EFFECT OF DIFFERENT VACUUM PRESSURES ON OOCYTE RECOVERY AND IN VITRO MATURATION OF OOCYTES COLLECTED BY ULTRASOUND GUIDED TRANS VAGINAL FOLLICULAR ASPIRATION IN CATTLE

T H E S I S

Submitted

In partial fulfillment of the requirements for the Degree of

M A S T E R O F V E T E R I N A R Y S C I E N C E

IN

ANIMAL REPRODUCTION, GYNAECOLOGY AND OBSTETRICS

BY

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DECLARATION OF STUDENT

I hereby declare that the experimental Research work and interpretation of the thesis entitled **"EFFECT OF DIFFERENT VACUUM PRESSURES ON OOCYTE RECOVERY AND IN VITRO MATURATION OF OOCYTES COLLECTED BY ULTRASOUND GUIDED TRANS VAGINAL FOLLICULAR ASPIRATION IN CATTLE"**or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

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Place: *Akola*

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF PLATES

LIST OF ABBREVIATIONS

Abbreviation		full form
%	:	Percent
/	:	Per
+	:	Plus
<	:	Less than
=	:	is equal to
>	:	Greater than
±	:	more than less than
≤	:	Less than or equal
≥	:	More than or equal
°F	:	Degree Farenheit
µl	:	microliter
^{O}C	:	Centigrade
CD	:	Critical difference
µg	:	microgram
cm/s	:	centimeter per second
CRD	:	Completely randomized design
BO	:	Brackett and Oliphant
eCG	:	Equine chorionic gonadotropin
OPU	:	Ovum pick-up
TVOR	:	Trans- vaginal oocyte retrieval
et al.	:	And others/ And Association
BFF	:	Bovine follicular fluid
Fig.	:	Figure
FSH	;	Follicle stimulating hormone
GnRH	:	Gonadotropin releasing hormone
hCG	:	Human Chorionic Gonadotropin
hr/hrs	:	hour/hours
i.e.	:	that is
TL Base	:	Tyrode –lactate base
RPM	:	Revolution per minute
IVP	:	*In vitro* production
IU	:	International units
IU/kg	:	International units per kilogram
TCM-199	:	Tissue culture medium -199
IVM	:	*In vitro* maturation
kg	:	kilogram
LH	:	Luteinizing hormone
mg	:	milligram
Ml	:	milliliter
mm	:	millimeter
ml	:	milliliter
IVF	:	*In vitro* fertilization

n	:	number of animals
ng/ml	:	nanogram/ milliliter
nmol/L	:	nano-moles per litre
NS	:	non-significant
P4	:	Progesterone value
pH	:	Power of hydrogen ion
mm	:	Millimeter
min.	:	Minute
Pvt	:	Private
MI	:	Metaphase first
GnRH	:	Gonadotrophin releasing hormone
MII	:	Metaphase second
LN_2	:	Liquid nitrogen
SE	:	Standard error
SEM	:	Standard error of mean
LH	:	Luteinizing hormone
CL	:	Carpus luteum
O_2	:	Oxygen
mM	:	Millimolar
ng	:	Nanogram
BSA	:	Bovine serum albumin
CO_2	:	Carbon –di-oxide
COCs	:	Cumulus oocyte complexes
DPBS	:	Dulbecco's phosphate buffered saline
ET	:	Embryo transfer
FBS	:	Fetal bovine serum
FCS	:	Fetal calf seum
FSH-p	:	Porcine follicle stimulating hormone
Hepes	:	N-2 Hydroxyethylpiperazine –N'-2-ethane sulfonic acid
E_2	:	17 –β oestrodiol
GV	:	Germinal vescicles

CHAPTER - I

INTRODUCTION

Livestock plays an important role in Indian economy. About 20.5 million people depend upon livestock for their livelihood. Livestock contributes 16% of the income of small farm household as against an average of 14% for all rural livelihood. It also provides employment to about 8.8% of the population in India. Livestock sector contributes 4.11% GDP and 25.6% of total agricultural GDP. According to Livestock Census 2012 Annual Report 2014-15 Department of Animal Husbandry, Dairying and Fisheries, Ministry of Agriculture, Government of India cattle population of India was 190.9 million and India ranks second in cattle population in throughout world (Tamizhkumaran *et.al*., 2016). Rapid spread of livestock with advanced production potential is the need of hour to meet the demand of feeding for expanding population of world. Assisted reproductive technology (ART) tools like, artificial insemination (AI), embryo transfer technology (ETT), *in vitro* embryo production (IVEP), embryo sexing, sex sorted semen technology and somatic cell nuclear transfer/cloning (SCNT)animal production, will help in faster multiplication of high producing livestock. These reproductive technologies turn around the livestock productivity in developed countries, however the success rate and cost factor are prohibitive for adopting them in developing countries (Gupta *et al*., 2015).

Productivity is the key to growth and reproduction is the pillar of animal production. Reproductive incompetence is one of the most important cause of economic losses in animal industries and it is realized throughout the world. To meet future needs and to be able to sustain agricultural production, agriculture research and its application, there is a need to use all emerging technologies specially the modern reproductive biotechnologies (Verma *et al*., 2012). In modern reproductive technologies, we first came to know about embryo transfer technology before in vitro embryo production due to some advantages of embryo transfer technology like increase the number of offspring sired from superior females, faster genetic progress, exportation and importation of embryos are easier than with live animals. But due to some disadvantages like this procedure

can only be done every 45-60 days, requires more straws of semen, higher cost in relation to boarding donor for more days, pregnant animals are not eligible for this technology, cost of chemicals, hormones and failure of super ovulatory response. (http://www.transova.com/tog-blog/in-vitro-fertilization-embryo-transfer-a-comparison).

To overcome these disadvantages and to improve the population of superior genetic livestock a new technology emerging i.e. *in vitro* embryo production (IVEP) through in vitro maturation, in vitro fertilization and in vitro culture of oocytes. *In vitro* embryo production technologies not only help in production of high genetic merit animals but also provide an excellent source of embryo for emerging biotechnologies like embryo sexing, cloning, nuclear transfer etc. (Galli and Lazzar., 2008, Manik *et al*., 2003).

Regardless of continuous attempts to improve bovine embryo production, its efficacy is still low since only 30-40% blastocysts development has been obtained from oocytes after *in vitro* maturation, fertilization and embryo culture (Sirard *et.al*. 2006). Even though several advantage of *in vitro* embryo productions, initial application in both cattle and buffaloes has been limited by the ability to recover oocytes. In earlier days, for in vitro embryo production (IVEP) slaughter house ovaries were used but due to some religious and sentimental issues in India slaughter of cattle was banned. Due to unavailability of cattle ovaries, a new recent development of low invasive ultrasound guided trans vaginal oocyte recovery (TVOR) and ovum pick up (OPU) technology has removed those difficulties to a large extent. The repeated recovery of oocytes permits production of more embryos than might be possible by standard embryo transfer practice (Galli and Lazzari, 2008; Manik *et al.*, 2003).

Ultrasound guided transvaginal follicular aspiration for recovering bovine oocytes was originally established by Pieterse *et al*. (1988). The ultrasound guided transvaginal ovum pick up (OPU) technique which overcomes some disadvantages of superovulation and use of ovaries obtained after slaughter, combined with in vitro embryo production (IVEP), has become a popular technique due to its potential commercial application. OPU is most flexible and

repeatable technique to produce embryos from nearly any given live donor. It can be used in cattle of high genetic value for obtaining valuable oocytes. (Yang *et al.*, 2005)

The birth of live offspring using in vitro fertilization has been reported in cattle (Brackett *et al*. 1982), goat (Hanada, 1985), pig (Cheng *et al*., 1986), sheep (Crozet *et al*., 1987) and buffalo (Suzuki *et al*., 1992). An ultrasound guided aspiration of bovine follicular oocytes was first proposed by a Danish teamworkers (Callesen *et al.,* 1987). In 1988, in vivo oocyte collection by transvaginal ultrasound-guided follicle aspiration (Ovum Pick-Up: OPU) was first established in cattle by a Dutch team (Pieterse *et al*., 1988). These researchers demonstrated that the repeated oocyte collection by OPU could be performed without risks to health and reproductive activity.

Ultrasound-guided transvaginal oocyte retrieval (TVOR) is the most commonly employed technique for harvesting oocytes for in vitro maturation, fertilization and in vitro culture from live animals. In cattle, it has been reported that the recovery rate and the quality of oocytes obtained through TVOR is dependent upon a number of factors such as hormonal stimulation (Goodhand *et al.*, 1999), time interval between successive aspiration (Gibbons *et al.*, 1994; Broadbent et al., 1997; Garcia and Salaheddine, 1998; Goodhand *et al.*, 1999); the combination of needle gauge and vacuum pressure applied (Fry *et al*., 1997; Hashimoto *et al*., 1999 and Ward *et al*., 2000) and the experience of the operator (Rath, 1993).

Vacuum pressure is also an important factor which is responsible for quality, quantity and developmental competence of oocytes. Recovery rate of oocytes and percentage of good quality oocytes were decreased as the vacuum pressure increased with increase in the percentage of denuded oocytes. Variation in the aspiration pressure affects oocyte recovery rate and quality of oocytes (Antosik *et al*., 2007; Brussow *et al*., 1997; Ward *et al.*, 2000). There is indirect relationship between vacuum pressure and oocyte quality, oocyte quantity and its developmental competence.

There are various factors responsible for the production of good quality and quantity of embryo in which cumulus oocyte complexes (COCs) plays an important role in, *in vitro* maturation, *in vitro* fertilization and *in vitro* culture of oocytes. During in vitro maturation cumulus cells play an important role in hormonal regulation because oocytes themselves do not have receptors for estradiol and gonadotropins (Lawerence *et al*., 1980). Cumulus cells give nutrients which are required for oocyte maturation through gap junction between ooplasm and cumulus cells (Dekel *et al*., 1984). Cumulus oocyte complexes (COCs) can synthesize growth hormones and these growth hormones proliferates cumulus cells during in vitro maturation (Izadyar *et al*., 1999).

During in vitro fertilization, due to hardening of zona pellucida and incomplete cytoplasmic maturation, denuded oocytes have low fertilizability because of disconnection of junctional complexes (Schroeder and Eppig, 1984).Fertilization rate and development to blastocyst stage is higher in cumulus enclosed oocytes during in vitro maturation in the presence of FSH, LH and estradiol (Saeki *et al.,* 1991). The percentage of cleaved oocytes was reduced, if denuded oocytes were matured *in vitro* (Sirard *et al*., 1988). Cumulus cell accelerates oocyte growth and developmental competence. Therefore, the oocyte stimulation of cumulus cell function is important for its subsequent development (Su *et al.,* 2004).

Scant information is available on in vitro embryo production in indigenous cattle. The aim of this work was to develop method of transvaginal oocyte retrieval by ultrasound machine and in vitro embryo production in indigenous cattle with following objectives.

1. Study the effect of different vacuum pressures on quality and recovery of oocyte collected by ultrasound guided transvaginal follicular aspiration.

2. Study the in vitro maturation of morphologically classified oocyte collected by ultrasound guided transvaginal follicular aspiration.

CHAPTER - II

REVIEW OF LITERATURE

Oocyte collection is usually performed by the isolation of follicles, dissection, aspiration or slicing of follicles or oviductal flushing. The recovery technique will vary according to whether collection is to be made from slaughtered animal or from live animal on a single or repeated occasion. In early period, laparoscopic technique was used for oocyte collection but it requires surgical procedure with anaesthetic.

The other procedure for recovery of oocyte from the live animal is ultrasonographic oocyte aspiration and today ovum pick up by ultrasonography is very flexible technique that can be used for several weeks for collection of oocytes from the same cow. However, several factor can affect the recovery rate of oocyte including needle type, puncturing frequency, hormonal treatment of animals, vacuum pressure during aspiration and operators experience. Effect of vacuum pressure is very much important for the recovery of oocytes, quality of oocytes and its developmental competence.

With all these above factors, present study was designed to study the **"Effect of different vacuum pressures on oocyte recovery and in vitro maturation of oocytes collected by ultrasound guided trans vaginal follicular aspiration in cattle."**

Pieterse *et al.,* (1991) reported OPU from natural cycling cows. They performed OPU in 21 cows over a three-month period. All visible follicles larger than 3 mm were punctured and aspirated three times during the oestrous cycle on Day 3 or 4, Day 9 or 10 and Day 15 or 16. They reported that mean (± SEM) oestrous cycle length after repeated follicle puncture was 22.2 ± 0.3 days and mean total number of punctured follicles per oestrous cycle was 12.6 ± 0.3. The maximum ($P<0.05$) number of follicles punctured (5.1 ± 0.3) for ovum pick-up

was on Day 3 or 4 of the oestrous cycle. The overall recovery rate of 541punctured follicles was 55 %.

Gibbons *et al.* (1994) studied the effects of once- versus twice-weekly transvaginal follicular aspiration on bovine oocyte recovery and embryo development. They aspirated the oocytes at negative vacuum pressure 75 mmHg and the oocytes were examined for morphology and used for *in vitro* maturation and fertilization. They observed that the oocytes from the twice-weekly and twice-weekly plus FSH aspiration groups generated a higher percentage of Grade-l quality embryos than the once-weekly group. They concluded that in commercial bovine oocyte aspiration, more transferable embryos can be generated from twice-weekly aspirations than from once-weekly aspiration.

Kruip *et al.* (1994) studied the effects of puncturing on follicle recruitment and on the number of oocytes recovery. Dairy cows (n=10) were punctured over a period of 5 month. They punctured and aspirated 14.5 ±0.4 follicles per session, and 8.0 ± 0.3 oocytes were recovered. A mean of 16 per cent of the oocytes developed into transferable embryos with a pregnancy rate of 40 per cent. The results did not differ between the months of the experiment, indicating that the transvaginal puncturing method can be used successfully over a 5 month period. No detrimental effects were observed after clinical and post-mortem examinations, nor did breed, age or reproductive status appear to affect the results.

Loony *et al.* (1994) employed ultrasound-guided follicular aspiration and *in vitro* embryo production techniques to retrieve oocytes and produce embryos from 200 problem donor female cattle that had not been successful in conventional embryo production. They reported on an average 6.3 oocytes per aspiration with weekly protocol. Total 1,006 retrieval sessions were performed; 9,120 follicles were aspirated and 6,344 oocytes were retrieved (69.6%). They concluded that ultrasound-guided follicular aspiration and IVEP was successful in producing pregnancies from non-productive problem donors.

Meintjes *et al*. (1995) studied transvaginal aspiration of oocytes from hormone treated pregnant beef cattle for in vitro fertilization. They observed that the percentage of oocytes recovered from pregnant donor cows treated with 40 mg

of FSH is greater than the pregnant donors cow treated with 20 mg of FSH. They concluded that this procedure proved to be effective and repeatable for viable oocyte recovery and safe for pregnant donors, because luteal function was maintained and no fetal loss resulted during pregnancy.

Stubbings and Walton (1995) conducted experiment to test the effect of ultrasonically-guided follicle aspiration on estrous cycle and follicular dynamics in Holstein cows. They aspirated all visible follicles ≥ 5 mm in diameter and the oocytes were aspirated once during the second and fourth cycle, with no aspirations during cycles 1, 3 and 5 in 4 Holstein cows. They reported that the mean inter-estrus interval for all non-aspirated cycles was 21.1 ± 0.8 day which was shorter ($P < 0.01$) than that for cycles with follicular aspiration (25.0 ± 0.7) days. The number of follicular waves, the size of ovulatory follicles and luteal phase plasma progesterone concentrations were unaffected by a single, mid-cycle aspiration of all visible follicles.

Bols *et al.* (1996) studied the effects of aspiration vacuum and needle diameter on cumulus oocyte complex morphology and developmental capacity of bovine oocytes. They observed that the highest oocyte recovery rates are obtained when using the thickest needle (18-g) regardless of the aspiration vacuum. They concluded that for all needle diameters, the proportion of oocytes surrounded by a compact cumulus decreases progressively as the vacuum increases.

Brüssow *et al.* (1997) studied on five different pressures 30, 60, 125, 250 and 375 mmHg and aspirated oocytes from different follicles and the morphology of oocytes was determined after aspiration. They observed that while increasing the aspiration pressure from 30 mmHg to 375 mmHg resulted in a decrease in oocytes recovery rate. They concluded that a higher aspiration pressure provokes an increase in the number of denuded oocytes. These results prove that variation in aspiration pressure affects oocytes recovery rate and COCs quality.

Fry *et al*. (1997) used various needle sizes (17 and 20-g), needle types (single and double lumen) and aspiration pressures (25, 50, 75 and 100 mmHg).They observed that the recovery of oocytes (Classes A, B and C) were significantly higher using a 17-g needle than a 20-g needle. As the vacuum

pressure increased so the recovery rate of number of oocytes reached a maximum at a calculated vacuum pressure with an increased incidence of denuded oocytes at higher vacuum pressures. On the basis of this experiment they concluded that as the vacuum pressure increases, the quality of oocytes decreases.

Klossok *et al.* (1997) reported OPU with once weekly interval by 6.5 MHz probe with 62 cm long needle carrier and with aspiration force of 30ml/minute in 24 dairy (Holstein Friesian) cows. They aspirated on an average 9.5± 3.9 follicles (X ± SD) per animal / session resulting in recovery of 5.0 ± 3.3oocytes / animal / session.

Garcia and Salaheddin (1998) studied effects of repeated ultrasound-guided transvaginal follicular aspiration on bovine oocyte recovery and subsequent follicular development. Aspirations were performed in Holstein-Friesian heifers every once weekly or twice weekly starting on Days 3 or 4 of the oestrous cycle. They observed that aspiration-induced follicle waves were indicated by an increased number of follicles > 4 mm seen within 2 d of the procedure. They concluded that follicle aspiration appears to induce and synchronize follicle waves, and when it is done twice a week it is associated with higher number of harvestable follicles and more oocytes recovered than when done once a week. These results can be attributed to the aspiration of a newly recruited pull of follicles 3 or 4 d after the first aspiration and before the establishment of follicular dominance and regression of subordinate follicles.

Hasler (1998) observed that the effect of influence of pressure on the quality of oocytes recovered from slaughter house ovaries. He used three different negative vacuum pressures (100, 75, 50 mmHg). From this study he concluded that higher percentage of oocytes were damaged when aspiration was conducted at 100 mmHg compared to 75 or 50 mmHg.

Carlin *et al*. (1999) observed that effects of ultrasound guided transvaginal follicular aspiration (TVFA) on oocyte recovery and hormonal profiles before and after GnRH treatment. They observed that follicle numbers after GnRH varied most among treatment groups for follicles < 9 mm. Progesterone concentrations increased during the 18-day period. From these results they suggest that long-term

transvaginal follicle aspiration affects progesterone, LH and FSH profiles and ovarian dynamics in cows.

Goodhand *et al*. (1999) carried out experiment with ultrasound guided transvaginal aspiration, either once a week or twice a week. Significantly fewer follicles per heifer per week were counted (14.7 ± 2.3 vs 27.4 ± 3.1) and aspirated (12.0 ± 2.0 vs 21.8 ± 2.7) in heifers on the once-weekly than twice weekly aspiration treatment. They also reported doubling the aspiration frequency led to an approximate 1.5- fold increase in the total number of oocytes collected per week, but the effect was not significant. There were no significant differences between treatments in the total number of oocytes recovered per week (5.6 ± 1.2 vs 8.9 ± 1.5).

Hashimoto *et al*. (1999) conducted experiments to increase the collection efficiency of bovine cumulus-oocyte-complexes (COCs) by transvaginal aspiration, the effects of aspiration pressure and needle diameter on bovine follicular oocyte collection were assessed. They observed that the when oocytes were collected from live cows, the highest recovery rates for category 1 (4 or more-layer of cumulus cells) and category 2 (between 1 and 3 layers of cumulus cells) were obtained using an 18-gauge needle and 40 mmHg pressure, and 21-gauge needle and 80 mmHg pressure. They also observed that the proportion of category 1 oocytes collected from live cows was lower than from slaughtered cows when 18-gauge needles at 80 mmHg. From these results they concluded that the combination of aspiration pressure and needle diameter is crucial for COC collection, and they suggest that optimal aspiration conditions for ovaries of slaughtered cows are not necessarily applicable to live cows.

Galli *et al.* (2001) used 115 mmHg of negative vacuum pressure for OPU with flow rate of 20 to 25 ml/min. With this setting they estimated oocyte recovery rate 50 to 60%. This setting compromises between a good recovery rate and minimal damage of the cumulus oocyte complex. On the basis of experiment, they concluded that higher aspiration pressure increases the total no of oocytes recovered but also increases the percentage of denuded or damaged oocytes.

Seneda *et al.* (2001) evaluated the relationship between follicle size and oocytes recovery by using ultrasound guided follicle aspiration. They aspirated follicles at65 to 70 mmHg of negative vacuum pressure. Aspirated follicles were divided into two groups on the basis of diameter of follicles, diameter ≤ 4 mm were small follicles and diameter ≥ 4mm were large follicles. The recovered oocytes from each group were kept separate and used for in vitro maturation, fertilization, and culture to the blastocyst stage. They observed that the recovery rate was greater in follicles ≤4mm.They concluded that routine aspiration of small follicles (≤4mm) could increase the number of oocytes available for in vitro development.

Manik *et al*. (2002) reported that transvaginal ultrasound-guided aspiration of follicles from Indian buffaloes (Bubalus bubalis) with a reproductive problem. They used 90 mmHg of negative vacuum pressure for follicular aspiration. Fifty-six oocytes were recovered through aspiration of 165 follicles, with an overall recovery rate was of 35%. Out of these, the oocytes were graded as per quality of oocyte then in vitro matured and fertilized. In this study they demonstrated that, the feasibility of collecting oocytes by repeated OPU from clinically sub-fertile or infertile buffalo.

Manik *et al*. (2003) performed ovum pick-up (OPU) using an ultrasound with transvaginal convex transducer (5 MHz) and a vacuum pressure of 90 mm Hg. The follicles were characterized on the basis of diameter as small (3-5 mm), medium (6-9 mm) and large (≥10mm). After recovery of oocytes they were classified on the basis of COCs layers and homogenicity of cytoplasm. They observed that overall recovery rate of 59% (range 35-79%). Of these 32% were of grades A and B and rest of grades C and D. This study demonstrates that the use of OPU is important tool for obtaining developmentally competent oocytes.

Sasamoto *et al*. (2003) observed that the effect of twisting and type of aspiration needle on the efficiency of transvaginal ultrasound guided ovum pick up in cattle. Ultrasound guided OPU was performed in live cows using two type of needle with a vacuum pressure of 75 mmHg. They observed that needle type did not affect the oocyte recovery or cumulus attachment of recovered oocytes.

Viana *et al*. (2004) used two different ovarian follicular aspiration schedules i.e. once in a week (group I) and twice in a week (group II) in Gir cows for evaluation of oocytes recovery and embryo yield. Oocytes were aspirated at 80 mmHg of negative vacuum pressure. Follicles were divided in three classes according to size: small (<6mm), medium (6-9mm) and large (>9mm). They found more oocytes were recovered per session in group I as compared with group II. From this study they concluded that the twice a week successive trans vaginal follicular aspiration sessions is the preferred schedule for recovering greater quality COCs and maximizing in vitro embryo production in Gir cows.

Yang *et al*. (2005) studied to compare two different schemes, continuous scheme i.e. CS (once a week interval) and discontinuous scheme i.e. DCS (3 day and 10 day of oestrous cycle) of once-weekly ovum pick up (OPU) with ultrasound-guided follicular aspiration technique. They found that the total number of aspirated follicles using DCS was lower than that using CS, but the mean numbers of punctured follicles and recovered oocytes per session were higher in DCS than CS group. This research demonstrates that similar quantity and quality oocytes can be achieved, and the side effects on donors are lower in discontinuous scheme than continuous scheme and discontinuous scheme is superior to continuous scheme.

Antosik *et al.* (2007) studied the effect of different vacuum pressures on the oocytes recovery rate from follicles of >2mm diameter as well as their quality in heifers and cows. They aspirated the follicles in a vacuum pressure 150, 100, 70, 50, 30 mmHg, respectively. The mean number of recovered oocytes in heifers and cows was 13.3 and 8.4, respectively. They concluded that increase in vacuum pressure above 50 and 70 mmHg, the percentage of C quality oocytes increased while percentage of A quality oocytes decreased.

Li *et al*, (2007) used two aspiration schedules twice weekly aspiration and once weekly aspiration for collection of oocytes through transvaginal ovum pick up for *in vitro* embryo production in Nanyang Yellow cattle. They concluded that the oocytes were aspirated twice in a week yielded higher number of aspirated

follicles, recovered oocytes and viable oocytes per session than in second aspiration schedule once weekly aspiration.

Rust *et al*. (2009) conducted study to investigate the possible effect of season on in vitro embryo production (IVEP) in sub-fertile beef cows. Animals were subjected to a once a week ultrasound guided oocyte recovery procedure (OPU) at negative vacuum pressure 60 mmHg. As for recovery rate, oocyte quality and embryo production, no significant differences could be established. They observed that the embryo production per OPU session per cow showed a definite seasonal pattern with low production during hotter months, a constant increase during the colder months and a peak in a rainy season.

Sakhong *et al*. (2012) studied ultrasound-guided transvaginal follicular aspiration and development of vitrified-thawed Thai indigenous beef cattle (Bos indicus) oocytes after in vitro fertilization. All the oocytes were aspirated at vacuum pressure 120 mmHg. They reported that OPU in Thai indigenous beef cattle without hormonal pre-stimulation can routinely done for an extended period of time. The age of donor does not affect the number and quality of oocytes aspirated, post-thawed viability and the number of embryos developing after IVF.

Su *et al*. (2012) studied the effect of donor age on the developmental competence of bovine oocytes retrieved by ovum pick up. They studied on the cumulus–oocyte complexes (COCs) were collected by 10 consecutive ovum pick up (OPU) sessions with a 4-day interval followed by in vitro maturation, fertilization and embryo development. They observed that cleavage rates (CR) and blastocyst rates (BR) were higher in the young cows than those in the middle-aged and old cows. They concluded that donor age of oocytes could affect developmental competence of oocytes recovered by OPU through the action of steroid hormonal balance on follicle development.

Silva *et al.* (2013) conducted the experiment on effect of heat stress on development, quality and survival of embryos produced in vitro from Nellore and Jersey cattle. Nellore and Jersey cattle embryos were submitted to heat stress (96 hours post insemination, 41^0C, 6 hours), developmental capacity of embryo was assessed at day 7. They reported that heat stress decreased the percentage of

Jersey blastocyst development, but did not find significant effect on embryo development in Nellore cow. They concluded that the detrimental effects of heat stress were dependent upon embryo from which breed and were more evident in Bos Taurus embryos than in Bos Indicus embryos.

Saini *et al*. (2015) studied developmental competence of different quality bovine oocytes retrieved through ovum pick-up following in vitro maturation and fertilization, in which all the oocytes were aspirated at 90 mmHg. All the aspirated oocytes were graded into two groups on the basis of morphological appearance of individual cumulus oocyte complexes (COCs). They observed that the cleavage rate in Group 1 (grade A and grade B oocytes) COCs was significantly higher than that of Group 2(grade C and grade D oocytes). The development of the presumed zygotes in Group 2 oocytes proceeded up to 8- to 16-cell stages only, while in Group 1 it progressed up to morulae (35.38 ± 7.11%) and blastocyst stage s (9.70 ± 3.15 %), indicating their better developmental potential.

2.2 Maturation and developmental competence:-

Harper and Bracket (1993) observed the bovine blastocyst development after *in vitro* maturation in a defined medium with epidermal growth factor and low concentrations of gonadotropins. They observed that varying concentrations of EGF in combination with low concentrations of either FSH or LH for IVM increased the proportions of oocytes reaching the blastocyst stage over conditions afforded by each gonadotropin alone. They concluded that combination of EGF with low concentrations of gonadotropins during IVM enabled subsequent blastocyst development in comparison to those having high concentrations of FSH or LH.

Goodhand *et al*. (1999) studied in vivo oocyte recovery and in vitro embryo production from bovine donors aspirated at different frequencies or following FSH treatment. Simmental heifers' follicles were aspirated at negative vacuum pressure 70 to 80 mmHg. Oocytes were graded, washed, matured and embryo development was observed. Twice weekly aspiration significantly increased the number of oocytes that cleaved, but the effect of FSH treatment was

not significant. They observed that the number of transferable morulae plus blastocysts produced per heifer per week was higher from animals aspirated twice a week or once a week following FSH treatment than from animals aspirated once a week without FSH treatment. They concluded that FSH treatment of bovine oocyte donors aspirated once a week enabled a similar number of transferable embryos to be produced per donor per week as aspiration twice a week without FSH treatment.

Holm *et al.* (1999) studied static in vitro production system for production of bovine blastocyst by using slaughtered cow ovaries. They used TCM-199 +cow serum (CS) with incubation for 22 to 25 hours under paraffin oil at 38.6° C in 5 % CO_2 incubator for maturation of oocytes. They observed 78 ± 7 to 81 ± 5 percent cleavage rate with SOF and co-culture medium, respectively.

Seneda *et al.* (2001) reported a study on relationship between follicle size and ultrasound-guided transvaginal oocyte recovery. They separated the aspirated follicles in two groups, group 1 (diameter < 4mm) and group 2 (>4mm). They observed that oocyte quality, cleavage rate and blastocyst development did not differ between different follicle sizes. Routine aspiration of small follicles (≤4mm) could increase the number of oocytes available for in vitro development.

Chian *et al*. (2002) studied the maturational and developmental competence of immature oocytes retrieved from bovine ovaries at different phases of folliculogenesis. They concluded that the maturational and developmental competence of immature oocytes is not affected by the phase of folliculogenesis.

Manik *et al.* (2003) reported a study on collection of oocytes through transvaginal ultrasound-guided aspiration of follicles in an Indian breed of cattle. Those oocytes were matured in maturation medium. Total 92 oocytes were recovered by aspiration of 157 follicles of which, 73 oocytes were cultured and fertilized, out of which 24 oocytes reached 2-4 cell stage at day 2 post-fertilization with a cleavage rate of 33%.

Viana *et al*. (2004) grouped animals in two groups on the basis of frequency of aspiration, group 1: once a week aspiration and group 2: twice a week aspiration. The collected oocytes were in vitro matured, in vitro fertilized

and in vitro cultured. They found that the cleavage rate did not differ in between both the groups but the percentage of embryo achieving the blastocysts stage was greater in group 2.

Vijayalakshmy *et al*. (2018) studied the effect of heat stress on folliculogenesis and quality of oocytes. They observed that heat stress and humidity during summer causes hyper prolactinaemia, suppressing the secretion of gonadotropins, which alters the ovarian steroidogenesis. It also affects folliculogenesis, follicular fluid microenvironment and oocyte quality. Hence, they conclude that in cattle the nutritional stress alters the feedback mechanism between oestradiol and LH surge which reduces the sensitivity of follicle to gonadotropins.

Jeena *et al*. (2019) studied the potential of different grades of cumulus-oocyte complexes (COCs) for in vitro maturation (IVM) and embryonic development. Abattoir derived oocytes were graded into grade A and B based on surrounding cumulus rings. Out of 1050 ovaries, a total number of 770 and 1360, were of grade A and B COCs, respectively, were aspirated. They observed that after IVM, grade A COCs had a significantly higher number of polar bodies as compared to grade B. They concluded that on IVF and embryo culture, grade A COCs produced the significantly higher rate of cleavage and blastocyst and as compared to grade B COCs.

2.3 Role of cumulus:-

Millions of germ cells add to health of one germ cell, number of somatic cells surrounding the germ cells in numerous layers with initiation of oocyte growth and the neighbouring somatic cell start to multiply and flattened granulosa cells developed cuboidal. After gathering of thousands of granulosa cells, extra cellular space increases to form a cavity known as antrum. Two particular populations of granulosa cells exist, cumulus granulosa that surrounds the oocyte and mural graulosa which consist of the inner layers of follicle wall. According to Anderson and Alberthi (1976) these heterologous contacts are

designed by cellular processes which extend from zona pellucida and end on oocyte membrane where gap junction is formed. This gap junction is intermediaries of cell to cell communication and metabolic cooperation in variety of system (Pitts, 1977 and Gilula *et al*., 1972). Thus, the association of cumulus cells with oocytes looks critical for regulation of oocyte maturation (Hillensjo *et al*., 1979).

According to Eppig (1979) throughout maturation cumulus cells play understanding role by simplifying essential product and by sending helpful signals to oocytes via gap junction.

Lawerence *et al*. (1980) stated that oocytes do not have receptors for gonadotropin and oestradiol which are present only in adjacent follicle cell. During maturation cumulus cells play an important role in hormonal regulation.

Dakel *et al*. (1984) observed that immature bovine oocytes are surrounded by several layers of tightly adherent, cumulus cells. The nutrients required for oocytes maturation were transported into the ooplasm via the gap junction between the ooplasm and cumulus cells.

Schroeder and Eppig (1984) stated that denuded oocytes have low fertilizability due to the hardening of zona pellucida and incomplete cytoplasmic maturation due to the disconnection of junctional complexes.

De felici *et al*. (1985) specified that the granulosa cells avoid spontaneous hardening of zona pellucida caused by production of cortical granules which decline oocyte fertility.

Downs *et al*. (1986) observed that the zona pellucida of denuded oocytes becomes hard than that of matured in vitro in presence of FCS.

Shioya *et al.* (1988) classified bovine oocytes on the basis of cumulus cells i.e. class A oocytes, with compact, dense cumulus cells; class B, partially naked oocytes with thin cumulus layer or small remnants of cumulus cells and class C, naked oocytes. They concluded that maturation rate was higher in class A oocytes whereas lower in class C oocytes.

Sirard *et al*. (1988) reported that in case of cows, if denuded oocytes were matured in vitro, the percentage of oocytes that cleave or form pronuclei after fertilization is reduced.

Saeki *et al*. (1991) observed that cumulus enclosed oocytes show high fertilization rates and development to blastocyst stage when matured in vitro in the presence of FSH, LH and oestradiol.

Izadyar *et al*. (1999) mentioned that growth hormone can be synthesized by COCs throughout in *vitro* maturation while these growth hormones increase the proliferation of cumulus cell during in *vitro* maturation.

Vozzi *et al*. (2000) stated that the intercellular communication intermediated by gap junction between cumulus cells are essential for the normal oocyte meiotic maturation.

Su *et al*., (2004) studied that cumulus cell promotes oocyte growth and developmental competence over difficult bi-directional interactions with the oocytes. Therefore, the oocyte stimulation of cumulus cell function is critical for its subsequent development.

Diaz *et al*. (2007) reported that particular characteristics and function of mural and cumulus cells compartments within the follicle is to raise an optimal microenvironment for appropriate endocrine (mural) and developmental (cumulus oocyte complex) function.

CHAPTER III

MATERIALS AND METHODS

The present study on "Effect of different vacuum pressures on oocyte recovery and in vitro maturation of oocytes collected by ultrasound guided transvaginal follicular aspiration in cattle" was carried out in the Department of Animal Reproduction, Gynaecology and Obstetrics, Post Graduate Institute of Veterinary and Animal Sciences, Akola from January to July 2019 for a period of six month.

Location and climate of the experimental station: -

Akola is located in sub-tropical region having extreme climatic condition situated at latitude of 22^0 to 24^0north longitude of 77.62 east and at an altitude of 307.415 meters above the mean sea level (M.S.L.). The minimum temperature in winter and maximum temperature in summer is being 15^0 C and 47^0 C, respectively with an average annual rainfall of 390.7 mm.

Table 3.1 Minimum and maximum temperature and relative humidity from January 2019 to July 2019: -

Months	Temp. max	Temp. min	Relative humidity I (morning) (%)	Relative humidity II (evening) (%)
Jan-19	28.5	10.5	69	27
Feb-19	32.2	15.6	53	22
Mar-19	36.7	17.0	44	20
Apr-19	42.1	22.2	41	22

May-19	43.1	26.4	39	19
Jun-19	40.1	24.7	60	33
Jul-19	32.1	20.9	84	61

The monthly temperature and relative humidity data from January 2019 to July 2019 was recorded at Meteorological Observatory Department of Agronomy Dr PunjabRao Deshmukh Krishi Vidyapeeth, Akola.

3.1 Materials

3.1.1 Buffer and Chemicals

Dulbacco's phosphate buffer saline (DPBS) was procured from Gibco laboratories, Life Technologies Inc, Grand Island, NY, USA.

3.1.2 Sera

Fetal Calf Serum (FCS) was procured from Gibco laboratories, Life Technologies Inc, Grand Island, NY, USA. Serum was heat inactivated at 56°C for 30 min, it was then frozen in small aliquots at – 20 °C.

3.1.3 Media

a) BO- IVF Bioscience, media

The media for in vitro maturation, sperm washing, in vitro fertilization and embryo development was procured from IVF Bioscience media from IVF Limited T/A IVF Bioscience, Falmouth, Cornwall, United Kingdom i.e. BO-IVM for in vitro maturation of immature oocytes; BO-Semen Preparation for washing of sperm for fertilization; BO-IVF for in vitro fertilization of matured oocytes; BO-Wash for washing of fertilized oocytes before culture; BO-IVC for in vitro embryo development; BO-Oil for overlaying of drops.

Table no. 3.2 Ovum pick up media (For follicular aspiration)

Sr. no	Ingredients	Quantity
1.	DPBS	96 ml
2.	FCS @ 4 %	4 ml
3.	Heparin @ 25 IU/ml	500 µl
4.	Gentamycin @ 5µg/ml (40mg/ml conc.)	12.5 µl

Table no. 3.3 Oocyte washing media

Sr. no.	Ingredients	Quantity
1.	DPBS	96 ml
2.	FCS @ 4 %	4 ml
3.	Gentamycin @ 5ug/ml (40mg/ml conc.)	12.5 ul

3.1.4 Antibiotic stock

Gentamycin Sulphate purchased from Laborate Pharmaceuticals India Ltd. (40 mg/ml of distilled water)

3.1.5 Disposable plastic ware

Disposable tissue culture flasks, culture plates, petri dishes, culture tubes, pipettes, syringes and hypodermic needles were obtained from Corning glass works, Corning, NY, USA and Becton Dickinson Co. 1950 William Drive, Oxnard CA, USA.

3.1.6 Equipments

Ultrasound machine pie medical 240 parus vet, The Netherland, vacuum pump K-MAR-5100 IVF Ultra Quiet, test tube heater V-FTH-2012, Cook medical technology William, Australia Pvt. Ltd., Veterinary oocyte aspiration biopsy attachment, disposable needle 19 G x 2'' 1.1 x 50 mm, TERUMO medical corporation, Tokyo, Japan, Sony electronic thermal printer, Sony, Japan, table top centrifuge, Biofuge Primo R Heraeus, Germany, Water jacketed CO_2 Incubator, CI/003, Thermo Electron Corporation, USA, Trinocular Stereo Zoom Microscope, (SMZ800) Inverted microscope, (TE300, Nikon Eclipse), Japan.

3.1.7 Miscellaneous

Membrane filters 0.22 micron were obtained from Millipore Corporation, USA. Pasture pipettes were purchased from local market, glass slides and cover slip were procured from Blue star, Polar Industrial Corporation, Bombay.

3.2 METHODOLOGY

3.2.1. Selection of animals

For the present study total 9 cows were selected, 2 Red Kandhari cows from Instructional Livestock Farm Complex, Post Graduate Institute of Veterinary and Animal Sciences, Akola and 7 Sahiwal cows from Department of Animal Husbandry and Dairy Sciences, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, for ultrasound guided transvaginal ovum pick up and in vitro embryo production.

3.2.2 Clinical examination

Selected animals were screened for brucellosis and tuberculosis. Reproductive tract of all selected cows was examined by per rectal examination and the cyclicity of ovaries examined per rectally. All the animals were dewormed and vaccinated prior to research work.

3.2.3 Animal Management-

Red Kandhari cows were kept at Livestock Instructional Farm Complex, Post Graduate Institute of Veterinary and Animal Sciences, Akola and Sahiwal cows were kept at Department of Animal Husbandry and Dairy Sciences, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola under the direct management of

Department of Animal Reproduction, Gynaecology and Obstetrics, Post Graduate Institute of Veterinary and Animal Sciences, Akola. The animals were fed on mixed ration consisting of concentrate, dry and green roughages as per the routine management practices of farm.

3.2.4 Preparation of oocyte pick up media and oocyte washing media -

The flushing medium i.e. Dulbecco's phosphate buffered saline (DPBS) containing 5 ug/ml Gentamycin and 4% Fetal Calf Serum (FCS) with 25 IU/ml heparin was used for aspiration of the oocytes as per table no. 3.2. For washing of oocytes DPBS containing 4% FCS and 5 ug/ml Gentamycin was used as per table no. 3.3. Prepared media was filtered with 0.22 micron filter paper and placed in water bath at 37°C before aspiration of follicles from experimental cows.

3.2.5 Preparation of maturation drops: -

Before 2 hours of aspiration session, maturation drops of 50µl size were prepared with BO-IVM in 35 mm petri dish, overlay with BO-OIL and placed in CO_2 incubator at 38.8 °C and 6 % CO_2 for equilibration.

3.2.6 Oocyte collection by ultrasound guided transvaginal ovum pick up technique-

At the time of collection of oocytes, the animal was restrained in travis and then the vulval lips were cleaned with lugol's iodine. An epidural anesthesia of 5 ml of 2% lignocaine hydrochloride was administered immediately before follicle aspiration.

Before each ovum pick up session, the ultrasonographic ovarian examination was carried out for study of follicular population with the help of a high quality real-time, B-mode diagnostic ultrasound scanner equipped with a 5.0 MHz sector transducer designed for transvaginal ovum pick up (Pie medical- 240 Parus vet, Philipswea, The Netherland). During scanning the relevant images was

frozen and measurements were taken. The total number of follicles were categorized on the basis of their diameter as small (<5 mm), medium (6-9 mm) and large (> 10 mm) were studied (Manik *et. al.* 2002).

At the time of follicular aspiration, the area of experiment was prepared for darkness to obtain the fine details, necessary for interpretation of images. The operator manipulates the ovary and the transducer, so that follicles to be punctured were positioned on the puncture (aspiration) line.

The short-beveled needles were connected to plastic tubing by means of stainless-steel connector. This system was connected to a plastic 50 ml centrifuge tube and a suction pump with a variable suction pressure (Cook suction pump, Australia). The suction pump was successfully adjusted at three different vacuum pressures (80 mm Hg, 90 mmHg and 100 mm Hg) for aspiration of oocytes. During the experiment vacuum pressure was applied till the follicle disappeared from the monitor with a foot operated control system. The procedure was repeated for the adjacent follicle and the needle was then withdrawn under continued suction and was flushed with a ovum pick up medium.

3.2.7 Effect of frequency of aspiration i.e. once/twice weekly on ovum pick up-

To study the effect of frequency of aspiration on oocytes recovery rate, the experimental cows were aspirated weekly (7 days interval) and twice weekly (3 to 4 days interval).

3.2.8 Searching of oocytes-

The oocytes along with the follicular fluid were aspirated in 50 ml centrifuge tube and placed in water bath at 37 °C, allowed to settle the oocytes at bottom of tube. After 10 to 15 min. supernatant was discarded to remove excess media and debris, remaining fluid was mixed with oocyte washing media and poured in squared 90 mm petri dish for searching of oocytes. The oocytes were searched from the dish under Zoom Stereo microscope. All the collected oocytes were washed in oocyte washing media and evaluated under phase contrast

microscope (200 X) and classified as per the compactness of cumulus cells around the oocytes.

3.2.9 Classification of oocytes: -

After searching of oocytes grading was done on the basis of compact cumulus oocyte complex (COC) layers and homogenicity of cytoplasm of oocytes and the oocytes were classified into 3 grades (Plate 4)

a) **Grade A (good): -** COCs with at least 4 to 5 layers of cumulus cells and with homogenous cytoplasm.

b) **Grade B (fair): -** COCs with 2 to 4 layers of cumulus cells and with homogenous cytoplasm.

c) **Grade C (poor): -** Oocytes partially denuded of cumulus cells and/or with irregular shrunken cytoplasm.

Plate 1 : Ultrasound guided transvaginal follicular aspiration

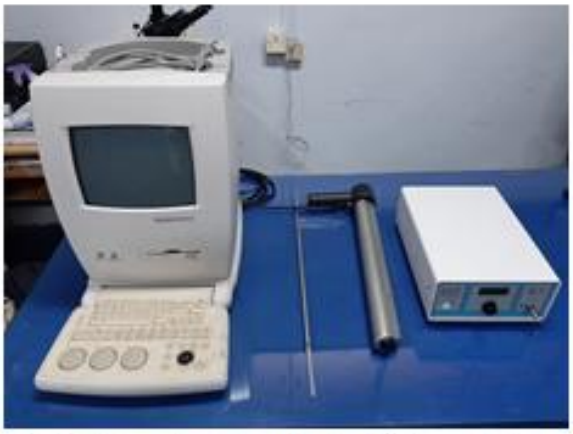
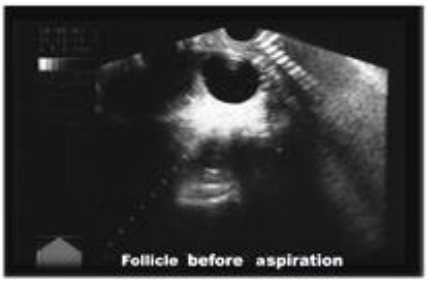
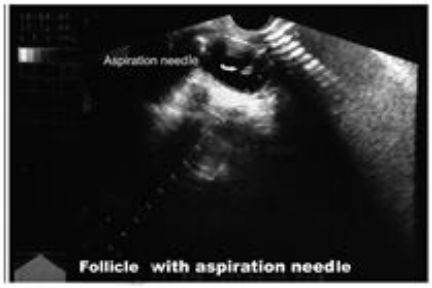


Plate 2 : Ultrasound machine and probe with biopsy attachment





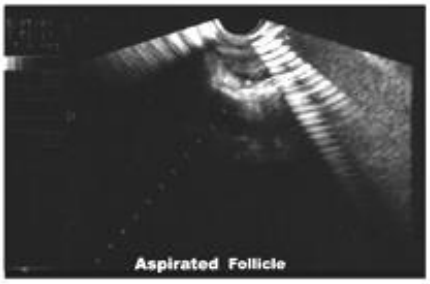


Plate 3 : Transvaginal oocyte retrieval (TVOR) by ultrasound

Plate 4 : Morphological classification of immature oocytes

3.2.10 <u>In vitro maturation</u>: -

Petri dish of BO-IVM drops was taken out from CO_2 incubator. All the graded oocytes were washed 2-3 times in oocyte washing media in 35 mm petri dish. All graded washed oocytes were transferred to separate pre equilibrated maturation medium drops in 35 mm petri dish, marked each drop for

identification of oocytes and placed in CO_2 incubator at 38.8 °C and 6 % CO_2 for 24 hrs.

3.2.11 Preparation of fertilization drops: -

Before 2 hours of fertilization, the BO-IVF drops of 50 µl size were prepared in 35 mm petri dish and overlay with BO-OIL. Prepared petri dish was placed in CO_2 incubator at 38.8 °C and 6 % CO_2 for equilibration.

3.2.12 Preparation of semen washing media: -

For washing of semen, add 2 ml of BO-Semen Preparation media in two separate 15 ml centrifuge tube and placed in water bath for equilibration at 37°C.

3.2.13 Preperation of Matured oocyte : -

Petri dish of matured oocytes and BO-IVF drops was taken out from CO_2 incubator. Matured oocytes were washed in drops of fertilization medium and transferred to separate BO-IVF medium drops. The BO-IVF dishes were placed in the CO_2 incubator at 38.8 °C and 6 % CO_2.

3.2.14 Semen preparation: -

The frozen semen straw was taken out of the liquid nitrogen and transferred immediately into the 37°C sterile water container for 30 sec. for thawing of semen straw. Thawed semen straw was placed immediately on the heated flow cabinet bench top. Semen straw was dried with a piece of sterile gauge and laboratory seal of semen straw was cut and this end was inverted in 500 µl Eppendorf tube and then sealing plug was cut, allowing the semen to flow into the 500 µl Eppendorf tube. The collected thawed semen transferred to BO- Semen preparation media in 15 ml centrifuge tube and semen centrifuged for 7 minutes at 2400 RPM. The supernatant was discarded and remaining sperm suspension approximately 350 ul was mixed with preheated BO- Semen preperation media. The semen was again centrifuged for 5 minutes at 2200 RPM. After centrifugation the supernatant was removed as much as possible. The motility of semen was evaluated during centrifugation under a microscope on a warmed glass slide and the sperm concentration was calculated to make the final concentration 2.0 x 10^6 spermatozoa/ ml in the IVF medium.

3.2.15 To calculate the correct volume of semen for fertilization of oocytes:

Uniform 25 µl sample of the sperm suspension was taken and mixed with 25 µl cold distilled water in Eppendorf tube. The 10 µl of mixture was taken and transferred to the Sperm Processors (makler) counting chamber. Under microscope the number of sperm in 10 squares was counted corresponding to the number of sperm in millions per ml ($x10^6$/ml) in the sperm suspension. The dilution was 1:2 as 25µl of sperm suspension was mixed with 25µl of cold water. Therefore, the number of sperm counted in 100 squares was by 10, and multiplied by 2 to obtain the actual sperm concentration $x10^6$/ml.

3.2.16 In vitro fertilzation (IVF)

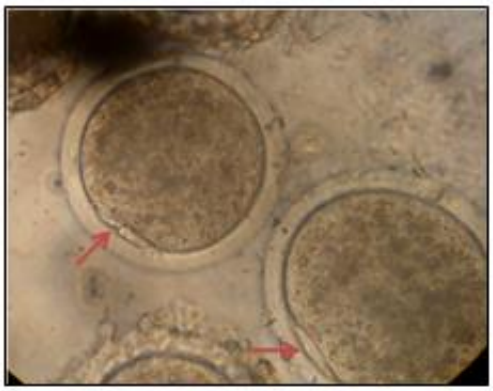
The BO-IVF dish was taken out from CO_2 incubator and calculated volume of sperm suspension was added to each fertilisation drop with the final sperm concentration as $2x10^6$/ml. The fertilization dish in the CO_2 incubator for co-incubation of sperm and oocytes at 38.8°C and 6 % CO_2 for 16-20 hours.

3.2.17 Preparation of BO- Wash media dish: -

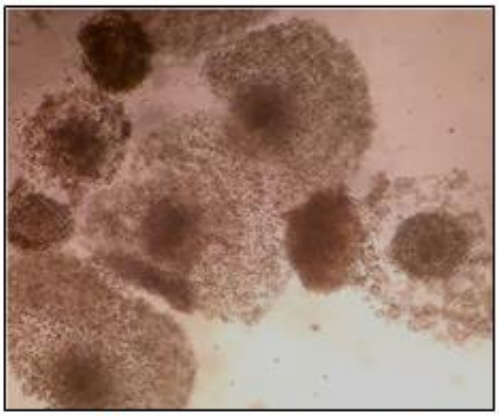
BO- Wash media 3 ml was transferred into 15 ml tube and preheated in water bath at 37°C. BO-Wash media was taken in 35 mm petri dish according to requirement for washing of fertilized oocytes.

3.2.18 Culture dish preparation: -

BO-IVC drops (50µl) were prepared in 35 mm petri dish and overlay with BO-Oil and placed in the CO_2 incubator for equilibration at 38.8°C and 6 % CO_2 for 2 hours.



Oocytes with polar body



Expanded matured oocytes (cumulus oocyte complexes)

Plate 5 : *In vitro* maturation of oocytes

3.2.19 In vitro culture (IVC)

The BO-IVF dish was taken out from the incubator following 16-20 hours of incubation. Preheated BO-Wash media was taken in 35 mm petri dish and the presumptive zygotes were transferred into the fresh preheated BO-Wash media. The presumptive zygotes were denuded with the help of pipette by repeated pipetting and the zygotes were washed with BO-Wash media many times as necessary to remove all cumulus cells and attached spermatozoa. Prepared IVC dish was taken out from the CO_2 incubator and washed denuded zygotes were transferred into the IVC drops and placed in CO_2 incubator for further development at 38.8°C temperature and 6 % CO_2. Cleavage rate was checked at every 48 hours of culture, up to 7 days.

3.2.20 Scoring of Cleavage-

The *in vitro* fertilized oocytes were observed under inverted microscope and the cleavage rate was calculated by using the following formula.

$$\% \text{ Cleaved} = \frac{\text{Number cleaved}}{\text{Total no. of oocytes}} \times 100$$

3.3 Statistical Analysis

The data collected during the present studies for different parameters were statistically analyzed, as per Snedecor and Cochrane (1994).

CHAPTER - IV

RESULT AND DISCUSSION

Many researchers studied on the effect of vacuum pressure on recovery of oocytes, quality of oocytes and its developmental competence, but there is lot of controversy in the results. There was effect of different parameters on recovery and quality of oocytes like effect of pressure, heat stress, relative humidity, season, nutrition, environment, operators experience, frequency of aspiration. The effect of different vacuum pressure and frequency of aspirations on recovery rate of oocytes, quality of oocytes and developmental competence was studied in present experiment.

1) Oocyte recovery by transvaginal oocyte retrieval method: -

a) Effect of different vacuum pressure on oocyte recovery and quality of oocytes

The paucity of information concerning the aspiration vacuum pressure is inconsistent. To date, the aspiration vacuum pressure has usually been expressed in millimeter of mercury and has varied between 40 and 400 mmHg. However, the exact aspiration vacuum pressure at the top of the needle depends on the construction of the ovum pick up device, the length and the diameter of the tubing system and the diameter of the needle. To study the effect of vacuum pressure on recovery rate and quality of oocytes three different aspiration vacuum pressures (80, 90 and 100 mmHg) were applied.

In the present study, total 60 sessions of follicular aspiration were carried out in 9 animal by using three different vacuum pressure (80, 90 and 100 mmHg). The obtained results are presented in table 4.1 and 4.2.

By using 80 mmHg vacuum pressure, total 9 animals were aspirated in 18 sessions, in which total 64 follicles (7.11 follicles/animal and 3.61±0.40 follicles/session) were aspirated. The mean number of small, medium and large size follicles 51 (2.83±0.37), 11 (0.66±0.16) and 2 (0.11±0.07) were observed,

respectively. The total 34 oocytes (3.77±0.61 oocytes/animal and 1.88±0.31 oocytes/session) were collected, in which the mean number of A, B and C quality

Table 4.1 Mean number of different size of ovarian follicles collected with three different vacuum pressure

Vacuum pressure	Number of sessions	Number of Animal aspirated	Number. of different size of follicles aspirated			Total follicles	Number of follicles/ Animal	Number of follicles/ sessions
			Small (<5mm) (per/session)	Medium (6-10mm) (per/session)	Large (>10mm) (per/session)			
80	18	9	51 (2.83±0.37) [a]	11 (0.66±0.16)	2 (0.11±0.07)	64 (3.61±0.40)	7.11	3.61±0.40
90	16	5	22 (1.37±0.31) [b]	12 (0.75±0.14)	5 (0.31±0.15)	39(2.43±0.31)	7.8	2.43±0.31
100	26	9	63 (2.42±0.27) [a]	21 (0.88±0.14)	10 (0.38±0.11)	94(3.61±0.28)	10.44	3.61±0.28

Different superscript indicates significant difference between means of different rows ($P < 0.05$)

Table 4.2 Effect of different vacuum pressure on oocytes recovery by using OPU technique

Vacuum pressure	Number of Animal	Number of sessions	Number of follicles	Number of qualities of recovered oocytes			Total number. of oocyte (per session)	Oocyte /Animal	Oocyte /Session	Recovery rate (%)
				A	B	C				
80	9	18	64	6 (0.33±0.11)	8 (0.44±0.16)	20 (1.11±0.24)	34 (1.88±0.31)	3.77±0.61	1.88±0.31	53.12
90	5	16	39	13 (0.81±0.27)	6 (0.37±0.17)	4 (0.25±0.11)	23 (1.43±0.44)	4.6±0.81	1.43±0.44	58.97
100	9	26	94	19 (0.73±0.20)	10 (0.38±0.11)	28 (1.07±0.29)	57 (2.19±0.52)	6.33±1.19	2.19±0.52	60.63

oocytes 6 (0.33±0.11), 8 (0.44±0.16) and 20 (1.11±0.24), respectively were observed. The average oocytes recovery by using 80 mmHg vacuum pressure was 53.12%.

By using 90 mmHg vacuum pressure, total 5 animals were aspirated in 16 sessions, in which total 39 follicles (7.8 follicles/animal and 2.43±0.31 follicles/session) were aspirated. The mean number of small, medium and large size follicles 22 (1.37±0.31), 12 (0.75±0.14) and 5 (0.31±0.15) respectively were observed. The total 23 oocytes (4.6±0.8 oocytes/animal and 1.43±0.44 oocytes/session) were collected, in which the mean number of A, B and C quality oocytes 13 (0.81±0.27), 6 (0.37±0.17) and 4 (0.25±0.11) respectively were observed. The average oocytes recovery by using 90 mmHg was 58.97 %.

By using 100 mmHg, total 9 animals were aspirated in 26 sessions, in which total 94 follicles (10.44 follicles/animal and 3.61±0.28 follicles/session) were aspirated. The mean number of small, medium and large size follicles 63 (2.42±0.27), 21 (0.88±0.14) and 10 (0.38±0.11) respectively were observed. The total 57 oocytes (6.33±1.19 oocytes/animal and 2.19±0.52 oocytes/session) were collected, in which the mean number of A, B and C quality oocytes 19 (0.73±0.20), 10 (0.38±0.11) and 28 (1.07±0.29) respectively were observed. The average oocytes recovery by using 100 mmHg was 60.63%.

The results revealed that by using 100 mmHg vacuum pressure, 3.61±0.28 follicles/session were aspirated. In which, average 2.19±0.52 oocytes/session were collected, so the total oocytes recovery rate was 60.63%, which was comparatively better than 80 mmHg and 90 mm Hg vacuum pressure, which had oocytes recovery rate as 53.12% and 58.97% respectively. However, there was no significant difference between 80 mmHg, 90 mmHg and 100 mm Hg vacuum pressure for ovum pick up.

By using 90 mmHg of vacuum pressure, small follicles 22 (1.37±0.31) were significantly lower than 80 mmHg 51 (2.83±0.37) and 100 mmHg 63 (2.42±0.27) negative vacuum pressure ($P<0.05$). Total number of follicles were

lower at 90 mmHg 39 (2.43±0.31) negative vacuum pressure than 80 mmHg 64 (3.61±0.40) and 100 mmHg vacuum pressure 94 (3.61±0.28).

Total number of follicles per animal were higher in 100 mmHg (10.44) vacuum pressure than 80 mmHg (7.11) and 90 mmHg vacuum pressure (7.8) in present study, but there is no significant difference observed. However, Manik *et al*. (2003) reported that the mean number of follicles per animals per session 6.8±0.7 in Karan Fries cattle were lower than present study. The difference in the number of aspirated follicles may be due to effect of variation in individual donor, breed, season, nutrition, environmental factors, physiological stress on follicular populations. (Kruip *et al*., 1994)

In present study, overall small size follicles were significantly higher at 80 mmHg and 100 mmHg than 90 mmHg vacuum pressure. The present result of follicular aspiration is in accordance with the study of Manik *et al*. (2003) who reported that, the overall small size follicles (5.20±0.50) were greater than medium size follicles (0.70±0.10) and large size follicles (1.00±0.20).

Seneda *et al*. (2001) also reported that number of small size follicles (276) were higher than large size follicles (154) which is an accordance with present study. They also reported that oocytes recovered from small size follicles (72.2%) were significantly higher than oocytes recovered from large size follicles (50%). By using ultrasound guided TVOR method when small follicles were higher in proportion, it could increase in vitro embryo production by increasing number of oocytes available for development.

Gupta *et al.* (2006) observed that mean number of small size follicles (2.2±0.3) were higher than medium size (0.6±0.2) and large size follicles (0.9±0.1) in Murrah buffaloes, which was similar to present study.

There was no significant difference in different quality (A, B and C) of oocytes on different vacuum pressures (80, 90 and 100 mmHg) in the present study. By using 100 mmHg pressure, the total number of oocytes 57 (2.19±0.52), were higher than 80 mmHg 34 (1.88±0.31) and 90 mmHg vacuum pressure 23 (1.4±0.44).

In present study, by using 100 mmHg vacuum pressure the number of oocytes per animal (6.33±1.19) were higher than 80 mmHg (3.77±0.61) and 90 mmHg vacuum pressure (4.6±0.8). However, Goodhand *et al.* (1999) observed that mean number of oocytes per animal per session as 5.6±1.2 from Simmental heifers which is similar to number of oocytes per animal at 90 mmHg and 100 mmHg vacuum pressure in present study.

By using 100 mmHg vacuum pressure the total number of oocytes per session (2.19±0.52) were higher than 80 mmHg (1.88±0.31) and 90 mmHg vacuum pressure (1.43±0.44). The mean number of oocytes collected per session in present study was lower than that of Gibbons *et al*. (1994), which was 6.8±2.0 oocytes per session from multiparous angus cows. The lower number of oocytes per session found in present study may be due to aspiration of smaller number of follicle in comparison to other study. It may be due to individual animal variation and operators skill.

Garcia and Salaheddine (1998) aspirated, total 12.4±6.1 number of follicles and recovered 5.4±3.7 oocytes per session from 96 sessions, which were higher than present study which may be due to the follicles were aspirated 3 or 4 day after the first aspiration and before the establishment of follicular dominance and regression of subordinate follicles.

However, Manik *et al*. (2002) and Manjunath *et al*. (2008) reported lower number of oocyte recovery per session in buffaloes than recorded in present study. The primary cause for lower oocyte recovery in buffaloes compared with that in cattle, might be due to lower follicular populations of all size categories in buffaloes.

By using 100 mmHg vacuum pressure, the overall recovery rate (60.63%) was higher than 80 mmHg (53.12%) and 90 mmHg vacuum pressure (58.97%). It may be due to a greater number of aspiration sessions and a greater number of aspirated follicles by using 100 mm Hg vacuum pressure than 80 mm Hg and 90 mmHg negative vacuum pressure.

In present study, C quality oocytes were higher in number as compared to A quality and B quality oocytes by using 80 mmHg and 100 mmHg of vacuum pressure. Hashimoto et al. (1999) and Carlin et al. (1999) also reported that, C quality oocytes were higher in number than A quality and B quality oocytes by using 80 mmHg pressure which is similar to present study. It may be due to increase in vacuum pressure. If the pressure increases, the total number of oocytes increases but quality of oocytes decreases (Galli *et al.,* 2001; Bols *et al*., 1996; Fry *et al.*, 1997).

In present study, A quality oocytes were higher than B quality and C quality oocytes by using 90 mmHg vacuum pressure. However, Manik *et al*. (2003) and Seneda *et al.* (2001) found that A quality oocytes were lower than B quality and C quality oocytes. The A quality oocytes were lower it may be due to needle gauge and the vacuum pressure have been reported which affect the quality of oocytes.

Table 4.3 Mean ± SE of different quality of oocytes recovered and total recovery rate

Total number of aspiration sessions	Total number of follicles observed	Total number of oocytes recovered	Reco-very rate (%)	Grading of oocytes		
				A	B	C
60 (6.67±0.87)	197 (21.89±1.83)	114 (12.67±1.19)	57.86	38 (4.23±0.66)^{ab}	24 (2.67±0.44)^{b}	52 (5.78±0.64

Different superscript indicates significant difference between means of different columns (P< 0.05)

The overall follicle observed, total number of oocytes recovered in 60 different aspiration session is presented in table 4.3. in which total 197 (21.89±1.83) follicles were available for puncture. Total mean number (n=114) of oocytes recovered were 12.67±1.19 with the recovery rate 57.86%. Out of total

number of oocytes recovered A quality, B quality and C quality oocytes were 38 (4.23±0.66), 24 (2.67±0.44) and 52 (5.78±0.64) respectively.

In present study A quality (4.23±0.66) and C quality (5.78±0.64) oocytes were significantly higher than B quality (2.67±0.44) oocytes ($P<0.05$). However, Seneda *et al*. (2001) also found that there was a significant difference between A quality, B quality and C quality oocytes recovery.

In present study, by using 80 mmHg, 90 mmHg and 100 mmHg vacuum pressure overall recovery rate was 57.86%. Manik *et al*. (2003) also reported that by using 90 mmHg vacuum pressure overall recovery rate was 59 % which is accordance with present study. However, overall recovery rate of oocytes observed by Manik *et al.* (2002) was lower than present study. This slight variation in results may be due to low follicular population and high rate of atresia among the growing follicles.

Sendag *et al.* (2008) used FSH stimulation before aspiration of follicle and observed that overall recovery rate was 77.3% which was higher than present study. It may be due to hormonal stimulation with FSH resulting the availability of large number of follicles for puncture.

In present study, overall recovery rate was lower than reported that Fry *et al*. (1998) which was 77.3 %. This slight variation in results may be due to hormonal stimulation with GnRH as it increases the number of follicles and it allows recruited antral follicles follow their growth during the early selection process.

This difference in the results of other studies may be due to effect of nutrition, breed, environmental temperature, effect of humidity on follicular population of various size and total recovery rate of oocytes. (Manik *et al.* 2003)

b) Effect of breed on oocyte recovery rate and quality of oocytes

In this experiment, breed wise (Red Kandhari and Sahiwal) recovery rate and quality of oocytes were studied at different vacuum pressures (80, 90 and 100 mmHg).

In Red Kandhari cows, from 6 aspiration sessions total number of follicles 13 (2.16±0.65) were punctured by using 80 mmHg negative vacuum pressure from which oocytes (0.83±0.30) were recovered with the total recovery rate of 38.46%. Out of total oocytes recovered, number of A, B and C quality oocytes were 0 (0), 0 (0) and 5 (0.83±0.30), were respectively. (Table 4.4)

Table 4.4 Breed wise (Red Kandhari and Sahiwal) on recovery rate and quality of oocytes on different vacuum pressure

Breed of animal	Aspiration pressure (mmHg)	Number of aspiration sessions	Total number of follicles (n) Punctured	Total number of oocytes recovered (n)	Grading of oocytes			Recovery rate (%)
					A	B	C	
Red Kandhar cattle (n=2)	80	6	13(2.16±0.65)	5(0.83±0.30)	0(0)	0(0)	5(0.83±0.30)	38.46
	90	8	17(2.12±0.35)	13(1.62±0.73)	7(0.875±0.47)	4(0.5±0.32)	2(0.25±0.16)	76.47
	100	6	17(2.83±0.60)	10(1.66±0.61)	3(0.5±0.22)	2(0.33±0.21)	5(0.83±0.40)	58.82
	Total	**20**	**47**	**28(1.4±0.35)**	**10**	**6**	**12**	**59.57**
Sahiwal cattle (n=7)	80	12	51(4.25±0.35)	29(2.41±0.35)	6(0.5±0.15)	8(0.66±0.22)	15(1.25±0.32)	56.86
	90	8	22(2.75±0.52)	10(1.5±0.53)	6(0.75±0.31)	2(0.25±0.16)	2(0.25±0.16)	45.45
	100	20	77(3.85±0.31)	47(2.4±0.65)	16(0.80±0.25)	8(0.40±0.13)	23(1.10±0.36)	61.03

	Total	**40**	**150**	**86** **(2.2±0.35)**	**28**	**18**	**40**	**57.33**

In total 8 aspiration sessions, 17 follicles (2.12±0.35) were punctured by using 90 mmHg negative vacuum pressure from which 13 oocytes (1.62±0.73) were recovered with the total recovery rate of 76.47%. Out of total oocytes recovered, number of A, B and C quality oocytes were 7 (0.875±0.47), 4 (0.5±0.32) and 2 (0.25±0.16), respectively.

In total 6 aspiration sessions, 17follicles (2.83±0.60) were punctured by using 100 mmHg negative vacuum pressure from which 10 oocytes (1.66±0.61) were recovered with the total recovery rate of 58.82%. Out of total oocytes recovered, number of A, B and C quality oocytes were 3 (0.5±0.22), 2 (0.33±0.21), 5 (0.83±0.30), respectively.

In Red Kandhari cows, total recovery rate (76.47%) by using 90 mmHg vacuum pressure was higher than 80 mmHg (38.46%) and 100 mmHg vacuum pressure (58.82%).

In Sahiwal cows, from total 12 aspiration sessions total 51 follicles (4.25±0.35) were punctured by using 80 mmHg negative vacuum pressure from which 29 oocytes (2.41±0.35) were recovered with the total recovery rate of 56.86%. Out of total recovered oocytes, number of A, B and C quality oocytes were 6 (0.05±0.15), 8 (0.66±0.22) and 15 (1.25±0.32) respectively. (Table 4.4)

In total 8 aspiration sessions, 22 follicles (2.75±0.52) were punctured by using 90 mmHg negative vacuum pressure from which 10 oocytes (1.5±0.53) were recovered with the total recovery rate of 45.45%. Out of total oocytes recovered, number of A, B and C quality oocytes were 6 (0.75±0.31), 2 (0.25±0.16) and 2 (0.25±0.76), respectively.

In total 20 aspiration sessions, 77 follicles (3.85±0.31) were punctured by using 100 mmHg negative vacuum pressure from which 47 oocytes (2.4±0.65) were recovered with the total recovery rate of 61.03%. Out of total oocytes recovered, number of A, B and C quality oocytes were 16 (0.80±0.25), 8 (0.40±0.13) and 23 (1.10±0.36), respectively.

In Sahiwal cows out of all three vacuum pressures, by using 100mmHg pressure the total recovery rate (61.03%) was higher than 80 mmHg (56.86%) and 90 mmHg (45.45%).

Overall recovery rate in Red Kandhari cows at three different vacuum pressure was 59.57%, and that of Sahiwal cows was 57.33% with no significant difference observed between overall recovery rate of these two breeds of cattle.

Bos indicus cattle have more follicular waves and higher proportion of small follicles than Bos Taurus Segerson *et al.* (1984) and Biase *et al*. (2008) stated that in zebu cows, there is variation among oocyte yield in different breeds, which seems to be related to gene sequence that affects the follicular population and oocyte yield.

In present study number of recovered oocytes per session from Red Kandhari cattle was 1.4±0.35 and that of Sahiwal cattle was 2.2±0.35. However, Pontes *et al*. (2004) reported that number of recovered oocytes per aspiration session from Gir cows (17.1±4.5) seemed to be higher than present study.

Viana *et al*. (2004) reported that number of oocytes per session in once a week and twice a week were observed as 8.9±0.8 and 7.0±0.7, respectively, which is higher than present study. These variations in results may be due to stress on individual animal because of to environmental temperature in different geographical area.

c) Effect of frequency of aspiration (i.e. once or twice week) on follicular number and oocyte recovery

The study was carried out to compare cumulus oocyte complex recovery and follicular development after once or twice weekly ultrasound guided transvaginal follicular aspiration. The results are presented in table 4.5 and 4.6.

1) Once a week aspiration-

In once a week aspiration session, total 9 animals were aspirated in 34 sessions, in which total 114 follicles (12.66 follicles/animal and 3.35±0.27 follicles/session) were aspirated. The observed mean number of small, medium

and large follicles were 83 (2.44±0.26), 23 (0.67±0.12) and 8 (0.23±0.07), respectively. A total of 55 (1.67±0.28) oocytes (6.11±1.01 oocytes/animal and

Table 4.5 Different sizes and total number of follicles present on ovary during once a week and twice a week session of aspiration

Freq. of aspiration	Number of sessions	Number of Animals aspirated	Number of follicles aspirated			Total number of follicles	Number of follicles/ Animal	Number of follicles/ sessions
			Small	Medium	Large			
Once a week	34	9	83 (2.44±0.26)	23 (0.67±0.12)	8 (0.23±0.07)	114 (3.35±0.27)	12.66	3.35±0.27
Twice a week	26	9	56 (2.15±0.32)	19 (0.69±0.14)	8 (0.30±0.09)	83 (3.15±0.29)	9.22	3.19±0.29

Table 4.6 Effect of frequency of aspiration (i.e. once a week and twice a week) on recovery of oocytes quality by using OPU technique

Frequency of aspiration	Number of Animals (n)	Number of sessions (n)	Number of follicles (per session)	Grading of oocytes			Total oocytes recovered	Oocyte /Animal	Oocyte /Session	Recovery rate (%)
				A	B	C				
Once a week	9	34	114 (3.35±0.27)	19 (0.55±0.13)	13 (0.38±0.11)	23 (0.69±0.18)	55 (1.64±0.28)	6.11±1.01	1.64±0.28	48.24
Twice a week	9	26	83 (3.19±0.29)	19 (0.73±0.21)	13 (0.52±0.14)	27 (1.08±0.26)	59 (2.26±0.50)	6.55±0.89	2.26±0.50	71.08

1.64±0.28 oocytes/session) were collected, in which the number of A, B and C quality oocytes were 19, 13 and 23, respectively. The mean number of A, B and C quality oocytes per sessions were 0.55±0.13, 0.38±0.11 and 0.69±0.18, respectively. The average oocytes recovery by once a week aspiration was 48.24%.

2) Twice weekly aspiration-

In twice a week aspiration session, total 9 animals were aspirated in 26 sessions, in which total 83 follicles (9.22 follicles/animal and 3.19±0.29 follicles/session) were aspirated. The mean observed number of small, medium and large follicles were 56 (2.15±0.32), 19 (0.69±0.14) and 8 (0.30±0.09), respectively. A total of 59 oocytes (6.55±0.89 oocytes/animal and 2.26±0.50 oocytes/session) were collected, in which the number of A, B and C quality oocytes were 19, 13 and 27, respectively. The mean number of A, B and C, quality oocytes per session were 0.73±0.21, 0.52±0.14 and 1.08±0.26, respectively. The average oocytes recovery by twice-weekly aspiration was 71.08%.

The results revealed that by using once a week aspiration session, 3.35±0.27 follicles/session and with twice a week aspiration session 3.19±0.29 follicles/session were aspirated. In with once a week aspiration session, average 1.64±0.28 oocytes/session and with twice a week aspiration session average 2.26±0.50 oocytes/session were collected. The total oocytes recovery rate was 71.08% in twice a week aspiration session, which is comparatively higher than once a week aspiration, which had 48.24% oocytes recovery rate.

In present study, number of follicles observed per session were not significantly different between once a week and in twice a week (3.35 ±0.27 Vs 3.19±0.29). However, Chaubal *et al*. (2006) reported that number of follicles per session in once a week (7.8±2.4) were higher than the twice a week (6.5±2.4).

Li *et al.* (2007) observed that mean number of aspirated follicles per session in once a week (9.1±1.7) were lower than twice a week (12.1±2.6) and reported that the difference may be due beginning of follicular wave on day 0 and at 3rd day there is follicular differentiation and subordinate follicles undergo

atresia by day 4 of wave emergence. Many follicles in follicle pool at day 7 after wave emergence would be in advance stage of atresia; therefore, higher degree of follicles would be already atretic during once weekly protocol. (Singh and Adams, 1998)

In present study, number of follicles per animal was higher in once a week (12.66) than twice a week (9.22) follicular aspration. However, Imai *et al*. (2006) reported that number of follicles per animal was higher in twice a week (33.4±12.0) than once a week (26.9±7.8). It may be due to performing the aspiration session twice a week has greater chances to aspirate many ovarian follicles.

The total recovery rate and oocyte per session was higher in twice a week (71.08 %; 2.26±0.50) than once a week (48.24 %; 1.64±0.28) follicular aspiration. In other parameters there is no significant difference observed between once and twice a week aspiration session, which may be due to unequal aspiration session were studied in both the group in present study.

Garcia *et al*. (1998) reported that oocytes recovered per session were higher in twice a week (7.7 ± 4.5) than once a week (5.4 ± 3.7) follicular aspiration which is similar to findings of present study. However, Gibbons *et al*. (1994) and Imai *et al*. (2006) observed that there was no significant difference in oocytes recovery per session in once a week and twice a week follicular aspiration.

Viana *et al*. (2004) and Chaubal *et al*, (2006) reported that oocytes recovery per session were higher in once a week (8.9 ± 0.8; 4.6 ± 1.9) than twice a week (7.0 ± 0.7; 3.9 ± 2.1) respectively. Garcia *et al*. (1998) reported that if the follicle aspiration was done then it induces and synchronizes a new follicular wave and when it is done in twice a week it will yields higher number of follicles and oocytes recovery than once a week.

3) Developmental competence of morphologically classified oocytes: -

In this experiment, the developmental competence of morphologically classified oocytes were studied at three different vacuum pressures i.e. 80 mmHg, 90 mmHg and 100 mmHg vacuum pressure in 60 aspiration sessions.

Table 4.7 Developmental competence of morphologically classified oocytes with different vacuum pressures

Vacuum pressure (mm Hg)	Number of sessions	Total number of recovered oocytes	Total number of cleaved oocytes	Grading of oocytes		
				A quality	B quality	C quality
80	18	34 (1.88±0.31)	11 (0.61±0.14)	5 (0.27±0.10)	3 (0.16±0.90)	3 (0.16±0.09)
90	16	23 (1.4±0.44)	4 (0.25±0.11)	2 (0.12±0.08)	1 (0.06±0.06)	1 (0.06±0.06)
100	26	57 (2.19±0.52)	5 (0.19±0.07)	2 (0.07±0.05)	0 (0)	3 (0.11±0.06)
Total	**60**	**114**	**20**	**9**	**4**	**7**

By using 80 mmHg negative vacuum pressure, total 18 sessions were performed in which 34 (1.88±0.31) number of oocytes were recovered. Out of total number of oocytes recovered, 11 (0.61±0.14) oocytes were cleaved. From

total cleaved oocytes, number of A, B and C quality cleaved oocytes were 5 (0.27±0.10), 3 (0.16±0.90) and 3 (0.16±0.09) respectively.

By using 90 mmHg negative vacuum pressure, total 16 sessions were performed in which 23 (1.4±0.44) oocytes were recovered. Out of total oocytes recovered, 4 (0.25±0.11) oocytes were cleaved. From total cleaved oocytes, number of A, B and C quality cleaved oocytes were 2 (0.12±0.08), 1 (0.06±0.06) and 1 (0.06±0.06) respectively.

By using 100 mmHg negative vacuum pressure, total 26 sessions were performed in which 57 (2.19±0.52) oocytes were recovered. Out of total number of oocytes recovered, 5 (0.19±0.07) oocytes were cleaved. From total cleaved oocytes, number of A, B and C quality cleaved oocytes were 2 (0.07±0.05), 0 (0) and 3 (0.11±0.06) observed respectively.

In present study out of all three different vacuum pressure, number of cleaved oocytes 11 (0.61±0.14) as well as number of A quality 5 (0.27±0.10) cleaved oocytes were higher at 80 mmHg negative vacuum pressure than 90 mmHg and 100 mmHg negative vacuum pressure.

Out of total cleaved oocytes, A quality oocytes were higher than B and C quality oocytes. As the vacuum pressure increased, the total number of oocytes recovered increased but the quality of oocytes i.e. developmentally competent oocytes decreased. The quality of oocyte decreased due to loss of cumulus cells from oocytes at higher vacuum pressure.

Table 4.8 Developmental competence of morphologically classified oocytes

Grade of oocyte	**Total number of recovered oocytes**	**Total number of matured oocytes**	**Total number of fertilized oocytes (%)**	**Total number of cleaved oocytes**	**Cleavage rate (%)**	**2-4 cell (%)**	**4-8 cell (%)**	**8-16 cell (%)**	**16-32 cell (%)**
A	38	38	31	9	29.03	2	0	4	3

B	24	24	19	4	21.05	1	1	2	0
C	52	52	39	7	17.94	4	2	1	0
Total	**114**	**114**	**89 (78.07)**	**20**	**22.47**	**7 (35)**	**3 (15)**	**7 (35)**	**3 (15)**

Out of 114 recovered oocytes, 38 A quality oocytes were matured and fertilized out of which 31 oocytes were in good condition. From fertilized oocytes, the number of oocytes that cleaved upto 2-4 cells stage, 4-8 cells stage, 8-16 cells stage and 16-32 cells stage were 2, 0, 4 and 3, respectively.

Out of 114 recovered oocytes, 24 B quality oocytes were matured and fertilized out of which 19 oocytes were in good condition. From fertilized oocytes, the number of oocytes that cleaved upto 2-4 cells stage, 4-8 cells stage, 8-16 cells stage and 16-32 cells stage were 1, 1, 2 and 0, respectively.

Out of 114 recovered oocytes, 52 C quality oocytes were matured and fertilized out of which 39 oocytes were in good condition. From fertilized oocytes the number of oocytes that cleaved upto 2-4 cells stage, 4-8 cells stage, 8-16 cells stage and 16-32 cells stage were 4, 2, 1 and 0, respectively.

In this experiment, that the developmental competence of morphologically classified oocytes was studied on the basis of layers of cumulus cells. Out of total number of oocytes, 114 oocytes were recovered, 114 oocytes were matured and 89 oocytes were fertilized. Out of total number of fertilized oocytes, 20 (22.47%) oocytes that cleaved upto 2-4 cell stage, 4-8 cells stage, 8-16 cell stage and 16-32 cells stage. The number of oocytes that cleaved upto, 2-4 cell stage, 4-8 cells stage, 8-16 cell stage and 16-32 cells stage were 7(35%), 3(15%), 7 (35%) and 3(15%), respectively.

Out of total number of oocytes, A quality oocytes cleaved at higher level than B and C quality. It may be due to greater number of cumulus cells surrounding the zona pellucida in A quality of oocytes than B and C quality, the

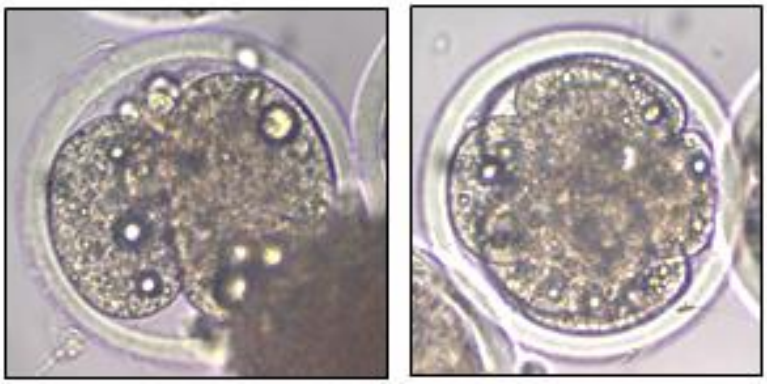
number of cumulus cells layer is important for development of oocytes. (Lonergan *et al*. 1992)

In present study, overall cleavage rate observed was 22.47%. However, Manik *et al*. (2003); Bols *et al*. (1995) and Sakhong *et al*. (2012) reported overall cleavage rate as 33%, 69% and 38.55% respectively, which was higher than present study.

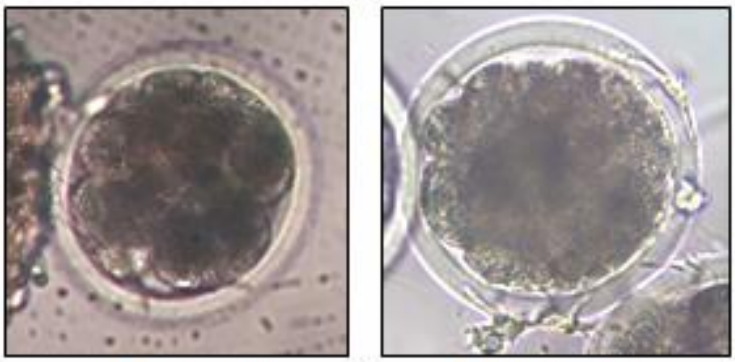
In present study cleavage rate was lower than the other studies, which may be due to, aspiration of higher number of small size follicles than medium and large size follicles. The developmental competence of oocytes from small follicles, being used in present work is lower than oocyte aspirated from large follicles. So due to a greater number of small follicles, the cleavage rate was low as compared to other studies. Bos indicus cattle have more follicular waves and higher proportion of small follicles than Bos Taurus. (Figueiredo *et al*. 1997; Viana *et al.* 2000 and Segerson *et al*.1984). These oocytes might have been recovered at too early stage before acquiring complete development competence, therefore, they might lack of some follicular factors which are required for development at competence of oocytes (Lonergan *et al*.1994 and Blondin and Sirard, 1995)

Present research work was done in summer months at high environmental temperature up to 44 - 45 °C (Table number. 3.1)**,** the cleavage rate was low which may be due to heat stress. The environmental temperature is very much important for follicular formation, oocyte quality and its developmental competence. Heat stress suppresses follicular dominance and changes in follicular growth, during summer at high temperature the oocyte development was ceased (Wolfenson *et al*. 2000 and Roth *et al*. 2002).

Rust *et al*. (2009) also reported that the seasonal effect on in vitro embryo production is very important and its effect on efficiency of in vitro embryo production. In summer months, the number of follicles and size of follicles were lower than cooler months. Also, lower number of oocytes were recovered during hotter months than cooler months. The recovery rate of of A quality oocytes was higher during cooler months and lower in hotter months.



Two cell embryo | Four cell embryo



Eight cell embryo | Sixteen cell embryo

Plate 6 : Different stages of *in vitro* developed cattle embryos

CHAPTER V

SUMMARY AND CONCLUSIONS

The present study on ‘’Effect of different vacuum pressures on oocyte recovery and in vitro maturation of oocytes collected by ultrasound guided trans vaginal follicular aspiration in cattle.” was conducted at Department of Animal Reproduction Gynaecology and Obstetrics, Post Graduate Institute of Veterinary and Animal Sciences, Akola.

For present research work total 9 animals were selected of 2 different breeds (Red Kandhari cows n=2 and Sahiwal cows n=7). From all the selected animals, oocytes were aspirated by using ultrasound guided transvaginal follicular aspiration at three different negative vacuum pressure i.e. 80 mmHg, 90 mmHg and 100 mmHg vacuum pressure. All aspirated oocytes were graded in three different categories on the basis of cumulus cells surrounding by oocyte i.e. A grade oocyte (4-5 layers of cumulus cells); B grade oocyte (2-4 layers of cumulus cells); C grade oocyte (partially denuded and shrunken cytoplasm). The oocytes were matured, fertilized and cultured in vitro and embryonic development was studied.

5.1 Oocyte recovery: -

5.1.1 Effect of different vacuum pressure on oocyte recovery and quality of oocytes

a) Effect of different vacuum pressure on different size of follicles, quality of oocytes and its recovery rate.

By using 80 mmHg vacuum pressure, total 9 animals were aspirated in 18 sessions, in which total 64 follicles were aspirated. The mean number of small, medium and large size follicles were recorded as 51 (2.83±0.37), 11(0.66±0.16) and 2(0.11±0.07), respectively. Total 34 oocytes were collected, with mean number of A, B and C quality oocytes were recorded as 6(0.33±0.11),

8(0.44±0.16) and 20(1.11±0.24), respectively. The average oocytes recovery by using 80 mmHg vacuum pressure was 53.11%.

By using 90 mmHg vacuum pressure, total 5 animals were aspirated in 16 sessions, in which total 39 follicles were aspirated with mean number of small, medium and large size follicles as 22(1.37±0.31), 12(0.75±0.14) and 5(0.31±0.1), respectively. Total 23 oocytes were collected, with mean number of A, B and C quality oocytes recorded as 13(0.81±0.27), 6(0.37±0.17) and 4(0.25±0.11), respectively. The average oocytes recovery by using 90 mmHg was 58.97 %.

By using 100 mmHg, total 9 animals were aspirated in 26 sessions, in which total 94 follicles were aspirated with mean number of small, medium and large size follicles recorded as 63(2.42±0.27), 21(0.88±0.14) and 10(0.38±0.11), respectively. Total 57 oocytes were collected, with mean number of A, B and C quality oocytes recorded as 19(0.73±0.20), 10(0.38±0.11) and 28(1.07±0.29), respectively. The average oocytes recovery by using 100 mmHg was 60.63%.

The results revealed that by using 100 mmHg vacuum pressure, the total oocytes recovery rate was 60.63%, which was comparatively better than 80 mmHg (53.12%) and 90 mm Hg (58.97%) vacuum pressure. However, there was no significant difference among 80 mmHg, 90mmHg and 100 mm Hg vacuum pressure for ovum pick up.

In this experiment, total 9 animals were aspirated in 60 aspiration sessions in which total 197 (21.89±1.83) follicles were available for aspiration. Total mean number of oocytes recovered were 114(12.67±1.19) with the recovery rate of 57.86%. In present study the mean number of A quality (4.23±0.66) and C quality (5.78±0.64) oocytes were significantly higher than B quality (2.67±0.44) oocytes ($P<0.05$).

5.1.2 Breed wise (Red Kandhari and Sahiwal) recovery rate and quality of oocytes with different vacuum pressure

In this experiment, breed wise (Red Kandhari and Sahiwal) recovery rate and quality of oocytes were studied at different vacuum pressures(80, 90 and 100 mmHg). Total 60 aspiration sessions (20 in Red Kandhari and 40 in Sahiwal) were performed with three different vacuum pressures.

In Red Kandhari cattle, from 6 aspiration sessions 13(2.16±0.65) follicles were aspirated by using 80 mmHg vacuum pressure in which total 5(0.83±0.30) oocytes were recovered with the total recovery rate of 38.46%. The mean number of A, B and C quality oocytes were recorded as 0(0), 0(0) and 5(0.83±0.30), respectively.

In total 8 aspiration sessions, 17(2.12±0.35) follicles were aspirated by using 90 mmHg vacuum pressure in which total 13(1.62±0.73) oocytes were recovered with the total recovery rate as 76.47%.The mean number of A, B and C quality oocytes were recorded as 7(0.87±0.47), 4(0.5±0.32) and 2(0.25±0.16), respectively.

In total 6 aspiration sessions, 17(2.83±0.60) follicles were aspirated by using 100 mmHg vacuum pressure, in which total 10(1.66±0.61) oocytes were recovered with the total recovery rate of 58.82%. The mean number of A, B and C quality oocytes were recorded as 3(0.5±0.22), 2(0.33±0.21) and 5(0.83±0.30), respectively.

In Red Kandhari cows, out of all three vacuum pressures by using 90 mmHg vacuum pressure the total recovery rate (76.47%) was higher than 80 mmHg (38.46%) and 100 mmHg vacuum pressure (58.82%).

In Sahiwal cattle, from total 12 aspiration sessions 51(4.25±0.35) follicles were aspirated by using 80 mmHg vacuum pressure, in which total 29(2.41±0.35) oocytes were recovered with the total recovery rate of 56.86%.The mean number of A, B and C quality oocytes were recorded as 6(0.05±0.15), 8(0.66±0.22) and 15(1.25±0.32) respectively.

In total 8 aspiration sessions, (2.75±0.52) follicles were aspirted by using 90 mmHg vacuum pressure in which total 10(1.5±0.53) oocytes were recovered with the total recovery rate as 45.45%. The mean number of A, B and C quality oocytes were recorded as 6(0.75±0.31), 2(0.25±0.16) and 2(0.25±0.6), respectively.

In total 20 aspiration sessions, 77(3.85±0.31) follicles were aspirated by using 100 mmHg vacuum pressure in which total 47(2.4±0.65) oocytes were recovered with the total recovery rate as 61.03%. The mean number of A, B and

C quality oocytes were recorded as 16(0.80±0.25), 8(0.40±0.13) and 23(1.10±0.36), respectively.

In Sahiwal cattle out of all three vacuum pressures, by using 100mmHg pressure the total recovery rate (61.03%) was higher than 80 mmHg (56.86%) and 90 mmHg (45.45%).Overall recovery rate in Sahiwal cattle at three different pressure was 57.33%.

Overall recovery rate in Red Kandhari and sahiwal cows at three different vacuum pressure was almost similar (59.57% vs 57.33%) difference in overall recovery rate of these between two breeds of cows.

5.1.3. Effect of frequency (i.e. once or twice week) of aspiration on OPU-

a) Once a week aspiration-

Total 9 animals were aspirated in 34 sessions, in which total 114 follicles (12.66 follicles/animal and 3.35±0.27 follicles/session) were aspirated. The mean number of small, medium and large size follicles were recorded as 83(2.44±0.26), 23(0.67±0.12) and 8(0.23±0.07), respectively. Total 55(1.67±0.28) oocytes (6.11±1.01 oocytes/animal and 1.64±0.28 oocytes/session) were collected, in which the number of A, B and C quality oocytes were recorded as 19, 13 and 23, respectively. The mean number of A, B and C quality oocytes per sessions were recorded 0.55±0.13, 0.38±0.11 and 0.69±0.18, respectively. The average oocytes recovery by once a week aspiration was 48.24%.

b) Twice weekly aspiration-

Total 9 animals were aspirated in 26 sessions, in which total 83 follicles (9.22 follicles/animal and 3.19±0.29 follicles/session) were aspirated. The mean number of small, medium and large follicles were observed as 56(2.15±0.32), 19(0.69±0.14) and 8(0.30±0.09), respectively. Total 59 oocytes (6.55±0.89 oocytes/animal and 2.26±0.50 oocytes/session) were collected, in which the number of A, B and C quality oocytes were recorded as 19, 13 and 27, respectively. The mean number of A, B and C, quality oocytes per session were recorded as 0.73±0.21, 0.52±0.14 and 1.08±0.26, respectively. The average oocytes recovery by twice-weekly aspiration was 71.08%.

The results revealed that by using once and twice a week aspiration session 3.35±0.27 and 3.19±0.29 follicles/session were aspirated. In once a week aspiration session average 1.64±0.28 oocytes/session and in twice a week average 2.26±0.50 oocytes/session were collected, so the total oocytes recovery rate was 71.08% in twice a week, which was comparatively better than once a week aspiration technique, which had 48.24% oocytes recovery rate. However, there was no significant difference in recovery rate between once and a twice week aspiration.

5.2. Developmental competence of oocytes :

5.2.1. Developmental competence of morphologically classified oocytes with different vacuum pressures

The developmental competence of morphologically classified oocytes was investigated at three different vacuum pressures i.e. 80 mmHg, 90 mmHg and 100 mmHg vacuum pressure in 60 aspiration sessions.

In present study out of all three different vacuum pressure, number of cleaved oocytes were higher at 80 mmHg 11(0.61±0.14) vacuum pressure than 90 mmHg 4(0.25±0.11) and 100 mmHg 5 (0.19±0.07) vacuum pressure.

Total 114 oocytes were recovered which were matured and 89 oocytes were fertilized. Out of total fertilized oocytes, A, B and C quality cleaved oocytes were recorded as 9 (29.3%), 4 (21.05%) and 7 (17.94%), respectively and total number of cleaved oocytes were 20 (22.47%). Out of total cleaved oocytes, 2-4 cells stage, 4-8 cells stage, 8-16 cells stage and 16-32 cells stage oocytes were recorded as 7(35%), 3(15%), 7 (35%) and 3(15%), respectively.

Out of 114 recovered oocytes, number of A, B and C quality oocytes were recorded as 38, 24 and 52 respectively. Oocytes in good condition (n=3) were fertilized. From which number of cleaved oocytes developing upto 2-4 cells stage, 4-8 cells stage, 8-16 cells stage and 16-32 cells stage were recorded as 2, 0, 4 and 3, respectively, with overall cleavage rate of 29.03% (9/31)

Out of recovered 24 oocytes of B quality, 19 were fertilized which were in good condition. The number of cleaved oocytes developing upto 2-4 cells stage, 4-8 cells stage, 8-16 cells stage and 16-32 cells stage were recorded as 1, 1, 2 and 0, respectively, with overall cleavage rate of 21.05% (4/19)

Out of recovered 52oocytes of C quality 39 were fertilized which were in good condition. The number of cleaved oocyte developing upto 2-4 cells stage, 4-8 cells stage, 8-16 cells stage and 16-32 cells stage were recorded 4, 2, 1 and 0, respectively, with overall recovery rate of 17.94% (7/52).Out of total number of oocytes, A quality oocytes cleaved at higher rate than B and C quality oocytes. In present study, overall cleavage rate was 22.47%

CONCLUSIONS

1. Total recovery rate of oocytes was higher at 100 mmHg vacuum pressure than 80 mmHg and 90 mmHg vacuum pressure.
2. In breed wise study, total oocyte recovery rate was nearly similar in Red Kandhari and Sahiwal cows.
3. Total number of oocytes, recovery rate of oocytes, mean number of A quality oocytes and oocytes per session were higher in twice a week than once a week aspiration.
4. In morphologically classified oocytes cleavage rate was higher in A grade oocyte than B and C grade oocytes.

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THESIS ABSTRACT

a)	**Title of the thesis (in Capital letters)**	**EFFFCT OF DIFFERENT VACUUM PRESSURES ON OOCYTE RECOVERY AND IN VITRO MATURATION OF OOCYTES COLLECTED BY ULTRASOUND GUIDED TRANS VAGINAL FOLLICULAR ASPIRATION IN CATTLE.”**
b)	**Full name of student**	**Harkal Satish Bhaousaheb**
c)	**Name and address of Major advisor**	**Dr. C.H. Pawshe** Associate Professor and Head Department of Animal Reproduction, Gynaecology and Obstetrics PGIVAS, Akola
d)	**Degree to be awarded**	M.V.Sc. (Animal Reproduction)
e)	**Year of award of degree**	2019
f)	**Major subject**	Animal Reproduction, Gynaecology and Obstetrics
g)	**Total number of pages In the tthesis**	53
f)	**Number of words in the Abstract**	353
g)	**Signature of Student**	
h)	**Signature, Name and address of forwarding authority**	

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ABSTRACT

The present experiment entitled “effect of different vacuum pressures on oocyte recovery and in vitro maturation of oocytes collected by ultrasound guided trans vaginal follicular aspiration in cattle.” The main objective of present study was to standardized a method of transvaginal oocyte retrieval by using ultrasound machine.In this study, oocytes were recovered by ultrasound guided transvaginal follicular aspiration from live indigenouscattle’s at three different pressures (80mmHg, 90 mmHg and 100 mmHg). Total recovered oocytes were graded in three different categories on the basis of layers of cumulus cells present over zona pellucida i.e. A quality oocyte (4 to 5 layers of cumulus); B quality oocytes (2 to 4 layers of cumulus); C quality oocyte (partially denuded and shrunken cytoplasm). All graded oocytes were in vitro matured, fertilized and in vitro cultured and development of embryo was observed every 48 hours.

To study the effect of vacuum pressure on average oocyte recovery, three different (80, 90 and 100 mmHg) vacuum pressure was used. At three different vacuum pressure total 114 oocytes recovered from 197 follicles with the overall recovery rate was 57.86%. It was observed that, by using 100 mmHg vacuum pressure, 2.19 ± 0.52 oocytes per session were collected which was comparatively higher than 80 mmHg (1.88±0.31) and 90 mmHg (1.43 ±0.44) vacuum pressure. The average oocyte recovery was 60.63% by using 100 mmHg vacuum pressures, however there was no significant difference between 80, 90 and 100 mmHg pressure.

Out of total number of oocytes, 114 oocytes were recovered, 114 oocytes were matured and 89 oocytes were fertilized. Out of total number of fertilized oocytes, A quality cleaved oocytes 9 (29.3%), B quality cleaved oocytes 4 (21.05%), C quality cleaved oocytes 7 (17.94%) and total number of cleaved oocytes 20 (22.47%) were observed. Out of total cleaved oocytes,2-4 cells stage, 4-8 cells stage, 8-16 cells stage and 16-32 cells stage oocytes 7(35%), 3(15%), 7 (35%) and 3(15%) were observed, respectively.

It is concluded that, total recovery rate of oocytes was higher at 100 mmHg vacuum pressure.In morphologically classified oocytes cleavage rate was higher in A grade oocyte than B grade and C grade oocytes.

<u>प्रबंध सारांश</u>

१.	**प्रबंधाचे शिर्षक**	: **:"गाइंमध्ये अल्ट्रासाऊंड पध्दतीने योनीद्वारे स्त्रीबीजकोषांमधून अस्पिरेशन पध्दतीने स्त्रीबीजांच्या पुनर्प्राप्तीवर आणि त्यांच्या परिपक्वतेवर विविध ऋण दाबांमुळे होणारा परिणाम."**
२.	**विद्यार्थ्याचे पूर्ण नांव**	: **हारकळ सतिश भाऊसाहेब**
३.	**मुख्य मार्गदर्शकाचे नांव व पत्ता**	: **डॉ . चैतन्य एच. पावशे** सहयोगी प्राध्यापक व विभागप्रमुख, पशुजननशास्त्र विभाग,स्नातकोत्तर पशुवैद्यक व पशुविज्ञान संस्था, अकोला
४.	**प्रदान केली जाणारी पदवी**	: एम.व्ही.एसस्सी.
५.	**पदवी प्रदान करण्याचे वर्ष**	: २०१९
६.	**मुख्य विषय**	: पशुजननशास्त्र
७.	**प्रबंधामधील एकुण पाने**	: 53
८.	**प्रबंध सारांशामधील एकुण शब्द**	: 336
९.	**विद्यार्थ्याची सही**	:
१०.	**प्रबंधक कार्यवाहीस्तव पाठविणाऱ्या अधिकाऱ्याची सही, नाव व पत्ता**	:

(चैतन्य एच. पावशे)

प्राध्यापक व विभागप्रमुख,

पशुजननशास्त्र विभाग,स्नातकोत्तर

पशुवैद्यक व पशुविज्ञान संस्था, अकोला

सारांश

सदर प्रयोगात गाइंमध्ये अल्ट्रासाऊंड पध्दतीने योनीद्वारे स्त्रीबीजकोषांमधून अस्पिरेशन पध्दतीने स्त्रीबीजांच्या पुनर्प्राप्तीवर आणि त्यांच्या परिपक्वतेवर विविध ऋण दाबांमुळे होणाऱ्या परिणामाचा अभ्यास केला गेला आहे. सध्याच्या अभ्यासाचा मुख्य उद्देश अल्ट्रासाऊंड मशीनचा वापर करून योनीद्वारे स्त्रीबीजांच्या पुनर्प्राप्तीची एक पद्धत प्रमाणित करणे होते. या अभ्यासामध्ये तीन वेगवेगळ्या ऋणदाबावर (८०मिमीएचजी, ९०मिमीएचजी आणि १०० मिमीएचजी) देशी गाइंमधून अल्ट्रासाऊंड मशीनच्या मदतीने योनीद्वारे स्त्रीबीजकोषामधून अस्पिरेशन पध्दतीने स्त्रीबीजांची पुनर्प्राप्ती करण्यात आली. झोना पेलूसीडावर अस्तित्वात असलेल्या क्युम्युलस पेशींच्या थरांच्या आधारे एकूण पुनर्प्राप्त स्त्रीबीजांचे तीन वेगवेगळ्या प्रकारात वर्गीकरण केले गेले. अर्थात अ गुणवत्ता स्त्रीबीज (४ ते ५ थर क्युम्युलस पेशी), ब गुणवत्ता स्त्रीबीज (२ ते ४ थर क्युमुलस पेशी), क गुणवत्ता स्त्रीबीज (एकही क्युम्युलस पेशींचा थर नाही आणि संकुचित पेशी द्रव्य). सर्वश्रेणी बद्ध स्त्रीबीजांची शरीरबाह्य परिपक्वता, फलित आणि संवर्धनासाठी ठेवल्या आणि गर्भाचा विकास दर दर ४८ तासांनी तपासण्यात आला.

सरासरी स्त्रीबीजांच्या पुनर्प्राप्तीवरील ऋण दाबाचा अभ्यास करण्यासाठी तीन विविध ऋण दाबाचा वापर केला गेला. तीन वेगवेगळ्या ऋण दाबावर १९७ स्त्रीबीजकोषयांमधून ११४ स्त्रीबीजे संकलित करण्यात आली आणि त्याला एकूण पुनर्प्राप्तीदर हा ५७.८६ टक्के इतका होता. असे आढळून आले कि १०० मिमीएचजी ऋण दाबाचा वापर करून प्रत्येक सत्रामध्ये २.१९+-०.५२ स्त्रीबीजे एकत्रित केले गेले जे तुलनात्मकपणे ८० मिमीएचजी (१.८८+-०.३१) आणि ९० मिमीएचजी (१.४३ +- ०.४४) ऋणदाबापेक्षा जास्त होता. १०० मिमीएचजी ऋण दाबाचा वापर करून स्त्रीबीजांची पुनर्प्राप्तीची सरासरी ६०. ६३ टक्के होती, तथापि ८०, ९० आणि १०० मिमीएचजी ऋण दाबामध्ये कोणताही फरक नव्हता.

एकून स्त्रीबीजकोषांमधून ११४ स्त्रीबीजे काढली गेली, त्यापैकी ११४ स्त्रीबीजे परिपक्व झाले आणि ८९ स्त्रीबीजे फलित केली गेली. एकूण फलित स्त्रीबीजांपैकी अ गुणवत्ता क्लीव्हड स्त्रीबीज ९ (२९. ३० टक्के), ब गुणवत्ता क्लीव्हड स्त्रीबीज ४ (२१.०५ टक्के), क गुणवत्ता क्लीव्हड स्त्रीबीज ७ (१७.१९टक्के) आणि एकूण क्लीव्हड स्त्रीबीजे २० (२०.४७ टक्के) आढळली. एकूण क्लीव्हड स्त्रीबीजांपैकी २ ते ४ पेशीस्तर ,४ ते ८ पेशीस्तर, ८ ते १६ पेशीस्तर आणि १६ ते ३२ पेशी स्तर स्त्रीबीजे ७ (३५ टक्के), ३ (१५ टक्के), ७ (३५ टक्के) आणि ३ (१५ टक्के) अनुक्रमे आढळले.

वर्तमान प्रबंधावरून असा निष्कर्ष काढला आहे कि, स्त्रीबीजांचा एकूण पुनर्प्राप्तीदर १०० मिमीएचजी ऋण दाबावर जास्त होता. बाह्यस्वरुपावरून वर्गीकरण केलेल्या क्लीव्हड अ गुणवत्ता स्त्रीबीजांचा दर हा ब गुणवत्ता व क गुणवत्ता स्त्रीबीजांपेक्षा जास्त होता.