories and Number of Oocytes Complexes	29
3	Effects of different maturation media on IVM rate of cow oocytes	32
4	In vitro fertilization of cow COCs based on pronuclei (PN) formation with different doses of gonadotropin hormone	35

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE NO.
1	Flow diagram of collection, evaluation, in vitro maturation, and fertilization of cow oocytes	27
2	Total number of follicles and number of follicles aspirated per ovary	30
3	Normal and abnormal number of COCs per ovary	31
4	Effects of media on IVM rate of cow oocytes	34
5	In vitro fertilization of cow COCs based on pronuclei (PN) formation	36

LIST OF PLATES

PLATE NO.	TITLE	PAGE NO.
1	Representative photograph of ovaries without corpus luteum and with corpus luteum (A)	19
2	Representative photograph of aspiration of cow oocyte (A,B)	20
3	Representative photograph of different grades of COCs (A,B,C,D)	21
4	Representative photograph of different level of COCs expansion during in vitro maturation (A,B,C)	23
5	Representative photograph of frozen Semen collection (A,B)	24
6	Representative photograph of insemination with Frozen Semen (A)	25
7	Representative photograph of in vitro fertilization based on pronuclei (PN) formation (A,B,C,D)	26

LIST OF APPENDICES

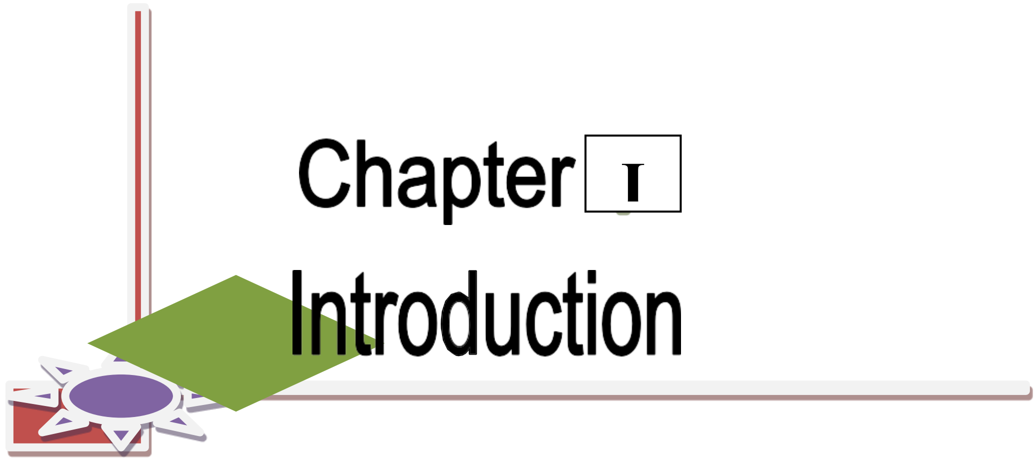
APPENDIX NO.	TITLE	PAGE NO.
1	Analysis of variance (ANOVA) for total number of follicle in ovary with CL and without CL	49
2	Analysis of variance (ANOVA) for number of visible follicle aspirated in ovary with CL and without CL	49
3	Analysis of variance (ANOVA) for normal (Grade A and Grade B) COCs in ovary with CL and without CL	49
4	Analysis of variance (ANOVA) for abnormal (Grade C and Grade D) COCs in ovary with CL and without CL	50
5	Analysis of variance (ANOVA) for total number of COCs in ovary with CL and without CL	50

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
Gm	= Gram

LIST OF ABBREVIATIONS AND SYMBOLS (cont'd)

ABBREVIATION	FULL WORD
IVF	= *In Vitro* Fertilization
IVM	= *In Vitro* Maturation
IVC	=*In Vitro* Culture
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
GVBD	= Germinal Vesicle Break Down



Chapter I

Introduction

CHAPTER-I

INTRODUCTION

1.1 Background

Cattle are economically very important and promising animal in the developing countries like Bangladesh. Cattle play a major role in the achievement of economic growth in developing countries. In recent years many economic activities are taking place encircling livestock resources. Share of Livestock in Agricultural GDP (Current prices) 13.10 % (DLS 2021). Apart from providing food, fiscal support, employment generation cattle play many remarkable contributions including production of hide, leather oriented exportable goods, draft power and fuel. Bangladesh is an over populated country with its 160 million people, always face hurdle to meet up milk and meat demand domestically. The production capabilities of indigenous cattle breeds are very low or not up to the mark. Total milk production in fiscal year 2020-2021was 119.85 lakh metric ton whereas the demand was 154.94 lakh metric ton (DLS 2021).

1.2 State of the Problems

Genetic improvement of cattle can be achieved by in vitro fertilization and maturation of cow oocyte. Though the genetic up gradation by artificial insemination of indigenous cattle with semen of exotic breeds has been practiced for four decades but there are no such eye catching achievements that could meet our expectation level. To meet up the growing demand of meat and milk and to reduce generation interval the existing cattle breed must be genetically upgrade by applying Assisted Reproductive Technologies ARTs such as in vitro fertilization, maturation, multiple ovulation and embryo transfer (MOET), and *in vitro* embryo production (IVEP) (Rahman *et al.*2018)**.** Assisted Reproductive Technologies could be most reliable techniques and could bring a revolutionary change in rapid embryo production consequently genetic up gradation of cattle by reducing generation interval.

1.3 Justification of the study

For successful *in vitro* embryo production (IVEP), *in vitro* maturation of oocytes is the first and important step. Oocyte maturation may be defined as those events that render the oocyte capable of fertilization and able to initiate the program that directs pre-implantation embryonic development. To establish a basic protocol of *in vitro* maturation, fertilization and culture of cattle oocytes for the first time in history of genetic improvement of cattle in Bangladesh an experiment was carried out in 2005 and it was found that the *in vitro* produced zygotes did not develop beyond the 8-cell stages of embryos (Das *et al*., 2006).The maturation of oocyte depends on various factors such as condition of ovaries, follicle size, oocyte quality, culture media, hormone supplementation, aspiration pressure during collection, and time between collection and processing of ovaries. Islam *et al.* (2007) found that few of the embryos developed up to the morula stage.

Culture media plays a vital role in in vitro maturation of oocyte ultimately proper fertilization and complete development of embryo. Maturation rate varies greatly in different culture medium. The reasons for variation in maturation rate among studies might be due to variation in basic media and amount of hormone or protein supplementation in it used for oocyte maturation (Rahman *et al*., 2018). Suboptimal culture conditions during IVM can affect oocyte competency and overall viability of the resulting embryo. Across different species, multiple culture conditions have been used without a consensus of the optimal conditions for oocyte maturation (Bahrami *et al.* 2022). Different culture media such as TCM-199 (Kharche *et al.*2006; Amer *et al.*2008), SOF (Totey *et al.* 1992), minimum essential medium (MEM) (Ravindranatha *et al.*2001) and Ham's F-10 (Totey *et al*. 1993) have been used for IVM of mammalian oocytes elsewhere. Among them, TCM 199 is the most widely used culture medium for such purposes (Arunakumari *et al.*, 2007).

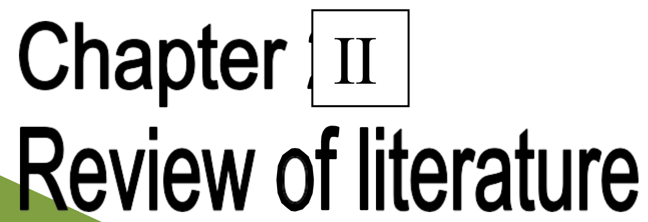
Supplementation of reproductive hormones in maturation media is essential because it improves IVM rate of mammalian oocytes. Follicle stimulating hormone, gonadotropin hormone, lutenizing hormone are some of the hormones commonly used and have substantial effect on IVM of cattle oocyte. In IVM, gonadotropins are the main stimulator

of the oocyte development and FSH being deemed vital for the oocytes becomes qualified to be *in vitro* fertilized (Lu *et al.*, 2014; Khan *et al.*,2015). Basic media supplemented with different hormone have different effect on maturation rate of oocytes. Even supplementation of same hormone with different amount has conspicuous effect on maturation rate and subsequent development after *in vitro* fertilization (IVF).

1.4 **Objectives**

Therefore the present study was undertaken with three objectives

1. To compare the oocyte recovery rate between the ovaries with and without corpus luteum.
2. To find out suitable amount of gonadotropin hormone supplementation in basic medium for optimum *in vitro* maturation and fertilization rate of cow oocyte in Bangladesh.
3. To identify an effective basic medium.


Chapter II
Review of literature

CHAPTER-II

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 works 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)

Covelo *et al.* (2022) collected the ovaries and reproductive tract were sectioned and placed in a thermos containing sterile saline and antibiotics (100 IU/cm^3 penicillin, 100mg/cm^3 streptomycin) at 36˚C. Then, they were transported to the laboratory within 30 min after the animals were slaughtered. Oocyte aspiration was performed with a 5 or 10 cm^3 syringes and an 18 G sterile hypodermic needle. Then the follicular fluid was stored in a 15 cm^3 Falcon tube in a thermostatic bath at 34˚C and, after decanting for a few minutes, they were placed in petridishes for observation. The oocytes were selected using a magnifying glass by evaluating their general appearance, cytoplasm, and the cumulus cells that surrounded them.

Abdulkarim *et al. (*2021) collected the ovaries of buffaloes with seemingly normal reproductive organs within 30 minutes of slaughter and evisceration. Within 1-2 hours of slaughter, the ovaries were kept in warm normal saline with (100 IU penicillin and 100 g streptomycin/ml) and transported to the lab. Aspiration of medium-sized ovarian follicles yielded cumulus-oocyte complexes (COCs) (2-8 mm). For the study, COCs with evenly granulated oocytes surrounded by multi-layered compact cumulus cells were chosen and washed three times in phosphate buffer saline. A 100 drop of TCM 199 supplemented with 10% heat-inactivated fetal calf serum, 10 g/ml LH, 5 g/ml FSH, and 1 g/ml estradiol-17 is given to each of the 10-15 COCs. Mineral oil is used to cover the drops, which are then incubated for 20-22 hours at 38.5°C in the air with a maximum humidity of 5 percent CO_2.

Rahman *et al*. (2018) collected the ovaries of indigenous zebu cows from local slaughter house after slaughtering within 2 hrs. and carried to the laboratory in a thermo flask containing warm normal saline (37°C, 0.9% sodium chloride solution,(w/v).In the laboratory, the ovaries were rinsed three times with warm (37°C) normal saline. The follicular fluid of 2 to 8 mm diameter follicles was aspirated using an 18 gauge needle (TERUMO®, Beijing, China) fitted with a 10 ml disposable plastic syringe (JMI Syringes and Medical Devices Ltd ®, Chauddagram, Comilla, Bangladesh).

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.

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 30ºC) to the laboratory within 1 to 3 hours in a thermo flask. 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 pre-puberal gilt from local abattoir and transported to the laboratory in pre-warmed (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 thermo flask 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 Use of Different Culture Media in *in vitro* maturation (IVM)

Sirin *et al*. (2021) Incubated COCs randomly in tissue culture media–199 (TCM-199) with10% royal jelly (10RJ, n=179) and 10% fotal calf serum (0RJ, n=172 oocytes) for 22h at 39 ºC under 5% CO_2 in humidified air at 95%. The nuclear maturation stages were determined by examining the oocytes under the inverted microscope. The proportion of oocytes reaching metaphase-I (MI) stage in the 0RJ and 10RJ groups was 19% and 20%, respectively. The rate of oocytes reaching the anaphase-I (AI) stage in both groups was determined as 2%. On the other hand, 1% of the oocytes de-veloped up to the telephase-I (TI) stage in both groups. The maturation rate in 10RJ media (78%) was similar when compared with 0RJ media (77%). Metaphase-II (MII) stage oocytes the 10RJ media did not affect the expansion rates of cumulus cells when compared to 0RJ media.

Saha (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-IIstage.

Khandoker *et al*. (2017) conducted experiment using TCM-199 basic medium and supplemented with different levels of BSA (2mg/ml, 4mg/ml and 6 mg/ml) for the maturation of Cumulous Oocyte Complexes in where TCM 199 supplemented with 0mg/ml BSA 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 nuclear 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) was 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.

Lee *at al.* (2016) conducted experiment using TCM 199 medium containing 10 % FBS was utilized as a control group. The serum free media include the IVDM 101 and IVD 101 that are supplemented with energy source and cell growth factors. In order to compare in vitro development rates between medium containing serum (i.e., control group) versus media free of serum supplementation (i.e., IVDM101 and IVD 101), numbers of embryos developed to 2-cell, blastocyst, and hatched blastocyst were monitored. The cleavage rates of 2-cell embryos in reference to Hanwoo oocytes were 86.7, 92.9, and 90.1 % in the control group, IVDM101 medium and IVD101 medium, respectively which indicates that the IVDM101 medium and IVD101 medium may result favorable outcomes.

Morshed *et al.*(2014) conducted experiment on culture conditions for *in vitro* maturation of abattoir derived oocytes of native zebu cows of Bangladesh where TCM-199 (With Earle's salts, L-glutamine and sodium bicarbonate) was the basic media for oocyte maturation. Bicarbonate buffered TCM 199 supplemented with 0.011 mg/ml sodium pyruvate, 5 mg/ml FSH, 1 mg/ml estradiol and 0.01 ml/ml penicillin-streptomycin solution was used for maturation. Moreover, as protein supplement, the culture medium was supplemented either with 10% FBS (v/v) or 5 mg/ml BSA according to the experimental design.

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%).

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.

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.

Tareq *et al*. (2012) 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) was 34.43, 35.03 and 34.65% in puncture, slicing and aspiration techniques, respectively.

Hoque *et al*. (2011) reported that 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.

Hakan *et al*. (2010) used tissue culture medium (TCM-199, Gibco/Invitrogen, Grand Island, NY) supplemented with 0.2 mM pyruvate, 0.5 µg/ml of FSH (Sioux Biochemicals, Sioux City, IA), 5 µg/ml LH (Sioux Biochemicals, Sioux City, IA), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco/Invitrogen, Grand Island, NY) as the main medium for IVM. Additionally, maturation medium was also supplemented with 10% FCS (FCS group), 10% SSS (Irvine Scientific, Santa Ana, CA) (SSS group) or 10% SR1 (S-0638) (SR1 group). SSS contains HSA, alpha and beta globulins. SR1 is composed of heat treated BSA, heat treated bovine transferrin and bovine insulin.

Gomez *et al.* (2008) prepared three culture media, B2 + 5% fetal calf serum (FCS) on Vero cells, synthetic oviduct fluid (SOF) + 5% FCS, and SOF + 20 g L1 bovine serum albumin (BSA), on the developmental competence of the embryos and their ability to survive on vitrification /warming. The culture of embryos in SOF + FCS and in Vero cells + B2 allowed obtaining more Day 6 and Day 7 blastocysts, and a higher % of Day 7 blastocysts vitrified than culture in SOF + BSA. Contrarily, on Day 8, more blastocysts were vitrified in SOF + BSA than in SOF + FCS. Blastocysts quality affected development after vitrification/warming, and Day 7 embryos showed higher survival rates than their Day 8 counterparts. Day 7 blastocysts produced in Vero cells or in SOF + BSA survived at higher rates than those produced in SOF + FCS at 24 and 48 h after warming.

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).

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).

Andrew *at al*. (2000) used the Methods for supporting maturation of bovine cumulus oocyte complexes (COCs) in vitro have employed Tissue Culture Medium 199 (TCM199) supplemented with 10% serum, gonadotropins, and estradiol-17b. These conditions support meiotic maturation of approximately 90% of oocytes and set the stage for high fertilization frequencies and development through to the blastocyst stage. An obvious concern exists, however, regarding the inherent undefined qualities of serum. To characterize specific requirements for oocyte maturation, serum-free systems must be developed. This has been the subject of recent intensive research, and several groups have reported successful development of bovine embryos through to the blastocyst stage following oocyte maturation in serum-free conditions.

Smetanina *et al*. (2000) Studied the capacity of the cattle oocyte for the resumption of meiosis and the achievement of metaphase II in various protein-free culture media (DMEM, TCM-199, Ham's F-10, and Ham's F-12) and the pattern of influence of the estrous serum on thein vitro development of fertilized cattle oocytes, with special reference to the time of its addition to the synthetic oviduct fluid containing BSA. In

the first experimental series, it was shown that the highest number of oocytes (76.1%) resumed meiosis in DMEM medium. Meiosis was not resumed in Ham's F-12. Intermediate results were obtained for TCM-199 (55.1%), which is commonly used for the maturation of cattle oocytesin vitro and for Ham's F-10 (51.7%). The oocytes reached metaphase II in DMEM at a higher rate (45.3%) than in TCM-199 or Ham's F-10 (29.4 and 8.6%, respectively).

Izquierdo *et al.(*1999) used the TCM199/HEPES (M-2520, Sigma) containing 2.2 mg/mL $NaHCO_3$ and 50 ug/mL gentamicin supplemented with 10 ug/mL FSH (OVAGEN, Inmuno Chemicals Products LTD, Auckland, New Zeland), as a oocyte maturation medium. 10 J&~L LH (supplied by J.F. Beckers, IRSIA Research Unit, University of Liege, Belgium), 1).@nL 178 estradiol (E-2257, Sigma) and 20% heat-inactivated estrous goat serum (EGS, 56°C for 30 min). The CGCs were cultured in groups of 9 to 11 in 50 p,L culture drops for 27 h at 38.5"C in 5% C@ in air with maximum humidity.

Eckert (1995) used TCM 199 basic medium and was supplemented with hormones during maturation in vitro and either estrous cow serum (ECS), bovine serum albumin (BSA) at various concentrations or polyvinyl-alcohol (PVA). Fertilization in vitro was carried out using frozen-thawed semen or one bull in Fert-talp containing heparin, hypotaurin and epinephrine and either 6 mg/ml BSA or 1 mg/ml PVA. In vitro culture up to the blastocyst stage was performed in TCM 199 supplemented with ECS, BSA or PVA. The first experiment investigated the influence of different medium-supplements (ECS, BSA or PVA) on nuclear maturation and revealed no significant differences among treatment groups or between categories of COC (63.9% to 74.9% and 48.9% to 77.0%, respectively).

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.

For in vitro maturation (IVM) and culture (IVC), and for the swim-up procedure of sperm, modified Menezo B2 medium (MMM) was used by Aurich *et al.* (1994). For fertilization, Tyrode's lactate (TL) medium was used as described by Bavister (1995). An MMM stock solution was prepared every 2 weeks according to Menezo (1976), but without glucose, pyruvate, amino acids, vitamins and proteins, which were supplemented directly before use. Amino acids were not added as single compounds, but in the form of amino acid supplements for MEM (Minimum Essential Medium) or BME (Basal Medium Eagle) tissue culture medium.

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.

2.3 Effect of Hormone Supplementation in *In Vitro* Maturation

Cantanhede *et al.*(2021) worked on Bovine cumulus-oocyte complexes (COCs) where COCs underwent IVM in TCM199 medium with Earle's salts, supplemented with 2.0 mM L-glutamine, 10% fetal bovine serum, antibiotics, and 0.05 IU/mL porcine follicle stimulating hormone (FSH+) or vehicle control (CTL) medium for 22 h. the effect of FSH on the expansion of cumulus cells, estradiol production, and oocyte nuclear maturation was observed ex vivo, thus reinforcing the seminal effects of this hormone on cellular hallmarks of oocyte maturation. Cumulus cells mediate FSH signaling pathways that contribute to the acquisition of developmental competence. The gene expression profile and physiology of cumulus cells are subject to extracellular cues and interactions with the oocyte. When supplemented in IVM media, FSH promotes the increase of cumulus cell expansion, resumption of meiosis, and steroid hormone production.

Bahrami *et al.* (2019) reported that Supplementation of maturation medium with hormones, such as FSH, is a common practice, primarily due to the known role of FSH in recruiting follicles *in vivo*. However, addition of a large range of FSH concentrations

to IVM medium is reported in the literature, from 0.5–20 μg/mL, and these had varying effects on bovine COC maturation. 17β-Estradiol is also commonly added to bovine COC maturation medium. The rationale for its addition is that 17β-estradiol has a regulatory role in mammalian ovarian function and is present in follicular fluid at a concentration of1.5 μg/mL. Bovine maturation medium is often supplemented with approximately this concentration of 17β-estradiol, despite reports that addition of 1 μg/mL 17β-estradiol actually reduces nuclear maturation of bovine oocytes and oocytes of other mammalian species.

Matsuo *et al.* (2017) prepared hormone supplemented Medium 199 Earle's salts (12340-030, Gibco) with 10 % (v/v) fetal bovine serum (FBS, 26140- 087, Life Technologies) inactivated at 56 ◦C for 30 min, 0.2 mM sodium pyruvate (P5280, Sigma-Aldrich, St. Louis, MO, USA), 50 μgmL−1 gentamicin sulfate (G3632, Sigma Aldrich) and 20 μgmL−1 FSH from porcine pituitary (F2293, Sigma-Aldrich) was used as a basal medium for IVM. Thirty COCs were cultured in 300 μL of IVM medium supplemented with steroid hormone in a well of a 48-well dish (150687, Thermo Fisher Scientific, USA) for 22 h at 39 ◦C in a humidified atmosphere of 5 % CO2 in air.

Do *et al.* (2016) reported that immature oocytes are subjected to in vitro culture when they are influenced by various hormones, proteins and growth factors. The most popular in vitro maturation (IVM) medium to use to support bovine oocyte in vitro maturation is tissue culture medium 199 (TCM199) supplemented with gonadotropin hormones and fetal calf serum (FCS).

Lekola *et al.* (2016) reported that media supplemented with hormone have remarkable effect on maturation rate. When immature oocytes grouped as T1, T2, T3 were randomly allocated into TCM 199 + 10% foetal bovine serum (FBS) maturation media supplemented with three concentrations of hormones as treatments (T). The T1 group was matured in the presence of 0.5 μg follicle stimulating hormone (FSH)/ml, 5 mg luteinising hormone (LH)/ml and 2 μg estradiol (E2)/ml. The T2 group was matured in 1 μg FSH, 6 mg LH and 2.5 μg E2/ml. The T3 group was matured in 1.5 μg FSH, 7 mg LH and 4.5 μg E2/ml. The maturation rate of oocytes was determined by the protrusion of the first polar bodies 24 h after maturation.

Hussain *et al.*(2015) placed Oocytes with A, B and C categories in micro drop at the rate of 10 oocytes per 50 ml of the three different maturation media, TCM-199, Ham's F-12 and Ham's F-10 containing same additives (Sodium pyruvate: 0.0009g/ml, Gentamicin: 50µg/ml, L-glutamine: 0.0001g/ml, Porcine FSH: 0.05µg/ml, Follicular Fluid: 1ml/10ml and HIOCS 1ml/ 10ml, in all the media) which had been equilibrated at 38.5°C in a CO2 incubator.

Smetanina *et al.* (2014) studied the effects of FSH in high concentrations on in vitro maturation of oocytes cultured in protein-free MEM-α compared to the efficiency of two hormones, FSH and PMSG, in oocyte maturation in vitro, and studied the possibility of normal in vitro development of oocytes maturation without hormones to see whether they could reach the late pre implantation stage. They found that the oocytes underwent in vitro maturation in a medium with three concentrations of FSH. Increasing FSH concentration by 1-2 orders of magnitude in comparison with the basal concentration significantly inhibited the development of pre implantation embryos without deteriorating the quality of the resultant embryos evaluated by the number of cells in a blastocyst. Presumably, FSH in high concentrations was harmful primarily for oocytes with low viability, which led to a sort of selection and hence, to worse quantitative parameters of their development to the late pre implantation stages.

Farag *et al.* (2009) evaluated the effect of addition of hormone combinations (PMSG + hCG + E2) alone or with fetal bovine serum (FBS) to culture media (TCM199 or RPMI – 1640), on IVM of Egyptian sheep oocytes (COCs or CDOs). The effect of type of culture media and oocyte quality on IVM of sheep oocytes was also investigated. The findings indicated that the addition of hormones combined with FBS to the basic medium (TCM-199) significantly improved the IVM of COCs as compared to the control (medium alone) (41.25 vs. 3.50, respectively). Supplementing TCM – 199 with hormones alone (PMSG+HCG+E2) insignificantly increased the IVM of COCs compared to the control (14.75 vs. 3.50, respectively).However, supplementing TCM – 199 medium with hormones alone or hormones combined with FBS did not improve the IVM of denuded oocytes. On the other hand, the addition of hormones combined with FBS to RPMI medium significantly.

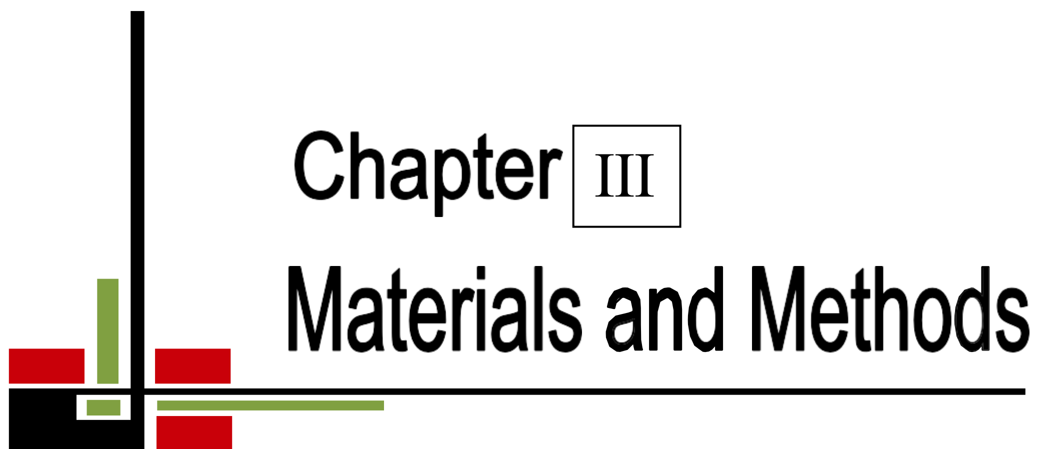
Anguita *et al.* (2007) matured oocytes in TCM 199 medium supplemented with 275 mg/ml sodium pyruvate, 146 mg/ml L- glutamine, 10% (v/v) steer serum, 10 mg/ml 0-LH, 10 mg/ml FSH, 1mg/ml 17-b estradiol, 400 mM cysteamine and 50 mg/ml gentamycin with 5% CO_2 ,5% O_2 and 90% N_2 at 38.5ºC for 27 h and the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

Gilchrist *et al.* (2004) 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 Gonadotropin during IVM have variable effects on nuclear maturation.

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 follicle Stimulating Hormone 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, and20 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% versus99+/-4%).

Izadyar *et al.* (1996) showed that at 4 and 8 h the percentage of oocytes in GV stage after Growth Hormone (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 1000 mg/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.

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).


Chapter III
Materials and Methods

CHAPTER-III

MATERIALS AND METHODS

The experiment was carried out at the Animal Nutrition, Genetics and Breeding Laboratory, Department of Animal Nutrition, Genetics and Breeding, Sher-e-Bangla Agricultural University, Dhaka.

3. Preparation of the laboratory

All the necessary equipment were properly installed and checked before starting the experiment. Finally all equipment cleaned and sterilized with 70% alcohol. There usable equipment's were properly washed with distilled water, later dried and covered with aluminium foil, sterilized and finally kept in a cleaned and sterilized chamber until use. All the essential disposable equipment as well as media, chemicals and reagents were made readily available before starting the experiment.

The list of equipment's are mentioned below-

a) Microscope
b) CO_2 incubator
c) Laminar Air Flow Cabinet
d) pH meter
e) Weighing balance
f) Disposal 10 ml syringes
g) Disposal 18 G needles
h) Sterilized rubber gloves
i) Sterilized beaker
j) Sterilized measuring 100 ml cylinder
k) Culture dishes of 35mm
l) Pasture pipette
m) Petridishes (90mm)
n) Water bath

o) Sterilized test tube 10ml

p) Glass microscope

q) Distilled water

r) Digital pipette

s) Paraffin oil

t) Tissue culture media 199 (TCM-199) for maturation

u) Measuring scale

v) Scissors

w) Watch glass

x) Forceps

3.1 Preparation for Ovary Collection

3.1.1 Collection and Processing Of Ovaries

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 liter of saline. The solution was warmed at 25 to 30ºC and kept in a thermos box to maintain this temperature during transporting the ovaries from slaughterhouse to laboratory. Dulbecco‟s phosphate buffered saline (D-PBS) solution was also prepared by adding one pack of PBS salt (Sigma Chemical Co.,USA) in one liter of distilled water. Then it was sterilized in autoclave and stored in refrigerator for further use.

3.1.2 Trimming of ovaries

The ovaries were kept in collection vial containing 0.9% Physiological saline in a thermo flask at 25 to 30ºC and transported to the laboratory within 2 to 3 hrs. of slaughter. The ovaries were then transferred to the sterilized petridishes containing same saline. The ovaries were rinsed thoroughly by physiological saline solution at 25ºC and recorded for the presence or absence of corpusluteum (CL).In the laboratory each ovary was trimmed to remove the surrounding tissue and overlying bursa.

3.2 Evaluation of Ovary

After collection and trimming, ovaries were evaluated on the basis of CL presence and absence.

A

Plate 1.Representative photograph showing Ovaries without corpus luteum and with corpus luteum (arrow indicates the CL and without CL).

3.3 Collection of oocyte and evaluation of COCs

3.3.1 **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.

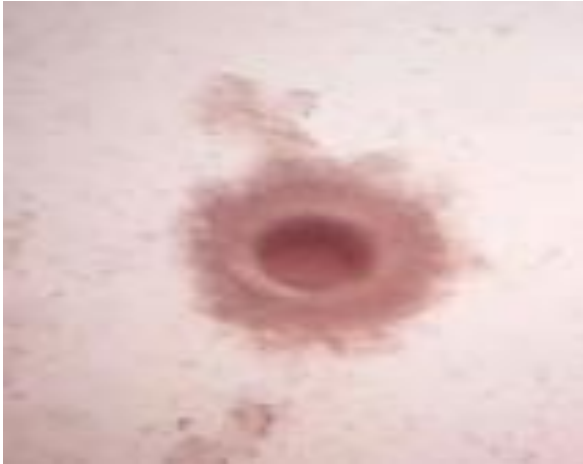
3.3.2 **Aspiration:** The 10 ml syringe was loaded with D-PBS (1.0-1.5ml), and the needle (18 G) was put in the ovarian parenchyma near the vesicular follicles and all 2-6 mm diameter follicles were aspirated. After aspirating the follicles from one ovary, it was precipitated for 10 minutes (Nolasco *et al.,* 2013) then the upper part was removed and the lower part was entered into a 90 mm petridish, avoiding damage to the cumulus cells as shown in **Plate** 2.

A

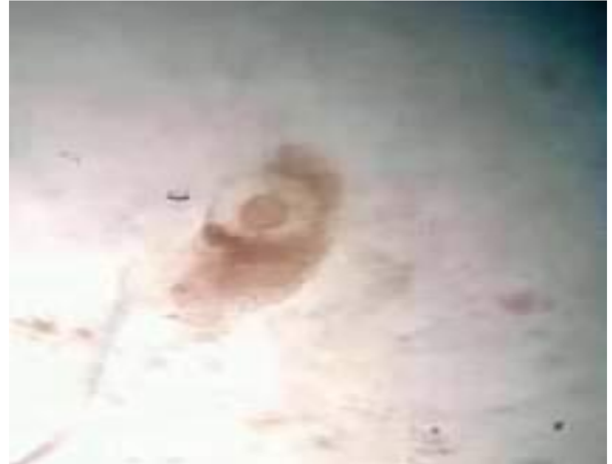
B

Plate 2. Representative photograph (A+B) showing aspiration of cow oocyte

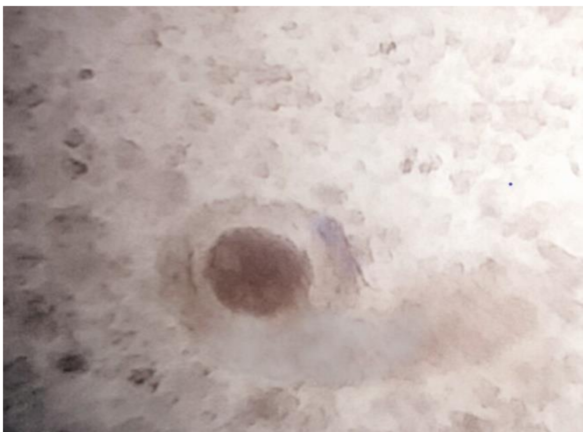
3.3.3 COCs grading: The COCs were searched and graded under microscope at 10x magnification. The COCs were classified into 4 grades on the basis of cumulus cells and nucleus as described by Khandoker *et al.* (2001). The grades were; Grade A: oocytes completely surrounded by cumulus cells; Grade B: oocytes partially surrounded by cumulus cells; Grade C: oocytes not surrounded by cumulus cells and Grade D: degeneration observed both in oocytes and cumulus cells.



Grade-A



Grade-B



Grade-C

Grade-D

Plate 3. Representative photograph showing different grades of COCs

Grade-A= COCs completely surrounded by cumulus cells,

Grade-B= COCs partially surrounded by cumulus cells,

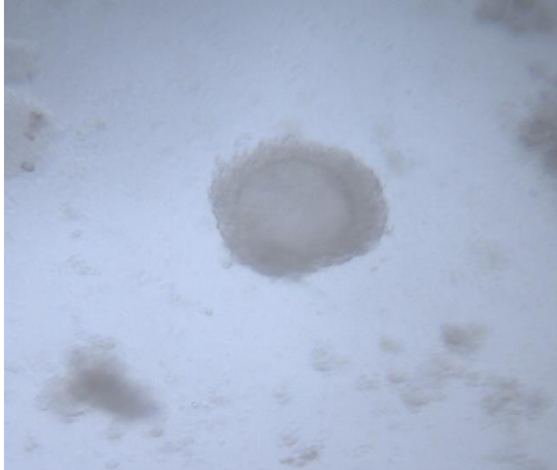
Grade-C= Oocytes not surrounded by cumulus cell and

Grade-D= Degeneration observed both in cumulus cells and oocytes.

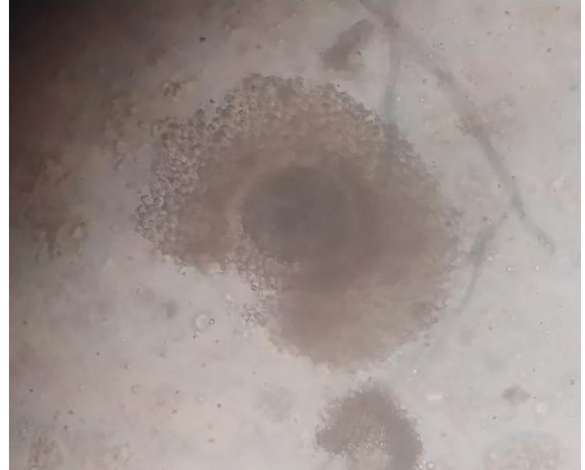
Grade A and Grade B considered as Normal COCs and taken for further study
Grade C and Grade D considered as Abnormal COCs and discarded

3.4. *In vitro* maturation (IVM) of COCs

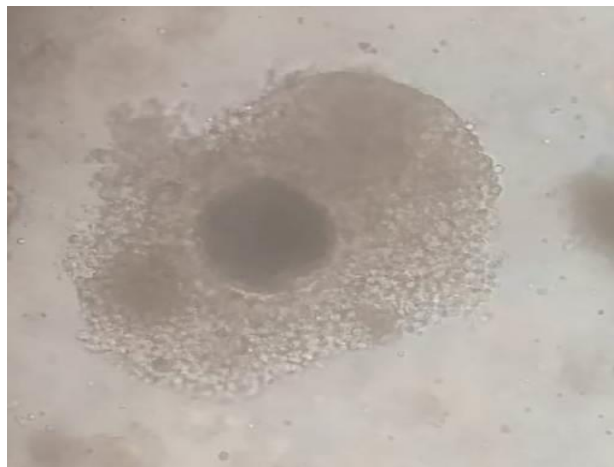
The maturation medium, TCM-199 (Sigma Chemical Co., USA) was prepared and the pH of all media was adjusted to 7.4 on the day of oocyte collection and sterilized by passage through a 20 µm filter. About 2.5-3.5 ml of the medium was poured into 35 mm culture dishes. This culture dishes were used for washing of COCs then 1-4 drops (depending on number of oocytes) of 100 µl of medium were poured in another culture dish and the COCs were transferred to the droplets and covered with paraffin oil. Finally, the dishes with droplet were kept in an incubator at 38.5^{0}C with 5% CO_2 in humidified air .After 48 hours culture of COCs in maturation medium, the level of maturation was checked.



A



B



C

Plate 4.Representative photograph showing different level of COCs expansion during *in vitro* maturation

A: expansion COCs at level 1

B: expansion COCs at level 2

C: expansion COCs at level 3

3.5 *In vitro* fertilization (IVF) cow oocyte

3.5.1 Culture Medium preparation

The fertilization medium, Brackett and Oliphant (BO) was prepared and its pH was adjusted to 7.8 on the day of use. Finally it was sterilized by passing through a 20 µm Sartorius Minisart filter.

3.5.2 Semen collection

Frozen Semen was collected from laboratory.

A

B

Plate 5. Representative photograph showing Frozen Semen collection

A: collection of frozen semen from liquid nitrogen cane

B: cutting of laboratory end of frozen semen straw

3.5.3 Semen preparation (sperm capacitation)

Each frozen semen straw was removed from liquid nitrogen cane and immersed in a 37^0C waterbath for 30- 40s.The straw was removed from waterbath and wiped it to dry, laboratory end of straws were cut. Only the sperm suspension was expelled in to the center of a flattened 100µl droplet of semen dilution. The sperm concentration was finally adjusted to 10^6 per ml by adding semen dilution (BO + 10µg/ml ovurelin). Then, depending on the number of matured COCs, 1-4 insemination droplets (100µl) 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 incubation.

3.5.4 Insemination (incubation with sperm)

After 48 hours of maturation, the half of the matured COCs was proceed to fertilization and other half was used for nuclear maturation test. Two 35 mm culture dishes were filled with COCs washing solution and the COCs were washed 3 times. About 15-20 COCs with minimum volume of medium were transferred to each of the sperm drops prepared previously and then incubated for 4-6 hours in incubator at 38.50^0C with 5% of CO_2 in humidified air.

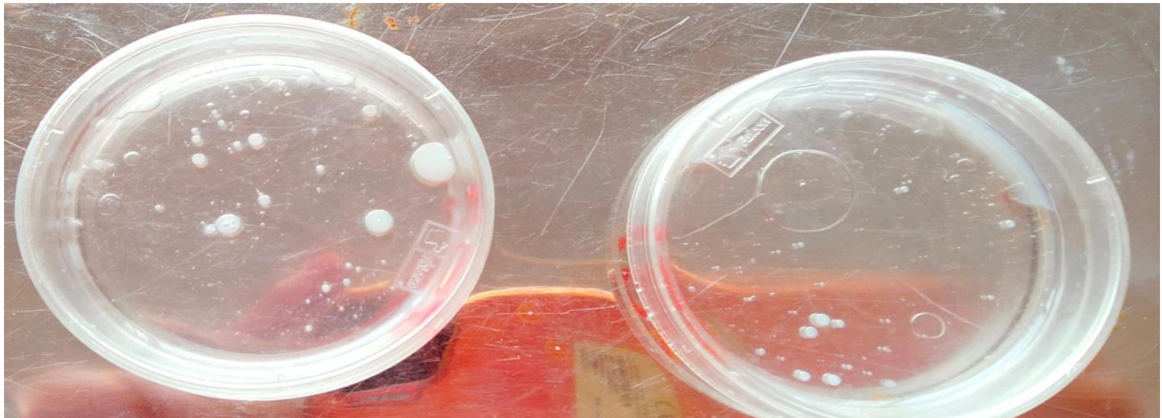


Plate **6. Representative photograph showing insemination with frozen semen**

3.6 Evaluation the fertilization rate

After 5 hours of incubation, all the COCs from each drop were denuded from cumulus cells by repeated pipetting.

a) Oocyte with two PN – normal fertilization

b) Oocyte with one PN – asynchronous PN development/parthenogenetic activation or one PN was obscured by lipid droplets

c) Oocyte with more than two PN – polyspermia

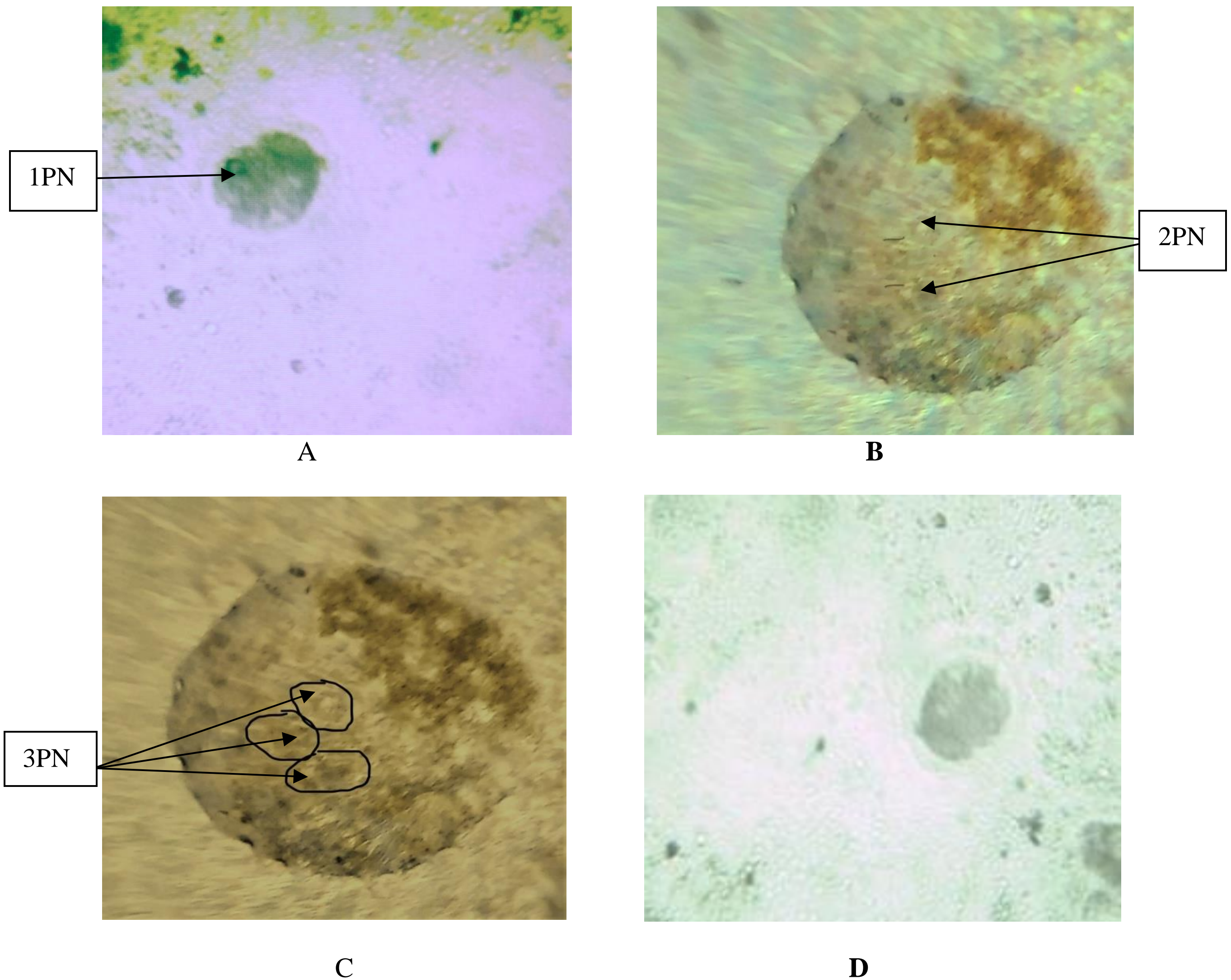


Plate 7. Representative photograph showing *in vitro* fertilization based on pronuclei (PN) formation

A: Oocyte with one PN **B:** Oocyte with two PN

C: Oocyte with three PN **D:** Oocyte with no PN

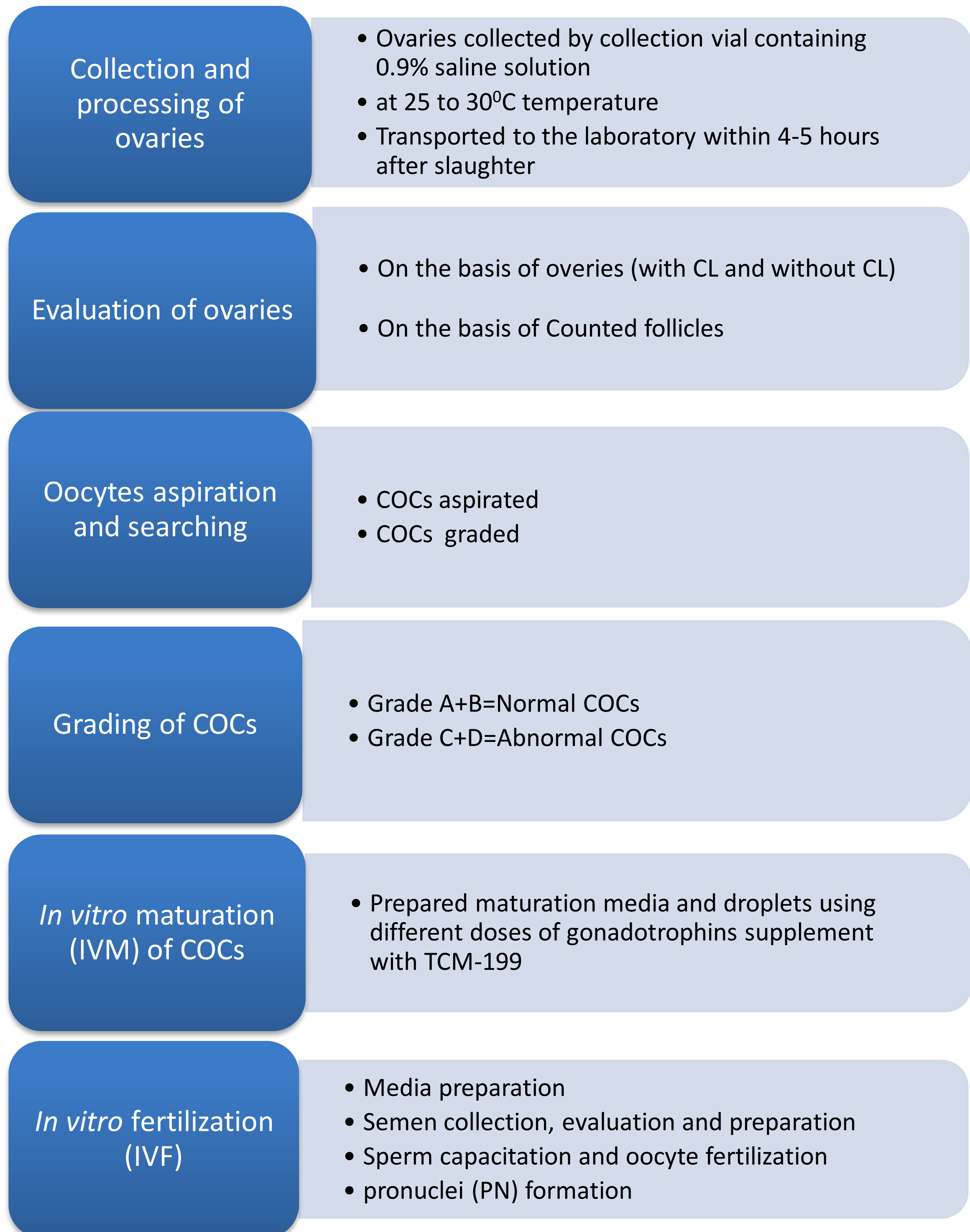


Figure 1. Flow diagram of collection, evaluation, *in vitro* maturation, and fertilization of cow oocytes

3.7 Preparation of maturation medium (TCM-199)

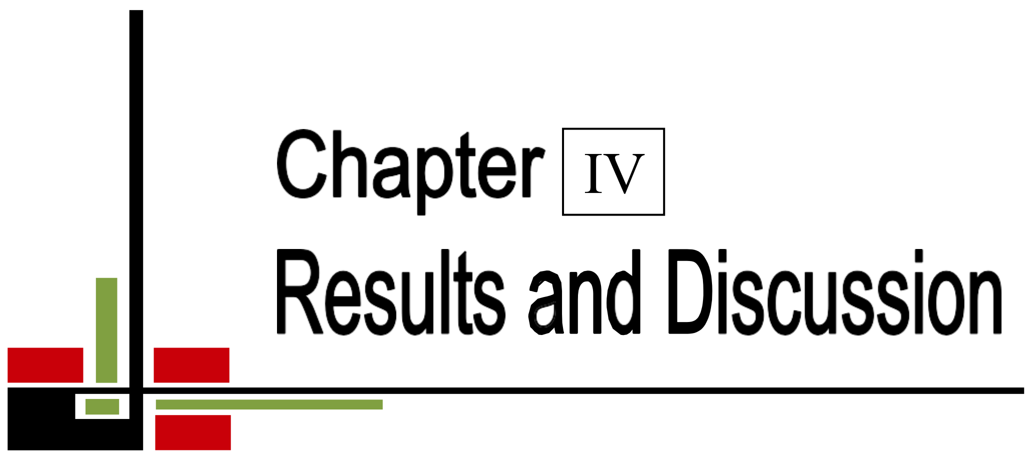
Table 1: Maturation medium (TCM-199)

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

Media TCM199 supplemented with gonadotropin hormone was prepared to evaluate the effect of media and hormone. Media was prepared using TCM199 supplemented with various doses of gonadotropin (0.5, 1, 5, 10 and 15 µg/ml) and TCM-199 without any supplementation was used as a control. 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 ml pore filter.

3.8 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).



Chapter IV

Results and Discussion

CHAPTER -IV

RESULTS AND DISCUSSION

4.1 Ovarian Categories of collected samples

A total 127 ovaries were collected form slaughter house and oocytes are retrieved through aspiration method. Among 127 ovaries a number of 74 belonged without CL and 53 with CL. The numbers of 670 follicles were recorded on the surface of the ovaries (Appendix-1).

Table 2: Ovarian categories, number of follicle and COCs collected per overy

Ovarian type	Total	Total number of visible follicle (Mean ±SE)	Number of follicle aspirated (Mean ±SE)	Collected COCs per overy (Mean± SE)		
				Normal	Abnormal	Total
Ovary with Corpus Luteum	53	$4.34^{b}\pm0.13$ (226)	$3.05^{b}\pm0.13$ (162)	$0.69^{b}\pm0.09$ (37)	$1.76^{a}\pm0.08$ (92)	$1.98^{b}\pm0.16$ (137)
Ovary without Corpus Luteum	74	$6.00^{a}\pm0.28$ (444)	$4.59^{a}\pm0.27$ (340)	$3.08^{a}\pm0.21$ (228)	$1.45^{b}\pm0.08$ (106)	$4.61^{a}\pm0.25$ (337)

Means with different superscripts within the column differ significantly ($p<0.05$).
Figure in the parenthesis indicates the total number.

Numbers of aspirated follicles were 502. Among them 162 were obtained with a mean of (3.05±0.13) per ovary from Ovary with Corpus Luteum and 340 from Ovary without Corpus Luteum with a mean of (4.59±0.27) per ovary (Table 2), (Figure.2) and (Appendix-2).Which was significantly higher ($p<0.05$) than that of CL containing ovaries. This result was comparable with the observation of Rahman *et al*. (2018) (4.0) who collected oocytes from ovary of indigenous zebu Cows by aspiration collection techniques. In the present

study the calculated mean number of oocytes obtained per ovary is sharply higher than that of previous study.

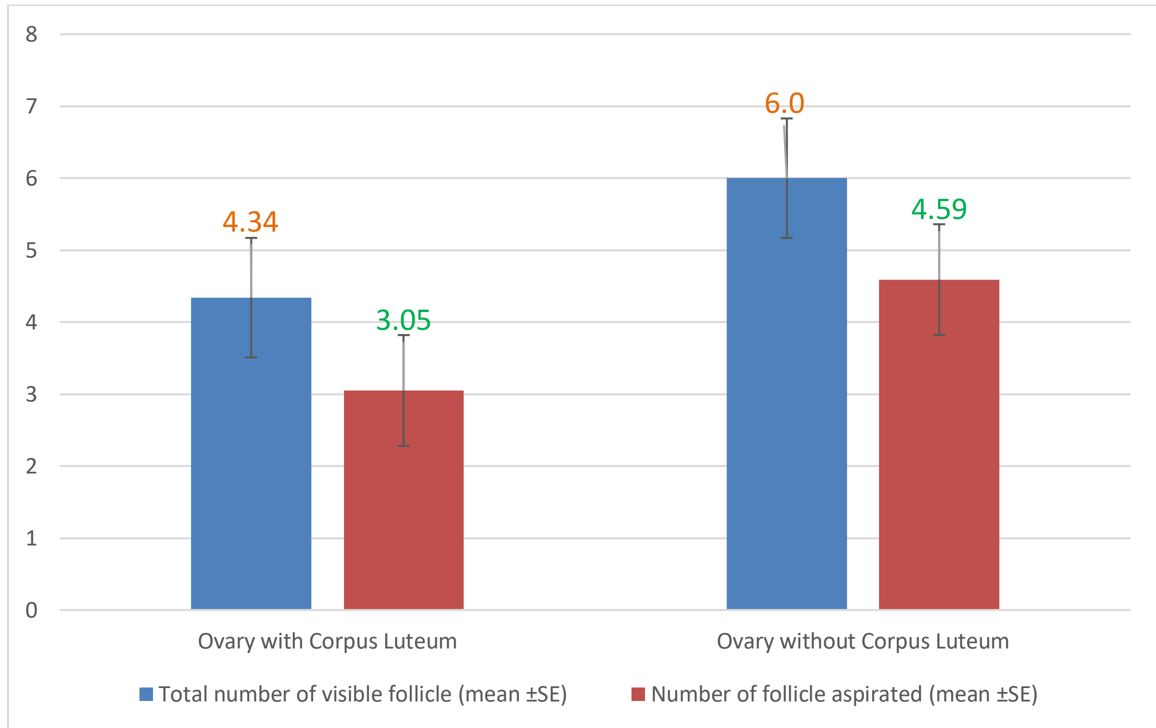


Figure 2.Total number of follicles and number of follicles aspirated per ovary

The mean number of retrieved oocytes per ovary by Morshed *et al.* (2014) was 3.0 and the mean number of oocytes collected from each ovary by Singha *et al*. (2015) was 3.35. Choudhury *et al*.(2017) found the mean number of oocytes collected from each ovary was 1.52.whereas Talukder *et al.*(2008) recorded the mean number of retrieved oocytes per ovary was 5.4.This mean value is higher than that of present study. Skill of follicle aspirators might be one of the main reasons behind the variations in oocyte retrieval rate among studies. Besides seasons of oocytes retrieval and cyclic status of cows may influence the oocyte retrieval rate from ovaries (Dode and Adona, 2001). An increase in FSH level in blood would be another influential factor for the number of oocytes retrieved (Fortune, 1994). Furthermore, nutrition and temperature may influence the Gonadotropin concentrations and affect the population of follicles and number of oocytes retrieved (Zeitoun *et al.*, 1996).The causes of higher number of follicles found in ovaries without CL than those of CL containing group were understood well as it fits the endocrinological

explanation. All female mammals are born with a huge number of follicles. As puberty approaches, follicles are tends to declines rapidly and only a small proportion of them will be used during the animal's reproductive life. Changes in the microenvironment such as the pH and hormonal concentration probably occur as the follicles evolve into the primary stage but these are probably effects rather than causes (Webb *et al*., 1999). Growth initiated of follicles has variously been attributed with hormonal triggers (gonadotropins), ochastic process (fluctuation in the internal signal follicle) and external inhibitory control growing follicles (Webb *et al*., 1999). The balance between the gonadotropins (FSH and LH) and steroid (estrogen and progesterone) might be the important criteria in this process. A highest number of follicles were found in ovaries without CL might be due to the optimum level of gonadotropins.

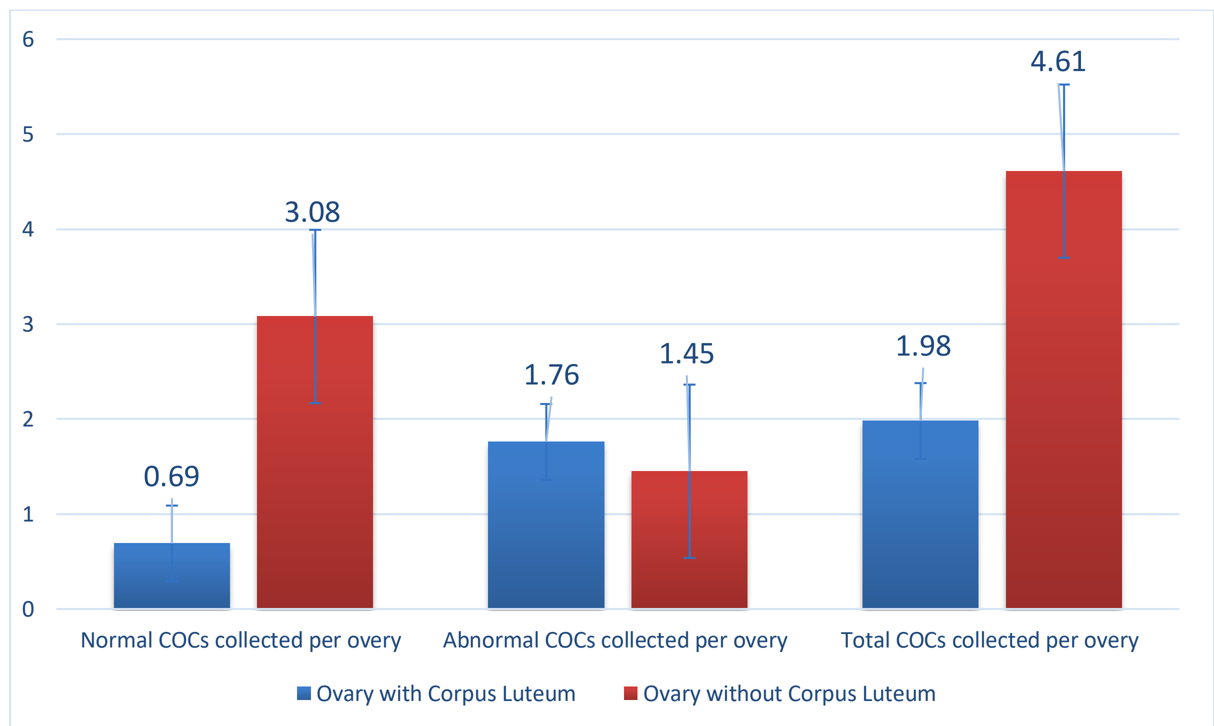


Figure 3. Normal and abnormal number of COCs per ovary

The collected number of COCs higher in Ovary without Corpus Luteum (4.61±0.25) compared to Ovary with Corpus Luteum (1.98±0.16) (Appendix-5). When the COCs were classified as normal and abnormal groups, the highest number of normal COCs were found

in Ovary without Corpus Luteum (3.08±0.21) than that of Ovary with Corpus Luteum(0.69±0.09) (Figure.3) and (Appendix -3).

4.2 Determination of an effective basic medium for oocytes maturation

The result of in vitro maturation of COCs after 48 h cultured in different levels of gonadotropin hormone is presented in table 3.The maturation rates of oocytes were 42.25%, 52.50%, 60.13%, 62.25%, 65.71%, 61.15% in TCM 199 (control) and TCM 199 supplemented with different doses of Gonadotropins (0.5, 1,5,10 and 15 µg/ml). The deference in maturation rate was significant between TCM 199 and Gonadotropin 10 µg/ml + TCM 199 media ($P<0.05$).

Table 3. Effects of different maturation media on IVM rate of cow oocytes

Maturation medium	Number of oocytes cultured	Number of oocytes matured	Maturation rate (%) (mean±SE)
CM-199 (Control)	46	20	42.25^{f}±3.20
TCM 199+Gonadotropin (0.5 µg/ml)	40	21	52.50^{e}±2.11
TCM 199+Gonadotropin (1 µg/ml)	30	18	60.13^{d}±2.73
TCM 199+Gonadotropin (5µg/ml)	25	14	62.25^{b}±2.60
TCM 199+Gonadotropin (10µg/ml)	35	23	65.71^{a}±2.74
TCM 199+Gonadotropin (15µg/ml)	40	25	61.15^{c}±1.15

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

In this experiment 10 µg/ml Gonadotropin supplemented TCM 199 was found to be the best medium for higher rate of maturation (65.71^{a}±2.74%) (Figure.4). A gradual increase in maturation rate was found when dose of Gonadotropin increased gradually up to

10μg/ml but if the dose increased to 15 μg/ml the maturations rate declines to $61.15^c \pm 1.15\%$. In IVM, gonadotropins are the main stimulator of the oocyte development and FSH being deemed vital for the oocytes becomes qualified to be *in vitro* fertilized (Lu *et al.*, 2014; Khan *et al.*, 2015). The present maturation rate is lower than previous study reported by Singha *et al.* (2015) (74.6%) in indigenous zebu cows. but comparatively lower maturation rate was reported by Morshed *et al.* (2014) (53.8%) in indigenous zebu cows. TCM 199 is one of the effective media widely used for oocyte maturation. But TCM199 supplemented with hormone (Gonadotropin) is found to be more effective and have noticeable influence on oocyte maturation. In the present study it is found that the maturation rate in media TCM 199 supplemented with Gonadotropin (10 μg/ml) was quite higher than that of control one. Even TCM 199 supplemented with 0.5, 1, 5 μg/ml the maturation rates are found to be better. But the effect of TCM 199 supplemented with and Gonadotropin (10μg/ml) resulted best maturation rate in the present study. This variation in maturation rate among studies might be due to variation in basic media and percentage of hormone supplementation in it used for oocyte maturation. 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 (Goswami 2002). The production and secretion of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) is controlled by the Gonadotropins hormone. Both LH and FSH have a direct effect on the ovary: FSH stimulates follicle development, while LH induces ovulation and luteinization. Gonadotropins are found to trigger cumulus cells to synthesize some molecules capable to initiate germinal vesicle breakdown (GVBD) as meiosis activating sterols (Tsafriri *et al.*, 2005).Addition of reproductive hormones in maturation media is necessary for the improvement of IVM rate of mammalian oocytes. Addition of FSH (Chauhan *et al.*, 1998), PMSG (Roy *et al.*, 1968), LH and oestradiol (Nandi *et al.*, 2002) to maturation media has been made to improve the developmental competence of *in vitro* matured oocytes Oestradiol has been found to improve the completion of maturational changes and also to support the synthesis of presumed male pronuclear growth factor (Fukui and Ono, 1989).

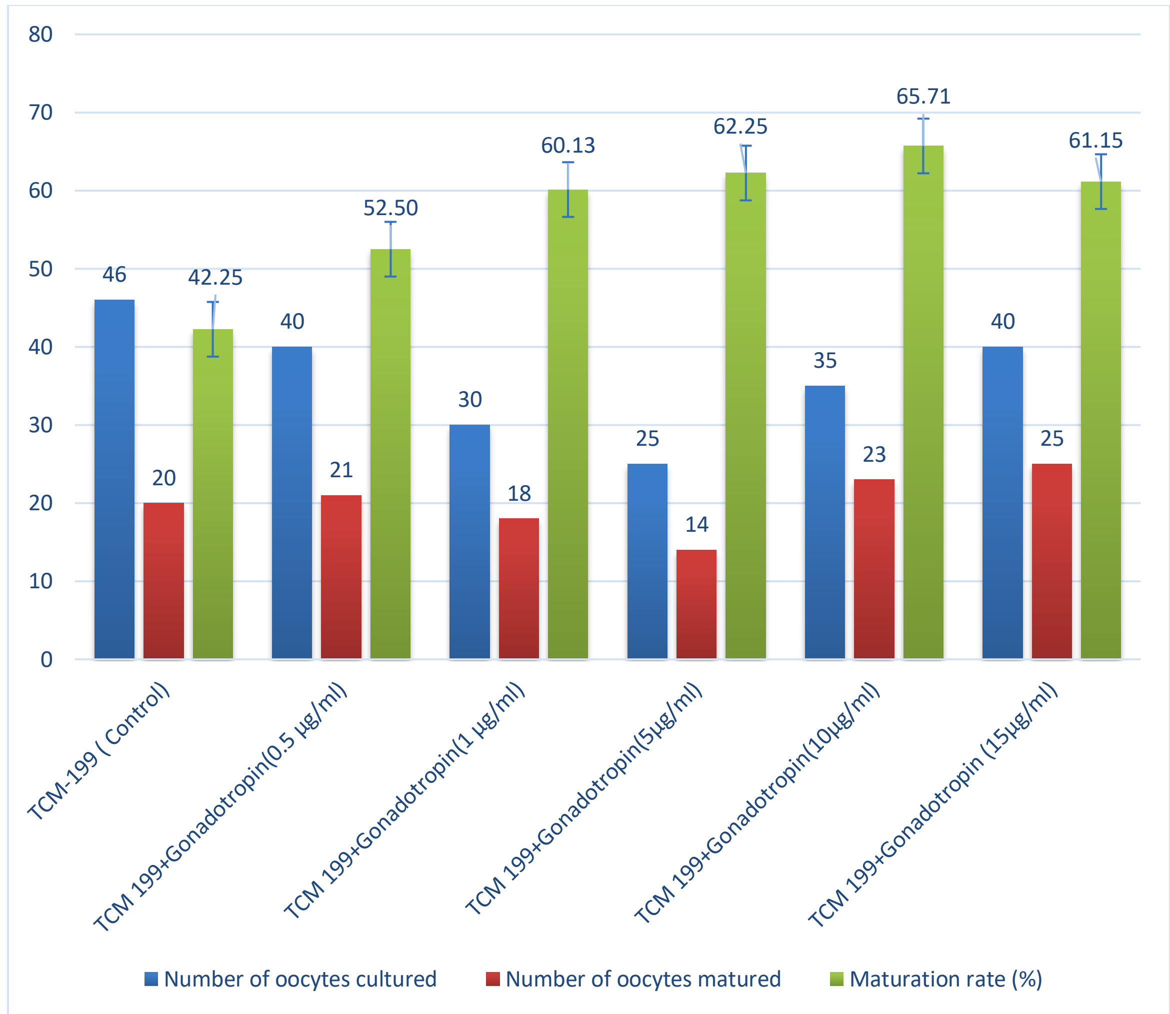


Figure 4. Effects of media on IVM rate of cow oocytes

4.3 In vitro fertilization of cow COCs by using frozen semen

After maturation, oocytes in TCM-199 supplemented different doses of Gonadotropin (10 µg/ml) were fertilized with frozen semen and the rates of pronuclei formation is summarized in Table 4. It was observed that significantly ($p<0.05$) higher percentage of normal fertilization (formation of 2 pronuclei) was observed in Gonadotropin (10 µg/ml) supplemented media (40.14%) than the control (22%).

Table 4: *In vitro* fertilization of cow COCs based on pronuclei (PN) formation with different doses of gonadotropin hormone.

Supplements	Total no. of oocytes	Fertilization rate (%) based on pronuclei (mean± SE)			
		2PN	1PN	>2PN	NPN
TCM-199 (Control)	25	22.00^{e}±7.6 (6)	20.67^{a}±5.59 (5)	25.67^{a}±2.14 (8)	30.67^{c}±5.21 (6)
TCM 199+Gonadotropin (0.5 µg/ml)	16	30.00^{d}±4.7 (6)	20.61^{a}±4.33 (4)	10.11^{b}±5.03 (2)	35.11^{b}±5.02 (4)
TCM 199+Gonadotropin (1 µg/ml)	38	33.37^{c}±5.35 (11)	20.35^{a}±7.01 (8)	14.10^{c}±4.45 (8)	31.67^{c}±4.20 (11)
TCM 199+Gonadotropin (5µg/ml)	41	37.04^{b}±5.19 (20)	14.70^{b}±5.18 (5)	9.17^{d}±4.13 (3)	38.42^{a}±4.13 (13)
TCM 199+Gonadotropin (10µg/ml)	40	40.14^{a}±5.12 (16)	11.14^{b}±2.21 (10)	9.10^{d}±4.21 (8)	30.23^{c}±4.22 (6)
TCM 199+Gonadotropin (15µg/ml)	32	34.15^{c}±2.22 (11)	10.15^{c}±2.22 (9)	8.23^{e}±1.12 (6)	34.12^{b}±3.32 (6)

Means with different superscripts within the column differ significantly ($p<0.05$).
Figure in the parenthesis indicates the total number.

In vitro fertilization of cow COCs based on pronuclei (PN) formation are stated in table 4 and figure 5. *In vitro* fertilization is a multi-factorial event that makes maintaining a consistent condition for all replicates difficult. This emphasizes the need for a stable condition to maintain for the IVF system .moreover nature of semen (whether it is fresh or frozen) used play a vital role in fertilization. The fertilization rate is directly dependent on the maturation of oocytes. After comparing the fertilization rate among the groups of oocytes matured in TCM-199 supplemented with different doses of Gonadotropin (0.5, 1,5,10 and 15 µg/ml) and non-supplemented (control), it can be suggested that supplementation in maturation media with Gonadotropin (10 µg/ml) yielded better results than control on further fertilization. Thus the fertilization rates in this study showed a

significant differences between supplemented (Gonadotropin 10µg/ml + TCM-199) (40.14[a]±5.12) and control (22.00d±7.6) (Figure 5). The observed fertilization rates (22.28-40.14%) support the observation of Khandoker *et al.* (2012) (23.28-30.52%) who worked on *in vitro* fertilization of buffalo COCs using cattle spermatozoa. but the data are poorer than the observation of Covelo *et al.*(2022) when worked on Follicular Fluid on *in vitro* Maturation and Fertilization of Bovine Oocytes (53.40-79.45%.).

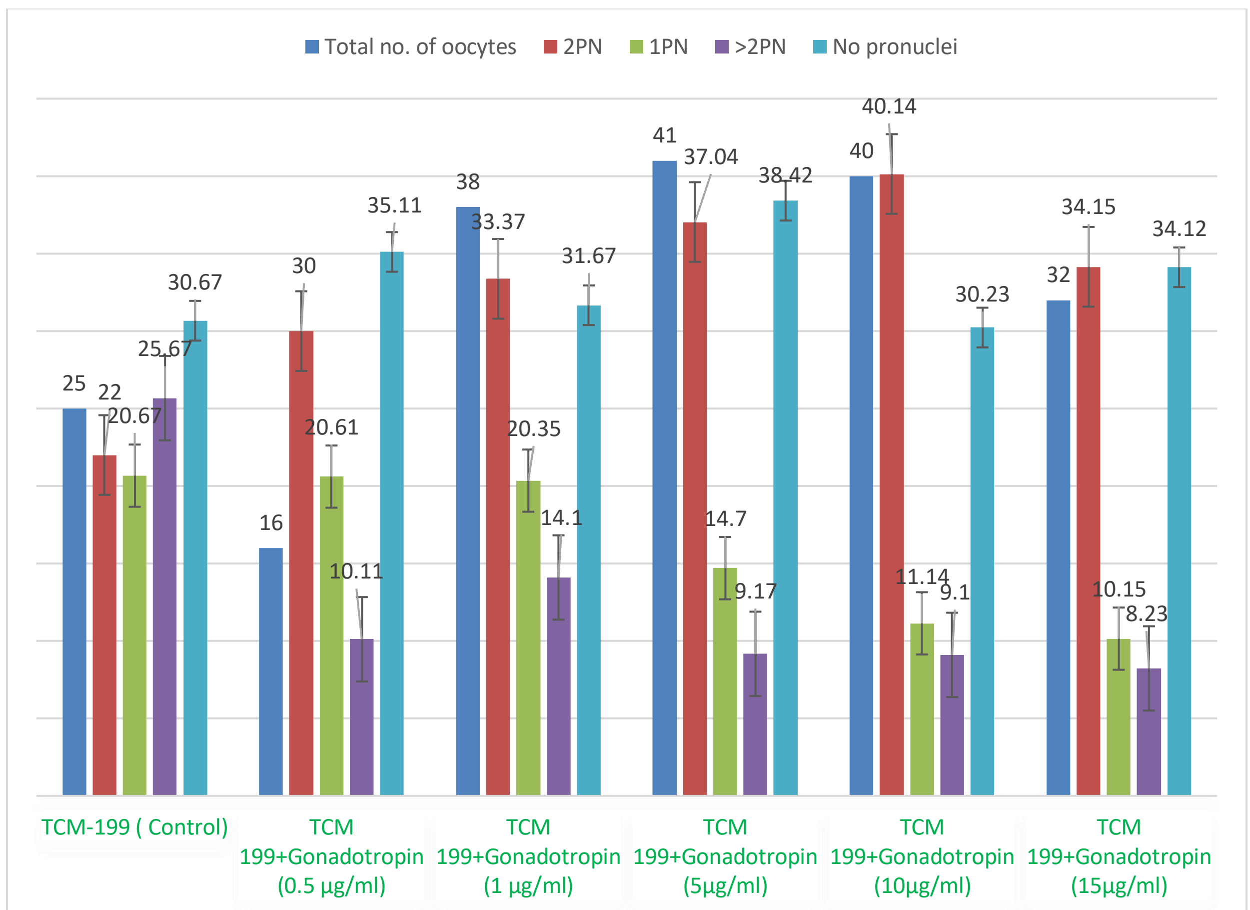


Figure-5. In vitro fertilization of cow COCs based on pronuclei (PN) formation

Chapter V

Summary and conclusion

CHAPTER-V

SUMMARY AND CONCLUSION

The research work was carried out with a view to identify an effective basic medium and to find out a suitable dose of gonadotropin hormone supplementation in basic medium for optimum in vitro maturation and fertilization rate of cow oocytes in Bangladesh. In this research, 502 follicles were aspirated out of 670 follicles on the surface of both ovaries from corpus luteum (CL) group (Luteal phase) and without CL (Follicular phase). Significantly higher ($p<0.05$) number of follicles were aspirated per ovary in ovaries without CL (4.59±0.27) than in CL containing ovaries (3.05±0.13). Higher numbers of COCs were found in ovaries without CL (4.61^{a}±0.25) than ovaries with CL (1.98±0.16), the significantly higher ($p<0.05$) number of normal COCs was found in ovaries without CL than those ovaries with CL with the mean of (3.08±0.21) and (0.69±0.09) follicles per ovary respectively. The result indicates that, ovary without CL is a good source of normal grade oocytes for *in vitro* maturation of bovine oocytes. The maturation rate of oocytes in media TCM 199 supplemented with a dose of Gonadotropin10 µg/ml hormone showed the good result (65.71%) then the control (TCM 199) (42.25±3.20). Even the result is comparatively higher than the results of other doses (0.5, 1, 5, and 15 µg/ml). A gradual increase in maturation rate was found when dose of Gonadotropin increased gradually up to 10µg/ml but when the dose exceeds 10 µg/ml the maturation rate falls to 61.15%.The present study suggest that TCM 199 supplemented with a dose of Gonadotropin10 µg/ml hormone would be the most effective media for better maturation rate. The result of this experiment indicates that Gonadotropin (10 µg/ml) could be used as a supplement in TCM-199 maturation media. Both the culture medium and hormone plays a crucial role in maturation and fertilization of cow oocytes. The better the maturation rate the better the fertilization and subsequent development of embryo.

Recommendations

From the present observation this can be concluded that selection of a suitable oocytes culture medium is crucial for optimal results in cattle *in vitro* maturation / *in vitro* fertilization. For IVM, ovaries without CL could be used in TCM199 basic medium supplemented with Gonadotropin (10 µg/ml) may be used as a basic medium for optimum IVM rate of cow's oocytes in Bangladesh.

References

REFERENCES

Abdulkarim, A.R., Balboula, A., Badr, M., Bedir, W., Elmetwaly, M.and M., Zaabel,S. (2021). Comparing in vitro maturation rates in buffalo and cattle oocytes and evaluating the effect of cAMP modulators on maturation and subsequent developmental competence. *Mansoura Vet.Med J.* **22** (3):136-140.

Andrews, J.C. and Bavister, B.D, (1988). A rapid serum motility bioassay procedure for quality-control testing of water and culture media. ***J.*** *Vitr. Fertil Emb. Trans.* **5**: 67-75.

Anguita, B., Jiménez, M. A., Izquierdo, D., Mogas, T.and Paramio, M. (2007). Effect of oocyte diameter on meiotic competence, embryo development, p34 (cdc2) expression and MPF activity in prepubertal goat oocytes.*Theriogenology*.**67***:* 526-536.

Amer, H.A., Hegab, A.O. and Zaabal, S.M. (2008). Effects of ovarian morphology on oocyte quantity and quality, granulosa cells, *in vitro* maturation, and steroid hormone production in buffaloes. *J. Anim. Reprod*. **5**:55-62.

Arunakumari, G., Vagdevi, R., Rao, B.S., Naik, B.R., Naidu, K.S., Kumar, R.V.S. and Rao, V.H. (2007).Effect of hormones and growth factors on *in vitro* development of sheep preantral follicles. *Small Rumin. Res*. **70**:93-100.

Asad, L.Y., Rahman, A.N.M.I., Hossain, M.M. and Akter, M. (2016). Effect of bovine serum albumin and follicular fluid on *in vitro* maturation, fertilization and development of goat embryos. Ph.D. Thesis, Department of Animal Breeding and Genetics, Bangladesh Agricultural University.

Avery, B., Bavister, B.D. and Greve, T. (1998). Development of bovine oocytes, *in vitro matured* in alchemically defined protein-free medium, supplemented with different amino acid formulations. *Theriogenology*.**49**: 306-31.

Bahrami, M., Pauline, A. and Cottee (2022). Culture conditions for in vitro maturation of oocytes -A review, *Reprod. & Breed.* **2**(2):31-36,

Bahrami, M., Morris, M.B. and Day, M.L. (2019). Amino acid supplementation of a simple inorganic salt solution supports efficient *in vitro* maturation (IVM) of bovine oocytes. *Sci. Rep.* **9:** 117-139.

Bormann, C., Ongeri, E. and Krisher, R., (2003). The effect of vitamins during maturation of caprine oocytes on subsequent developmental potential in vitro. *Theriogenology.* **59***:*1373-1380.

Brinsko, S.P., Ball, B.A. and Ellington, J.E. (1995). In vitro maturation of equine oocytes obtained from different age groups of sexually mature mares, *Theriogenology*. **44** (4):461-469.

Cantanhede, L.F., Santos-Silva, C.T., Moura, M.T., Ferreira-Silva, J.C., Oliveira, J.M.B., Gonçalves, D.N.A., Teixeira, Á.A.C., Wanderley-Teixeira, V. and Oliveira, M.A.L. (2021). Follicle-stimulating hormone mediates the consumption of serum-derived glycogen by bovine cumulus-oocyte complexes during in vitro maturation. *Veterinary World*.**14** (9): 2512-2517.

Chauhan, M.S., Katiyar, P.K., Singla, S.K., Manik, R.S. and Madan, M.L. (1997). Production of buffalo calves through *in vitro* fertilization. *Indian J. Anim. Sci.* **67**:306-308.

Choi, W., Banerjee, J., Falcone, T., Bena, J., Agarwal, A. and Sharma, R., (2007) Oxidative stress and tumor necrosis factor-alpha-induced alterations in metaphase II mouse oocyte spindle structure. *J. Fertil. & Steril.* **88**: 1220-1231.

Choudhary, S., Kumaresan, A., Kumar, M., Chhillar,S., Malik, H., Kumar, S., Kaushik, S.K., Datta, T.K. and Mohanty, A.K. (2017). Effect of recombinant and native buffalo OVGP1 on sperm functions and in vitro embryo development: a comparative study. *J. Ani.. Sci. & Biotech.* **8** (1): 69–100.

Chr. A. and Hahn, J. (1994). In vitro maturation, fertilisation and culture of bovine oocytes in a modified Menezo B_2 medium. *Ani.Rep.Sci.* **35**(3):0–162.

Covelo, I., Puente, M.A. and Tartaglione, C.M. (2022). Influence of Follicular Fluid on in Vitro Maturation and Fertilization of Bovine Oocytes. *Open J. Anim. Sci.* **12:** 118-128.

Crozet, N., Ahmed-Ali, M. and Dubos, M. P. (1995). Developmental competence of goat oocytes from follicles of different size categories following maturation, fertilization and culture in vitro. *Reproduction,* **103**(2):293–298.

Das, Z.C., Siddiqui, M.A.R., Bhattacharjee,R., Bhuiyan, M.M.U. and Shamsuddin, M. (2006). In vitro fertilization using zebu and crossbred bull semen. Bangladesh Society for Veterinary Education and Research (BSVER) Publication 31: 31. (Abstract)

Dell'Aquila, M..E., Fusco, S., Lacalandra, G.M. and Maritato, F. (1995) *In vitro* maturation and fertilization of equine oocytes recovered during the breeding season, *Theriogenology.* 45(3): 547-560,

DLS. (2021). Report on Achievements in Livestock Sector during 2020-2021, Department of Livestock Sector.

Do, V.H., Walton, S. and Taylor-Robinson, A.W. (2016). Improvements to In Vitro Culture Media for Use in Bovine IVF. *J Vet Sci Anim Husb.* **4**(2): 205.

Dode, M..A. and Adona, P.R. (2001). Developmental capacity of Bos indicus oocytes after inhibition of meiotic resuption by 6-dimethylaminopurine. *Anim. Reprod. Sci.* **65** (3-4): 171-180.

Eckert, J. and Niemann, H. (1995). In vitro maturation, fertilization and culture to blastocysts of bovine oocytes in protein-free media. *Theriogenology.* **43** (7): 1211-1225.

Farag, I.M., Girgis, S.M., Khalil , W.K., Hassan, B. N.H.A., Sakr, A.A.M., Abd Allah , S.M. and Ali, N. I. (2009).Effect of hormones, culture media and oocyte quality on in vitro maturation of Egyptian Sheep oocytes. *J Appl. Biosc.* **24**: 1520 -1534.

Ferdous J. (2006). Collection, grading and evaluation of goat cumulus-oocyte-complex (COCs) in view of *in vitro* maturation, fertilization and culture. M.S. Thesis, Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh.

Fukui, Y. and Ono, H. (1989). Effects of sera, hormones and granulosa cells added to culture medium for *in vitro* maturation, fertilization, cleavage and development of bovine oocytes. *J. Reprod. Fertil*. **86**: 501-506.

Garcia, M., Beatriz, Fernández, G., Lauro, Dickson and Hinrichs, K. (2017). Effect of calcium, bicarbonate, bovine serum albumin and medium pH on spontaneous acrosome reaction in equine sperm. *Reprod.* 149 (1): 87-99.

Gilchrist, R.B., Ritter, L.J., Cranfield, M., Jeffery, L.A., Amato, F., Scott, S.J., Myllymaa,S., Kaivo-Oja, K., Lankinen, D.G., Mottershead, Groome, N.P. and Ritvos, O. (2004). Immunoneutralization of growth differentiation factor 9 reveals it partially accounts for mouse oocyte mitogenic activity. *Biol. Reprod*. 71:732-739.

Gomez, E., Rodrıguez, A. Munoz, M., Caamano, J.N., Hidalgo, C.O., Moran, E., Facal, C. and Diez, C. (2008). Serum free embryo culture medium improves in vitro survival of bovine blastocysts to vitrification. *Theriogenology.* **69**: 1013–1021.

Goswami, P.C. (2002). Collection and grading of bovine cumulus oocyte complexes (COCs) from slaughterhouse ovaries in- view of *in vitro* maturation, fertilization and culture. MS Thesis, Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh.

Hakan, S. and Burcu, U. (2010). Replacement of Fetal Calf Serum with Two Different Synthetic Sera in in Vitro Maturation Medium: Effects on Maturation, Fertilization and Subsequent Development of Cattle Oocytes in Vitro. *Uludag Univ. J. Fac. Vet. Med.* **29**(2): 51-56.

Hoque, S.A.M., Kabiraj, S.K., Khandokar, M.A.M.Y., Mondal, A. and Tareq, K.M.A. (2011). Effect of Collection techniques on cumulus oocyte complexes (COCs) recovery, *in vitro* maturation and fertilization of goat oocytes. *African J. Of Biotech.* **10**(45):9177-9181.

Hoque, S.A.M., Khandoker, M.A.M.Y., Kabiraj, S.K., Asad, L.Y., Fakruzzaman.M. and Tareq, K.M.A. (2012). Effect of goat follicular fluid on *in vitro* production of embryos in Black Bengal goats. *J. Appl. Anim. Sci.* **2**:287-294.

Izadyar, F., Colenbrander, B. and Bevers, M. M. (1996). *In-vitro* maturation of bovine oocytes in the presence of growth hormone accelerates nuclear maturation and promotes subsequent embryonic development. *Mol. Reprod. Develop.***45**: 372-377.

Izquierdo, D., Villamediana, P., Palomo, M., Mogas, T. and Paramio, M. (1998). Effect of sperm capacitation and fertilization media on IVF and early embryo development of prepubertal goat oocytes. *Theriogenology.* **49** :1501-1513.

Islam, M.R., Khandoker, M.A.M.Y., Afroz, S., Rahman, M.G.M. and Khan, R.I. (2007). Qualitative and quantitative analysis of goat ovaries, follicles and oocytes in view of *in vitro* production of embryos. *J.Zhejiang Univ*. **8**: 465-469.

Khan, D.R., Guillemette, C., Sirard, M.A. and Richard, F.J. (2015). Transcriptomic analysis of cyclic AMP response in bovine cumulus cells. *Physiol Genomics.* **47**(9):432-42.

Khandoker, M.A.M.Y., Imai, K., Takahashi, T. And Hashizume, K. (2001). Role of gelatinase on follicular atresia in the bovine ovary. *Biol. Repord*. **65**:720-732.

Khandoker, M.A.M.Y., Jahan,N. Asad, L.Y., Hoque, S.A.M. and Ahmed, S. (2011). Qualitative and quantitative analysis of buffalo ovaries, follicles and oocytes in view of *in vitro* production of embryos. *Bang. J. Anim.Sci*.**409**:23-27.

Kharche, S. D., Goel, A. K., Jindal, S. K. and Sinha, N. K. (2006). A note on *in-vitro* maturation of caprine oocytes using different concentrations of estrous goat serum. *Small Rumin.Res* **64**: 186-89. Lee, J.Y., Jung, Y.G. and Seo, B.B. (2016). Effects of culture media conditions on production of eggs fertilized in vitro of embryos derived from ovary of high grade Hanwoo.*J. Anim. Sci .& Tech.* 58:11.

Lekola, K.P.M., Ngambi J.W., Nkadimeng, M., Mphaphathi, M.L. and Nedambale, T.L. (2016). Effects of various concentrations of gonadotropins and 17β estradiol on the in vitro maturation of cattle oocytes selected using brilliant cresyl blue staining. *S. Afr. J. Anim. Sci*. vol. 46.

Lonergan, P., Monaghan, P., Rizos, D., Boland, M. and Gordon, I. (1994). Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in-vitro. *Molec. Reprod. & Dev.* **37:** 48-53.

Lu, C.L., Wang, T.R., Yan, L.Y., Xia, X., Zhu, Xu., Li, R., Zhao, H.C., Yan, J., Yin, T.L., Jin, H.Y., Zhang, Y., Yhang, W.X., Feng, H.L. and Qiao, J. (2014). Gonadotropin-mediated dynamic alterations during bovine oocyte maturation in vitro. *Biol. Reprod.* **91**:1-9.

Matsuo, M., Sumitomo, K., Ogino, Chihiro, Gunji, Yosuke, Nishimura, Ryo, and Hishinuma, Mitsugu. (2017). Three-step in vitro maturation culture of bovine oocytes imitating temporal changes of estradiol-17 β and progesterone concentrations in pre-ovulatory follicular fluid. *Arch. Anim. Breed.* **60:** 385-390.

Mondal, A., Khandoker, M.A.M.Y., Rahman, A.H.M.S., Apu, A.S. and Pervage, S. (2008). *In vitro* production of goat embryos. *Bang. J. Anim. Sci*. **37**:1-9.

Morshed, N.S.M., Bhuiyan, M.M.U., Rahman, M.M., Singh, J.K. and Juyena, N.S (2014). Culture condition for *in vitro* maturation of abattoir derived oocytes of native zebu cows in Bangladesh. *J. Emb. Trans.* **29**: 201-206.

Nandi, S., Raghu, H.M., Ravindranatha, B.M. and Chauhan, M.S. (2002). Production of buffalo *(Bubalus bubalis)* embryos *in vitro*. Premises and promises. *Reprod.Dom. Anim.* **37**: 65-74.

Palma, G.A., Müller, M. and Brem, G. (1997). Effect of insulin-like growth factor I (IGF-I) at high concentrations on blastocyst development of bovine embryos produced in vitro. *J. Reprod. Fertil.* **110***:* 347-353.

Pawshe, C.H., Palanisamy, A., Taneja, M., Jain, S.K. and Totey, S.M. (1996). Comparison of various maturation treatments on *in vitro* maturation of goat oocytes and their early embryonic development and cell numbers. *Theriogenology.* **46**: 971-982.

Rahman, M.M., Morshed, S.M.N., Juyeana, N.S. and Bhuiyan M.M.U. (2018). Determination of an effective media and its hormone and protein Supplementation for in vitro maturation of oocytes of indigenous zebu cows *Bangl. J. Vet. Med.*16 (1): 45-51.

Rath, D., Long, C. R., Dobrinsky, J. R.,Welch, G. R., Schreier, L. L. and Johnson, L. A. (1995). In vitro production of sexed embryos for gender pre-selection: high-speed sorting of X-chromosome-bearing sperm to produce pigs after embryo transfer. *J. Anim. Sci.* **77**:3346-3352.

Ravindra, J.P., Manjunatha, B.M., Gupta, P.S., Devaraj, M. and Nandi, S. (2008). Oocyte recovery by ovum pick up and embryo production in river buffaloes *(Bubalus bubalis). Reprod. Dom. Anim.* **43:**477-480.

Ravindranatha, B.M., Nandi, S., Gupta, P.S.P. and Sharma, P.V. (2001). Comparison of three different media on maturation of buffalo oocytes *in vitro. Indian J. Anim. Sci.* **71**: 841-843.

Rizos, Dimitrios,Ward, Fabian, Duffy, Boland, Maurice, Lonergan, and Pat. (2004). Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Molec. Reprod.dev.***61:** 234-48.

Rodriguez, G. E., Lopez, B. M., Mertens, M. and Paramio, M. (2004). Effects on in vitro embryo development and intracellular glutathione content of the presence of thiol compounds during maturation of prepubertal goat oocytes. *Molec. Reprod. & Dev.* **65***:*446-453.

Roy, A., Razada, B.C., Tiwari, R.B.I., Andey, M.D.P., Yadav and Sengupta, B.P. (1968). Effect of management on fertility of buffalo cows bred during summer. *Indian J. Vet. Sci.* **38**: 554-560.

Saha, M. (2018). Effect of bovine serum albumin on *in-vitro* maturation of bovine oocyte. MS Thesis, Department of Animal Breeding and Genetics, Sher-e-Bangla Agricultural University, Dhaka.

Saha, S., Khandoker, M.A.M.Y., Asad, L.Y., Reza, A.M.M.T. and Hoque, A. (2014). Effect of fresh and frozen semen on *in vitro* fertilization and subsequent development of goat embryos. *Iran. J. Appl. Anim. Sci*. **4**:325-330.

Singh, L.R., Rao, K.S. and Mohan, K.M. (2013). Oocyte retrieval methods, grade and percentage of oocytes in goats. *Int. J. Mol. Vet. Res*.**3**:4-6.

Sirard, M. A. and Ali (2002). Effect of the absence or presence of various protein supplements on further development of bovine oocytes during in *vitro* maturation. *Biol. Reprod*. **66**:901–905.

Sirard, M.A. (2001) Resumption of meiosis: Mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology*. **55**: 1241-1254.

Sirin, E. and Kuran, M. (2021). In vitro maturation of bovine oocytes may use royal jelly as protein source in the culture media. *Large Anim. Rev.* **27**: 135-141.

Smetanina, I. G.; Tatarinova, L. V.and Krivokharchenko, A. S. (2014). Effects of Hormones on *in Vitro* Maturation of Cattle Oocytes. *Bull. Exp. Biol. Med.* **157**(5): 634–636.

Smetanina, I. G.; Tatarinova, L. V. and Krivokharchenko, A. S. (2000). Influence of the culture medium composition on cattle oocyte maturation and embryo genesis in vitro. *Russ. J. Dev. Biol*. **31:** 113–116.

Tareq, K.M., Akter, Q.S., Khandoker, M.A. and Tsujii, H. (2012). Selenium and vitamin E improvethe in vitro maturation, fertilization and culture to blastocyst of porcine oocytes. *J. Reprod. Dev.* **58**: 621-628.

Totey, S.M., Pawshe, C.H. and Singh, G.P. (1993). Effects of bull and heparin and sperm concentrations on *in vitro* fertilization of buffalo (*bubalus bubalis*) oocytes matured *in vitro. Theriogenology.* **39**: 887-898.

Totey, S.M., Singh, G., Taneja, M., Pawshe, C.H. and Talwer, G.P. (1992). *In vitro* maturation, fertilization and development of follicular oocytes from buffalo (*Bubalus babulis*). *J. Reprod. Fertil.* **95**: 597-607.

Tsafriri, A., Cao, X., Ashkenazi, H., Mottola, S., Popliker, M. and Pomerantz, S.H. (2005). Resumption of oocyte meiosis in mammals: On models, meiosis activating sterols, steroids and EGF like factors. *Mole. Cellular Endocr.* **234**:37-45.

Webb, R., Campbell, B.K., Garveric, H.A. and Gong, J.G. (1999). Molecular mechanisms regulating follicular recruitment and selection. *J.Reprod. Fert.* **45**:123-126.

Zeitoun, M.M., Rodriguez-Martinez, H. and Randel, R.D. (1996) Effects of season on ovarian follicular dynamics in Brahman cows. *Theriogenology* **45**: 1577-1581.

Zuelke, K. A. and Brackett, B. G. (1990). Luteinizing hormoneenhanced in vitro maturation of bovine oocyte with and without protein supplementation. *Biol. Reprod.***43**:784-787.

Appendices

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	1.21	117.86	1.03	6.03	0.0001
Within Category	113	460.80	3.90		
Total	114.31	580.54			

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	1.21	77.64	0.64	4.94	0.00001
Within Category	113	410.01	3.51		
Total	114.31	490.02			

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	1.21	160.16	1.30	7.17	0.00001
Within Category	113	235.08	2.02		
Total	114.31	420.38			

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	1.21	3.02	0.02	1.67	0.0078
Within Category	113	40.57	0.19		
Total	114.31	40.81			

Significant ($P<0.05$)

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	1.21	110.51	0.90	2.3	0.0005
Within Category	113	340.60	2.86		
Total	114.31	457.01			

Significant ($p<0.05$)