DEVELOPMENTAL POTENCY OF IMMATURE OOCYTES HARVESTED FROM CRYOPRESERVED SHEEP OVARY

BY

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(J-15-MV-448)

Thesis submitted to Faculty of Postgraduate Studies

in partial fulfillment of the requirements

for the degree of

MASTER OF VETERINARY SCIENCE

IN

VETERINARY PHYSIOLOGY

Division of Veterinary Physiology and Biochemistry
Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu
Main Campus, Chatha, Jammu – 181102

2017

CERTIFICATE-I

This is to certify that the thesis entitled **"Developmental potency of immature oocytes harvested from cryopreserved sheep ovary"** submitted in partial fulfillment of the requirements for the degree of **Master of Veterinary Science** in subject of **Veterinary Physiology** to the Faculty of Post-Graduate Studies, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu is a record of bonafide research, carried out by **Miss Vaishali Sharma, Registration No. J-15-MV-448,** 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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ABSTRACT

Title of Thesis	:	Developmental potency of immature oocytes harvested from cryopreserved sheep ovary
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The cryopreservation of reproductive cells and tissues is a growing field of research and has many applications in biomedical and reproductive research. It allows to preserve the germ plasm of high yielders, old aged animals with reproductive inefficiency, valuable animals suffering from diseases and the animal species on the verge of extinction. Cryopreservation of ovary containing immature follicle is an innovative way of preservation of female gametes. In the present study, an attempt was made to study the developmental potency of immature oocytes harvested from cryopreserved ovaries. The sheep ovaries were obtained from local slaughter house of Jammu and randomly divided into two groups i.e. fresh and cryopreserved ovaries. A total of 114 ovaries were cryopreserved for one month in liquid nitrogen (LN_2) using 10% dimethyl sulfoxide (DMSO) as a cryoprotectant. After cryopreservation, the number and nature of oocytes retrieved, histological as well as viability of follicular cells and maturation competence of the harvested oocytes were assessed and compared with those of fresh ovaries. No significant differences were observed in oocyte retrieval rates, histology of ovarian tissue and maturation rate of oocytes harvested from fresh and cryopreserved ovaries. However, only 39.76% of the retrieved oocytes from cryopreserved ovaries were able to undergone *in vitro maturation* (*IVM*) successfully. The findings of the study may provide helpful tool for further investigation aiming at cryopreservation of ovaries and assisted reproductive technique.

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With heartiest reverence to my family, I admire the confidence bestowed on me by my parents, Mr. Tirath Sharma and Mrs. Sunita Sharma, sister Shafali Sharma, brother-in-law Vijay Sharma and niece Mishti Sharma. My indebtedness to my parents is beyond expression, as next to Lord Shiva I owe everything of my life to them and without their blessings it would have been an impossible task to complete this study.

I owe my deep sense of gratitude to all those whom I could not mention here for their love and affection throughout the course of study enabling me to achieve this goal.

4/8/17

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Signature of Major Advisor **Signature of Student**

DETAILS OF CHAPTERS

CHAPTER	PARTICULARS	PAGE NO.
I	INTRODUCTION	1-4
II	REVIEW OF LITERATURE	5-18
III	MATERIALS AND METHODS	19-26
IV	RESULTS	27-36
V	DISCUSSION	37-42
VI	SUMMARY AND CONCLUSION	43-45
	APPENDIX	46-48
	REFERENCES	49-65
	VITA	

LIST OF TABLES

Table No.	Particulars	Page No.
4.1.1	Number of COC's harvested from fresh and cryopreserved sheep ovary.	27
4.1.2	Usable quality COC's harvested from surface and inner follicles from fresh and cryopreserved sheep ovaries.	29
4.1.3	Non-usable quality COC's harvested from surface and inner follicles from fresh and cryopreserved sheep ovaries.	30
4.2.3	Effect of cryopreservation on viability of follicular cells.	33
4.3.1	Comparison of maturation of usable oocytes harvested from fresh and cryopreserved ovaries.	34
4.3.2	Comparison of maturation of oocytes harvested from surface and inner follicles of cryopreserved ovaries.	36

LIST OF FIGURES

Figure No.	Particulars	Page No.
4.1.1	Bar diagram showing COC's harvested from surface and inner follicles of fresh and cryopreserved ovary.	28
4.1.2	Bar diagram showing usable quality COC's harvested from surface and inner follicles from fresh and cryopreserved sheep ovaries.	29
4.1.3	Bar diagram showing non-usable quality COC's harvested from surface and inner follicles from fresh and cryopreserved sheep ovaries.	30
4.2.3	Bar diagram showing effect of cryopreservation on viability of follicular cells.	33
4.3.1	Bar diagram showing maturation of usable oocytes harvested from fresh and cryopreserved ovaries.	35
4.3.2	Bar diagram showing maturation of oocytes harvested from surface and inner follicles of cryopreserved ovaries.	36

ABBREVIATIONS

%	-	Percent
˚C	-	Degree celcius
BSA	-	Bovine serum albumin
CO_2	-	Carbon dioxide
COC	-	Cumulus oocyte complex
CPA	-	Cryoprotectant Agent
DPBS	-	Dulbecco's phosphate buffer saline
FBS	-	Fetal bovine Serum
FF	-	Follicular fluid
Fig	-	Figure
FAO	-	Food and Agricultural Organization
FM	-	Freezing media
gm	-	Gram
h	-	hours
J&K	-	Jammu and Kashmir
IVF	-	In vitro fertilization
IVM	-	In vitro maturation
MPF	-	Maturation promoting factor
mol	-	Molar
M-199	-	Medium-199
mg	-	Milli gram
$MgCl_2$	-	Magnesium chloride
M- II	-	Metaphase II

ml	-	Milli litre
mM	-	Mili Molar
min	-	minutes
MPF	-	Maturation Promoting Factor
mRNA	-	Messenger Ribonucleic acid
NSS	-	Normal saline solution
Nos.	-	Numbers
OCM	-	Oocyte collection media
PBS	-	Phosphate Buffer Saline
rpm	-	Revolutions per minute
TCM-199	-	Tissue culture medium-199
TGF-ß	-	Transforming growth factor-beta
µg	-	Microgram
µl	-	Microlitre
µM	-	micro Molar
No.	-	Number

LIST OF PLATES

Plate No.	Particulars	After page no.
4.1.1	Photograph showing COC's harvested from surface follicles of fresh ovary.	30
4.1.2	Photograph showing COC's harvested from surface follicles of cryopreserved ovary.	30
4.1.3	Photograph showing COC's harvested from inner follicles of fresh ovary.	30
4..1.4	Photograph showing COC's harvested from inner follicles of cryopreserved ovary.	30
4.2.1a	Photograph showing freshly collected sheep ovary.	31
4.2.1b	Photograph showing cryopreserved ovary.	31
4.2.1c	Photograph showing longitudnal section of fresh ovary.	31
4.2.1d	Photograph showing longitudnal section of cryopreserved ovary.	31
4.2.2a	Photograph showing haematoxylin and eosin stained sections of fresh ovaries.	32
4.2.2b	Photographs showing haematoxylin and eosin stained sections of cryopreserved ovaries.	32
4.2.3a	Photograph showing results of trypan blue staining of follicular cells of fresh ovary.	32
4.2.3b	Photograph showing results of trypan blue staining of follicular cells of cryopreserved ovary.	32
4.3.1	Photograph showing oocytes collected from fresh ovaries.	34
4.3.2	Photograph showing proliferation of granulosa cells after *IVM* of oocytes collected from fresh ovaries.	34
4.3.3	Photograph showing oocytes collected from cryopreserved ovaries.	35
4.3.4	Photograph showing oocytes after *IVM* of cryopreserved ovaries.	35

Chapter - 1

INTRODUCTION

CHAPTER-I

INTRODUCTION

In today's world animal plays an important role in human's life, in one or another way humans are dependent on animals. But from few years it has been seen that there is rapid and continuous decline in certain wild as well as domestic animal species and some of them are on the verge of extinction. Extinction of mammalian species is irreversible process and has been increasing from past few years due to human activities like habitat destruction and over hunting (Holt and Pickard, 1999). So it is necessary to protect them and develop some rescue strategies to ensure their survival. Genetic resources banks of male and female gametes and embryos can be created in order to preserve the genetic diversity and assure survivability and conservation of endangered wild animal species and farm animal breeds (Wildt, 2000). Out of many ways to preserve the genetic diversity, one is to cryopreserve the male and female gametes. Cryopreservation of reproductive cells and tissues is a growing field of research and has many application for advancement of biomedical and reproductive research. It allows us to preserve the germ-plasm of high yielders, old aged animals with high reproductive efficiency, valuable animals suffering from disease and the animal species on verge of extinction.

Cryopreservation of semen is the most popular way of storing the male germplasm due to its simple collection methods and ease of application (Holt and Pickard, 1999; FAO, 2007). Cryopreservation of semen of highly superior and endangered males allows the maintenance of hetero-zygosity and minimize the transmission of veneral disease (Johnston and Lacy, 1995; Andrabi and Maxwell, 2007). Cryopreservation of semen is easy as the spermatozoa in it contains very less amount of water and is protected against physical injuries and can be isolated from unhealthy/ dead male in emergency situation (Zamboni, 1987). However, the major disadvantage of the technology is that the protocols used to conserve semen cannot be applied to all species (Woods *et al*., 2004). Moreover, it preserves only one half of the genome of a species.

Cryopreservation of oocytes is a way of preserving the female gametes. Oocyte cryopreservation for desired period of time plays an important role in the field of

biomedical research. At birth, the mammalian ovaries contain a large pool of primordial oocytes. After puberty, daily a number of follicles enter folliculogenesis process, in which small primordial follicle containing immature oocytes grow into large preovulatory follicle that ovulate during estrus period (Hirshfield, 1991) or end in its atresia. Development of primordial follicle involves cellular growth, proliferation and differentiation (Braw, 2002).

Oocyte cryopreservation is beneficial for transportation of germplasm as it avoids injury involved in live animal transportation (Pereira and Marques, 2008). Oocyte cryopreservation offers many advantages such as preservation of fertility of high genetic value females and endangered species. Oocyte can be preserved at germinal vesicle stage and metaphase II stage. The metaphase II oocytes are very fragile and sensitive to low temperature due to its large size, water content and presence of spindle (Bernard and Fuller, 1996). During freezing or thawing process the spindle apparatus is damaged due to intracellular ice formation (Boiso *et al*., 2002). They also have low permeability to cryoprotectants (Woods *et al*., 2004). Whereas the germinal vesicle stage oocytes without spindle are less prone to chromosomal and microtubular damage during cryopreservation and they survive better than metaphase II oocytes (Winiger and Kort, 2002). In certain species like dog collection of oocytes, *in vitro maturation*, and *in vitro fertilization* is difficult (Luvoni *et al*., 2006). The cryopreserved oocytes give disappointing results because of problems encountered during fertilization and embryonic development (Bernard and Fuller, 1996). It is also reported that there is reduction in rate of hatching of blastocyst (Van der Elst *et al*., 1998). Experiments revealed that the survival rate of developed fetuses from cryopreserved mouse oocytes was very low (3%) (Nakagata, 1992). The freezing of germinal vesicle stage oocytes would be alternative to the metaphase II oocytes to avoid aneuploidy, however hardening of zona and damage to cytoskeleton may be observed. So live birth using frozen thawed germinal vesicle oocyte is very less. Hence, the *in vitro* maturation of cryopreserved oocytes is to be mastered.

Cryopreservation of isolated follicles have some advantage over oocyte preservation like direct monitoring of the follicles during *in vitro* culture (Abir *et al*.,

1999), assessment of follicular quality and quantity after cryopreservation of known number of follicles (Amorim *et al*., 2003).

At birth, ovaries contain large number of primary oocytes arrested in prophase of meiosis I and are surrounded by single layered pregranulosa/ granulosa cells to form primordial follicles. Those follicles remain viable after animal's death for several hours (Silva *et al*., 2000). The development of primordial follicle involves cellular growth, proliferation and differentiation (Braw, 2002). The fully grown oocyte is enclosed by several layers of cumulus cells held together by gap junctions and are called cumulus oocyte complex (Russell and Salustri, 2006). During the growth of follicle there is formation of fluid filled cavity adjacent to oocyte called antrum and causes rise in pituitary FSH due to disintegration of corpus luteum. In response to higher FSH, antral follicles start secreting estrogen and inhibin which have negative feedback effect on FSH (Ziegler., 2007). Follicles with fewer FSH will not be able to develop further and show retardation and become atretic. Eventually, only one follicle will be viable called dominant follicle which finally ovulates. These millions of primordial follicles present in ovarian cortex can be cryopreserved for future use (Paris *et al*., 2004). It would be great practical benefit to rescue these follicles and cryopreserving for future use or *in vitro* culture (Amorim *et al*., 2003) in order to get an extra source of gametes that can be used in future. The limited success with the methods concerning the cryopreservation of oocyte and follicles has proposed research on cryopreservation of immature oocytes present in primordial follicles.

The cryopreservation of ovarian tissue is a new methodology to conserve the female fertility. Cryopreservation of ovarian tissue in human beings was tried by several scientists (Oktay and Karlikaya, 2000; Donnez *et al*., 1998). It is an alternative to cryopreservation of isolated oocytes and embryos, since ovarian tissue contains large number of primordial follicles with oocytes at germinal vesicle stage are relatively inactive and undifferentiated, therefore less prone to cryo-injury. So it would be beneficial to cryopreserve these oocytes contained in ovarian tissue/ whole ovary as they require a period of maturation for inducing nuclear and cytoplasmic changes before undergoing fertilization and early embryo development. Follicular cells protect oocytes

during cryopreservation and the follicular fluid provides nutrition for the growth of the oocytes. The work on ovarian tissue cryopreservation was performed in rabbit (Smith, 1952) and rat (Parkes and Smith, 1953; Deansely, 1954). Till date the technique has not been tried in any domestic animal species in the country. Therefore keeping in view of above knowledge, the present research work had been performed on sheep. As mutton is consumed in large quantities in Jammu and Kashmir, it would be very beneficial to increase the number of sheep by cryopreserving the immature oocytes followed by their *in vitro* maturation, fertilization and development of fetuses. The research work was performed under following objectives:

1. To compare the compact cumulus oocyte complexes (COCs) harvested from surface follicles with those from follicles present inside the cortex of cryopreserved sheep ovaries.
2. Assessment of the quality of frozen- thawed sheep ovarian tissue.
3. Assessment of maturation of collected oocytes from cryopreserved ovaries after *in vitro* culture.

Chapter - 2

REVIEW OF LITERATURE

CHAPTER-II

REVIEW OF LITERATURE

Female fertility is a function of ovary to produce mature oocytes. The functional unit of ovary is the follicle that contains oocytes surrounded by granulosa cells and theca cells. Maturation of oocytes depends on the production of hormones by somatic cells surrounding the oocytes as well as a few paracrine and autocrine growth and differentiation factors produced by oocyte and other cells.

The ovary contains a non- renewable reserve of germ cells. However Johnson *et al*. (2004) contradicted the above statement by suggesting that there are germ stem cells present in the surface epithelium of ovary. They also reported that origin of these stem cells are from bone marrow (Johnson *et al*., 2005), the hypothesis is yet to be confirmed. However, traditional belief is that ovary of animals contains a decreasing reserve of oocytes from fetal life till their non-reproductive life. The oocyte quantity reaches its peak during the later stages of gestation but reduced to approximately $½_0^{th}$ of the original number at the time of its puberty. During the reproductive life of the female animals a few hundred oocytes ovulate and rest lakhs of oocytes go through atresia and degenerate.

During the maturation process, the follicles grow and go through primordial, primary, secondary and preantral follicle and subsequently develop an antral cavity. At the antral stage most of the follicles undergo atresia, but a few of them reach preovulatory stage under the influence of pituitary hormones (gonadotropins). Following preovulatory surge one or few follicles release mature oocytes ready for fertilization (Mc Gee and Hsueh, 2000). The factors trigger the transformation of primordial follicles to primary stage is still unknown. But the culture of ovarian tissue removes the inhibition present *in vivo* and transforms the primordial follicle into growing stage (Hovatta *et al*., 1997; Wandji *et al*., 1996; Wandji *et al*., 1997). The proliferation of granulosa cells is confirmed by the production of PCNA (Proliferating Cell Nuclear Antigen). When the oocytes are surrounded by three to six layers cells with theca cells and blood vessels, the follicles exposed to factors circulating in blood. Then under the influence of hormones and growth factors, the follicles grow with proliferation and differentiation of granulosa

cells into mural granulosa cells and cumulus cells. The maturation of oocyte results due to communication between oocyte and surrounding granulosa cells.

Oogenesis starts in embryonic life with the formation of primordial germ cells. Cytokines like Kit-Ligand (KL) and transforming growth factor β (TGFβ) influence their migration to genital ridge where they form the oogonia. They expand through mitotic division and undergo last round of DNA replication before they enter meiosis and becomes oocytes. During intiation of meiosis, the oocytes are surrounded by one layer of pregranulosa cells to form primordial follicle (Gosden, 1995). The process of formation of fertilizable M II oocyte from a primordial germ cell requires more than 200 days and its volume increases by 100 fold (Gougeon, 1996; Picton *et al*., 1998). There are two components of oocyte maturation: 1. Longer Cytoplasmic maturation and 2. Shorter nuclear maturation.

Cytoplasmic maturation

Cytoplasmic maturation involves several events to make oocyte fit for fertilization. It includes accumulation of mRNAs and proteins, cytoplasmic recognization and epigenetic modification.

During maturation of oocytes huge amount of RNA is deposited in cytoplasm, out of which 8% is mRNA (Gosden, 2002). During maturation, different polyadenylation of mRNAs patterns are observed. mRNAs with longer poly A tail are transcribed immediately and those with shorter tail are masked and stored for future use. The masked mRNAs contain an adenylation control element which regulates the expression of masked ones (Fox *et al*., 1989; Verroti *et al*., 1996).

During oocyte growth replication of mitochondria with its DNA molecule takes place (Wassarman and Josefowicz, 1978). Proliferation of ribosomes and golgi bodies take place to secrete more protein for formation of zone pellucida (Mehlmann *et al*., 1995). At the time of maturation of oocytes, pores appear in nuclear membrane. A large pale nucleus (Germinal vesicle) is formed at the end of maturation. Large amount of glycogen, lipid droplets and proteins are stored in cytoplasm during this growth period.

Epigenetic modification is a major component of cytoplasmic maturation and most studied form of imprinting DNA methylation (Obata and Kono, 2002).

Nuclear maturation

Nuclear maturation is depicted by breakdown of GV. This follows the 1st meiotic division i.e. expellation of 1st polar body and formation of mature metaphase II oocyte. At GV oocyte stage, the cytoplasm has achieved the maturity without nuclear maturation. Nuclear maturation achieved by completion of 1st meiotic division which is triggered by activation of maturation promoting factor (MPF). This MPF, a complex of catalytic and regulating subunits is involved in nuclear envelope breakdown; chromosome condensation and arrangement of cytoskeleton (Gautier and Maller, 1991; Moreno and Nurse 1990; Fulka *et al* 1992; Wu *et al*., 1997). Synthesis and relocation of cyclin-B form cytoplasm to nucleus and dephosphorylation of catalytic subunit of MPF convert the GV stage oocyte to M II stage. Luteinizing hormone (LH) surge lowers the cAMP production in oocyte which triggers the dephosphorlyation of MPF to activate it (Millar and Russell, 1992) cellular equivalent to viral oncogene mos (c-mos) is another regulator of MPF (Sagata *et al*., 1989 a, b).

When the follicles enter the growing stage, the oocyte starts to grow and communicate with surrounding granulosa cells. The GCs communicate with each other by connexions composed gap junctions. Connexion 37 & 43 are two most important connexions required for the communication in between cells (Eppig, 1982). Connexion lacking leads to arrest of follicular growth at early stage, prevention of formation a mature Graafian follicle and occurrence of premature leutinization (Ackert *et al*., 2001; Simon *et al*., 1997).

2. 1 Oocyte collection methodology and retrieval of oocytes

The collection of oocyte is prerequisite for *in vitro* fertilization (IVF) and other assisted reproductive techniques (ART). There are several techniques of oocyte collection namely, follicular aspiration, ovary slicing (Wani *et al*., 2000; Zeinoaldini *et al*., 2013), mincing method (Shioya *et al*., 1988) in abattoir derived ovaries. Ovary slicing technique provides cumulus-oocyte complexes (COCs) of better quality and the number of

harvested oocyte collected is higher compared to follicle aspiration (Wani *et al*., 2000; Amiridis *et al*., 2012). Sheep ovum pick-up systems are applied in living animals using minimal invasive procedures such as laproscopic ovum pick-up (LOPU) technique guided by laproscopy. Oocyte recovery with centrifugation is a method of oocyte recovery using modified falcon tubes (Davachi *et al*., 2011).

Deka *et al*. (2015) studied the effect of aspiration and slicing method on *in vitro* maturation of bovine oocytes. He found that number of oocytes matured were recovered more by aspiration method than slicing method.

John *et al*. (2015) recovered caprine oocyte by aspiration, slicing and puncture method. He observed highest percentage of oocyte recovery by slicing and puncture followed by aspiration.

Singh *et al*. (2013) studied the efficiency of three different collection techniques-aspiration, slicing and dissection technique on percentage and grades of oocytes in goats. Oocyte retrieval percentage was highest in slicing technique followed by dissection and aspiration.

Zeinoaldini *et al*. (2013) compared the recovery rate of sheep oocyte harvested from slaughter house by four methods; ovarian puncture, aspiration technique, slicing, and oocyte recovery via centrifugation (ORC). He observed that oocytes recovery rate using oocyte recovery with centrifugation (ORC) using modified falcon tubes was higher compared to slicing, aspiration and puncturing method.

Sekhar *et al*. (2012) recovered bovine oocytes by slicing, aspiration and post aspiration slicing methods. He observed oocyte retrieval percentage was higher in slicing technique followed by post aspiration slicing and aspiration methods.

Davachi *et al*. (2011) recovered sheep oocytes by a new oocyte recovery method "Oocyte Recovery with centrifugation (ORC)" using modified Falcon tubes and centrifuged at 750 rpm for 5 minutes. He observed that the percentage of oocyte recovery was higher in ORC as compared with aspiration method.

Hoque *et al*. (2011) collected goat cumulus oocyte complexes (COCs) by three techniques - puncture, slicing, and aspiration method. The total number of COCs per ovary as well as number of abnormal COCs per ovary was higher in puncture and slicing method followed by aspiration technique.

Majeed *et al*. (2011) studied the effect of collection techniques on recovery and *in vitro* maturation of Black Iraqi goat oocytes. He found that the, number of oocytes harvested and maturation of oocytes was more in aspiration method compared to slicing method.

Talukder *et al*. (2011) observed that the number of normal cumulus oocyte complexes per sheep ovary was significantly higher in ovaries without corpus luteum and number of abnormal cumulus oocyte complexes was higher in ovaries with corpus leuteum.

Kakkassery *et al*. (2010) recovered bovine oocytes by 3 methods: aspiration, slicing and puncture method. Oocyte retrieval percentage was higher in puncture method followed by aspiration and slicing.

Pawshe *et al*. (1994) recovered goat oocyte by aspiration, slicing and puncturing methods. Oocytes recovered per ovary were higher by aspiration method than puncturing or slicing and good quality usable oocytes were obtained by slicing compared to aspiration or puncturing methods.

2.2 Grading of collected oocytes

John *et al*. (2015) graded the retrieved caprine oocytes into A, B, C and poor quality oocytes. He observed higher percentage of A and B grade oocytes by puncture followed by aspiration and slicing. Grade C oocytes by aspiration and slicing followed by puncture method. Poor quality oocytes were retrieved by slicing followed by aspiration and puncture methods.

Singh *et al*. (2013) graded the retrieved goat oocytes into A, B, C and D grade oocytes. He observed maximum yield of grade A and B oocytes by aspiration and dissection technique. Grade C and D oocytes were retrieved by slicing technique. Overall

yield of oocytes were retrieved by slicing technique compared to aspiration and dissection methods.

Sekhar *et al*. (2012) graded the retrieved bovine oocytes into A, B, C and D based on cell layers and homogenesity. He observed greater percentage of grade A oocyte by slicing method, grade B oocyte by post aspiration slicing, grade C oocytes by slicing and grade D by aspiration method.

Kakkassery *et al*. (2010) graded the retrieved bovine oocytes into A, B, C oocytes. Higher percentage of grade A oocytes observed in slicing followed by puncture and aspiration. Grade B oocytes percentage was higher in slicing followed by puncture and aspiration method. Grade C oocytes percentage was higher in slicing followed by aspiration and puncture methods.

Based on the cellular attachment and homogenesity, the oocytes are classified as grade A, B, C and D (Chauhan *et al*., 1998). Grade A: COCs with unexpanded cumulus cells having at least 5 layers of cumulus cells with homogenous cytoplasm of bovine oocytes. Grade B: COCs with 2-4 layers of cumulus cells and with homogenous cytoplasm. Grade C: Oocytes partially denuded of cumulus cells and with irregular shrunken cytoplasm. Grade D: Oocytes completely denuded of cumulus cells with irregular shrunken cytoplasm.

Kobayashi *et al*. (1994) studied the influence of epidermal growth factor and transforming growth factor- alpha on in vitro maturation of cumulus cell- enclosed bovine oocytes in a defined medium. He observed an increase in number of fertilized ova that developed to the blastocyst stage and use of epidermal growth factor and transforming growth factor alpha yielded high quality of mature bovine oocytes for *in vitro* fertilization.

2.3 Cryopreservation of oocytes, follicles and ovaries

Henry *et al*. (2016) studied the effect of anti- apoptotic drugs on transport and freezing media of sheep ovaries. He used anti- apoptotic drugs (2-VAD-FMK, a pan-caspase inhibitor or sphinosine-1 phosphate (SIP). It was seen that after 2 days of culture

Sphingosine- 1 phosphate improved the quality of primordial follicles, higher densities of morphologically normal and proliferation primordial follicles were found.

Fakhrildin *et al*. (2015) investigated the impact of sheep ovarian tissues cryopreservation using different cryoprotectants, ethylene glycol(EG), propendiol (ProH) and glycerol (GLY) supplemented with sucrose and mannitol and techniques(vitrification or rapid cryopreservation) on ability of oocytes for *in vitro* fertilization (IVF) and early embryonic development. Post xenotransplantation to mice bodies revealed significant increase in % of IVF in control group (without freezing) as compared with other groups. Lowest IVF% was observed in group using rapid cryopreservation.

Milkich *et al*. (2012) used metal container for vitrification of mouse ovaries, as a clinical grade model for human ovarian tissue cryopreservation after different times and transport. He developed an enclosed metal vessel which has advantage of a faster heat transfer, when in contact with the tissue. It was observed that at temperature 37˚C, helped to maintain normal primordial and primary follicle morphology for upto 4 hours after collection and vitrification in a metal container.

Fathi *et al*. (2011) investigated the effects of two vitrification methods on sheep ovarian tissue. The sheep ovarian tissue vitrification by two step methods was more effective than four step method.

Gerritse *et al*. (2011) developed an assay to monitor the extent of cryodamage on bovine ovarian tissue by different cryopreservation protocols. The assay measured the glucose and lactate metabolism of ovarian tissue fragments *in vitro* and determines the extent of cryo damage in cryopreserved ovaries. Submersion of intact ovaries in DMSO prior to freezing thawing resulted in complete protection of glucose/ lactate metabolism of cortex. Perfusion without submersion resulted in partial protection of cortex, subcortex and medulla. While combination of both resulted in protection of all 3 ovarian tissue layers.

Zhang *et al*. (2011) assessed the cryopreservation induced cytotoxicity of vitrification solution by using Lactate dehydrogenase (LDH) assay. LDH levels were

compared by analysis of variance (ANOVA). It was observed that there was no significant difference in the levels of LDH amongst the control group.

Arav *et al*. (2009) observed ovarian function six years after whole sheep ovarian cryopreservation. Total 36 antral follicles were counted by transalumination, 4 germinal vesicle oocytes were aspirated and matured *in vitro* to metaphase II.

Selvaraj *et al*. (2009) and (2010) reported the first pregnancy and birth in India of healthy male babies after the transfer of embroys generated by intracytoplasmic sperm injection into thawed human oocytes.

Neto *et al*. (2008) compared the effects of different concentration of cryoprotectants, equilibration method and post seeding freezing rate on doe rabbit ovarian tissue. He observed that equilibration method and cryoprotectants concentration have no effect on the proportion of normal follicles.

Baudot *et al*. (2007) investigated a study regarding outcomes of whole sheep ovaries cryopreservation. Ovaries were perfused with VS_4 (Vitrification solution artery and cooled by quenching in LN_2 in less than a minute. Following rewarming, a dye exclusion test indicated about 60% of small follicles were viable while histological analysis showed about 48% of primordial follicles were normal.

Amorim *et al*. (2006) studied the efficiency of cryoprotectants like DMSO, EG, PROH and Glycerol to cryopreserved primordial follicles. Higher follicular survival was reported when DMSO and EG were used.

Arav *et al*. (2005) studied the function of cryopreserved whole sheep ovaries both *in vitro* and *in vivo* by transplanting frozen thawed intact ovaries in sheep by artery and vein anastomosis. He found that the whole ovaries and the follicles survived cryopreservation and the functioned normally after transplantation.

Cecconi *et al.* (2004) tried cryopreservation of ovarian cortex with slow freezing protocol using DMSO and EG as cryoprotectant. He observed the follicles who had survived thawing grew up to antral follicle. The percentage of healthy COCs harvested from the DMSO group was significantly higher than EG and during the culture oocyte

increased in size but maximum number of oocytes are arrested at germinal vesicular stage.

Fabbri *et al*. (2001) investigated the effect of exposure time of cryoprotectants and the sucrose concentration in the freezing solution on human oocyte survival after thawing. He found a significantly higher survival rate of oocytes with double sucrose concentration in the freezing solution and the longer exposure time to cryoprotectants significantly increased the oocyte survival rate.

2.4 Histological status of cryopreserved tissue

Al-Soudy *et al*. (2016) cryopreserved immature and mature camel oocytes by open pulled straw vitrification. Results indicated non- significant difference in maturation rate between vitrified immature oocytes and non- vitrified (control) group. However average rate of extrusion of first polar body was significantly reduced in vitrified immature oocytes compared to non- vitrified group. Morphological abnormalities occurred more in vitrified immature oocytes as compared to vitrified mature oocytes. Survival rate of vitrified immature oocytes was less than vitrified mature oocytes.

Hanh *et al*. (2016) cryopreserved whole porcine ovaries by slow freezing and vitrification method. The oocytes maturation were evaluated by extrusion of 1^{st} polar body. He observed the % of viable oocytes was highest in vitrification method compared to slow freezing method.

Fauque *et al*. (2016) used trypan blue staining technique to assess the quality of cryopreserved sheep ovaries. The percentage of unstained follicles was lower after freezing/ thawing than before cryopreservation.

Choi *et al*. (2015) studied the effect of DMSO based solution on trehalose on the cryopreservation of a whole sheep ovary and evaluated its use as an efficient cryoprotectant. On histological assessment it was seen that DMSO free ovarian structure remained largely intact compared to fresh control groups and significant damage was observed in ovaries of DMSO group, TUNEL assay and mRNA transcript showed apoptosis parameter in fresh group was lowest amongst rest of the groups.

Du *et al*. (2015) studied the protective effects of DMSO –free solution based on trehalose on the cryopreservation of whole sheep ovary. Histological assessment indicated no change in the structure of DMSO- free ovaries, however significant damage observed in ovaries with DMSO group.

Ting *et al*. (2013) studied the morphological and functional preservation of preantral follicles after vitrification of rhesus macaque ovarian tissue in a closed system. Two vitrification solutions containing ethylene glycol plus glycerol and ethylene glycol plus DMSO. Dense stroma and intact preantral follicles were observed using 27% glycerol and 27% EG and 0.8% polymers without cooloing in LN_2 and a two step warming.

Merdassi *et al*. (2012) examined the viability and quality of ovarian tissue after cryopreservation in sheep ovarian tissue. Follicular viability was assessed by trypan blue staining and it was seen that there was decrease in viability after freezing- thawing. A significant negative correlation between the % of morphologically normal follicles and cytotoxicity was observed.

Purohit *et al*. (2012) studied the effects of vitrification on immature and *in vitro* matured, denuded and cumulus compact goat oocytes and their subsequent fertilization. He reported the number of oocytes retaining normal morphology was significantly higher for cumulus compact oocytes compared to denuded oocytes. Similarly *in vitro* maturation of oocytes was highest for non-vitrified control oocytes.

Campos *et al*. (2011) studied the effect of storage duration on cryopreserved ovarian tissue using fresh and frozen thawed samples on human ovaries. It was observed that there was no difference between the groups in follicular density, which was assessed in haematoxylin and eosin sections. Follicular viability was greater in fresh tissues compared with the cryopreserved tissues.

Milenkovic *et al*. (2011) cryopreserved whole sheep ovary by slow freezing protocol using DMSO as cryoprotectant. After rapid thawing, viability was assessed by ovarian *in vitro* perfusion, cell culture histology and fluroscent live dead assay. It was

seen that production of cyclic AMP and progesterone was slightly higher in DMSO group and tissues were well preserved in DMSO group.

Zhang *et al*. (2011) compared the efficacy of vitrification and conventional freezing of whole ovaries and observed vitrification was better than conventional and rapid freezing method.

Wallin *et al*. (2009) cryopreserved the whole ovary of sheep and assessed its viability and function. Ovaries were flushed with either cryoprotectants (Propendiol FROZEN-PROH) or Ringer Acetate (FROZEN- RA) followed by slow freezing. Assessment was done by light microscopy, biochemical response, viability assay. Microscopy showed well preserved morphology with the presence of small follicles.

Bilska *et al*. (2008) studied the morphology and function of cryopreserved whole sheep ovaries after heterotopic autotransplantation, also determined the ability to respond to *in vivo* FSH-treatment and fertilization ability of retrieved oocytes. It was seen that the morphology of autotransplanted and ovaries was similar, proliferating cells were detected in follicles and the rate of apoptosis was minimal in ovaries of control and autotransplanted ovaries.

Wang *et al*. (2008) vitrified mouse ovaries and human ovarian cortex following self-developed needle immersed vitrification (NIV) method. He found the primordial follicles in humans and mouse ovarian tissue were well preserved and number of normal morphological primary and secondary follicles were greater in the NIV method of preservation compared to those in slow freezing group.

Courbiere *et al*. (2005) reported cryopreservation technique by vitrification of whole ovaries with their vascular pedicles in sheep by using 2 cryoprotectant solutions. No significant difference in follicle viability or normal primordial follicle rate was observed between ovaries exposed and non- exposed to cryoprotectants solution. There was also no significant difference observed before and after vitrification with 2 cryoprotectant solutions.

Segino *et al*. (2005) studied the *in vitro* follicular development of cryopreserved mouse ovarian tissue. Ovaries obtained from 3 week old female mice were cryopreserved by rapid freezing method. Preantral follicles isolated from frozen thawed ovarian tissue were cultured for 12- 16 days. The preantral follicles isolated from frozen/ thawed mouse ovarian tissue developed slowly compared with the freshly prepared preantral follicles.

Demirci *et al*. (2001) studied the follicular viability and morphology of sheep ovaries after exposure to cryoprotectants and cryopreservation with different freezing protocols. It was observed that optimum survival rate of primordial follicles were obtained in sheep by slow cooling protocol with semiautomatic seeding at 2M of DMSO.

Cecconi *et al.* (1999) cultured preantral ovarian follicle isolated from pre-pubertal sheep ovaries in presence of FSH and under 20% and 5% oxygen concentration. He observed follicles were growing with addition of FSH and at low oxygen concentration.

Hovatta *et al*. (1999) cryopreserved the human ovarian tissue using dimethylsulfoxide and propanediol–sucrose as cryoprotectants. He observed no difference between oocytes appearance after freezing and thawing with both cryoprotectants.

Saunders and Parks (1999) studied the effect of cryopreservation on the cytology and fertilization rate of *in vitro* matured bovine oocytes. He observed an increase in occurrence of cytological abnormalities in oocytes on freezing.

2.5 Maturation of oocytes

Normal fertility of females depends upon the proper development of the oocyte. This growth is achieved just prior to ovulation, when the maturation of oocytes occurs. Oocyte maturation is the release of meiotic arrest that allows oocyte to advance from prophase I to metaphase II of meiosis. Oocytes within the ovary are arrested in prophase I of meiosis until the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), stimulate the follicular growth and development which then triggers the resumption of meiosis up to metaphase II. Oocytes are subsequently again held in meiotic arrest until fertilization, when meiosis is complete. Oocyte maturation involves a

complex interaction of several important intracellular, paracrine and structural factors, cyclic adenosine monophosphate (cAMP) and gap junctions.

Sara *et al*. (2015) studied the effect of different concentration of serum on *in vitro* maturation of bovine oocytes. Maturation rate was significantly higher when rat estrous serum or sheep serum was added to controlled group.

Yasmin *et al*. (2014) studied the effect of serum free culture medium containing silk protein sericin on maturation and fertilization of sheep oocyte. COCs maturated without Bovine serum albumin (BSA), supplementation of 0.1% sericin increased the rate of maturation to metaphase II and total fertilization of oocytes.

Sreenivas *et al*. (2013) studied the effect of protein supplementation on *in vitro* maturation of sheep embryo. Maturation rate of oocyte was significantly higher in fetal bovine serum (FBS) supplemented group compared with BSA and wheat peptone supplemented groups.

Fasano *et al*. (2012) studied the effect of *in vitro* maturation of human oocytes before and after vitrification. Results showed survival rate after warming was similar in both the groups (before and after vitrification). However oocyte maturation rate per collected oocyte was significantly higher for oocytes matured before vitrification than for oocytes vitrified before *IVM.*

Sekhar *et al*. (2012) studied the efficacy of *in vitro* maturation of bovine oocytes in TCM 199, PMSG and follicular fluid. On the basis of degree of cumulus expansion maturation was classified as D0, D1 and D2 expansion and it was seen that higher percentage of grade A and B oocyte attained D2and D1 degree of cumulus expansion and grade C oocytes with D0 degree of cumulus expansion. D0 cumulus cells were slightly adhere to zona pelluicida. D1 70 per cent of cumulus cells homogenesity expanded. D2 all cumulus cells homogeneously spreaded.

Farag *et al*. (2010) investigated the effect of supplementation of protein additive (10% ovine amniotic fluid, 10% FBS and 10% sheep serum on *in vitro* maturation of

sheep oocyte. Supplementation of protein additives was very efficacious and is required for *in vitro* maturation of sheep oocytes.

Kakkassery *et al*. (2010) in vitro matured bovine oocytes in TCM-199 supplemented with LH, FSH, estradiol, pyruvate and foetal calf serum. He observed grade A oocytes showed no significant difference in maturation rate except slicing method. Lower maturation rate was observed in grade C oocytes in comparison to A and B in all three methods. Grade D oocyte failed to mature in all of retrieved method.

Shabankareh *et al*. (2010) studied the effect of different maturation media (estrus sheep serum, estrous goat serum, ovine follicular fluid and bovine follicular fluid) on *in vitro* maturation and *in vitro* fertilization of sheep oocyte. It was observed that basic culture medium with above serum supported better rates of *in vitro* maturation as compared to sheep and bovine follicular fluid.

Farag *et al*. (2009) studied the effect of hormones, culture media and oocyte quality on *in vitro* maturation of sheep oocytes. Results indicated that the addition of hormones improved the *in vitro* maturation of COCs as compared to control groups.

Motlagh *et al*. (2008) studied the *in vitro* maturation of sheep oocytes in different concentration of mare serum. Significantly higher maturation rate of oocytes were observed in all concentrations of mare serum compared to those without serum supplementation.

Cecconi *et al*. (1999) cultured preantral ovarian follicle isolated from pre-pubertal sheep ovaries in presence of FSH and under 20% and 5% oxygen concentration. He observed follicles were growing with addition of FSH and at low oxygen concentration.

Eppig and Brien (1996) tried to get matured oocytes from *in vitro* cultured oocytes collected from primordial follicle of newborn mouse ovaries. He observed 8 times increase in size of oocyte with addition of growth factors (epidermal growth factor) in culture medium.

Chapter – 3

MATERIALS AND METHODS

CHAPTER-III

MATERIALS AND METHODS

Cryopreservation of ovaries may be an alternative to isolate mature oocyte and can be used to preserve female germ-plasm of valuable as well as endangered animals. It enables preservation of immature oocytes along with other ovarian cells necessary for development and maturation of oocytes. The present research work was carried out to establish a protocol for cryopreservation of the sheep ovaries for effective retrieval of immature oocytes for studying their *in vitro* developmental potency.

Materials

3.1 Glass ware and plastic ware

All the glasswares used in the present investigation were made of high grade pyrogen free glass. The glasswares, wherever used, were thoroughly cleaned and rinsed with double distilled water and then heat sterilized at 180^0C for 2 h. The plastic wares included disposable petri dishes (large 90 mm, small 35 mm diameter) were of Genaxy Scientific Pvt Ltd, New Delhi. The 15 ml and 50 ml sterile plastic centrifuge tubes were purchased from Abdos LDPE, Roorkee, Uttarakhand. Used 0.22µm filters were procured from Millipore Corporation, Bedford, MA, USA. Disposable nontoxic and non-pyrogenic plastic syringes of assorted sizes were procured from Dispo Van, HMD Ltd, Banglore.

3.2 Equipments

3.2.1 Microscopes

3.2.1a Stereo zoom microscope

Low magnification stereo zoom microscope (Trinocular stereozoom MSZ-TR Mac Olympus) was used for searching of the aspirated oocytes and for evaluating the quality of the oocytes.

3.2.1b Inverted microscope

An inverted microscope (Magnus Invi, Olympus) was used for the examination of morphological status and growth of the cultured oocytes. The microscope was equipped with programmable still photography and video recording.

3.2.1c CO_2 incubator

The culture of oocytes was carried out in a CO_2 incubator (MAC, MSW-129) at 38.5^0C with 5% CO_2 in air and 90-95 % relative humidity environment.

3.3 Chemicals and cell culture media

All media (TCM-199) and hormones (FSH F2293-1VL Follicle stimulating hormone from porcine pituitary, Estradiol E-8875-250 MG), Fetal bovine serum (FBS) RM9952-100 ML, Bovine serum albumin- Fraction V (BSA-V) CAS 9048-46-8 and Chemicals (NaCl, KCl, KH_2PO_4, Na_2HPO_4, D-Glucose, sodium pyruvate, cystamine etc) were procured from M/S Sigma chemicals Co. (St. Lous, MO, USA). Medium-199 with Earle's salts, L-glutamine and sodium bicarbonate M4530-500ML used in washing and equilibration media was procured from Hi-media Laboratories, Mumbai, India. Ethanol, xylene and paraffin wax used for tissue processing were of SDFCL, Mumbai, India.

3.4 Working environment

All the procedures were carried out in highly sterile condition under laminar air flow cabinet to avoid any bacterial or fungal contamination in the tissue culture laboratory of Division of Veterinary Physiology & Biochemistry, FVSc & AH, R.S. Pura. Water used for media preparation was from reverse osmosis milli-Q water system. All the working solutions/media excluding OCM (Oocyte Collection Media) were kept for at least 3-4 hours in CO_2 incubator at 37^0C, 5% CO_2, 21% O_2 and 95 % relative humidity (RH) for quenching before use. The stocks of all media were stored at 4^0C and used within fifteen days of preparation. Prepared OCM was kept at 37^0C just prior to work.

3.5 Sterilization procedures

All the media were sterilized by filtration through 0.22 µm filter. The glassware and micropipette tips were sterilized by autoclaving at 121^0C for half an hour. The petri dishes, centrifuge tubes, syringes were exposed to UV light for 15 minutes before use.

3.6 Preparations of media

The compositions of media used in the experiment i.e. oocytes collection medium (OCM), equilibration medium, thawing medium and maturation media are listed in Appendix I. All the prepared media excluding OCM were kept for at least 3-4 h in CO_2 incubator at 38.5^0C, 5% $CO_2$and 95% relative humidity for quenching before use. All the culture media were sterilized by filtration through 0.22 µm filter and stored at 4^0C and used within 15 days of preparation.

3.7 Preparation of sheep (estrus) serum

Blood was collected from jugular vein of sheep at standing heat (estrus) from Govt. Sheep Breeding Farm, Panthel, Reasi. The serums were separated and heat inactivated at 56^0C for 30 min, filtered through 0.22µm filters and stored at -20^0C until future use.

3.8 Collection of sheep follicular fluid

Sheep ovaries were obtained from local abattoir and transported to laboratory at 4^0C within 2 h of slaughter. After ovaries were washed with sterile cold saline, follicular fluid was aspirated from all the visible non-atretic, healthy surface follicles with an 18 gauge needle attached to a 5 ml syringe. The cellular debris was removed by centrifuging collected follicular fluid at 3000 rpm for 30 minutes. The collected supernatant was sterilized by filtration through 0.22µm syringe filter. Aliquot of 1ml of follicular fluid was made in sterile eppendrof tube and stored at -20˚C until further use.

3.9 Collection of ovaries

Sheep ovaries used in the experiment were collected from the local slaughter house, Gujjar Nagar, Jammu. The ovaries were transported to the laboratory in normal

saline solution (NSS, 0.85% NaCl) fortified with gentamycin (50µg/ml) in a thermo flask at ~38^{0}C within 2 hours of slaughter. In laboratory, tissue attached to the ovaries were trimmed out with scissors, and then washed in sterile NSS at 38^{0}C. The obtained ovaries were randomly assigned to fresh control and cryopreserved group.

3.10 Cryopreservation of ovaries:

The ovaries were cryopreserved by vitrification procedure of Courbiere *et al.* (2005) with minor modification. Vitrification is a simple and fast process by which water is prevented from forming ice due to the viscosity of a highly concentrated cryoprotectant cooled at an extremely rapid rate (Taylor, 1987). To reduce exposure to the toxic effect of cryoprotectant and prevent extreme dehydration in the study, cells were exposed to the cryoprotectants for a very short period. Dimethyl Sulfoxide (DMSO) was used as cryoprotectant in the present study due to its permeable nature.

The vitrification procedure followed in the study consisted of three equilibration steps in solutions with increasing concentrations of permeable cryoprotectant (CPA) DMSO dissolved in freezing medium (FM) i.e. HEPES buffered TCM-199 fortified with fetal bovine serum (Freezing media, DMSO: FBS: HEPES buffered TCM-199 :: 1:4:5). Ovaries were sequentially immersed for 5 minutes each in the equilibration medium prepared with HEPES buffered TCM-199 and freezing media at a ratio of 1:1, 1:2 and 0:1. The ovaries kept in sterile cryovials with FM were exposed to liquid nitrogen (LN_2) vapour for 5 minutes before putting those into liquid nitrogen (LN_2).

On completion of cryopreservation period of 1 month, the thawing of cryopreserved ovaries was done as per the procedure of Gook and co-workers (Gook *et al*., 2007). The procedure followed briefly as follows;

- Cryovials were taken out of LN_2 and immersed in water bath at 37^{0}C for 2 min
- The CPA was diluted by transferring the ovaries through thawing medium (TM); HEPES buffered TCM-199 with 20% serum and 1 mol/L of sucrose.

- The tissues were thawed serially in diluted thawing solution at a ratio of 1:1, 2:1, and 1:0 with HEPES buffered TCM-199. Each step of dilution protocol lasted 5 min.

- Then each ovary was washed twice in working OCM for 5 min at 37 °C.

3.11 Oocytes collection and processing

Oocytes were aspirated from all visible non-atretic surface follicles by an 18 or 20 gauge needle attached to 5 ml syringe containing OCM. The cumulus oocyte complex (COC) along with follicular fluid (FF) was pooled into a 50 ml sterile plastic tube and was allowed to settle for 10 min in BOD incubator at 37^0C. About $2/3^{rd}$ of supernatant was discarded gently. Finally the sediments were taken in a large sterile petridish (90mm). The searching of oocytes were carried out under stereo zoom microscope (Olympus). The COCs were evaluated and graded by the methods followed Kobayashi et al. (1994).

Usable quality: Compact cumulus oocyte complexes (COCs) with an unexpanded cumulus mass having more than 2 layers of cumulus cells and with homogenous evenly granulated ooplasm.

Unusable quality: oocytes partially or wholly denuded or with expanded or scattered cumulus cells or with an irregular ooplasm.

The oocytes of only usable quality were used for *in vitro* maturation (*IVM)*. COC was selected and washed in sterile petri dish (35mm) containing OCM. The COCs were washed in 8-10 drops with working OCM in a petri dish. Finally these oocytes were washed in 6-8 drops of maturation medium (MM) in a 90mm petri dish.

3.12 *In vitro* maturation of oocytes

Washed COC were cultured in 50µl droplets (7-10 oocytes/droplet) of maturation media (TCM-199 with HEPES buffer, 10% FBS, sheep serum 5%, 5% FF, BSA-V 3mg/ ml, 0.5µg/ml FSH-P 0.5µg/ml, estradiol 10µg/ml and cystamine 50 µM) in 35 mm sterile

petridish. The droplet was covered with warm non-toxic paraffin oil and cultured at 38.5^0C, 5% CO_2 and (95%) humidity for 27 h.

Assessment of maturation after in vitro culture of oocytes

The evaluation of maturation were based on the visual assessment of degree of cumulus expansion under inverted microscope (Olympus Magnus Invi) as described by Kobayashi *et al*., (1994).

Degree 0: No expansion

Degree 1: Cumulus cells are non-homogenously spread and clustered cells are still observed.

Degree 2: Cumulus cells are homogenously spread and clustered cells are no longer present.

Only degree 1 and 2 cumulus expanded oocytes are considered as matured ones.

3.13 Cryopreservation induced toxicity assessment

3.13.1 Histological analysis

Histological assessment of frozen thawed as well as freshly collected ovarian tissues was done by sectioning the tissues and staining with haematoxylin – eosin stain (Luna, 1968). The procedure followed is as follows;

- Fresh ovarian biopsy (day 0) and cryopreserved ovarian biopsy (1 month) specimens were fixed in 4% formaldehyde solution.
- Tissues were kept under running tap water overnight
- Ovarian tissues were dehydrated for removal of extractable water by slow substitution in the tissues with organic solvent using different concentration of alcohol serially- 50% (6h), 70% (overnight), 90% (6-8h), 95%(overnight), Absolute alcohol I (30min), absolute alcohol II(30min), Absolute alcohol III(30min), Absolute alcohol plus xylene (30min).

- Then ovarian tissues were cleared in xylene I (30min), xylene II (30min), xylene III (30min).
- Tissues were then put into melted in paraffin wax (57-58^{0}C) in different sets of paraffin, Paraffin I (15-20min), Paraffin II (15-20min), Paraffin III (15-20min).
- Blocks were made using L shaped mould. The tissue pieces were serially sectioned at a thickness of 4µm.
- The sections were then mounted on the slides with 6 sections omitted between each mounted section to prevent duplication in observation.

Staining of ovarian tissues

- The sections were deparaffinized in xylene (10-20min), rehydrated in 100% alcohol for 1-2 minutes, then 95% for 1-2 minutes and rinsed in tap water.
- The sections were stained with haematoxylin for 3-5 minutes and then washed in tap water.
- After washing section were differentiated with 1% HCl in 70% alcohol(1-2 dips). Slides were again washed in running tap water for 15 minutes and then stained in eosin for 1-4 minutes.
- Then stained tissues were dehydrate and differentiated using 95% alcohol 5-6 dips, 100% alcohol 5-6 dips.
- Slides were then cleared in xylene 2 times and mounted with mounting media (DPX).

Following staining with haematoxylin and eosin, the slides were analysed for any variation between fresh and cryopreserved ovarian tissues. Then the follicular developmental stages were classified according to classification method of Gougeon (Gougeon 1986). Briefly the follicles containing a single layer of flattened granulosa cells were regarded as primordial, those having one or more cuboidal granulosa cells around all or part of oocyte were identified as secondary follicles. Atretic follicles were

identified by oocyte fragmentation, eosinophilia of cytoplasm, pyknotic granulosa cells and or clumping of the chromatin.

3.13.2 Viability analysis of follicular cells

Viability of follicular cells of both fresh and cryopreserved ovaries was analysed using trypan blue dye following the method of Demirci *et al*. (2001) with minor modification, briefly.

- After aspiration, follicular fluid was collected in centrifuge tube and centrifuged at 3000 rpm for 10 min. The supernatant was discarded leaving behind the sediment at bottom of the tube.
- Follicular cell suspension was prepared by adding 1ml of PBS to the sediment.
- Working solution of trypan blue was prepared by mixing 0.5 ml of 0.4% trypan blue solution with 0.3 ml of PBS.
- 0.1 ml of cell suspension and 0.1ml of working solution were mixed thoroughly.
- The chambers of hemocytometer were charged with cell suspension & observed for their viability. Non-viable cells looked blue and live cells unstained

$$\text{Cell viability (\%)} = \frac{\text{Total viable cells (unstained)}}{\text{Total cells (stained and unstained)}} \times 100$$

3.14 Statistical analysis

For all the observed data in the present experiment, the standard procedures recommended by Snedecor and Cochran (2004) have been followed. The data were presented by showing mean and standard error. The significant differences of values for different parameters studied were assessed by chi square test. All the above calculations were carried out using SPSS software version 16.

Chapter - 4

RESULTS

CHAPTER-IV

RESULTS

The present research work was done to study the developmental competence of harvested oocytes from the cryopreserved ovary. A total of 234 numbers of ovaries were used in the experiment, out of which 114 ovaries cryopreserved for one month in different replications and rest used as standard.

4.1 Comparison of COC's harvested from fresh and cryopreserved sheep ovaries.

The COC's from surface and inner (submerged) follicles were collected by aspiration and mincing methods respectively. The number of oocytes harvested from surface and inner follicles are shown in Table 4.1.1 and Fig 4.1.1. It was observed that 1.688±0.117 number of COC's were harvested per fresh ovary whereas 1.694±0.202 number of COC's per cryopreserved ovary. The number of harvested COC's per ovary in both the groups were not varying significantly.

Table 4.1.1: Number of COC's harvested from fresh and cryopreserved sheep ovary.

Group	Total no. of COC's harvested per ovary	No. of COC's harvested from surface follicles per ovary	No. of COC's harvested from inner follicles per ovary
Fresh ovary	**1.688 ± 0.117**	**1.025 ± 0.007**	**0.665 ± 0.050**
Cryopreserved ovary	**1.694 ± 0.202**	**1.026 ± 0.117**	**0.668 ± 0.108**

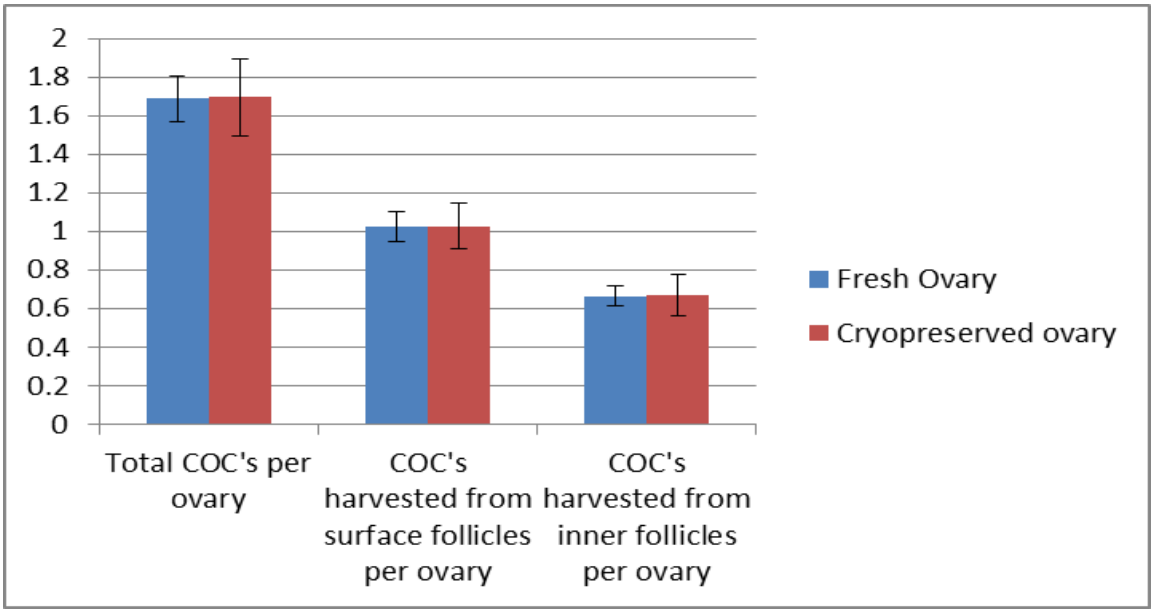


Fig. 4.1.1: Bar diagram showing COC's harvested from surface and inner follicles of fresh and cryopreserved ovary.

The number of COC's harvested from the surface follicles of fresh and cryopreserved ovaries were 1.025±0.007 and 1.026±0.117, respectively which were also not differing significantly with each other. The number of COC's harvested from the submerged follicles of fresh (0.665±0.050) and cryopreserved (0.668±0.108) ovaries were also not significantly differing with each other (Table 4.1.1).

The qualities of the harvested COC's were assessed as per Kobayashi *et al*., (1994) and categorized as usable and non-usable quality for *IVM*. The Table 4.1.2 revels that total usable quality COC's harvested per ovary from fresh and cryopreserved groups are 1.105±0.062 and 1.054±0.168 respectively. No significant variation was observed between the total number of COC's harvested from both the groups. The number of usable quality COC's harvested per ovary from the surface follicles of fresh and cryopreserved ovaries are observed to be non- significant statistically (0.729±0.053 vs 0.660±0.091) (Table 4.1.2). Similarly number of usable COC's harvested from inner follicles per fresh and cryopreserved ovary were also statistically non-significant with each other (Table 4.1.2). However, it is observed that number of usable COC's (total as well as COC's harvested from surface follicles), harvested per fresh ovary is more than that of the cryopreserved ovary. But the usable COC's harvested from inner follicles per

cryopreserved ovary was more than that of the fresh group. The above values are depicted in Fig. 4.1.2.

Table 4.1.2: Usable quality COC's from surface and inner follicles from fresh and cryopreserved sheep ovaries

Usable quality COC's per ovary			
Group	**Total**	**From Surface follicles**	**From Inner follicles**
Fresh ovary	**1.105±0.062**	**0.729±0.053**	**0.377±0.016**
Cryopreserved ovary	**1.054±0.168**	**0.660±0.091**	**0.394±0.085**

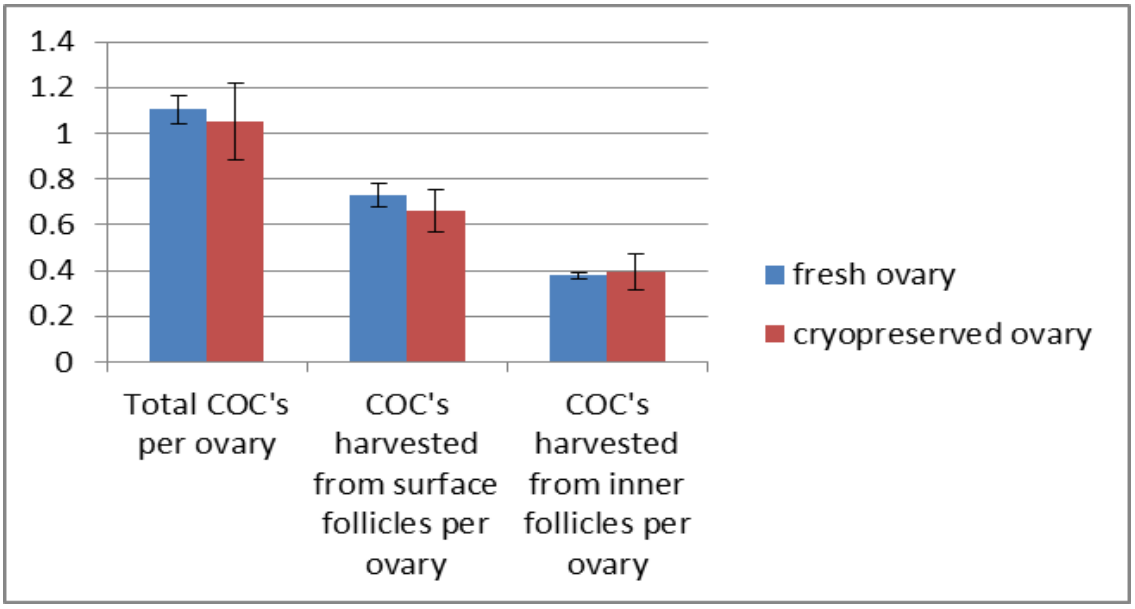


Fig. 4.1.2: Bar diagram showing usable quality COC's from surface and inner follicles from fresh and cryopreserved sheep ovaries.

Table 4.1.3 shows that the non- usable quality COC's harvested from surface as well as inner follicles of fresh and cryopreserved groups per ovary are not differing

significantly with their counterpart. However, statistically significant difference ($p < 0.05$) were observed between the harvest of non-usable COC's from surface and inner follicles of cryopreserved as well as fresh group of ovaries (Fig 4.1.3).

Table 4.1.3: Non-usable quality COC's harvested from surface and inner follicles from fresh and cryopreserved sheep ovaries.

Non-usable quality COC's per ovary			
Group	**Total**	**From Surface follicles**	**From Inner follicles**
Fresh ovary	**0.583±0.090**	**0.192±0.037[a]**	**0.391±0.061[b]**
Cryopreserved ovary	**0.640±0.089**	**0.206±0.041[a]**	**0.434±0.054[b]**

Mean ± SE bearing different superscript (a and b) in a row differing significantly ($p<0.05$).

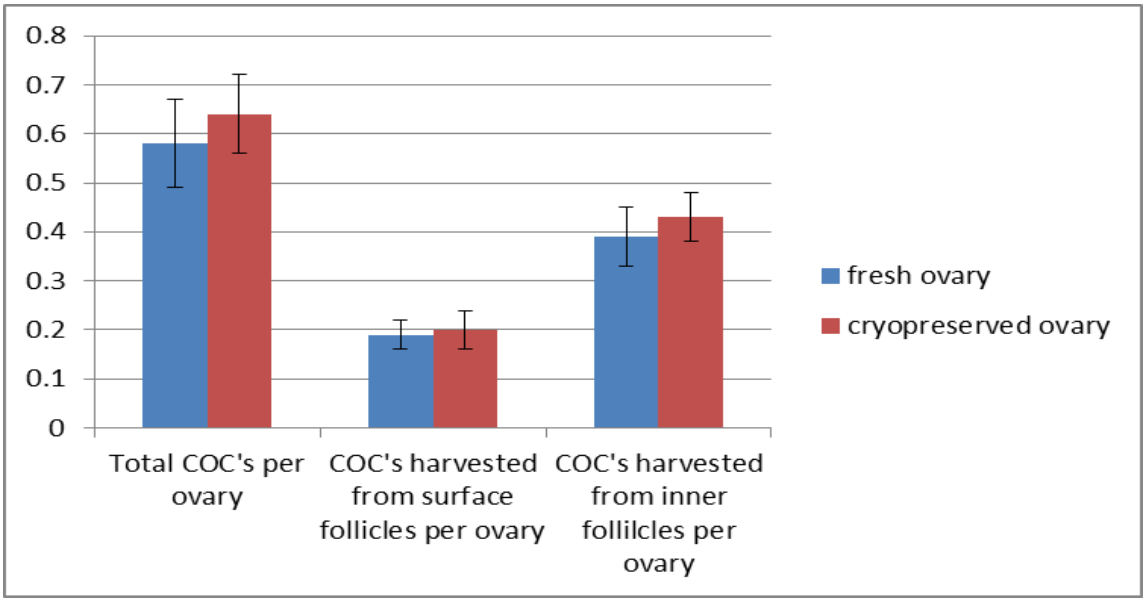


Fig. 4.1.3: Bar diagram showing non-usable quality COC's harvested from surface and inner follicles of fresh and cryopreserved sheep ovaries.

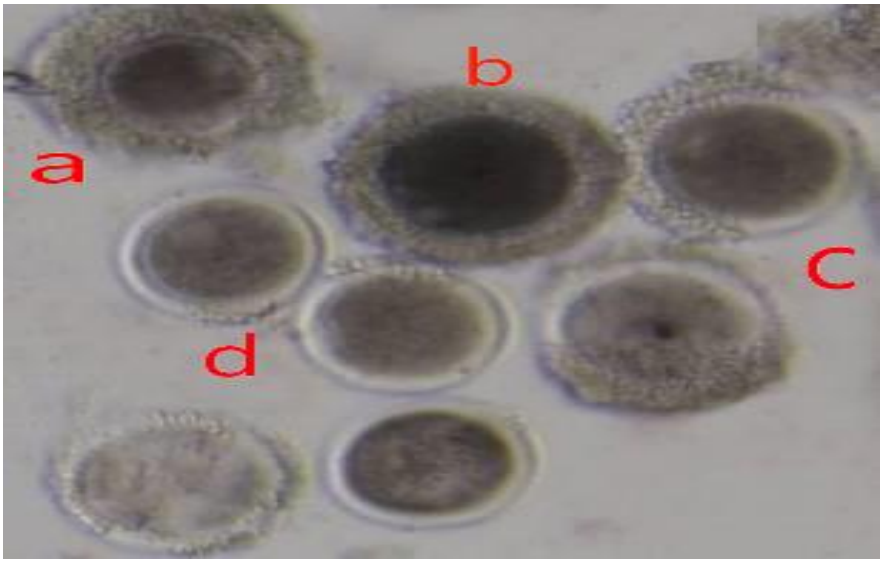


Plate 4.1.1: Photograph showing COC's harvested from surface follicles of fresh ovary (magnification x 200), a: oocytes surrounded by > 3 layers of granulosa cells, b: oocytes surrounded by 2-3 layers of granulosa cells, c: oocytes surroundedby 1-2 layers of granulosa cells, d: denuded).

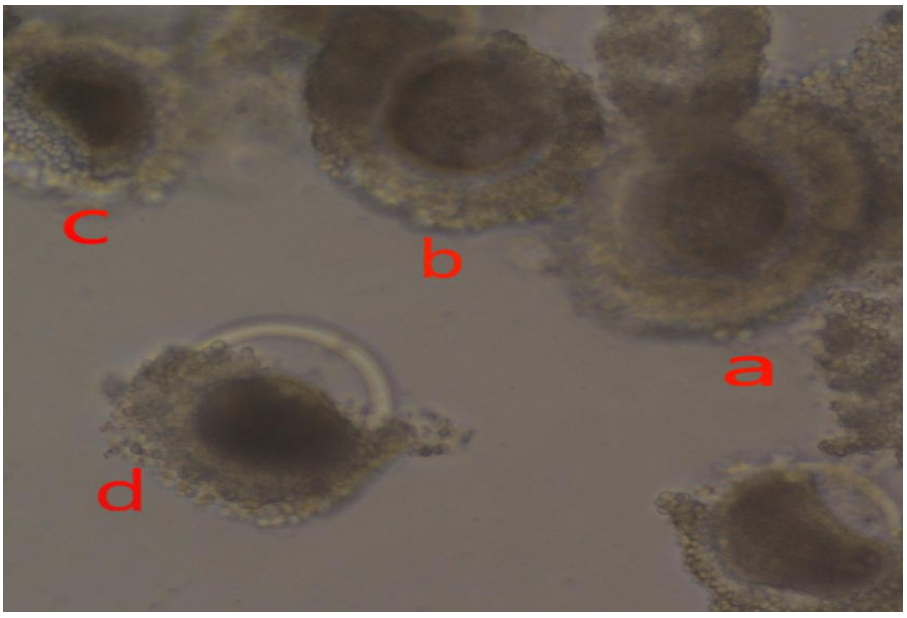


Plate 4.1.2: Photograph showing COC's harvested from surface follicles of cryopreserved ovary (magnification x 200), a: oocytes surrounded by > 3 layers of granulosa cells, b: oocytes surrounded by 2-3 layers of granulosa cells, c: oocytes surrounded by 1-2 layer of granulosa cells, d: denuded).

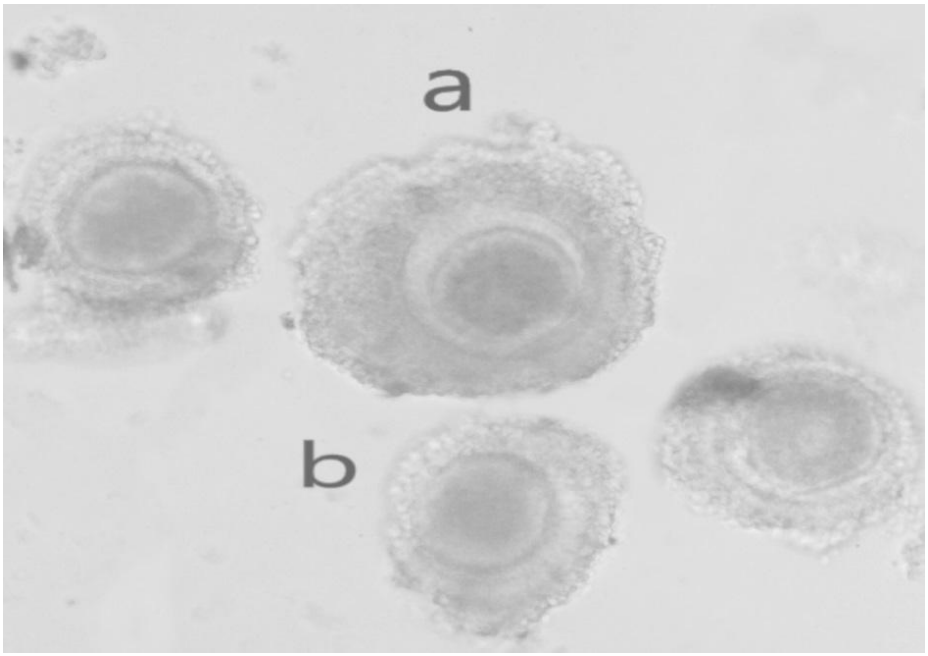


Plate 4.1.3: Photograph showing COC's harvested from inner follicles of fresh ovary (magnification x 200), a: oocyte surrounded by > 3 layers of granulosa cells, b: oocyte surrounded by2-3 layers of granulosa cells).

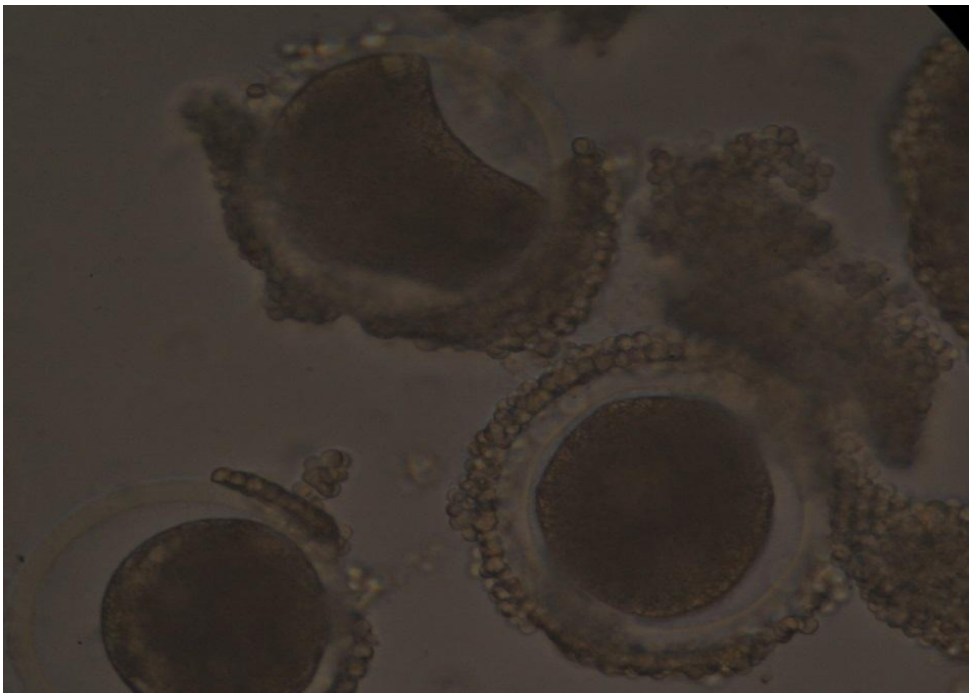


Plate 4.1.4: Photograph showing COC's harvested from inner follicles of cryopreserved ovary (magnification x 200).

The Plate 4.1.1 and 4.1.2 showing the photos of COC's (usable and non-usable) harvested from surface of fresh and cryopreserved ovaries respectively. Most of the harvested oocytes were having more than 4-5 layers of cumulus cells and homogenous cytoplasm without any intra cytoplasmic vacuoles. The collected oocytes were showing regular contour with basal membrane. However, some oocytes were observed to be denuded but with homogenous cytoplasm and clear boundary.

The COC's harvested from inner follicles of fresh and cryopreserved ovaries are shown in plate 4.1.3 and 4.1.4. The oocytes harvested from inner follicles of fresh ovary were having continuous layering of granulosa cells (more than 3) with homogenous cytoplasm and well defined contour but, the number of oocytes harvested from inner follicles of cryopreserved ovaries (Plate 4.1.3 and 4.1.4) were having irregular contour as well as irregular attachment of granulosa cells to the oocytes. However, more number of usable oocytes with standard morphology were harvested from inner as well surface follicles of both the fresh and cryopreserved ovaries.

4.2 Assessment of the quality of frozen-thawed sheep ovarian tissue:

The ovaries after collection from the slaughter house were divided into 2 groups namely fresh and cryopreservation ovary respectively. The cryopreservation of ovaries was done as per the method mentioned in chapter 3. After the completion of the scheduled duration of cryopreservation (1 month) 24 nos. of ovaries were taken out of LN_2. Those ovaries were thawed as per procedure mentioned in previous chapter and half of them used for study of gross change due to cryopreservation and other half used to study the histological changes.

4.2.1 Gross analysis:

The photographs of whole ovaries (fresh as well as cryopreserved) are given in Plate 4.2.1a and 4.2.1b. Similarly the longitudinal section of the ovaries (fresh and cryopreserved) are given in the Plate 4.2.1c and 4.2.1d. No change was observed in gross structures of both types of ovaries (fresh and cryopreserved). No significant change was observed in ovarian surface epithelium (peritoneal mesothelium) which covers the ovarian surface in both groups of specimen (fresh and cryopreserved ovaries). The

Plate 4.2.1a: Photograph showing freshly collected sheep ovary, arrows showing follicles.

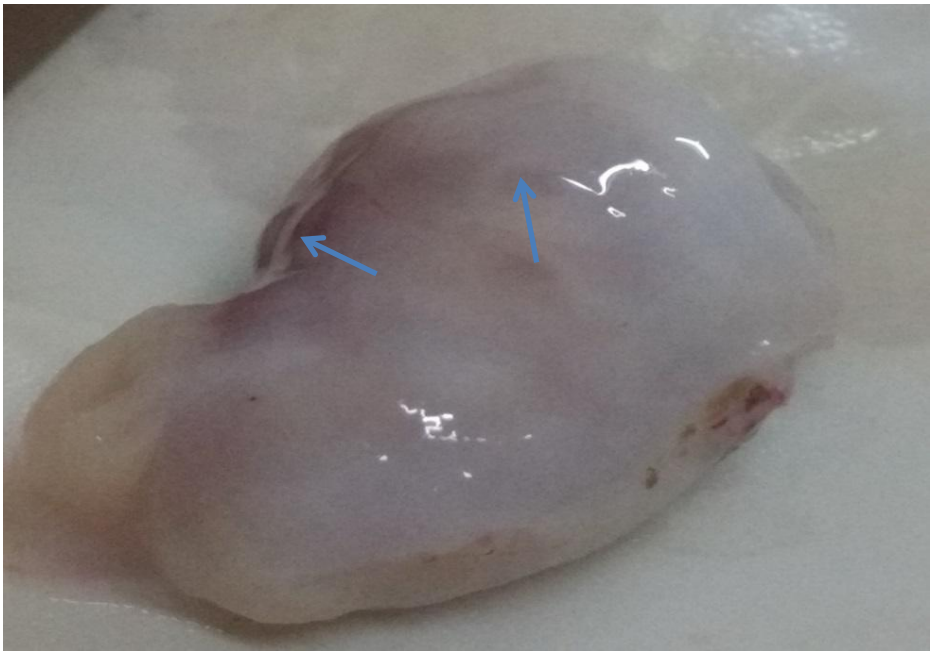


Plate 4.2.1b: Photograph showing cryopreserved sheep ovary, arrows showing follicles.

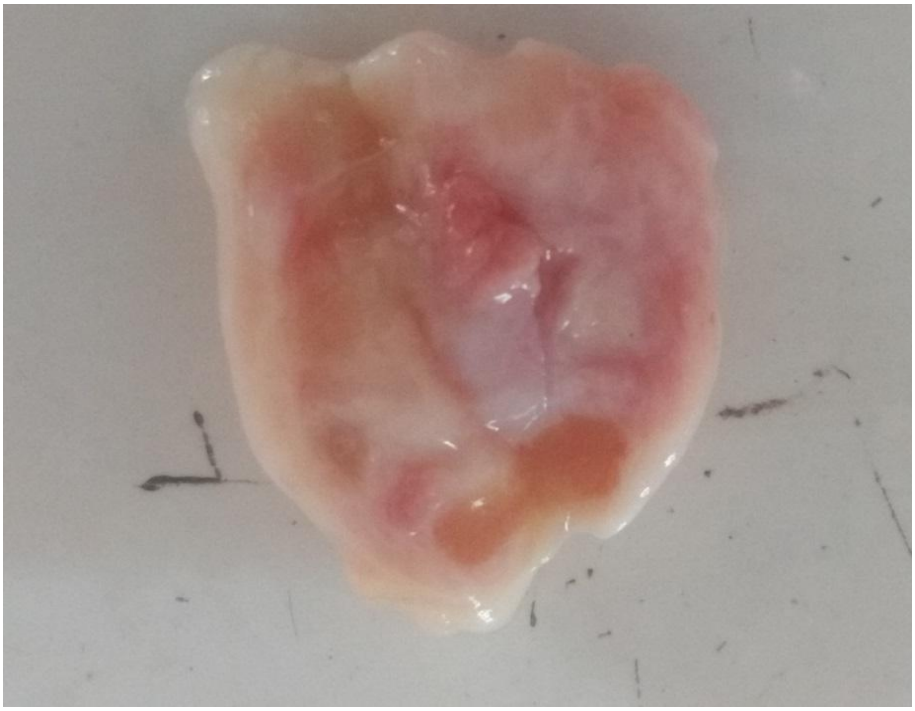


Plate 4.2.1c: Photograph showing longitudnal section of fresh ovary

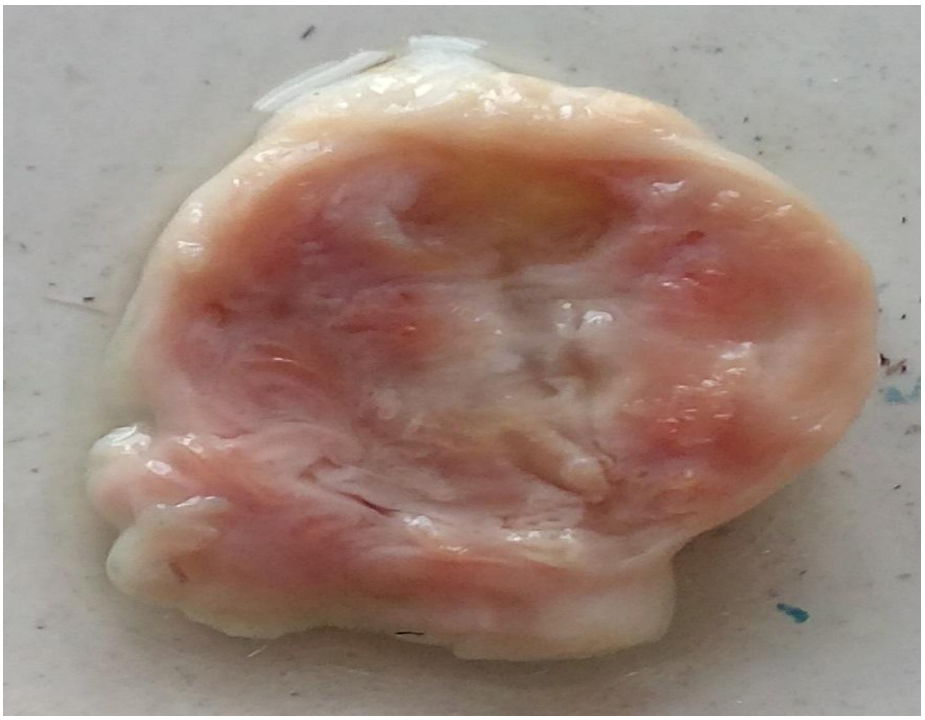


Plate 4.2.1d: Photograph showing longitudnal section of cryopreserved ovary.

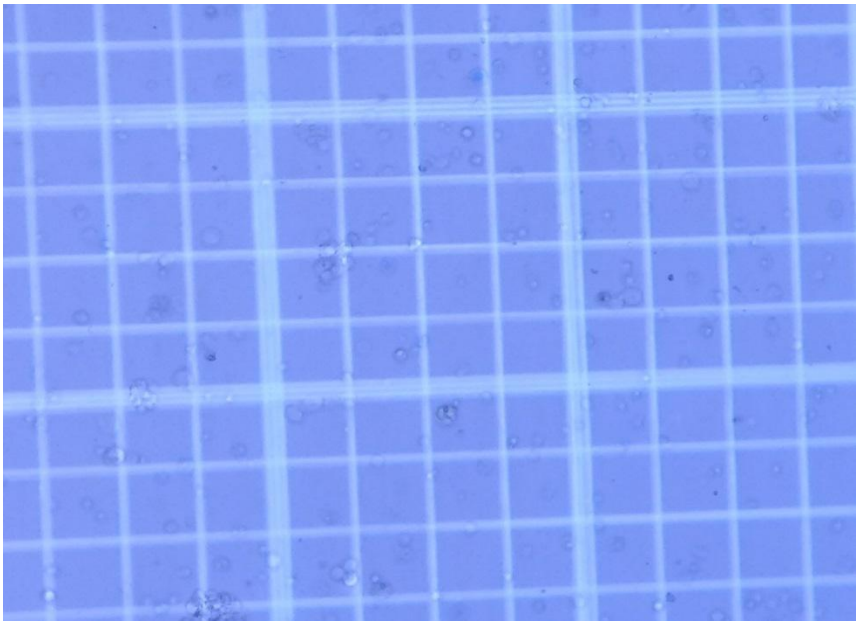


Plate 4.2.3a: Photograph showing result of trypan blue staining of follicular cells of fresh ovary: live cell unstained, dead cells- stained.

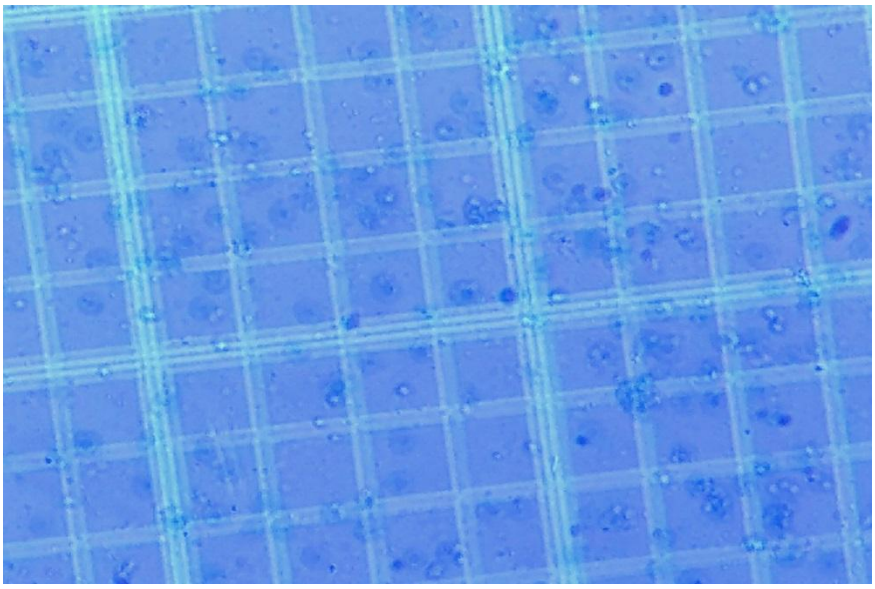


Plate 4.2.3b: Photograph showing result of trypan blue staining of follicular cells of cryopreserved ovary: live cells- unstained, dead cells- stained.

growing follicles protruding from the surface of the ovaries (fresh and cryopreserved) were observed to be intact and clearly visible. In both types of ovaries the surface looked smooth and undamaged.

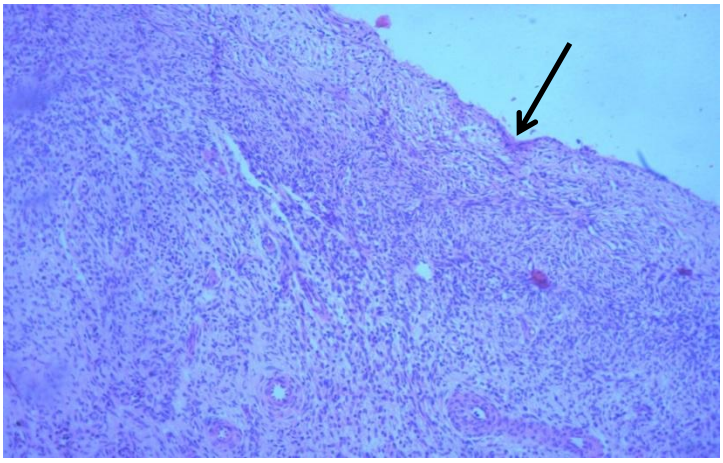
The longitudinal sections of the fresh as well as cryopreserved ovaries did not reveal any difference. There was no shrinkage of tissue in case of cryopreservation. In both the cases the cortex and medulla were well demarcated and clearly visible follicles in cortical mass. The follicles were normal sized according to their categories.

4.2.2 Histological analysis:

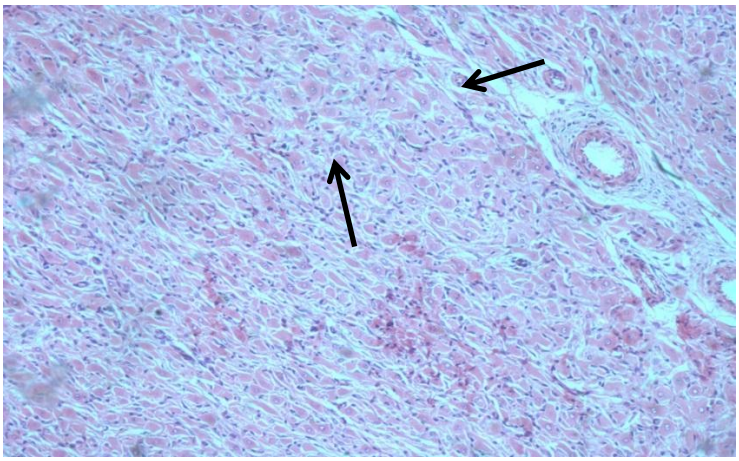
The follicular morphology in cryopreserved ovarian sections was similar to that in the control (fresh ovary). The haematoxylin and eosin (H&E) stained sections in fresh ovarian tissue demonstrated contact between oocytes and surrounding granulosa cells as well as between neighboring granulosa cells (Plate 4.2.2a and 4.2.2b). The oocyte's cytoplasm in these follicles showed a uniform distribution. The light microscopic examination of cryopreserved ovarian tissue sections showed intact follicles with centrally located oocytes and intact basement membrane surrounded by flattened theca cells. In some antral follicles the oocyte present eccentrically with intact cumulus oophorus and corona radiata. But a few samples of cryopreserved ovarian tissue sections exhibited cryo-induced damage like shrunken and vacuolated oocytes, space between follicles and stroma as well as in between stromal tissue at some fragments. In some fragments of the samples the granulosa cells disrupted from the theca cells. The follicles of different categories were scattered throughout the cortical tissue mass. In both the cases (fresh and cryopreserved) the ovarian surface epithelium and tunica albuginea observed to be normal.

4.2.3 Follicular cell viability analysis:

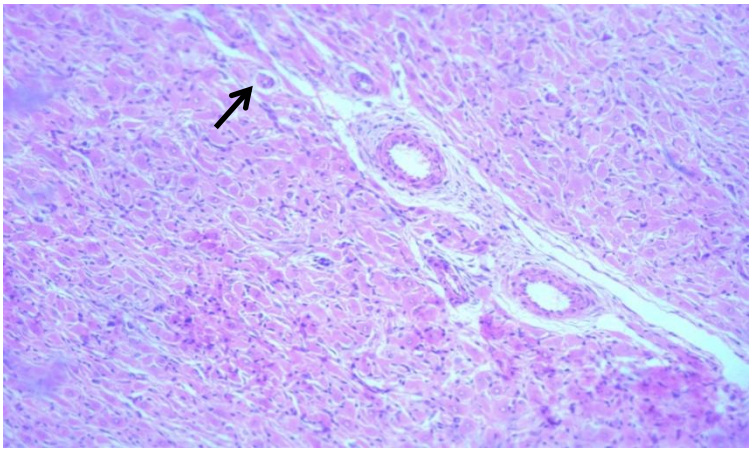
The examination of viability of follicular cells from fresh and cryopreserved ovary with trypan blue revealed that viability of cryopreserved cells were significantly ($P<0.05$) lower than that of the freshly collected ovaries. The number of viable follicular cells are presented in Table 4.2.3 and Figure 4.2.3. The follicular cell viability in freshly collected and ovaries cryopreserved for 1month are presented in Plate 4.2.3a and 4.2.3b.



X.

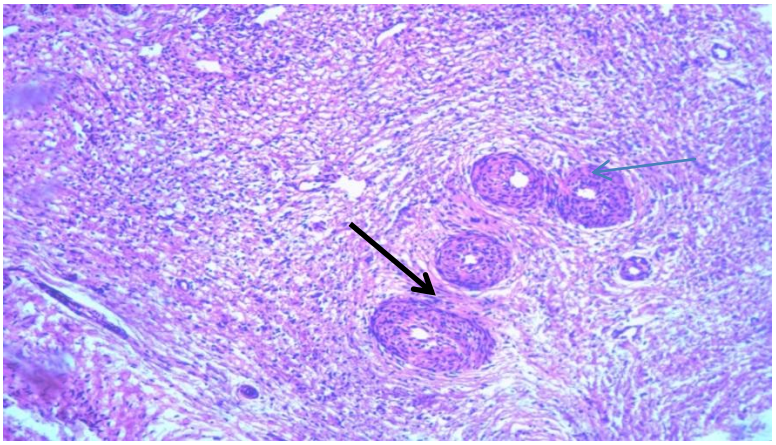


Y.

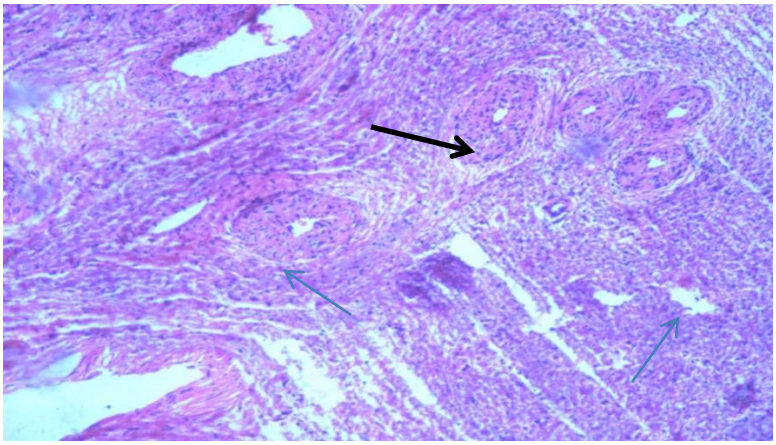


Z.

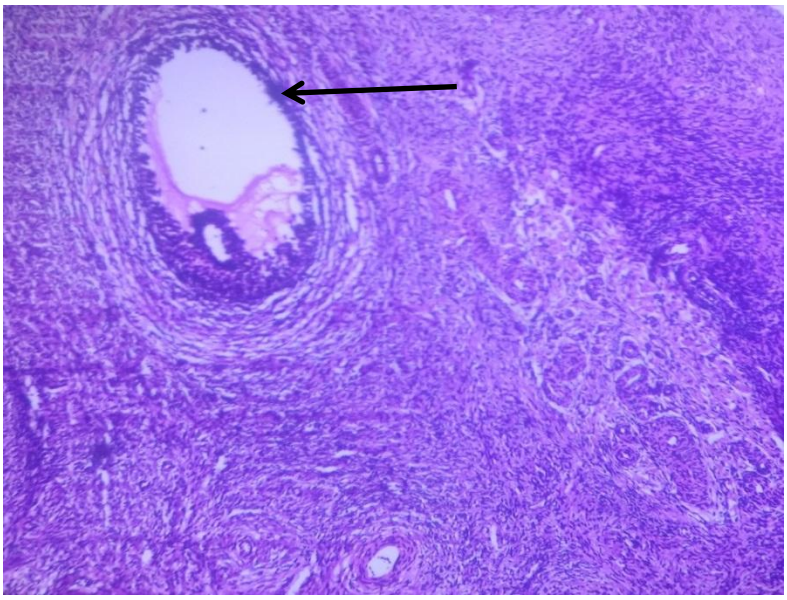
Plate 4.2.2a: Photograph showing H&E stained sections of freshly collected ovaries (magnification x 100). X- arrow showing intact epithelium, Y,Z- arrow showing primary follicles.



A.



B.



C.

Plate 4.2.2b: Photograph showing H&E stained sections of cryopreserved ovaries (magnification x 100). A,B- arrow showing secondary follicles, C- arrow showing antral follicle with oocyte.

Table 4.2.3: Effect of cryopreservation on viability of follicular cells.

Group	Live cells %	Dead cells %
Fresh ovary	73.817±1.106[b]	26.183±1.106[a]
Cryopreserved ovary	67.467±1.016[a]	32.533±1.016[b]

Mean ± SE bearing different superscripts (a and b) in a column differ significantly ($p < 0.05$).

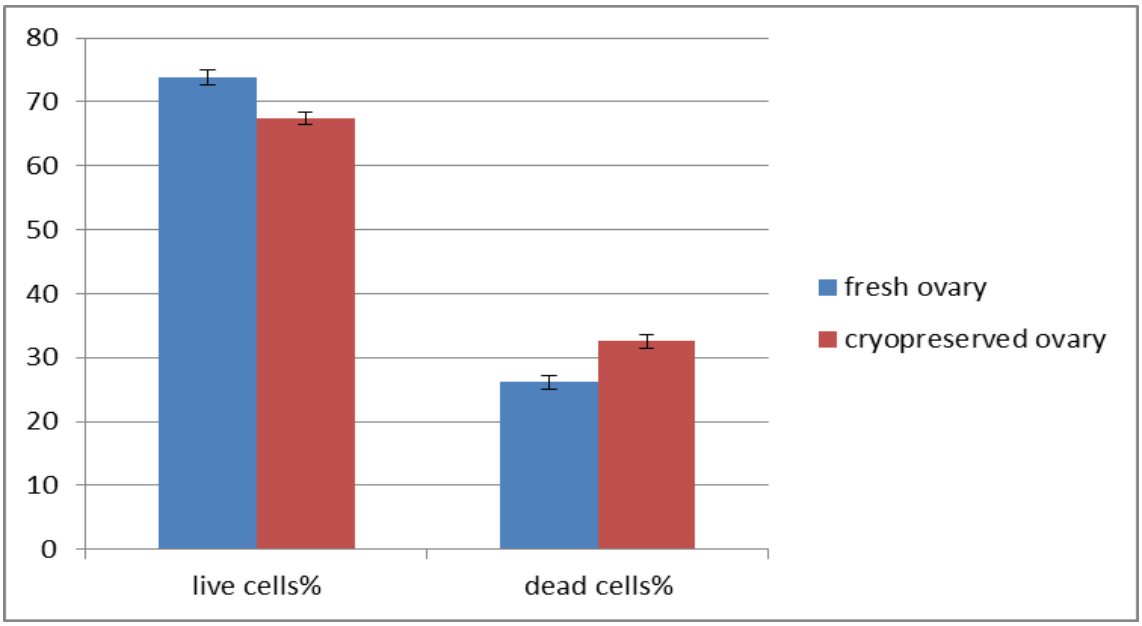


Fig. 4.2.3: Bar diagram showing effect of cryopreservation on viability of follicular cells.

4.3 Assessment of maturation of oocytes collected from cryopreserved ovaries after *in vitro* culture.

The oocytes aspirated from the surface follicles of the fresh as well as cryopreserved ovaries were categorized into usable and non-usable oocytes as mentioned

in chapter 3. 95 number of usable oocytes harvested from fresh ovary and 83 number of usable oocytes harvested from cryopreserved ovaries were used for *IVM* experiment. Out of them only 44.21% and 39.76% collected from fresh and cryopreserved ovaries respectively of used oocytes were undergone maturation process as evident from proliferation of granulosa cells. The statistical analysis revealed no significant difference in no. of maturation of oocytes harvested from fresh and cryopreserved ovary. The data in this regard are presented in Table 4.3.1 and Fig 4.3.1. The Plates 4.3.1 and 4.3.2 are showing the oocytes aspirated from the surface follicles of freshly collected ovaries. Most of the oocytes were of usable quality as evident from the layers of granulosa cells and cytoplasmic structure. After the 27 h of maturation the COC's showed extensive proliferation of granulosa cell layers. The granulosa cells did not exhibit much compactness between themselves.

Table 4.3.1: Comparison of maturation of usable oocytes harvested from fresh and cryopreserved ovaries.

Group	No. of oocytes used for *IVM*	No. of oocytes matured	%	Chi square value
Fresh ovary	**95**	**42**	**44.21**	**0.147 NS**
Cryopreserved ovary	**83**	**33**	**39.76**	

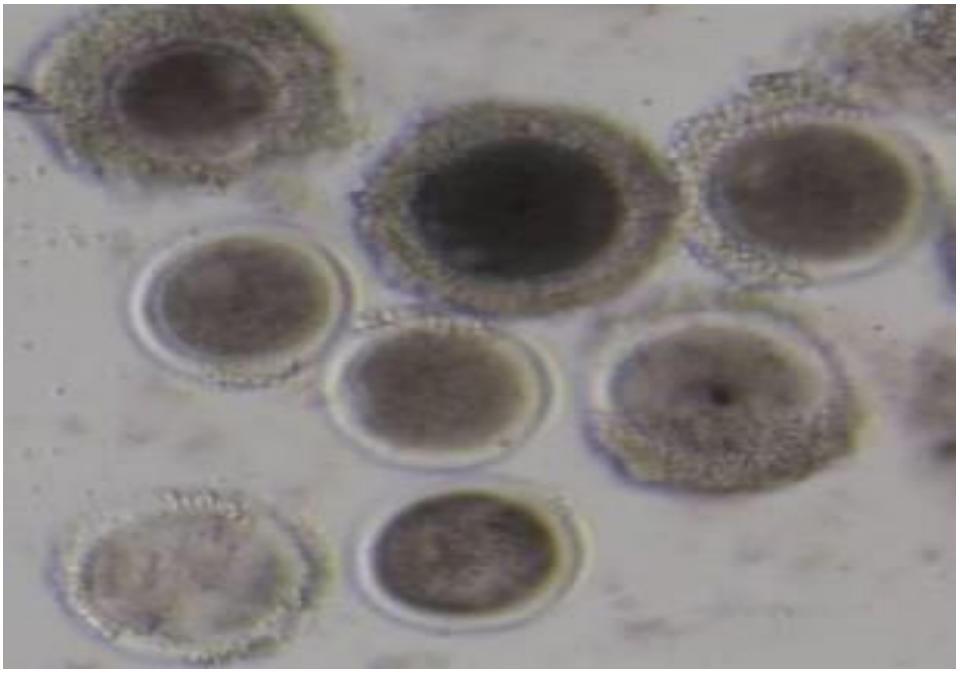


Plate 4.3.1: Photograph showing oocyte collected from fresh ovaries, before maturation (magnification x 200)

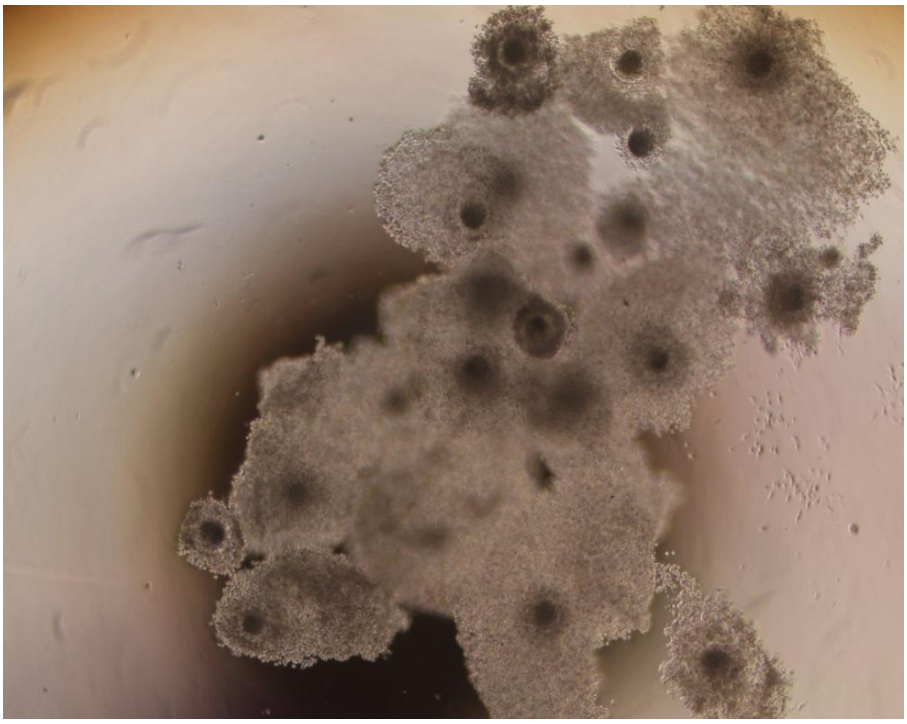


Plate 4.3.2: Photograph showing proliferation of granulosa cells after *IVM* of oocytes collected from freshly collected ovaries (magnification x 100).

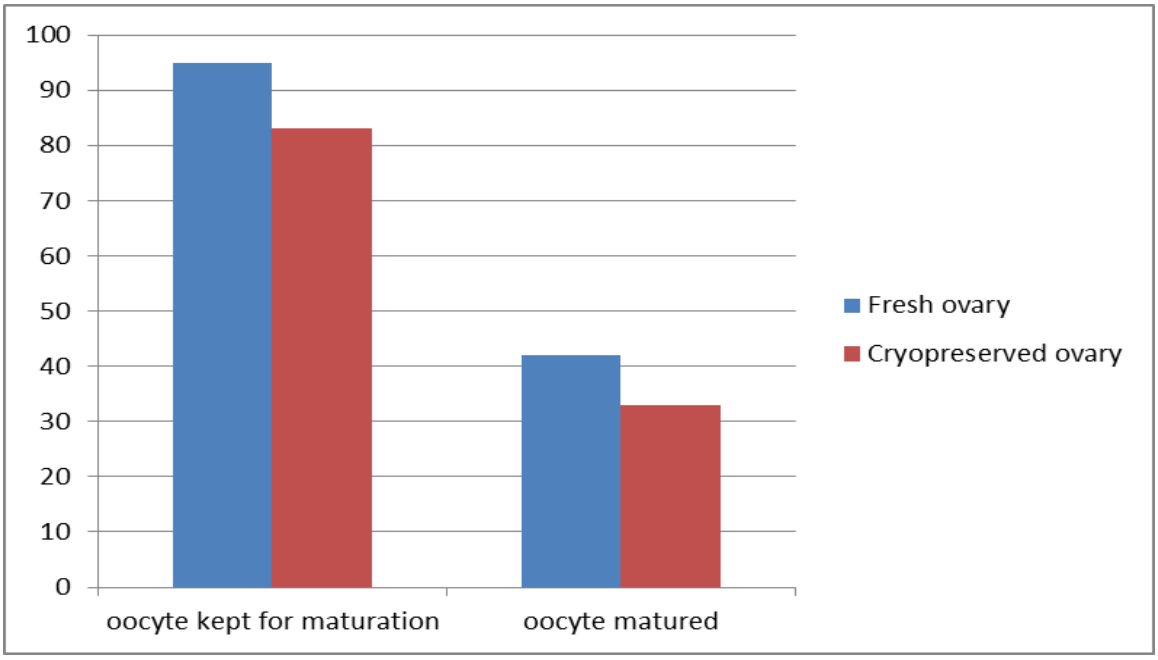


Fig. 4.3.1: Bar diagram showing maturation of usable oocytes harvested from fresh and cryopreserved ovaries.

The Plate 4.3.3 and 4.3.4 showing the COC's aspirated from the surface follicles of the cryopreserved ovaries and their morphology after the maturation process. In case of the cryopreserved ovary a number of COC's harvested were denuded type, hence not used for the maturation. The oocytes undergone the maturation process under ideal conditions exhibited proliferation of granulosa cells. Proliferated layers of granulosa cells were arranged more compactly than in comparison to matured oocytes collected from the fresh ovary.

The Table 4.3.2 and Fig 4.3.2 are showing the number of COC's matured after the *IVM*, harvested from surface and inner follicles of cryopreserved ovaries. The table reveals that 35.85% of oocytes collected from the surface follicles of cryopreserved ovaries undergone successful maturation. But the number of oocytes undergone successful maturation (26.67%) was less than oocytes undergone successful maturation harvested from inner follicles of cryopreserved ovaries. No statistical significant

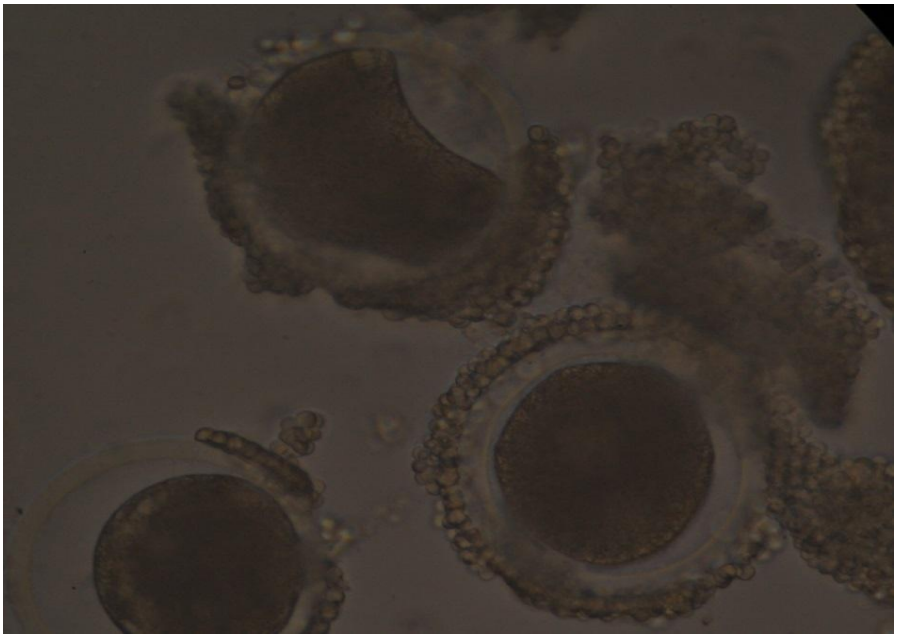


Plate 4.3.3: Photograph showing oocytes collected from cryopreserved ovaries, before maturation (magnification x 200).

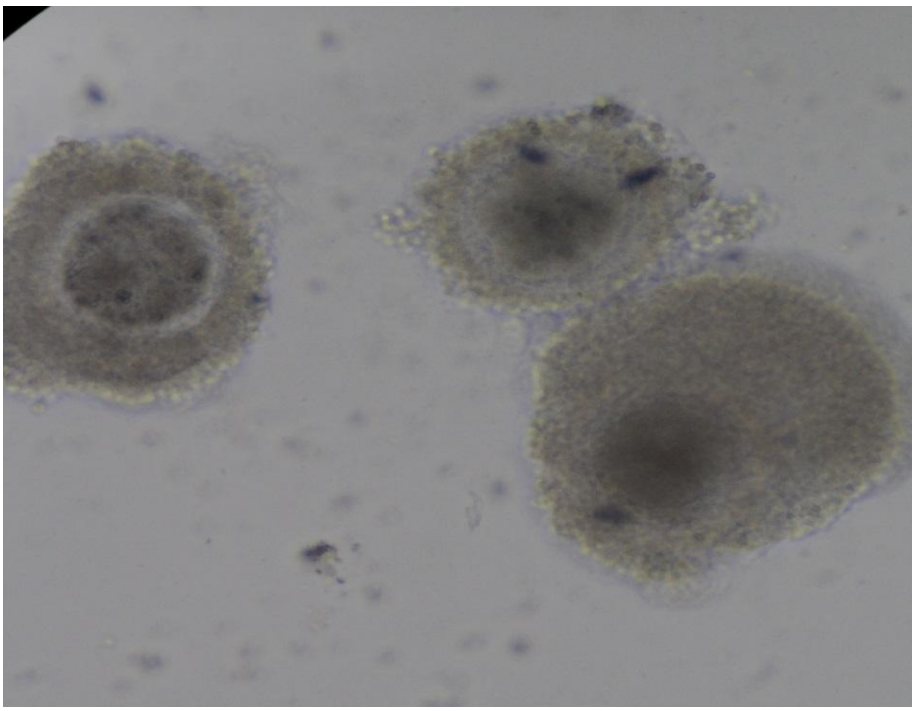


Plate 4.3.4: Photograph showing after *IVM* of oocytes collected from cryopreserved ovaries (magnification x 200).

difference was observed between the maturation of oocytes harvested from the surface and inner follicles of cryopreserved ovaries.

Table 4.3.2: Comparison of maturation of oocytes harvested from surface and inner follicles of cryopreserved ovaries.

Group	No. of oocytes used for *IVM*	No. of oocytes matured	%	Chi square value
Oocytes harvested from surface follicles	**53**	**19**	**35.85**	**0.382 NS**
Oocytes harvested from inner follicles	**30**	**8**	**26.67**	

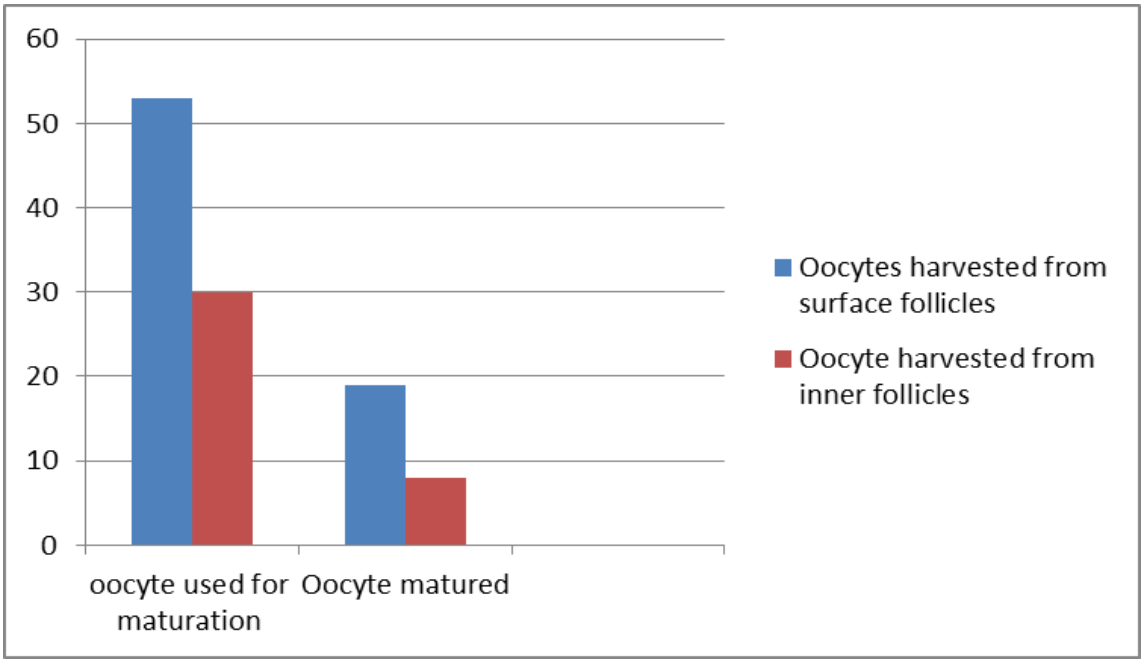


Fig. 4.3.2: Bar diagram showing maturation of oocytes harvested from surface and inner follicles of cryopreserved ovary.

Chapter – 5

DISCUSSION

CHAPTER-V

DISCUSSION

Cryopreservation of ovary containing the primordial follicles is an innovative way for the preservation of female gametes. The aim of present experiment was to cryopreserve ovaries and harvest immature follicles out of them to assess their developmental competence. Ovarian cryopreservation and autotransplantation were previously designed and applied in human and rats to protect and restore their reproductive function at the time of need. Earlier studies of animal ovarian autografts were done by various workers Aubard *et al*. (1999), Baird *et al*. (1999), Candy *et al*. (2000). In the year 1999, first orthotropic transplantation of frozen ovarian tissue was performed by Baird in animals (Baird *et al*. 1999). In sheep orthotropic transplantation was able to restore its endocrine function, fertility and also yield viable offspring (Gosden, 1994). In the present experiment the aim was to study the developmental potency of oocytes harvested from cryopreserved ovaries.

5.1 Recovery rate and morphological differences between harvested oocytes:

The oocytes were collected from ovaries (fresh and cryopreserved) by either aspiration (from surface follicles) and by mincing the ovarian tissues mechanically (from inner follicles). In the present study the rate of retrieval of total COC's were 1.688±0.117 and 1.694±0.202 per fresh and cryopreserved ovary respectively. The number of harvested COC's from inner follicles per ovary was less compared to those from surface follicles. Out of different techniques of oocyte collection, oocytes recovered per ovary by aspiration method produced better results as compared to other methods (Pawshe *et al*., 1994). The rate of retrieval in present study is in agreement with Majeed *et al*., (2011) who found the number of oocytes harvested was better by aspiration method than slicing method. The presence of corpus luteum might be a reason for less recovery of oocytes in the present study. Most of the ovaries used in the present study were having corpus luteum on their surface. Talukder *et al*., (2011) observed that number of normal COC's for sheep ovary was significantly higher in ovaries without corpus luteum (CL). The oocyte recovery rate in the present study was observed to be higher in aspiration

methodology than the mincing or slicing technique. This finding corroborates the findings of Kakkassery *et al*., (2010), but contradicts with the findings of Singh *et al*., 2013; Hoque *et al*., 2011 (goat); John *et al*., 2015; Sekhar *et al*., 2012 (bovine).

The previous reports suggest that highest oocyte recovery rate occurs using higher suction pressure and thicker needles (Rose and laky, 2013). In the present study application of uncontrolled manual pressure on the piston of the syringe might be the reason for the lower recovery rate of oocytes. Oocyte recovery rate was increased from 21 gauge needle to 18 gauge needle (Fry *et al*., 1997). In the present experiment as the ovaries were collected randomly from slaughter house, size of the ovaries was variable with more smaller sized follicles. Therefore, smaller sized needle was used to penetrate the follicles.

The observation in the present study indicates slightly lower oocyte recovery rate from cryopreserved ovary compared those out of fresh ovary. During the cryopreservation there is damage to the vascular system and tissues of the ovaries, (Onions *et al*., 2009). The ovarian tissue during cryopreservation might undergone some changes in the organization that result in lower retrieval of COC's. Hence the comparative less retrieval of COC's from cryopreserve ovary might be a function of cryoinjury, aspiration pressure and lumen size of needle.

The results of the present study revealed that the retrieval of usable quality COC's per cryopreserved ovary was at par with that for fresh ovary, but most of the usable COC's were derived from the surface follicles. Aspiration pressure and needle size play important roles in harvesting of good number of better quality oocytes. As aspiration pressure increased the recovered oocytes were increasing retrieval of denudal cells (Bols *et al*., 1997; Fry *et al*., 1997; Bols *et al*., 1995). It is also reported that the loss of cumulus cells occurred at low aspiration pressure with use of larger diameter needles. The high pressure caused during aspiration with hand held syringe might resulted in denuded oocytes. The naked oocytes are less likely to mature, fertilize or to cleave as embryos (Goud *et al*., 1998; Hwang *et al*., 2000). The result of the present study indicates that more number of usable quality COC's were harvested from the surface follicles than the

inner follicles. The mechanical mincing of the ovarian tissue might be responsible for damaging more number of follicles trapped in the inner mass of cortex.

The result of the present study in agreement with Kakkassery *et al.* 2010; Sekhar *et al*., 2012; John *et al.* 2015. The above studies reported aspiration of oocyte result in lower grade of oocytes. But result of present study contradicts the findings of Singh *et al*. (2013) that maximum yield of best quality oocytes was retrieved by aspiration method. The COC's are embedded in the wall of the follicle with physical connection with granulosa cells and zona pellucida of the oocyte. During aspiration the cumulus cells remain attached to the theca interna layer, the force produced on the cumulus cells and oocytes while inside the follicular wall. Therefore, the loss of cumulus covering is more likely to occur during the aspiration process (Bols *et al*., 1997). The entry of COC's into the tip of the needle normally involves a change in direction that may result in turbulent flow before entry into the needle. This period of turbulent flow exerts stronger forces on COC's in different direction. The strength of these forces increases with increased flow rate. So these forces provides possible explanation for loss of cumulus cells to different degree.

5.2 Cryoprservation of ovary and histological analysis:

In the present study an attempt has been made to cryopreserve the whole sheep ovary with 10% DMSO and increased quantity FBS (40%) containing cryoprotectant solution. The result of the study demonstrates satisfactory result. The high concentration of cryoprotectants was used to reduce the critical cooling rate to keep the cooling rate down (Fuller, 2004). The water in the cells was replaced by cryoprotectants solution. The cryoprotectants molecules check the ice formation and limit the diffusion within the tissues. The use of cryoprotectants may be toxic to tissues and level of toxicity depends upon the incubation temperature. DMSO penetrates the ovarian tissues more rapidly at 4˚C (Newton *et al*., 1998). The DMSO is less toxic to the tissues at lower temperature than other vitrified cryoprotectants (Hovatta *et al*., 1999 and Wallin *et al*., 2009).

In the experiment the sheep ovaries were selected for cryopreservation due to their texture which contains dense fibrous stroma and higher primordial follicle content.

It is difficult to introduce the cryoprotectants to the core of the ovary. Therefore, it was tried to introduce the cryoprotectants solution by dipping ovaries in cryoprotectants solution in an ascending concentration. The shorter infiltration time (5min each) of use of the cryoprotectant solutions facilitates a moderate penetration. Zhang *et al*., (2012) reported that a shorter perfusion period with low pressure may not ensure the complete saturation of tissues causing the formation of intracellular and extracellular ice physically damaging to cell and a high perfusion pressure with long perfusion period may rupture the vessels and create toxicity to the cells. The result of this experiment indicates the infiltration timing of cryoprotectants solution might be ideal for cryopreserving sheep ovary. The first cryopreservation of whole sheep ovary was done following slow cooling technique (Bedaiwy *et al*., 2003). In their study the ovaries were perfused with 10% FBS and 1.5 M DMSO in Leibovitz L-15 media and cryopreserved by controlled freezing -140^0C before putting them in LN_2 which yielded good result. Imhof *et al*., (2006) also cryopreserved sheep ovaries using RPMI-1640 solution containing 1.5M DMSO and 10% human albumin as a cryoprotectant solution. After grafting, the ovaries were found to function normally. Courbiere *et al*., (2005) successfully vitrified whole ovary using cryoprotectants solution in a stepwise increase in its concentration and observed few anomalies in follicles. In the present study the procedure of cryopreservation was somewhat similar to above procedure and also observed fewer abnormality in morphology of follicles. In the present study in few specimen, space between follicle and stromal tissues as well as in between stromal tissue was observed which might be due to defective tissue sectioning and processing.

In terms of biocompatibility, the cytotoxic impact of DMSO (Martino *et al*., 1996) on follicles and follicular cells was found to be acceptable from the result of the study. It has been reported that huge amount of trehalose were synthesized in cell exposed to stressful conditions (Jain and Roy 2009). A lot of stress might be produced during equilibration on ovarian tissues with different concentration of cryoprotectant solution as well as during cryopreservation. The processes might have resulted in release of huge amount of trehalose. The released trehalose might have neutralized the negative effect of DMSO on ovarian tissue during cryopreservation. Trehalose also inhibits rapid changes in osmotic pressure which reduces osmotic shock and swelling. Trehalose also

helps in stabilizing the cell membrane by preventing the ice formation (Branca *et al*., 2005). According to some scientists trehalose helps in maintenance of cell bioactivity and DNA integrity (Zhao *et al*., 2013 and Nguyen *et al*., 2013). Post cryopreservation follicular cell viability was 65% as evident from trypan blue test. So the technique followed could conserve more than half of the follicular population in a whole ovary. Trypan blue test for ovarian cell follicular cell viability is not an extensive ultrastructual study of oocyte status. In the study the counting of partially and fully denuded oocytes was not done as it could not be confirmed either they resulted from thermal damage, faulty aspiration and/ or due to ischemia. Many previous workers reported vitrification procedure as the best cryopreservation technique for preservation of whole ovary in human (Martinez- Madrid *et al*., 2004) and ewes (Bedaiwy *et al*., 2003; Revel *et al*., 2004), rat (Sugimoto *et al*., 2000) and mice (Salehmia 2002; Migishima *et al*., 2003). The spaces observed in histological cryopreserved samples might be due to temperature variation between LN_2 temperature (-196˚C) and the transition temperature of thawing. In the present study, followed thawing protocol yielded good result.

5.3 Assessment of maturation of harvested oocyte:

Oocyte maturation is a prolong process during which the oocyte acquires the competence to be fertilized and undergo embryogenesis. A lot of progress has been made to get healthy embryos with *in vitro* matured oocytes. But the underline mechanisms regulating folliculogenesis and oocyte maturation are aid to be fully understood (Gougeon 2010). Many a factor affecting *in vitro* maturation of oocytes Son and coworkers (2010) reported that priming of ovarian immature oocytes with FSH or hCG-H prior to oocyte retrieval improves oocyte maturation rate. Similarly the size of the follicles is also important for *IVM*. The oocytes retrieved from small antral follicle is not at all affected by the presence of dominant follicle (Chian, 2004). Previous workers reported that the cumulus cells, composition of maturation media and culture condition are prime aspects which effects the *IVM* (Du *et al*.,2005; Lornage 2006).

The cryopreservation techniques has been improvised to preserve the oocyte. The *in vitro* maturation of cryopreserved immature oocyte requires prior warming. The cryopreservation have some adverse effect on the maturation capacity of the oocytes. The

cryopreserved oocytes failed to develop due to critical disturbances of cell components (Coticchio *et al*., 2004).

In the present study out of 83 number of oocytes retrieved from cryopreserved ovary only 33 (39.76%) of them had successfully undergone the maturation process, which was statistically non-significant with the number of oocytes retrieved from fresh ovary successfully matured. The work of Cao *et al*., (2009) is in agreement with the present finding. The oocyte maturation rates significantly reduced when oocytes are vitrified at immature GV stage (Fasano, 2010). This study have been performed with less optimal materials and hence the results may not be comparable with others.

In the maturation media along with hormones (FSH and estradiol) the estrus sheep serum FBS were incorporated to yield higher maturation rate of oocytes. The application of FSH accelerates the follicular growth (Cecconi *et al*.,1999). Increased use of serum might have role in minimizing the cryoinjury to the follicles and maximize maturation rate of oocytes retrieved from the cryopreserved ovary. The result is supported by the study of Motlagh *et al*., (2008) who reported that higher maturation rate of oocytes were observed with use of mare serum in maturation media. Work of Farag *et al*., (2010) also corroborates the result of present study, as he observed supplementation of protein additives was very efficacious for *in vitro* maturation of sheep oocytes. Sreenivas *et al*., (2013) also observed increased maturation rate of oocytes with increased supplementation of FBS in the media.

In the present study no difference was observed in the rate of maturation of oocytes harvested from freshly collected ovary and cryopreserved ovary. This might be due to less or no cryoinjury imparted to oocytes encapsulated in the ovaries with use of freezing media having more serum content along with different hormones.

Chapter - 6

SUMMARY AND CONCLUSION

CHAPTER-VI

SUMMARY AND CONCLUSION

The present study was conducted in the Division of Veterinary Physiology and Biochemistry, Faculty of Veterinary Sciences and Animal Husbandry, Shere-e-Kashmir University of Agriculture Sciences and Technology of Jammu (SKUAST-J), R.S. Pura. The study was conducted from September, 2016 to May, 2017 to establish a protocol for cryopreservation of sheep ovaries for effective retrieval of immature oocytes for studying their *in vitro* developmental potency.

The ovaries were collected from local saughter house jammu and transporated to laboratory in NSS fortified with gentamycin (50µg/ ml) in a thermo flask at 38˚C within 2 h of slaughter. A total number of 234 ovaries were used in the experiment out of which 114 ovaries were cryopreserved for one month in LN_2 submerged in freezing medium containing 10% DMSO as a cryoprotectant. Before putting into the LN_2 the ovaries were sequentially treated for 5 min each with equilibration medium of 3 different composition. After cryopreservation the ovaries were thawed as per Gook *et al*., (2007). The fresh and thawed ovaries were washed twice with working OCM for 5 min each at 37˚C. The oocytes were aspirated from all visible non-atretic surface follicles. The oocytes from inner follicles/ submerged follicles were collected by mincing the ovarian cortex mechanically. The harvested oocytes were searched under stereozoom microscope and transferred to OCM droplets (50µl). The harvested COC's were evaluated and graded as per methods of Kobayashi *et al*., (1994). The COC's were washed several times in working OCM followed by washings in maturation media. The washed COC's were cultured in 50µl droplets of maturation media (FBS 10%, sheep serum 5%, follicular fluid 5%, BSA fraction-V 3mg/ml, FSH-P 0.5µg/ml, cystamine 50µM, estradiol 10µg/ml) in 35mm sterile petridish. The droplets were covered with non-toxic paraffin oil and placed for 27 h at 38.5˚C, 5% CO_2 and 95% humidity. After the maturation period the evaluation of maturation were done by visual assessment of cumulus expansion under inverted microscope as per methods of Kobayashi *et al*., (1994).

The histological assessment of frozen thawed ovarian tissue as well as freshly collected ovarian tissue were done by sectioning the tissues and staining them with haematoxylin and eosin stain (Luna, 1968). Whereas, the viability of follicular cells of both fresh and cryopreserved ovaries was analyzed using trypan blue (Demirci *et al*. 2001).

The rate of retrieval of total oocytes from fresh and cryopreserved ovaries (1.688±0.117 vs 1.694±0.202) were not significantly differ from each other. The number of harvested COC's from inner follicles per ovary was less compared to those from surface follicles. The lower retrieval of usable COC's might be due to use of smaller size needle (20 gauge), higher manual aspiration pressure and presence of corpus luteum on the ovaries. In the present study more denuded oocytes were harvested from inner follicles of cryopreserved ovary which might be a result of change in organization of ovarian tissues during cryopreservation, mechanical mincing of ovarian tissues as well as applied aspiration pressure and lumen size of the needle.

No significant difference was observed between gross and histological analysis of ovary and ovarian tissue of both groups. The texture and size of follicles of cryopreserved ovary remained unchanged. They looked similar to fresh ovary. The cryopreserved ovarian tissue section exhibited fewer morphologically abnormal follicles. The microscopic study revealed creation of vacuole or space between stromal tissues which might a result of defective tissue processing. Use of DMSO as cryoprotectant proved to be beneficial as it is producing stress on cells, the stressful condition leads to synthesis of large amount of trehalose in the cells. Trehalose helps in cell membrane stabilization and maintains bioactivity and DNA integration during the cryopreservation. In terms of biocompatibility the toxic impact of DMSO as well as cryopreservation on follicles and follicular cells were acceptable. The post cryopreservation follicular cell viability was observed to be more than 65%. So the technique followed for the cryopreservation in the present study could conserve more than half of the follicular population in a whole ovary. But as trypan blue test for study of follicular cell viability is not an extensive ultrastructual study of oocyte status, further work must be done to assess the oocyte status after cryopreservation of whole ovary.

In the present study approximately 40% of oocyte retrieved from cryopreserved ovaries have successfully undergone the maturation which is *at par* with the number of oocytes harvested from fresh ovary. The use of immature oocytes, incorporation of few hormones (FSH and estradiol 17ß only) in the maturation medium might be the reasons for the low maturation rate of oocytes in the study. It has been reported that oocyte maturation rate significantly reduced when oocytes are cryopreserved at immature stage. The maturation rate of retrieved oocytes from cryopreserved whole ovary may be increased by addition of more number of hormones, proteins and growth factors. Hence, further work is needed in this regard.

Conclusion

It is concluded from the study that the ovaries collected from slaughtered animals as well as immediately after the death (normal or accidental) of the animals can be cryopreserved for a long period, after which the oocytes collected from those successfully used for IVM/ *IVF*. The present study has explored the possibility to preserve the pool of immature follicles inside the ovary itself by cryopreserving the whole ovary and observed that this is possible with the use of effective cryoprotective agent DMSO. The study also revealed that 40% of immature oocytes harvested from cryopreserved ovaries have successfully undergone *IVM*. It has been also observed that, *IVM* treatment can provide a unique and seldom investigated source of non- leutinized granulosa cells. As these cells at earlier stage of development react differently from leutinized granulosa cells, the increased knowledge about factors secreted from these cells may provide information to improve *IVM* of oocytes retrieved from cryopreserved ovaries.

Appendix- I

1. Composition/ concentration of oocyte collection media (OCM) - Stock solution:

Chemical	Concentration (mM)
NaCl	136.89
KCl	2.68
KH_2PO_4	1.46
NaH_2PO_4	8.09
$CaCl_2\,2H_2\,O$	0.90
$MgCl_2$	1.00
D-Glucose	5.5
Sodium Pyruvate	0.32

2. Composition/ concentration of working OCM (50ml)

Chemical	Composition
Sheep (estrus) serum 10%	5 ml
OCM	45ml
Bovine serum albumin	3mg/ml

3. Composition/ concentration of Equilibration Media

Equilibration Media I	
Chemical	**Composition**
Media (TCM-199)	1 part
Freezing media	1 part

Equilibration Media II	
Chemical	**Composition**
Media (TCM-199)	1 part
Freezing media	2 part

Equilibration Media III	
Chemical	**Composition**
Media (TCM-199)	0 part
Freezing media	1 part

4. Composition/ concentration of Freezing Media

Chemical	Composition
Dimethyl sulfoxide (DMSO)	1 part
Fetal bovine serum (FBS)	4 part
TCM-199 (with Earl's salt, 25mM HEPES, 0.01g L-glutamine	5 part

5. Composition/ concentration of Thawing Solution

Thawing Solution I	
Chemical	**Composition**
Freezing Media	1 part
Media (TCM-199)	1 part

Thawing Solution II	
Chemical	**Composition**
Freezing Media	2 part
Media (TCM-199)	1 part

Thawing Solution III	
Chemical	**Composition**
Freezing Media	1 part
Media (TCM-199)	0 part

6. Composition/ concentration of Maturation Media

Chemical	Composition
Fetal bovine serum	10%
TCM-199 (with Earl's salt, 25mM HEPES, 0.01g L-glutamine	
Follicular fluid	5%
Sheep serum	5%
Bovine serum albumin (BSA) Fracton-V	3mg/ml
Follicle stimulating hormone (FSH-P)	0.5µg/ml
Estradiol	10µg/ml
Cystamine	50µM

REFERENCES

REFERENCES

Abir, R., Roizman, P., Fisch, B., Nitke, S., Okon, E., Orvieto, R. and Ben Rafael, Z. 1999. Pilot study of isolated early human follicles cultured in collagen gels for 24 hours. *Human Reproduction*, **14**: 1299-1301.

Ackert, C. L., Gittens, J. E., Brien, M. J., Eppig, J. J. and Kidder, G. M. 2001.Intecellular communication via connexin43 gap junctions is required for ovarian folliculogenesis in the mouse. *Developmental Biology*, **233** (2): 258-270.

Al-Soudy, A., Sayed, E. and Hussein, E. H. A. 2016. Cryopreservation of immature and mature camel oocytes by open pulled straw vitrification. *Arab Journal of Biotechnology*, **19** (2).

Amiridis, G. S. and Csehb, S. 2012. Assisted reproductive technologies in the reproductive management of small ruminants. *Animal Reproduction Science*. **130**: 152-161.

Amorim, C. A., Goncalves, P. B. and Figueiredo, J. R. 2003. Cryopreservation of oocytes from pre-antral follicles. *Human Reproduction*, **9**: 119-129.

Amorim, C. A., Rondina, D., Lucci, C. M., Goncalves, P. B., Figueiredo, J. R. and Giorgetti, A. 2006. Permeability of ovine primordial follicles to different cryoprotectants. *Fertility and Sterility*, **85**: 1077-1081.

Andrabi, S. M. and Maxwell, W. M. 2007. A review on reproduction biotechnologies for conservation of endangered mammalian species. *Animal Reproduction Science*, **99** (3): 223-243.

Arav, A., Gavish, Z., Elami, A., Natan, Y., Revel, A., Silber, S., Gosden, R. G. and Patrizio, P. 2009. Ovarian function six years after cryopreservation and transplantation of whole sheep ovaries. *Reproductive Biomedicine Online*, **20**: 48-52.

Arav, A., Revel, A., Nathan, Y., Bor, A., Gacitua, H., Yavin, S., Gavish, Z. and Uri, M. 2005. Oocyte recovery, embryo development and ovarian function after cryopreservation and transplantation of whole sheep ovary. *Human Reproduction*, **20** (12): 3554-3559.

Aubard, Y., Piver, P., Congie, Y., Fermeaux, V., and Driancourt, M. A. 1999. Orthotopic and heterotopic autografts of frozen thawed ovarian cortex in sheep. *Human Reproduction*, **14**: 2149-2154.

Baird, D. T., Webb, R., Campbell, B. K., Harkness, L. M. and Gosden, R. G. 1999. Long-term ovarian function in sheep after ovarieoctomy and transplantation of autografts stored at -196˚C. *Endocrinology*, **140**: 462-471.

Baudot, A., Courbiere, B., Odagescu, V., Salle, B., Massardier, J. and Lorange, J. 2007. Towards whole sheep ovary cryopreservartion. *Cryobiology*, **55** (3): 236-248.

Bedaiwy, M. A., Jeremias, E. and Gurunluoglu. 2003. Restoration of ovarian function after autotransplantation of intact frozen-thawed sheep ovaries with microvascular anastomosis. *Fertility Sterility*, **79** (3): 594-602.

Bernard, A. and Fuller, B. J. 1996. Cryopreservation of human oocytes: a review of current problems and perspectives. *Human Reproduction*, **3**: 193-207.

Bilska, A. T. G., Banerjee, J., Yazici, I., Bilski, J. and Sharma, R. K. 2008. Morphology and function of cryopreserved whole ovine ovaries after heterotropic autotransplantation. *Reproductive Biology and Endorinology*, **6** (16).

Boiso, I., Marti, M. and Santalo, J. 2002. The meiotic spindle configuration of human oocytes cryopreserved in the germinal vesicle and metaphase II stage. *Human Reproduction*, **17**: 1885-1891.

Bols, P. E. J., Ysebaert, M. T. and Van den Dobbelsteen, J. J. 1995. Effects of needle tip bevel and aspiration procedure on the morphology and developmental capacity of bovine compact cumulus oocyte complexes. *Therionology*, **47**: 1221-1236.

Bols, P. E. J., Ysebaert, M. T., Van, S. and Kruif, A. 1997. Effects of needle tip bevel and aspiration procedure on the morphology and developmental capacity of bovine compact cumulus oocyte complexes. *Theriogenology*, **47:** 1221-1236.

Branca, C., Maccarrone, S. and Magazu, S. 2005. Tetrahedral order in homologous disaccharide-water mixtures. *Journal of Chemical Physics*, **122**.

Braw, T. R. 2002. The intiation of follicular growth: The oocyte or somatic cell? *Molecular and Cellular Endocrinology*, **22**: 11-18.

Campos, J. R.., Silva, R., Carvalho, B. R., Aparecida, A., and Carolina, A. 2011. Cryopreservation time does not decrease follilcular viability in ovarian tissue frozen for fertility preservation. *Clinical Science*, **66** (12): 2093-2097.

Candy, C. J. and Whittingham, D. J. 2000. Restoration of normal reproductive lifespan after grafting of cryopreserved mouse ovaries. *Human Reproduction*, **15**: 1300-1304.

Cao, Y., Xing, Q., Zhang, Z. G., Wei, Z. L. and Cong, L. 2009. Cryopreservation immature and in vitro matured human oocytes by vitrification. *Repoductive Biomedicine Online*, **19**: 369-373.

Cecconi, S., Barboni, B., Coccia, M. and Mattioli, M. 1999. *In vitro* development of sheep preantral follicles. *Biology of Reproduction*, **60**: 594-601.

Cecconi, S., Capacchietti, G., Russo, V., Berardinelli, P., Mattioli, M. and Barboni, B. 2004. *In vitro* growth of preantral follicles isolated from cryopreserved ovine ovarian tissue. *Biology of Reproduction*, **70**: 12-17.

Chauhan, M. S., Singla, S. K., Patla, P., Manik, R. S. and Madan, M. L. 1998. *In vitro* maturation and fertilization, and subsequent development of buffalo (Bubalus bublais) embryos: effect of oocyte quality and type of serum. *Journal of Reproduction and Fertility Development*, **10**: 173-177.

Chian, R. C., Tan, J., Kato, O. and Nagai, T. 2004. High survival rate of bovine oocytes matured *in vitro* following vitrification. *Journal of Reproduction and Development*, **50**: 685-696.

Choi, M. H., Gong, S. P. and Lim, J. M. 2015. Retrieval of porcine ovarian follicles by different methods. *Asian- Australian Journal of Animal Sciences*, **21** (3): 353-357.

Coticchio, G., Bonu, M. A. and Flamigni, C. 2004. Oocyte cryopreservation: a biological perspective. *European Journal of obstetrics and gynecology and Reproductive Biology*, **1**: 2-7.

Courbiere, B., Valentina, O., Boudot, A., Massardier, J., Salle, B. and Lornage, J. 2005. Cryopreservation of the ovary by vitrification as an alternative to slow- cooling protocols. *Fertility and Sterility*, **86** (3).

Davachi, N. D., Kohram, H. and Zeinoaldini, S. 2011. Effect of the presence of corpus luteum on the ovary and the new oocyte recovery method on the oocyte recovery rate and meiotic competence of ovine oocytes. *African Journal of Biotechnology*, **10** (47): 9706-9709.

Deansely, R. 1954. Immature rat ovaries grafted after freezing and thawing. *Journal of Endocrinology*, **11** (2): 197-200.

Deka, S. S., Kalita, D. J., Sara, S. and Dutta, D. J. 2015. *In vitro* maturation rate of oocytes collected from indigenous cows of Assam after slaughter. *Indian Journal of Animal Research*, **49** (6): 869-871.

Demirci, B., Lornage, J., Salle, B., Franck, M. and Guerin, F. 2001. Follicular viability and morphology of sheep ovaries after exposure to cryoprotectants and cryopreservation with different freezing protocols. *Fertility and Sterility*, **75** (4).

Donnez, J. and Bassil, S. 1998. Indications for cryopreservation of ovarian tissue. *Human Reproduction*, **4**: 248-259.

Du, A., Kadoch, I. J., Bourcigaux, N., Tachdjian, G., Frydman, R. and Frydman, N. 2005. In vitro maturation for the treatment of infertility associated with polycystic ovarian syndrome: the French experience. *Human Reproduction*, **10**: 587-592.

Du, T., Chao, L., Zhao, S., Chi, L., Li, D., Shen, Y., Shi, Q. and Deng, X. 2015. Successful cryopreservation of whole sheep ovary by using DMSO- free cryoprotectant. *Jouranl of Assisted Reproduction and Genetics*, **32**: 1267-1275.

Eppig, J. J. 1982. The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation and cumulus expansion. *Developmental Biology*, **89** (1): 268-272.

Eppig, J. J. and Brien, M. J. O. 1996. Development of *in vitro* mouse oocytes from primordial follicles. *Biology of Reproduction*, **54**: 197-207.

Fabbri, R., Porcu, E., Marsella, T., Rocchetta, G., Venturoli, S., Flamigni, C. Human oocyte cryopreservation: new perspectives regarding oocyte survival. 2001. *Human Reproduction*, **16**: 411-416.

Fakhrildin, M. B. and Aljuaifri, A. A. I. 2015. Effects of sheep ovarian tissue vitrification and transplantation on *in vitro* fertilization and early embryonic development of oocytes. *International Journal of Current Research in Biosciences and Plant Biology*, **2** (5): 167-173.

FAO 2007: The state of the world's animal genetic resources for food and agriculture, *Food and Agriculture Organization of United Nations*, Commission on Genetic Resources for Food and Agriculture, Rome, Italy.

Farag, I. M., Girgis, S. M. Khalil, W. K. B., Hassan, N. H. A., Sakr, A. A. M and Ali, N. I. 2009. Effect of hormones, culture media and oocyte quality on *in vitro* maturation of Egyptian sheep oocytes. *Journal of Applied Biosciences*, **24**: 1520-1534.

Farag, L. M., Girgis, S. M., Khalil, W. K. B., Sakr, A. M. and Ali, N. I. 2010. Effect of protein additives on *in vitro* maturation of Egyptian sheep oocytes with reference

to seasonal variation effects on yield and quality of oocytes. *Journal of American Science*, **6** (10).

Fasano, G., Demeestere, I. and Englert, Y. 2012. *In vitro* maturation of human oocytes: before and after vitrification. *Journal of Assisted Reproduction and Genetics*, **29**: 507-512.

Fasano, G., Vannin, A. S., Delbaere, A. and Englert, Y. 2010. Cryopreservation of human failed maturation oocytes shows that vitrification gives superior outcomes to slow cooling. *Cryobiology*, **61**: 243-247.

Fathi, R., Valojerdi, M. R., Eimani, H., Hasani, F., Yazdi, P.E. and Tahaei, L. S. 2011. Sheep ovarian tissue vitrification by two different dehydration protocols and needle immersing methods. *Cryoletters*, **32** (1): 51-56.

Fauque, P., Amor, A. B., Joanne, C., Agnani, G. and Roux, C. 2016. Use of trypan blue staining to assess the quality of ovarian cryopreservation. *Fertility and Sterility*, **87** (5).

Fox, C.A., Sheets, M.D. and Wickens, M.P. 1989. Poly (A) addition during maturation of frog oocytes: distinct nuclear and cytoplasmic activities and regulation by sequence UUUUUUAU. *Genes and Development*, **3** (12B): 2151-2162.

Fry, R. C., Niall, E. M. and Reynolds, J. 1997. The collection of oocytes from bovine ovaries. *Theriogenology*, **47**: 977-987.

Fuller, B. J. 2004. Cryoletters, **25**: 375-388. [PubMed: 15660165].

Fulka, J., Jung, T. and Moor, R. M. 1992. The fall of biological maturation promoting factor (MPF) and histone H1 kinase activity during anaphase and telophase in mouse oocytes. *Molecular Reproduction and Development*, **32** (4): 378-382.

Gautier, J. and Maller, J. L. 1991. Cyclin B in Xenopus oocytes: implications for the mechanism of pre- MPF activation. *The EMBO Journal*, **10** (1): 177-182.

Gerritse, R., Beerendonk, C. C. M., Westphal, J. R., Bastings, L., Braat, D. D. M. and Peek, R. 2011.Glucose/lactate metabolism of cryopreserved intact bovine ovaries as a novel quantitative marker to assess tissue cryodamage. *Reproductive Biomedicine Online*, **23**: 755-764.

Gook, D. A., Hale, L. and Edgar, D. H. 2007. Live birth following transfer of a cryopreserved embryo generated from cryopreserved oocyte and cryopreserved sperm: Case report, *Journal of Assisted Reproduction and genetics*. **24**: 43-46.

Gook, D. A., Osborn, S. M., Archer, J., Lutjen, P. J. and McBain, J. C. 2004. Follicle development following cryopreservation of human ovarian tissue. *European Journal of Gynecology Reproductive Biology*, **113**: 60-62.

Gosden, R. G. 1995. Oocyte development throughout life. In: Gametes, The oocyte, pp.19-149: Grudzinskas, J. G.; Yovich, J. L. (Eds), *Cambridge Reviews in Human Reproduction.* Gosden, R. G. 2002. Oogenesis as a foundation for embryogenesis. *Mollecular and Cellular Endocrinology*, **186** (2): 149-153.

Gosden, R. G., Baird, D. T. and Webb, R. 1994. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at -196˚C. H*uman Reproduction*, **9**: 597-603.

Gosden, R. G., Mullan, J., Picton, H. M., Yin, H. and Tan, S. L. 2002. Current perspective on primordial follicle cryopreservation and culture for reproductive medicine. *Human Reproduction*, **8**: 105-110.

Goud, P. T., Goud, A. P., Qian, C. and Sutter, P. 1998. *In vitro* maturation of human germinal vesicle stage oocytes: role of cumulus cells and epidermal growth factor in the culture medium. *Human Reproduction*, **17**: 39-43.

Gougeon, A. 1996. Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocrinology of Metabolism*, **81** (4): 1401-1405.

Gougeon, A. 2010. Human ovarian follicular development: from activation of resting follicles to preovulatory maturation. *Animal Endocrinology*, **71** (3): 132-143.

Hanh, V. N., Viet, L. N. and Nghia, S. H. 2016. Successful *in vitro* maturation of oocytes collected from whole porcine ovaries vitrification. *Electronic Journal of Biology*, **12** (4): 422-427.

Henry, L., Fransolet, M., Labied, S., Blacher, S., Masereel, M. C., Nisolle, M. and Munaut, C. 2016. Supplementation of transport and freezing media with anti-apoptotic drugs improves ovarian cortex survival. *Journal of Ovarian Research*, **9**: 4.

Hirshfield, A. N. 1991. Development of follicles in the mammalian ovary. *International Review of Cytology*, **124**: 43-101.

Holt, W. V. and Pickard, A. R. 1999. Role of reproductive technologies and genetic resource banks in animal conservation. Review Reproduction, **4** (3): 143-150.

Hoque, M. A. S., Kabiraj, K. S., Khandoker, Y., Mondal, A. and Tareq, A. M. K.2011. Effect of collection techniques on cumulus oocyte complexes recovery, *in vitro* maturation and fertilization of goat oocytes. *African Journal of Biotechnology*, **10** (45): 9177-9181.

Hovatta, O., Silye, R., Abir, R., Krausz, T. and Winston, R. M. 1997. Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long term culture. *Human Reproduction*, **12** (5): 1032-1036.

Hovatta, O., Wright, C., Krausz, T., Hardy, K. and Winston, R. M. L. 1999. Human primordial and secondary ovarian follicles in long- term culture: effect of partial isolation. *Human Reproduction*. **14**: 2519-2524.

Hwang, J-L., Lin, Y. H. and Tsai, Y-L. 2000. *In vitro* maturation and fertilization of immature oocytes: a comparative study of fertilization techniques. *Journal of Assisted Reproduction and Genetics*. **17**: 39-43.

Imhof, M., Bergmeister, H. and Lipovac, M. 2006. Orthotopic microvascular anastomosis of whole cryopreserved ovine ovaries resulting in pregnancy and live birth. *Fertility Sterility*, **92** (1): 374-381.

Jain, N. K. and Roy, I. 2009. Effect of trehalose on protein structure . *Protein Science*, **18** (1): 24-36.

John, A., Joseph, M., Vijayakumaran, V. and Manoj, C. J. 2015. Effect of oocyte retrieval techniques on yield and quality of caprine oocytes. *IOSR Journal of Agriculture and Veterinary Science*, **8** (4): 50-52.

Johnson, J., Bagley, J., Skaznik, W.M., Lee, H. J., Adams, G. B., Niikura, Y., Tschudy, K. S., Tilly, J. C., Forkert, R. and others. 2005. Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell*, **122** (2): 303-315.

Johnson, J., Canning, J., Kaneko, T., Pru, J. K. and Tilly, J. L. 2004. Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature*, **428** (6979): 145-150.

Johnston, L. A. and Lacy, R. C. 1995. Genome resource banking for species conservation: selection of sperm donors. *Cryobiology*, **32** (1): 68-77.

Kakkassery, P. M., Vijayakumaran, V. and Sreekumaran, T. 2010. Effect of cumulus oocyte complex morphology on *in vitro* maturation of bovine oocytes. *Journal of Veterinary and Animal Sciences*, **41**: 12-17.

Kobayashi, K., Yamashita, S. and Hochi. 1994. Influence of epidermal growth factor and transforming growth factor alpha on *in vitro* maturation of cumulus cells enclosed bovine oocytes in defined medium. *Journal of Reproduction and Fertility*, **100**: 439-446.

Lornage, J. and Salle, B. 2006. Ovarian and oocyte cryopreservation. *Current Opinion in Obstetrics and Gynecology*, **19**: 390-394.

Luna, L. G. 1968. Manual of histological staining methods of Armed Forces Institute of Pathology. 3rdEdn. McGraw Hill Book Co., New York. Pp. 153-173.

Luvoni, G. C., Chigioni, S. and Beccaglia, M. 2006. Embryo production in dogs: from in vitro fertilization to cloning. *Reproduction in Domestic Animals*, **141** (4): 286-290.

Majeed, A. F., Saied, I. H. and AL-Saigh, M. N. 2011. Effect of collection techniques on recovery and *in vitro* maturation of Black Iraqi Goats oocytes. *Al-Anbar Journal of Veterinary Sciences*, **4** (2).

Martinez-Madrid, B., Defrere, S. and Donnez, J. 2004. Freeze- thawing intact human ovary with its vascular pedicle with a passive cooling device. *Fertility Sterility*, **82**: 1390-1394.

Martino, M., Morabito, F. and Messina, G. 1996. Fractional infusions of cryopreserved stem cells may prevent DMSO-induced major cardiac complications in graft recipients. *Haematologica*, **81** (1): 59-61.

McGee, E. A. and Hsueh, A. J. 2000. Initial and cyclic recruitment of ovarian follicles. *Endocrinology Reviews*, **21** (2): 200-214.

Mehlmann, L. M., Terasaki, M., Jaffe, L. A. and Kline, D. 1995. Reorganization of cytoplasmic reticulum during meiotic maturation of the mouse oocyte. *Developmental Biology*, **170** (2): 607-615.

Merdassi, G., Mazoyer, C., GUERIN, F. J., Salle, B. and Lornage, J. 2012. Examination of viability and quality of ovarian tissue after cryopreservation using simple laboratory methods in ewe. *Reproductive Biology and Endocrinology*, **9**: 78.

Migishima, F., Suzuki-Migishima, R., Song, S. Y. Kuramochi, T. and Nishijima, M. 2003. Successful cryopreservation of mouse ovaries by vitrification, *Biology Of Reproduction*. **68**: 881-887.

Milenkovic, M., Wallin, A. and Brannstrom, M. 2011. Whole sheep ovary cryopreservation: evaluation of a slow freezing protocol with dimethylsulfoxide. *Journal of Assisted Reproduction and Genetics*, **28**: 7-14.

Milkich, A. B., Marques, L., Rodrigues, L., Lothammer, N. and Frantz, N. 2012. The use of a metal container for vitrification of mouse ovaries, as a clinical grade model for human ovarian tissue cryopreservation, after different times and temperatures of transport. *Fertility Preservation*, **29**: 1267-1271.

Millar, J. B. and Russell, P. 1992.The cdc25 M-phase inducer: an unconventional protein phosphatase. *Cell*, **68** (3): 407-410.

Moreno, S. and Nurse, P. 1990. Substrates for p34cdc: *in viv*o veritas? *Cell*, **61** (4): 549-551.

Motlagh, M. K., Shahneh, A. Z., Daliri, M. and Gharagozlou, F. 2008. *In vitro* maturation of sheep oocytes in different concentrations of mare serum. *African Journal of Biotechnology*, **7** (18): 3380-3382.

Nakagata, N. 1992.Production of normal young following insemination of frozen-thawed mouse spermatozoa into fallopian tubes of pseudopregnant female. *Journal of Experimental Animal Science*, **84**: 585-519.

Neto, V., Samuel, B., Lornage, J., Bottollier, B. and Joly, T. 2008. Effects of different freezing parameters on the morphology and viability of preantral follicles after cryopreservation of doe rabbit ovarian tissue. *Fertility and Sterility*, **89** (3).

Nguyen, T.M., Kikuchi, K. and Nakai, M. 2013. Effect of trehalose on DNA integrity of freeze dried boar sperm, fertilization and embryo development after intracytoplasmic sperm injection. *Theriogenology*, **80** (9): 1033-1044.

Obata, Y. and Kono, T. 2002. Maternal primary imprinting is established at a specific time for each gene throughout oocyte growth. *The Journal of Biological Chemistry*, **277** (7): 5285-5289.

Oktay, K. and Karlikaya, G. 2000. Ovarian function after transplantation of frozen, banked autologous ovarian tissue. *New England Journal of Medicine*, **342**: 1919.

Onions, V. J., Webb, R., McNeilly, A. S. and Campbell, B. K. 2009. Ovarian endocrine profile and long-term vascular patency following heterotropic autotransplantation of cryopreserved whole ovine ovaries. *Human Reproduction*, **24**: 2845-2855.

Paris. M. C., Snow, M., Cox, S. L. and Shaw, J. M. 2004. Xenotransplantation: a tool for reproductive biology and animal conservation? *Therionology*, **61**: 277-291.

Parkes, A. S. and Smith, A. U. 1953. Regeneration of rat ovarian tissue grafted after exposure to low temperatures. *Proceedings of the Royal Society B: Biological Science,* **140** (1-4): 455-470.

Pawshe, C. H., Totey, S. M. and Jain, S. K.1994. A method of three methods of recovery of goat oocytes for *in vitro* maturation and fertilization. *Theriogenology*, **42** (1): 117-125.

Pereira, R. M. and Marques, C. C. 2008. Animal oocyte and embryo cryopreservation. *Cell Tissue Bank*, **9** (4): 267-277.

Picton, H., Briggs, D. and Gosden, R. 1998. The molecular basis of oocyte growth and development. *Molecular and Cellular Endocrinology*, **145** (1-2): 27-37.

Purohit, G. N. Meena, H. and Solanki, K. 2012. Effects of vitrification on immature and *in vitro* matured, denuded and cumulus compact goat oocytes and their subsequent fertilization. *Journal of Reproduction and Infertility*, **13** (1): 217-227.

Revel, A., Elami, A. and Bor, A. 2004. Whole sheep cryopreservation and transplantation. *Fertility Sterility*, **82** (6): 1714-1715.

Rose, B. I., and Laky, D. C. 2013. A comparison of the cook single lumen immature ovum IVM needle to the Steiner-Tan pseudo double lumen flushing needle for oocyte retrieval for *IVM*. *Journal of Assisted Reproduction and Genetics*. **30**: 885-860.

Russell, D. L. and Salustri, A. 2006. Extracellular matrix of the cumulus- oocyte complex. *Seminars in Reproductive Medicine*, **24**: 217-227.

Sagata, N., Daar, I., Oskarson, M., Showalter, S. D. and Vande, W. G. F. 1989 a. The product of the mos proto-oncogene as a candidate "initiator" for oocyte maturation. *Science*, **254** (49): 643-646.

Sagata, N., Watanabe, N., Vande, W. G. F. and Ikawa, Y. 1989 b. The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature*, **342** (6249): 512-518.

Salehnia, M. 2002. Autograft of vitrified mouse ovaries using ethylene glycol as cryoprotectant. *Experimental Animals*, **51**: 509-512.

Sara, H. S. and Reza, R. A. 2015. Effects of different concentrations of serums on *in vitro* maturation of bovine oocytes. *Biosciences Biotechnology Research Asia*, **12** (2): 1151-1156.

Saunders, M. K. and Parks, J. E. 1999. Effects of cryopreservation procedures on the cytology and fertilization rate of in vitro matured bovine oocytes. *Biology of Reproduction*, **61:** 178-187.

Segino, M., Iked, Mario., Hirahara, F. and Sato, K. 2005. *In vitro* follicular development of cryopreserved mouse ovarian tissue. *Reproduction Research*, **130**: 187-192.

Sekhar, K. T., Solmon, K. G. and Rao, S. K. 2012. Efficacy of in vitro maturation of bubaline oocytes in TCM-199, TCM-199+ PMSG and follicular fluid. *Indian Journal of Animal Reproduction*, **33** (1).

Selvaraj, P., Selvaraj, K. and Srinivasan, K. 2009. Successful birth of the first frozen oocyte baby in India. *Journal of Human Reproductive Sciences*, **2** (1): 41-44.

Selvaraj, P., Selvaraj, K. and Srinivassan, K. 2010. First successful birth of twins in India following the transfer of vitrified oocytes. *Journal of Human Reproductive Sciences*, **3** (1): 44-48.

Shabankareh, K. H. Sarsaifi, K. and Mehrannia, T. 2010. *In vitro* maturation of ovine oocytes using different maturation media: effect of human menopausal serum. *Journal of Assisted Reproduction and Genetics*, **28**: 531-537.

Shioya, Y. M., Kuwayama, M., Fukushima, S. and Hanada, A. 1988. *In vitro* fertilization and cleavage capability of bovine follicular oocytes classified by cumulus cells and matured *in vitro*. *Theriogenology*, **30**: 489.

Silva, J. R., Lucci, C. M., Carvalho, F. C., Bao, S. N., Costa, S. H., Santos, R. R. and Figueiredo, J. R. 2000. Effect of coconut water and Braun-Collins solutions at different temperature and incubation times on the morphology of goat preantral follicles preserved *in vitro*. *Therionogenology*, **15**: 809-822.

Simon, A. M., Goodenough, D. A. and Paul, D. L. 1997. Female infertility in mice lacking connexion 37. *Nature*, **385** (6616): 525-529.

Singh, R., Sadasiva, R. and Mohan, M. K. 2013. Oocyte retrieval methods, grade and percentage of oocytes in goats. *International Journal of Molecular Veterinary Research*, **3** (2): 4-6. Smith, A. U. 1952. Culture of ovarian granulosa cells after cooling to very low temperatures. *Experimental Cell Research*, **3**: 574-583.

Smith, A.U. 1952. Culture of ovarian granulosa cells after cooloing to very low temperatures. *Experimental Cell Research*, **3**: 574-583.

Snedecor, G. W. and Cochran, W. G. 2004. Statistical Methods, 8th Edition., Oxford and BH Pub. Co., Kolkata.

Son, W. Y. and Tan, S. L. 2010. Laboratory and embryological aspects of hCG- primed in *vitro maturation* cycles for patients with polycystic ovaries. *Human Reproduction Update*, **16**: 675-689.

Sreenivas, D., Kaladhar, G. K., Yaria, S. N., Thomas.V. and Samy, A. P. 2013. Effect of protein supplementation on *in vitro* maturation of sheep oocytes and *in vitro* culture of preimplantation with alpha tocopherol supplementation in CR1aa Medium on sheep embryos to blastocyst stage. *Journal of Allergy and Therapy*, **4** (2).

Sugimoto, M., Maeda, S., Manabe, N. and Miyamoto, H. 2000. Development of infantile rat ovaries autotransplanted after cryopreservation by vitrification. *Theriogenology*, **53**: 1093-1097.

Talukder, M. N. S., Iqbal, A., Khandoker, M. A. M. Y. and Alam, M Z. 2011. Collection grading and evaluation of cumulus-oocyte-complexes for *in vitro* maturation in sheep. *The Bangladesh Veterinarian*, **28** (11): 31-38.

Taylor, M. J. 1987. Physio-chemical principles in low temperature biology. In: Grout, B. W. W., Morris, G. J. (Eds.), The effects of low temperatures on Biological systems. *Edward Arnold, London*, 3-71.

Ting, A. Y., Yeoman, R. R., Campos, J. R., Lawson, M. S., Mullen, S. F., Fahy, G. M. and Zelinski, M. B. 2013. Morphological and functional preservation of pre-antral follicles after vitrification of macaque ovarian tissue in a closed system. *Human Reproduction*, **28** (5): 1267-1279.

Van der Elst, J., Amerijckx, Y. and Van Steirteghem A. 1998. Ultra- rapid freezing of mouse oocytes lowers the cell number in the inner cell mass of 5 day old in vitro cultured blastocysts. *Human Reproduction*, **10**: 202-212.

Verroti, A. C., Thompson, S. R. Wreden, C., Strickland, S. and Wickens, M. 1996. Evolutionary conservation of sequence elements controlling cytoplasmic polyadenylation. *Proceedings of the National Academy of Sciences* U.S.A, **93** (17): 9027-9032.

Wallin, A., Ghahremani, M. and Brannstrom, M. 2009. Viability and function of the cryopreserved whole ovary: *in vitro* studies in the sheep. *Human Reproduction*, **1** (1): 1-11.

Wandji, S. A., Sren, V., Nathanielsz, P. W., Eppig, J. J., Fortune, J. E. 1997. Initiation of growth of baboon primordial follicles *in vitro. Human Reproduction*, **12** (9): 1993-2001.

Wandji, S. A., Sren, V., Voss, A. K., Eppig, J. J. and Fortune, J. E. 1996. Initiation *in vitro* growth of bovine primordial follicles. *Biology of Reproduction*, **55** (5): 942-948.

Wang, Z. G., Xu, Z .R. and Yu, S. D. 2008. Effects of oocyte collection techniques and maturation media on *in vitro* maturation and subsequent embryo development in goat. *Journal of Animal Sciences*, **52** (1): 21-25.

Wani, N. A., Wani, G. M., Khan, M. Z. and Salahudin, S. 2000. Effect of oocyte harvesting techniques on in vitro maturation and in vitro fertilization in sheep. *Small Ruminants Research*, **36**: 63-67.

Wassarman, P. M. and Josefowicz, W. J. 1978. Oocyte development in the mouse: an ultrastructural comparison of oocytes isolated at various stages of growth and meiotic competence. *Journal of Morphology*, **156** (2): 209-235.

Wildt, D. E. 2000. Genome resource banking for wildlife research, management, and conservation. ILAR J, **41**: 228-234.

Winiger, J. D. and Kort, H. I. 2002. Cryopreservation of immature and mature human oocytes. *Seminars in Reproductive Medicine*, **20**: 45-49.

Woods, E. J., Benson, J. D., Agca, Y. and J. K. 2004. Fundamental cryobiology of reproductive cells and tissues. *Cryobiology*, **48** (2): 146-156.

Wu, B., Ignotz, G., Currie, W. B. and Yang, X. 1997. Dynamics of maturation-promoting factor and its constituents proteins during *in vitro* maturation of bovine oocytes. *Biology of Reproduction*, **56** (1): 253-259.

Yasmin, C., Otoi, T., Setiadi, M. A. and Karja, N. W. K. 2014. Maturation and fertilisation of sheep oocytes cultured in serum-free medium containing silk protein sericin. *Acta Veterinaria Hungarica*, **63** (1): 110-117.

Zamboni, L. 1987. The ultrastructual pathology of the spermatozoon as a cause of infertility: the role of electron microscopy in the evaluation of semen quality. *Fertility sterility*, **48**: 711-734.

Zeinoaldini, S., Jafari, Z., Sarmast, F., Torbati, E. and Davachi, N. D. 2013. Different harvesting techniques used in ovine *in vitro* embryo production. *Scimetr*, **1** (1): 38.

Zhang, J. M., Sheng, Y., Zhi, Y.,Wang, H. Y. and Chen, Z. J. 2011. Cryopreservation of whole ovaries with vascular pedicles: vitrification or conventional freezing? *Journal of Assisted Reproduction and Genetics*, **28**: 445-452.

Zhang, J. M., Zhang, Y. C. and Ruan, L. H. 2012. Optimizing cryoprotectant perfusion conditions for intact ovary: a bovine model. *Journal of Assisted Reproduction and Genetics*, **29** (11): 1255-1260.

Zhao, J. and Bao, J. 2013. Trehalose maintains bioactivity and promotes sustained release of BMP-2 from lyophilized CDHA scaffolds for enhanced osteogenesis in vitro and in vivo. *PLoS ONE*, **8** (1).

Ziegler, D. 2007. Roles of FSH and LH during follicular phase: insight into the natural cycle IVF. *Reproductive Bio Medicine Online*, **15** (5): 508.

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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 **"Developmental potency of immature oocytes harvested from cryopreserved sheep ovary"** submitted by **Vaishali Sharma** Regd. No. **J-15-MV-448**.

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