STUDIES ON FOLLICULAR OOCYTE RETRIEVAL AND *IN VITRO* MATURATION IN GOAT

By

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Thesis submitted to Faculty of Veterinary Sciences and Animal Husbandry
in partial fulfilment of the requirements
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IN
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Division of Veterinary Gynaecology and Obstetrics
Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu
Main Campus, Chatha, Jammu-180009

2023

CERTIFICATE – I

This is to certify that the thesis entitled **"Studies on follicular oocyte retrieval and *in vitro* maturation in goat"** submitted in partial fulfillment of the requirements for the degree of **Masters of Veterinary Sciences** in **Veterinary Gynaecology and Obstetrics** to the Faculty of **Veterinary Sciences and Animal Husbandry**, Sher-e Kashmir University of Agricultural Sciences and Technology of Jammu, is original work and has similarities with published work not more than minor similarities as per UGC norms of 2018 adopted by the University. Further, the level of minor similarities has been declared after checking the manuscript with URKUND software provided by the University.

The work has been carried out by Mr. **Raashid Lateef Dar,** Registration No. **J-20-MV-631,** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. It is further certified that help and assistance received during the course of thesis investigation have been duly acknowledged.

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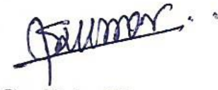

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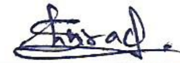

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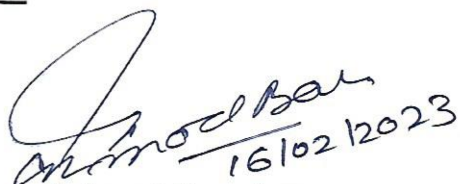

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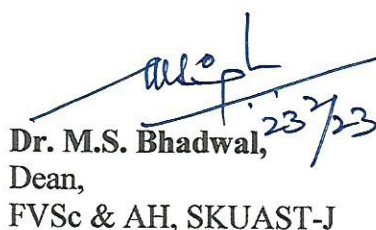

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ABSTRACT

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ABSTRACT

The present study was conducted to know the effect of oocyte harvesting techniques on recovery rate of oocytes along with the effect of follicular size and different additives on *in vitro* maturation (IVM) of follicular oocytes retrieved from abattoir derived goat ovaries. Ovaries were collected from municipal slaughter house, Jammu in two distinct seasons, viz., winter and summer. The study was done in three phases viz., Phase-I: Study the effect of oocyte collection techniques (aspiration, puncture and slicing) on recovery rate of oocytes, Phase-II: Effect of follicular size on *in vitro* maturation percentage of oocytes and Phase-III: Effect of Epidermal growth factor (EGF) and Cysteamine on rate of *in vitro* maturation of oocytes. In phase I, the recovery rate of good, fair, and total oocytes by slicing (2.94 ± 0.06, 2.85 ± 0.03, 6.46 ± 0.03) and Puncture (1.23 ± 0.04, 1.64 ± 0.07, 4.12 ± 0.14) was significantly higher ($p<0.05$) than the aspiration (0.74 ± 0.06, 0.85 ± 0.05, 2.94 ± 0.13) technique. Comparatively recovery of good and fair and total oocytes was significantly higher ($P<0.05$) for slicing than puncture and aspiration technique. The mean number of good, fair, and total oocytes recovered in winter season was significantly higher ($P<0.05$) than in summer season. The mean number of good, fair and total oocytes recovered from left ovaries did not differ significantly than right ovaries. The mean number of good, fair, and total oocytes yield in ovaries without corpus luteum was significantly higher ($P<0.05$) than ovaries with corpus luteum. In phase-II, the *in vitro* maturation percentage of oocytes based on cumulous cell expansion and 1st polar body extrusion was significantly higher ($p<0.05$) for large follicles (64.73%, 41.58%) than medium (46.47%, 28.23%) and small follicles (42.00%, 26.66%), respectively. In phase-III, the maturation percentage based on cumulous cell expansion and 1st polar body extrusion for EGF containing Group 1, Cysteamine containing Group 2 and control Group 3 was 69.61 and 46.07, 48.57 and 31.41, 46.23 and 29.03 percent, respectively. The maturation percentage based on cumulous cell expansion and 1st polar body extrusion was significantly higher ($P<0.05$) for EGF containing medium than Cysteamine containing medium and control medium.

Key Words: oocyte collection techniques, oocytes, follicular size, *in vitro* maturation, Epidermal growth factor (EGF), Cysteamine, goat ovaries.

Signature of Major Advisor

Signature of the Student

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ABBREVIATIONS AND SYMBOLS

CL	Corpus luteum
Fig.	Figure(s)
FSH	Follicle Stimulating Hormone
GnRH	Gonadotrophin-releasing hormone
EGF	Epidermal Growth Factor
IU	International Unit
L	Litre (s)
LH	Luteinizing Hormone
mcg	Microgram(s)
mg	Milligram (s)
min	Minute (s)
ml	Millilitre (s)
mm	Millimeter
mM	Mili mole
mmHg	Milimeters of Mercury
%	Per cent
@	At the rate of
±	Plus or minus
^{0}C	Degree centigrade
D0	Degree Zero
D1	Degree One
D2	Degree Two
≥	More than or equal
>	More than
<	Less than
χ^2	Chi-square
%	Per cent
=	Equal to
±	Plus or minus
ANOVA	Analysis of variance
BP	Bard parker
CCE	Cumulus cell expansion
CCs	Cumulus cells
CE	Cumulus expansion
CO_2	Carbon dioxide

COCs	Cumulus oocyte complexes
conc.	Concentration
DPBS	Dulbecco's phosphate-buffer saline
PBS	Phosphate buffer saline
et al.	And others
FBS	Fetal bovine serum
FCS	Fetal calf serum
FSH	Follicle stimulating hormone
g	Gram
GDP	Gross domestic product
GOI	Government of India
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
h	Hour(s)
HM	Holding media
i.e.	That is
I.U	International unit
IVC	In vitro culture
IVEP	In vitro embryo production
IVF	In vitro fertilization
IVM	In vitro maturation
IVMFC	In vitro maturation, fertilization and culture
IVP	In vitro production
MI	Metaphase-I
MII	Metaphase-II
min.	Minute (s)
µM	Micromolar
ng	Nanogram
no.	Number
PB	Polar body
sec.	Second (s)
TCM-199	Tissue Culture Medium-199
DMM	Defined maturation medium
viz.	As follows
w/v	Weight by volume
µg	Microgram
µl	Micro liter

Chapter-I

Introduction

CHAPTER – I

INTRODUCTION

The goat is a versatile animal that has long been used to provide milk, meat, skin, and expensive hair in the form of long lustrous mohair and pashmina. Goat milk and meat are high-biological-value animal protein sources for human diet. The goat is often called as a "running dairy" since it can be milked as needed.

India has the highest goat population in the world. With the largest population (148.9 million; 20th Livestock census, 2019) among Asian countries and the world, India has a large and diverse goat genetic resource. In developing countries like India, goat farming generates income and employment while also providing nutrition to poor, small and marginal farmers as well as landless laborers. In South Asia and South-East Asia, India has supplied exceptional goat germplasm for goat improvement.

In order to maximise reproductive effectiveness in this essential domestic animal, effective use of reproductive biotechnology techniques such as oocyte harvesting and freezing for long term *in vitro* preservation is critical. The following are some of the benefits of harvesting ovaries for embryo production technology: (i) significant production of embryos from superior genetic value females through oocyte recovery from prepubertal, pregnant and even dead or slaughtered goats. (ii) low-cost embryos can be produced for basic research on developmental biology and physiology. (iii) used as an approach for the rescue of some endangered animal species by interspecific embryo transfer.

The potential benefits of *in vitro* embryo production techniques include greater genetic gain obtained, reduced chances of disease transmission and easiness in transportation of embryos compared to animals. The success in application of reproductive technologies such as cloning by nuclear transfer, egg and sperm sexing and production of transgenic animals is critically dependent on the expertise in the basic reproduction techniques such as *in vitro* maturation (IVM) and *in vitro* culture (IVC) of embryos.

Ovaries of slaughtered animals are the most common and inexpensive source of primary oocytes for large scale production of embryos through IVM-IVF (Agarwal *et al*., 1995). The important aspect of an oocyte recovery method is to maximize the yield of good quality oocytes (evenly granulated cytoplasm surrounded by several layers of compact cumulus cells) recovered per ovary, which can be used for IVM and IVF (Sherazi *et al*., 2005). Modern laboratory techniques have been introduced for collection of oocytes from the ovaries of slaughtered animals, which include slicing, puncture of ovary and aspiration of follicle techniques, by which it is possible to obtain good quality oocytes and many numbers (Wani *et al*., 2000).

Maturation of oocytes is the process by oocytes achieve competence to undergo fertilization and to transform into embryo. The primary oocytes of goat undergo arrest at the diplotene stage of meiosis at birth like other mammals. When removed from their follicles and cultured under *in vitro* conditions, they are able to resume meiosis spontaneously (Gilchrist and Thompson, 2007). For normal fertilisation and embryonic development, the goat oocytes must achieve both nuclear and ooplasmic maturation. The meiotic arrest at diplotene stage is removed during maturation process and meiosis resumes to progress to metaphase II. Cytoplasm undergoes changes like protein and m-RNA and protein synthesis and its accumulation (Rodriguez and Farin, 2003).

An oocyte must first be seen as normal before it may be expected to mature *in vitro*. Cumulous cell (CC) investment around the zona pellucida (ZP), absence of fractured ZP and absence of vesicles in the ooplasm are all signs of a healthy egg. It is thought that the existence of more and compact layers of CCs is preferable. An excellent goat oocyte will be golden, golden-yellow or brownish in colour and the ooplasm will be granulated (Rajikin *et al*., 1994). The size of an oocyte also matters when it comes to maturation. According to De Smedt *et al*. (1992), 86 percent of goat oocytes from follicles 2 to 6 mm in diameter advanced to MII, but just 24 percent of oocytes from follicles 1-1.8 mm advanced to MII.

The cumulus oophorus is only found in eutherian mammalian oocytes. It is made up of a granulosa cell (GC) mass that surrounds the oocyte. The deposition of proteoglycan matrix causes the cumulus oophorus to enlarge *in vivo* following ovulation.

Hyaluronic acid is the most abundant carbohydrate in this muco-elastic matrix (Salustri *et al*., 1999). The CCs are known to provide nutrients (Haghight and Van Winkle, 1990), energy substrates (Sutton *et al*., 2003), and/or messenger molecules for oocyte growth (Buccione *et al*., 1990), as well as mediate hormone effects on the cumulous oocyte complexes (COC) (Buccione *et al*., 1990; Zuelke and Brackett, 1990). When compared to denuded or corona-enclosed oocytes, cumulus investment boosts fertilisation and embryo development rates *in vitro* (Tanghe *et al*., 2003). In goats, similar developmental patterns were identified (Rajikin *et al*., 1994). When goat oocytes were surrounded by more than five layers of CCs, they matured significantly more ($p<0.001$) than when they were surrounded by less than five layers of CCs and denuded oocytes (Rahman *et al*., 2006). In comparison to *in vivo* production techniques, the success rate of *in vitro* production of embryos is less. The quality and quantity of IVF/IVP embryos can be improved by increasing the efficiency of *in vitro* maturation procedures. The various factors which are involved in the successful IVM of goat oocytes are follicle, follicular fluid or cells, composition of IVM medium, age of the donor goat and the culture conditions (Rahman *et al*., 2008a). Das *et al*. (1996a) dissected follicles from goat ovaries and classified them into small (upto 2.0 mm), medium (2.0-5.0 mm) and large (> 5.0 mm) grades. Morphological parameters like cumulous cell expansion, extrusion of polar body and increased perivitelline space (Kruip *et al.,* 1983) are taken into account to evaluate the maturation. Goat oocytes matured *in vitro* in presence of EGF had greater cumulous cell expansion, high maturation and fertilization rates than the control oocytes (Nagar and Purohit, 2005). Cysteamine is a low molecular weight thiol that which increases the intra-cytoplasmic oocyte Glutathione (GSH) concentration and improves embryonic development rates (De Matos and Furnus, 2000). Keeping in view the above mentioned facts, the present study was planned with the following objectives:

Objectives

1. To study the effect of harvesting techniques on recovery rate of oocytes from goat ovaries.

2. To study the effect of follicular size on *in vitro* maturation of goat oocytes.

3. To study the effect of different additives on *in vitro* maturation of goat oocyte.

Chapter-II

Review of Literature

CHAPTER – II

REVIEW OF LITERATURE

Goat ovaries from slaughtered animals are the most economical and abundant source of primary oocytes and they were frequently employed for *in vitro* maturation (Tajik and Esfanabadi, 2003; Wang *et al.,* 2007a; Mahesh *et al.,* 2013; Widayati and Pangestu 2020; Maksura *et al.,* 2021). The use of ovaries harvested from slaughter house animals as a source of oocytes for IVM-IVF enables for large-scale production of embryos for use in the development of novel biotechnologies like cloning and genetic engineering (Gordon, 2003).

2.1 Collection of Ovaries

2.1.1 Media and time interval for ovary transportation

Kharche *et al.* (2005) used PBS supplemented with antibiotics for transport of caprine ovaries.

Nager and Purohit (2005) used DPBS at 39°C and transported ovaries within 2 hours to laboratory.

Wani and Nowshari (2005) reported that the proportion of oocytes reaching M II stage did not differ between ovaries collected and stored in normal saline solution at room temperature for 12 hours and those collected in warm normal saline solution and processed immediately after arrival in the laboratory.

Wang *et al.* (2007) transported the ovaries to laboratory within 2 hours in 0.9% saline solution.

Wani *et al* (2013) used a warm normal saline solution supplemented with 50ug per ml Gentamicin sulphate for the transport of ovaries to the laboratory within 1-2 hours.

Asad *et al.,* (2016) used warm 0.9% physiological saline and transported ovaries within 5-6 hours.

2.1.2 Temperature of transport media

Caprine ovaries were transported from abbatoir to laboratory within temperature range of 25-30°C (Pawshe *et al.,* 1993) or 30-35°C (Tajik and Esfandabadi, 2003; Widayati and pangestu, 2020) or 35-37°C (Kharche *et al.*, 2009; Borah *et al.*, 2018; AL-Nuaimi *et al.*, 2020) or 39°C (Mahesh *et al.*, 2013)

2.2 Follicular Status

Das *et al.* (1996a) dissected small (upto 2.0 mm), medium (2.0-5.0 mm), and large (> 5.0 mm) follicles from goat ovaries. Based on cumulus investment, compactness, and ooplasm granulity, they graded oocytes recovered from follicles as good, fair, or poor.

Suri *et al.* (2006) evaluated the ovarian follicular and oocyte ratio in Gaddi goats of various reproductive stages and discovered that the follicle and oocyte ratio of primordial follicle was found maximum in prepubertal (1.13±0.07) and minimum in senile phase (0.67±0.07).

Islam *et al.* (2007) in their studies on surface follicular population and oocyte collection reported the number of surface follicles as 4.40 ± 0.17 and corresponding values for total, normal and abnormal oocytes collected as 1.85 ± 0.08, 1.06 ± 0.06 and 0.79 ± 0.06, respectively.

Suri and Kalita (2009) classified follicles as small (1-3 mm), medium (3-6 mm), and large (> 6 mm) visible follicles on the ovarian surface.

According to Alsafy and El- Shahat (2011), the presence of CL influenced ovarian dimensions and weights, with the latter being higher in the CL containing ovary (P<0.05). When compared to the left ovary, the average number of big follicles was considerably (<P0.05) higher in the right ovary.

Khandoker *et al.* (2011) reported that the left (7.25±0.31) ovaries had more follicles (P<0.05) than the right (6.22±0.32) ovaries. In addition, the number of detected

follicles, aspirated follicles, COCs, and normal COCs were substantially (P<0.05) higher in CL ovaries than in CL ovaries.

Dar (2014) classified goat ovaries into three groups viz., ovaries containing less than 5 surface follicles, 5 to 10 surface follicles and more than ten surface follicles and reported the mean oocyte recovery rate of 2.45 ± 0.06, 5.05± 0.07 and 8.86 ± 0.11 from three groups of follicular populations, respectively. The parallel values in terms of quality of oocytes recovered were 0.48 ± 0.04, 0.84 ± 0.4 and 1.12 ± 0.03, 1.67 ± 0.04, 2.02 ± 0.04 and 1.35 ± 0.04 and 3.48 ± 0.07, 3.63 ± 0.08 and 1.74 ± 0.11 as good, fair and poor quality for three groups of follicle populations, respectively.

Gabr *et al.* (2019) reported that in the breeding season, the number of follicles and oocytes/ovary (P<0.001), as well as the number/ovary and proportion of oocytes at compact (P<0.0001) and denuded (P<0.05) stages, were all higher than in the non-breeding season.

2.3 Retrieval of Oocytes

Agarwal *et al.* (1992) found that the Puncture approach recovered more good quality oocytes from goat ovaries than the Slicing (64 percent) and Aspiration (54-55 percent) techniques. In comparison to follicular puncture, surface puncture recovered 30% more oocytes. In the monsoon and winter seasons, they found an average oocyte recovery rate of 13.4 and 8.2, respectively. However, when compared to the monsoon season, the percentage of good quality oocytes was higher in the winter.

Dutta *et al.* (1993) recovered higher average number of sheep oocytes by slicing (7.36) than puncture (5.50) and aspiration (2.17) with higher good quality oocytes in slicing (38.8%) as compared to aspiration (20.4%) or dissection (28.8%) and proposed that slicing was the best method for oocyte recovery due to the oocyte yield as well as its time economy.

Das *et al.* (1996b) used slicing, dissection, follicle puncture, and aspiration to retrieve oocytes from sheep ovaries gathered at a local abattoir. The average number of oocytes recovered per ovary was 10.33, 11.13, 4.41, and 2.75. 1.32, 10.63, 1.32, and 1.77

minutes were spent processing each unit. The slicing method was shown to be the most effective for oocyte retrieval in ovines.

Sharma *et al.* (1996) collected oocytes from goat ovaries using the ovarian surface piercing technique to analyse the chronological events during *in vitro* maturation of goat oocytes.

Wani *et al.* (2000) reported significantly higher average number of oocytes recovery by slicing (9.5 ±0.4) and puncture (9.5 ±0.45) than by the aspiration method (6.8 ±0.3) in sheep. However, there was higher percentage of good quality oocytes in the aspiration (64.4%) as compared to the puncture (54.7%) or slicing (54.3%).

Wani *et al.* (2002) reported that puncture of the ovarian surface with a hypodermic needle is more effective for recovering oocytes in smaller ovaries of prepubertal sheep, whereas aspiration is better for larger ovaries.

Shirazi *et al.* (2005) studied the effect of the oocyte collection techniques (aspiration and slicing) on the quantity and quality of immature ovine oocyte recovered during the non-breeding season and observed that the total number and good quality oocytes recovered per ovary in ewes by aspiration and slicing were 8.1 and 1.8 and 6.3 and 3.3, respectively. However, the corresponding values for ewe lambs were 3.7 and 0.8 and 4 and 1.7 and concluded that slicing technique produced more good quality oocytes per ovary than aspiration in both ewe and ewe lambs during the non-breeding season.

Yadav *et al.* (2007) compared the efficacy of various procedures for recovering oocytes from prepubertal goat ovaries and discovered that puncture was the most effective approach, followed by slicing and aspiration, in terms of yielding high-quality oocytes.

Islam *et al.* (2007) in their analysis on goat ovaries found that left ovaries contained slightly higher (2.55±0.14) mean number of aspirable (>2 mm diameter) follicles per ovary than those of right ovaries (2.52±012). They used the aspiration technique for collection of oocytes and found an almost same COCs number in right and

left ovaries, although the higher number of normal COCs were recorded in left [(1.06±0.09) per ovary].

Wang *et al.* (2007) reported a yield of larger (P<0.05) number of oocytes per ovary from slicing (6.3) and puncturing (5.8) in Boer goat than aspiration I (2.9) and aspiration II (3.1). Aspiration I and II differ in fact that the former used an 18-g needle with a syringe, whereas the latter used a vacuum pump to apply a continuous pressure (60 mm Hg) to aspirate the oocytes. On placing a representative sample of excellent-quality oocytes (good and fair) generated by each method through the IVM/IVF/IVC protocol, the rate of oocytes reaching the M II stage was significantly lower (P>0.05) when slicing techniques were utilized.

Paramio (2010) stated that in adult goat ovaries, oocytes are recovered by follicle aspiration technique selecting follicles bigger than 3 mm diameter while from prepubertal goat ovaries, slicing the ovary allows collection of more (6.05) oocytes per ovary than by follicle aspiration (1.27) though the morphological quality is lower.

Hoque *et al.* (2011) stated that puncture and slicing yielded considerably more (p<0.01) number of total COCs per ovary (4.22 and 4.14, respectively) than that of aspiration (3.28), however, a significantly higher (p<0.01) number of normal COCs per ovary was yielded in aspiration (2.48) than those of puncture (1.85) and slicing (1.91) techniques.

Singh *et al.* (2013) studied oocyte retrieval methods, grade, and percentage of oocytes in goats. The slicing approach yielded considerably more (50.57%) of the 437 oocytes retrieved in this study, followed by the dissection technique (37.07%) and aspiration technique (12.53%). The mean yield of oocytes per ovary was found to be 3.93 ± 0.11, 4.44 ± 0.06 and 3.59 ± 0.07 by aspiration, slicing and puncture, respectively. Slicing yielded significantly higher number of oocytes per ovary. Percentage yield of culturable quality oocytes (A, B and C) by different retrieval methods were 78.18, 76.05 and 81.39 respectively by aspiration, slicing and puncture.

AL-Nuaimi *et al.* (2020) explored the role of oocyte collection techniques on the recovery and quality of oocytes from Iraqi goat ovaries (Capra hircus). The results

showed that the average total number of oocytes retrieved per ovary was significantly increased P<0.05) in slicing (6.8±0.11) than punctures (4.14±0.06) and aspiration (2.80±0.03) techniques. The rate of oocytes collected per ovary were 4.58±0.08 of which 2.01±0.12 (40.07%), 1.20±0.03 (28.93%) and 1.29±0.02 (30.99%) were good, fair and poor oocytes respectively. The average number and percentage of good grade oocytes were significantly increased (P<0.05) by slicing 3.56±0.04 (52.35%) than puncture 1.75±0.15 (42.55%) and the aspiration 0.72±0.08 (25.32%) techniques.

2.4 Grading of oocytes

To classify cumulous cells, a stereo-zoom microscope with magnifications of 40X to 60X is typically utilised. Intact cumulous cells and homogenous cytoplasm were the most dependable morphological criteria for COC selection (Wang *et al.* 2007b; Kharche *et al.*, 2008b). According on their cumulus investments, Datta *et al.* (1993) categorised recovered oocytes from sheep ovaries as good, moderate, or poor.

Das *et al.* (1996b) assigned Good, Fair, and Poor grades to the retrieved sheep oocytes based on the types of cumulus investments, compactness, and ooplasm granulity.

Yadav *et al.* (2007) graded the recovered oocytes from goat ovaries as Excellent (A), Good (B), Fair (C), and Poor (D) based on cumulus investments and cytoplasmic dispersion.

Mondal *et al.* (2008) categorized the COCs into two groups: normal COCs (grades A and B) and abnormal COCs (grades C and D). Within the typical COCs, grade A COCs matured at a much greater rate (P<0.01) than grade B COCs (71.70%: 51.52%).

Majeed *et al.* (2019) reported that in case of slaughtered Iraqi black goats, a high recovery rate of fair oocyte (Grade B) 53.4% (1220/ 2285) was obtained followed by good oocyte (Grade A) 31.4% (719/ 2285) and poor oocyte (Grade C) 15.4% (346/ 2285).

AL-Nuaimi *et al.* (2020) categorized the retrieved oocytes into three grades based on the number of cumulus cells surrounding them and the homogeneity of the ooplasm:

oocytes with at least three layers of cumulus cells, oocytes with less than three layers of cumulus cells and denuded oocytes.

2.5 Different factors affecting oocyte recovery

Various factors influence the quality of retrieved oocytes, including the animal's species, age, nutritional state (body condition), and environmental factors such as season, cyclicity of animals at the time of slaughter, developmental stage, functional status, and follicle size (diameter). The oocyte retrieval procedure, the presence or lack of corpus luteum in the ovary, and the side of the ovary all lead to variations in results. (Nandi *et al.*, 2001; Zoheir *et al.*, 2007; Arangasamy *et al.*, 2008; Nandhi and Kumar, 2008).

2.5.1 Effect of season on the recovery rate of oocytes

Initial oocyte quality and a number of linked factors such as environmental causes, heat stress, nutrition, and management techniques assist fertilization and subsequent embryo development (Hansen *et al.*, 2010). Heat stress in the summer season causes ovarian oocyte reduction in crossbreds, which affects the recovery rate and quality of oocytes (Rocha *et al.,* 1998), and heat stress in the winter season causes ovarian oocyte reduction in crossbreds, which affects the recovery rate and quality of oocytes (Rocha *et al.*, 1998; Raj *et al.*, 2016). Heat stress impairs follicular growth and oocyte formation at the early stages (Torres-Junior *et al.*, 2008), as well as delaying follicle selection and lengthening the follicular wave, thereby lowering oocyte quality (Badinga *et al.*, 1993; Roth *et al.*, 2001). In a study shown by Cognie (1999) the number of recovered oocytes from sheep and goat ovaries during breeding season were one and two oocytes, respectively. Furthermore, results revealed no significant differences between numbers of recovered oocytes in breeding and non-breeding seasons. Nandhi and Kumar (2008) observed in sheep that the recovery rates of good (6.3 ± 1.4 vs. 1.6 ± 0.4) and total (11.3 ± 2.4 vs. 4.3 ± 1.1) number of oocytes per ovary were significantly higher in breeding season. However, there was no significant difference in recovery rates of average (3.3 ± 1.0 vs. 1.7 ± 0.5) and poor (1.7 ± 0.8 vs. 0.9 ± 0.3) oocytes in breeding and non-breeding seasons. Davachi *et al.* (2014) recorded highest number of oocytes irrespective of

recovery technique in breeding season in sheep which might reflect the optimum level of 13 gonadotropins and steroids.

Dar *et al.* (2014) recorded a significantly higher ($P<0.05$)) mean number of good (1.94±0, 07), fair (2.21±0.07), poor (1.21±0.04) and total (5.38±0.13) in winter season than summer and rainy season in goat.

Sarkar *et al.* (2020) in their studies on comparative seasonal assessment on the quality of Black Bengal goat oocytes revealed that the average number of follicles and oocytes recovered were higher in the summer than in the winter, with grade A and B oocytes recovery more in the summer than in the winter, and grade C and D oocytes recovery faster in the winter than in the summer.

Souza-Fabjan *et al.* (2021) in their studies on effect of reproductive seasonality on *in vitro* embryo production outcomes in adult goats concluded that the during breeding season, oocyte developmental competence improves, resulting in increased cleavage and blastocyst yield, whereas embryo quality remains consistent over time.

2.5.2 Recovery rate of oocytes from right and left ovaries

Islam *et al.* (2007) reported that the COCs were found to be greater in the left ovaries (2.42±0.14 per ovary) than in the right ovaries (2.32±0.12 per ovary). When the COCs were divided into normal and abnormal groups, the left ovary had a higher number of normal COCs than the right ovary, which validates the previous result of Islam *et al.* (2007) in goat.

Rao *et.al* (2010) studied developmental competence of oocytes recovered from postmortem ovaries of the endangered Indian Blackbuck and they found that there was no significant difference in the recovery of total and culture grade oocytes from left ovaries (11.8 ± 2.1 and 8.5 ± 1.2) and right ovaries (10.0 ± 1.6 and 7.0 ± 1.4, respectively.

Alsafy and El-Shahat (2011) recorded the mean number of COCs (2.0±0.13 and 1.2±0.09) in right and left ovary, respectively in sheep.

Talukder *et al.* (2011) carried out collection, grading, and assessment of cumulous-oocyte complexes for *in vitro*-maturation in sheep and found that the difference in the number of normal, abnormal and total COCs per ovary between left (1.7 ± 0.17, 0.6 ± 0.10, 2.3 ± 0.18, respectively) and right (1.6 ± 0.12, 0.7 ± 0.13 and 2.2 ± 0.19, respectively) was not significant (P>0.05).

Khandokar *et al.* (2011) reported that a total of 916 follicles were recorded on the surface of the ovaries in buffaloes, and 806 follicles were aspirated from the surface of both (right and left) ovaries, with 385 obtained with a mean of 5.66±0.25 per ovary from right ovaries and 421 obtained with a mean of 6.19±0.24 per ovary from left ovaries.

Mahesh *et al.* (2013) studied the effect of side of ovary on oocyte recovery from goat ovaries using different collection techniques viz, aspiration, slicing and puncture and they reported the mean recovery rate of 4.06 ± 0.31 and 4.12 ± 0.22 oocytes using aspiration, 6.57 ± 0.39 and 6.62 ± 0.55 oocytes using slicing and 6.48 ± 0.58 and 6.59 ± 0.52 oocytes using puncture techniques from left and right ovaries, respectively.

Asad *et al.* (2016) in his analysis on Goat ovaries observed that the length (cm) of right ovaries (1.19±0.09) was significantly (p<0.05) higher than left ones (1.15±0.04). The number of normal oocytes was found significantly higher (p<0.05) in left ovaries.

2.5.3 Effect of corpus luteum on recovery rate of oocytes

The presence of CL on ovary might affect oocyte recovery due to higher levels of progesterone hormone. Negative feedback to anterior pituitary gland by circulating progesterone prevents gonadotrophin secretion and ultimately follicular degeneration occurs (Webb *et al.*, 1999). The cause of a low number of oocytes per ovary with a CL is likely because follicular development is restricted by lutein cells which occupy a great portion of the ovary and the presence of CL may inhibit the growth of follicles and increases their atresia (Hafez, 1993).

Wani *et al.* (1999) reported that ovary with a CL produced substantially fewer oocytes than ovary without a CL in sheep.

Vassena *et al.* (2003) studied the morphology and developmental competence of COCs in relation to the phase of follicle development and the presence of corpus luteum or dominant follicle in the ovary. The presence of corpus luteum or dominant follicle in the ovary had no effect on the mean number of oocytes that developed to the blastocyst stage.

Talukder *et al.* (2011) reported that the number of normal (1.9±0.11) and total (2.5±0.14) COCs per ovary were considerably ($P<0.05$) higher in ovaries without CL (1.2 0.36 and 2.0 0.30, respectively) than in those with CL (1.2±0.36 and 2.0±0.30) in case of sheep. However, the number (0.80±0.13) of aberrant COCs per ovary was considerably ($P<0.05$) higher in grade A (6.9±2.05) COCs than in grade B (53.1±1.27) COCs.

Khandokar *et al.* (2011) in buffaloes found that out of a total of 806 aspirated follicles, 630 were retrieved from ovaries without CL (Follicular phase) and 176 from ovaries with CL (Luteal phase). The number of follicles aspirated per ovary in ovaries without CL (6.78±0.18) was significantly larger ($p<0.05$) than in CL-containing ovaries (4.09±0.26).

Davachi *et al.* (2011) divided the ovaries into two groups: one with functional CL and the other without. The oocytes were extracted using an aspiration pump or an ORC. They were cultivated in TCM-199 for 24 hours after oocyte recovery. The mean number of oocyte recovery per ovary in group 1 ovaries (1.8 ± 0.01 via aspiration and 3.84 ± 0.05 via ORC) was lower ($P<0.05$) than group 2 ovaries (2.2 ± 0.00 via aspiration and 5.43 ± 0.01 via ORC). The presence of CL on the ovaries resulted in a decrease in the quality and number of oocytes.

Sahoo and Singla (2013) examined the recovery rate and quality of oocytes recovered from abbatoir derived ovaries with and without corpus luteum. They divided recovered oocytes into four grades: grade A, grade B, grade C, and grade D, and discovered that the recovery rates of different grades of oocytes from ovaries with corpus luteum and ovaries without corpus luteum are same. They further reported the percentage of grade A, B, C and D oocytes from ovaries with and without corpus luteum are 23.6 ±

1.4 and 24.7 ± 1.6, 45.7 ± 1.2 and 46.6 ± 1.2,21.4 ±2,0 an d19.9 ± 1.3 and 7.8 ± 1.3 and 9.5 ± 2.9, respectively.

Mahesh *et al.* (2013) observed that the oocyte recovery was significantly lower in CL containing ovaries than that of ovaries without CL in aspiration (2.92 vs 4.57), puncture (5.89 vs 6.78) and slicing (5.40 vs 7.02) methods. However, the presence of CL did not affect the oocyte's ability to reach the MII stage (75.31% vs 76.67%).

Dar (2014) studied in the effect of CL on oocytes recovery rate in goat and recorded mean number of good, fair, poor and total in with CL ovary (0.97±0.06, 1.30±0.06, 1.08±0.03 and 3.36±0.10 respectively) and without CL ovary (1.84±0.05, 2.15±0.05, 1.47±0.04 and 5.47±0.10), respectively.

Rahman *et al.* (2016) in goats observed that ovaries without CL yielded a higher number of usable oocytes.

2.6 *In vitro* oocyte maturation

2.6.1 Maturation Media

The proportion of oocytes that reach metaphase II and become fertile, as well as the subsequent embryonic development, is affected by the culture medium employed in IVM (Bavister *et al.*, 1992). Gonadotropins, steroid hormones, pyruvate, serum from diverse sources, growth factors, and antibiotics are added to complex tissue culture media during maturation.

2.6.2 TCM 199

TCM-199 (tissue culture medium-199: HEPES modification, with Earl's salt, L-Glutamate, and 25 mM HEPES without sodium bicarbonate) was the most commonly used culture medium for the maturation of caprine oocytes. It was supplemented with hormones, serum and other substances at various concentrations (Pawshe *et al.*, 1994; Pawshe *et al.*, 1996; Zhou and Zang, 2006; Mahesh *et al.*, 2013; Kharche *et al.*, 2009; Maksura *et al.*, 2021; Fathi and Elkarmoty, 2021). Pawshe *et al.* (1996) found that the combination of M-199 with FSH, LH, and Estradiol 17, supplemented with 10% FCS,

was the most efficacious medium for *in vitro* maturation and subsequent embryonic development of goat oocytes in a comparative study of M-199 and Hams F-12 medium supplemented with or without serum and hormones for supporting *in vitro* maturation of goat oocytes.

2.7 Culture conditions

2.7.1 Temperature

Immature goat oocytes were found to be highly sensitive to temperature changes by De Smedt *et al.* (1992). Although 37˚C temperature is optimal for performing *in vitro* maturation in mammals, best results could be obtained when normal basal body temperature of the respective species could be provided (Eppig., 1996).

In case of goat, the most practiced temperatures were 39°C (Majeed *et al.,* 2019; Widayati *et al.,* 2020; Nagar and Purohit, 2005; Fathi and Elkarmoty, 2021), 38.5˚C (Talukder *et al.,* 2011; Wang *et al.,* 2007a; Mahesh *et al.*, 2013; Maksura *et al.,* 2021; Arya *et al.*, 2021) and 38˚C (Younis *et al.,* 1991; Pawshe *et al.,* 1993; Pawshe *et al.,* 1994).

2.7.2 Gas phase

The two most prevalent gas mixes used during *in vitro* gamete maturation were 5% CO_2, 5% O_2, and 90% N_2 or 5% CO_2 in 95% air, with the latter being the more common (Eppig., 1996).

In goats, majority of investigators used 5% CO_2 in air (Pawshe *et al.,* 1993; Mahesh *et al.,* 2013; Sharma *et al.,* 1996; Kharche *et al.*, 2009; Nagar and Purohi., 2005; Talukder *et al.,* 2011; Maksura *et al.,* 2021).

2.7.3 Humidity

Many of the workers used maximum humidity (around 95-100 @) for IVM of goat oocytes and obtained good maturation rates (Pawshe *et al.,* 1993; Sharma *et al.,* 1996; Nagar and Purohit, 2005; Zhou and Zang, 2006). High humidity of 90 to 100 percent was opined by Gilchrist and Thompson, 2007 as being ideal for *in vitro*

maturation of oocytes as it prevented evaporation and thus maintained osmolality. At 95 percent humidity, the highest rate of oocyte maturation was obtained.

2.7.4 Culture Duration

Wang *et al.* (2007); Widayati *et al.* (2020) and Maksura *et al.* (2021) cultured oocytes *in vitro* for 24 hours with good results. A maturation rate of 80% was reported by Kharche *et al.* (2009) after 27 hours of IVM. Preantral oocytes were cultured by Zhou and Zang (2006) from preantral follicles for 48 hours with 54.08% maturation rate.

2.7.5 Effect of hormones on *in vitro* maturation of Oocytes

Pawshe *et al.* (1996) reported that in goats, the combination of M-199 with FSH, LH, and E2 supplemented with 10% FCS was shown to be the most effective medium for *in vitro* maturation and subsequent embryonic development of the media, sera, and hormone combinations evaluated.

Mogas *et al.* (1997) in their studies on adult and pre-pubertal goats found 72.4 and 64.1 percent maturation rates of oocytes in TCM-199 supplemented with FSH, LH, and estradiol-17, as well as estrus goat serum (EGS).

Tajik and Esfandabadi (2003) cultivated oocytes in TCM-199 with penicillin, streptomycin, 10, 15, or 20% FBS, ESS, or EGS and found maturation rates of 74-94 percent in all concentrations of the three sera tested. However, no significant differences were found across concentrations or between sera, and nearly no maturation (4%) was detected in medium without protein addition. It was concluded that these sera could be replaced for one another for IVM of caprine oocytes at the concentrations tested.

Wang *et al.* (2007a) investigated the effect of oocyte collection procedures and media on IVM and found that the media containing TCM-199 combined with 10% FCS and 1 IU/ml FSH resulted in an 81.7 percent oocyte maturation rate.

Shirazi *et al.* (2012) reported that the cleavage rate was significantly higher when IVM medium was supplemented with FBS than BSA in goat. Similarly, the overall blastocyst rates were significantly higher in FBS supplemented groups.

Wahjuningsih *et al.* (2014) investigated the effects of adding Goat Follicular Fluid to the media on cumulus cell expansion and oocyte nucleus metaphase-II. Immature oocytes of the highest grade were matured *in vitro* for 26 hours using TCM 199 + 10% FCS supplemented with varied amounts of Goat Follicular Fluid: 0%, 5%, 7.5%, 10%, 12.5% [v/v].

Maksura *et al.* (2021) studied effects of estradiol on *in vitro* maturation of buffalo and goat oocytes. In both species, 17-estradiol-treated oocytes had a faster cumulus expansion rate than control oocytes (0 g/mL treated). The percentage of oocytes developed to the metaphase II (MII) stage rose in buffalo in a concentration-dependent manner of 17-estradiol. Estradiol also had a beneficial effect on the nuclear maturation of goat oocytes *in vitro.*

Widayati and Pangestu *et al.* (2020) reported that FSH supplementation significantly improves oocyte maturation and yields mature oocytes for future embryo development *in vitro* in goat. The results showed that FSH supplementation significantly increased oocyte maturation rate (65.21±7.261% vs. 43.25±6.23%). Furthermore, the rate of degenerated oocytes was lower in the medium with FSH supplementation (3.21±0.25% vs. 10.15±3.1%).

2.7.6 Effect of additives on *in vitro* maturation of oocytes

EGF was discovered in the ovary of cattle and is thought to carry out cellular tasks through EGF receptors (Yoshida *et al.* 1998). Increased cAMP production by cumulus oocyte complexes induced the germinal vesicle to break down, resulting in EGF stimulation of IVM (Downs *et al.* 1991). EGF could act on the cumulus cells surrounding the oocyte and/or the oocyte itself, as mRNA for the EGF receptor was discovered in the bovine oocyte (Banwell and Thompson, 2008) and could be extrapolated to goat oocytes. Growth factors have been shown to bind to membrane and cytoplasmic high-affinity receptors, boosting the production of signals and second messengers (Druker *et al.* 1989).

Down *et al.* (1991) found that EGF aids in the breakdown of the germinal vesicle and increases the rate of maturation of denuded oocytes to the MII stage.

Pawshe *et al.* (1998) found that when EGF and IGF-1 were used, the percentage of cleavage and blastocyst development was considerably higher ($p<0.05$) than in the control group (38.5 percent and 14.8 percent).

Dinesh and Purohit (2004) discovered that combining EGF and IGF-1 on the maturation medium of buffalo oocytes resulted in a higher maturation rate (83.52%) than IGF-1 alone (67.08%) and EGF alone (63.64%), and significantly ($p<0.05$) higher than the control (46.30%), as well as a higher Fertilization rate (48.62%) than IGF-1 alone (36.36%) and significantly ($p<0.05$) higher than (15.00 percent).

Nagar and Purohit (2005) in their research on the effect of epidermal growth factor on the maturation of goat oocyte discovered that in comparison to the control group, EGF supplementation resulted in a significantly ($P<0.01$) greater proportion of oocytes reaching M II. In media supplemented with 10, 20, 50, 100, and 0 (control) ng ml-1 of EGF, the proportion of oocytes that reached M II was 55.63 percent, 64.5 percent, 52.35 percent, 49.18 percent, and 34.07 percent, respectively.

Wang *et al.* (2007) found in his studies on boer goat that oocytes matured in EGF or FCS supplemented TCM-199 medium in presence of FSH had a significantly larger proportion of blastocysts than the other treatments ($P <0.05$) and concluded that TCM-199 medium supplemented by EGF or FCS in the presence of FSH is suitable for *in vitro* maturation of oocytes.

Shabankareh and Zandi (2010) studied developmental potential of sheep oocytes cultured in different maturation media and reported that the addition of Cysteamine to DMM supplemented with EGF and IGF-I improved the mean blastocyst formation compared medium without Cysteamine. The combination of EGF + IGF-I + and Cysteamine in UDMM resulted in a higher proportion of cleavage and greater morula and blastocyst yields than DMM + EGF + IGF-I + Cysteamine or SDMM + EGF + IGF-I + Cysteamine.

Wani *et al.* (2012) reported that the EGF-supplemented media matured at a substantially greater rate (84.9%) than the cysteamine-supplemented (69.6%) or control mediums (67.2 percent). Fertilization and cleavage rates in the control, cysteamine, and

EGF supplemented media were 73.7 percent, 49.5 percent, 72.3 percent, 52.7 percent, and 76.5 percent, 55.5 percent, respectively. No significant differences were found ($p > 0.05$). Morula formation rates were significantly higher ($p<0.05$) in the EGF supplemented (38.7%) and cysteamine supplemented (36.7%) media than in the control (22.1%) medium. Finally, the data suggest that EGF has a role in *in vitro* maturation and that EGF and cysteamine both induce extra embryonic development in sheep.

An *et al.* (2018) in a study on goat oocytes found that those in medium supplemented with Cysteamine had higher Fertilization (56.1 percent vs. 72.1 percent), cleavage (36.7 percent vs. 44.8 percent), and blastocyst development (1.7 percent vs. 4.2 percent) than those in gonadotropin basal maturation medium ($P< 0.05$). Supplementing with Cys+LIF further increased Fertilization (81.6%), cleavage (54.9%), and blastocyst development (6%; $P< 0.05$).

Borah and Biswas (2019) investigated the effect of medium additions on goat oocyte maturation *in vitro.* The IVM rate of oocytes on the basis of cumulus cells expansion and nuclear maturation was found to be significantly higher (P less than 0.05) with EGF + IGF-1 (88.74 ± 1.85% and 61.71 ± 1.61%) than with EGF + sodium pyruvate (82.86 ± 0.97% and 54.62 ± 1.88%), EGF + cysteine (78.58 ± 1.45% and 49.02 ± 1.52%) and without additive (control) (75.27 ± 1.58% and 43.03 ± 1.48%).

2.8 Evaluation of *in vitro* maturation of oocyte

2.8.1 Based on cumulous cell expansion and 1st polar body extrusion

The CCs are recognised to provide nutrients (Haghight and Van Winkle, 1990), energy substrates (Sutton *et al.*, 2003), and/or messenger molecules for oocyte growth (Buccione *et al.,* 1990), as well as mediate hormone effects on the COC (Buccione *et al.*, 1990; Zuelke and Brackett, 1990). When CCs were eliminated before maturation *in vitro*, bovine oocytes showed no maturation or a low maturation rate (Fukui and Sakuma, 1980; Zhang *et al.,* 1995). The cumulous cells were recognised to provide nutrition, energy substrates, and messenger molecules for oocyte growth, as well as signal the influence of hormones on the cumulous oocyte complex (Buccione *et al.*, 1990).

The degree of cumulous cell expansion was graded by Chauhan *et al.* 1997 as zero when there was no expansion, one when cumulous cells were homogenously spread but clustered cells were still visible, and two when cumulous cells were homogenously spread but clustered cells were no longer visible. The degree of cumulous expansion after IVM was graded by Gupta *et al.* (2005) as zero, one, two, and three. For ooplasmic development, optimal growth of cumulous bulk seems to be necessary (Chen *et al.,* 2007). Cumulative cell expansion has been found to be related to Fertilization rate and developmental potential in oocytes, therefore it's thought to be significant for complete oocyte maturation (Cox *et al.*, 1993).

Kakkassery *et al.* (2010) examined bovine oocytes in the culture drops after 24 h of culture under zoom stereo microscope for maturation associated changes such as expansion and mucification of cumulus cells. The oocytes were denuded by vortexing and examined for extruded polar bodies.

Mahesh *et al.* (2013) removed the cumulus cells from the caprine oocytes by treating them with hyaluronidase (100 IU/ml) for 10 minutes and repeatedly passing them through a narrow bore glass pipette. The first polar body was then inspected for extrusion.

Widayati and Pangestu (2020) evaluated the matured oocytes for the morphology based on the expansion of cumulus cells and PB1 extrusion. After that, 600 oocytes were stained with 1% aceto-orcein to analyse maturation based on chromosomal configuration and nuclear membrane breakdown. When oocytes entered metaphase II, they were regarded as mature.

Chapter-III

Materials and Methods

CHAPTER – III

MATERIALS AND METHODS

3.1 Experimental location

The present research work was carried out at Division of Veterinary Gynaecology and Obstetrics, Faculty of Veterinary Sciences and Animal Husbandry, R.S. Pura, Jammu.

3.1.1 Seasons

The period of study was divided into two distinct seasons viz: 1. Winter: November to February 2. Summer: March to June

The experiment was conducted from November, 2021 to June, 2022.

3.1.2 Experimental material

Ovaries of goat were collected from municipal slaughterhouse, Jammu.

3.1.3 Chemicals, media and additives

All chemicals and media used in the experiment were obtained commercially from Sigma Chemical Company (St. Louis, MO, USA) and EGF from Invitrogen (Thermo Fisher Scientific) and Penicillin Streptomycin from Gibco (Life Technologies).

3.1.4 Plastic wares

Plastic wares like petri dish, plastic tubes and other plastic wares were purchased from Polylab Industries Pvt. Ltd, Haryana. Micron membrane filters (0.22 µm) were obtained from Millipore Co, USA.

3.1.5 Composition of various media

All the media used in present study were supplemented with penicillin (100 IU/ml) and streptomycin (100ug/ml) and sterilized by passing through 0.22 µm Millipore filters prior to use.

3.1.5.1 Handling media

It was used for washing and handling of cumulus oocyte complexes. To make a volume of 100 ml, the following components were mixed in proportion given against each:

Component	**Volume**
TCM 199 with HEPES	90 ml
FBS	10 ml

3.1.5.2 Oocyte Maturation media I (10 ml)

Component	**Volume**
TCM-199	9 mL
Foetal bovine serum	1 mL
pFSH	50µg
Penicillin	100 IU/ml
Streptomycin	100 µg/ml
Epidermal growth factor	50 ng/ml

3.1.5.3 Oocyte Maturation media II (10 ml)

Component	**Volume**
TCM-199	9 mL
Fetal bovine serum	1 mL
pFSH	50µg
Penicillin	100 IU/ml
Streptomycin	100 µg/ml
Cysteamine	100 µM/ml

The study was conducted in three phases namely Phase-I: Study the effect of oocyte collection techniques viz. slicing, puncture and aspiration on recovery rate of

oocytes, Phase-II: Effect of follicular size on *in vitro* maturation percentage of oocytes and Phase III: Effect of Cysteamine and epidermal growth factor on rate of *in vitro* maturation of oocytes.

3.2 Phase 1

300 ovaries were collected from local slaughter house and 100 ovaries were subjected to each collection technique viz. slicing, puncture and aspiration.

3.2.1 Collection of Ovaries and their Processing

Goat ovaries (classified into left and right) were brought from municipal slaughter house, Jammu in thermos containing physiological saline (0.9%, w/ v, NaCl) and antibiotic (100 µg/ml streptomycin and 100 IU/ml penicillin) at 37°C within an hour of slaughter (Plate 1). In the laboratory, the surrounding tissue and overlying bursa were removed from each ovary. The ovaries were rinsed by physiological saline and 70% alcohol followed by three washings in DBPS with antibiotics (100 µg/ml streptomycin and 100 IU/ml penicillin). Ovaries were classified for the presence or absence of corpus luteum. The diameter of surface follicles was measured with the help of vernier calliper and accordingly classified into three groups: small (<2 mm), medium (2-4 mm), large (>4 mm) (Dar, 2014). The ovaries were then transferred in a beaker containing holding medium.

3.2.2 Retrieval of Oocytes

Each ovary was handled individually, and oocytes were collected by one of the following methods:

3.2.2.1 Aspiration

A 20-gauge needle was attached to a 5 ml sterile disposable plastic syringe containing 2 ml holding medium to aspirate visible surface follicles on the ovaries (Plate 2A). Aspirated follicular fluid was then transferred to a sterile 90 mm Petri dish.

3.2.2.2 Puncture

A sterile 18-gauge hypodermic needle was used to puncture the whole ovarian surface while the ovary was held totally submerged in holding media in a 90 mm Petri dish (Plate 2B).

3.2.2.3 Slicing

A hemostat was used to attach to the base of ovary to hold it firmly in place and 2-3 mm deep incisions were made throughout the whole ovarian surface, while the ovary was held totally submerged in holding media in a 90 mm Petri dish (Plate 2C).

In all the three harvesting methods, the Petri dishes were left undisturbed for 5 minutes to allow the oocytes to settle. The number of oocytes collected against each approach were recorded using a stereo-zoom microscope.

Based on types of cumulus investments, their compactness and ooplasm granulity, oocytes were graded as good, fair, and poor (Das *et al.,* 1996b).

Good: Those with more than three layers of cumulous cells surrounding the oocyte and uniform granulation of cytoplasm.

Fair: Those with less than three layers of cumulous cells surrounding the oocyte and uniform granulation of cytoplasm.

Poor: Those with no cumulus cells surrounding the oocyte.

The oocytes were washed three times by using washing media.

3.3 Phase II

The effect of size of follicles on *in vitro* maturation percentage of oocytes was studied. For this 300 ovaries were used. Oocytes were retrieved from small (<2mm), medium (2-4mm) and large (>4mm) follicles.TCM-199 +Fetal bovine serum+ FSH + Penicillin-streptomycin were used as the media for *in vitro* maturation of oocytes.

3.3.1 *In vitro* maturation

50 µl IVM droplets were formed in a polystyrene culture dish (3.5 mm x 10 mm), covered with mineral oil, and pre-equilibrated in a Carbon dioxide (CO_2) incubator for a minimum of 2 h at 38.5°C under 5% CO_2. The usable oocytes were washed four times in washing medium, and 10 COCs were added to each droplet and allowed to mature for 27 hours at 38.5°C in a humidified atmosphere with 5% CO_2. Maturation media was prepared freshly before each trial.

3.3.2 Evaluation of *in vitro* maturation

Evaluation of oocytes was done on the basis of cumulous cell expansion and extrusion of 1st polar body (Plate 3).

3.3.2.1 Cumulous cell expansion

After incubation period, the oocytes were evaluated for maturation based on the degree of cumulus cell expansion as per Kobayashi *et al.* (1994) and categorized as:

Degree 0 (D0): No expansion observed

Degree 1(D1): Cumulus cells were homogenously spread, and clustered cells still present.

Degree 2(D2): Cumulus cells were homogenously spread, and clustered cells no longer present.

3.3.2.2 1st polar body extrusion

The oocytes were denuded of the cumulous cell by pipetting with 0.2% hyaluronidase in PBS at 20-22 hr. post maturation.

Under bright field of inverted phase contrast microscope under 200X and 400X, the denuded oocytes were examined for the presence of polar body.

3.4 Phase III

The effect of epidermal growth factor (EGF) and cysteamine on *in vitro* maturation percentage based on cumulus cell expansion and appearance of 1st polar body was studied. A total of 300 usable oocytes were utilized for *in vitro* maturation. Three groups of media were used. First group (n=100 oocytes) containing TCM-199 + FBS + FSH + Penicillin-Streptomycin + EGF media was used for maturation. Second group (n=100 oocytes) containing TCM-199 + FBS + FSH + Penicillin-Streptomycin + Cysteamine media was used for maturation. Third group (n=100 oocytes) containing TCM-199 + FBS + FSH + Penicillin-Streptomycin was kept as control.

3.4.1 Collection of ovaries: as in phase I

3.4.2 Harvesting of oocytes

Procedure was same as in Phase-II and the oocytes were collected by slicing method.

3.4.3 *In vitro* maturation of oocytes

In this phase, the procedure for maturation was same as in Phase-II but, the effect of EGF and Cysteamine supplementation in the maturation medium was studied and compared with the control medium.

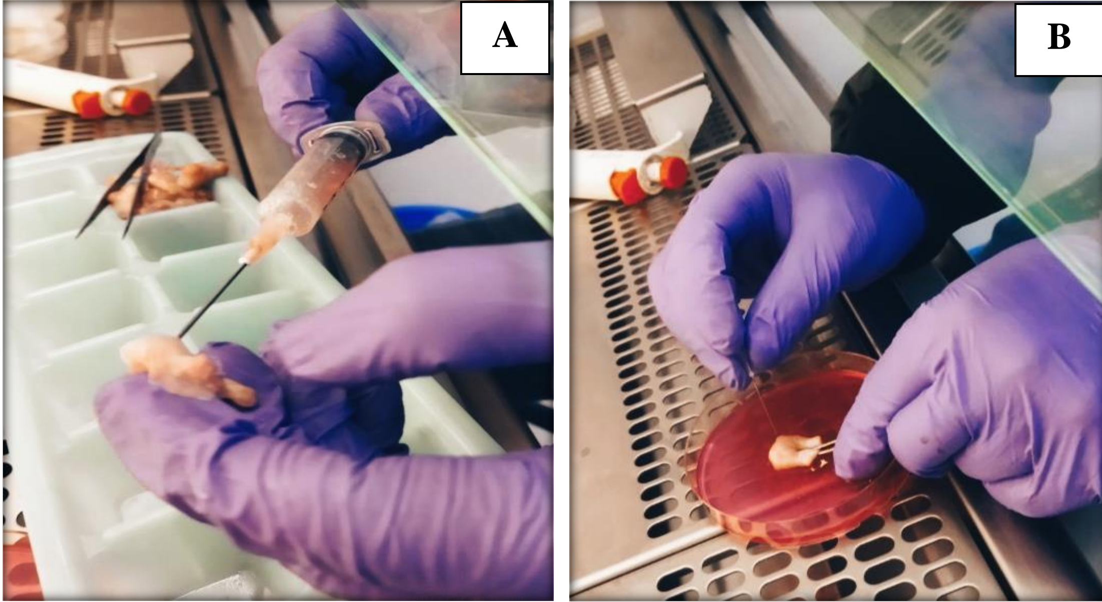
3.5 Statistical analysis

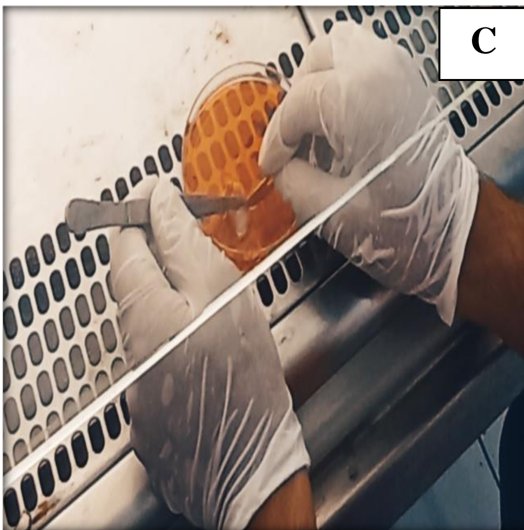
The data in Phase-I and II were analysed by Paired-T test and Chi-Square test with significance determined by Wilcoxon Signed Ranks test at p value less than 0.05 (Software SPSS Version-13). The percentage data in phase-II and III were analysed by simple proportion test at p value less than 0.05.

PLATE-1

Goat ovaries collected from slaughter house

PLATE-2





Different oocyte collection techniques

A) Aspiration technique

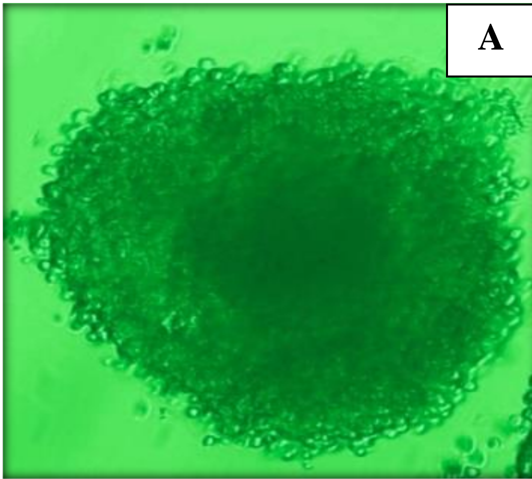
B) Puncture technique

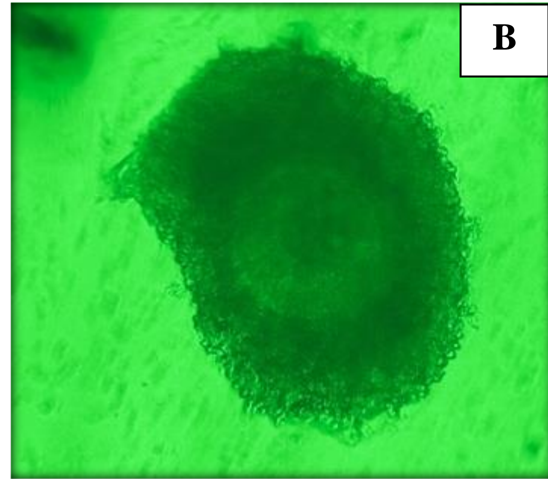
C) Slicing technique

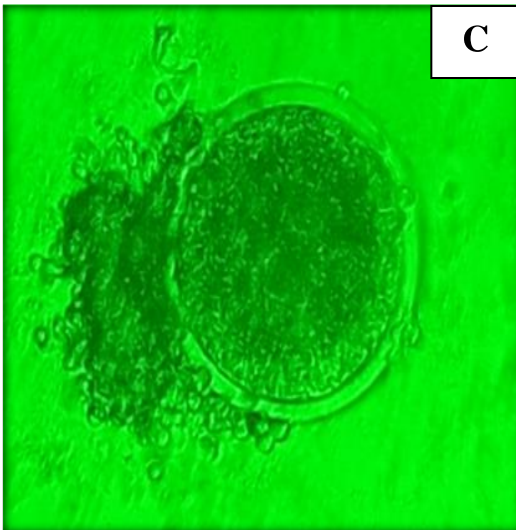
PLATE-3

Evaluation of *in vitro* maturation status of oocytes

PLATE-4







Grading of oocytes

A) Good quality oocytes with many layers of compact cumulus cells

B) Fair quality oocytes with less than three layers of cumulus cells

C) Poor quality oocytes with denuded cumulus cells

Results

CHAPTER – IV

RESULTS

The present study was conducted to know the effect of oocyte harvesting techniques on recovery rate of oocytes along with the effect of follicular size and different additives on *in vitro* maturation (IVM) of follicular oocytes retrieved from abattoir derived goat ovaries. The study was divided into three phases: I) Study the effect of harvesting techniques (Aspiration, puncture, and slicing) on oocyte recovery rate. II) Study the effect of size of follicles on *in vitro* maturation percentage of oocytes. III) Study the effect of EGF and Cysteamine on IVM percentage of oocytes collected from >2mm diameter follicle. The results are shown phase wise as.

4.1 PHASE I

In this phase, the effect of three collection techniques viz; Slicing, Puncture and Aspiration on the recovery rate of good, fair, poor and total oocytes per ovary was studied. A total of 300 ovaries were used in this phase, 100 ovaries were allotted for each collection technique. The oocyte was collected and graded based on quality status as Good, Fair and Poor under stereo-Zoom microscope (Plate 4 A B C).

4.1.1 Effect of oocyte collection techniques on recovery rate of oocytes

The mean number of good, fair, poor and total oocytes recovered per ovary using slicing method was 2.94 ± 0.06, 2.85 ± 0.03, 0.67 ± 0.04 and 6.46 ± 0.11 respectively. The corresponding values were 1.23 ± 0.04, 1.64 ± 0.07, 1.25 ± 0.02 and 4.12 ± 0.14 for puncture and 0.74 ± 0.06, 0.85 ± 0.05, 1.35 ± 0.03 and 2.94±0.13 for aspiration method (Table 1 and Fig.1). The number of good, fair and total oocytes collected per ovary by slicing and puncture was significantly higher ($p<0.05$) as compared to aspiration technique. The recovery of poor oocytes was significantly higher in case of aspiration and puncture technique than slicing technique. Comparatively, slicing method yielded a higher number of good, fair, and total oocytes in comparison to other two methods. The mean number of poor-quality oocytes was significantly higher ($P<0.05$) than good and

fair quality oocytes in aspiration technique. The mean number of fair quality oocytes was significantly higher ($P<0.05$) than good and poor-quality oocytes in puncture technique. The mean number of good and fair quality oocytes was significantly higher ($P<0.05$) than poor quality oocytes in slicing technique.

Table 1: Mean number of good, fair, poor quality and total oocytes harvested by aspiration, puncture and slicing techniques from goat ovaries

S. No.	Technique	No. of ovaries	No. of oocytes recovered	Oocyte quality			
				Good	Fair	Poor	Total
1	Aspiration	100	294	0.74 ± 0.06^{aA} (25.21%)	0.85 ± 0.05^{aA} (28.87%)	1.35 ± 0.03^{bC} (45.92%)	2.94 ± 0.13^{a}
2	Puncture	100	412	1.23 ± 0.04^{bA} (29.85%)	1.64 ± 0.07^{bB} (39.81%)	1.25 ± 0.02^{bA} (30.34%)	4.12 ± 0.14^{b}
3	Slicing	100	646	2.94 ± 0.06^{cB} (45.52%)	2.85 ± 0.03^{cB} (44.21%)	0.67 ± 0.04^{aA} (10.27%)	6.46 ± 0.03^{c}
Overall		300	1352	1.64±0.04	1.78±0.03	1.09±0.04	4.51±0.08

Means with superscripts a, b, c within a column differ significantly at $P<0.05$
Means with superscripts A, B, C differ significantly row-wise at $P<0.05$
Figures in parentheses indicate percentage

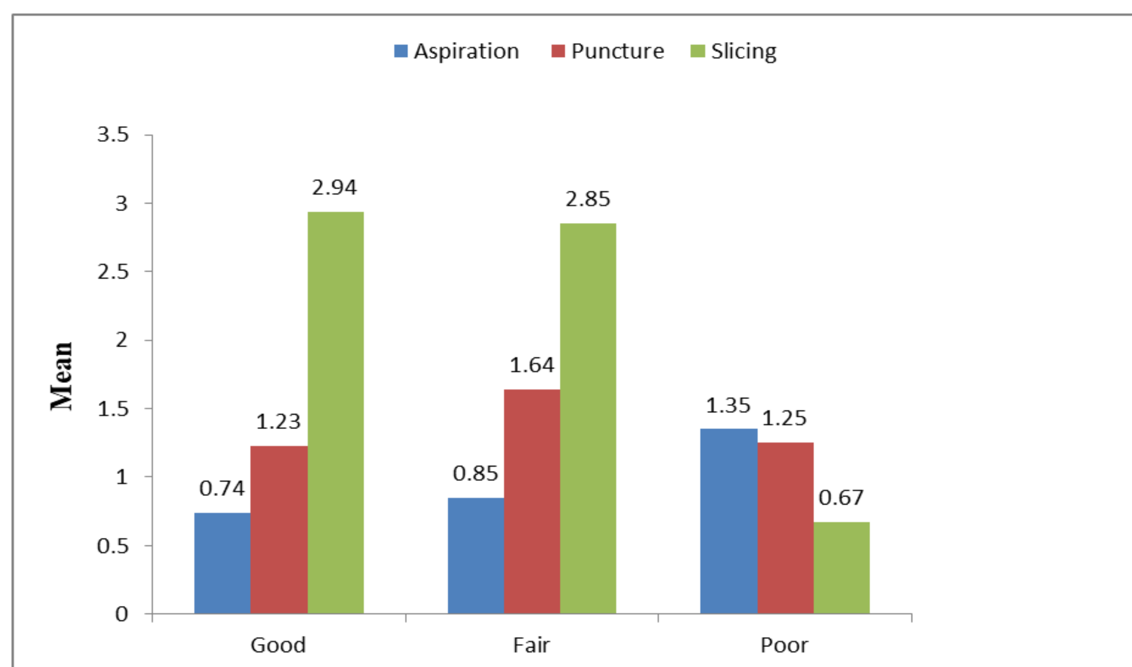


Fig. 1: Mean number of good, fair, poor quality and total oocytes harvested by aspiration, puncture and slicing techniques from goat ovaries

4.1.2 Effect of season on the recovery rate of oocytes

The mean number of good, fair, poor and total oocytes recovered in winter season was 1.76 ± 0.05, 2.14 ± 0.03, 1.26 ± 0.05 and 5.16 ± 0.15 respectively and in case of summer season was 1.04 ± 0.02, 1.32 ± 0.04, 1.51 ± 0.04 and 3.94 ± 0.11 respectively (Table 2 and Fig. 2). The number of good, fair, and total oocyte recovered in winter season was significantly higher than the number of good, fair, and total oocytes recovered in summer season. The mean number of poor-quality oocytes was significantly higher (P<0.05) than good and fairy quality oocytes in summer season, but the mean number of fair quality oocytes was significantly higher (P<0.05) than good and poor-quality oocytes in winter season.

Table 2: Mean number of good, fair, poor quality and total oocytes recovered from goat ovaries during winter and summer season

S. No.	Season	No. of ovaries	No. of oocytes recovered	Oocyte quality			
				Good	Fair	Poor	Total
1	Winter	143	737	1.76 ± 0.05^{bB} (34.11%)	2.14 ± 0.03^{bC} (41.48%)	1.26 ± 0.05^{aA} (24.41%)	5.16 ± 0.15^{b}
2	Summer	157	615	1.04 ± 0.02^{aA} (26.54%)	1.32 ± 0.04^{aB} (33.69%)	1.51 ± 0.04^{bC} (39.77%)	3.87 ± 0.11^{a}
Overall		300	1352	1.40±0.05	1.73±0.06	1.38±0.04	4.51±0.08

Means with superscripts a, b, c within a column differ significantly at P<0.05
Means with superscripts A, B, C differ significantly row-wise at P<0.05
Figures in parentheses indicate percentage

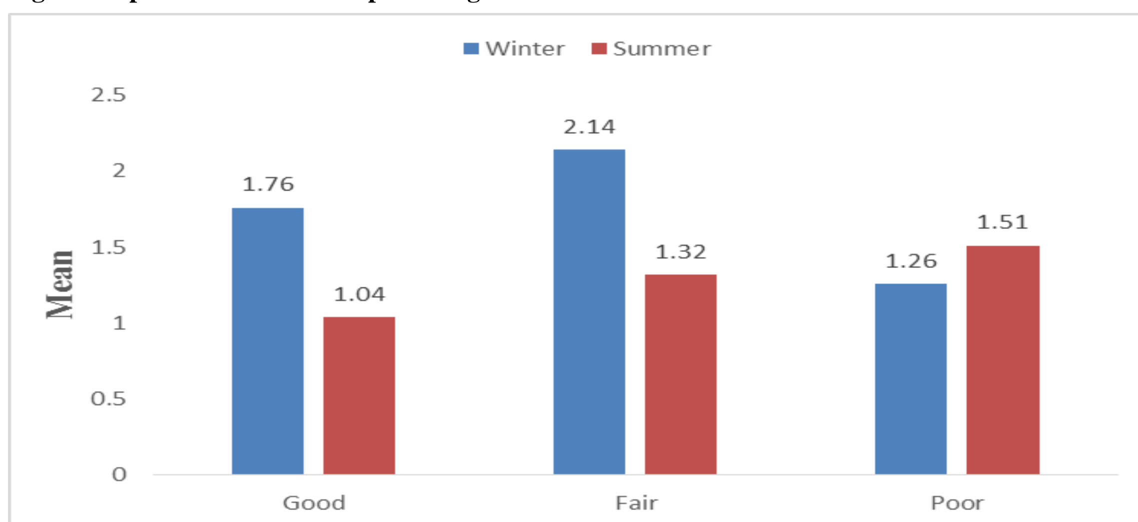


Fig. 2: Mean number of good, fair, poor quality and total oocytes recovered from goat ovaries during winter and summer season

4.1.3 Recovery rate of oocytes from right and left ovaries

The recovery rate of oocytes from left (n=141) and right (n=159) ovaries is shown in Table-3 and Fig.3. The number of good, fair, poor and total oocytes recovered from left ovaries (1.51 ± 0.01, 1.73 ± 0.01, 1.40 ± 0.02, 4.64 ± 0.02) did not differ significantly than right ovaries (1.36 ± 0.02, 1.79 ± 0.01, 1.23 ± 0.01, 4.38 ± 0.03). The mean number of fair quality oocytes was significantly higher ($p<0.05$) than good and poor-quality oocytes in right and left sided ovary.

Table 3: Mean number of good, fair, poor quality and total oocytes recovered from left and right goat ovaries

S. No.	Side of ovary	No. of ovaries	No. of oocytes recovered	Oocyte quality			
				Good	Fair	Poor	Total
1	Left	141	654	$1.51{\pm}0.01^{aA}$ (32.54%)	$1.73{\pm}0.01^{aB}$ (37.28%)	$1.40{\pm}0.02^{aA}$ (30.18%)	$4.64{\pm}0.02^{a}$
2	Right	159	698	$1.36{\pm}0.02^{aA}$ (30.97%)	$1.79{\pm}0.01^{aB}$ (40.77%)	$1.23{\pm}0.01^{aA}$ (28.26%)	$4.38{\pm}0.03^{a}$
Overall		300	1352	1.43±0.03	1.76±0.04	1.31±0.03	4.51±0.08

Means with superscripts a, b, c within a column differ significantly at P<0.05
Means with superscripts A, B, C differ significantly row-wise at P<0.05
Figures in parentheses indicate percentage

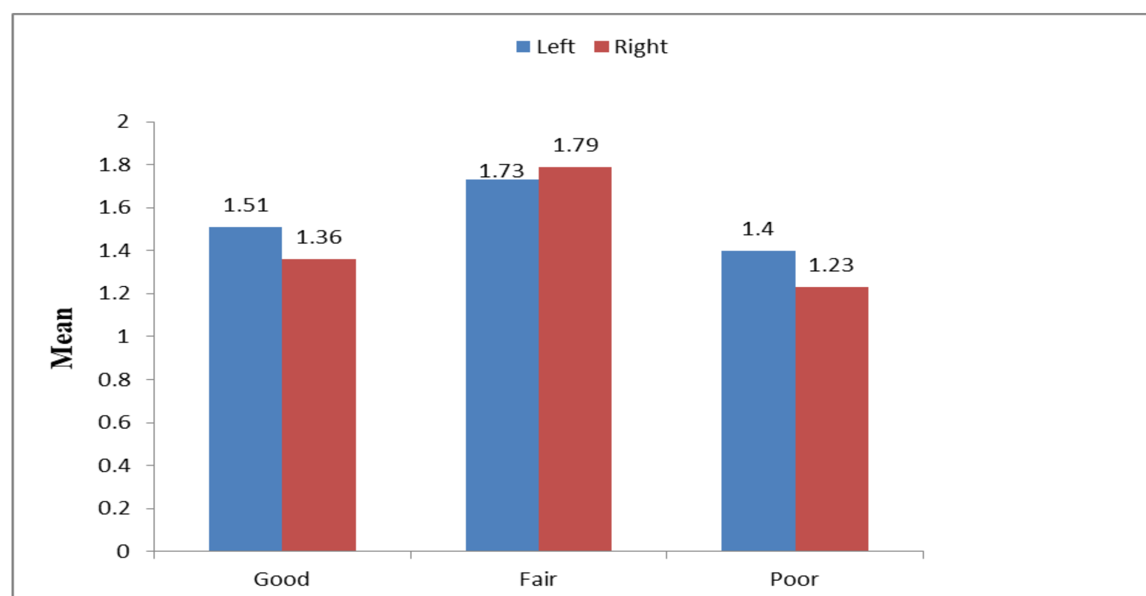


Fig. 3: Mean number of good, fair, poor quality and total oocytes recovered from Right and Left goat ovaries

4.1.4 Effect of corpus luteum on recovery rate of oocytes

The mean number of oocytes collected per ovary from ovaries containing corpus luteum (n=113) and without corpus luteum (n=187) is presented in table 4 and Fig. 4. The number of good, fair, poor and total oocytes recovered per ovary from ovaries without corpus luteum was significantly higher (2.11 ± 0.05, 2.05 ± 0.06, 1.62 ± 0.07, 5.78 ± 0.11: p <0.05) than ovaries containing corpus luteum (0.79 ± 0.05, 1.26 ± 0.06, 1.19 ± 0.04, 3.24 ± 0.08). The recovery of fair and poor-quality oocytes was significantly higher (P<0.05) as compared to good quality oocytes in ovaries having CL, however the mean fair and poor-quality oocytes did not differ significantly with each other. The mean number of good and fair quality oocytes recovered from ovaries without CL was significantly higher (p<0.05) as compared to poor-quality oocytes.

Table 4: Mean number of good, fair, poor quality and total oocytes recovered from goat ovaries with or without Corpus luteum

S. No.	Ovary Characteristic	No. of ovaries	No. of oocytes recovered	Oocyte quality			
				Good	Fair	Poor	Total
1	With CL	113	366	0.79 ± 0.05^{aA} (24.38%)	1.26 ± 0.06^{aB} (38.89%)	1.19 ± 0.04^{aB} (36.73%)	3.24 ± 0.08^{a}
2	Without CL	187	986	2.11 ± 0.05^{bB} (36.50%)	2.05 ± 0.06^{bB} (35.45%)	1.62 ± 0.07^{bA} (28.05%)	5.78 ± 0.11^{b}
Overall		300	1352	1.45±0.04	1.65±0.05	1.40±0.05	4.51±0.08

Means with superscripts a, b, c within a column differ significantly at P<0.05

Means with superscripts A, B, C differ significantly row-wise at P<0.05

Figures in parentheses indicate percentage

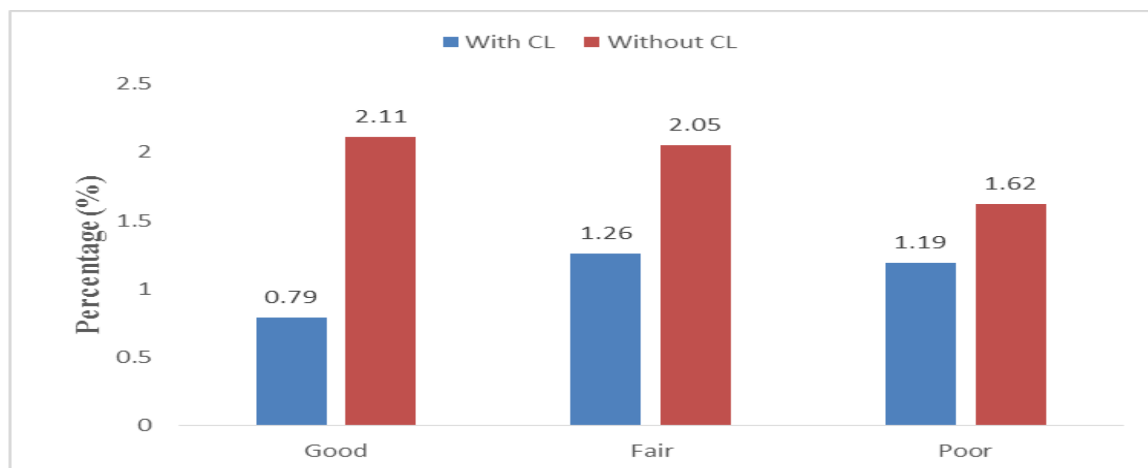


Fig. 4: Mean number of good, fair, poor quality and total oocytes recovered from goat ovaries with or without Corpus luteum

4.2 PHASE II

4.2.1 Effect of follicular size on *in vitro* maturation of oocytes

In this phase, the study was conducted to evaluate the effect of size of follicle on the rate of *in vitro* maturation of oocytes. A total of 300 ovaries were used in this phase to collect oocytes by puncturing of small, medium, and large follicles separately in 35 mm petri dishes. The pooling of oocytes from small and large follicles was done on each day separately to evaluate the rate of maturation percentage of respective oocytes. In this phase, 140, 170 and 190 oocytes retrieved from small, medium, and large follicles respectively were matured. The *in vitro* maturation percentage of oocytes based on cumulus cell expansion (Plate 5) is given in Table 5 and Fig. 5 and based on 1st polar body extrusion (Plate 6) is given in Table 6 and Fig. 6. The *in vitro* maturation percentage of oocytes based on degree 2 cumulous cell expansion was significantly higher ($p<0.05$) for large follicles (64.73%) than medium (46.47%) and small follicles (43.57%). However, oocytes harvested from small and medium sized follicles showed higher percentage of D0 (25.00%, 14.70%) and D1 (38.57%, 38.82%) degree of maturation as compared to corresponding values for oocytes collected from large follicles (D0=12.10%, D1=23.16%). The *in vitro* maturation percentage of oocytes based on 1st polar body extrusion was significantly higher ($p<0.05$) for large follicles (41.58%) than medium (28.23%) and small follicles (28.57%).

Table 5: IVM percentage of oocytes collected from small, medium, and large follicles based on cumulous cell expansion

S. No.	Size of follicle	Total oocytes	Degree of cumulous cell expansion (%)		
			D0	D1	D2(mature)
1	Small (≤2mm diameter)	140	35 (25.00^{b})	54(38.57^{b})	61 (43.57^{a})
2	Medium (2-4mm diameter)	170	25 (14.70^{a})	66 (38.82^{b})	79 (46.47^{a})
3	Large (>4mm diameter)	190	23 (12.10^{a})	44 (23.16^{a})	123 (64.73^{b})

Percentages with in same column with different superscript differ significantly ($P<0.05$)

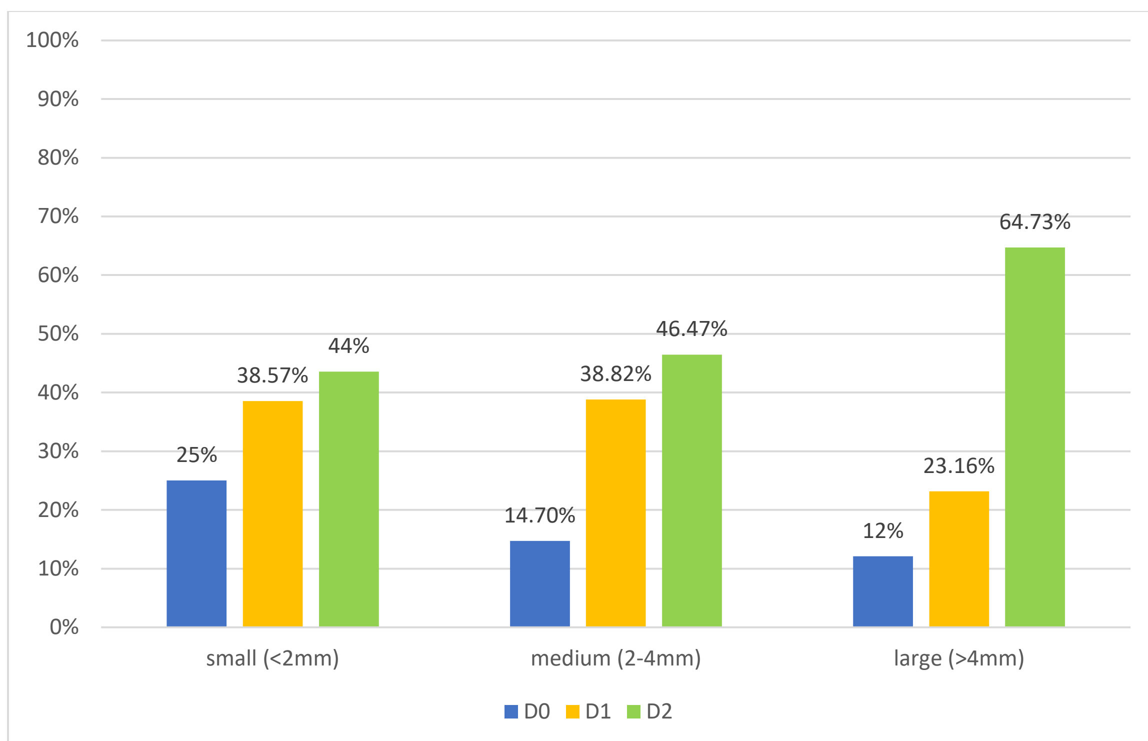


Fig. 5: IVM percentage of oocytes collected from small, medium and large follicles based on cumulous cell expansion

Table 6: IVM percentage of oocytes collected from small, medium, and large follicles based on 1st polar body extrusion

S. No.	Size of follicle	Total oocytes	Matured Oocytes (%)	1st Polar body extrusion (%)
1	Small (≤2mm diameter)	140	61 (43.57^{a})	40 (28.57^{a})
2	Medium (2-4mm diameter)	170	79 (46.47^{a})	48 (28.23^{a})
3	Large (>4mm diameter)	190	123 (64.73^{b})	79 (41.58^{b})

Percentages within same column with different superscript differ significantly (P<0.05)

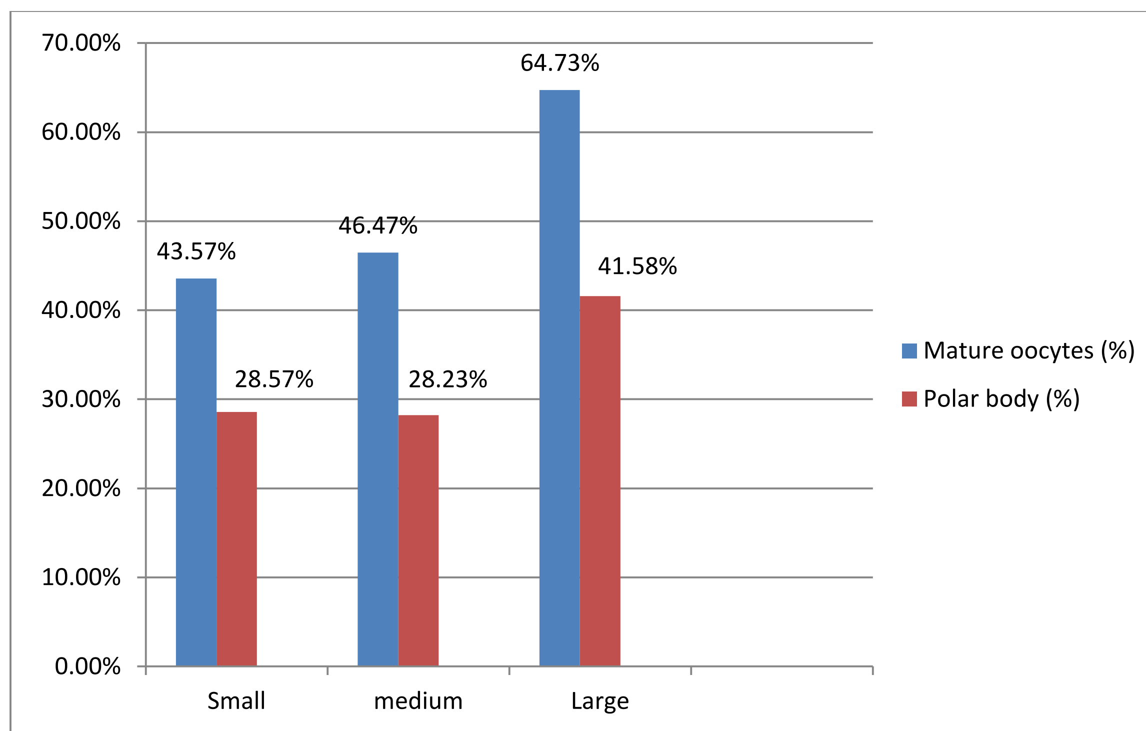


Fig. 6: IVM percentage of oocytes collected from small, medium and large follicles based 1st polar body extrusion

4.3 PHASE III

4.3.1 Effect of Epidermal growth factor and Cysteamine on *in vitro* maturation percentage of oocytes

In this phase, the study was done to evaluate the effect of epidermal growth factor and Cysteamine on the maturation percentage of goat oocytes. The oocytes were collected by Slicing technique. The usable oocytes harvested using slicing method were pooled and allotted to three maturation media viz. Group 1, Group 2 and Group 3(control) media. The stage of maturation was assessed based on cumulus cell expansion as D0, D1 and D2 under inverted phase contrast microscope at 200-400x magnification with D2 degree of expansion considered as mature oocytes. The extrusion of 1st Polar body was also used for assessment of the *in vitro* maturation of oocytes. During this study period, a total of 300 oocytes were assessed out of which 102 oocytes were allotted to Group 1, 105 to group 2 and 93 to Group 3 (control). The results based on cumulous cell expansion (Plate 7) and 1st polar body extrusion (Plate 8) are presented in Table 7 and

Fig. 7 and Table 8 and Fig. 8, respectively. The maturation percentage based on cumulous cell expansion (D2) for EGF containing Group 1, Cysteamine containing Group 2 and control (Group 3) was 69.61, 48.57 and 46.23 percent respectively. The degree of cumulus cell expansion (D2) was significantly higher ($p<0.05$) for EGF containing Group 1 than Cysteamine containing Group 2 and control (Group 3). The maturation percentage based on 1st polar body extrusion for EGF (Group1), Cysteamine (Group2) and control (Group 3) was 46.07, 31.41 and 29.03 percent respectively.

Table 7: IVM percentage of oocytes in three maturation media based on cumulous cell expansion

S. No.	Medium used	Total Oocytes	Degree of cumulous cell expansion (%)		
			D0	D1	D2(mature)
1	Group 1(EGF)	102	10(9.8^{a})	21(20.59^{a})	71(69.61^{b})
2	Group 2 (Cysteamine)	105	20(19.04^{b})	34(32.38^{b})	51(48.57^{a})
3	Group 3 (Control)	93	21(22.58^{b})	29(31.18^{b})	43(46.23^{a})

Percentages within same column with different superscript differ significantly (P<0)

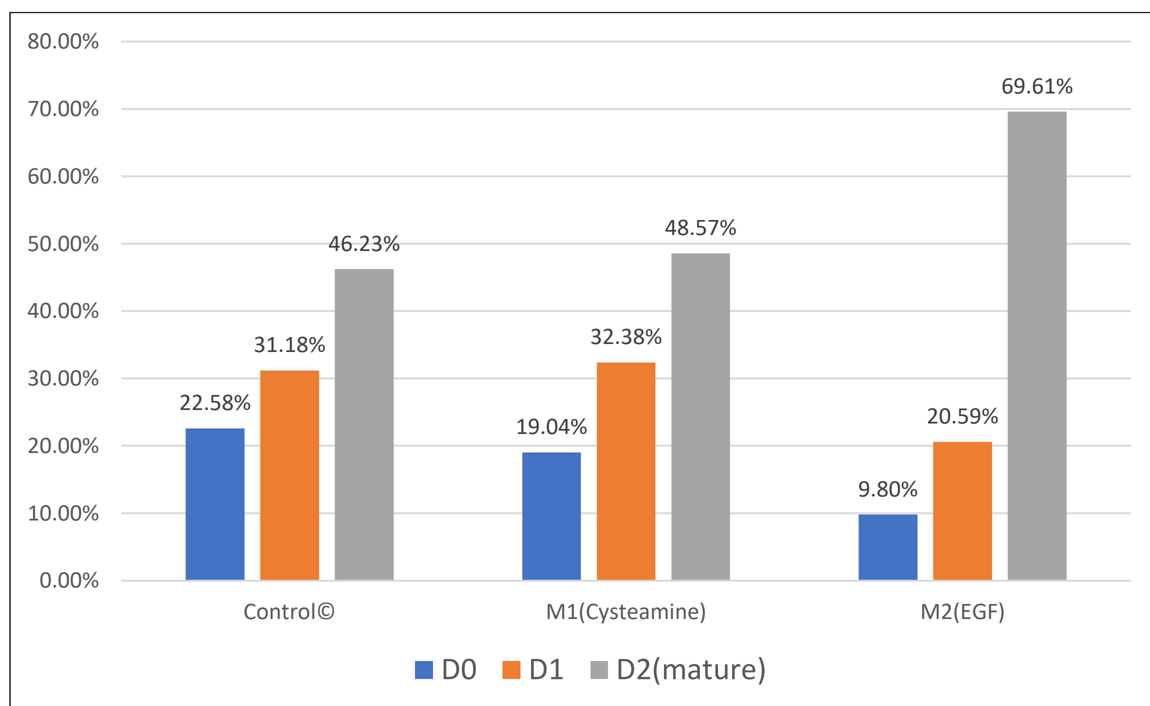


Fig. 7: IVM percentage of oocytes in three maturation media based on cumulus cell expansion

Table 8: IVM percentage of oocytes in three maturation media based on 1st polar body extrusion

S. No.	Medium used	Total oocytes	Matured Oocytes (%)	1st Polar body extrusion (%)
1	Group 1(EGF)	102	71(69.61^{b})	47(46.07^{b})
2	Group 2 (Cysteamine)	105	51(48.57^{a})	33(31.41^{a})
3	Group 3 (Control)	93	43(46.23^{a})	27(29.03^{a})

Percentages within same column with different superscript differ significantly ($P<0.05$)

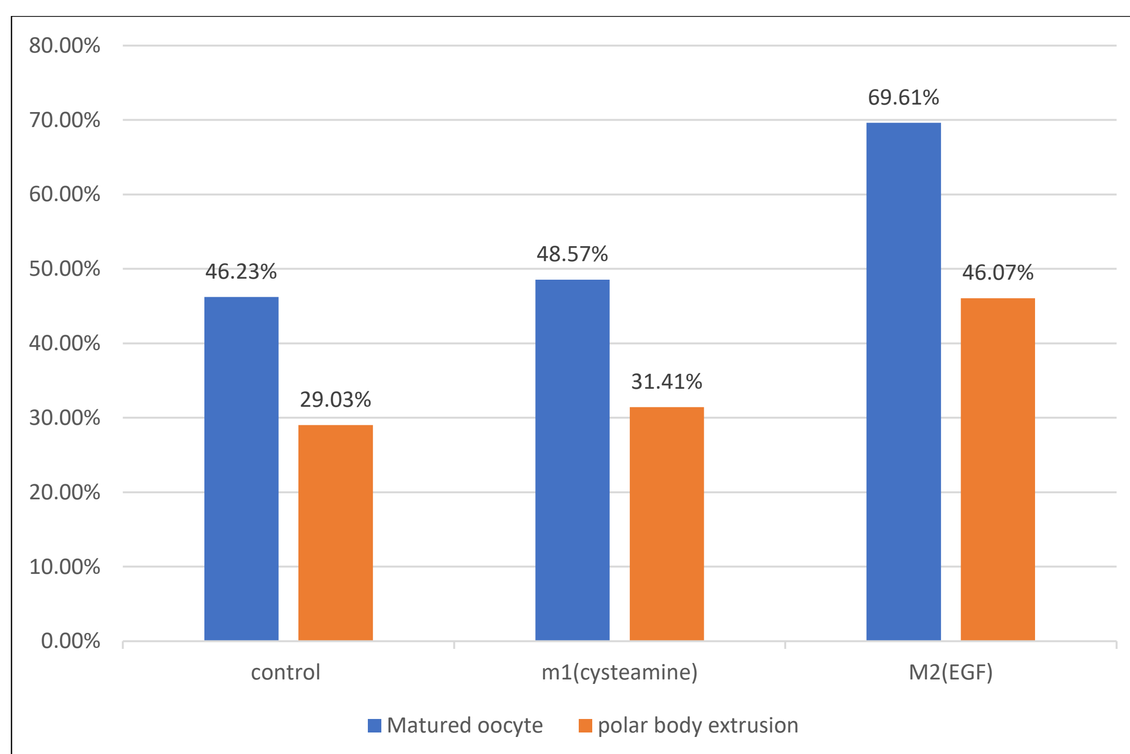
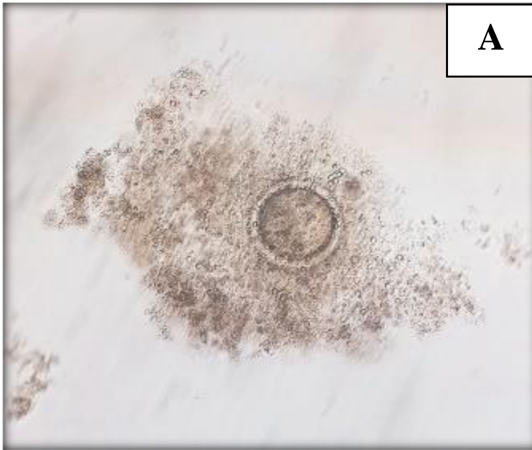
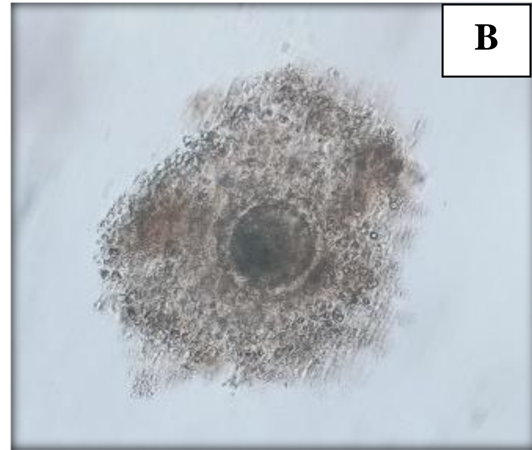
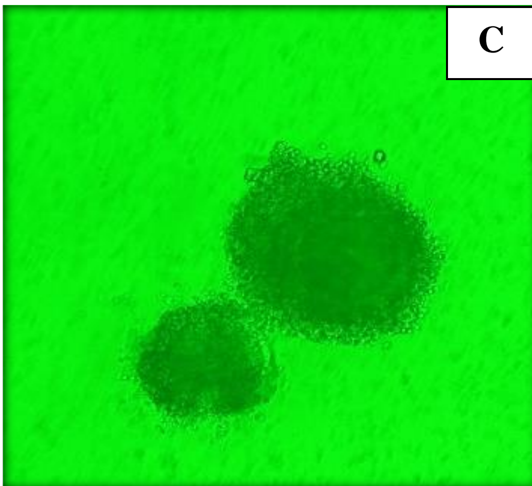


Fig. 8: IVM percentage of oocytes in three maturation media based on 1st polar body extrusion

PLATE-5







***In vitro* maturation on the basis of cumulous cell expansion (Phase II)**

A) Degree 2 cumulus cell expansion

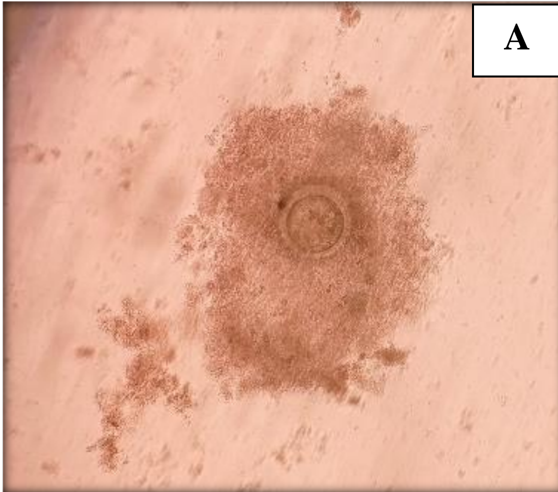
B) Degree 1 cumulus cell expansion

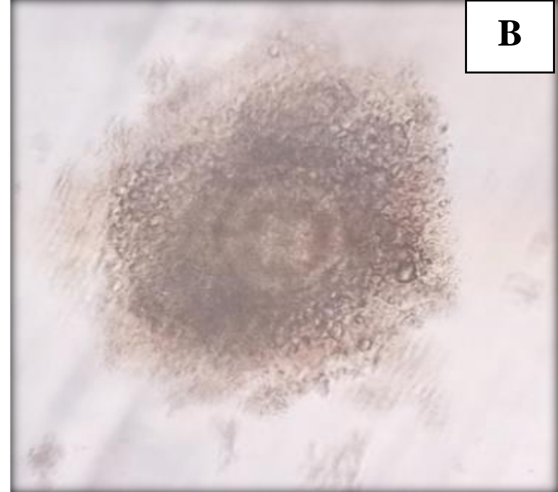
C) Degree 0 cumulus cell expansion

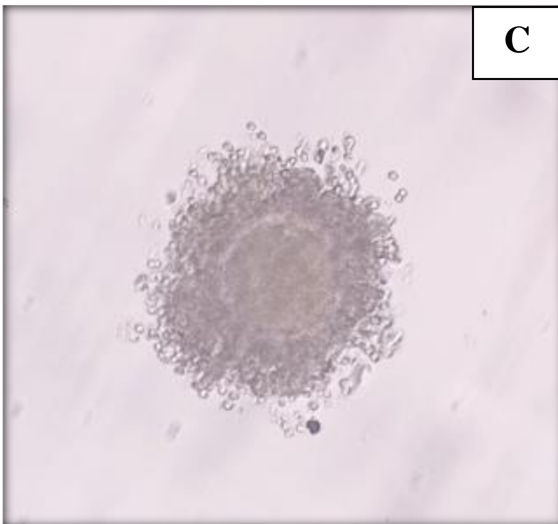
PLATE-6

Presence of 1st polar body extrusion after *in vitro* maturation (Phase II)

PLATE-7







***In vitro* maturation on the basis of cumulous cell expansion (Phase III)**

A) Degree 2 cumulus cell expansion

B) Degree 1 cumulus cell expansion

C) Degree 0 cumulus cell expansion

PLATE-8

Presence of 1st polar body after *in vitro* maturation of oocytes (Phase III)

Chapter-V

Discussion

CHAPTER – V

DISCUSSION

The study was divided into three phases. In phase-I, the effect of harvesting techniques (Aspiration, puncture, and slicing) on oocyte recovery rate was studied. In phase-II, the effect of size of follicles on *in vitro* maturation percentage of oocytes was studied and in phase-III, the effect of EGF and Cysteamine on IVM percentage of oocytes was studied.

5.1 PHASE I

5.1.1 Effect of oocyte collection techniques on recovery rate of oocytes

An important aim of oocyte collection method from the ovaries collected from slaughterhouse is to maximize the total number of oocytes and the yield of good quality oocytes at low cost, which can be used for IVM, IVF and IVC. In the present study the overall mean number of total oocytes recovered per ovary was 4.51 ± 0.08. The overall mean number of good, fair, and poor-quality oocytes recovered per ovary were 1.64 ± 0.04, 1.78 ± 0.03 and 1.09 ± 0.04 respectively. The mean oocyte recovery recorded in the present study is in close agreement with the studies of Pawshe *et al.* (1994), Wang *et al.* (2007a), Hoque *et al.* (2011), Singh *et al.* (2013), Rameez *et al.* (2017) and Nuaimi *et al.* (2020) in goats. On contrary to present result, Wani *et al.* (1999) in sheep, Rahman *et al.* (2009) in goat have reported relatively higher whereas, Nagar and Purohit (2005) and Arangasamy *et al.* (2008) reported relatively lower mean recovery rate in goat and buffalo.

The number of good, fair, poor and total oocytes recovered per ovary using slicing method was 2.94 ± 0.06, 2.85 ± 0.03, 0.67 ± 0.04 and 6.46 ± 0.11 respectively. The corresponding values were 1.23 ± 0.04, 1.64 ± 0.07, 1.25 ± 0.02, 4.12 ± 0.14 for puncture and 0.74 ± 0.06, 0.85 ± 0.05, 1.35 ± 0.03, 2.94 ± 0.13 for aspiration method. Comparatively, the recovery of good, fair, and total oocytes was significantly higher ($p<0.05$) for slicing than puncture and aspiration technique. Similar findings have been

reported by Das *et al.* (1996a), Wang *et al.* (2007a) and Singh *et al.* (2013) in goats. The recovery of poor oocytes was significantly higher in slicing technique than aspiration and puncture technique. Slicing technique produces more debris and required more washings. As a result, a number of COCs become denuded from cumulus cells due to repeated washing and ultimately result in a lower number of normal COCs (Mahesh *et al.,* 2014). Higher oocyte recovery in slicing technique may be due to release of oocytes from both surface as well as from deeper cortex (Das *et al.,* 1996a). Furthermore, in case of slicing, incisions are given along the whole ovarian surface using a scalpel blade in which all sizes of surface follicles are harvested (Rahman *et al.,* 2016).

The higher recovery rate of good, fair and total oocytes by puncture than aspiration technique (Rahman *et al.*, 2016) is in accordance with present study. The higher oocyte yield in puncture method compared to aspiration in the present study might be due to extra pressure applied during puncture which may release oocytes from small and medium sized follicles (Rao and Mahesh, 2012). The lower recovery rate of good, fair and total oocytes by aspiration may be because of the fact that oocytes remain firmly attached to the small and medium sized follicles before cumulus expansion and cannot be aspired, but can be easily recovered from the small follicles through slicing method (Wani *et al.,* 2000).

In contract to present findings, a significantly higher recovery rate of total oocyte per ovary for aspiration compared to puncture or slicing technique was reported in goat (Pawshe *et al.,* 1994); in sheep (Shirazi *et al.,* 2005) and in prepubertal goats (Majeed *et al.*, 2011). On the other hand, Hoque *et al.* (2011) observed that puncture technique is superior to slicing and aspiration techniques in terms of oocyte recovery. The lower recovery rate of total and usable oocytes by aspiration may be due to the fact that oocytes remain firmly attached to the small and medium sized follicles before cumulus expansion and cannot be aspired, but can be easily recovered from the small follicles through slicing method (Wani *et al.,* 2000).

5.1.2 Effect of season on the recovery rate of oocytes

The mean number of good, fair, poor and total oocytes recovered in winter and summer season were 1.76 ± 0.05, 2.14 ± 0.03, 1.26 ± 0.05, 5.16 ± 0.15 and 1.04 ± 0.02, 1.32 ± 0.04, 1.51 ± 0.04, 3.87 ± 0.11 respectively. The number of good, fair, and total oocytes recovered in winter season was significantly higher ($p<0.05$) than the number of good, fair and total oocytes recovered in summer season. The present results are comparable to the findings of Dode *et al.* (2001) in cattle, Zoheir *et al.* (2007) and El-Naby *et al.* (2013) in buffalo. The mean number of poor-quality oocytes was significantly higher ($P<0.05$) than good and fairy quality oocytes in summer season but the mean number of fair quality oocytes was significantly higher ($P<0.05$) than good and poor-quality oocytes in winter season.

The present findings are in accordance with the previous study of Dar (2014) who recorded mean good (1.94±0, 07), fair (2.21±0.07) and total (5.38±0.13) oocytes in winter season. Hussain *et al.* (2005) also reported significantly higher ($P<0.05$) recovery of average number of oocytes in winter in both cattle (4.4±0.07 vs 3.41±0.15) and buffaloes (2.2±0.1 vs 1.6±0.16) than summer. In accordance with the results of the present study, Raj *et al.* (2016) in crossbred cows reported significantly higher good quality oocytes in winter season as compared to summer season.

Summer has a detrimental effect on both recovery and quality of oocytes (Samad and Raza, 1999; Jamil *et al.,* 2008; Manjunatha *et al.*, 2009a) and Davachi *et al.* (2014) as oocyte function is compromised during oogenesis by heat stress leading to recovery of more poor and fair quality oocytes in summer season. In goats, heat stress leads to reduced plasma concentrations of oestradiol and lowers follicular oestradiol concentration, aromatase activity and LH receptor level, and delays ovulation (Ozawa *et al.,* 2005).

On contrary to present study, Cognie (1999) in sheep reported that the oocyte recovered per ovary and the number and incidence of good quality oocytes were not affected by season. Tasripoo *et al.* (2013) in buffalo also did not observe any seasonal (summer, rainy and winter) effects on the number of oocytes. Sarkar *et al.* (2020) in their

studies on comparative seasonal assessment on the quality of Black Bengal goat oocytes revealed that the average number of follicles and oocytes recovered were higher in the summer than in the winter, with grade A and B oocytes recovery more in the summer than in the winter, and grade C and D oocytes recovery faster in the winter than in the summer.

Variations in the oocyte yield among different studies are due to differences in geographical location in relation to the status of animals slaughtered, season of ovary collection, number of ovaries processed and techniques used by different workers and criteria for selecting ovaries at slaughterhouse (Sharma and Loganathasamy, 2007). In addition, environmental factors other than photoperiod e.g., feed availability, rainfall, temperature and humidity variations may affect the breeding season (Prasad and Bhattacharyya, 1979).

5.1.3 Recovery rate of oocytes from right and left ovaries

The mean number of good, fair, poor, and total oocytes yield did not differ significantly between right and left sided ovaries. The mean number of fair quality oocytes was significantly higher ($p<0.05$) than good and poor-quality oocytes in right and left sided ovary. The present findings are in accordance with earlier reports by Singh *et al.* (2013) and Mahesh *et al.* (2013) in goats and Talukder *et al.* (2011) in sheep.

However, the present study contradicts with the reports of Alsafy and El-Shahat (2011) and Majeed and Adel (2015) in sheep who recovered significantly higher ($P<0.05$) number of oocytes from right than left ovaries. Asad *et al.* (2016) in goat recorded significantly higher ($P<0.05$) number of normal oocytes (1.18±0.10) in left than right ovaries (1.11±0.09).

5.1.4 Effect of corpus luteum on recovery rate of oocytes

The mean number of good, fair, poor and total oocytes yield in ovaries without corpus luteum (2.11 ± 0.05, 2.05 ± 0.06, 1.62 ± 0.07, 5.78 ± 0.11) were significantly higher ($P<0.05$) than ovaries with corpus luteum (0.79 ± 0.05, 1.26 ± 0.06, 1.19 ± 0.04 3.24 ± 0.08). The recovery of fair and poor-quality oocytes was significantly higher

(P<0.05) as compared to good quality oocytes in ovaries having CL, however the mean fair and poor-quality oocytes did not differ significantly with each other. The mean number of good and fair quality oocytes recovered from ovaries without CL was significantly higher (p<0.05) as compared to poor-quality oocytes.

The present findings are in accordance with the studies of Talukder *et al.* (2011) in sheep; Rao and Mahesh (2012), Jamil *et al.* (2008) and Mahesh *et al.*, (2014) in buffalo; Wani *et al.* (1999), Islam *et al.* (2007), Mahesh *et al.* (2013) and Dar (2014) in goat who observed significantly higher number of good, fair and usable oocyte in without CL ovary than with CL ovary. Wani *et al.* (1999) indicated that the significantly lower yield of oocytes from ovary with a CL than ovary without CL (p<0.05) might be due the possibility of animal being pregnant, infertile or in diestrus phase in which corpus luteum covers the major portion of ovary. The presence of CL decreases the oocyte yield per ovary which may be due to the fact that major portion of ovary is occupied by lutein cells (Alsafy and El-Shahat, 2011). Hafez (1993) reported that a CL inhibits the growth of follicles and increases their atresia which may be a secondary factor responsible for lower oocyte recovery from ovaries with CL, since significantly reduced follicular population in ovaries with corpus luteum.

However, there was no significant differences in the developmental competence of oocytes collected from follicles in the presence or absence of the corpus luteum by Vassena *et al.* (2003). Sahoo and Singla (2013) also reported that recovery rate of different grades of oocytes from ovaries containing corpus luteum does not differ from ovaries without corpus luteum. Varisanga *et al.* (1998) in contrast to present study found significantly higher (p<0.01) oocyte yield from CL containing ovaries than those without CL in bovines.

5.2 PHASE II

5.2.1 Effect of follicular size on *in vitro* maturation percentage of oocytes

In vitro maturation is highly affected by follicular size and quality of oocytes. Only meiotically competent oocytes can be used for IVM, which limits the number of embryos produced from a particular donor. The *in vitro* maturation percentage of oocytes

based on degree 2 cumulous cell expansion was significantly higher ($p<0.05$) for oocytes retrieved from large follicles (64.73%) than medium (46.47%) and small follicles (43.57%) in the present study.

Similar findings were observed by Wani *et al.* (2013) who found the maturation percentage of 52.44 and 85.20 percent for oocytes collected from small (<2mm) and large (≥2mm) diameter follicles based on cumulous cell expansion in sheep. Majeed *et al.* (2019) observed the maturation percentage for small (2-4 mm) and large (5-8 mm) follicles to be 31.05 and 46.99 percent in goats. Qian *et al.* (2001) observed that rate of porcine oocytes with expanded cumulus cells collected from antral follicles of size 5 mm, 4-4.9 mm, 3-3.9 mm and 2-2.9 mm diameter was 90.5, 89.7, 85.4 and 67.4 percent. Alsaadoon *et al.* (2021) observed that the maturation percentage of sheep oocytes harvested from small, medium and large follicles was 18.50, 29.57 and 38 percent respectively. The present results are in accordance with the findings of El- Ratel *et al.* (2017) who observed that the maturation percentage of maturation percentage of bovine oocytes retrieved from small follicles (≤8mm) and large follicles (>8mm) was 62.50 and 78.99 percent respectively. The present results contrast with the findings of Sarwar *et al.* (2020) who reported that the maturation percentage of oocytes harvested from 2-6 mm follicles and >6 mm follicles did not differ significantly and was 92.2 percent and 92.9 percent respectively.

The *in vitro* maturation percentage of oocytes based on 1st polar body extrusion was significantly higher ($p<0.05$) for large follicles (41.85%) than medium (28.57%) and small follicles (26.70%). The present results are in accordance with the findings by Crozet *et al* (1995) who observed the percentage of oocytes reaching M II stage from small (2-3 mm), medium (3.1-5 mm) and large (> 5mm) to be 70, 83 and 97 percent, respectively in goat. The percentage of oocytes reaching M-II stage after 28h of maturation was 73 and 87 percent collected from small and large follicles in camel (Khatir *et al.,* 2007). The lower maturation percentage observed in small follicle oocytes (42.44%) in the present study may be due to species difference. Iwata *et al.* (2004) reported the attainment of M-II stage after 18 hours of maturation of bovine oocytes was

34 and 65 percent for small and large follicles respectively, which in close agreement with the current study.

The developmental stage of the follicle and growth of the oocyte go hand in hand. Sirard *et al.* (2006) stated that follicular size greatly affects the quality of oocyte obtained during ovulation and quality of embryo obtained. Follicular size therefore influences the oocyte quality, potentially involving mRNA or protein stores as factors involved in determing the oocyte competence (Krisher, 2004). The oocyte diameter is directly related to the diameter of follicle and oocyte continue to grow, even in follicles having a diameter of >10 mm (Arlotto *et al.*, 1996). Studies have shown that oocyte having a diameter of less than 110 µm are still in the growth phase (Fair *et al.*, 1995). These small oocytes are less capable of developing post-fertilization resulting in lower rates of blastocyst formation. There are high chances of these oocytes undergoing chromosomal aberrations during maturation stage (Armstrong, 2001; Lechniak *et al.*, 2002). In buffalo, Yousaf and Chohan (2003) reported poor *in vitro* maturation percentages (32% and 32.7%) to metaphase stage for oocytes retrieved from 2 to <3 and from 3 to <3 mm follicles, respectively, whereas significantly (P<0.05) more oocytes from 4 to <6 and from 6 to <8 mm follicles reached MII (67.1% and 79.1, respectively).

5.3 PHASE III

5.3.1 Effect of Epidermal growth factor and Cysteamine on *in vitro* maturation percentage of oocytes

The maturation medium and the selection of hormones, protein supplements, growth factors and different antioxidants for IVM play an important role in subsequent IVF and *in vitro* development (Pawshe *et al.,* 1996). Thus, the effect of EGF and Cysteamine on IVM was studied. In our study, the maturation percentage of oocytes based on cumulous cell expansion in three media i.e., EGF supplemented media (Group 1), Cysteamine supplemented media (Group 2) and control (Group 3) was 69.61, 48.57 and 46.23 percent respectively. Significantly, higher (p<0.05) maturation percentage in EGF supplemented media was observed than Cysteamine supplemented and control media.

Wani *et al.* (2012) observed that maturation percentage recorded in EGF supplemented medium (84.9%) was significantly higher than in the cysteamine supplemented (69.60%) and control medium (67.20%) which is in accordance with the findings in the present study. Lower percentage of cumulous cell expansion was observed than the present study by Nagar and Purohit (2005) in goat oocytes (42.25%) who used 10 ng EGF/ml in TCM-based medium. Borah and Biswas (2019) observed cumulous expansion of 82.86 ± 0.97% in goat oocytes supplemented with EGF + sodium pyruvate-based media which is similar to the findings in the present study. Mohammadi *et al.* (2007) observed that the maturation percentage of oocytes in control and cysteamine (200µM/ml) supplemented media was 60 and 68 percent respectively, in mouse which was closely related to our findings. No significant difference in the overall rate of maturation of sheep oocytes with or without cysteamine supplementation (88.11% vs. 87.53%) was observed by Shanmugasandaram and Devanathan (2007) which is in accordance with the present study. Reed *et al.* (1993) reported that the maturation percentage of porcine oocytes in control was 50.70 percent and in EGF containing medium was 80.90% which is in close agreement with the present findings. Yadav *et al.* (2013) observed that the percentage of oocytes with nuclear maturation using 10 ng/ml EGF in TCM-based media was 60 percent in goats. Harper and Brackett (1993) reported 89.10 percent cumulus cell expansion when 10 ng/ml EGF + LH was used in the maturation media of bovine oocytes. Cervik *et al.* (2011) observed 58.60 percent cumulous cell expansion in bovine oocytes on using 10 ng EGF / ml in TCM-based media along with hormones.

In the present study, the maturation percentage on the basis of polar body extrusion for EGF (Group1), Cysteamine (Group 2) and control (Group 3) was 46.07, 31.41 and 29.03 percent respectively. Borah and Biswas (2019) observed polar body extrusion of 54.62 ± 1.88 percent in goat oocytes supplemented with EGF + sodium pyruvate-based media which is similar to the findings in the present study. They also observed polar body extrusion of 43.03 ± 1.48 percent in media containing cysteamine. Talukdar *et al.* (2020) observed that medium containing 25 ng/mL leptin in 50 ng/mL IGF-I + 10 ng/mL EGF showed the rate of cumulus expansion and polar body extrusion as 66.67% and 28.57%, respectively.

EGF has been reported to have some positive effects on IVM of oocytes in cattle (Lonergan *et al.,* 1996; Izadyar *et al.,* 1998), pig (Abeydeera *et al.*, 2000) and sheep (Guler *et al.,* 2000). EGF was reported to be present in the ovary of cattle and performed cellular functions through EGF receptors (Yoshida *et al.*, 1998). Stimulation of IVM by EGF could be due to enhanced cAMP production by the cumulus oocyte complexes which induced breakdown of the germinal vesicle (Downs *et al.*, 1991). EGF was reported to be a mediator of the mitogenic activity of FSH in the granulosa cells (Roy and Greenwald, 1991). EGF might act on the cumulus cells surrounding the oocyte and/or on the oocyte itself since mRNA for the EGF receptor was stated to be present in the bovine oocyte (Banwell and Thompson, 2008) which could be extrapolated to goat oocytes. The goat cumulus cells were found to express EGF receptor (Gall *et al.*, 2004) and EGF being a polypeptide with potent mitogenic activity was reported to trigger signalling through the MAPK (mitogen-activated protein kinase) pathway during IVM in goat COCs (Gall *et al.,* 2005).

Cysteamine is a low molecular weight thiol which, when present during IVM of oocytes and IVC of embryos, increases the intracytoplasmic oocyte glutathione (GSH) concentration and enhances embryo development rates (De Matos *et al.*, 1995; Luvoni *et al.*, 1996; De Matos and Furnus, 2000). GSH participates in various mechanisms such as amino acid transport, protein synthesis, reduction of disulphides and protection against oxidative damage. The glutathione content of goat oocytes seemed to be a good indicator for ooplasmic competence and that the addition of cysteamine to a defined IVM medium improved caprine IVP (Cognie *et al.*, 2003).

Chapter-VI

Summary and Conclusions

CHAPTER – VI

SUMMARY AND CONCLUSIONS

The present study was conducted to know the effect of oocyte harvesting techniques on recovery rate of oocytes along with the effect of follicular size and different additives on *in vitro* maturation of follicular oocytes retrieved from abattoir derived goat ovaries. Ovaries were collected from municipal slaughter house, Jammu in two distinct seasons, viz., winter and summer. The study was done in three phases viz., Phase-I: Study the effect of oocyte collection techniques (slicing, puncture and aspiration) on recovery rate of oocytes, Phase-II: Effect of follicular size on *in vitro* maturation percentage of oocytes and Phase III: Effect of Epidermal growth factor (EGF) and Cysteamine on rate of *in vitro* maturation of oocytes.

The number of good, fair and total oocytes collected per ovary by slicing and puncture was significantly higher ($p<0.05$) as compared to aspiration technique. Comparatively, slicing method yielded a higher number of good, fair, and total oocytes in comparison to other two methods.

The mean number of good, fair, poor and total oocytes recovered in winter season was 1.76 ± 0.05, 2.14 ± 0.03, 1.26 ± 0.05 and 5.16 ± 0.15 respectively and in case of summer season was 1.04 ± 0.02, 1.32 ± 0.04, 1.51 ± 0.04 and 3.87 ± 0.11 respectively. The mean number of good, fair and total oocytes recovered in winter season was significantly higher than those recovered in summer season.

The recovery rate of good, fair, poor and total oocytes did not differ significantly between right (1.36 ± 0.02, 1.79 ± 0.01, 1.23 ± 0.01, 4.38 ± 0.03) and left (1.51 ± 0.01, 1.73 ± 0.01, 1.40 ± 0.02, 4.64 ± 0.02) ovaries.

The recovery rate of good, fair, poor and total oocytes from ovaries without CL was significantly higher ($p<0.05$) than from ovaries with CL.

The cumulus expansion rate of oocytes derived from small, medium and large sized follicles was 43.57, 46.47 and 64.73 percent, respectively. The 1st polar body

extrusion rate of oocytes derived from small, medium, and large sized follicles was 28.57, 28.23 and 41.58 percent respectively.

The *in vitro* maturation percentage of oocytes matured in Group 3 (control), Group 2 (Cysteamine supplemented) and Group 1 (EGF supplemented) medium was 46.23, 48.57 and 69.61 percent respectively based on cumulus cell expansion and 29.03, 31.41 and 46.07 percent respectively based on 1st polar body extrusion. The maturation percentage was significantly higher ($p<0.05$) in Group 1 medium than in Group 2 and Group 3 (control) medium. Therefore, keeping the above results in view, it was concluded that:

CONCLUSION

- Slicing technique was best method in terms of oocytes retrieved per ovary but puncture technique can also be utilized for collection of goat oocytes.
- Winter season was better for collection of good quality oocytes than summer season and ovaries without corpus luteum were better for collection of oocytes from goat ovaries.
- Follicles of greater than 2 mm diameter were better for *in vitro* maturation of goat oocytes.
- Supplementation of epidermal growth factor should be encouraged for in *vitro* maturation of goat oocytes.

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CERTIFICATE – IV

Certified that all the necessary corrections as suggested by the External Examiner/ Evaluator and the Advisory Committee have been duly incorporated in the thesis entitled **"Studies on follicular oocyte retrieval and *in vitro* maturation in goat"** submitted by Mr. **Raashid Lateef Dar**, Registration No. **J-20-MV-631.**

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