Sudan University of Science & Technology

College of Graduate Studies

Evaluation of Ovarian Potential for In vitro Embryo Production in Indian Buffalo at Haryana State, India

تقويم كفاءة المبايض لإنتاج جنين الأنابيب في الجاموس الهندي بمقاطعة هريانا الهندية

A Thesis submitted in Fulfillment of the Requirement for PhD degree in Veterinary Medicine (Theriogenology)

By

Eias Elzein Ibrahim Osman

B.V.Sc - University of Nyala (2009)

M.V.Sc – Sudan University of Science & Technology (2014)

Supervisor

Dr. Rakesh Kumar Sharma

Principal Scientist – Division of Physiology & Reproduction

Central Institute for Research on Buffaloes, India

Co. Supervisor

Dr. Majdi Elnaim Badawi

Associate Professor – Department of Animal Medicine & Surgery, College of Veterinary Medicine, Sudan University of Science & Technology, Sudan

July 2020

DEDICATION

I dedicate this study to my Supervisor, Co-Supervisor, Family, Wife, Sons and all Veterinarians in Sudan.

ACKNOWLEDGMENT

My first and last thank to Allah who gave me the ability to accomplish this study. My deep thanks are to my Supervisor Dr. Sharma, Co-supervisor Dr. Majdi, CV Raman, University of Nyala and CIRB for helping me to accomplish this study. My great thanks are to my Family, Wife, Sons, Izdeen, Logman, Fathirahman and Mohammed Arab for help.

Table of contents

List of Tables

List of figures

List of Abbreviations

Abbreviations	Stand for
AI	Artificial Insemination
ET	Embryo Transfer
IVEP	*In vitro* Embryo Production
IVM	*In vitro* Maturation
IVF	*In vitro* Fertilization
IVC	*In vitro* Culture
BCS	body condition score
%	Percentage
Kg	Kilogram
cm	Centimeter
LH	Luteinizing Hormone
CL	Corpus Luteum
WM	washing medium
$^{\circ}$C	Degree Centigrade
ORR	Oocyte recovery rate
No	Number
FSH	Follicle Stimulating Hormone
SOF	Synthetic oviduct fluid
FCS	Fetal calf serum
ECS	Estrous Cow Serum
NBCS	New born Calf Serum
SCS	Superovulated Calf Serum
ACS	An estrous Cow Serum

BSA	Bovine Serum Albumin
hCG	human Chorionic Gonadotropin
eCG	Equine Chorionic Gonadotropin
EGF	Epidermal Growth Factor
FGF	Fibroblast Growth Factor
IGF	Insulin like Growth Factor
ITS	Insulin, Transferrin Sodium selenite
TCM	Tissue Culture Media
mM	Milli mol
buFF	buffalo Follicular Fluid
µg	microgram
ml	Millilitre
CO_2	Carbon dioxide
BO	Bracket Oliphant
TALP	Tyrode's Albumin Lactate Pyruvate
mRNA	Messenger Ribonucleic Acid
CRaa	Charles Rosenkrans amino acid
CZB	Chatot Ziomek Bavister
HECM	Hamster Embryo Culture Medium
OPU	Ovum Pick Up
pH	potential of Hydrogen
sec	second
MHz	megahertz
N	North
E	East
m	Metre

Fig	Figure
µL	Micro litter
h	Hours
RVCL	Research Vitro Cleave Medium
CR	Cleavage rate
CIRB	Central Institute for Research on Buffaloes
SPSS	Statistical Package for Social Sciences
SE	Stander Error
g	Gram
PBS	Phosphate Buffer Saline

List of Appendices

List of publications

No	Publications
1.	*In vitro* Embryo Production in Indian Buffalo.
2.	Determine the effect of ovarian and non ovarian factors on follicular population in Murrah buffalo using Ultrasoundgraphy technique.
3.	Influence of different stages of corpus luteum on ovary size, oocytes grades and follicular population in Indian buffaloes.
4.	Evaluation of ovarian potential for *In vitro* embryo production in Indian buffaloes.

ABSTRACT

The present study was conducted at the Central Institute for Research on Buffaloes, Haryana, India to evaluate the ovarian potential for *in vitro* embryo production in Indian buffaloes. This study comprised two parts. In the first part, 296 samples of buffalo ovaries were used to determine the ovaries weights (using electronic scale balance), lengths, widths, thicknesses, follicular population (using electronic Vernier calipers), oocytes grades, oocytes recovery rate, oocytes index, cleavage rate and blastocyst rate. The samples were collected from Delhi slaughterhouse and classified into two groups; group with a corpus luteum (CL) and a group without CL. The group of ovaries having CL was classified into three groups: ovaries having CL in early stage, middle stage and late stage. There was significant difference ($P < 0.05$) in CL stages groups with ovary weight and size. The ovaries having CL in late stage showed the highest mean of ovary weight (5.03±0.17 g), length (2.35±0.05 cm), width (1.74±0.05 cm) and thickness (1.48±0.03 cm) as compared to that having CL in early (3.38±0.24 g, 2.09±0.09 cm, 1.41±0.06 cm and 1.20±0.02 cm) and middle stage (4.03±0.24 g, 2.14±0.09 cm, 1.54±0.06 cm and 1.47±0.04 cm). No significant difference was observed on follicular population between ovaries bearing CL and without CL. The number of small, large follicles and average number of follicles per ovary were found significantly higher in

ovaries weighting more than 5g (1.76 ± 0.37 , 0.41±0.51 and 2.59±0.48) as compared to less than 3g (1.01±0.11 , 0.22±0.04and 1.80±0.10) and 3 to 5g (1.32±0.12 , 0.36±0.05 and 2.22±0.14). The number of small follicles in the large size ovaries was significantly higher (1.43±0.12) as compared to small size ovaries (0.90±0.10). No significant difference was observed between number of medium, large follicles and ovary size. There were no significant differences in oocytes recovery per ovary, oocytes index, cleavage, blastocyst rates between the ovaries with and without CL. Also, presence or absence of CL and its stages were not affected significantly on oocytes grades. In the second part, 160 female Murrah buffaloes were used and divided into two groups (pregnant80 and non pregnant 80) to monitor the effect of body condition score (BCS), age, pregnancy, present or absent of CL and ovary localization on follicular population using ultrasonography technique. Animals with BCS 4 having a higher total number of follicles per ovary (9.85±0.74) than those with BCS 3 (6.78±0.69). There was no significant difference in a number of small and large follicles between animals with BCS 3 and BCS 4. The pregnant buffaloes have more medium size follicles (1.96 ± 0.17) than the non pregnant ones (1.48± 0.19). There was no significant difference between the number of small follicles, large follicles and total number of follicles in pregnant and non pregnant animals. The right ovaries have lower number of large follicles (0.81±0.08) as compared to left ovaries (0.91±0.09). Also, the

ovaries contained CL have significantly higher number of medium follicles (1.95±0.17) than the without CL (1.39±0.18).No significant difference on follicular population between different ages of buffaloes. In conclusion, this study showed that the ovarian and non ovarian factors influenced on follicular population in Murrah buffaloes. The ovaries of buffaloes have a good potential for *In vitro* embryo production.

المستخلص

أجريت الدراسة الحالية في المعهد المركزي لإبحاث الجاموس بولاية هريانا الهندية لتقييم كفاءة المبايض لأنتاج جنين الأنابيب في الجواميس الهندية. تألفت هذه الدراسة من جزئين. في الجزء الاول أستخدمت 296 عينة مبايض من الجواميس الهندية لتحديد أوزان المبايض (بأستخدام الميزان الألكتروني) وطولها وعرضها وسمكها وتعداد الجريبات فيها (بأستخدام مقياس فيرنر الألكتروني) وتصنيف الخلايا البيضية ومعدل أستردادها ومؤشرها ومعدل التشطر و معدل كيسة الاريمة. العينات جمعت من مسلخ دلهي و قسمت الي مجموعتين: مجموعة تحتوي علي جسم أصفر و مجموعة لاتحتوي علي جسم أصفر. مجموعة المبايض التي تحتوي علي جسم أصفر صنفت الي ثلاثة مجموعات: مبايض تحتوي علي جسم أصفر في المرحلة المبكرة و المرحلة الوسطي و المرحلة المتاخرة. هنالك أختلاف معنوي ($P < 0.05$) في المجموعات التي تحتوي علي مراحل جسم أصفر مع وزن و حجم المبيض. المبايض التي تحتوي علي جسم أصفر في المرحلة المتأخرة أظهرت أعلي متوسط في وزن (0.17 ±5.03 جرام) وطول (0.05± 2.35 سم) و عرض (0.05 ± 1.74 سم) و سمك المبيض (0.03 ± 1.48 سم) عند مقارنتها بتلك التي تحتوي علي جسم أصفر في المرحلة المبكرة (الوزن 0.24 ± 3.38 جرام، الطول 0.09 ± 2.09 سنتمتر، العرض 0.06±1.41 سم والسمك 0.02± 1.20 سم) و الوسطي (الوزن 0.24±4.03 جرام ، الطول 0.09±2.14 سم ، العرض 0.06 ± 1.54 سم والسمك 0.04±1.47 سم). لا يوجد أختلاف معنوي في تعداد الجريبات بين المبايض التي تحتوي علي جسم أصفر او بدون جسم أصفر. عدد الجريبات الصغيرة والكبيرة و متوسط الجريبات في المبيض وجدت اعلي معنويا ($P < 0.05$) في المبايض التي تزن اكثر من 5 جرام (الصغيرة 0.37 ± 1.76 الكبيرة0.51

±0.41 والمتوسط 0.48± 2.59) عند مقارنتها بتلك التي تزن اقل من 3جرام (الصغيرة 0.11±1.01 ،الكبيرة 0.04±0.22 والمتوسط 0.10±1.80) والتي تزن مابين 3− 5 جرام (الصغيرة0.12 ± 1.32 ، الكبيرة 0.05 ±0.36 والمتوسط 0.14±2.22). عدد الجريبات الصغيرة في المبايض كبيرة الحجم اعلي معنويا (0.12±1.43) عند مقارنتها بالمبايض صغيرة الحجم (0.10±0.90) ($P < 0.05$). لايوجد أختلاف معنوي ملحوظ بين عدد الجريبات المتوسطة و الكبيرة و حجم المبيض. ليس هنالك أختلاف معنوي بين معدل أسترداد ومؤشر الخلايا البيضية ، ومعدل التشطر ومعدل كيسة الاريمة مابين المبايض التي تحتوي علي جسم أصفر والتي لا تحتوي علي جسم اصفر. ايضا وجود او غياب الجسم الاصفر و مراحله لايؤثر معنويا علي تصنيف الجريبات. في الجزء الثاني من التجربة أستخدمت ١٦٠ أنثي جواميس المراح و قسمت بالتساوي الي مجموعتين (٨٠ حامل و ٨٠ غير حامل) للكشف عن تأثير الحالة الجسمانية و العمر و الحمل و وجود او عدم وجود الجسم الأصفر و موقع المبيض علي تعداد الجريبات بأستخدام تقنية الموجات فوق الصوتية. الحيوانات التي تمتلك حالة جسمانية بدرجة 4 تمتلك اعلي مجموع لعدد الجريبات للمبيض (0.74± 9.85) من تلك بحالة جسمانية بدرجة 3 (0.69± 6.78). ليس هنالك اختلاف معنوي في عدد الجريبات الصغيرة و الكبيرة بين الحيوانات التي تمتلك حالة جسمانية بدرجة 3 و 4. الجواميس الحوامل تحتوي علي عدد اكثر من الجريبات المتوسطة (0.17± 1.96) من تلك الغير الحامل (0.19 ±1.48). ليس هنالك اختلاف معنوي بين عدد الجريبات الصغيرة والكبيرة و العدد الكلي للجريبات في الحيوانات الحوامل و الغير حوامل. المبايض اليمني تمتلك عدد اقل من الجريبات الكبيرة (0.08±0.81)عند مقارنتها بالمبايض اليسري (0.09±0.91). ايضا المبايض التي تحتوي علي جسم أصفر لديها عدد اكثر معنويا ($P < 0.05$) من الجريبات المتوسطة (0.17± 1.95) من تلك بدون جسم

أصفر (0.18± 1.39). لايوجد أختلاف معنوي في تعداد الجريبات بين الاعمار المختلفة للجواميس. في الختام ، هذه الدراسة أظهرت بان العوامل المبيضية و الغير مبيضية تؤثر علي تعداد الجريبات في جواميس المراح. مبايض الجواميس تمتلك كفاءة جيدة علي إنتاج جنين الأنابيب .

INTRODUCTION

Biotechnologies are being used in animal and agriculture to improve production, and to develop specialized food products and pharmaceutical products. Manipulations of reproductive processes are necessary to accomplish these goals. The application of the first reproductive biotechnology is artificial insemination (AI). The dairy industry also remains the number one user of embryo transfer technology (ET). Recently the dairy industry has also adopted the field of *in vitro* embryo production (Munjunatha and Devaraj, 2006).

In vitro Embryo Production (IVEP) a reproductive technique that supplement AI in the genetic improvement of local cattle breeds (Hernandez-Fonseca *et al.*, 2002). IVEP permits the preservation of genetic potential of sub-fertile or dead animals (Deleuze *et al*., 2009) by the creation of a gene bank with oocytes recovered from slaughterhouses (Seidel and Seidel, 1989) for the improvement of livestock productivity (Huang and Rosenwarks, 2012).

The embryo production is carried out through a combination of techniques of collection of immature oocytes, *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC). However, the significantly contributing factors in the success of IVEP are the quality and number of collected oocytes (Kumar and Anand, 2012).

In vitro production technologies not only help in production of high genetic merit animals, but also consider an excellent source of embryos for

emerging biotechnologies such as embryo sexing, cloning, nuclear transfer, transgenesis etc. Furthermore, it allows analyzing developmental potential of embryos, including the pattern of gene expression, epigenetic modifications and cytogenetic disorders during the development (Galli and Lazzari, 2008).

Objectives of study:

1. To determine the follicular population and oocytes recovery rate in the ovaries of slaughtered Indian buffaloes for *in vitro* embryo production.
2. To evaluate the effects ovarian factors such as corpus luteum, size and weight of the ovary on follicular population and oocyte recovery rate.
3. To investigate the influence of non ovarian factors such as age, body condition score (BCS) and pregnancy on follicular population in Indian buffaloes (Murrah) using Ultrasonography technique.

CHAPTER I

LITERATURE REVIEW

1.1 Indian buffalo:

The buffaloes are in the order of *Artiodactyla*, the cloven-hooved mammals, genus *Bubalus* and species *bubalis*. Two main species of buffalo are found in the world; the Asiatic (water) buffalo (*Bubalus bubalis*) and the African buffalo (*Syncerus caffer*). The types of buffalo are different habitats and chromosome numbers. The population of buffaloes is about 170 million head in the world (Perera *et al.*, 2005). Out of this 97 % of them are water buffaloes and are mainly found in the Asian region. Riverine buffaloes are characterized by black colour and have long curled horns (e.g. Murrah breed) and the Swamp buffaloes are dark grey, but may also be black, black and white, or even all white and have long, gently curved horns. Riverine buffaloes (70% of the total world population) are reared in high numbers in South Asia, especially in India and Pakistan. The name 'swamp' has probably arisen from their preference for wallowing in stagnant water pools and mud holes (Subasinghe *et al.*, 1998). Swamp buffaloes are found mainly in southern China Sri Lanka, and the South-East Asia countries of Thailand, Philippines, Indonesia, Vietnam, Burma (Myanmar), Laos, Cambodia and Malaysia (Chantalakhana and Falvey, 1999).

India has about 95 million buffalo's represents 56.5% of the world buffalo population. India is the first country in the world for rearing buffalo's production about 134 million tons of milk (Borghese, 2005).

India is the first country in Asia for scientific and technological development in buffalo nutrition, production, reproduction, biotechnologies and genetic improvement. It is possesses the best River milk breeds in Asia e.g. Murrah, Nili-Ravi, Surti and Jaffarabadi, which originated from the North-Western states of India and have a high potential for milk and milk fat production in addition to use as a work animal and as a supplementary stock for meat production (Borghese, 2005).

1.2 Types of buffalo breeds in India:

1.2.1 Murrah:

It is black, massive and stocky animals and has heavy bones and tightly curved short horn. The high at withers of adult male and female are 142 and 133 cm, respectively. The body weight of adult male is 750kg while in the female is 650 kg. The Murrah breed is originated in central of harayana and then spread to the Punjab, Ravi and Sutley valleys, North Sind and Uttar Pradesh. It has been exported to Brazil, Bulgaria and many countries of Eastern Asia (Moioli and Borghese, 2005). Sethi (2003) was reported that the age of the Murrah at first calving is 50.6±2.0 months, lactation duration is 305 days and calving interval is 479±33 days.

1.2.2 Jaffarabadi:

The Jaffarabadi breed is originated from Gujarat, in India. It is characterized by black colour, Massive and long-barreled conformation. Horns are long, heavy and sometimes they cover the eyes. Height at withers of adult male and female are 142 and 140 cm, respectively. The body weight of adult male range from 600 to 1500 kg and adult female weight as much as 700-800 kg (Moioli and Borghese, 2005).

Sethi (2003) was reported that the age of the Jaffarabadi at first calving is 1925±196 days, lactation length is 320.1±11.6 days and calving interval is 509.8±20.1 days.

1.2.3 Bhadawari:

This is an improved local breed. It is the result of selection of Indian breeds of buffalo. It is raised in the Agra and Etawa districts of Uttar Pradesh and in Bhind and Morena districts of Madhya Pradesh.

It is characterized by copper colored coat, scanty hair which is black at the roots and reddish brown at the tip. Sometimes it is completely brown. The neck presents the typical white colour ring. Tail switch is white or black and white. Horns are short and grow backwards.

Height at withers of adult male and female are 128 and 124 cm, respectively. The body weight of adult male is 475 kg while in the adult female is 425 kg (Moioli and Borghese, 2005).

Sethi (2003) was reported that the age of the Jaffarabadi at first calving is 48.6±0.58 months, lactation length is 272±4days and Calving interval is 478±11days.

1.2.4 Mehsana:

The existence of the Meshana breed in north Gujarat, India, is referred to since1940. This breed is the result of selection of Indian breeds of buffalo. The characteristics of this breed are intermediate between Surti and Murrah. Horns are sickle-shaped but with more curve than the Surti. The udder is well developed and with prominent milk veins. The body weight of adult male and female are 570 and 430 kg, respectively (Moioli and Borghese, 2005). Sethi (2003) reported that the average milk yield per animal per day in Mehsana buffaloes was ranges from 4.37 to 4.81 kg.

1.2.5 Nagpuri:

It is an improved local breed as the result of selection of Indian breeds of buffaloes. It is characterized by black colour and sometimes there are white markings on the face, legs and switch long horns are 50-65 cm long, flat-curved and carried back near to the shoulders. Nasal flap is mostly absent and even if present is very short. The height at withers of adult male and female are 140 and130 cm, respectively .The body weight in adult male is 522 kg while in the female is 408 kg. This breed is raised in the Nagpur,

Wardha and Berar districts of Madhya Pradesh. (Cockrill, 1974; Moioli and Borghese, 2005).

1.2.6 Surti:

The Surti breed found in north Gujarat (India) since 1940. It is one of the most important breeds in Gujarat and in Rajasthan. The population of this breed is about 500 000 head (Moioli and Borghese, 2005).

It is characterized by black colour coat, black or reddish skin and two white chevrons on the chest. Horns are flat, of medium length, sickle shaped and are directed downward and backward, and then turn upward at the tip to form a hook. The udder is well developed, finely shaped and squarely placed between the hind legs. The tail is fairly long, thin and flexible ending in a white tuft. The height at withers of adult male and female are 131 and 124 cm respectively. The body weight in adult male is 700 kg while in female is 550-650 kg (Moioli and Borghese, 2005). Sethi (2003) reported that the age at first calving is 53.2±1.7 months lactation length is 311±7 days and the calving interval is 510±16 days.

1.2.7 Toda:

This breed is raised in the Nilgiris hills of Madras. It is characterized by light or dark grey colour; horns are set wide apart with recurved tip inwards, outward and forward. The height at withers of adult male and female are 160 and 150 cm respectively .The body weight in adult male is 380 kg white in

female is 380 kg (Moioli and Borghese, 2005). Sethi (2003) reported that the average lactation length is 198.6±2.8 days and average calving interval is15.74±0.4 months.

1.3 Reproduction in Buffaloes :

The buffalo reproduction in the world is considered as short day breeder and its reproductive efficiency is greatly and adversely influenced by biometeorological factors such as day length, ambient temperature, relative humidity (Singh *et al.*, 2000; Ribeiro *et al*., 2003). However, in equatorial zone buffalo can show estrous cycle throughout the year when adequate nutrition is provided to maintain the reproductive efficiency (Zicarelli, 1994; Zicarelli, 1997; Baruselli, 2001). Although buffaloes are polyestrous, they exhibit a distinct seasonal variation in display of estrus, conception rate, and calving rate. In Indian buffaloes the reproductive efficiency was better during winter compared to summer months due to environmental factors (Madan,1988; Tailor *et al*.,1990). Similarly, breeding frequency in Pakistani buffaloes was highest during winter, decreased in autumn and spring, and was lowest in the summer (Shah,1988). Furthermore, environmental factors play a significant role in seasonality of reproduction in Italian buffaloes (Singh *et al*., 2000). The stress and adverse environmental factors have direct effect on neuroendocrine set up resulting in hyperprolactinemia, reduced pulsatile gonadotropin secretion, poor follicular maturation and poor oestradiol production culminating in poor heat expression and anoestrus

condition (Aboul-Ela and Barkawi, 1988; Palta *et al*., 1997; Phogat *et al*., 1997).Jainudeen (1988) reported that under nutrition coupled with high ambient temperature were implicated with anoestrus condition in buffaloes during summer. However poor availability of green fodder is not the sole factor responsible in seasonality of poor breeding in Pakistani buffaloes .Also Jainudeen (1988) found that, non-availability of good quality nutritious food along with reduction in voluntary feed intake during thermal stress was attributed towards poor reproductive efficiency.

Season of calving has a profound effect on service period (Madan, 1988; Singh *et al*., 1993). In India, buffaloes calving in late winter and early summer have lower reproductive efficiency compared to those calving during other periods (Singh and Nanda , 1993 ;Singh *et al*., 2000) ; and these calving during autumn season had shorter anoestrus period than other seasons calves (Tailor *et al*.,1997). Singh and Nanda (1993) registered that the resumption of ovarian activity after calving was significantly delayed in buffaloes that calved from February to May (116–148 days) compared to the rest of the year (38–64 days). The mean service period of buffaloes calving from December to June was significantly higher (140 days) than calving between July to November (< 110 days). The high service period during the former group was associated with more silent estruses (Prakash *et al*., 2005). Furthermore, conception rate was low between February and August and number of services per conception was higher in buffaloes calving summer

than those calving in the other seasons (Madan, 1988). Similarly, in Italy, buffaloes calving during the non-breeding season have a long post-partum period and they resume cyclicity only during the following breeding period (Zicarelli *et al*., 2007). The postpartum anestrus was divided into a temporary (less than150 days) and deep anestrus (more than 150 days) according into the time elapsed between calving and conception (Zicarelli, 1997). It was also classified to superficial and deep anestrus depending upon the presence or absence of follicular turn over (Presicce *et al*., 2005). In Egyptian buffaloes, El-Wishy (2007) reported that the effect of calving season on post-partum acyclicity was conflicting. Barkawi *et al.* (1996) recorded that the effect of season resulted in longer interval of calving during hot months compared to cold ones.

The normal estrous cycle in buffaloes can vary from 16 to 28 days with a duration of estrous 10-20 hours during the breeding season (Vale and Ohashi, 1994 ; Baruselli *et al*.,1997).The interval between onset of estrous and LH surge is 1-12 hours in buffaloes and ovulation occurs between 18-40 hours after the LH surge (Prakash *et al*., 2005). The interval from estrous end to ovulation has been reported to be between 12-24 hours in different Indian breeds of swamp buffaloes where as in Murrah buffalo the onset of estrus to ovulation time is 28-60 hours (Raut and Kadu , 1988 ;Prakash *et al*., 2005).

In Nagpuri buffaloes, seasonal suppression (summer versus winter) of reproductive function has been documented by a shorter duration of estrus (8–10 versus 18 hours) and apparent prolongation of the interval from estrus to ovulation (15.8±0.4 versus 14.9±0.4 hours) (Raut and Kadu, 1988). Incidence of summer anestrous has been reported to be between 36-60% (Singh *et al*., 1989). In more recent study, an incidence of 63% and 83% of summer anestrus were reported in nomadic and housed rural buffaloes, respectively (Brar and Nanda, 2004).

Singh and Singh (1985) recorded that the incidence of true anestrous (defined as the absence of large follicles and CL in the ovaries) was 78% in July and 14% in November. The summer anoestrus has been found to be associated with smooth inactive ovaries without any follicle or CL may be due to lower follicular reserve in acyclic buffaloes than cyclic buffaloes (Razdan *et al*., 1981; Nanda *et al*., 2003). Also poor quality of oocyte during summer season is also responsible for poor reproductive efficiency in buffaloes (Das *et al*., 1996; Nandi *et al*., 2001).

1.4 Factors influencing the follicular population and oocyte recovery rate:

1.4.1 Diameter of the ovaries:

Length, width and thickness of the ovaries were measured as pole to pole, surface to surface and hilus to the free border, respectively, with the help of a vernier caliper. Linear measurements of the ovaries were made by a

vernier caliper using the average of three measurements (Bukar *et al*., 2006). Jablonka-Shariff *et al* (1993) reported that the length, width and thickness were higher in ovaries which have CL than those which have no CL in sheep.

1.4.1.1 Ovary length:

The length of the ovary was taken as the distance from anterior pole to posterior pole along an axis parallel to the ovarian mesenterial attachment (Bukar *et al*., 2006). Asad *et al* (2016) found that the length of right ovary (1.19±0.09) was significantly ($p<0.05$) higher than the left on goat. Also Sarker (1993) reported that the right ovaries were more normal physiological than left ovaries.

1.4.1.2 Ovary width:

Width of the ovary was taken as the greater distance from the medial to the lateral surfaces or borders (Bukar *et al*., 2006). Asad *et al* (2016) registered that the width was significantly ($p<0.05$) higher in ovaries with CL than those of ovaries without CL.

1.4.1.3 Ovary thickness:

Thickness of the ovary was recorded as the greatest distance along an axis vertical to the longitudinal axis (base) at its center expressed in centimeter (Razzaque *et al*., 2008).

1.4.1.4 Ovary weight:

Weight of the ovaries was taken on the electronic (digital) mono pan balance and expressed in gram (Bukar *et al*., 2006).

The weights of ovaries were increased significantly during the luteal phase compared to the follicular phase; this increase might be due to the presence of corpus luteum (Fields and Fields, 1996). Osman and Shehata (2005) found that the corpus luteum represents 30.1% of the ovarian weight in buffalo. The disproportion between the right and left ovary was also reported by Trigal *et al*. (2014). Indeed, studies of Ginther *et al*. (2013) showed that ovulations were more frequent on the right ovary. This greater physiological activity on the right ovary would be responsible for the increase of its weight (Ginther *et al*., 2013).

1.4.2 Localization of the corpus luteum:

The corpus luteum (CL) plays a central role in regulating the estrous cycle and maintaining the pregnancy. These functions are performed largely by progesterone, which is the main steroid synthesized by the CL, a transient endocrine gland (Gregoraszczuk, 1994). The formation, maintenance, regression, and steroidogenesis of the CL are among the most significant and closely regulated events in mammalian reproduction. At the end of its life span, the CL undergoes a process of regression, leading to its disappearance from the ovary and allowing the initiation of a new cycle. Mamy *et al* (2011)

found that the CL was present on right ovaries than left ovaries because normal physiological explanation of ovarian activity is that right ovaries are more active than left.

1.4.3 Age determination:

Buffalo longevity under natural conditions is unknown, but under intensive farming conditions they have potential of living to an age of 25 years or more. Age determination of a buffalo can be done by means of dentition (Hornsveld, 1996). Also Hornsveld (1996) reported that buffalo dentition set consists of four lower incisors, no upper incisors and three premolars on either side of the upper and lower dental arches.

The first lower incisor appears at an age of two years, the second at three years the third around four and a half years and the fourth at five years of age.

1.4.4 Body condition Score (BCS):

The body condition score (BCS) system is a subjective scoring method of evaluating the energy reserves of dairy animals. It which provides a better understanding of the biological relationship between body fat, milk production and reproduction that helps in adopting the optimum managemental practices to derive maximum production and maintain better health status (Gransworthy, 1988). It is based on evaluation of the outer appearance of the animal that interacts with its body fat reserves and

therefore is directly influenced by energy balance. It gives an immediate appraisal of the body state of the animal and is readily incorporated in operational decision making (Gransworthy, 1988). BCS systems have been developed earlier by many scientists (Jefferies, 1961) using 0 to 5 scales in ewes and (Lowman *et al*., 1976) using scale graded from 0 to 5 in beef cattle.

Anitha *et al* (2011) reported that the eight locations grade system which used in dairy cows includes; tail head to pin bones, spinous processes of the lumbar vertebrae, depression between the spinous and transverse processes, transverse processes of lumbar vertebrae, point between 12th and 13th ribs, sacral crest, depression between sacral crest and hooks and depression between hooks and pins.

1.5 *In vitro* embryo production (IVP):

The *in vitro* production of embryos involves retrieval of oocytes from ovaries of slaughtered animals or live animals. The (IVP) consists three phases that is: *in vitro* maturation (IVM) of oocyte, *in vitro* fertilization (IVF) and *in vitro* culture (IVC) (Noakes *et al*., 2009).

1.5.1 Collection of ovaries and handling:

Specimens of ovaries collected from slaughterhouse are the cheapest and the most abundant source of primary oocytes for large scale production of embryos through IVM and IVF (Nandi *et al*., 2006).

After slaughter of female buffaloes, the left and right ovaries were excised and placed in separate conical tubes containing washed medium (WM) and transport to the laboratory at 35-37°C within next 2 hours after slaughter. All cystic ovaries were excluded from studies (Wang *et al*., 2007).

1.5.2 Evaluation of ovaries:

Ovaries are the primary reproductive organs in the female because they produce the female gametes. Numerous follicles are produced during the reproductive cycles in ruminants (Fitzpatrick and Entwistle, 1997; Cerri *et al*., 2009; Lauderdale, 2009). The ovary of the ruminants contains several hundred growing follicles. Mcnatty *et al* (1982); Kaulfuss *et al*., (1994) found that an average of 44 visible vesicular follicles at different stages during the estrus cycle and the number of follicles did not different significantly between the left and right ovaries.

Depletion of the ovarian reserve is associated with reproductive senescence in mammalian females and there is a positive relationship between the size of the ovarian reserve and the number of antral follicles on the surface of the ovary (Cushman *et al*., 2009). Normally cow and buffalo produce one ovum per estrus cycle whereas sheep and goat produce one or more ova each estrus cycle (Waldron 1999).

Trigal *et al* (2014) reported that there was disproportion between the right and left ovary in cow. Indeed, many studies (Pierson and Ginther, 1987 ;Ginther *et al*., 2013) showed that the ovulations is more frequent on the

right ovary. This greater physiological activity on right ovary would be responsible for the increase of its weight (Ginther *et al*., 2013).

After collection and trimming ovaries were evaluated on the basis of the many parameters, such as measurement of weight, length and width of ovaries. Individually right and left, ovaries with or without CL were weighed per gram using a digital balance and the weight was recorded in tabular form (Asad *et al*., 2016). The length and width of right and left ovaries in the presence or absence of CL were measured with a digital slide calipers per cm (Asad *et al*., 2016).

1.5.3 Collection of oocytes from slaughtered animal:

Kumar and Anand (2012) described methods used for the collection of oocytes from ovaries of slaughtered animal, these methods are;

1.5.3.1 Aspiration technique:

This method is commonly employed because of the convenience associated with its application. Aspiration of oocytes is done using a needle attached to a 10-ml syringe and to avoid disruption of the surrounding cumulus cells; an 18- gauge needle is used. Possible toxicity associated with syringes containing rubber plungers and siloxane lubricants is avoided by washing and sterilizing glass syringes under the stringent conditions used for tissue culture glassware. For livestock, the use of plastic disposable syringes is acceptable (Kumar and Anand, 2012). The oocytes are aspirate from individual ovaries after carefully removing the extraneous tissues and place

in Petri dish containing 1 ml of PBS media. That was prepared according to Gordon (1994). Also, Rao and Mahesh (2012) reported that oocytes were aspirated from the visible follicles present on the ovarian surface. Oocytes were aspirate using 22 gauge needle fixed to 5 ml disposable syringe containing 1-2 ml of PBS media.

1.5.3.2 Puncturing or dissecting technique:

In this method the ovaries are placed in a sterile glass Petri dish containing 2 ml of PBS media. All the visible follicles are carefully subject to blunt dissection using forceps and the remaining ovarian tissues are removed after a brief rinsing. Then follicles are rupture and their fluid is allowed to flow into the PBS media in the Petri dish (Singh *et al.,* 2013).

1.5.3.3 Slicing technique:

The ovaries are held firmly with the help of forceps in a sterile glass Petri dish containing 2 ml of PBS media. The ovaries are sliced into thin sections with a blade fixed to the artery forceps. The oocytes containing PBS media are placed in petri dish and examined under sterozoom microscope (Abid *et al.*, 2011).

1.5.4 Evaluation and grading of oocytes:

Oocytes were examining under sterozoom microscope and classifying depending upon their compaction, number of cumulus cell layers and

homogeneity of ooplasm are regarding according to Alves *et al*(2014), into 4 categories:

1-Grade I (GI): oocytes with more than 4 layers of bunch of compact cumulus cells mass with evenly granulated cytoplasm.

2- Grade II (GII): Oocyte with at least 2–4 layers of compact cumulus cell mass with evenly granulated cytoplasm.

3- Grade III (GIII): Oocyte with at least one layer of compact cumulus cell mass with evenly granulated cytoplasm.

4- Grade IV (GIV): Denuded oocyte with no cumulus cells, incomplete layer of cumulus cell or expanded cells and having dark or unevenly granulated cytoplasm.

1.5.5 Oocyte recovery rate:

Abid *et al* (2011) calculated the oocyte recovery rate by the following formula:

ORR = (No of recovered oocytes / total No of ovaries).

1.5.6 In vitro maturation (IVM):

In vitro maturation (IVM) of an oocyte is an important reproductive technology for generating mature oocytes which are capable of supporting pre-implantation and post-implantation development of embryos to term(Gilchrist and Thompson, 2007). Oocytes maturation is the most critical step towards successful in vitro embryo production. The culture medium and

selection of protein supplements and hormones for IVM play an important role in the subsequent maturation rate and embryonic development following IVF (Bavister *et al*., 1992). Several factors such as addition of FSH, LH and their combination to culture media had been considered for maximizing success (Saeki *et al*., 1991).

In bovine, there are different laboratories and workers who have their own protocol for in vitro maturation. The most widely used medias employed to perform IVM includes Ham's F 10a, tissue culture medium 199 with and without serum and synthetic oviduct fluid (SOF), these medias are complex and may be supplemented with fetal calf serum (FCS), estrus cow serum (ECS), new born calf serum (NBCS) superovulated cow serum (SCS), anestrus cow serum (ACS) or bovine serum albumin (BSA) (Gandhi *et al*., 2000). Maturation medias are also supplemented with pituitary FSH and/or LH (gonadotropins) with estrdial-17α or extra gonadotropin hormones like human chorionic gonadotropins (hCG) or equine chorionic gonadotropin (eCG). Also some laboratories prefer to add growth factors like epidermal growth factor (EGF) (Nedambale *et al*., 2004), EGF plus fibroblast growth factor (FGF), insulin like growth factor (IGF), insulin, transferrin sodium selenite (ITS) (Galli *et al*., 2001).

In buffaloes the most IVM medias used for maturation of oocyte are tissue culture media (TCM-199) and Ham's F-10. Further common used TCM-199 that supplemented with 10% FBS + 0.81 mM sodium pyruvate +

5% buffalo follicular fluid (buFF) + 50 µg/ml gentamycin sulfate and 5 µg/ml porcine FSH. This media achieved 82.3% of maturation rates for 24 hours incubation in a CO_2 incubator (5% CO_2 in air, 90-95% relative humidity) at 38.5°C (Kumar *et al*., 2007).

1.5.7 In vitro fertilization (IVF):

In bovine, the natural fertilization takes place in the reproductive tract of the female when male and female gametes meet and combine. Spermatozoa fuse with oocytes in the oviduct and this relatively long journey through the uterus serves to select the fastest swimming sperm, which is often that with the longest flagella. However, it is not simply a matter of sperm meeting egg, sperm must undergo capacitation in order to fertilize the egg successfully. Sperm capacitation means that it is prepared to undergo the acrosome reaction when it meets the zona pellucida of the ovum. It involves stripping many of the seminal plasma proteins from the head of the sperm which were attached during ejaculation (Holt *et al.*, 2010).

Parrish (2014) observed that capacitation takes 4-6 hours in cow, but these time points are not as well defined in vivo due to many factors specific to the uterine environment (Rodriguez-Martinez ,2007). The afore mentioned acrosome reaction involves fusion of the plasma and acrosomal membranes of the sperm head, which allows proteolytic enzymes to leave the sperm head and help break down a small area of the zona pellucida to assist the sperm entry into the perivitelline space. The entry of a single

sperm initiates a cascade of events inside the oocyte leading to the zona block which should prevent any other sperm from penetration. This is important to present oocyte from fertilization by more than one sperm (Rodriguez-Martinez, 2007).

In buffaloes, the IVF is the most critical step of the IVEP procedures and requires appropriate preparation of sperm and oocyte, as well as culture conditions that are favorable to the metabolic activity of the male and female gametes. The suggested media used for IVF are BO and TALP, which contain motility enhancing substance such as caffeine or theophylline (Bavister, 1995).

For successful fertilization of oocytes, good sperm preparation is the essential and crucial step. Different methods tried for separation of good motile sperm like swim-up or percoll based separation system (Mahadevan, 1984).

In bovines, the capacitation occurs basically in the oviduct during the period of estrus and this is confirmed by presence of heparin-like glycosaminoglycan in the oviduct fluid (First and Parrish, 1987). High rates of IVF has been achieved by addition of heparin (Brackett and Zuelke, 1993) or its combination with penicillamine, hypotaurine and epinephrine, Ca^{++} ionophore A23187 with or without caffeine and high ionic strength media (Brackett et *al*., 1982). Presently the efficiency of IVF is approximately 80% for the cattle (First and Parrish, 1987).

1.5.8 In vitro embryo culture (IVC):

The developing embryo (both in vivo and in vitro) goes through several recognizable stages of growth. A one-celled structure where male and female pronuclei have fused is called a zygote (Senger, 2005). In the first few mitotic divisions, growth is only in cell number, as the original cytoplasm of the oocyte is divided among the daughter cells. Each blastomere possesses the quality of totipotency, meaning that it could be induced to become any fetal or adult cell type. After the fourth cell cycle (when the embryo is made up of 8 cells), the embryo undergoes the maternal to embryonic transition which means that the embryo is charged with the task of making its own mRNA instead of using the stock piled maternal mRNA present in the oocyte (Senger, 2005).

The embryonic genome is highly methylated (and thus unavailable for transcription) in the gamete, so it must be both de- and re-methylated (Zhao *et al.,* 2010; Kepkova *et al.,* 2011). After the blastomeres become too numerous to count (16 cell stage, at the earliest), the embryo is called a morula. Further, this is usually the time at which the cells become differentiated into cells which make up the inner cell mass and those of the trophectoderm. Thus, although they are still pluripotent, they are totipotent no longer. The inner cell mass gives rise to the embryo proper as well as

other extra embryonic membranes, and the trophectoderm becomes the chorion, or the fetal contribution to the placenta (Kepkova *et al.*, 2011). The final developmental milestone is signaled when the embryo develops a fluid-filled cavity called a blastocoels around which primitive endoderm will enclose the yolk sac. Then the blastocyst stages is consist initial stage, in vivo or in vitro produced embryos are evaluated and transferred to appropriately timed recipients (Kepkova *et al.*, 2011).

After the end of incubation of zygote and prior to transfer to the in vitro culture droplets, presumed zygotes were washed four times in embryo culture medium (mCR_2aa containing 0.8% BSA) and cultured in this medium in a humidified CO_2 incubator at 38.5 °C for up to 9-10 days to get the blastocyst. The produced embryos were examined under an inverted microscope, to record the number of cleaved embryos at 8-16 cells which could either be transferred to synchronized recipients for producing live offspring or cryopreserved for future use. (Enchaparambil *et al*., 2014).

There are various systems available for IVC of zygotes. These includes co-culture with various types of cells such as bovine oviduct epithelial cells (BOEC) (Eyestone and First, 1989), cummulus cells or tropoblastic vesicles, established cell line, buffalo rat liver (BRL) cells or vero cells. The chemically defined medias like SOF, CR1a, Chatot Ziomek Bavister medium CZB (Chatot *et al.*, 1989), hamster embryo culture medium-6 (HECM-6), and G1.1/G2.2 were used for IVC (Krisher *et al*., 1999) . SOF is

the medium used very commonly by different laboratories and workers. These defined media generally require low oxygen tension (5% oxygen) to yield higher blastocyst rate. Lonergan *et al* (1999) and Vanroose *et al* (2001) observed that culturing bovine oocytes in SOF and in SOF plus BSA using 5 % oxygen was increased the blastocyst yield on Day 8 compared with those medias by 20% oxygen.

Secretions of the female reproductive tract have several amino acids that can be used as energetic substrate for the embryo (Bavister, 1995). The use of amino acids in serum-free culture media improves embryo development (Lee *et al*., 2004), probably through an antioxidant action, controlling pH and osmolarity (Gaedner, 1998). Amino acids can also reduce the stress and cell fragmentation caused by IVC (Donnay *et al*., 1997).

The oocyte yield in ovum pick up OPU, IVM, IVF and IVC depends upon species and breed of animal used for experiment, number of follicles available for aspiration, OPU session interval, stage of estrus cycle, environmental consideration, oocytes handling, quality of media (pH osmolarity), O_2 concentration in incubator and culture conditions of the oocytes need to be taken into account when need to rescue genetic material from females. Due to the above mentioned factors potential of Indian crossbred cattle to act as oocyte donors can be expected to differ from that of exotic breeds (Donnay *et al*., 1997).

1.6 Evaluation of female reproductive tract by using ultrasonography technique:

Ultrasonography has become an important diagnostic tool for evaluating the female reproductive system, where it is possible to view the entire reproductive system in a non-invasive manner (Carriere *et al*., 2002). Also using this new technology to identify non-pregnant dairy cows and heifers early after AI may play a key role in management strategies to improve reproductive efficiency and profitability (Raja *et al*., 1994).

Ultrasound covers the spectrum of sound frequencies exceeding 20,000 cycles/ sec, which is the maximum frequency perceived by the human ear. The sound waves are emitted and pass through tissues or are reflected back to the transducer (crystals). The transducer, also called an ultrasound probe, emits pulses of sound waves between 2.0 to 20.0 megahertz (MHz). The reflected sound waves or echo are received and converted into electrical impulses, processed and displayed through an oscilloscope and cathode ray tube according to the type of ultrasound machine used. The image is created based on the amplitude, frequency and time that take for the ultrasound signal to return from the examined organ to the transducer, as well as the type of tissue which the sound travels through. Data are shown as a two dimensional vector of pixels (picture elements or pixels) whose intensity is represented on a grey-scale. The brightness of a pixel corresponds to the

amplitude of that individual echo signal. Brightness is represented by shades of gray extended from white to black. Tissue reflections that are very dense result in white pixels, whereas reflections of intermediate density result in gray pixels. The clinical descriptive terms are based on the way the ultrasound wave is reflected back to the transducer. Therefore, each tissue has a particular echogenicity. Tissues or organs that reflect high-frequency sound waves are described as echogenic; thus, depending on the structure of the tissue, the intensity of the gray-scale may vary from non-echogenic (black) to echogenic (bright) (Pierson *et al*., 1988; O'Brien, 2007).

The acoustic image or aligned echo depends on the property of the reflection of the waves in the tissues and the position of the ultrasonic beam. The ultrasonic beam causes the molecules in the tissues to vibrate. Ultrasounds must overcome a specific resistance called acoustic impedance which may be estimated by multiplying the density of tissue by the propagation speed of sound; the average speed in soft tissues is 1.540 m per second. The boundary between two tissues of different impedance is called acoustic interface. The ability in the propagation of the sound depends on the distance between molecules; if the molecules are close to one to another or if there are a greater number of molecules per unit volume, then the sound is transmitted faster. Each representation of the echoes that returns is recorded on a video monitor, so the image may then be interpreted by the clinician

(Pierson *et al*., 1988; Ginther, 1998; Mattoon and Nyland, 2015; Blond and Buczinski, 2009).

1.6.1 Types of ultrasound:

1.6.1.1 Real time B-mode:

A two-dimensional diagnostic ultrasound presentation of echo producing interfaces; the intensity of the echo is represented by modulation of the brightness (B) of the spot, and the position of the echo is determined from the angular position of the transducer and the transit time of the acoustical pulse and its echo. B mode ultrasound provides a great deal of information in a short period of time, allowing a dynamic anatomic diagnosis. A two-dimensional image in a series of images that are generated quickly, give an idea of movement structure analysis in real time. The signals are repeatedly transmitted, received and processed so the image of the organ is continually updated. Method-mode ultrasonography is most widely used for the examination of the reproductive tract of cattle and other large animals including the water buffalo (Pierson *et al*., 1988; Ginther, 1998 Edmondson *et al*., 1986; Fissore *et al*., 1986).

1.6.1.2 A-Mode:

The so called amplitude mode produces a one dimensional display of echo amplitudes for various depths, depicted as a line graph where the axes are amplitude and depth. The principal use of the A-mode ultrasonography is

to evaluate the fat and lean portions of meat animals. Although A-mode has also been used for pregnancy diagnoses, real time B-mode is most commonly used for this purpose (Pierson *et al*., 1988; King, 2006).

1.6.1.3 M-Mode:

The motion mode is an adaptation of B-mode ultrasonography and is used to evaluate moving structures such as the heart. The change in reflector depth at different times is displayed as a simple line graph where the axes are depth and time (Pierson *et al*., 1988; King, 2006).

1.6.1.4 Doppler:

Doppler ultrasonography uses the motion of blood toward, away or at an angle to the transducer to construct dazzling multicolor images of flow patterns (red= toward the transducer; blue= away from the transducer). Doppler is useful to evaluate blood flow in the fetal heart, corpus luteum formation and ovulation, among other clinical applications (Pierson *et al*., 1988; Ginther, 1998; Ginther and Utt, 2004).

1.6.2 Ultrasonographic examination of uterus:

Sarabia, (2004) reported that the tubular genitalia of water buffalo are generally more muscular and firmer, and the uterine horns are more coiled than cow. The body of the uterus is shorter (1–2 cm) than that of the cow (2–4 cm). The cervix of the water buffalo is smaller (length 3–10 cm, diameter 1.5–6.0 cm) and the cervical canal is more tortuous than that of the cow. The average number of cervical folds in water buffalo is three (Sarabia, 2004) .In

addition, the broad ligament seems to be tighter compared to cattle, which makes it difficult to fully retract and expose the uterine horns during routine rectal examinations of the non-pregnant buffalo female (Sarabia, 2004) . Evaluation of uterus has been suggested that the uterine horn be divided into four or five segments to be able to describe the location of an embryo or of a specific pathological change when a longitudinal section of the uterus is observed (Ginther, 1998). If the transducer is placed away from the longitudinal axis, then, the image will show different cross sections of the uterine horn. Physiological changes are evaluated based on parameters such as echogenicity, vascularity and edema of the uterine wall as well as the accumulation of fluid in the lumen of the uterine horn.

The echogenicity of the uterine wall increases during estrus due to the increased edema and uterine tone. Vascularity and edema are characterized as non-echogenic areas within the uterine wall, and denote the days of the estrous cycle dominated by estrogens. Occasionally, physiological accumulation of fluid in the uterine lumen can be observed during estrus in female buffaloes as observed in cattle (Honparkhe *et al*., 2004; Beal *et al*., 1992; Des coteaux *et al*., 2006).

1.6.3 Ultrasonographic examination of ovary:

Vittoria, (1997) registered that the buffalo ovary has smaller size and lighter weight compared with than cattle (2.5 cm vs. 3.7 cm of length and 3.9 g vs. 8.5 g of weight, respectively).The morphological appearance of the CL

has also been described as deeply embedded in the ovarian stroma and of a smaller size than in cattle (Senatore *et al*., 2002; Roy and Mullick, 1964; Drost, 2007). Evaluation of ovary is important to emphasize that the transducer must be located in close contact with the ovary to avoid artifacts resulting from the transducer not making contact or in contact with other tissues. In order to obtain high quality images, it is necessary for the clinician to manipulate the ovary and the transducer to adequately differentiate structures present at certain time. The image of a follicle observed on the screen is characterized by its round shape, commonly smaller than 8 mm, containing anon-echogenic fluid surrounded by a thin echogenic wall. On the other hand, the corpus luteum (CL) typically appears as an echogenic structure (the CL appears as a gray structure on the screen) ranging in size from 13-16 mm in diameter in its mature state depending on the day of the estrous cycle As mentioned earlier, CL in buffalo are smaller than in cattle (Drost, 2007) , and they do not tend to protrude from the ovarian parenchyma which makes it difficult to positively identify by rectal palpation (Drost, 2007).

1.6.4 Ultrasonographic examination of the pregnant reproductive tract of the female buffalo:

Ultrasonography has been used widely for pregnancy diagnosis and fetal gender determination in different species (Peter *et al*., 1992; Kahn, 1990). Through the use of ultrasonography, fetal and placental development and

viability can be monitored and embryonic or fetal mortality can be diagnosed and confirmed early in gestation. Traditionally, pregnancy diagnosis in buffaloes has been performed by rectal palpation of the uterus similar to cattle, but results are variable depending on the skill of the clinician (Peter *et al*., 1992; Kahn, 1990) .Early pregnancy diagnosis can be done efficiently with ultrasonography to detect problem animals and to facilitate decision making in a timely manner in order to minimize economic losses generated by low pregnancy rates, increased days open and low calving rates. Ultrasonography is a non-invasive method that allows pregnancy detection in buffaloes at around Day 25-33 of gestation (Karen *et al*., 2007), unlike transrectal palpation which allows early diagnosis between 35-60 days, depending on the skills of the clinician (Karen et al., 2011) .Nevertheless, the results depend on the type of equipment used, quality of the image and resolution, type of transducer used, and the experience and interpretation of the operator (Stroud, 1994). The results can also be influenced by the age of the animal and the number of calving, making early diagnosis easier in heifers than in pluriparous animals (Stroud, 1994).

Images of early pregnancy by ultrasonography, allows the visualization of non- echogenic fluid in the lumen of the uterine horn. The pregnant horn must be symmetrical and spherical, which corresponds to fluid in the allantoic cavity. The presence of an embryo inside the cavity confirms the diagnosis, but viability can only be confirmed through visualization of a

heartbeat, it is accepted that the fetal heartbeat can be detected by ultrasonography between Days 25 and 28 of gestation in buffaloes (Herrera *et al*., 2007; Glatzel *et al*., 2000; Sharma *et al*., 2012).

CHAPTER II

MATERIALS AND METHODS

2.1 Study area:

The study was conducted during 2018 – 2019 at Central Institute for Research on Buffalo, Hisar Haryana, India, located between Latitude: 29°09′14″ N Longitude: 75°43′22″ E and Elevation above sea level: 216 m.

2.2 City climate:

The climate in Hisar city is characterized by hot summer and cool winter and it lies at the outer margins of the monsoon region, 1600 km away from the Indian ocean (Anurag *et al* .,2017). It has a semi arid subtropical climate. South westerly monsoon current in the summer, brings rain generally from the last week of June to the middle of September. From October to the end of June next, the weather remains extremely dry, except for few light showers received due to westerly disturbances. About 80% of annual precipitation is received in the south-west monsoon season. Summer is very hot (maximum temperature about 45° C or sometimes even more) and winter is fairly cool (minimum temperature around 1 to 2° C or sometimes less). Occasionally temperature may fall below 0 $^{\circ}$C in the month of December and January (Anurag *et al*., 2017).

2.3 Experiment Design:

2.3.1 Experiment 1: In buffaloes ovaries collected from slaughterhouse

2.3.1.1 Media preparation:

All chemicals and media used in the present study were obtained from Sigma Aldrich (St. Louis, MO, USA), and the ware plastic was from Falcon (Paignton, UK), unless stated. Media and reagents were prepared using standard protocol of embryo laboratory technique at the Central Institute for Research on buffaloes. All the media were filtered using 0.22 µm pore size filter (Durapure® membrane filter, Carrigtwohill, Ireland) and culture medium was routinely equilibrated in an incubator at 38.5°C with 5% CO_2 in humidified air for at least 2 hrs before use.

2. 3.1.2 Ovaries collection:

Two hundred and ninety six buffalo ovaries were collected immediately after slaughtering from Delhi slaughterhouse and transported to the laboratory in an insulated container containing 0.9% normal saline with antibiotics 400 IU/ml penicillin and 500 µg/ml streptomycin at 32‑37°C within 4‑5 h. In the laboratory, all tissues attached to the ovaries were removed by the scissor and all the ovaries were washed twice in a saline solution containing antibiotics (Dharmendra *et al*., 2011). After wash all ovaries were classified into two groups: group of ovaries with CL and

without CL. The group of ovaries having CL as classified into: Group having CL in early stage, middle stage and late stage.

2.3.1.3 Determination of ovaries weights:

Ovaries were weighed using an electronic scale balance and expressed in gram (Kouamo *et al*., 2014)

2.3.1.4 Determination of ovaries lengths:

Ovaries lengths were measured using electronic Vernier calipers as the distance from anterior pole to posterior pole and expressed in cm (Samad and Raza, 1999)

2.3.1.5 Determination of ovaries widths:

Ovaries widths were measured using electronic Vernier calipers as the greater distance from the medial to the lateral surfaces and expressed in cm (Bukar *et al*., 2006)

2.3.1.6 Determination of ovaries thicknesses:

Ovaries thicknesses were measured using electronic Vernier calipers as the greatest distance along an axis vertical to the longitudinal axis and expressed in cm (Razzaque *et al*., 2008).

2.3.1.7 Determination of follicular population:

For each ovary, visible follicles were counted and follicles size was measured with electronic Vernier calipers. Follicles were classified into 3 categories: small (<3 mm), medium (3 to 8 mm) and large (> 8 mm) (Baki Acar *et al*., 2013).

2. 3.1.8 Oocytes collection:

Oocytes were collected by an aspiration of surface follicles (2–8 mm diameter) using 18-gauge disposable needle attached to a 10 ml syringe (Henke Saas Wolf GmBH, Tuttingen, Germany) in aspiration medium consisting of TCM-199 and 0.6% (v/w) bovine serum albumin (BSA) (figure 2.9). The follicular fluid was collected in a tube and kept for 15 minutes. The sediment was collected in 60 mm petri dish and oocytes were searched under a stereo zoom microscope (Dharmendra *et al*., 2011).

2. 3.1.9 Grading of oocytes:

The oocytes were graded as: grade A: having evenly granulated homogenous ooplasm with cumulus cells more than 4 compact layers, grade B: having evenly granulated homogenous ooplasm with 2 to 3 layers of cumulus cells, grade C: having evenly granulated homogenous ooplasm with less compact cumulus cells and grade D: having irregular dark ooplasm and highly expanded cumulus cells. Grade A and B Oocytes were used for IVM (Alves *et al.*, 2014).

2.3.1.10 Oocyte Recovery Rate per ovary (ORR):

The oocyte recovery rate was calculated by the following formula:

ORR = (No of recovered oocytes / total No of ovaries) (Abid *et al.*, 2011).

2.3.1.11 Oocyte index:

It was calculated as an index using the formula [(A x 1 + B x 2 + C x 3 + D x 4) / Total number of oocytes recovered] as described by (Baki Acar *et al*., 2013). Index values that approach one reflects good quality oocytes.

2. 3.1.12 In vitro maturation of oocytes (IVM):

Oocytes were washed three times with the washing medium (TCM-199+10% FBS+0.81 mM sodium pyruvate+50 µg/ml gentamycin sulphate) and then twice with the IVM medium (TCM-199+10% FBS+5 µg/ml porcine FSH+1 µg/ml estradiol-17β+0.81 mM sodium pyruvate+5% buffalo follicular fluid+50 µg/ml gentamycin sulphate). The washed cumulus oophorus complexes were then placed in 80-µl droplets (15–20 oocytes/droplet) of the IVM medium, covered with sterile paraffin oil, in a 35 mm Petri dish (Becton, Dickinson and Co., Lincoln Park, NJ, USA) and cultured for 24 h in a CO_2 incubator (5% CO_2 in air, 90–95% relative humidity) at 38.5°C (Dharmendra *et al*., 2011).

2.3.1.12.1 Identification of mature oocytes

After 24 h of incubation the maturation of oocytes was assessed based on the degree of cumulus expansion. Expansion of Cumulus Oophorus Complexes was characterized by its sticky nature and enlargement of the cumulus mass to at least 2-3 diameters from the zona pellucida (de Loos *et al*., 1992).

2. 3.1.13 In vitro fertilization of oocytes (IVF):

The matured oocytes were washed three times in BO medium having 10 µg/ml heparin, 137.0 µg/ml sodium pyruvate and 1.942 mg/ml caffeine sodium benzoate then transferred to 50 µL droplets (15–20 oocytes/droplet) of the IVF medium (BO medium containing 10 mg/ml fatty acid-free BSA).Thereafter two straws of frozen semen brought from semen frozen laboratory at the CIRB were thawed in thermos contain hot water 37˚C then washed twice with BO medium. The spermatozoa in 50 µL of the IVF medium (3 million spermatozoa/ ml) were then added to the droplets containing the oocytes, covered with sterile mineral oil and placed in a CO2 incubator (5% CO2 in air) for 18 h at 38.5°C (Dharmendra *et al*., 2011).

2. 3.1.14 In vitro culture of fertilized oocytes (IVC):

Presumptive zygotes were denuded from cumulus cells and the extra spermatozoa by gentle pipetting and washed three times in IVC washing medium (T_2) supplemented with 1% fatty acid-free bovine serum albumin (BSA).Then fertilized oocytes were cultured in IVC (Research Vitro Cleave Medium (K-RVCL-50; Cook, Brisbane, Queensland, Australia) by making two to three droplets in 35mm petri dish and overlaid with mineral oil, at 38.5 ˚C and 5% CO2 in a humidified incubator for 8 days (Dharmendra *et al*., 2011).

1.3.1.15 Embryo identification and evaluation:

Cleavage embryos were identified according to their cell number during specific time as 1- cell on day 1, 2-cells on day 2, 4-cells on day 3, 8-cells on day 4, 16-cells on day 5 as well as morula and blastocyst stages on day 6 and 7 respectively (Linder and Wright ,1983) (figures 2.13 and 2.14)

1.3.1.16 Cleavage rate (CR):

The cleavage rate was calculated as follows:

Cleavage rate (CR) = number of cleavage / total of oocytes inseminated X 100 (Abid *et al.*, 2011).

1.3.1.17 Blastocyst rate:

The blastocyst rate was calculated as follows:

Blastocyst rate = number of Blastocyst / total of oocytes inseminated X 100.

2.3.2 Experiment 2: In live Murrah buffaloes at Buffaloes farm in the Central Institute for Research on Buffaloes

2.3.2.1 Experimental animals:

The study was conducted at buffalo's farm in Central Institute for Research on Buffaloes (CIRB) during period from September, 2018 to March, 2019. One hundred and sixty female Murrah buffaloes with average age of 2- 11 years were used

The animals were maintained in a semi intensive housing system at CIRB animal farm and were fed a balanced ration consisting of green fodder, wheat straw and concentrates with specific mineral mixture developed by the institute.

2.3.2.2 Determination of the animal's body condition scoring:

Animal body condition scoring was determined according to the method descriped by (Anitha *et al*., 2011) from 1-5 score.

2.3.2.3 Trans-rectal ultrasonography examination:

Ultrasonography was performed using a portable real-time B-mode transrectal ultrasound scanner (PVF -738F, TOSHIBA, SSA220 Japan) with an intra operative 7.0 MHz micro convex transducer. Each ovary was scanned at several planes by maneuvering the transducer along its surface to identify the ovarian structures. Positions and sizes of follicles (≥ 3mm) were

measured after freezing the image using inbuilt calipers. However, follicles < 3mm were counted by seeing the image on the ultrasound screen appears as black circular structure surrounded by echogenic ovarian tissues. Follicular population, localization of CL and uterus structure were examined and the diagnosis was made on basis of echogenicity of image. Pregnancy was confirmed by the presence of a visible fetus on the viewing screen of the ultrasound machine which was irregularly shaped echogenic structure surrounded by non echogenic black color image in the uterus (Sharma *et al*., 2012).

2.4 Statistical analysis:

The BCS, age, pregnancy, CL, ovary localization, ovary weight and ovary size were expressed as mean ± SE. The Data were analyzed using SPSS (Statistical Package for Social Sciences) Version 18.The difference between means were detected by ANOVA and Duncan's Multiple Range Test. The differences were significant at $P < 0.05$ (Kouamo *et al*., 2014). While, the effect of CL on oocytes recovery, oocytes index, cleavage and blastocyst rates were determined as percentage values according to method described by (Shabankareh *et al*.,2015).

CHAPTER III

RESULTS

3.1 Effect of non ovarian factors on follicular population:

The Effect of non ovarian factors on follicular population is presented in Table 3.1. The mean number of small follicles in animals having BCS 4.0 (7.35±0.69) were significantly higher (P<0.05) than those of animals having BCS 3.0 (4.47±0.64). However, no significant difference was observed between medium and large follicles in animals having BCS of 3 and 4. The total number of follicles per ovary was significantly greater (P<0.05) in animals having BCS 4 (9.85±0.74) as compared to those having BCS 3 animals 6.78±0.69. Age of animal did not have any significant difference effect on follicular population. Number of medium follicles was significantly higher (P<0.05) in pregnant animals when compared with non-pregnant cyclic animals. However, there was no significant difference in the number of small follicles, large follicles and total number of follicles between pregnant and non-pregnant animals.

3.2 Effect of presence and absence of CL and ovary localization on follicular population:

The effect of presence and absence of CL and ovary localization on follicular population is presented in Table 3.2. The ovaries having CL had more number of medium size follicles (1.95±0.17) as compared to ovary

without CL (1.39±0.18)and difference was significant (P<0.05). There was no significant difference in the total number of follicles present in both ovaries. The right ovary was having significantly lower (P<0.05) number of large follicles than left ovary (0.28±0.04 vs.0.43±0.05). This decrease has no effect on the total number of follicle per ovary. No significant differences were observed between numbers of small follicle, large follicle and ovary localization at the present study.

3.3 Effect of presence and absence of CL, ovary weight and size on follicular population:

The effects of the presence and absence of CL, weight and size of the ovary on follicular population are presented in Table 3.3. No significant difference was observed between ovaries with CL and ovaries without CL on follicular population. The number of small (1.76±0.37) and large follicles (0.41±0.51) was found to be significantly higher (P<0.05) numbers in ovaries having weight more than 5g as compared to ovaries weight between 3-5g and less than 3g. No significant difference was observed between the number of medium follicles and ovaries weight. The average number of follicles per ovary was significantly higher (P<0.05) in ovaries having weight more than 5g (2.59±0.48) as compared to ovaries had weight between 3-5g (2.22±0.14) and less than 3g (1.80±0.10) respectively. Upon comparison of ovary size, we found that the number of small follicles in large size ovaries (1.43±0.12) was significantly higher (P<0.05) as compared

to small size ovaries (0.90±0.10). Overall, the average number of follicles per ovary in large size ovaries (2.25±0.13) was shown significantly higher ($P<0.05$) than small size ovaries (1.76±0.10). Otherwise no significant difference was observed between number of medium, large follicles and ovary size.

3.4 Effect of presence and absence of CL on oocytes grades:

The effect of the presence and absence of CL on oocyte grade is presented in table 3.4. The results showed that there is no significant difference observed between presence and absence of CL and oocytes grades.

3.5 Effect of CL on oocytes recovery, oocytes index, cleavage and blastocyst rates:

The effect of CL on oocytes recovery, oocytes index, cleavage and blastocyst rates are presented in table 3.5. The result showed the mean number of oocytes recovered obtained from ovaries with CL (145) was lower than that of ovaries without CL (335). The mean number of cleavage obtained from ovaries with CL (23) was lower as compared with those without CL (55). The oocytes recovery rate per ovary, oocytes index, percentages of cleavage and blastocyst were not different between groups.

3.6 Effect of CL stages on ovary weight and size:

The effect of CL stages on ovary weight and size were presented in table 3.6. The results showed significantly difference ($P<0.05$) in type of CL groups with ovary weight and ovary size. Ovaries having CL in late stage showed highest mean of ovary weight (5.03±0.17), length (2.35±0.05), width (1.74±0.05) and thickness (1.48±0.03) over the CL in early and middle stage.

3.7 Effect of CL stages on follicular population:

The **Effect of CL stages** on follicular population is presented in table 3.7. The results showed no significant difference between type of CL and follicular population.

3.8 Effect of CL stages on oocytes grades:

The **Effect of CL stages** on oocytes grades is presented in table 3.8. The results showed no significant difference between type of CL and oocytes grades.

Table 3.1: Effect of BCS, age and pregnancy on follicular population (in vivo) in murrah buffaloes:

Factors		NO. of animals	Mean ± SE Number of follicles			
			Small	Medium	Large	Total of follicle No / ovary
BCS	Good (3)	72	4.47±0.64[a]	1.64±0.16	0.67±0.07	6.78±0.69[a]
	V. Good (4)	88	7.35±0.69[b]	1.77±0.18	0.73±0.08	9.85±0.74[b]
	P < 0.05	**-**	**0.00**	**0.59**	**0.58**	**0.00**
Age (years)	2 - 5	123	5.55±0.56	1.63±0.14	0.66±0.06	7.85±0.58
	6 - 12	37	7.11±1.02	1.92±0.28	0.84±0.14	9.86±1.14
	P < 0.05	**-**	**0.18**	**0.33**	**0.16**	**0.10**
Pregnancy	Pregnant	80	5.79±0.72	1.96±0.17[b]	0.73±0.08	8.48±0.80
	Non-pregnant	80	5.70±0.65	1.48±0.19[a]	0.71±0.07	7.89±0.66
	P < 0.05	**-**	**0.92**	**0.05**	**0.91**	**0.57**

a,b,c in each column different letters (a, b) indicated significant difference between group ($p<0.05$). N=number of buffalo cow

NO = Number SE = Standard Error

Table 3.2: Effect of CL and ovary localization on follicular population (in vivo) in murrah buffaloes:

Factors		NO. of animals	Mean ± SE Number of follicles			Total of follicle No / ovary
			Small	Medium	Large	
CL	Present	80	5.81±0.73	1.95±0.17[b]	0.73±0.09	8.49±0.80
	Absent	80	5.76±0.65	1.39±0.18[a]	0.74±0.08	7.89±0.66
	P < 0.05	**-**	**0.95**	**0.02**	**0.91**	**0.56**
Ovary localization	Right	160	3.06±0.26	0.81±0.08	0.28±0.04[a]	4.15±0.27
	Left	160	2.72±0.26	0.91±0.09	0.43±0.05[b]	4.05±0.29
	P < 0.05	**-**	**0.36**	**0.44**	**0.01**	**0.80**

a,b,c in each column different letters (a, b) indicated significant difference between group ($p<0.05$). N=number of buffalo cow
NO = Number SE = Standard Error

Table 3.3: Effect of CL, ovary weight and ovary size on follicular population (in vitro) in Indian buffaloes:

			Mean ± SE Number of follicles			
Factors		**No. of ovaries**	**Small**	**Medium**	**Large**	**Average number of follicle /ovary**
CL	Present	109	1.26± 0.14	0.52 ±0.09	0.28±0.05	2.06±0.15
	Absent	187	1.15 ±0.1	0.56±0.07	0.30±0.04	2.01±0.11
	P < 0.05	**-**	**0.51**	**0.74**	**0.81**	**0.76**
Ovary weight(g)	<3	147	1.01±0.11[c]	0.57±0.08	0.22±0.04[c]	1.80±0.10[c]
	3-5	132	1.32±0.12[b]	0.54±0.09	0.36±0.05[b]	2.22±0.14[b]
	>5	17	1.76±0.37[a]	0.41±0.26	0.41±0.51[a]	2.59±0.48[a]
	P < 0.05	**-**	**0.03**	**0.80**	**0.05**	**0.01**
Ovary size(cm)	Small size	134	0.90±0.10[b]	0.60±0.08	0.26±0.04	1.76±0.10[b]
	Large size	162	1.43±0.12[a]	o.51±0.08	0.32±0.05	2.25±0.13[a]
	P < 0.05	**-**	**0.00**	**0.42**	**0.34**	**0.00**

(a, b) indicated significant difference between group (p<0.05). No =number CL = corpus luteum SE = Standard Error g = gram cm = centimeter

Table 3.4: Effect of CL on oocytes grades (in vitro) in Indian buffaloes:

Factors		No. of ovaries	Mean ± SE Oocytes grades A	B	C	D	Selected oocytes for IVEP. A and B
CL	Present	109	0.25±o.11	0.41±0.18	0.62±0.38	0.05±0.03	0.66±0.28
	Absent	187	0.27±0.19	0.84±0.60	0.68±0.50	0.00	1.11±0.78
	P < 0.05	**-**	**0 .94**	**0.59**	**0.93**	**0.06**	**0 .67**

No=number SE = Standard Error CL = corpus luteum

Table 3.5: Effect of CL on oocytes recovery, oocytes index, cleavage and blastocyst rates (in vitro) in Indian buffaloes:

Factors		No. of ovaries	No. of Oocytes recovery	Oocytes recovery rate / ovary	Oocytes Index	No. of Oocytes for IVEP. A and B	No. of Cleavage	Cleavage rate %	No. of Blastocyst	Blastocyst rate %
CL	Present	109	145	1.3	2.35	72	23	31.9	1	1.3
	Absent	187	335	1.7	2.23	207	55	26.6	1	0.5

No= number % = percent CL = corpus luteum

Table 3.6: effect of stage of CL on ovary measurements (in vitro) in Indian buffaloes:

Factors		No. of ovaries	Weight	Mean ± SE of ovary measurements Length	Width	Thickness
CL	Early stage	26	3.38 ± 0.24^{c}	2.09 ± 0.09^{c}	1.41 ± 0.06^{c}	1.20 ± 0.02^{c}
	Middle stage	23	4.03 ± 0.24^{b}	2.14 ± 0.09^{b}	1.54 ± 0.06^{b}	1.47 ± 0.04^{b}
	Late stage	60	5.03 ± 0.17^{a}	2.35 ± 0.05^{a}	1.74 ± 0.05^{a}	1.48 ± 0.03^{a}
	P < 0.05	**-**	**0.00**	**0.01**	**0 .00**	**0 .00**

(a, b) indicated significant difference between group ($p<0.05$) No =number SE = Standard Error
CL = corpus luteum

Table 3.7: Effect of CL stages on follicular population (in vitro) in Indian buffaloes:

			Mean ± SE Number of follicles			
Stages of CL		**No. of ovaries**	**Small**	**Medium**	**Large**	**Average number of follicle /ovary**
CL	Early stage	26	1.46±0.30	0.46±0.16	0.31±0.11	2.23±0.29
	Middle stage	23	1.13±0.29	0.61±0.18	0.26±0.14	2.00±0.35
	Late stage	60	1.22±0.18	0.52±0.13	0.28±0.06	2.02±0.20
	P < 0.05	-	**0 .69**	**0 .85**	**0 .95**	**0 .82**

No =number CL = corpus luteum

Table 3.8: Effect of CL stages on oocytes grades (in vitro) in Indian buffaloes:

Stages of CL		No. of ovaries	Mean ± SE of Oocytes grades				
			A	B	C	D	Selected Oocytes for IVEP. A and B
CL	Early stage	26	0.23±0.20	0.46±0.34	0.50±0.46	0.12±0.12	0.69±0.53
	Middle stage	23	0.39±0.27	0.52±0.38	0.35±0.27	0.09±0.09	0.91±0.64
	Late stage	60	0.20±0.15	0.35±0.25	0.78±0.65	0.0	0.55±0.39
	P < 0.05	-	**0 .79**	**0 .92**	**0.89**	**0 .29**	**0.88**

No=number SE = Standard Error CL = corpus luteum

CHAPTER IV

DISCUSSION

The present study demonstrated that the average number of small follicles were significantly higher ($P < 0.003$)in animals that had BCS 4.0 as compared to those having BCS 3.0. However, there was no significant difference in average number of medium and large follicles between these two groups. The results are similar to that found in cow (Kouamo *et al*., 2014). This has been attributed to low blood concentration of growth hormones such as Insulin like growth factor -1 (IGF-1). High plasma levels of IGF-1 resulting from improved nutrition, increases the sensitivity of granulosa cells to FSH stimulation (O'Callaghan and Boland, 1999). Ryan *et al*. (1994) also found a relationship between the blood concentration of IGF-1 and fat or thin animals presented low concentrations of IGF-1. The total number of follicles per ovary was significantly greater ($P<0.05$) in animals having BCS 4 as compared to those having BCS 3 animals. This effect was owing to more number of small follicles in the group. On the other hand the effect of age on follicular population did not show any significant difference between animals. This may be related to the age of the animals which between 2-5 years were heifers and have BCS grade 3. The results are similar to that reported in young steer and heifer's cow (Breier *et al*., 1988; Granger *et al*., 1989).The feed restriction causes a

decrease in hepatic concentration of IGF-I and lead to deceleration of follicular growth.

This study also demonstrated that the pregnancy status in buffaloes did not affect the follicular population. Further, an ovary bearing corpus luteum has more number of medium size follicles as compared to an ovary without CL. This might be due to more blood supply in the ovary and continuous follicular growth waves every 8 to 10 days without dominance during pregnancy despite the continuous production of progesterone (Ginther *et al.*, 1989). The presence of corpus luteum has a negative influence for the growth follicles larger than approximately 7mm after day 21 or 22 of pregnancy (Pierson and Ginther, 1989). The left ovary has significantly greater number of large follicles than the right one. The presence of the corpus luteum in the ovary may contribute to unfavorable condition for follicular growth. As a result, follicle regressed and led to lower COCs recovered from ovaries with a presence of corpus luteum (Hafez and Hafez, 2000).

The present study demonstrated that the number of small and large follicles were significantly higher in ovaries having a weight more than 5g as compared to ovaries weight 3-5g and less than 3g. The average number of follicles per ovary was significantly higher in ovaries having weight more than 5g as compared to ovaries had weight between 3-5g and less than 3g. This effect was due to high number of small and large follicles in

group ovaries of weight more than 5g. No significant effect was observed between number of medium follicles and ovary weight. This result agree with that of found in cow heifers (Cushman *et al.*,1999 ; Maya *et al*.,2005), indicated that the variation in ovarian weight is not only due to the weight of antral follicles and CL but there is a number of microscopic follicles and these follicles at least partially contribute to the weight of the ovary. The number of follicle per ovary was significantly higher (P <.004) in large size ovaries when compared with the small size ones. This effect was owing to more number of small follicles in group. Our results are in agreement with early report of Samad and Raza. (1999) in buffalo and Wani (1995) in sheep. This study demonstrated that no significant difference observed between presence or absence of CL and oocytes grades. The results are also similar to that reported in bovine (Kouamo *et al.*, 2014).

The mean of oocyte recovery rate obtained from ovaries with CL (1.3%) was significantly lower than that of ovary without CL (1.7%). Our results is similar to that found in buffalo by Das *et al* (1996) and Raza *et al* (2001) and who reported that the presence of a CL significantly reduces the number of ovarian follicles as well as the quality of the oocytes in buffaloes. Because the follicular development is restricted as lutein cells occupy most of the ovary (Kumar *et al*., 1997). Different studies have reported 0.4 - 3.85 good oocyte/ ovary in buffalo (Nandi *et al*., 2002). This

variation is due to the different methods used for COC recovery, seasonal effects, and variation in the reproductive status of the slaughtered buffaloes. However, when compared to cattle (10 oocyte /ovary) (Greve and Madison, 1991).The number of good oocyte/ovary in buffalo is lower which may be due to an inherently smaller number of primordial follicles and a higher frequency of atresia in buffalo (Drost, 2007).

Further, the percentages of cleavage and blastocyst were lower as compared with the number of the oocytes recovery. This result may be due to the effect of time taken on measuring the weight and size of the ovaries on the quality of oocytes.

The mean weight, length, width, thickness were significantly higher in ovaries having CL in late stage as compared with ovaries having CL in early and middle stages. This result may occur due to hyperplasty of fibroblast of the connective tissue and vascularity contributes to an increase in size of the CL (Jablonka-Shariff *et al.*, 1993).

CONCLUSION

- Murrah buffaloes with BCS 4.0 have higher number of follicles than those with BCS 3.0.
- Pregnancy increased number of the medium size follicles in Indian buffaloes.
- The right ovaries in Murrah buffaloes have lower number of large follicles than left ovaries.
- The ovaries of Murrah buffaloes having weight more than 5g contained more number of small and large follicles than those with ovary weight less than 5g.
- Absence of the CL caused high oocytes recovery rates in Indian buffaloes
- The ovaries with CL in late stage have higher weight and large size than those with CL in middle and early stage.
- Limitation of study prevented the inability to measure fertility by producing in vitro embryos.

RECOMENDATIONS

- The ovaries samples must be collected from pregnant Murrah buffaloes with a body condition score (BCS) 4.0.
- For in vitro embryo production, left ovaries should be collected because having higher number of follicles than the right ones.
- For in vitro embryo production the oocytes should be aspirated from ovaries without CL and have weight more than 5 gram.

REFERENCES

Abid, M., Muhammad, A and Sayed, M. (2011). In vitro maturation and fertilization of buffalo oocytes: the effect of recovery and maturation methods, Journal of veterinary Animal Science, 35(6): 381- 386.

Aboul-Ela, M. B. and Barkawi, A. H. (1988). Pulsatile secretion of LH in cycling buffalo heifers as affected by season and stage of estrous cycle. In: Proceedings of the 11th International Congress on Animal Reproduction and Artificial Insemination, Vol. 2, Dublin, Ireland, pp. 3.

Alves, B.G., Alves, A.K., Lucio, A.C., Martins, C.M., Silva, T.H. and Santos, R.M. (2014). Ovarian activity and oocyte quality associated with the biochemical profile of serum and follicular fluid from girolando dairy cows postpartum. Animal Reproduction Science. 1:1010-1016.

Anitha, A., Sarjan Rao, K., Suresh, J., Srinivasa Moorthy, P.R and Kotilinga Reddy, Y.(2011). A body Condition Score (BCS) system in Murrah Buffaloes. Buffalo Bulletin: 30.1.PP 79- 99.

Anurag, Anil, K., Raj, S., Diwan, S and Manoj, K. (2017). Long Term Trends in weather parameters at Hisar (Haryana): A Location in semi arid region of North West India: International Journal of Recent Scientific Research Vol. 8, Issue, 7, pp. 18087-18090.

Asad .L, A., Rahman . M ., Hossain and Akter. M. (2016). Ovarian category, follicles and oocytes analysis of goat ovaries in view of in vitro

production of embryos. International Journal of Animal Resources. 1:27 - 34.

Baki Acar, D., Birdane, M.K., Dogan, N. and Gurler, H. (2013). Effect of the stage of oestrus cycle on follicular population oocyte yield and quality, and biochemical composition of serum and follicular fluid in Anatolian water buffalo. Animal Reproductive Science. 137, 8-14.

Barkawi, A., Khattab, R. M., Mokhless, E. M. and El-Wardani, M. A. (1996). Patterns of ovarian activity influencing calving interval of Egyptian buffaloes in relation to season of calving, Bulgarian Journal of Agricultural Science, 2 : 49–53.

Baruselli, P. S., Mucciolo, R. G., Vistin, J. A., Viana, W. G., Arruda, R. P., Maduriera, E. H., Oliveira, C. A. and Molero- Filho, J. R. (1997). Ovarian follicular dynamics during estrus cycle in buffalo. Theriogenology, 47 : 1531-47.

Baruselli, P. S. (2001). Control of follicular development applied to reproduction biotechnologies in buffalo. In: Proceedings of the National Congress on Buffalo Breeding, Italy, Book of the Congress, pp.128–146.

Bavister, B. D., Rose-Hellekant, T. A. and Pinnyomminter, T. (1992). Development of in vitro and matured in vitro fertilized bovine embryos into morula and blastocyst in defined culture media. Theriogenology, 37:127-146.

Bavister, B.D. (1995). Culture of preimplantation embryos: facts and artifacts. Human Reproduction Update, 1: 91–148.

Beal, W.E., Perry, R.C and Corah, L .R. (1992). The use of ultrasound in monitoring reproductive physiology of beef cattle. Journal Animal Science;70: 924-929.

Blond, L and Buczinski, S. (2009). Basis of Ultrasound Imaging and the Main Artifacts in Bovine Medicine. Veterinary Clinical North American Food Animal Practice; 25:553-565.

Borghese A .(2005). Buffalo production and research. FAO Regional Office for Europe, REU Technical Series 67: 1-315.

Brackett, B.G. (1982). Normal development following in vitro fertilization in cow. Biology of Reproduction, 27:147-158.

Brackett, B.G. and Zuelke, K.A. (1993). Analysis of factors involved in the in vitro production of bovine embryos. Theriogenology, 39:43-64.

Brar, P. S and Nanda, A. S. (2004). Impact of conventional managemental practices on reproductive performance of rural buffaloes. Indian Journal of Animal Reproduction, 25: 94–96.

Breier, B.H., Gluckman, P.D and Bass, J.J .(1988) Influence of nutritional status and oestradiol-17b on plasma growth hormone, insulin-like growth factors-I and -II and the response to exogenous growth hormone in young steers. Journal Endocrinolology; 118: 243-250.

Bukar, M.M., Amin, J.D., Sivachelvan, M.N and. Ribadu, A.Y.(2006). Postnatal histological development of the ovaries and uterus and the attainment of puberty in female kid goats. Small Ruminant, Research, 65:200-208.

Carriere, P.D., Des Coteaux, L. and Durocher, J .(2002). Echographic evaluation of the bovine reproductive tract: Normal and abnormal development of ovarian follicles and corpus luteum, Veterinary Surgeons of Quebec, 32, 128-131.

Cerri, R. L A, Rutigliano, H. M., Chebel, R. C and Santos, J. E P (2009). Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. Reproduction. 137: 813 – 823.

Chantalakhana, C. and Falvey, L. (1999). Small Holder Dairying in the Tropics. International Livestock Research Institute (ILRI), Nairobi, Kenya. 462p.

Chatot, C.L ., Ziomek, C.A ., Bhavister, B.D., Lewis, J.L. and Torres, I. (1989). An improved culture medium supports development of random bread cell mouse embryos in vitro. Journal of Reproduction and Fertility, 86: 679-688.

Cockrill, W. (1974). The husbandry and health of the domestic buffalo .FAO, Rome 1 – 991.

Cushman, R.A., Desouza ,J.C., Hedgpeth, V.S and Britt, J.H.(1999). Superovulatory response of one ovary is related to micro- and

macroscopic population of follicles in contra lateral ovary of cow, Biology of reproduction. 60: pp349-354.

Cushman, R. A., Allan, M. F., Kuehn, L. A., Snelling, W. M ., Cupp, A. S. and Freetly, H. C.(2009). Evaluation of antral follicle count and ovarian morphology in crossbred beef cows: Investigation of influence of stage of the estrus cycle, age, and birth weight, Journal of Animal Science. 87: 1971- 1980.

Das, G. K., Jain, G. C., Solanki, V. S. and Tripathi, V. N. (1996). Efficacy of various collection methods for oocyte retrieval in buffalo. Theriogenology.46: 1403-1411.

de Loos, F., van Maurik, P., van Beneden, T and Kruip, T.A.M. (1992). Structural aspects of bovine oocyte maturation in vitro. Molecular Reproductive Development; 31: 208-214.

Deleuze, S., Goudet, G., Caillaud, M., Lahuec, C and Duchamp, G. (2009). Efficiency of embryonic development after Intra-Follicular and Intra-Oviduct transfer of in vitro and in vivo matured horse oocytes. Theriogenology, 72: 203-209.

DesCoteaux, L, Gnemmi, G and Colloton, J. (2006). Ultrasonography of the bovine female genital tract. Veterinary Clinical North American Food Animal Practice; 25:733-752.

Dharmendra, K ., Anand, T., Singh, K. P., Singh, M. K., Shah., R. A., Chauhan, M. S., Palta, P., Singla, S. K and Manik ,R. S. (2011) . Derivation of

buffalo embryonic stem-like cells from in vitro-produced blastocyst on homologous and heterologous feeder cells .Journal of Assisted Reproductive Genetic: 28:679–688.

Donnay, I., De Roover, R., Van Langendonckt, A., Massip, A. and Dessy, F. (1997). Overall efficiency of an experimental ovum pick-up program in cattle. Theriogenology, 47: 155.

Drost, M.(2007). Bubaline versus bovine reproduction. Theriogenology; 68: 447-449.

Edmondson, A.J., Fissore, R .A and Pashen, R.L,.(1986). The use of ultrasonography for the study of the bovine reproductive tract.Normal and pathological ovarian structures. Animal Reproductive Science;12:157-165.

El-Wishy, A. B. (2007). The postpartum buffalo endocrinological changes and uterine involution. Animal Reproduction Science, 97: 201–215.

Enchaparambil, M., Sadeesh , F. S., Ashok k. B ., Sumangali, M.K., Thirumaran , S. Y and Prem S. Y. (2014). Effect of growth factor and antioxidant on in vitro maturation of oocytes and cleavage rates of in vitro produced Indian buffalo (Bubalus bubalis) embryos. Veterinarski Arhiv , 84 (5):459-474.

Eyestone, W .H. and First , N.L. (1989). Co-culture of early cattle embryos to the blastocyst stage with oviduct tissue or in conditioned medium. Journal of Reproduction Fertility, 85: 715-720.

Fields, M.J. and Fields, P.A (1996). Morphological characteristics of the bovine corpus luteum during the estrus cycle and pregnancy. Theriogenology ,45: 1295–1325.

First, N. L. and Parrish, J .J. (1987). In vitro fertilization of ruminants. Second Int. Symposia. On Reproduction. in Domestic Ruminant. Journal of Reproduction Fertility, 34: 151-165.

Fissore, R.A., Edmondson, A.J and Pashen, R.L,(1986). The use of ultrasonography for the study of the bovine reproductive tract. II. Non-pregnant, pregnant and pathological conditions of the uterus. Anim Reprod Sci;12:167-177.

Fitzpatrick, L. A. and Entwistle, K. W. (1997). A comparison of dissected follicle numbers and follicle counts on the ovarian surface for the evaluation of ovarian follicular populations in Bos indicus cows. Animal Reproduction Science. 46: 179-186.

Gaedner, D.K. (1998). Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. Theriogenology, 49: 83-102.

Galli, C.,Crotti.G., Notari, C., Turini, P., Duchi, R and Lazzari, G. (2001). Embryo production by ovum pick-up from live donors. Theriogenology, 55: 1341–1357.

Galli, C. and Lazzari, G. (2008). The manipulation of gametes and embryos in farm animals. Reproductive In Domestic Animals, 43: 1-7.

Gandhi, A.P., Lane, M., Gradner, D .K and Krisher, R. L.(2000). A single medium supports development of bovine embryo throughout maturation, fertilization and culture. Human Reproduction, 15(2): 395-401.

Gilchrist, R. B. and Thompson, J. G. (2007). Oocyte maturation: Emerging concepts and technologies to improve developmental potential in vitro Theriogenology. 67: 6-15.

Ginther, O.J., Knopf, L. and Kastelic, J.P. (1989).Ovarian follicular dynamics in heifers during early pregnancy. Biology Reproduction. 41, 247-254.

Ginther, O.J.(1998). Ultrasonic imaging and Animal reproduction: Color Doppler Ultrasoundgraphy, Book 4. WI. USA.: Publishing 4343.

Ginther, O.J and Utt, M.D.(2004). Doppler ultrasound in equine reproduction: principles, techniques, and potential. Journal Equine Veterinary Science;24:516-526.

Ginther, O.J., Bashir, S.T., Santos, V.G. and Beg, M.A. (2013). Contralateral ovarian location between the future ovulatory follicle and extant corpus luteum increases the length of the luteal phase and number of follicular waves in heifers. Theriogenology 79 (7): 1130-1138.

Glatzel, P.S., Ali, A and Gilles, M. (2000). Diagnosis of early pregnancy in 30 water buffalo heifers by transrectal palpation with and without ultrasonography. Tier¨arztliche Umschau ; 55:329–332.

Gordon, I. (1994). Oocyte recovery and maturation. In: Laboratory Production of Cattle Embryos. Biotechnology in Agriculture. U.K. CAB International; 20-142.

Granger, A.L, Wyatt, W.E, Craig, W.M and Thompson, D.L, Hembry, F.G.(1989). Effects of breed and wintering diet on. growth, puberty and plasma concentrations of growth hormone and insulin like growth factor I in heifers. Domestic Animal Endocrinology; 6: 253-262.

Gransworthy, P.C. (1988). The effect of energy reserves at calving on performance of dairy cows, 157-170.

Gregoraszczuk,E.L. (1994). Is progesterone a modulator of luteal steroidogenesis in pig A tissue culture approach. Folia. Histochem. Cytobiology. 32:31–3.

Greve, T. and Madison, V. (1991) In vitro fertilization in cattle: a review. Reproduction Nutrition. Development; 31: 147-157.

Hafez, E. S and Hafez, E.B. (2000).Folliculogenesis, egg maturation, and ovulation. Reproduction in Farm Animals, 7th Edition, 68-81.

Hernandez-Fonseca, H.J., Sirisathien, S., Bosch, P., Cho, H.S., Lott, J.D., Hawkins, L.L., Hollett, R.B., Coley, S.L. and Brackett, B.G. (2002). Offspring resulting from direct transfer of cryopreserved bovine embryos produced in vitro in chemically defined media. Animal Reproductive. Science 69:151-158.

Herrera, P., Campo, E.and Denis R. (2007).Relationship between the size of structures fetal and time of pregnancy in river buffaloes. Rev Salud Anim; 29: 28-31.

Holt, W.V., Hernandez, M., Warrell, L., and Satake, N. (2010).The long and the short of sperm selection in vitro and in vivo: swim-up techniques select for the longer and faster swimming mammalian sperm. Journal of Evolution Biology 23(3): 598-608.

Honparkhe, M., Gandotra, V.K and Nanda, A.S.(2004). Ultrasonographic Measurements in Comparison with the Rectal Palpation and Echotexture of Reproductive Organs of Buffaloes (Bubalus bubalis) during Different Stages of the Estrous Cycle. Asian Australian Journal Animal Science;17:919-923.

Hornsveld, M. (1996). Applied anatomical aspects of the African buffalo. In BL Penzhorn, (Ed.), Proceedings of a symposium on the African Buffalo as a game ranch animal. Pretoria: Wildlife Group of the South African Veterinary Association. pp 6-20.

Huang, Y.J. and Rosenwarks, Z. (2012). In vitro fertilization treatment and factors affecting success. Best Practice Resource. Clinical Obstetric. Gynecology. 26: 777-788.

Jablonka-Shariff, A., Grazul-Bilska, A.T., Redmer, D.A., Reynold, L.P., (1993). Growth and cellular proliferation of ovine corpus luteum throughout the estrous cycle. Endocrinology, 133:1871-1879.

Jainudeen, M. R. (1988). Reproduction problems of buffaloes in the world, Proceedings of the Second World Buffalo Congress, vol. II New Delhi, India:189–196.

Jefferies, B.C. (1961). Body condition scoring and its use in management. Tasmanian Journal of Agriculture 32: 19-21.

Kahn, W.(1990). Sonographic managing of the bovine fetus. Theriogenology; 33:2.

Karen, A., Darwish, S and Ramoun, A . (2007). Accuracy of ultrasonography and pregnancy associated glycoprotein test for pregnancy diagnosis in buffaloes. Theriogenology; 68:1150-1155.

Karen, M. A., Darwish, S., Ramoun A .(2011). Accuracy of transrectal palpation for early pregnancy diagnosis in Egyptian buffaloes. Tropical Animal Health Production; 43:5 -11.

Kaulfuss, K. H., Richter, A. and Schulz, J. (1994). Pattern of ovarian follicle growth during the oestrus cycle in Mutton merino sheep monitored by laparoscopy. Reproduction. Domestic Animal. 29: 22-37.

Kepkova, K.V., Vodicka, P., Toralova, T., Lopatarova, M., Cech, S., Dolezel, R., Havlicek, V., Besenfelder, U., Kuzmany, A., Sirard, M.A., Laurincik, J., and Kanka, J. (2011). Transcriptomic analysis of in vivo and in vitro produced bovine embryos revealed a developmental change in cullin 1 expression during maternal-to-embryonic transition. Theriogenology 75(9): 1582-95.

King, A .M.(2006). Development, advances and applications of diagnostic ultrasound in animals. The Veterinary Journal; 171:408-420.

Kouamo . J, Dawaye. S.M , Zoli. A.P. and Bah. G.S. (2014). Evaluation of bovine (*Bos indicus*) ovarian potential for in vitro embryo production in the Adamawa plateau (Cameroon), Open Veterinary Journal, 4(2): 128-136.

Krisher, R.L., Lane, M. and Bavister B.D. (1999). Developmental competence and metabolism of bovine embryos cultured in semi-defined and defined culture media. Biology Reproduction, 60: 1345–1352.

Kumar, A., Solanki, V. S., Jindal, S. K., Tripathi, V. N. and Jain, G. C. (1997). Oocyte retrieval and histological studies of follicular population in buffalo ovaries. Animal reproduction science. 47: 189-195.

Kumar, D., Palta, P., Manik, R .S., Singla, S. K and Chauhan, M. S. (2007). Effect of culture media and serum supplementation on the development of in vitro fertilized buffalo embryos. Indian Journal of Animal Science **77**: 697–01.

Kumar, D and Anand, T.(2012). In Vitro Embryo Production in Buffalo: Basic Concepts. Journal of Buffalo Science, 1:50-54.

Lauderdale J. W. (2009). ASAS Centennial Paper: Contributions in the Journal of Animal Science to the development of protocols for breeding management of cattle through synchronization of estrus and ovulation. Journal Animal Science. 87(2): 801 - 812.

Lee, E.S., Fukui, Y., Lee, B.C., Lim, J.M. and Hwang, W.S. (2004). Promoting effect of amino acids added to a chemically defined medium on blastocyst formation and blastomere proliferation of bovine embryos cultured in vitro. Animal Reproduction science, 84: 257-267.

Linder, G.M .M. and Wrigth,R.W. (1983). Bovine embryo morphology and evaluation .Theriogenology, 20:407- 416.

Lonergan, P., O'Kearney-Flynn, M., Boland, M.P.(1999). Effect of protein supplementation and presence of an antioxidant on the development of bovine zygotes in synthetic oviduct fluid medium under high or low oxygen tension. Theriogenology, 51:1565-1576.

Lowman, B.G., Scott, N.A and Sommerville, S.H. (1976). Condition scoring of cattle. Bulletin East of Scotland College of Agriculture, No. 6.

Madan, M. L. (1988). Status of reproduction in female buffalo. In: Buffalo Production and Health: a compendium of latest research information based on Indian studies, ICAR Publication, New Delhi, India :89–100.

Mahadevan, M and Baker, G (1984).Assessment and preparation of semen for in vitro fertilization. In: Clinical In Vitro Fertilization Edited by: Wood C, Trounson A. Springer-Verlag, Berlin: 83-97

Mamy, K., Jahan, N., Asad, L., ,Hoque, S.A.M., Ahmed, S and Faruque, M.O.(2011). Qualitative and quantitative analysis of buffalo ovaries,

follicles and oocytes in view of in vitro production of embryos , Bangladesh journal of Animal Science ,409: 23-27.

Mattoon, J.S and Nyland, T.G.(2015). Fundamentals of Diagnostic Ultrasound. In Small Animal Diagnostic Ultrasound (Third Edition*)*, ed. JSMG Nyland, St. Louis: W.B. Saunders; 1-49.

Maya, M., Tsuyoshi, T., Hiromi, N., Sawa, Y., Seizo, H and Masafumi, T. (2005).The relationship between ovarian weight and follicular population in heifers, Journal of reproduction and development.51: pp 689-693.

Mcnatty, K. P., Gibb, M., Dobson, C., Ball, K., Coster, D., Heath D. and Thurley, D. C. (1982). Preovulatory follicular development in sheep treated with PMSG Assiut Journal of Agricultural Science, 40 (4):1-12 prostaglandin. Journal of reproduction and fertility 65: 111-123.

Moioli, B. and Borghese, A . (2005). Buffalo Breeds and Management Systems. Pages 51–76 in Borghese, A. (ed.) Buffalo Production and Research. REU Technical Series 67. Inter-regional Cooperative Research Network on Buffalo, FAO Regional Office for Europe, Rome.

Munjunatha, B. M. and Devaraj, M. (2006). Ovum pickup and in vitro embryo production in buffaloes and cattle: an alternative to the conventional embryo production approach. ICAR Winter School on Reproductive Techniques to Augment Fertility in Dairy Animals: 96-99.

Nanda, A. S., Brar, P. S and Prabhakar, S. (2003). Enhancing reproductive performance in dairy buffalo: major constraints and achievements. Reproduction Supply, 61: 27–36.

Nandi, S., Chauhan, M. S. and Palta, P. (2001). Effect of environmental temperature on quality and developmental competence in vitro of buffalo oocytes. Veterinary Recurrent, 148: 278–279.

Nandi, S., Raghu, H.M., Ravindranatha, B.M., Chauhan, M.S.(2002). Production of buffalo (Bubalus bubalis) embryos in vitro : premises and promises. Reproduction. Domestic. Animal., ;37: 65- 74.

Nandi, S, G., Kumar, V. and Chauhan, M.S. (2006). In vitro production of bovine embryos: We need to stop or proceed - a review. Agricultural. Review. 27(2):122-129.

Nedambale T.L., Dinnyes A., Groen W., Dobrinsky J.R., Tian X.C., Yang X. (2004). Comparison on in vitro fertilized bovine embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification. Theriogenology, 62, 437–449.

Noakes, D.E., Parkinson,T.J and England, G.C. (2009). Veterinary Reproduction and Obstetrics, 9th edition, WB Saunders, Elsevier.

O'Brien, J. W.D.(2007). Ultrasound–biophysics mechanisms. Progress in Biophysics and Molecular Biology ; 93:212-255.

O'callaghan, D. and Boland, M.P. (1999). Nutritional effects on ovulation, embryo development and establishment of pregnancy in ruminants. Animal Science. 68, 299-314.

Osman, A. M. and Shehata S. H. (2005). Effect of seasons on ovarian morphology and oocytes quality in slaughtered buffaloes. Assiut Veterinary Medicine Journal. 51: 314-330.

Palta, P., Mondal, S., Prakash, B. S. and Madan, M. L. (1997). Peripheral inhibin levels in relation to climatic variations and stage of estrous cycle in Buffalo (Bubalus bubalis). Theriogenology, 47: 989–995.

Parrish, J.J. (2014). Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin. Theriogenology 81(1): 67-73.

Perera, B.M.A., beyguawardena, O. H., Vale, W.G and Chantalakhana, C. (2005). Livestock and wealth creation - Improving the husbandry of animals kept by poor people in developing countries. Chapter 22: Buffalo. Livestock Production Programme. Natural Resources International Ltd. United Kingdom :pp 601.

Peter, A.T., Jakovljevic, S and Pierson, R. A. (1992). Use of real-time ultrasonography in bovine and equine reproduction. Compend Contin Education Practice Veterinary; 14:1116–1124.

Phogat, J.B., Smith, R.F and Dobson, H. (1997). The influence of stress on neuroendocrine control of the Hypothalamic-pituitary-ovarian axis-a review. Veterinary Bulletin, 67: 551-567.

Pierson, R.A. and Ginther, O.J. (1987). Follicular populations during estrus cycle in heifers. Influences of right and left sides and intraovarian effects of corpus luteum. Animal Reproduction Science 14 :177-186.

Pierson, R. A., Kastelic, J.P and Ginther, O.J.(1988). Basic principles and techniques for transrectal ultrasonography in cattle and horses. Theriogenology 29: 3-20.

Pierson, R.A and Ginther, O.J. (1989). Intraovarian effect of the corpus luteum on ovarian follicles during early pregnancy in Heifers. Animal reproduction Science, 15:53-60.

Prakash, B. S., Sarkar, M., Paul, V., Mishra, D. P., Mishra, A and Meyer, H. H. D. (2005). Postpartum endocrinology and prospects for fertility improvement in the lactating riverine buffalo (Bubalus bubalis) Livestock. Science, 98: 13-23.

Presicce, G. A., Senatore, E. M., De Santis, G. and Bella, A. (2005). Follicle turnover and pregnancy rates following oestrus synchronization protocols in Mediterranean Italian buffaloes (Bubalus bubalis). Reproductive Domestic Animal, 40: 443–447.

Raja Rajamahendran, Divakar J., and Ambrose, B,B .(1994).Clinical and research applications of real-time ultrasonography in bovine reproduction: A review. Canadian veterinary journal.; 35: 563-572.

Rao, M.M. and Mahesh, U.Y. (2012). Efficacy of different harvesting techniques on oocyte retrieval from buffalo ovaries. Buffalo Bulletin, 31 (4): 209-213.

Raut, N. V and Kadu, M. S. (1988). Observations on ovulation and its association with fertility in Berari (Nagpuri) buffaloes. In: 11th Int. Congress on Animal Reproduction and Artificial Insemination. 3:542.

Raza, A., Samad, H. A., Rehman, N. U. and Zia, E. U. H. (2001). Studies on in vitro maturation and fertilization of Nili-Ravi buffalo follicular oocyte International Journal of Agriculture and Biology.3: 503–506.

Razdan, M. N., Kaker, M. L and Galhotra, M. M. (1981). Serum luteinizing hormone levels of non-cycling buffaloes (Bubalus bubalis). Indian Journal Animal Science, 51: 286–288.

Razzaque, W.A.A., Sahatpure, S. K., Pawshe, C.H and Kuralkar, S.V. (2008). Biometry of ovaries and follicular count in cycling and non cycling Nagpuri buffaloes (Bubalus Bubalis). Journal of Buffalo Bulletin: 27.1:150-153.

Ribeiro, H. F. L., Vale, W. G., Andrade, V. J and Marques, J. A. P. (2003). Environmental effect on the ovarian postpartum activity in the buffaloes raised in low Amazon region, Brazil. Buffalo Journal, 19: 311–321.

Rodriguez-Martinez, H. (2007). Role of the oviduct in sperm capacitation. Theriogenology 68, Supplement 1: 138-146.

Roy, D.J and Mullick, D.N.(1964). Endocrine Function Of Corpus Luteum Of Buffaloes During Estrous Cycle. Endocrinology; 75:284-287.

Ryan, D.P., Spoon, R.A., Griffith, M.K. and Williams, G.L.(1994). Ovarian follicular recruitment, granulosa cell steroidogenic potential and growth hormone/ insulin-like growth factor-I relationships in suckled beef cows consuming high lipid diets: Effect of graded differences in body condition maintained during the puerperium. Domestic Animal Endocrinology. 11, 161-174.

Saeki, k., Hoshi, M.L and Leibfried-Rutledge, N. L.(1991). In vitro fertilization and development of bovine oocytes matured in serum-free medium. Biology Reproductive, 44: 256-260.

Samad, H.A. and Raza, A. (1999). Factors affecting recovery of buffalo follicular oocytes. Pakistan Veterinary Journal. 19:pp 56-59.

Sarabia, A .S.(2004). Basic anatomy and physiology of buffalo reproduction. . Presented at Precongress training on the use of reproductive biotechniques in water buffalo. 7th World Buffalo Congress, Philippines.

Sarker, M .K. (1993). Studies on the incidence of reproductive abnormalities in female goat *(Capra hircus)*. MSc thesis. Bangladesh Agricultural University, Mymensingh.

Seidel, G.E. and Seidel, S.M. (1989). Analysis of application of embryo transfer in developing countries. Theriogenology 31(1): 3-16.

Senatore, E.M., De Santis, G and Barile V. (2002). Corpus luteum measurements, echotexture and plasma progesterone in adult and puberal Mediterranean Italian buffalo (Bubalus bubalis). Theriogenology; 57:792.

Senger, P.L. (2005). 'Pathways to pregnancy and parturition.' 2nd review. endocrinology. (Current Conceptions: Pullman, WA) : p373.

Sethi, R.K. (2003). Improving rivering and swamp buffaloes through breeding. 4th Asian Buffalo Congress.

Shabankareh, H, K., Shahsavari, M, H and Moghaddam, G. (2015). In vitro developmental competence of bovine oocytes: Effect of corpus luteum and follicle size, Iran Journal of Reproductive Medicine; 13: 10; pp: 615-622.

Shah S.N.H. (1988). Comparative studies of seasonal influence on breeding behaviour ad conception rate of dairy buffalo and zebu cattle. In: Proceedings of the 11th International CD Congress on animal reproduction and artificial insemination. 3: 538–542.

Sharma, R, K., Singh, J.K., Khanna, S., Phulia, S. K., Sarkar, S.K and Inderjeet, S. (2012). Fetal age determination in Murrah buffaloes from days 22 through 60 with ultrasonography. Indian Journal of Animal Sciences 82 (4): 374–376.

Singh, G and Singh, G. B. (1985). Effect of month of calving on postpartum estrous interval and service period in Murrah buffaloes, Proceedings of the First World Buffalo Congress, Cairo, Egypt : 960–963.

Singh, G., Singh, G. B and Dhaliwal, G. S. (1989). Studies on reproductive status of rural buffaloes in summer. Indian Journal Animal Reproduction, 10 : 151–153.

Singh, R and Nanda, A. S. (1993). Environmental variables governing seasonality in buffalo breeding. Journal of Animal Science, 71: 119.

Singh, R., Singha, S. P. S and Setia, M. S. (1993). Circulating levels of thyroidal hormones during various reproductive phases in buffaloes (Bubalus bubalis), a longitudinal study. Buffalo Bull. 12: 7–10.

Singh, J., Nanda, A. S and Adams, G. P. (2000). The reproductive pattern and efficiency of female buffaloes. Animal Reproductive Science, 60–61: 593–604.

Singh, R.L., Rao, S.K. and Murali, M. K. (2013).Oocyte retrieval methods, grade and percentage of oocytes in goats. International Journal of Molecular Veterinary Resource, 3 (2): 4-6.

Stroud, B. K.(1994). Clinical applications of bovine reproductive ultrasonography. Compend Contin Education Practice Veterinary; 16:1085–1097.

Subasinghe, D.H.A., Horadagoda , N.U., Abeygunawardena, H. and Siriwardena, J.A. de S. eds. (1998). Water Buffalo: Improved utilization through new technologies; National Science Foundation, Colombo, Sri Lanka.

Tailor, S. P., Jain, L. S., Gupta, H. K and Bhatia, J. S. (1990). Oestrus and conception rates in buffaloes under village conditions. Indian Journal of Animal Science, 60 :1020–1021.

Tailor, S. P., Banerjee, A. K., Singh, B and Pathodiya, O. P. (1997). Factors affecting reproductive performance in Surti buffaloes. Indian Journal Dairy Science, 50 : 407–409.

Trigal, B, Díez, C., Muñoz, M., Caamaño, J.N., Goyache, F., Correia-Alvarez, E., Corrales, F.J., Mora, M.I., Carrocera, S., Martin, D. and Gómez, E. (2014). Elements of functional genital asymmetry in the cow. Reproductive. Fertility. Development. 26(4) : 493-501.

Vale, W. G and Ohashi, O. M. (1994). Problems of reproduction in buffaloes. Buffalos Journal (Suppl.), 2 : 103–122.

Vanroose, G., Vansoom, A and de Kruif, A. (2001). From co-culture to defined medium: state of the art and practical considerations. Reproductive Domestic Animal, 36: 25- 28.

Vittoria, A.(1997). Anatomy of the female genital tract in the buffalo. In ThirdCourse on Biotechnology of Reproduction in Buffaloes Caserta, Italy; 15-20.

Waldron D. (1999). Age at first estrus, ovulation rate, and age at anestrus in pubertal Boer Spanish and Spanish does. Small Ruminant Research, 31: 173-176.

Wang, Z.G., Song-Dong, Y. and Zi-Rong, X. (2007). Effects of collection methods on recovery efficiency, maturation rate and subsequent embryonic developmental competence of oocytes in Holstein cow. Asian-Australian Journal of Animal. Science. 20(4): 496-500.

Wani ,A .N. (1995). In vitro maturation and in vitro fertilization of ovine oocytes. MVSc thesis, Faculty of postgraduate studies Sher-e –Kashmir University of Agricultural Science and Technology, Srinagar

Zhao, J., Whyte, J and Prather, R.S. (2010). Effect of epigenetic regulation during swine embryogenesis and on cloning by nuclear transfer. Cell Tissue Resource 341(1) : 13-21.

Zicarelli. L. (1994). Management under different environmental conditions. Buffalos Journal, 2 : 17–38.

Zicarelli, L. (1997). Reproductive seasonality in buffalo. In: Proceedings of Third International Course of Biotechnology in Buffalo Reproduction, Napoli 6-10 October, Suppl. Bubalus Bubalis, 4 : 29–52.

Zicarelli, L., Di Palo, R., Neglia, G., Ariota, B., Varricchio, E and Campanile, G. (2007). Estimation of the intercalving period in Italian Mediterranean buffalo. Proceedings of the 8th World Buffalo Congress, Caserta, October : 19-22

APPENDICES

Appendix A: Different diagrams of study.

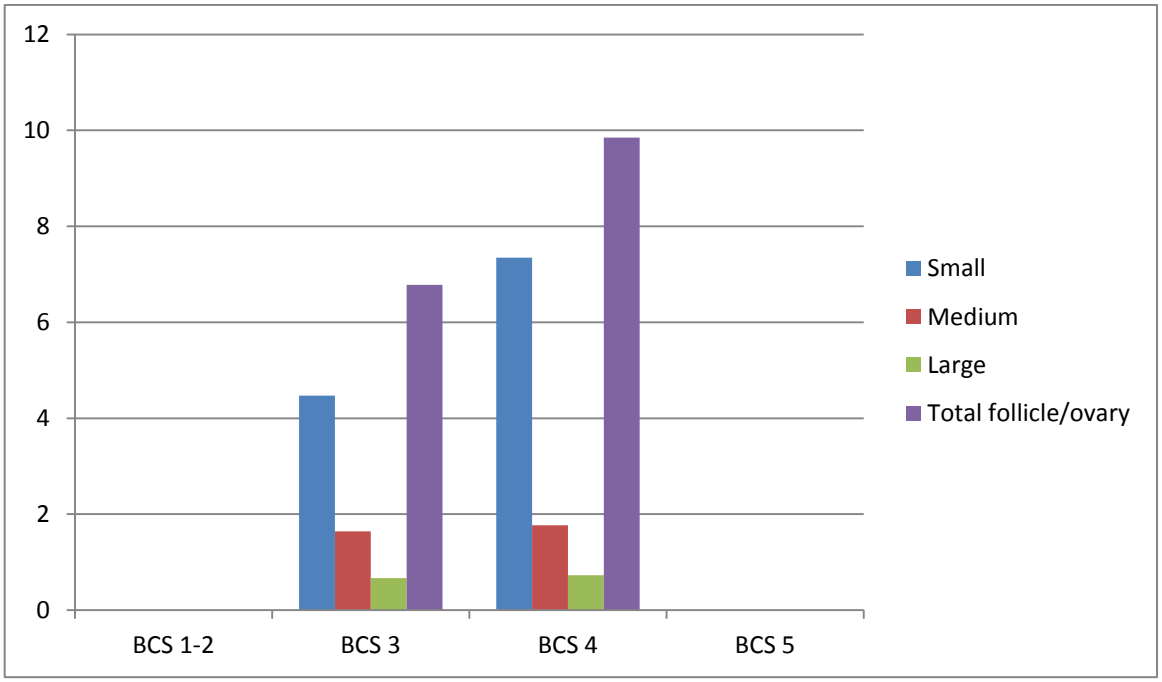


Fig -1 Relationship between BCS and follicular population in Murrah buffaloes

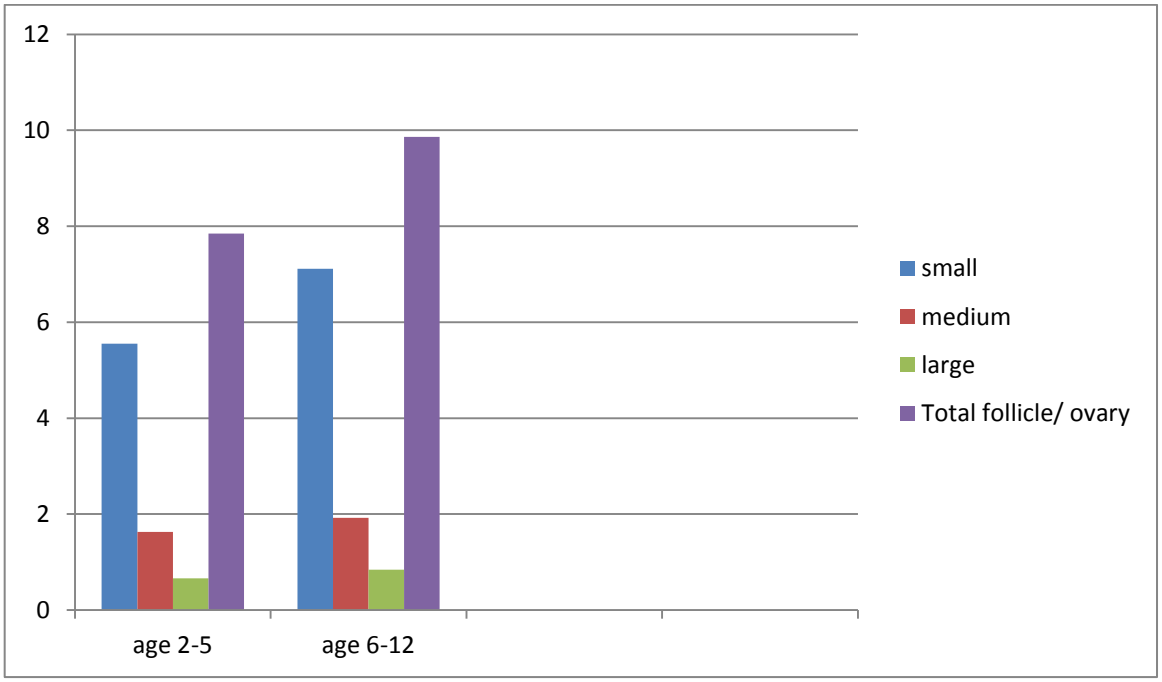


Fig – 2 Relationship between Age and follicular population in Murrah buffaloes

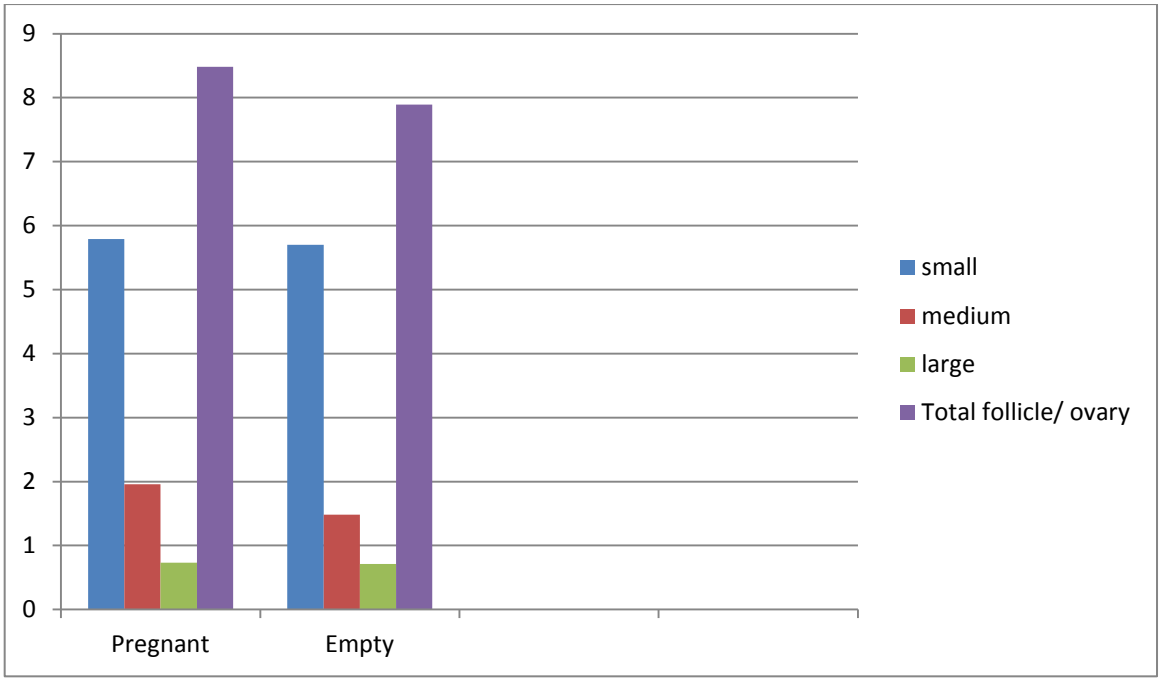


Fig -3 Relationship between pregnancy status and follicular population in Murrah buffaloes

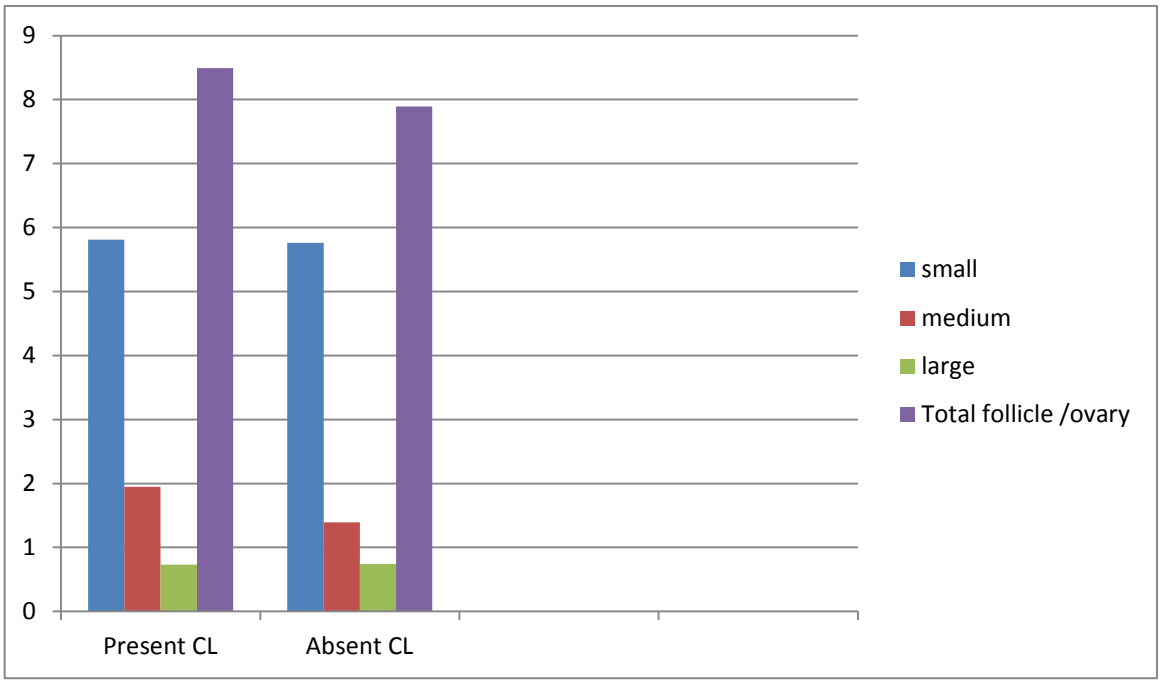


Fig -4 Relationship between present or absent of CL and follicular population in Murrah buffaloes

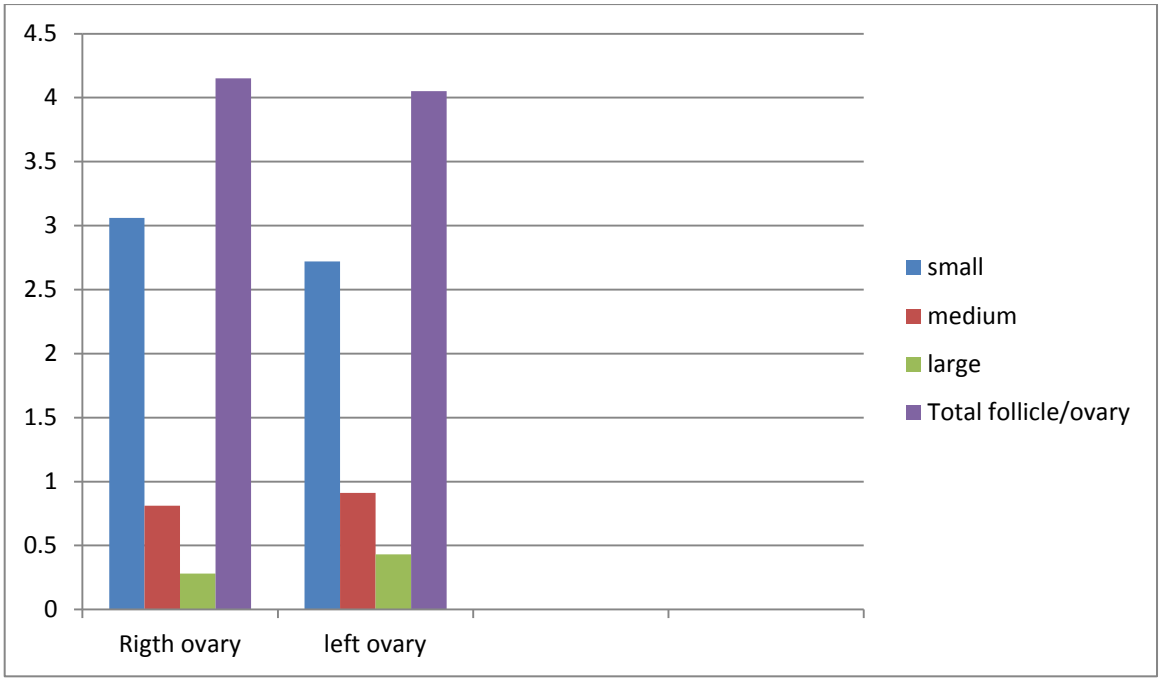


Fig -5 Relationship between ovary localization and follicular population in Murrah buffaloes

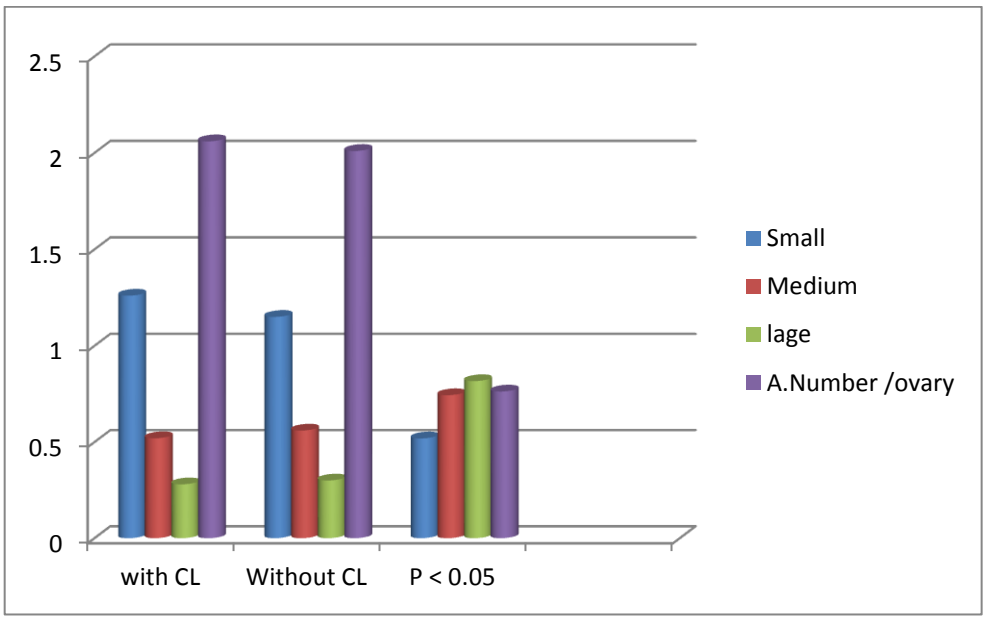


Fig – 6 Relationship between Present or absent of CL and follicular population

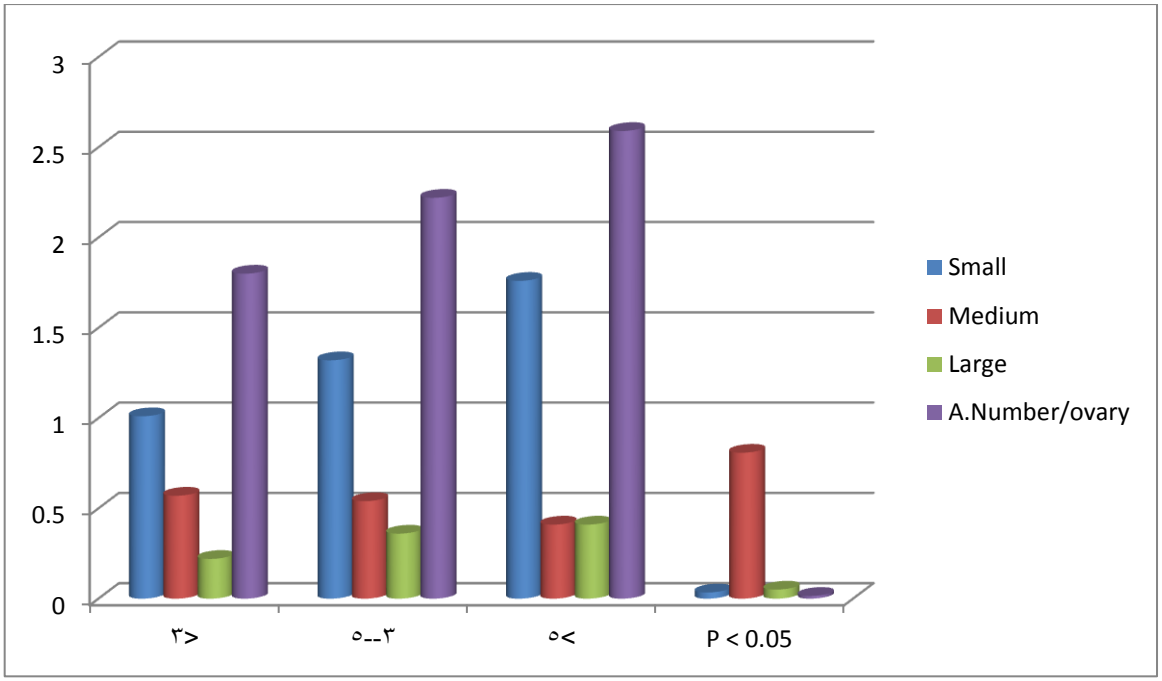


Fig – 7 Relationship between ovary weight and follicular population

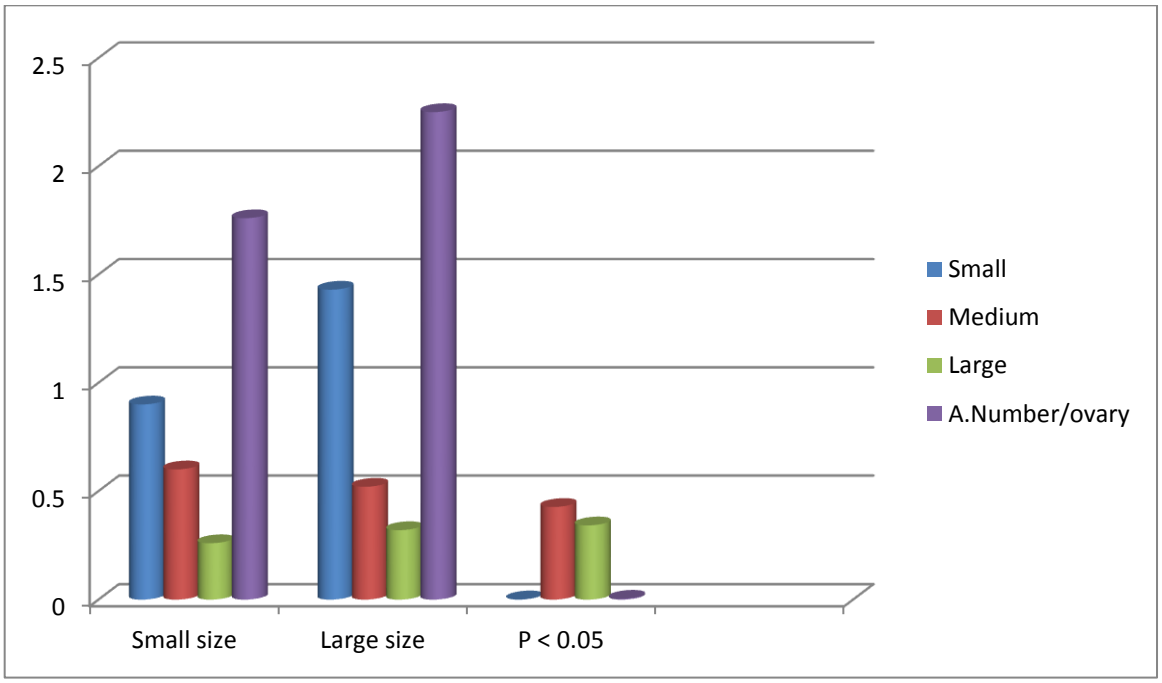


Fig – 8 Relationship between ovary size and follicular population

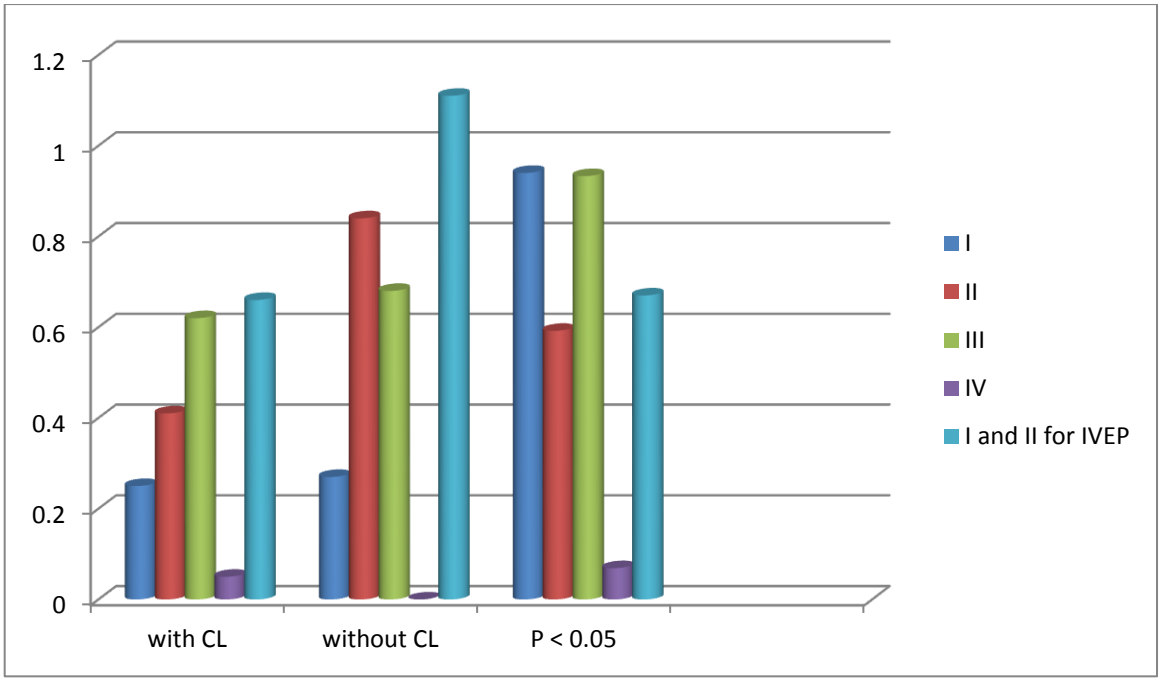


Fig – 9 Relationship between present or absent CL and oocytes grades

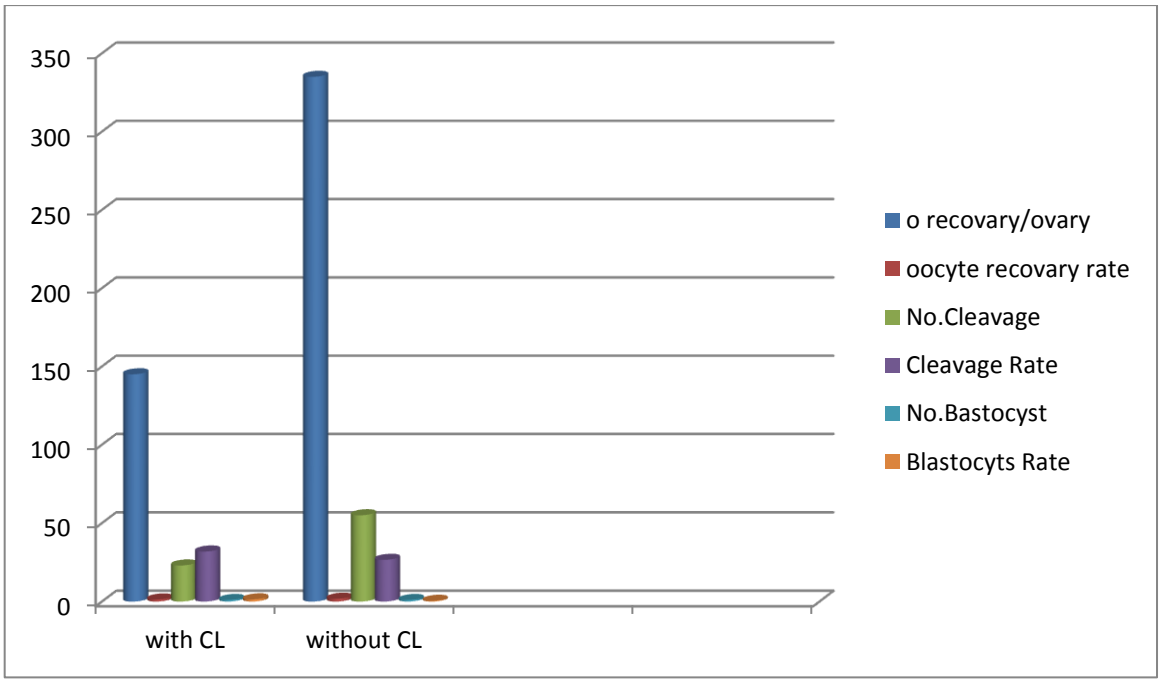


Fig- 10 Effect of CL on cleavage and blastocyst rates

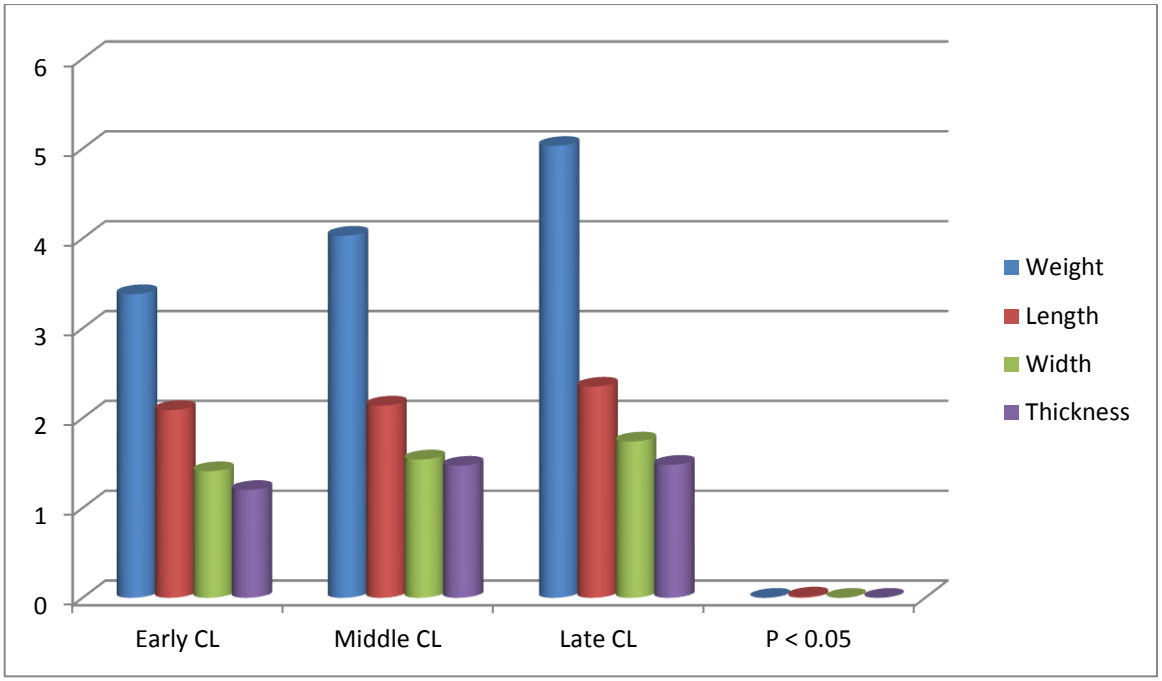


Fig-11 Relationship between types of CL, ovary weight and ovary size

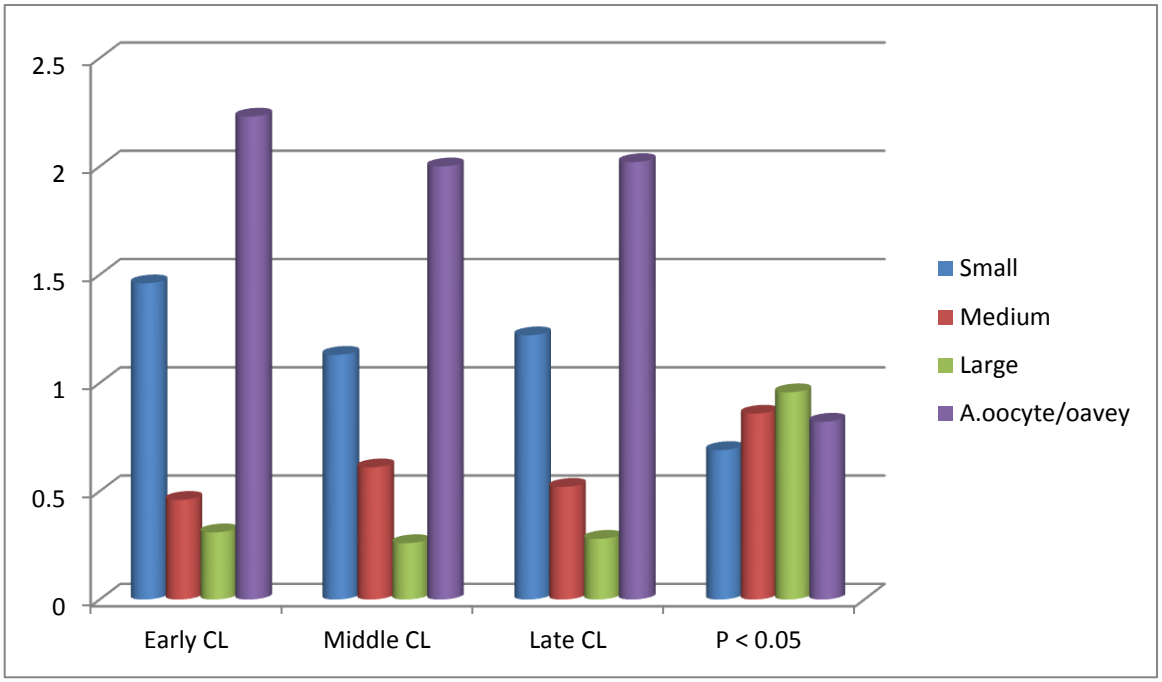


Fig- 12 Relationship between types of CL and follicular population

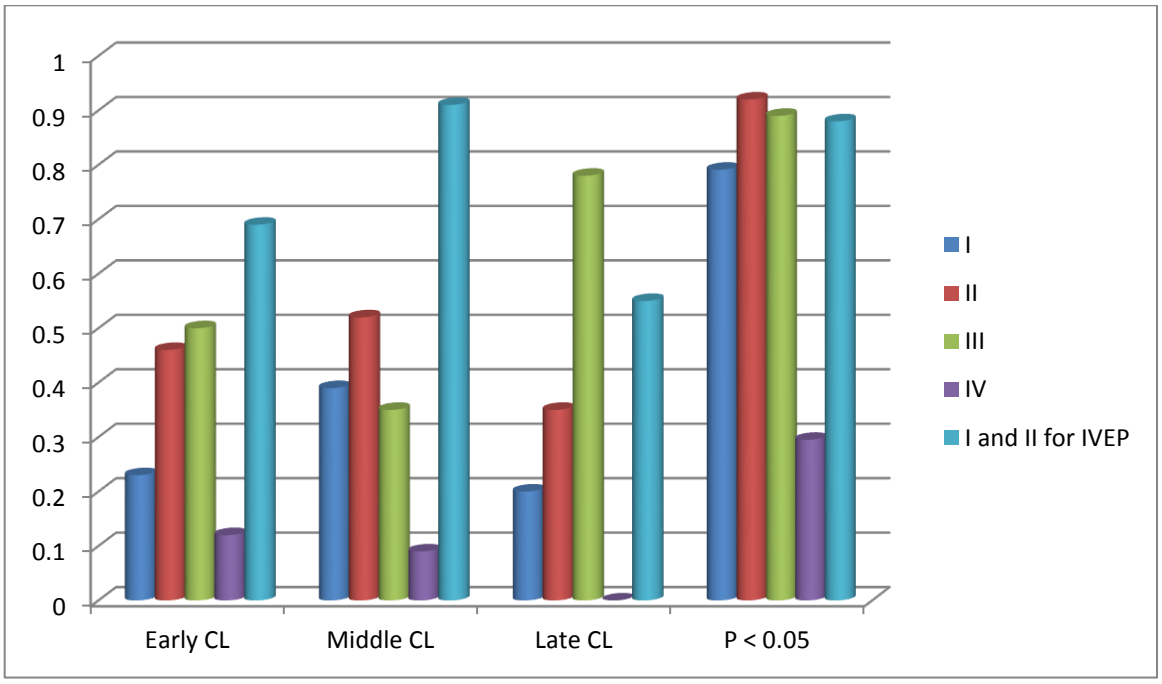


Fig- 13 Relationship between types of CL and oocytes grades

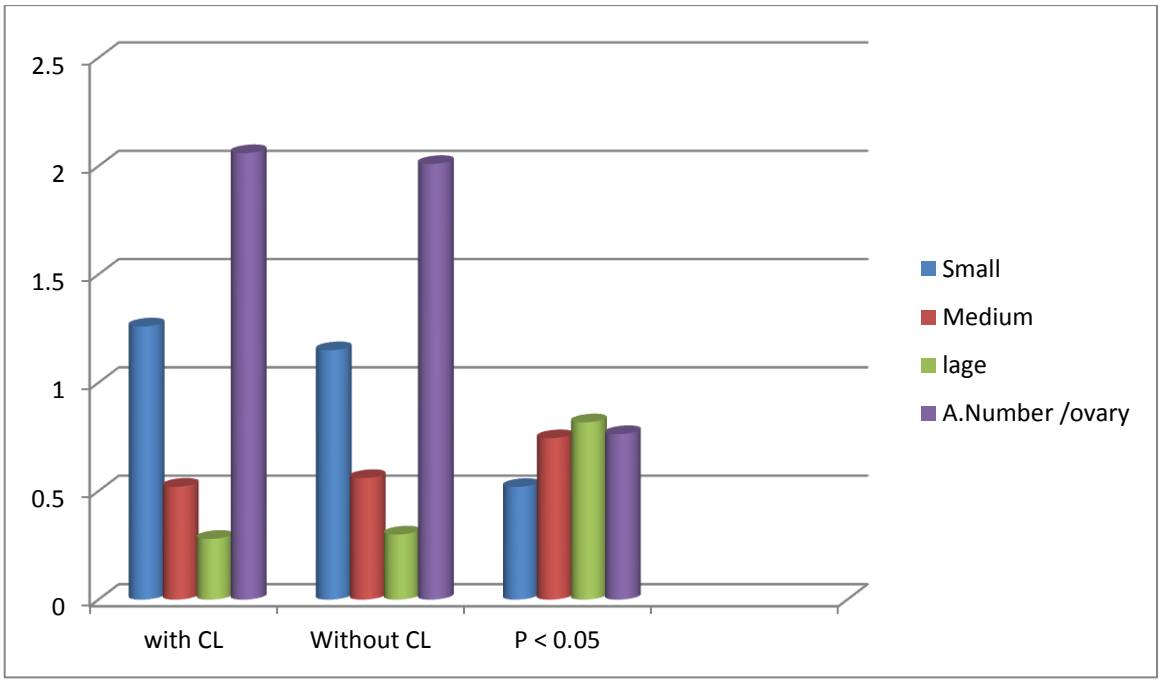


Fig – 14 Relationship between Present or absent of CL and follicular population

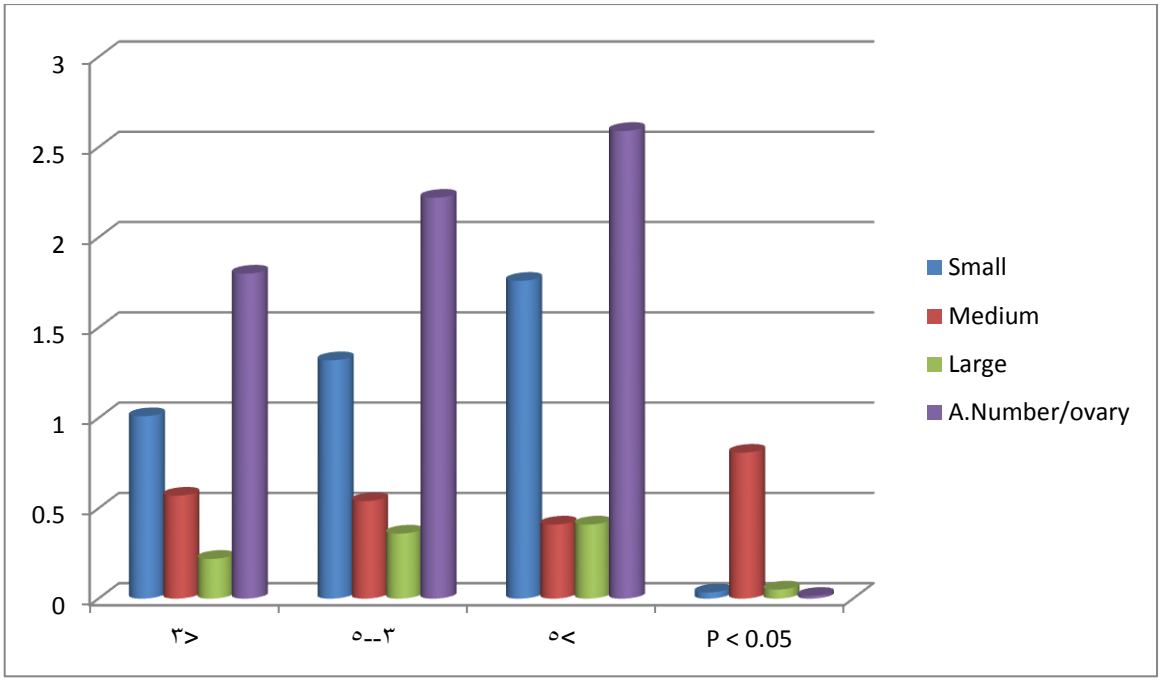


Fig – 15 Relationship between ovary weight and follicular population

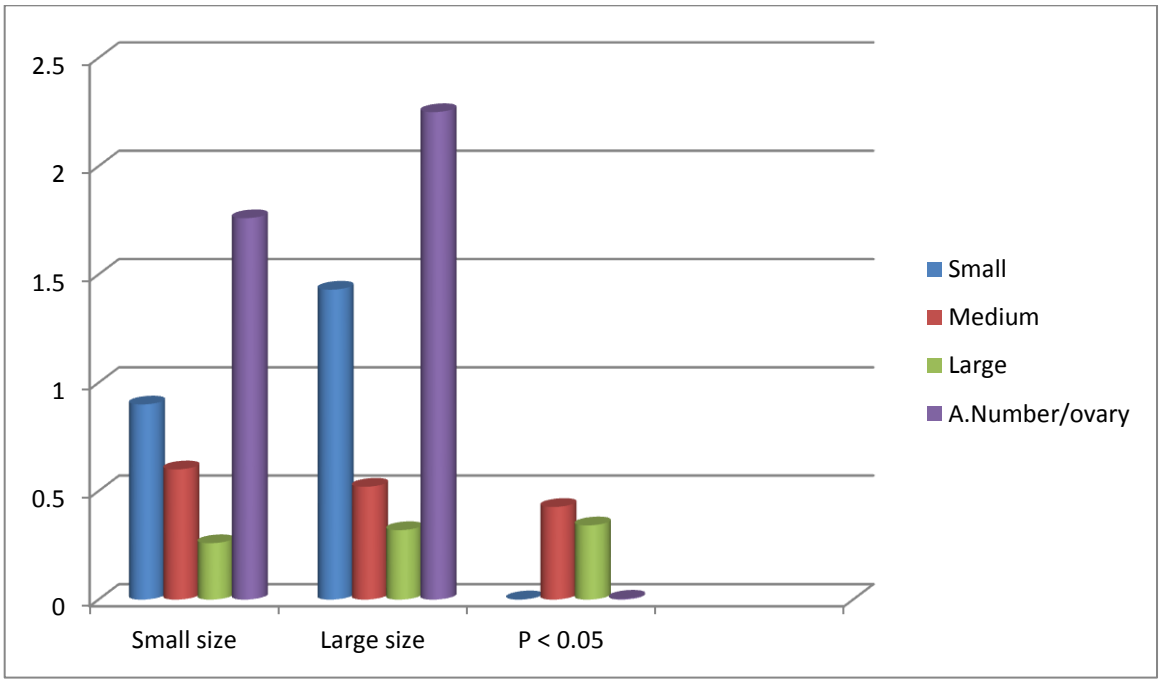


Fig – 16 Relationship between ovary size and follicular population

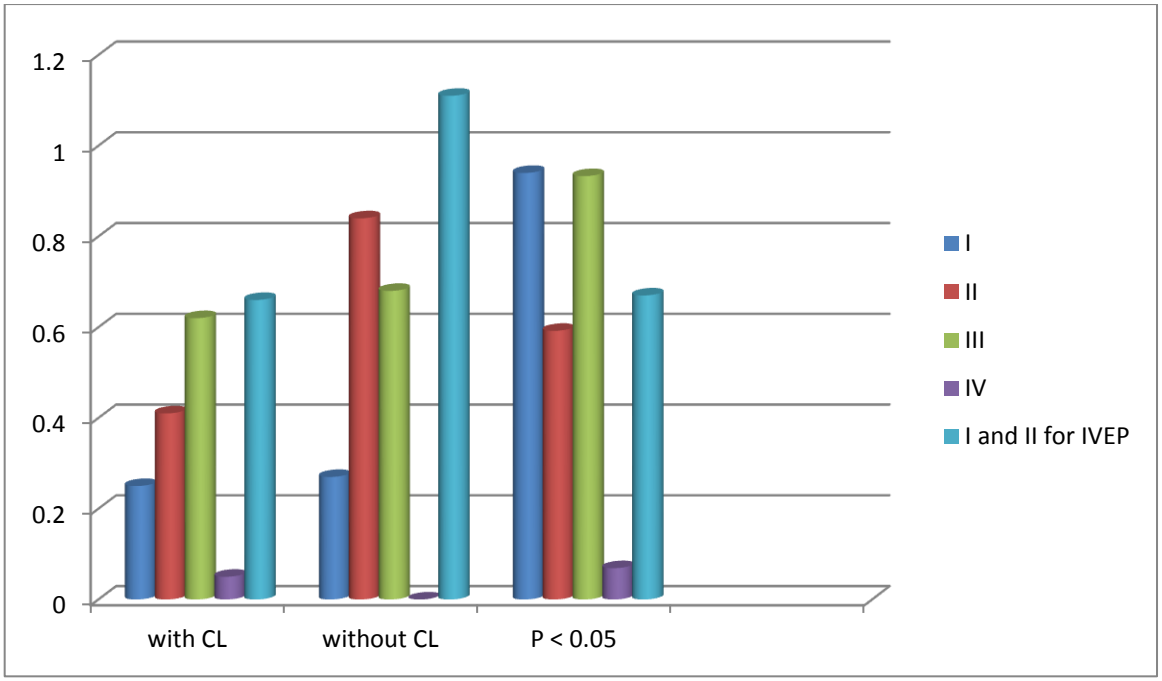


Fig – 17 Relationship between present or absent CL and oocytes grades

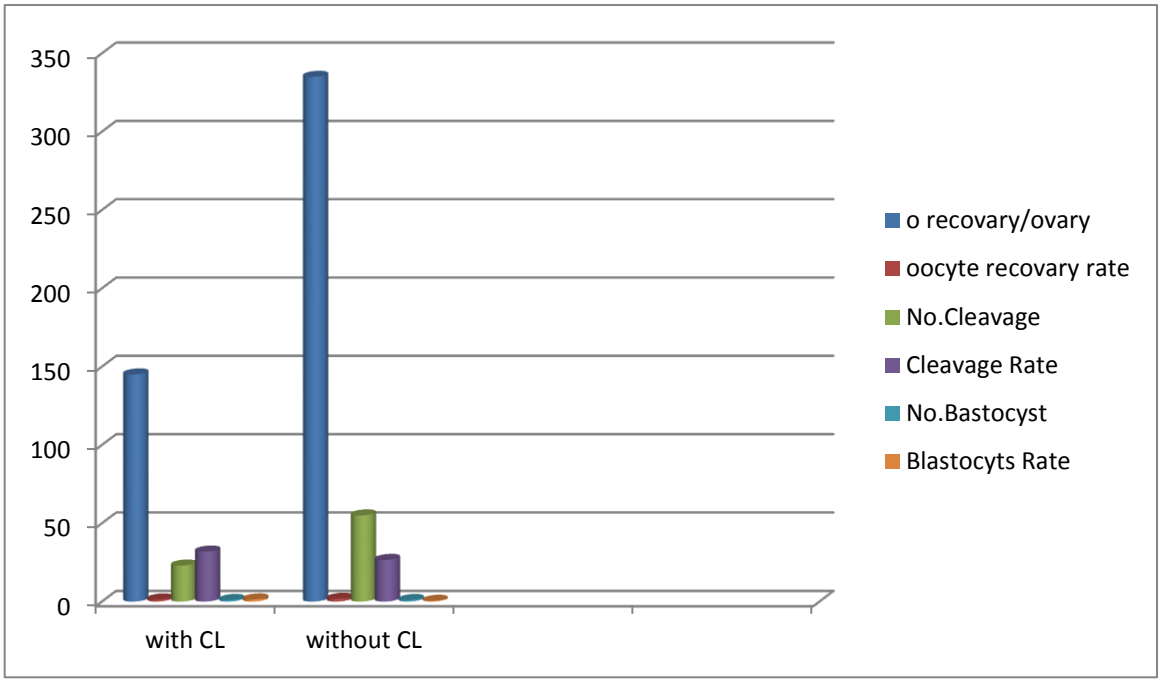


Fig- 18 Effect of CL on cleavage and blastocyst rates:

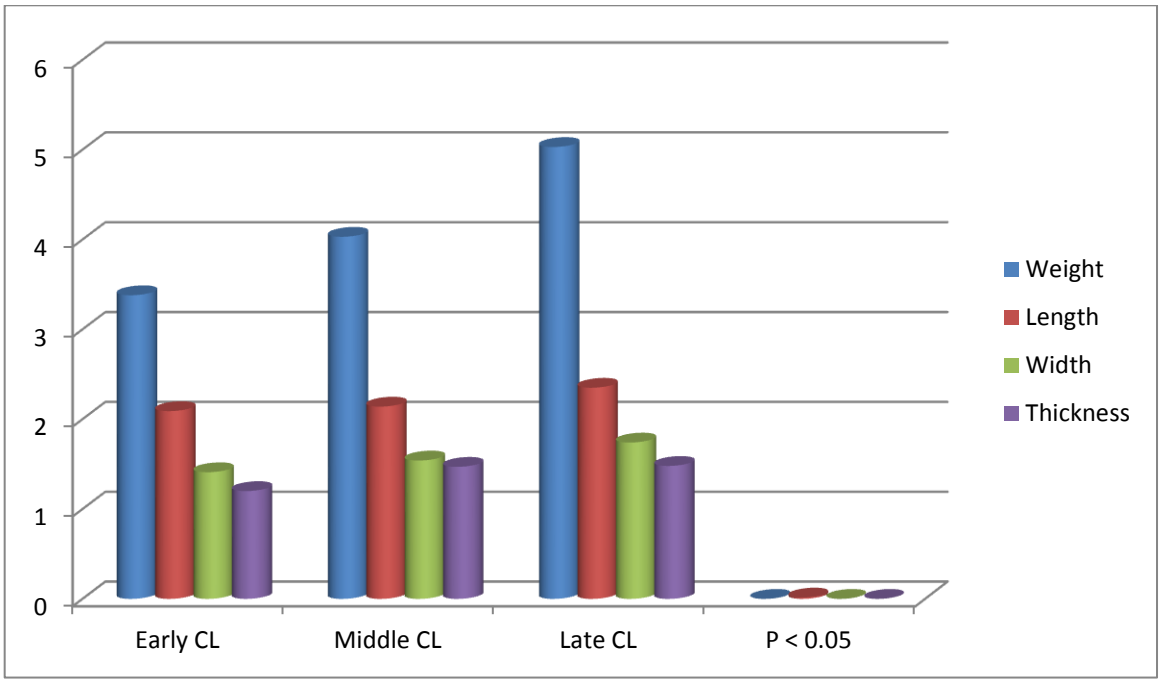


Fig-19 Relationship between types of CL, ovary weight and ovary size

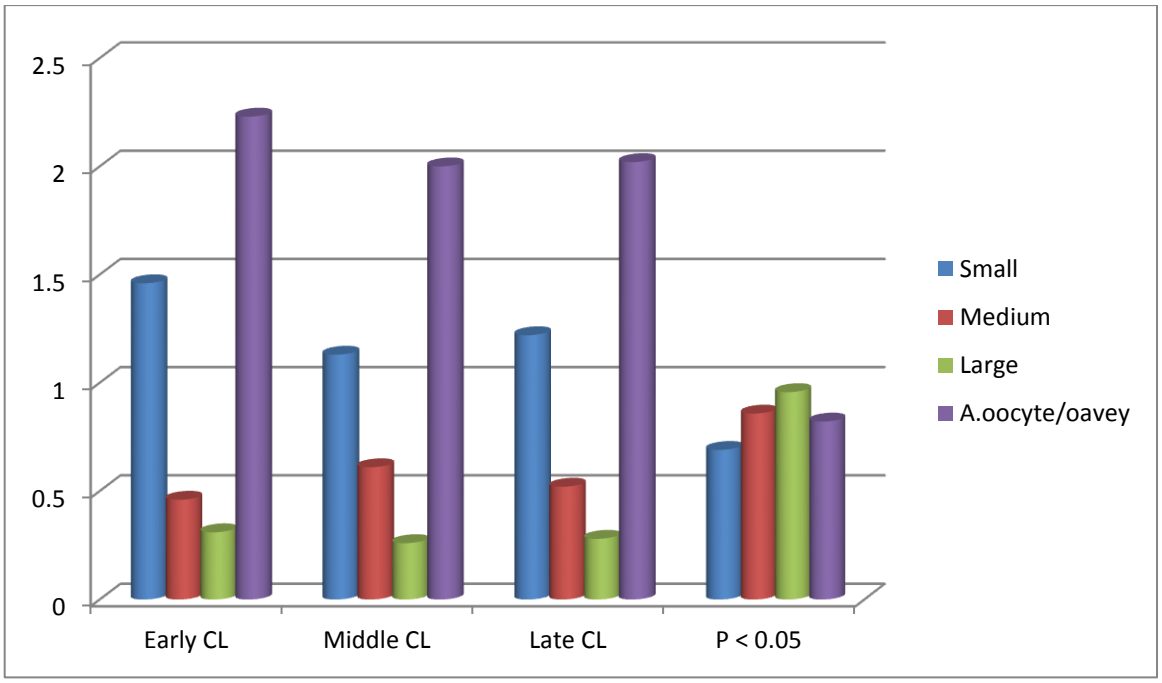


Fig- 20 Relationship between types of CL and follicular population

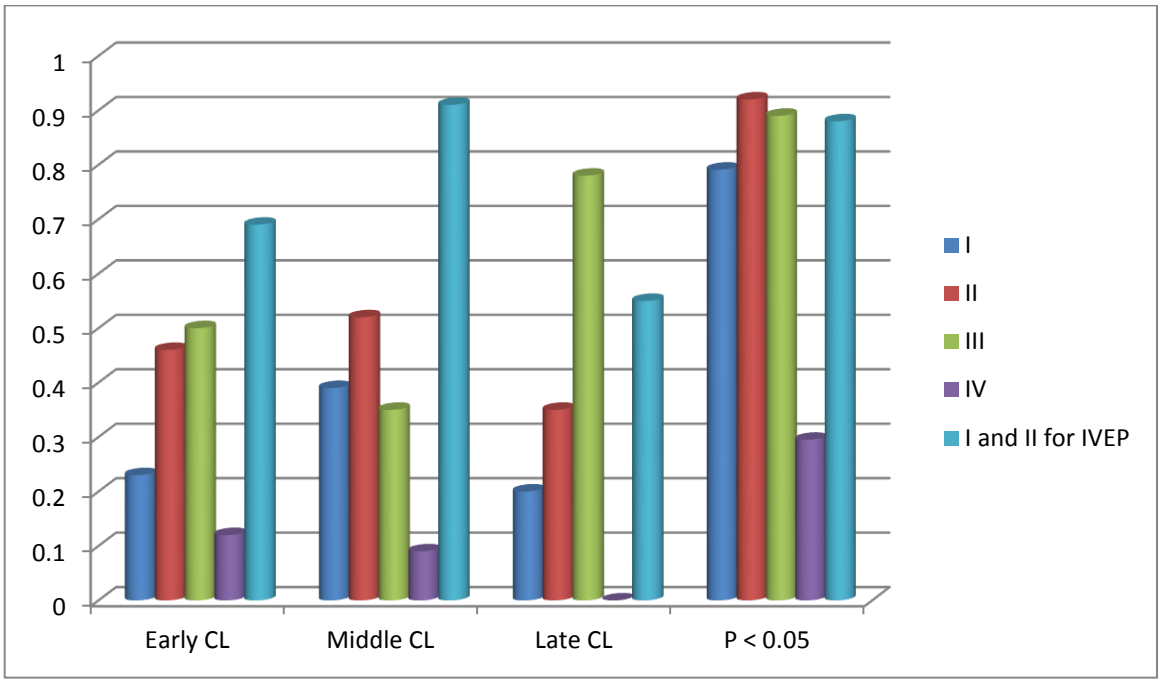


Fig- 21 Relationship between types of CL and oocytes grades

Appendix B: Media compositions

1- Ovaries washing medium:

Items	Quantity
Sodium chloride 0.9 %	9 g/L
Penicillin G	0.12 g/L
Distilled water	1000 mL

2-Tissue culture medium:

Items	Quantity
DMEM- F12	43.5 mL
Essential amino acid	500 µL
Vitamins	500 µL
FBS	5 mL
Ab / Antimycotic	500 µL
Total volume	**50 mL**

3- Aspiration or oocytes collection medium:

Items	Quantity
TCM – 199	40 mL
L – Glutamine	0.004 gm
BSA	0.27 gm
Ab/ Antimycotic	400 µL

4- Oocytes washing medium:

Items	Quantity
TCM – 199	36 mL
L – Glutamine	0.005 gm
FBS	5 mL
Na Pyruvate	0.0045 gm
Ab/ Antimycotic	500 µL
Total volume	**50mL**

5- In Vitro Maturation medium:

Items	Quantity
Washing medium	10 mL
FSH (prepared stock)	10µL
β estradial (prepared stock)	50 µL

6- In Vitro Fertilization medium:

Items Solution A (Yellow color)	Quantity 500 ML	200 ML
Na Cl	4.3092gm	1.7236mg
KcL	0.1974 gm	0.0789 mg
$CacL_2 2H_2O$	0.2171 gm	0.08684 mg
$MgcL_2 6H_2O$	0.0697 gm	0.02788 gm
$NaH_2PO_4 2HO$	0.0840 gm	0.0336 gm
Phenol red 0.5 %	100 µL	50 µL

Items Solution B (Pink color)	Quantity 200 ML
Na_2CO_3	2.5873 gm
Phenol red 0.5 %	40 µL

7- BO medium for IVF and sperm washing:

Items	Quantity
Solution A	38 mL
Solution B	12 mL
Heparin	12 µL
Sodium Pyruvate	0.0068 gm
Caffeine sodium bicarbonate	0.0971 gm
Total volume	**50 mL**

8- In Vitro Fertilization medium:

Items	Quantity
BO Medium	10 mL
BSA free fatty acid	0.1 gm

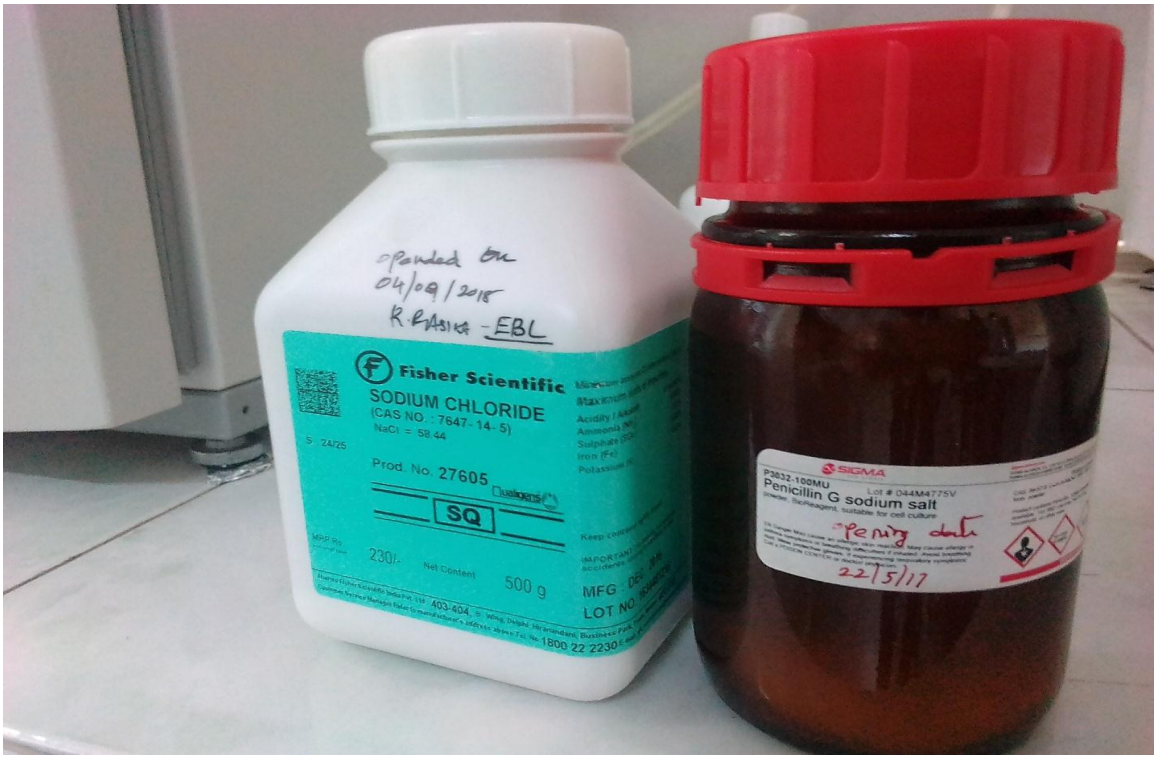
9- T_2 washing medium:

Items	Quantity
TCM	20 mL
FBS	4 mL
Sodium Pyruvate	0.0024 g
Ab	200 µL
L – Glutamine	0.002 g

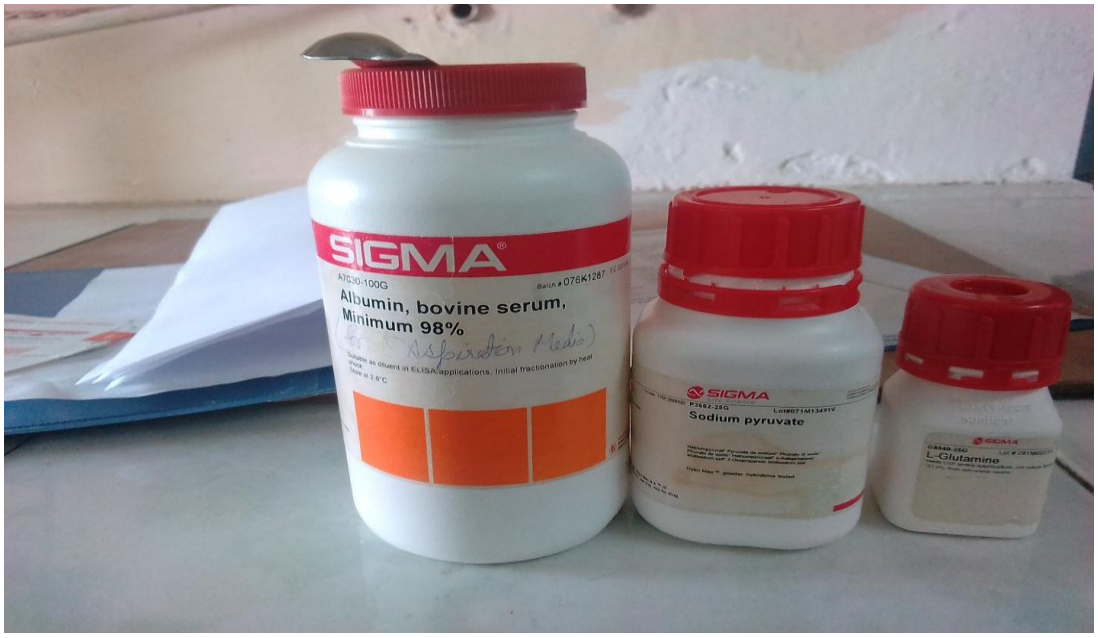
10- In Vitro Culture medium:

Items	Quantity
RVCL	1 mL
BSA (free fatty acid)	0.1 g

Appendix C: Media photos index



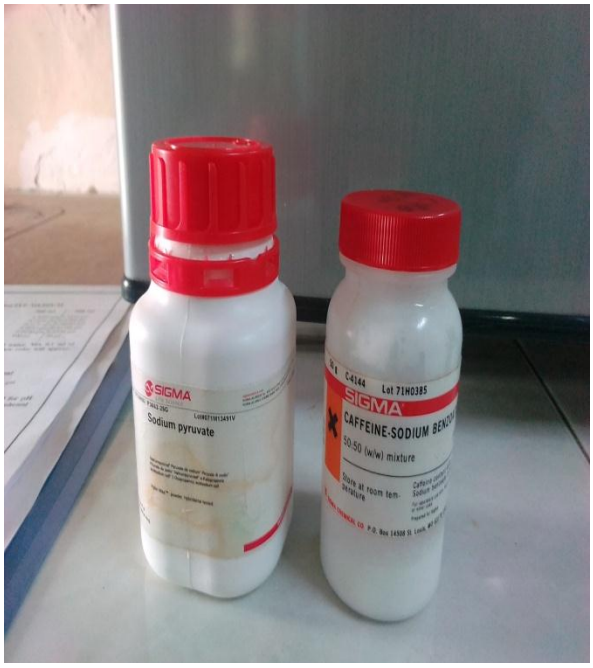
Ovaries washing medium composition: Sodium chloride and Penicillin G sodium salt.



Aspiration and washing medium composition: Albumin bovine serum, Sodium Pyruvate and L- Glutamine.

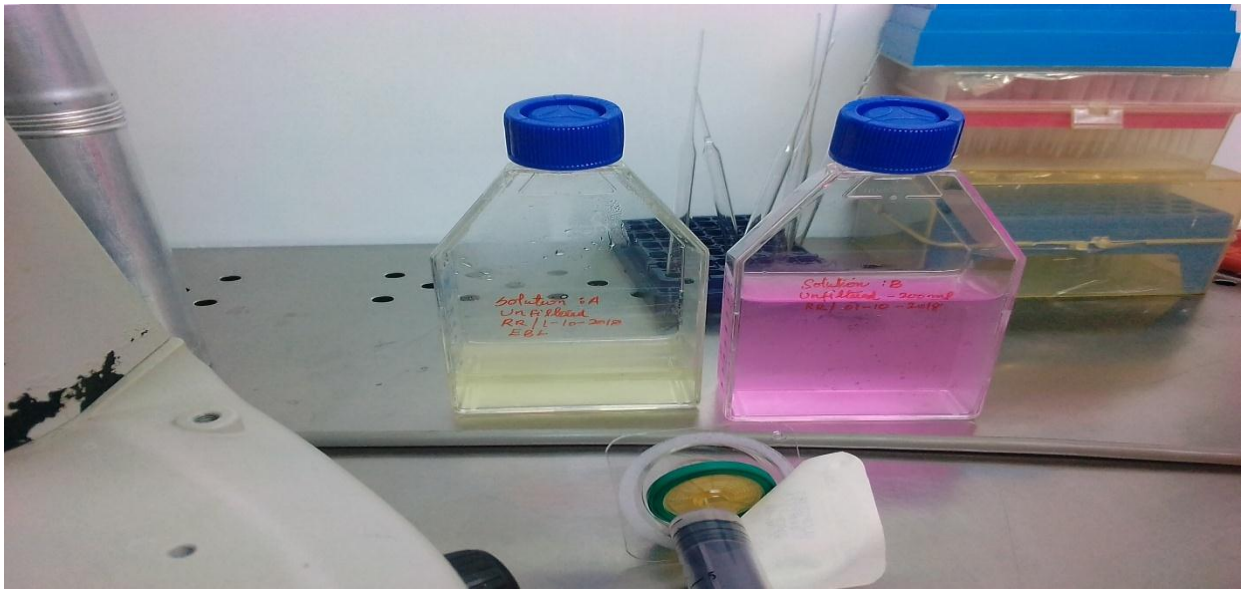
A **B**

In Vitro Maturation Medium (IVM) composition: A – FSH
B – β estradiol.



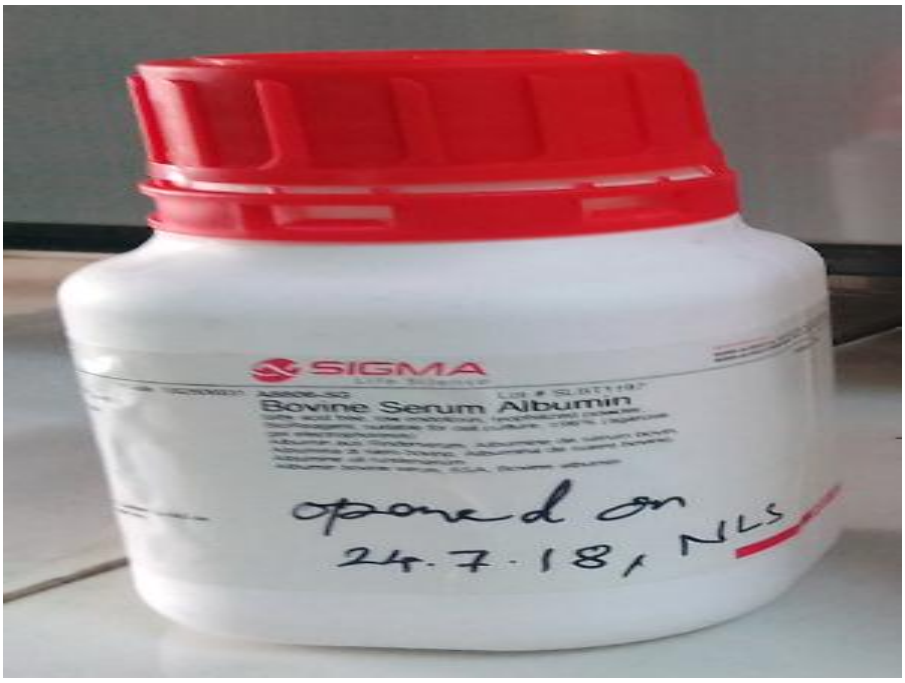
A

B



C

BO washing medium or(IVF washing medium) composition: A - Sodium Pyruvate and Caffeine sodium benzoate B – Heparin C – Solution A and B with filter 0.22 µnm.



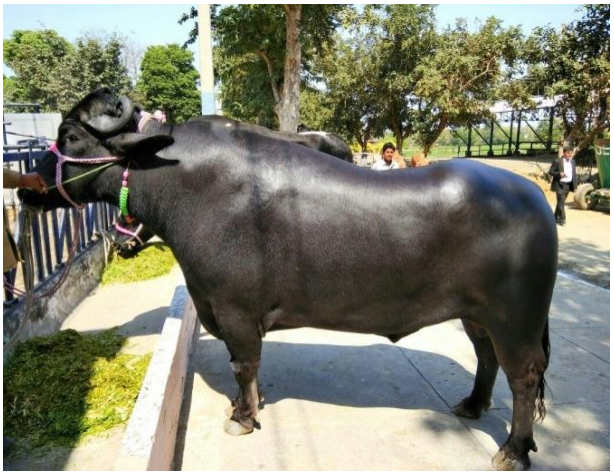
BO medium (IVF medium) composition: Bovine Serum Albumin (BSA) free fatty acid.

Referigator for keeping media compositions

Laminar for searching oocytes, IVM, IVF, IVC and embryo evaluation. Consisting of: 2- sterozoom microscopes, racks, tissue pipettes , tips , glass , mark pen and disinfection spray.

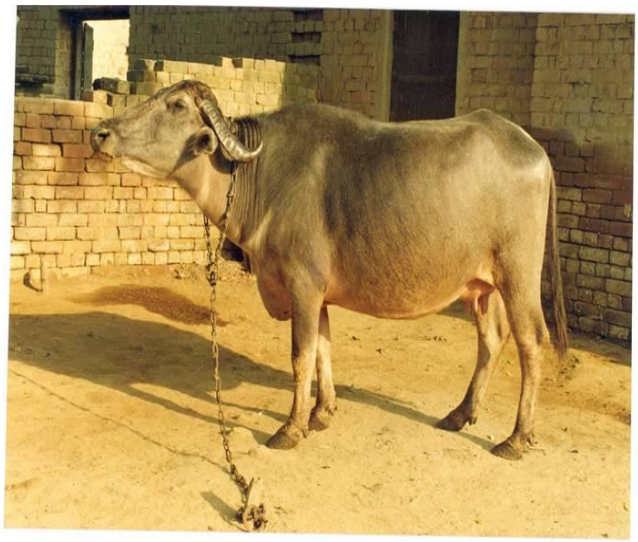
Appendix D: Indian buffalo breeds

1- Murrah



2- Jaffarabadi

3- Bhadawari



4- Mehsana

5- Nagpuri

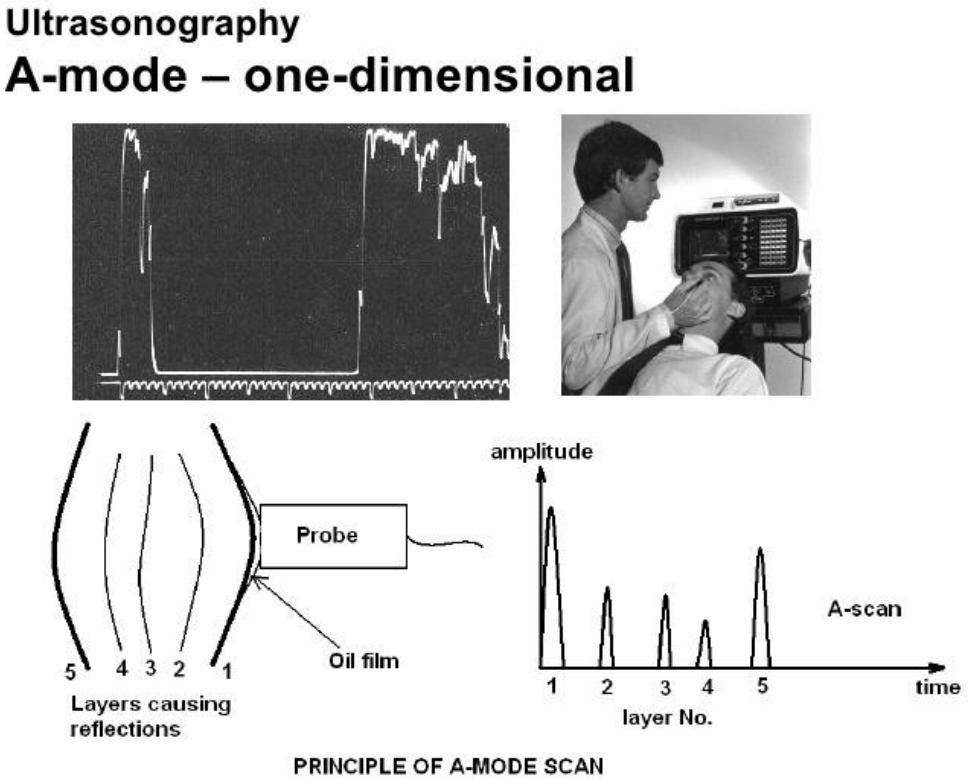
6- Surti

7- Toda

Appendix E: Types of Ultrasound

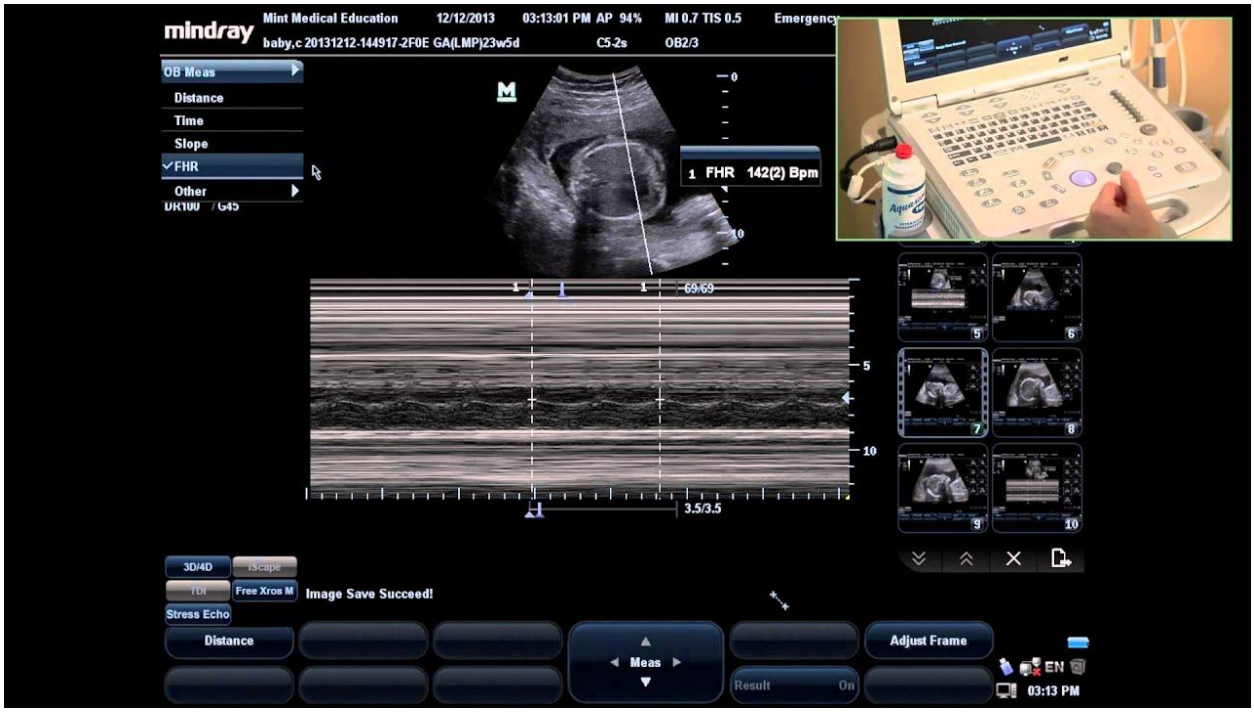
1- Real time B – mode

2- A – mode

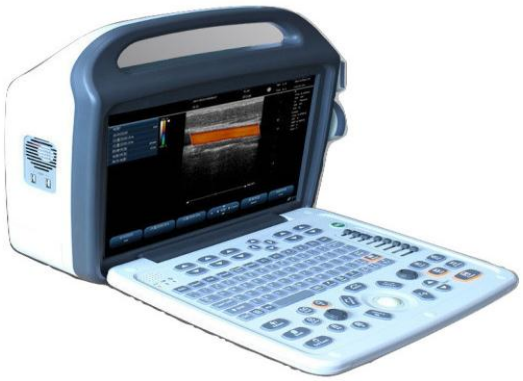


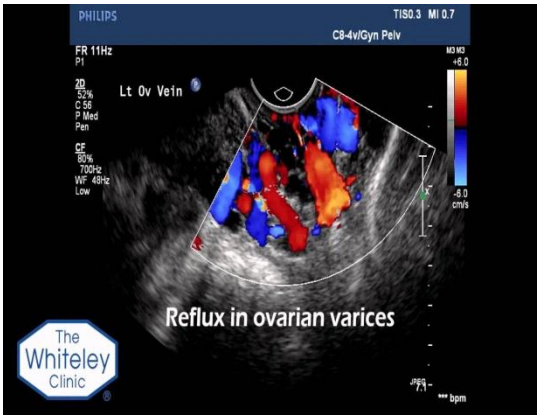
PRINCIPLE OF A-MODE SCAN

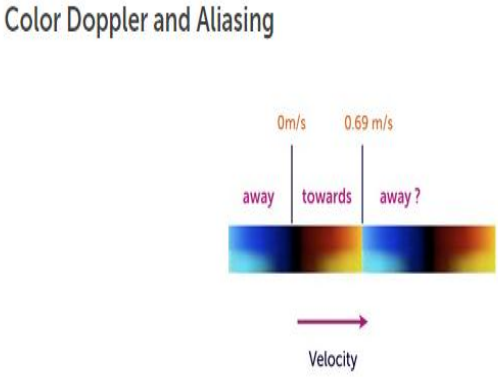
3- M – mode



4- Doppler







Appendix F:

Fig 1: Removal tissues attached to ovaries and washing ovaries with saline solution.

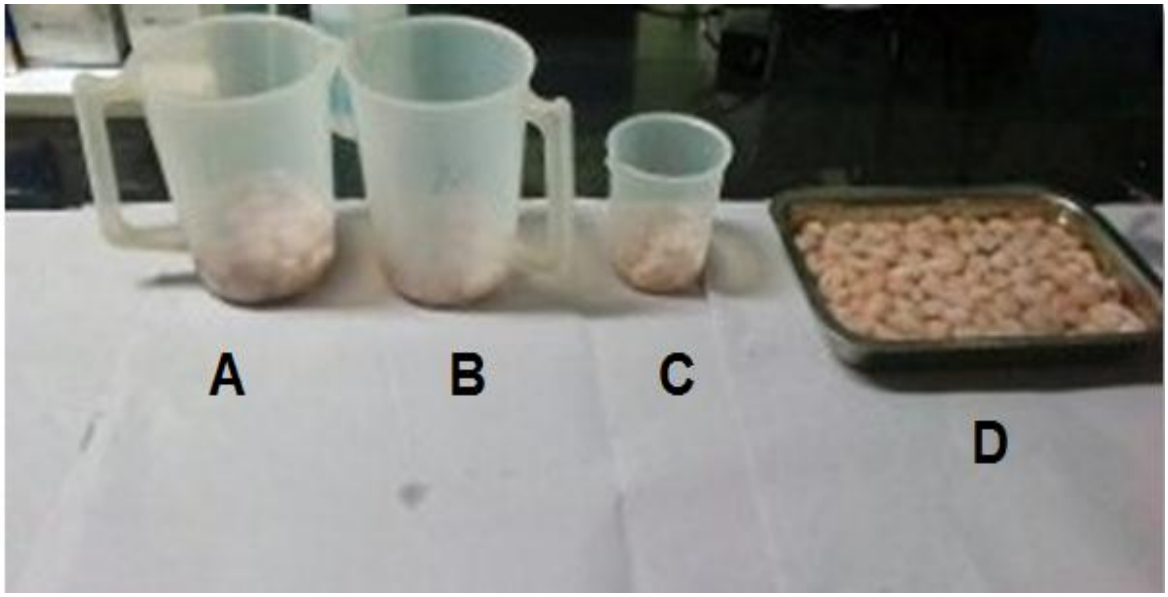


Fig 2: Classify ovaries from left to right: A: ovaries having CL in late stage B: ovaries have CL in middle stage C: ovaries having CL in early stage and D: ovaries without CL.

Fig 3: Ovaries with CL in different stages: A: early B: middle and C: late stage.

Fig 4: weight ovary by using an electronic scale balance.

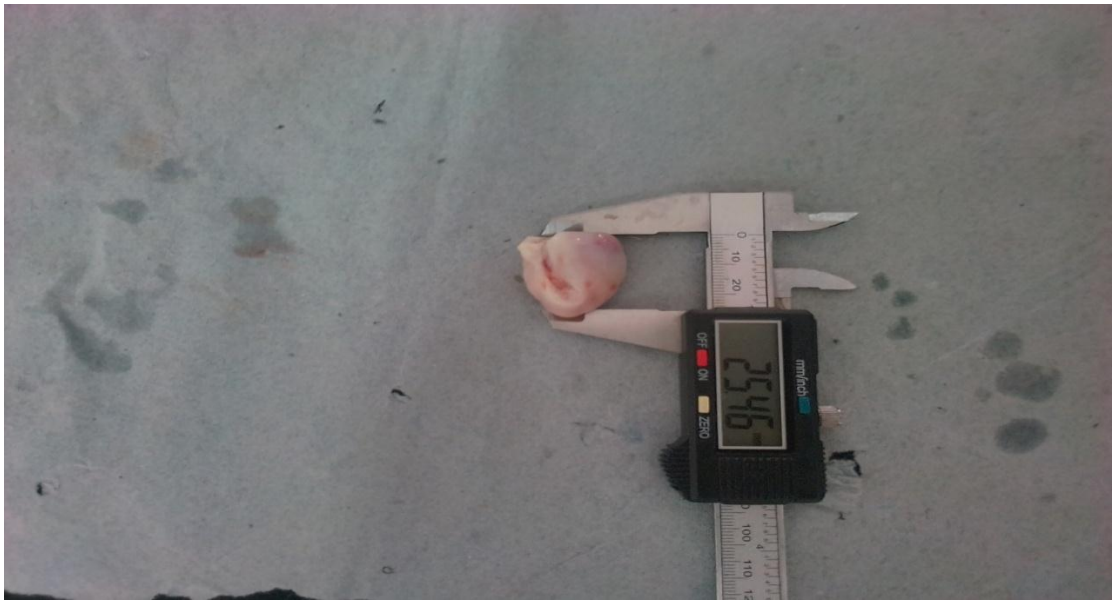


Fig 5: Measuring an ovary length by using Vernier calipers.

Fig 6: Measuring an ovary width by using Vernier calipers

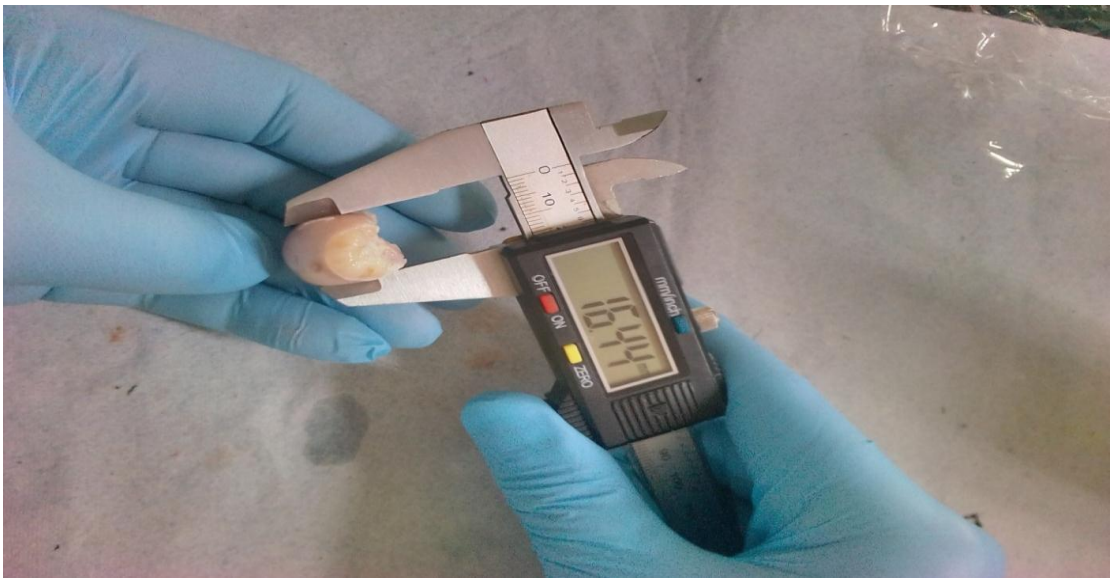


Fig 7: Measuring an ovary thickness by using Vernier calipers

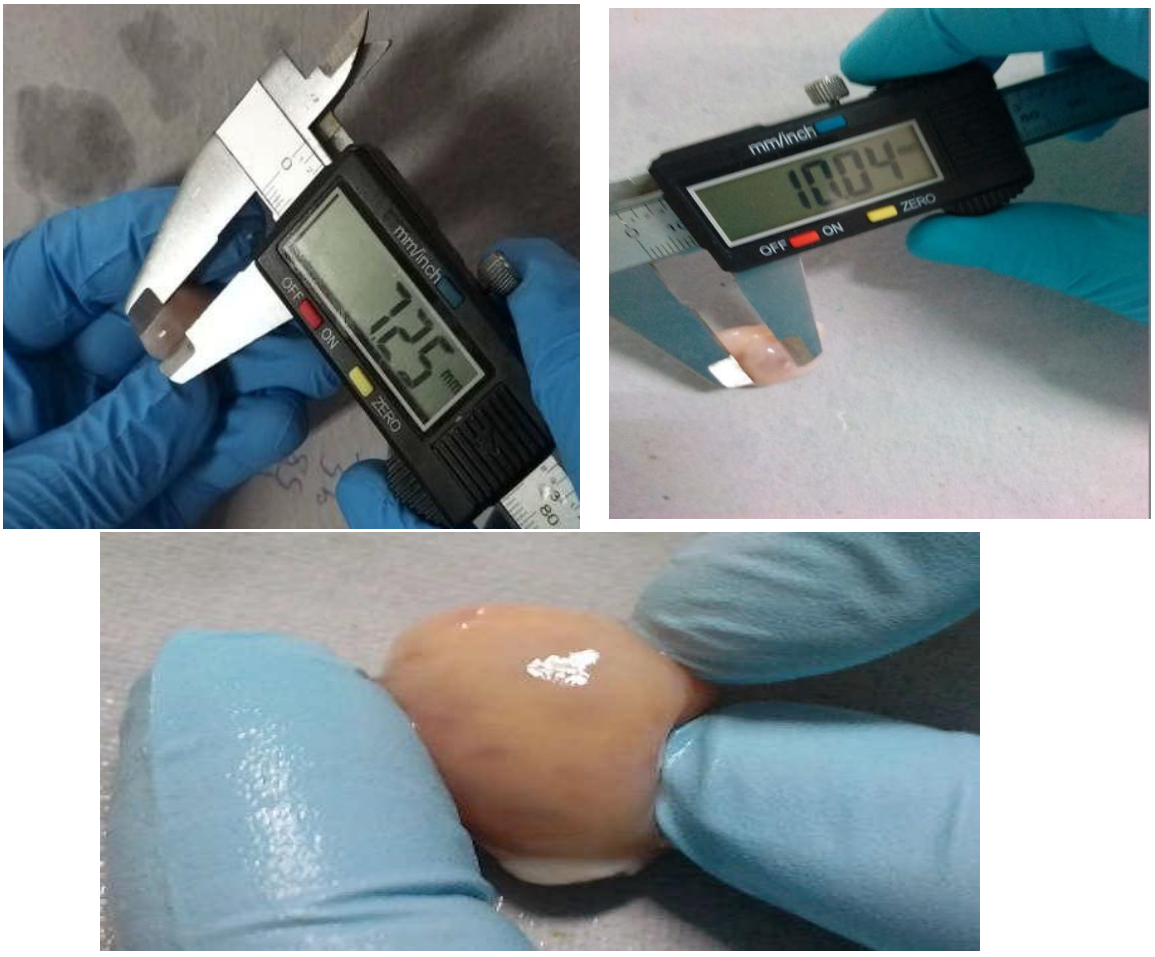


Fig 8: follicle size measuring by electronic Vernier calipers and count of visible follicles.

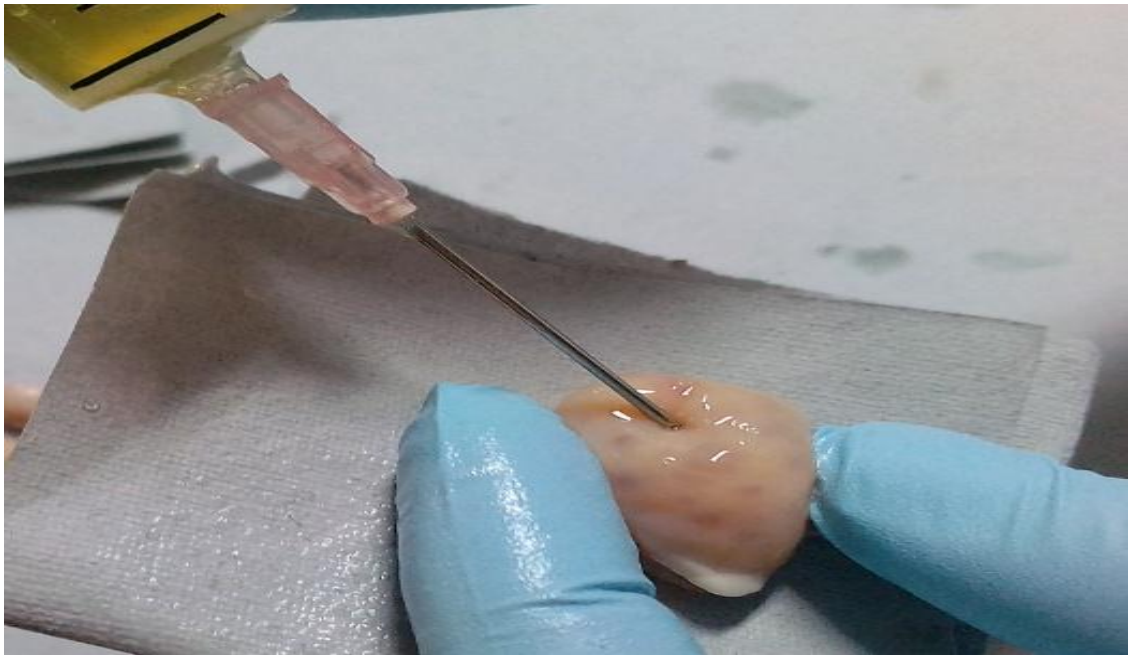


Fig 9: Aspiration method for oocytes collection.

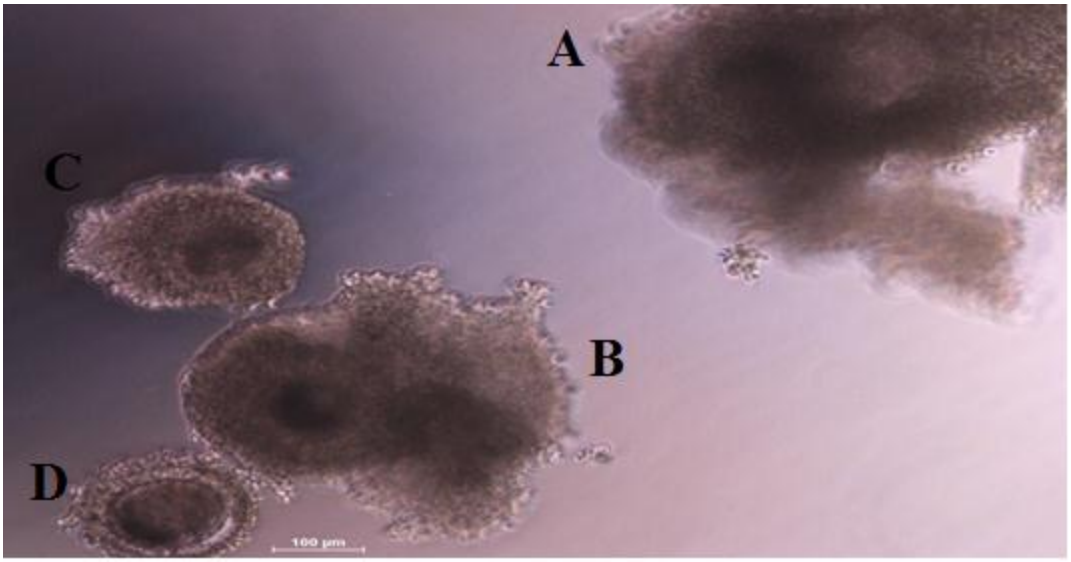


Fig 10: Oocytes grades, A, B, C and D.

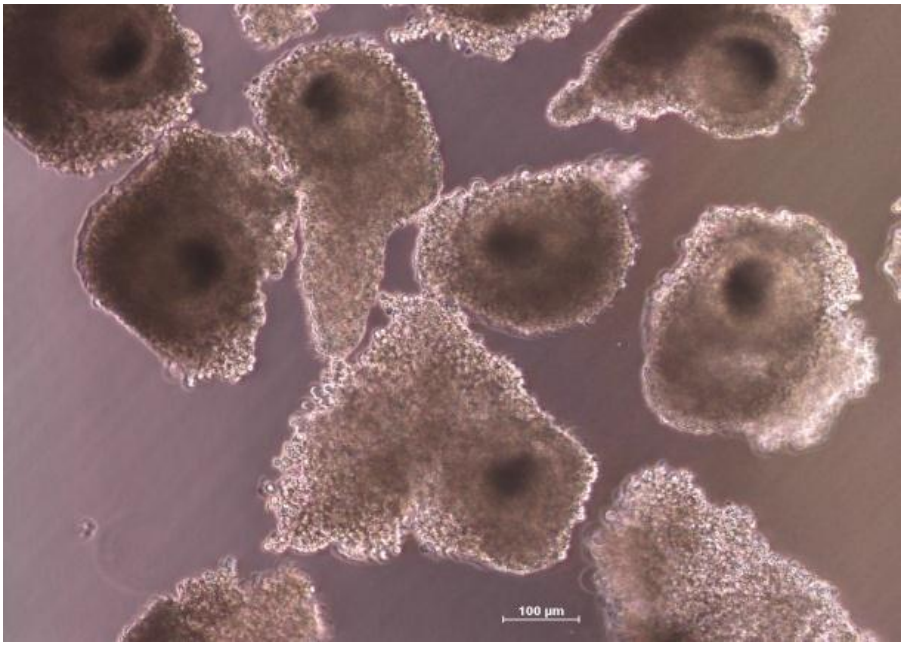


Fig 11: Oocytes before IVM under florescent microscope 10X

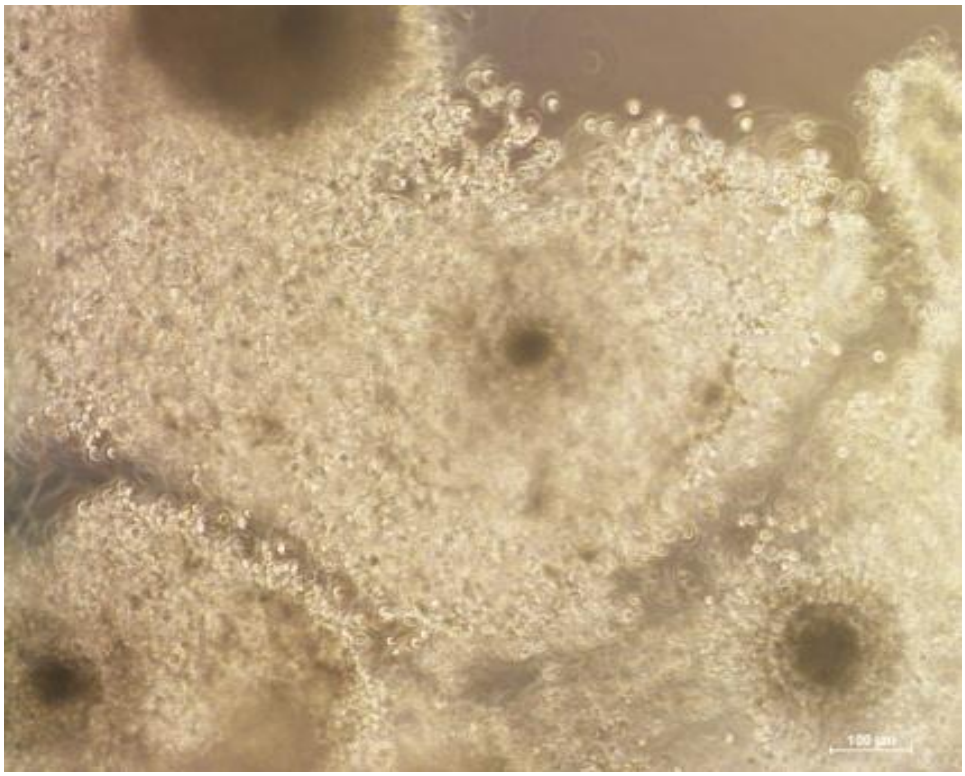


Fig 12: Oocytes after IVM under florescent microscope 10X.

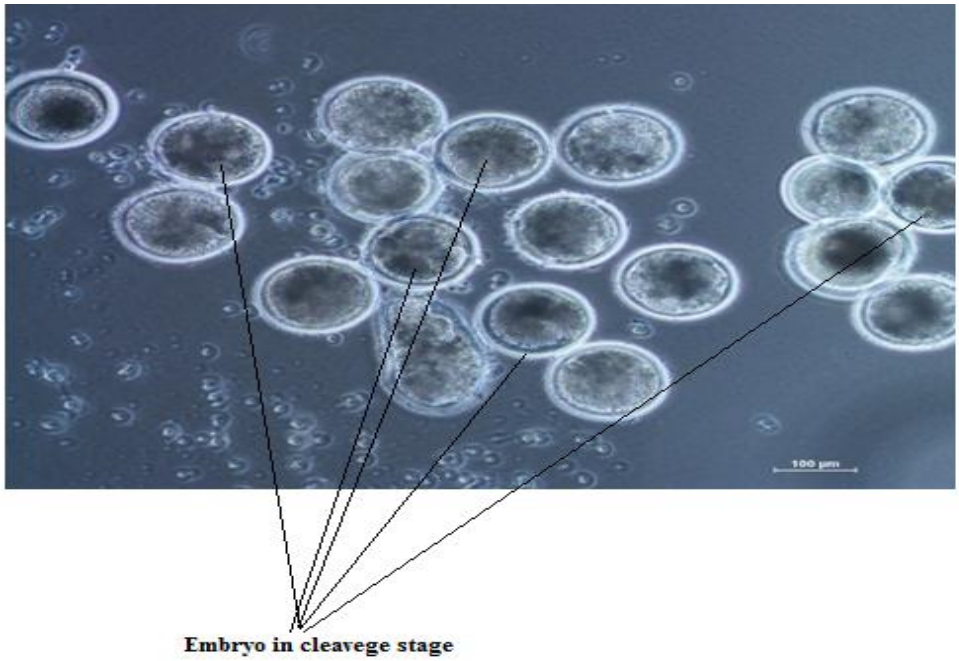


Fig- 13: Embryos in cleavage stage under florescent microscope 10X

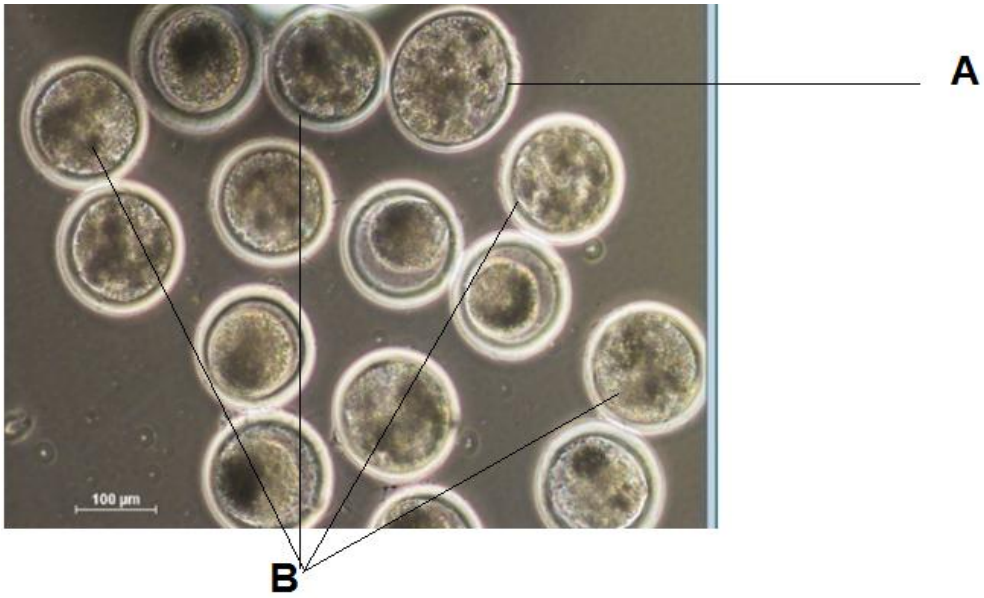


Fig 14: A: Embryo in blastocyst stage B: Embryos in cleavage stage under florescent microscope 10X

Fig 15: Indian buffaloes (Murrah) at farm of CIRB

Fig 16: Real-time B-mode trans-rectal ultrasound scanner with an intra operative 7.0 MHz micro convex transducer.

Fig 17: Follicle measured and pregnancy diagnosed using real- time B- mode transrectal ultrasound scanner

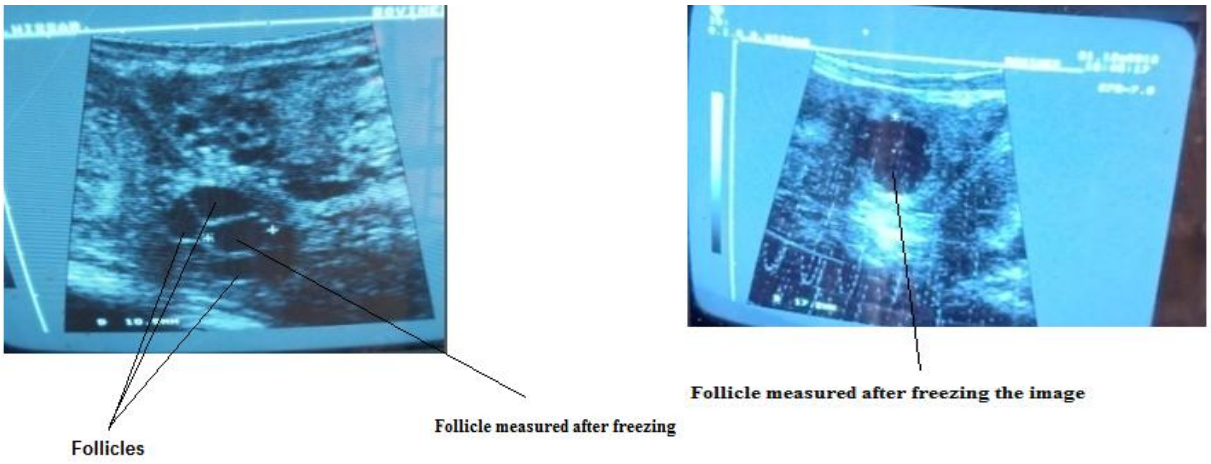


Fig 18: Follicle measured after freezing the image using inbuilt calipers.

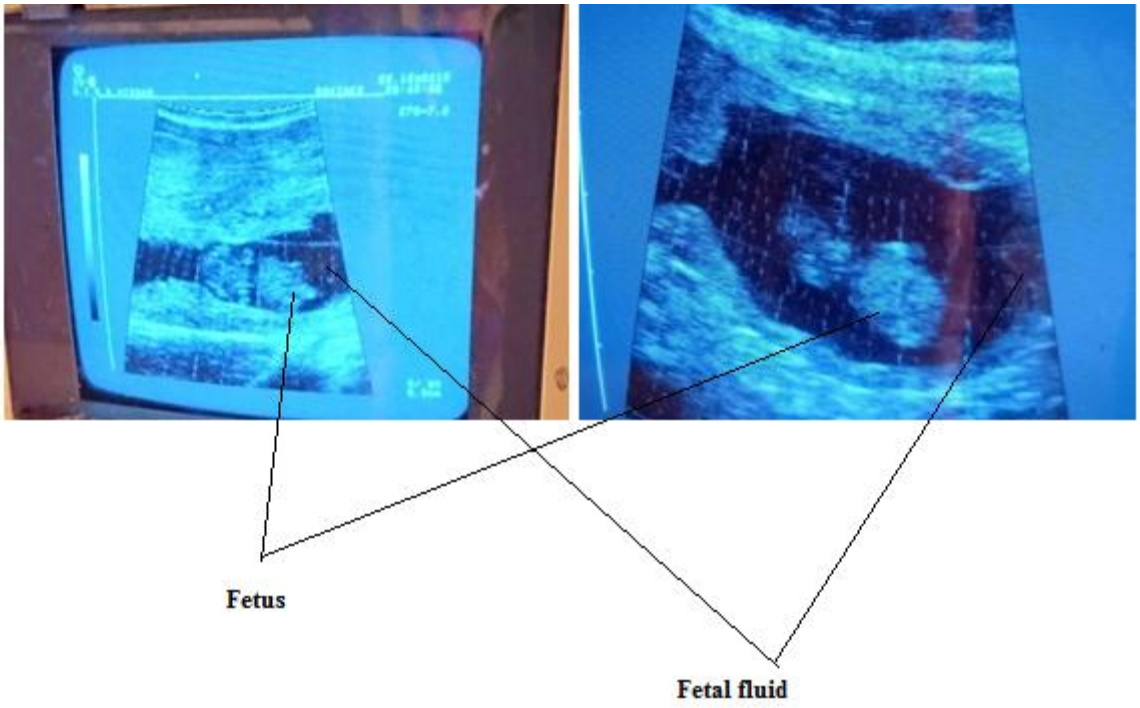


Fig 19: Pregnancy diagnosed by presence of fetus on viewing screen as irregularly shaped echogenic structure surrounded by non echogenic black color image in uterus.

P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Short Communication

In Vitro Embryo Production in Indian Buffalo

Eias Elzein, I Osman[1], Sharma RK[2] and Majdi E Badawi[3]

[1]Department of clinical studies, Faculty of Veterinary Science, University of Nyala, Nyala- Sudan
[2]Physiology and Reproduction Division, Central Institute for Research on Buffaloes, Hisar-125001, India
[3]Department of medicine and animal surgery, College of Veterinary Medicine, Sudan University of Sciences and Technology, Sudan
***Corresponding author:** eiasalzain66@yahoo.com

Article History: Received: November 07, 2017 Revised: January 21, 2018 Accepted: January 23, 2018

ABSTRACT

The embryo production is carried out through a combination of techniques of collection of immature oocytes, in vitro maturation (IVM), fertilization (IVF) and culture (IVC). Samples collected from slaughterhouse are the cheapest and the most abundant source of primary oocytes for large scale production of embryos through in vitro maturation (IVM) and in vitro fertilization. The culture medium and selection of protein supplements and hormones for IVM play an important role in the subsequent maturation rate, and embryonic development following IVF. The in vitro fertilization procedures in buffalo and requires appropriate preparation of sperm and oocyte, as well as culture conditions that are favorable to the metabolic activity of the male and female gametes. The presumptive zygotes are then cultured in vitro up to the blastocyst stage at which these could either be transferred to synchronized recipients for producing live offspring or cryopreserved for future use.

Key words: Buffalo, Maturation, Fertilization, Embryo culture

INTRODUCTION

The buffaloes are in the order of *Artiodactyla*, the cloven-hooved mammals, genus *Bubalus* and species *bubalis*. Two main species of buffalo are found in the world: the Asiatic (water) buffalo (*Bubalus bubalis*) and the African buffalo (*Syncerus caffer*). The two buffalo types are having different habitats and chromosome numbers. There are about 170 million buffaloes in the world (Perera *et al.*, 2005). Out of this 97 percent of them are water buffaloes and are mainly found in the Asian region. Riverine buffaloes are characterized by black colour and have long curled horns (e.g. Murrah Breed) and the Swamp buffaloes are dark grey, but may also be black, black and white, or even all white, have long, gently curved horns. Riverine buffaloes (70 percent of the total world population) are reared in high numbers in South Asia, especially in India and Pakistan. The name 'swamp' has probably arisen from their preference for wallowing in stagnant water pools and mud holes (Subasinghe *et al.*, 1998). Swamp buffaloes are found mainly in southern China Sri Lanka, and the South-East Asia countries of Thailand, the Philippines, Indonesia, Vietnam, Burma (Myanmar), Laos, Cambodia and Malaysia (Chantalakhana and Falvey, 1999).

India has about 95 million buffalo's represents 56.5 percent of the world buffalo population. India is the first country in the world for rearing buffalo's production (about 134 million tons of milk). India is also the first country in Asia for scientific and technological development in buffalo nutrition, production, reproduction, biotechnologies and genetic improvement. Moreover, India has implemented national programmes known green revolution¨ for increasing crop production for animals, the "white revolution" for increasing milk productivity to satisfy human needs for animal proteins and finally the "red revolution" for increasing meat production and supporting meat industry, especially from buffalos. India possesses the best River milk breeds in Asia e.g. Murrah, Nili-Ravi, Surti and Jaffarabadi, which originated from the north-western states of India and have a high potential for milk and milk fat production in addition to use as a work animal and as a supplementary stock for meat production. The IVEP permits the preservation of genetic potential of sub-fertile or dead animals **(**Deuleuze *et al.*, 2009) by the creation of a gene bank with oocytes recovered from slaughterhouses (Seidel and Seidel, 1989**)** for the improvement of livestock productivity. The purpose of this article is to summarize the steps of in vitro embryo production in Indian buffalo.

Cite This Article as: Elzein E, I Osman, RK Sharma and ME Badawi, 2018. In vitro embryo production in Indian buffalo. Inter J Vet Sci, 7(1): 18-20. www.ijvets.com

In Vitro embryo production

The in vitro production of embryos involves retrieval of oocytes from ovaries of slaughtered animals or live animals, in vitro maturation (IVM), fertilization (IVF) and culture (IVC) (Kumar and Anand, 2012). However, the significantly contributing factors in the success of IVEP are the quality and number of collected oocytes.

Ovaries collection and handling

Samples collected from slaughterhouse are the cheapest and the most abundant source of primary oocytes for large scale production of embryos through in vitro maturation (IVM) and in vitro fertilization (IVF) (Nandi *et al.,* 2006).

After slaughter, the left and right ovaries are excise and placed in separate conical tubes containing Washed Medium (WM) and transport to the laboratory at 35-37°C within next 2 hours after slaughter. All cystic ovaries are excluded from studies (Wang *et al*., 2007).

Oocytes collection from slaughtered animal ovaries

The in vitro production of embryos in buffalo involves retrieval of oocytes from ovaries of slaughtered animals. They are three methods used for the collection of oocytes from slaughtered animal ovaries as described by (Kumar and Anand, 2012) as follows:

1. Aspiration from surface follicles using 18-20G needle.
2. Puncturing or dissecting of prominent follicle.
3. Slicing of ovaries into small pieces.

The aspiration method is commonly employed because of the convenience associated with its application. Aspiration of oocytes is done using a needle attached to a 10-ml syringe. To avoid disruption of the surrounding cumulus cells, an 18- gauge needle is used. Possible toxicity associated with syringes containing rubber plungers and siloxane lubricants is avoided by washing and sterilizing glass syringes under the stringent conditions used for tissue culture glassware. For livestock, the use of plastic disposable syringes is acceptable.

Oocytes evaluation and regarding

Oocytes are examined under stereomicroscopy and classifying according to their compaction, number of cumulus cell layers and homogeneity of ooplasm according to (Alves *et al*., 2014), into 4 categories:

1. Grade I (GI): Oocytes with more than 4 layers of bunch of compact cumulus cells mass with evenly granulated cytoplasm.
2. Grade II (GII): Oocyte with at least 2–4 layers of compact cumulus cell mass with evenly granulated cytoplasm.
3. Grade III (GIII): Oocyte with at least one layer of compact cumulus cell mass with evenly granulated cytoplasm.
4. Grade IV (GIV): Denuded oocyte with no cumulus cells or incomplete layer of cumulus cell or expanded cells and having dark or unevenly granulated cytoplasm.

Oocyte in vitro maturation

Oocytes maturation is the most critical step towards successful in vitro embryo production. The culture medium and selection of protein supplements and hormones for IVM play an important role in the subsequent maturation rate, and embryonic development following IVF (Bavister *et al*., 1992). Several factors such as addition of FSH, LH and their combination to culture media had been considered for maximizing success (Saeki *et al.,* 1991). The most in vitro maturation medias used to maturation in vitro buffalo oocyte are tissue culture media (TCM-199), and Ham's F-10. But the most widely used TCM-199 supplemented with 10% FBS + 0.81 mM sodium pyruvate + 5% buffalo follicular fluid (buFF) + 50 µg/ml gentamycin sulfate and 5 µg/ml porcine FSH, achieving 82.3% maturation rates for 24 h incubation in a CO2 incubator (5% CO2 in air, 90-95% relative humidity) at 38.5°C (Kumar *et al*.,2007).

Oocyte in vitro fertilization

The in vitro fertilization is the most critical step of the IVEP procedures in buffalo and requires appropriate preparation of sperm and oocyte, as well as culture conditions that are favorable to the metabolic activity of the male and female gametes. The media used for IVF suggested are BO and TALP, which contain motility enhancing substance like caffeine or theophylline (Bavister, 1995).

On other hand for *in vitro* fertilization, generally TALP (Tyrode's modified medium; Parrish *et al.,* 1988) or BO (Brackett and Oliphant medium) is most widely used medium. For successful fertilization of oocytes, good sperm preparation is the essential and crucial step. Different workers tried different methods for separation of good motile sperm like swim-up (Lopata *et al*., 1976) or percoll based separation system. Sperms used for fertilization should pass through process of capacitation. Capacitation involves alterations of the sperm plasma membrane, which cause it to become unstable and to undergo vesiculation with the outer acrosomal membrane. In bovines, the capacitation occurs basically in the oviduct during the period of estrus and there is evidence that capacitation is caused by a heparin-like glycosamino-glycan in the oviductal fluid (First and Parrish, 1987). High IVF rates have been achieved by addition of heparin (Brackett and Zuelke, 1993) or its combination with penicillamine, hypotaurine and epinephrine, Ca^{++} ionophore A23187 with or without caffeine and high ionic strength media (Brackett *et al.,*1982).

In Vitro embryo culture

At the end of sperm- oocyte incubation, prior to transfer to the *in vitro* culture droplets, presumed zygotes were washed four times in embryo culture medium (mCR2aa containing 0.8% BSA) and cultured in this medium in a humidified CO2 incubator at 38.5 °C for up to 9-10 days to get the blastocyst, the embryo production rate was examined under an inverted microscope, to record the number of cleaved embryos at 8-16 cells which could either be transferred to synchronized recipients for producing live offspring or cryopreserved for future use. (Enchaparambil *et al.,* 2014).

REFERENCES

Alves BG, AK Alves, AC Lucio, CM Martins, TH Silva and RM Santos, 2014. Ovarian activity and oocyte quality associated with the biochemical profile of

serum and follicular fluid from girolando dairy cows postpartum. Anim Reprod Sci, 1: 1010-1016.

Bavister BD,1995. Culture of pre-implantation embryos. Human Reproductive; 1: 91-148. http://dx.doi.org/ 10.1093/humupd/1.2.91.

Bavister BD, TA Rose-Hellekant, and T Pinnyomminter, 1992. Development of in vitro and matured in vitro fertilized bovine embryos into morula and blastocyst in defined culture media. Theriogenology, 37: 127-146.

Brackett, BG and KA Zuelke, 1993. Analysis of factors involved in the *in vitro* production of bovine embryos. Theriogenology, 39: 43-64.

Brackett BG, *et al*., 1982. Normal development following *in vitro* fertilization in the cow. Biol Reprod, 27: 147-158.

Chantalakhana C and L Falvey, 1999. Small holder dairying in the tropics. International Livestock Research Institute (ILRI), Nairobi, Kenya 462p.

Deleuze S, G Goudet, M Caillaud, C Lahuec, G Duchamp, 2009. Efficiency of embryonic development after Intra-Follicular and Intra-Oviductal transfer of in vitro and in vivo matured horse oocytes Theriogenology, 72: 203-209.

Enchaparambil MSadeesh, F Shah, AK Balhara, SMK Thirumaran, S Yadav and PS Yadav, 2014. Effect of growth factor and antioxidant on in vitro maturation of oocytes and cleavage rates of in vitro produced Indian buffalo (Bubalus bubalis) embryos. Vet Arhiv, 84: 459-474.

Kumar and Anand, 2012. In vitro embryo production in buffalo: basic concepts. J Buffalo Sci, 1: 50-54.

Kumar D, P Palta, RS Manik, SK Singla, and MS Chauhan, 2007. Effect of culture. Indian J Anim Sci, 77: 697-01.

Lopata A, *et al*., 1976. A method for collecting motile spermatozoa from human semen. Fertil Steril, 27: 677-84.

Nandi S, VG Kumar and MS Chauhan, 2006. In vitro production of bovine embryos: We need to stop or proceed - a review. Agric Rev, 27: 122-129.

Parrish JJ, J Susko, M Parrish, A Winer and NL First, 1988. Capacitation of bovine sperm by heparin. Biol Reprod, 38: 1171–1180.

Perera BMAO, H Abeyguawardena, WG Vale and C Chantalakhana, 2005. Livestock and wealth creation - Improving the husbandry of animals kept by poor people in developing countries. Chapter 22: Buffalo. Livestock Production Programme. Natural Resources International Ltd United Kingdom. 601p. (cited from Vale WG, 2007: Effects of environment on buffalo Reproduction. Ital J Anim Sci, 6(Suppl 2): 130-142.

Saeki M, ML Hoshi and NL Leibfried-Rutledge, 1991. In vitro fertilization and development of bovine ocytes matured in serum-free medium. Biol Reprod, 44: 256-260.

Seidel GE and SM Seidel, 1989. Analysis of application of embryo transfer in developing countries. Theriogenology, 31: 3-16.

Subasinghe DHA, NU Hor adagoda, H Abeygunawardena and JA Siriwardena de S eds, 1998. Water Buffalo: Improved utilization through new technologies; National Science Foundation, Colombo, Sri Lanka.

Wang, ZG, Y Song-Dong and X Zi-Rong, 2007. Effects of collection methods on recovery efficiency, maturation rate and subsequent embryonic developmental competence of oocytes in Holstein cow. Asian-Aust. J Anim Sci, 20: 496-500.

International Journal of Veterinary Sciences and Animal Husbandry 2019; 4(2): 01-04

ISSN: 2456-2912
VET 2019; 4(2): 01-04

www.veterinarypaper.com
Received: 01-01-2019
Accepted: 04-02-2019

Eias Elzein I Osman
Department of clinical studies, Faculty of Veterinary Science, University of Nyala, Nyala-Sudan

Sharma RK
Physiology and Reproduction Division, Central Institute for Research on Buffaloes, Hisar, Haryana, India

Majdi E Badawi
Department of medicine and animal surgery, College of Veterinary Medicine, Sudan University of Sciences and Technology, Khartoum, Sudan

Correspondence
Eias Elzein I Osman
Department of clinical studies, Faculty of Veterinary Science, University of Nyala, Nyala-Sudan

Determine the effect of ovarian and non ovarian factors on follicular population in murrah buffalo using Ultrasound graphy technique

Eias Elzein I Osman, Sharma RK and Majdi E Badawi

Abstract
The aim of this study was to investigate the effect of age, body condition score (BCS), CL, pregnancy status and ovary localization on follicular population in Murrah buffalos using Ultrasound graphy technique. 160 female Murrah buffaloes (80 pregnant and 80 empty) with average age of 2- 11 years were used. All animals were maintained in a semi intensive system. The results obtained revealed that, ovaries of Murrah buffaloes with BCS 4 have higher number of follicles than those with BCS 3.The ovaries from pregnant animals have more number of medium size follicles as compared to those of non-pregnant buffaloes. The right ovaries in Murrah buffaloes have lower number of large follicles than left ovaries.

Keywords: Buffalo, ovarian, non ovarian factors, follicular population

1. Introduction
Ultrasound graphy technique is simple, non invasive imaging technique without side effect and has been used for various functions such as a tool in buffalo reproductive management (Giuseppina, 2012) [4]. Gyan *et al.* (2017) [6] reported that this technique has helped in diagnosis of various ovary and uterus diseases such as ovarian cyst and tumors, in addition to helped in predicting estrus in dairy animals after prostaglandin administration. Presicce *et al* (2003) [12] registrated that the ovarian follicular dynamics and follicular growth in buffalo was similar to that observed in cattle and was characterized by waves of follicular growth and regression. Ultrasound graphy has proved to be a valuable tool in assessing the status of ovarian structures such as follicle and corpus luteum in cyclic and non-cyclic buffaloes. For this purpose the current study was designed to investigate the influence of related factors such as age, body condition score (BCS), CL, pregnancy status and ovary localization on follicular population in Murrah buffalos using Ultrasound graphy technique.

2. Materials and methods
2.1Study area
The present research was conducted in year 2018 – 2019 at Central Institute for Research on Buffalo, Hisar Haryana, India, located between Latitude: 29°09′14″ N Longitude: 75°43′22″ E and Elevation above sea level: 216 m.

2.2 Experimental animals
The study was conducted at buffalo's farm in Central Institute for Research on Buffaloes (CIRB) during period from September, 2018 to January, 2019. 160 female Murrah buffaloes with average age of 2- 11 years were used.
The animals were maintained in a semi intensive system of housing at CIRB animal farm and were fed balance ration consisting of green fodder, wheat straw and concentration with specific mineral mixture developed by the institute.

2.3 Determination of animal's Body Condition Scoring
Animal body condition scoring was determined according to that method described by (Anitha *et al.*, 2011) [1] and classified animals from 1-5 score.

2.4 Transrectal Ultrasound graphy examination

Ultrasound graphy was performed by using a portable real-time B-mode transrectal ultrasound scanner (PVF -738F, TOSHIBA, SSA220 Japan) with an intra operative 7.0 MHz micro convex transducer. Each ovary was scanned several planes by maneuvering the transducer along it surface to ovary identity the ovarian structures. Positions and sizes of follicles (≥ 3mm) were measured after freezing the image using inbuilt calipers. However, follicles < 3mm were counted by seeing the image on the ultrasound screen appears as black circular structure surrounded by echogenic ovarian tissues. Ovary sizes, follicular population, localization of CL and uterus structure were examined and the diagnosis was made on basis of echogenicity of images. The pregnancy was confirmed by the presence of a visible fetus on viewing screen of the ultrasound machine which was irregularly shaped echogenic structure surrounded by non echogenic black color image in uterus (Sharma *et al*., 2012) [14].

3. Statistical Analysis

Data were analyzed using SPSS (Statistical Package for Social Sciences) Version 18. The analysis of variance and Duncan's test statistics were used to analyze appropriate data sets. Differences were significant at $P<0.05$ (Kouamo *et al*., 2014) [8, 9].

4. Results

4.1 Effect of non ovarian factors on follicular population

The Effect of non ovarian factors on follicular population was presented in Table 1. The number of small follicles in animals having BCS 4 (7.35±0.69) were significantly higher ($P<0.05$) as compared to animals having BCS 3 (4.47±0.64). However, no significant difference was observed between medium and large follicles in animals having different BCS. The total number of follicle per ovary was significantly greater ($P<0.05$) in animals having BCS 4 (9.85±0.74) as compared to BCS 3 animals 6.78±0.69. This effect was owing to more number of small follicles in the group. On the other hand, no significant different ($P<0.05$) was observed between the age of animal and the follicular population. Number of medium follicles was significantly higher ($P<0.05$) in pregnant animals when compared with non-pregnant cyclic animals. However, there was no significant different between the number of small follicles, large follicles and total number of follicle in pregnant and non-pregnant animals.

4.2 Effect of ovarian factors on follicular population

The effect of ovarian factors on follicular population was presented in Table 2. The ovary having CL had more number of medium size follicles (1.95±0.17) as compared to ovary without CL (1.39±0.18)and difference was significant ($P<0.05$). There was no significant difference in the total number of follicles present in both ovaries. Right ovary was having significantly lower ($P<0.05$) number of large follicles than left ovary (0.28±0.04 vs.0.43±0.05). This decrease has no effect on the total number of follicle per ovary. While, no significant observed between numbers of small follicle, large follicle and ovary localization at the present study.

Table 1: Means (± SE) values of BCS, age and pregnancy status

factors		NO	Number of follicles			Total of follicle / ovary
			Small	Medium	Large	
BCS	Thin (1-2)	0	0.0	0.0	0.0	0.0
	Good (3)	72	4.47±0.64a	1.64±0.16	0.67±0.07	6.78±0.69a
	V. Good (4)	88	7.35±0.69b	1.77±0.18	0.73±0.08	9.85±0.74b
	Fat(5)	0	0.0	0.0	0.0	0.0
	P < 0.05	-	0.003	0.594	0.587	0.003
Age	2 - 5	123	5-55±0.56	1.63±0.14	0.66±0.06	7.85±0.58
	6 - 12	37	7.11±1.02	1.92±0.28	0.84±0.14	9.86±1.14
	P < 0.05	-	0.180	0.333	0.166	0.102
Pregnancy status	pregnant	80	5.79±0.72	1.96±0.17b	0.73±0.08	8.48±0.80
	empty	80	5.70±0.65	1.48±0.19a	0.71±0.07	7.89±0.66
	P < 0.05	-	0.928	0.051	0.911	0.572

a,b,c In each column different letters (a, b) indicated significant difference between group ($p<0.05$). N=number of buffalo cow NO = Number SE = Standard Error BCS = Body Condition Score v= vary

Table 2: Means (± SE) values of CL and ovary localization

factors		NO	Number of follicles			Total of follicle / ovary
			Small	Medium	Large	
CL	Present	80	5.81±0.73	1.95±0.17^b	0.73±0.09	8.49±0.80
	Absent	80	5.76±0.65	1.39±0.18^a	0.74±0.08	7.89±0.66
	P<0.05	-	0.959	0.024	0.912	0.564
Ovary Localization	Right	160	3.06±0.26	0.81±0.08	0.28±0.04^a	4.15±0.27
	Left	160	2.72±0.26	0.91±0.09	0.43±0.05^b	4.05±0.29
	P<0.05	-	0.361	0.446	0.013	0.801

a,b,c In each column different letters (a, b) indicated significant difference between group ($p<0.05$). N=number of buffalo cow, NO = Number, SE = Standard Error, BCS = Body Condition Score

5. Discussion

The present study demonstrated that the average number of small follicles were significantly higher ($P<0.003$)in animals that had BCS 4.0 as compared to those having BCS 3.0. However, there was no significant difference in average number of medium and large follicles between these two groups. The results were similar to that found in cow (Kouamo *et al*., 2014) [8, 9]. This has been attributed to low Blood concentration of growth hormones such as Insulin like grow factor -1 (IGF-1). High plasma levels of IGF-1 resulting from improved nutrition, increases the sensitivity of granulosa cells to FSH stimulation (O'Callaghan and Boland, 1999) [10].

Ryan *et al.* (1994) [13] also found a relationship between the blood concentration of IGF-1 and fat or thin animals presented low concentrations of IGF-1. On the other hand the effect of age on follicular population showed no significant different between animals. May be this related to most of animals their ages between 2-5 years were heifers and have BCS grade 3. The results are similar to that reported in young steer and heifer's cow (Breier *et al.*, 1988; Granger *et al.*, 1989) [2, 5].The feed restriction causes a decrease in hepatic concentration of IGF-I and lead to deceleration of follicular growth.

This study also demonstrated that the pregnancy status in buffaloes did not affect the follicular population. Further, the ovary bearing Corpus luteum has more number of medium size follicles as compared to ovary without CL. This might be due to more blood supply in the ovary and continuous follicular growth waves every 8 to 10 days without dominance during pregnancy despite the continuous production of progesterone (Ginther *et al.*, 1989) [3]. The corpus luteum has a negative influence for the growth follicles larger than approximately 7mm after day 21 or 22 of pregnancy (Pierson and Ginther, 1989) [11]. The left ovary has significantly greater number of large follicles than right ovary. The presence corpus luteum in the ovary may contribute to unfavorable condition for follicular growth. As a result, follicle regressed and led to lower COCs recovered from ovaries with a presence of corpus luteum (Hafez and Hafez, 2000) [7].

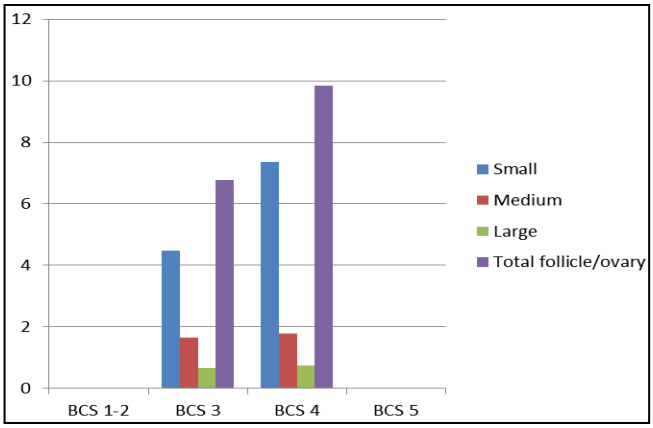


Fig 1: Relationship between BCS and follicular population in Murrah buffaloes

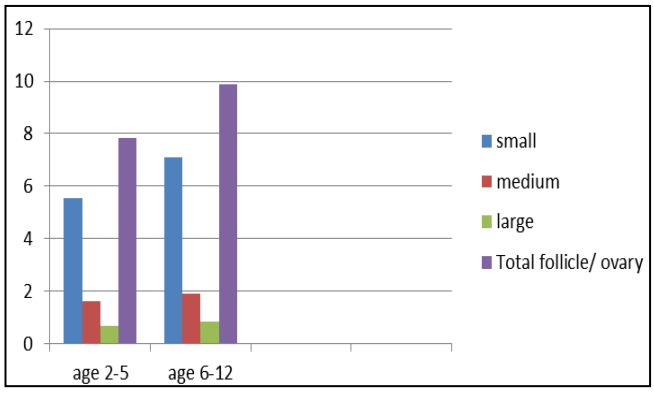


Fig 2: Relationship between Age and follicular population in Murrah buffaloes

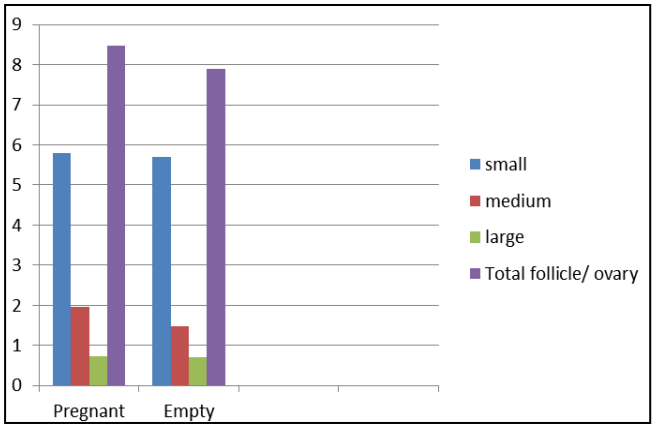


Fig 3: Relationship between pregnancy status and follicular population in Murrah buffaloes

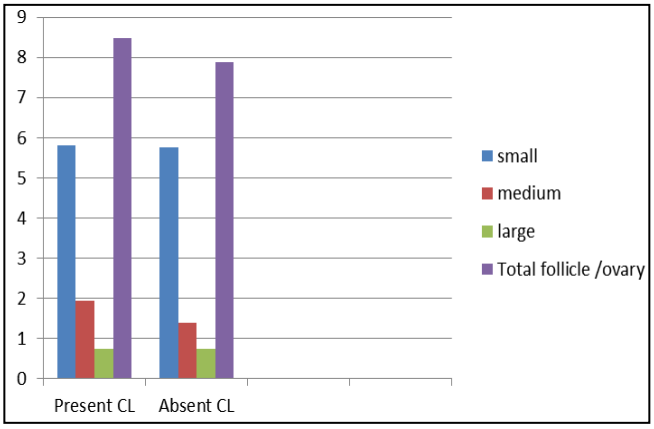


Fig 4: Relationship between present or absent of CL and follicular population in Murrah buffaloes

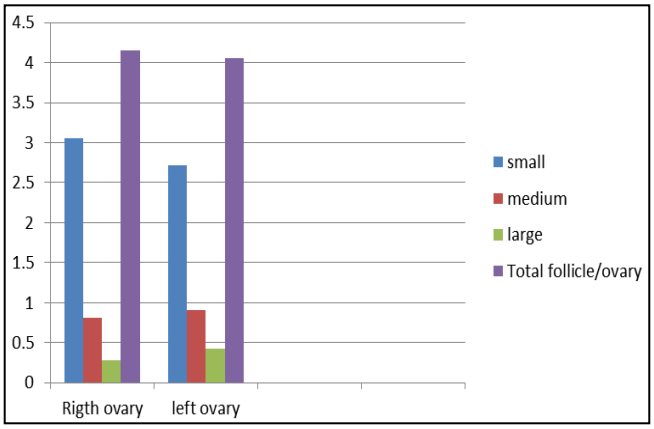


Fig 5: Relationship between ovary localization and follicular population in Murrah buffaloes

6. Conclusion

This study revealed that the ovaries of Murrah buffaloes with BCS 4 have higher number of follicles than those with BCS 3.The ovaries from pregnant animals have more number of medium size follicles as compared to those of non-pregnant buffaloes. The right ovaries in Murrah buffaloes have lower number of large follicles than left ovaries. Future studies are require to study any seasonal variation if any, between different BCS, age, pregnancy status, presence or absence of CL and ovary localization.

7. Acknowledgements
The author thanks the CIRB administration for allow him to did this research in the buffalo's farm and CV Raman fellowship for support this research.

8. References

1. Anitha A, Sarjan Rao K, Suresh J, Srinivasa Moorthy PR, Kotilinga Reddy Y. A body Condition Score (BCS) system in Murrah Buffaloes. Buffalo Bulletin. 2011; 1:79- 99.
2. Breier BH, Gluckman PD, Bass JJ. Influence of nutritional status and oestradiol-17b on plasma growth hormone, insulin-like growth factors-I and -II and the response to exogenous growth hormone in young steers. Journal Endocrinolology. 1988; 118:243-250.
3. Ginther OJ, Knopf L, Kastelic JP. Ovarian follicular dynamics in heifers during early pregnancy. Biology. Reproduction. 1989; 41:247-254.
4. Giuseppina MT. Ultrasonography and Reproduction in Buffalo. Journal of Buffalo Science. 2012; 1:163-173.
5. Granger AL, Wyatt WE, Craig WM, Thompson DL, Hembry FG. Effects of breed and wintering diet on. growth, puberty and plasma concentrations of growth hormone and insulin like growth factor I in heifers. Domestic Animal Endocrinology. 1989; 6:253-262.
6. Gyan S, Chandolia RK, Dutt R, Saini A, Dalal J, Malik RK. Comparative Study of Follicular Turn Over in Cyclic and Non-Cyclic Murrah Buffaloes through 2D Trans-Rectal Ultrasonography. International Journal of Current Microbiology and Applied Sciences. 2017; 6:3590-3602.
7. Hafez ES, Hafez EB. Folliculogenesis, egg maturation, and ovulation. Reproduction in Farm Animals, 7th Edition. 2000; pp. 68-81.
8. Kouamo J, Dawaye SM, Zoli AP, Bah GS. Evaluation of bovine (Bos indicus) ovarian potential for in vitro embryo production in the Adamawa plateau (Cameroon), Open Veterinary Journal. 2014; 4(2):128-136.
9. Kouamo J, Dawaye SM, Zoli AP, Bah GS. Evaluation of bovine (Bos indicus) ovarian potential for in vitro embryo production in the Adamawa plateau (Cameroon), Open Veterinary Journal. 2014; 4(2):128-136.
10. O'callaghan D, Boland MP. Nutritional effects on ovulation, embryo development and establishment of pregnancy in ruminants. Animal. Science. 1999; 68:299-314.
11. Pierson RA, Ginther OJ. Intraovarian effect of the corpus luteum on ovarian follicles during early pregnancy in Heifers. Animal reproduction Science. 1989; 15:53-60.
12. Presicce GA, Parmeggiani A, Sentore EM, Stecco R, Barille VL, De Maruo GJ, *et al.* Hormonal dynamics and follicular turnover in prepubertal Mediteranean Italian buffalo. Theriogenology. 2003; 8860:1-9.
13. Ryan DP, Spoon RA, Griffith MK, Williams GL. Ovarian follicular recruitment, granulosa cell steroidogenic potential and growth hormone/ insulin-like growth factor-I relationships in suckled beef cows consuming high lipid diets: Effect of graded differences in body condition maintained during the puerperium. Domestic Animal Endocrinology. 1994; 11:161-174.
14. Sharma RK, Singh JK, Khanna S, Phulia SK, Sarkar SK, Inderjeet S. Fetal age determination in Murrah buffaloes from days 22 through 60 with ultrasonography. Indian Journal of Animal Sciences. 2012; 82(4):374-376.

https://dx.doi.org/10.22161/ijeab.45.4 *ISSN: 2456-1878*

Influence of different stages of corpus luteum on ovary size, oocytes grades and follicular population in Indian buffaloes

Eias Elzein I Osman[1], Sharma. RK[2] and Majdi. E. Badawi[3], Dahiya S[2]., Prem, Y[2]., Pholia A[2]., Dharmendra K[2]., Naresh S[2]., Jerome A[2]., Pradeep K[2]., Monika S[2]., Nidhi R[2]., Abubaker W[2]., and Rasika R[2].

[1]Department of clinical studies, Faculty of Veterinary Science, University of Nyala, Nyala- Sudan.
[2]Physiology and Reproduction Division, Central Institute for Research on Buffaloes, Hisar-125001, India.
[3]Department of medicine and animal surgery, College of Veterinary Medicine, Sudan University of Sciences and Technology- Khartoum.
***Corresponding author:** eiasalzain66@yahoo.com

Abstract**—** *The aim of this study was to evaluate the effect of different stages of corpus luteum on ovary size, oocytes grades and follicular population. A total of 109 buffalo ovaries were collected from slaughterhouse and transported to laboratory for determination of ovaries weight, length, width, thickness, follicular population and oocytes grades. The results obtained revealed that the effect of types of CL on ovary weight and size showed significantly difference in type of CL groups with ovary weight and ovary size. Ovaries having CL in late stage showed highest mean of ovary weight, length, width and thickness over the CL in early and middle stage. Moreover, the results showed no significantly different in type of CL groups with follicular population and oocytes grades.*
Keywords**— *Buffalo, Ovary, Corpus luteum, Follicular population, oocytes grades.***

I. INTRODUCTION

Corpus luteum (CL) is an endocrine gland formed after ovulation of graffian follicle and contributes to regulate estrous cycle and maintenance of pregnancy (Schams and Berisha, 2004). In different stages of estrus cycle and pregnancy, corpus luteum has several stages in size and structure (fields and fields, 1996). Corpus luteum synthesizes and secretes hormones such as progesterone, estrogen, relaxin, oxytocin, vasopressin and inhibin (Fields, 1991). Progesterone is essential steroid hormone necessary for establishing of pregnancy in domestic animals (Tomac *et al*., 2011). Moreover, blood progesterone has useful tool to determine an appropriate time of insemination, monitoring of cyclicity and pregnancy diagnosis in buffaloes (Batra and Pandey, 1983) .The aim of the current study was to evaluate the effect of different stages of corpus luteum on ovary size, oocytes grades and follicular population.

II. MATERIALS AND METHODS

2.1 Study area:

The present research was conducted in year 2018 – 2019 at Central Institute for Research on Buffalo, Hisar Haryana, India, located between Latitude: 29°09′14″ N Longitude: 75°43′22″ E and Elevation above sea level: 216 m.

2.2 Experiment design

One hundred nine buffalo ovaries having CL were collected immediately after slaughtering from Delhi slaughterhouse and transported to the laboratory in an insulated container containing normal saline with antibiotics. In laboratory, all tissues attached to ovaries were removed and all ovaries were washed twice in saline solution containing antibiotics (Dharmendra *et al*., 2011). After wash all ovaries were classified into three groups: Group having CL in early stage, middle stage and late stage.

2.2.1 Determination of ovaries weights:

Ovaries were weighed by using an electronic scale balance and expressed in gram (Kouamo *et al*., 2014).

2.2.2 Determination of ovaries lengths:

Ovaries lengths were measured using electronic Vernier calipers as the distance from anterior pole to posterior pole and expressed in cm (Samad and Raza, 1999).

https://dx.doi.org/10.22161/ijeab.45.4 *ISSN: 2456-1878*

2.2.3 Determination of ovaries widths:

Ovaries widths were measured using electronic Vernier calipers as the greater distance from the medial to the lateral surfaces and expressed in cm (Bukar *et al*., 2006).

2.2.4 Determination of ovaries thicknesses:

Ovaries thicknesses were measured using electronic Vernier calipers as greatest distance along an axis vertical to the longitudinal axis and expressed in cm (Razzaque *et al*., 2008).

2.2.5 Determination of follicular population:

For each ovary, visible follicles were counted and follicle size was measured with electronic Vernier calipers. Follicles were classified into 3 categories: small (<3 mm), medium (3 to 8 mm) and large (> 8 mm) (Baki Acar *et al*., 2013).

2.2.6 Oocytes collection:

Oocytes were collected by aspiration of surface follicles (2–8 mm diameter) using 18-gauge disposable needle attached to a 10 ml syringe in aspiration medium. The follicular fluid was collected in tube and kept for 15 minutes. The sediment was collected in 60 mm Petri dish and oocytes were searched under stereo zoom microscope.

2.2.7 Grading of Oocytes:

Oocytes were graded as: A, B, C and D according to homogenous of ooplasm and cumulus cells layer.

III. STATISTICAL ANALYSIS

Data were analyzed using SPSS (Statistical Package for Social Sciences) Version 18. The analysis of variance and Duncan's test statistics were used to analyze appropriate data sets. Differences were significant at $P < 0.05$.

IV. RESULTS AND DISCUSSION

The effect of types of CL on ovary weight and size is presented in table 1. The results showed significantly difference ($P<0.05$) in type of CL groups with ovary weight and ovary size. Ovaries having CL in late stage showed highest mean of ovary weight (5.03±0.17), length (2.35±0.05), width (1.74±0.05) and thickness (1.48±0.03) over the CL in early and middle stage. The mean weight, length, width, thickness were significantly higher in ovaries having CL in late stage as compared with ovaries having CL in early and middle stages. This result may occur due to hyperplasty of fibroblast of the connective tissue and vascularity contributes to an increase in size of the CL (Jablonka-Shariff *et al*., 1993).

The effect of types of CL on follicular population is presented in table 2. The results showed no significant difference between type of CL and follicular population. May be due to progesterone mechanism which inhibits follicular growth through suppression of LH which is critical for continued growth to large follicles (Bartlewski *et al*., 2001). Campbell *et al* (1991) reported that the CL secreted inhibin hormone into ovarian venous blood which has widely affect on ovarian follicular growth. These results were different than that found by (Mervat and Marwa, 2019) in cow. This difference might be due to animal and environment.

The effect of type of CL on oocyte grades is presented in table 3. The results showed no significant difference between type of CL and oocyte grades.

Table -1 Means (± SE) values of early, middle and late stage of CL:

Factors		No. of ovary	Ovary size			
			Weight	**Length**	**Width**	**Thickness**
CL	Early CL	26	3.38±0.24^{c}	2.09±0.09^{c}	1.41±0.06^{c}	1.20±0.02^{c}
	Middle CL	23	4.03±0.24^{b}	2.14±0.09^{b}	1.54±0.06^{b}	1.47±0.04^{b}
	Late CL	60	5.03±0.17^{a}	2.35±0.05^{a}	1.74±0.05^{a}	1.48±0.03^{a}
	P < 0.05	-	**.000**	**.015**	**.000**	**.000**

a,b,c In each column different letters (a, b) indicated significant difference between group ($p<0.05$ No =number SE = Standard Error CL = corpus leutum

Table -2 Means (± SE) values of early, middle and late stage of CL:

Factors		No. of ovary	Number of follicles			
			Small	**Medium**	**Large**	**Average No**
CL	Early CL	26	1.46±0.30	0.46±0.16	0.31±0.11	2.23±0.29
	Middle CL	23	1.13±0.29	0.61±0.18	0.26±0.14	2.00±0.35
	Late CL	60	1.22±0.18	0.52±0.13	0.28±0.06	2.02±0.20
	P < 0.05	-	**.690**	**.858**	**.957**	**.821**

No =number SE = Standard Error CL = corpus leutum

https://dx.doi.org/10.22161/ijeab.45.4 *ISSN: 2456-1878*

Table -3 Means (± SE) values of early, middle and late stage of CL

			Oocytes grades				
Factors		**No. of ovary**	**I**	**II**	**III**	**IV**	**Selected oocytes for IVEP. I and II**
	Early CL	26	0.23±0.20	0.46±0.34	0.50±0.46	0.12±0.12	0.69±0.53
	Middle CL	23	0.39±0.27	0.52±0.38	0.35±0.27	0.09±0.09	0.91±0.64
	Late CL	60	0.20±0.15	0.35±0.25	0.78±0.65	0.0	0.55±0.39
CL	**P < 0.05**	-	**.791**	**.920**	**.890**	**.295**	**.880**

No=number SE = Standard Error CL = corpus leutum

V. CONCLUSION

From the present study, it is concluded that the highest ovary weight and size in ovaries with CL in late stage than others stages. So, the corpus luteum has a great effect on ovarian morphology without having effect on oocyte grades and follicular population in buffaloes.

ACKNOWLEDGEMENTS

The author thanks the CIRB administration for allow him to did this research in Embryo laboratory and CV Raman fellowship for support this research.

REFERENCES

[1] Baki Acar, D., Birdane, M.K., Dogan, N. and Gurler, H. (2013). Effect of the stage of estrus cycle on follicular population oocyte yield and quality, and biochemical composition of serum and follicular fluid in Anatolian water buffalo. Animal Reproductive Science. 137, 8-14.

[2] Bartlewski, P.M., Beard, A.P. and Rawlings, N.C. (2001). Ultrasonographic study of the effects of the corpus luteum on antral follicular development in unilaterally ovulating western white faced ewes. Animal reproduction Science; 65:231 – 244.

[3] Bukar, M.M., Amin, J.D., Sivachelvan, M.N and. Ribadu, A.Y. (2006). Postnatal histological development of the ovaries and uterus and the attainment of puberty in female kid goats. Small Ruminant, Research, 65:200-208.

[4] Batra, S.K. and Pandey, R.B.(1983). Luteinizing hormone and oestradiol- 17 β in blood plasma and milk during the estrous cycle and early pregnancy in Murrah buffaloes. Animal Reproduction Science, 5:247 – 257.

[5] Campbell, B.K., Picton, H.M., Mann, G. E., Mcneilly, A. S. and Baird, D.T. (1991). Effect of steroid and inhibin-free ovine follicular fluid on ovarian follicles and ovarian hormone secretion. Journal Reproduction and Fertility; 93:81 – 96.

[6] Fields, M .J and Fields, P.A. (1996). Morphological characteristics of bovine corpus luteum during estrous cycle and pregnancy. Theriogenology; 1295- 1325.

[7] Fields, P. A. (1991). Relaxin and other luteal secretory peptides: Cell localization and function in the ovary. In: familiari, G., Makabe, S., Motta, P.M (eds): Ultrastructure of the ovary. Electronic Microscopy on Biology and Medicine,9;177 – 198.

[8] Kouamo. J, Dawaye. S.M, Zoli. A.P. and Bah. G.S. (2014). Evaluation of bovine (Bos indicus) ovarian potential for in vitro embryo production in the Adamawa plateau (Cameroon), Open Veterinary Journal, 4(2): 128-136.

[9] Mervat, S.H. and Marwa, E. (2019). Evaluation of Corpora lutea effect on ovarian morphology, follicular population and biochemical profile in follicular fluid and blood of slaughtered cow. Assut Veterinary Medicine Journal, 5:1-9.

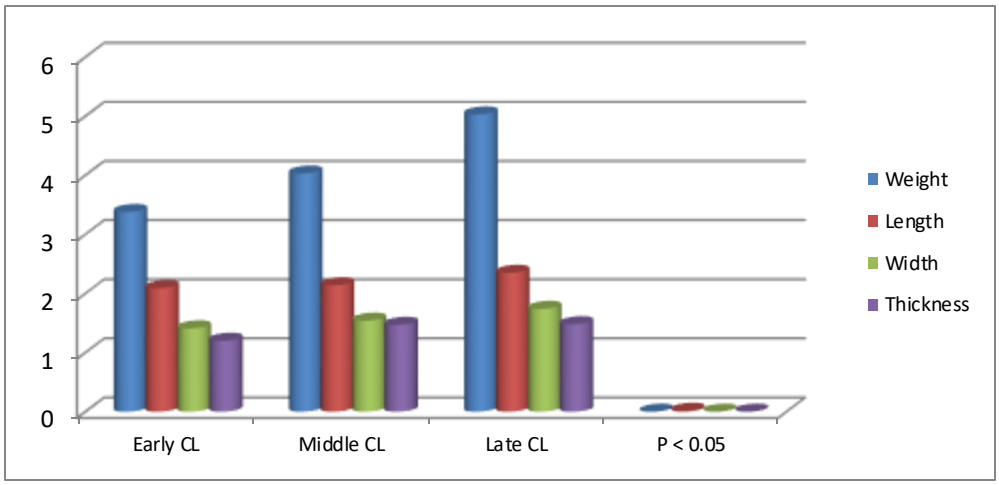
[10] Razzaque, W.A.A., Sahatpure, S. K., Pawshe, C.H and Kuralkar, S.V. (2008). Biometry of ovaries and follicular count in cycling and non cycling Nagpuri buffaloes (Bubalus Bubalis). Journal of Buffalo Bulletin: 27.1:150-153.

[11] Samad, H.A. and Raza, A. (1999). Factors affecting recovery of buffalo follicular oocytes. Pakistan Veterinary Journal. 19:pp 56-59.

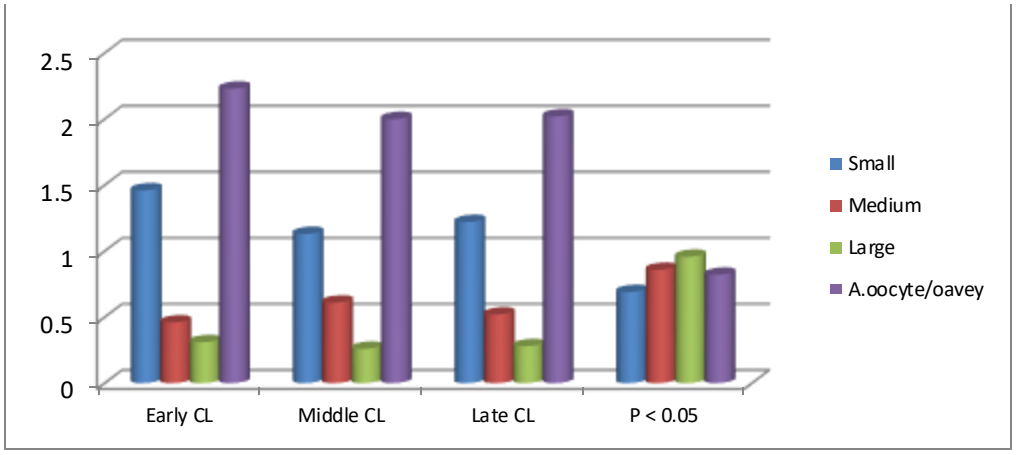
[12] Schams, D and Berisha, B. (2004). Regulation of Corpus luteum functions in cattle-An overview. Reproductive Dominant Animal; 241- 251.

[13] Tomac, J., Cekinovi, D. and Arapovic, J. (2011). Biology of the corpus luteum. Periodicum Biologorum; 113: 49 – 73.

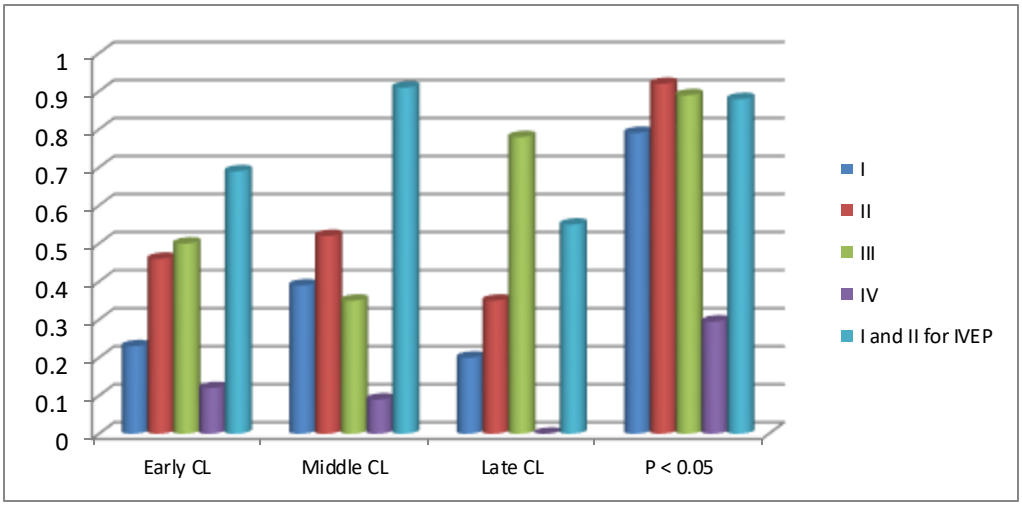
https://dx.doi.org/10.22161/ijeab.45.4 *ISSN: 2456-1878*



Relationship between types of CL, ovary weight and ovary size



Relationship between types of CL and follicular population



Relationship between types of CL and oocytes grades

ISSN 2664-4010 (Print) & ISSN 2664-6730 (Online)
South Asian Research Journal of Agriculture and Fisheries
Abbreviated Key Title: South Asian Res J Agri Fish

| Volume-1 | Issue-3 | Oct-Nov -2019 | **DOI: 10.36346/SARJAF.2019.v01i03.005**

Original Research Article

Evaluation of Ovarian Potential for in Vitro Embryo Production on Indian Buffaloes

Eias Elzein I Osman[1*], Sharma RK[2] and Majdi E Badawi[3]

[1]Department of clinical studies, Faculty of Veterinary Science, University of Nyala, A43, Nyala, Sudan
[2]Physiology and Reproduction Division, Central Institute for Research on Buffaloes, Sirsa Rd, Sector 14, Hisar, Haryana 125001, India
[3]Department of medicine and animal surgery, College of Veterinary Medicine, Sudan University of Sciences and Technology, Khartoum, Sudan

***Corresponding Author**
Eias Elzein I Osman

Article History
Received: 22.10.2019
Accepted: 31.10.2019
Published: 11.11.2019

Abstract: The aim of this study was to evaluate the effect of CL, ovary weight and ovary size, on follicular population, oocytes recovery, oocytes grades, oocytes index and cleavage rate on Indian buffalos. A total of 296 buffalo ovaries were collected from slaughterhouse and transported to laboratory for determination of ovaries weight, length, width, thickness, follicular population and oocytes grades, oocytes index and cleavage rate. The results obtained revealed that the number of small and large follicles were significantly higher in ovaries having weight more than 5g as compared to ovaries weight between 3-5g and less than 3g. The number of small follicles in large size ovaries was significantly higher as compared to small size ovaries. The mean number of oocytes recovery, number of oocytes for IVEP and number of cleavage, obtained from ovaries with CL was lower than that of ovaries without CL. No significant difference observed between presence and absence of CL, follicular population and oocytes grades.

Keywords: Indian buffalo, Ovary, follicular population, oocytes recovery, oocytes grades, embryo.

INTRODUCTION

Biotechnologies are being used in animal and agriculture to improve production, and to develop specialized food products and pharmaceutical products. Manipulations of reproductive processes are necessary to accomplish these goals. In this direction dairy industry was perfected the application of the first reproductive biotechnology is artificial insemination (AI). The dairy industry also remains the number one user of embryo transfer technology (ET). In addition, recently the dairy industry has also adopted the field of in vitro embryo production [1]. *In vitro* Embryo Production (IVEP) is reproductive techniques that supplement AI in the genetic improvement of local cattle breeds [2]. IVEP permits the preservation of genetic potential of sub-fertile or dead animals [3] by the creation of a gene bank with oocytes recovered from slaughterhouses [4] for the improvement of livestock productivity [5]. The embryo production is carried out through a combination of techniques of collection of immature oocytes, in vitro maturation (IVM), fertilization (IVF) and culture (IVC). However, the significantly contributing factors in the success of IVEP are the quality and number of collected oocytes [6]. In addition, in vitro production technologies not only help in production of high genetic merit animals, but also consider an excellent source of embryos for emerging biotechnologies such as embryo sexing, cloning, nuclear transfer, transgenesis etc. Furthermore, it allows analyzing developmental potential of embryos, including the pattern of gene expression, epigenetic modifications and cytogenetic disorders during the development [7]. Early stages of bovine embryo development show many similarities with human embryos. Therefore, bovine embryos are used as a model organism [8]. Also, IVP can be used to rescue and save irreplaceable genetic material following slaughter for infectious disease control or culling for other reasons [9]. There is no information available about evaluation of ovarian potential for In Vitro Embryo Production in study area. The objective of this study was to evaluate of ovarian potential for In Vitro Embryo Production on Indian buffaloes.

MATERIALS AND METHODS

Study Area

The present research was conducted in year 2018 – 2019 at Central Institute for Research on Buffalo, Hisar Haryana, India, located between Latitude: 29°09'14" N Longitude: 75°43'22" E and Elevation above sea level: 216 m.

Experiment Design

Medium Preparation

All chemicals and media used in the present study were obtained from Sigma Aldrich (St. Louis, MO, USA), and the ware plastic was from Falcon (Paignton, UK), unless stated otherwise. Media and reagents were prepared using standard protocol of embryo laboratory technique at Central Institute for Research on buffaloes, India under aseptic conditions. All media were filtered using 0.22 µm pore size filter (Durapure® membrane filter, Carrigtwohill, Ireland) and culture medium was routinely equilibrated in incubator at 38.5°C with 5% CO_2 in humidified air for at least 2 hrs before use.

Ovaries Collection

Two hundred ninety six buffalo ovaries were collected immediately after slaughtering from Delhi slaughterhouse and transported to the laboratory in an insulated container containing 0.9% normal saline with antibiotics 400 IU/ml penicillin and 500 µg/ml streptomycin at 32–37°C within 4–5 h. In laboratory, all tissues attached to ovaries were removed and all ovaries were washed twice in saline solution containing antibiotics [10]. After wash all ovaries were classified into two groups: group ovaries with CL and without CL.

Determination of Varies Diameters

Length

The length of the ovary was taken as the distance from anterior pole to posterior pole along an axis parallel to the ovarian mesenterial attachment expressed in centimeter [11].

Width

Width of the ovary was taken as the greater distance from the medial to the lateral surfaces or borders expressed in centimeter [12].

Thickness

Thickness of the ovary was recorded as the greatest distance along an axis vertical to the longitudinal axis (base) at its center or distance from attached to the free borders expressed in centimeter [13].

Weight

Weight of the ovary was taken on the electronic balance and expressed in gram [14].

Follicular Population

The visible follicles were counted and follicle size was measured with electronic Vernier calipers. Follicles were classified into 3 categories: small (<3 mm), medium (3 to 8 mm) and large (> 8 mm) [15].

Oocytes Collection

Oocytes were collected by aspiration of surface follicles (2–8 mm diameter) using 18-gauge disposable needle attached to a 10 ml syringe in aspiration medium consisting of TCM-199 and 0.6% (v/w) bovine serum albumin (BSA). The follicular fluid was collected in tube and kept for 15 minutes. The sediment was collected in 60 mm petri dish and oocytes were searched under stereo zoom microscope [10].

Oocytes Grading

The oocytes were graded as: grade A: having evenly granulated homogenous ooplasm with cumulus cells more than 4 compact layers, grade B: having evenly granulated homogenous ooplasm with 2 to 3 layers of cumulus cells, grade C: having evenly granulated homogenous ooplasm with less compact cumulus cells and grade D: having irregular dark ooplasm and highly expanded cumulus cells. Oocytes selected as grade A and B for IVM [16].

Oocyte recovery rate per ovary

The oocyte recovery rate was calculated by following formula: ORR = (No of recovered oocytes / total No of ovaries) [17].

Oocyte Index

It was calculated as an index using the formula [(G I x 1 + G II x 2 + G III x 3 + G IV x 4) / Total number of oocytes recovered] as described by [15]. Index values that approach one reflected good quality oocytes.

In Vitro Maturation of Oocytes

Oocytes were washed three times with the washing medium (TCM-199+10% FBS+0.81 mM sodium pyruvate+50 µg/ml gentamycin sulphate) and then twice with the IVM medium (TCM-199+10% FBS+5 µg/ml porcine FSH+1 µg/ml estradiol-17β+0.81 mM sodium pyruvate+5% buffalo follicular fluid+50 µg/ml gentamycin sulphate). The washed cumulus oophorus complexes were then placed in 80-µl droplets (15–20 oocytes/droplet) of the IVM medium, covered with sterile paraffin oil, in a 35 mm Petri dish (Becton, Dickinson and Co., Lincoln Park, NJ, USA) and cultured for 24 h in a CO_2 incubator (5% CO_2 in air, 90–95% relative humidity) at 38.5°C [10]. After 24 h of incubation the maturation of oocytes was assessed based on the degree of cumulus expansion. Expansion of COCs was characterized by its sticky nature and enlargement of the cumulus mass to at least 2-3 diameters from the zona pellucida [18].

In Vitro Fertilization

The matured oocytes were washed three times in BO medium having 10 µg/ml heparin, 137.0 µg/ml sodium pyruvate and 1.942 mg/ml caffeine sodium benzoate then transferred to 50 µL droplets (15–20 oocytes/droplet) of the IVF medium (BO medium containing 10 mg/ml fatty acid-free BSA).There after two straws of frozen-thawed ejaculated buffalo semen were washed twice with BO medium. The spermatozoa in 50 µL of the IVF medium (3 million spermatozoa/ ml) were then added to the droplets containing the oocytes, covered with sterile mineral oil and placed in a CO_2 incubator (5% CO_2 in air) for 18 h at 38.5°C [10].

In Vitro culture of fertilized oocytes

Presumptive zygotes were denuded from cumulus cells and the extra spermatozoa by gentle pipetting and washed three times in IVC washing medium (T_2) supplemented with 1% fatty acid-free bovine serum albumin (BSA).Then fertilized oocytes were cultured in IVC (Research Vitro Cleave Medium (K-RVCL-50; Cook, Brisbane, Queensland, Australia) by making two to three droplets in 35mm petridish and overlaid with mineral oil, at 38.5 ◦C and 5% CO_2 in a humidified incubator for 8 days [10].

Cleavage embryos were identified according to their cell number during specific time as 1- cell on day 1, 2-cells on day 2, 4-cells on day 3, 8-cells on day 4, 16-cells on day 5 as well as morula and blastocyst stages on day 6 and 7 respectively [19].

Cleavage Rate

The cleavage rate was calculated as follows: Cleavage rate (CR) = number of cleavage / total of oocytes inseminated X 100 [17].

Blastocyst Rate

The blastocyst rate was calculated as follows: Blastocyst rate = number of Blastocyst / total of oocytes inseminated X 100.

STATISTICAL ANALYSIS

The study data were analyzed by Student t- test analysis using SPSS Version 18. Statistically significant confidence interval was taken as $P<0.05$.

RESULTS

Effect of presence and absence of CL, ovary weight and size on follicular population:

The effects of presence and absence of CL, weight and size of ovary on follicular population are presented in Table-1. No significant difference was observed between ovaries with CL and ovaries without CL on follicular population. On the other hand, the number of small (1.76±0.37) and large follicles (0.41±0.51) found significantly higher ($P<0.05$) numbers in ovaries having weight more than 5g as compared to ovaries weight between 3-5g and less than 3g. Otherwise, no significant difference was observed between number of medium follicles and ovaries weight. The average number of follicles per ovary was found significantly higher ($P<0.05$) in ovaries having weight more than 5g (2.59±0.48) as compared to ovaries had weight between 3-5g (2.22±0.14) and less than 3g (1.80±0.10) respectively. This effect was due to high number of small and large follicles in group ovaries of weight more than 5g. Upon comparison of ovary size, we found that the number of small follicles in large size ovaries (1.43±0.12) was significantly higher ($P<0.05$) as compared to small size ovaries (0.90±0.10). Overall, the average number of follicles per ovary in large size ovaries (2.25±0.13) was shown significantly higher ($P<0.05$) than small size ovaries (1.76±0.10). This effect was owing to more number of small follicles in group. Otherwise no significant difference was observed between number of medium, large follicles and ovary size.

Effect of presence and absence of CL on oocytes grades

The effect of presence and absence of CL on oocyte grade is presented in table 2. The results showed that there is no significant difference observed between presence and absence of CL and oocytes grades.

Effect of present or absent of CL on oocytes recovery, oocytes index, cleavage and blastocyst rates

The effect of CL on oocytes recovery, oocytes index, cleavage and blastocyst rates are presented in table 3. The result showed the mean number of oocytes recovery obtained from ovaries with CL (145) was lower than that of ovaries without CL (335).

The mean number of cleavage obtained from ovaries with CL (23) was lower as compared with those without CL (55). Otherwise, the oocytes recovery per ovary, oocytes index, percentages of cleavage and blastocyst were not different between groups.

Table-1: Means (± SE) values of CL, ovary weight and ovary size:

Factors		No. of ovaries	Number of follicles			Average number of follicle /ovary
			Small	Medium	Large	
CL	Present	109	1.26± 0.14	0.52 ±0.09	0.28±0.05	2.06±0.15
	Absent	187	1.15 ±0.1	0.56±0.07	0.30±0.04	2.01±0.11
	P < 0.05	**-**	**.519**	**.744**	**.817**	**.763**
Ovary weight(g)	<3	147	1.01±0.11^{c}	0.57±0.08	0.22±0.04c	1.80±0.10^{c}
	3-5	132	1.32±0.12^{b}	0.54±0.09	0.36±0.05^{b}	2.22±0.14^{b}
	>5	17	1.76±0.37^{a}	0.41±0.26	0.41±0.51^{a}	2.59±0.48^{a}
	P < 0.05	**-**	**.034**	**.808**	**.050**	**.015**
Ovary size(cm)	Small size	134	0.90±0.10^{b}	0.60±0.08	0.26±0.04	1.76±0.10^{b}
	Large size	162	1.43±0.12^{a}	o.51±0.08	0.32±0.05	2.25±0.13^{a}
	P < 0.05	**-**	**.001**	**.427**	**.342**	**.004**

a,b,c in each column different letters (a, b) indicated significant difference between group (p<0.05). No =number CL = corpus luteum SE = Standard Error g = gram cm = centimeter

Table-2: Effect of presence or absence of CL (Means ± SE) on oocytes grades

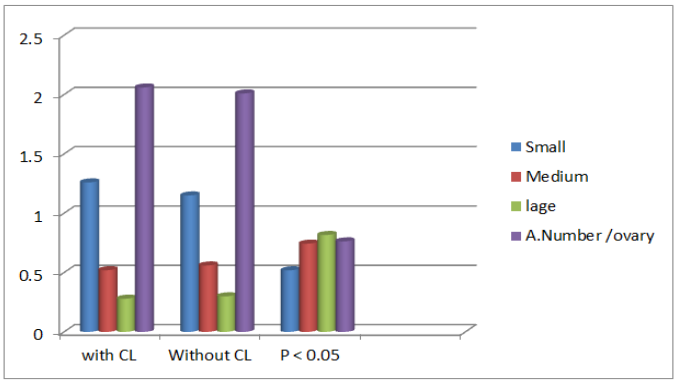
Factors		No. of ovaries	Oocytes grades				Selected oocytes for IVEP. I and II
			I	II	III	IV	
CL	Present	109	0.25±o.11	0.41±0.18	0.62±0.38	0.05±0.03	0.66±0.28
	Absent	187	0.27±0.19	0.84±0.60	0.68±0.50	0.00	1.11±0.78
	P < 0.05	-	**.940**	**.592**	**.933**	**.069**	**.670**

No=number SE = Standard Error CL = corpus luteum

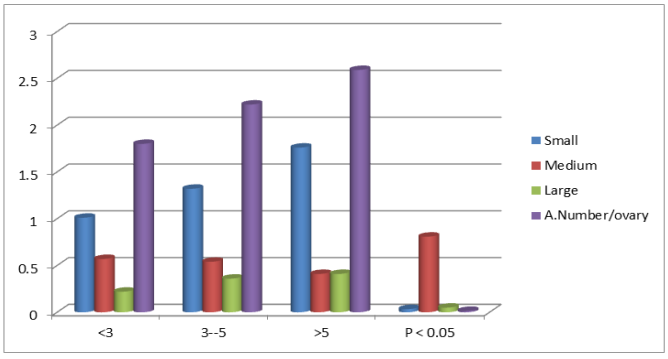
Table-3: Effect of CL on oocytes recovery, oocytes index, cleavage and blastocyst rates

Factors		No. of ovaries	No. of Oocytes recovery	Oocytes recovery rate / ovary	Oocytes Index	No. of Oocytes for IVEP. I and II	No. of Cleavage	Cleavage rate %	No. of Blastocyst	Blastocyst rate %
CL	Present	109	145	1.3	2.35	72	23	31.9	1	1.3
	Absent	187	335	1.7	2.23	207	55	26.6	1	0.5

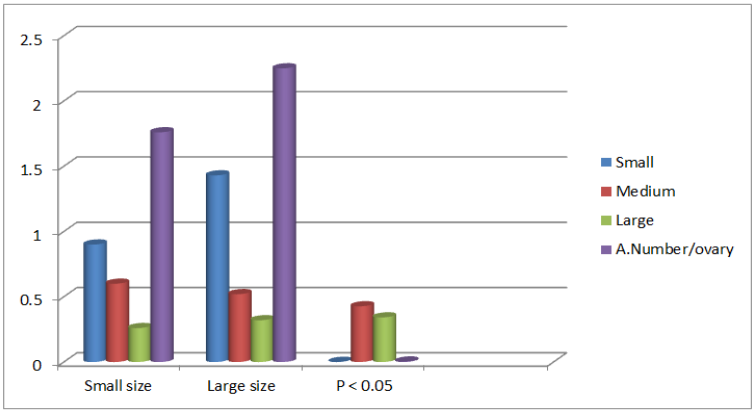
No= number SE = Standard Error % = percent CL = corpus luteum



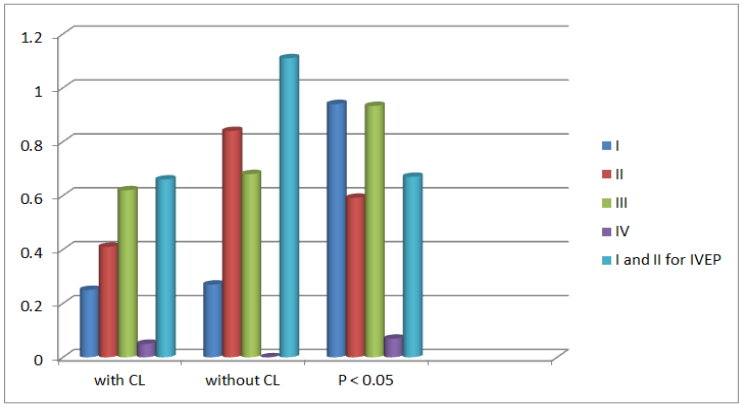
Relationship between Present or absent of CL and follicular population



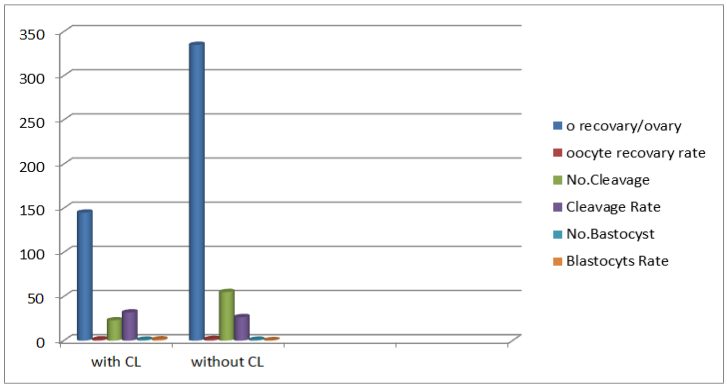
Relationship between ovary weight and follicular population



Relationship between ovary size and follicular population



Relationship between present or absent CL and oocytes grades



Effect of CL on cleavage and blastocyst rates

DISCUSSION

The present study demonstrated that the number of small and large follicles were significantly higher in ovaries having weight more than 5g as compared to ovaries weight 3-5g and less than 3g. No significant effect was observed between number of medium follicles and ovary weight. This result simulate to that of found in cow heifers [20, 21], indicated that the variation in ovarian weight is not only due to the weight of antral follicles and CL but there is number of microscopic follicles and these follicles at least partially contributed to the weight of the ovary. The number of follicle per ovary was significantly higher (P <.004) in ovaries have large size when compared with ovaries of small size. Our results are in agreement with early report of buffalo [11] and sheep [22]. This study demonstrated that no significant difference observed between presence or absence of CL and oocytes grades. The results are also similar to that reported in bovine [14].

The mean of oocyte recovery rate obtained from ovaries with CL (1.3%) was significantly lower than that ovary without CL (1.7%). Our results is similar to that found in buffalo by Das *et al.,* [23] and Raza *et al.,* [24] and they reported that the presence of a CL significantly reduces the number of ovarian follicles as well as the quality of oocytes in buffaloes. Because the follicular

development is restricted as lutein cells occupy most of the ovary [25]. Different studies have reported 0.4 - 3.85 good oocyte/ ovary in buffalo [26].

This variation is due to the different methods used for COC recovery, seasonal effects, and variation in the reproductive status of the slaughtered buffaloes. However, when compared to cattle (10 oocyte /ovary) [27].The number of good oocyte/ovary in buffalo is lower which may be due to an inherently smaller number of primordial follicles and a higher frequency of atresia in buffalo [28]. Further, the percentages of cleavage and blastocyst were lower as compared with the number of oocytes recovery. This result may be due to the effect of time taken on measuring the weight and size of the ovaries on the quality of oocytes.

CONCLUSION

This study revealed that the number of small and large follicles were significantly higher in ovaries having weight more than 5g as compared to ovaries weight between 3-5g and less than 3g. The number of small follicles in large size ovaries was higher than small size ovaries**.** The number of oocytes recovery, number of oocytes for IVEP and number of cleavage, obtained from ovaries with CL was lower than that of ovaries without CL. The buffaloes ovaries have a good potential for IVEP but less than cattle.

ACKNOWLEDGEMENTS

The author thanks the CIRB administration for allow him to did this research in the buffalo's farm and CV Raman fellowship for support this research.

REFERENCES

1. Munjunatha, B. M., & Devaraj, M. (2006). Ovum pickup and in vitro embryo production in buffaloes and cattle: an alternative to the conventional embryo production approach. *ICAR Winter School on Reproductive Techniques to Augment Fertility in Dairy Animals*, 96-99.
2. Hernandez-Fonseca, H. J., Sirisathien, S., Bosch, P., Cho, H. S., Lott, J. D., Hawkins, L. L., Hollett, R. B., Coley, S. L., & Brackett, B. G. (2002). Offspring resulting from direct transfer of cryopreserved bovine embryos produced in vitro in chemically defined media. *Animal Reproductive Science*, 69:151-158.
3. Deleuze, S., Goudet, G., Caillaud, M., Lahuec, C., & Duchamp, G. (2009). Efficiency of embryonic development after Intra-Follicular and Intra-Oviduct transfer of in vitro and in vivo matured horse oocytes. *Theriogenology*, 72: 203-209.
4. Seidel, G. E., & Seidel, S. M. (1989). Analysis of application of embryo transfer in developing countries. *Theriogenology*, 31(1): 3-16.
5. Huang, Y. J., & Rosenwarks, Z. (2012). In vitro fertilization treatment and factors affecting success. Best Practice Resource. *Clinical Obstetric. Gynecology*, 26: 777-788.
6. Kumar, D., & Anand, T. (2012). In Vitro Embryo Production in Buffalo: Basic Concepts. *Journal of Buffalo Science*, 1:50-54.
7. Galli, C., & Lazzari, G. (2008). The manipulation of gametes and embryos in farm animals. *Reproductive In Domestic Animals*, 43: 1-7.
8. Niemann, H., & Wrenzycki, C. (2000). Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent development. *Theriogenology,* 53: 21-34.
9. Hasler, J. F. (2003). The current status and future of commercial embryo transfer in cattle. *Animal Reproductive Science*, 79: 245-264.
10. Dharmendra, K., Anand, T., Singh, K. P., Singh, M. K., Shah., R. A., Chauhan, M. S., Palta, P., Singla, S. K., & Manik, R. S. (2011). Derivation of buffalo embryonic stem-like cells from in vitro-produced blastocyst on homologous and heterologous feeder cells. *Journal of Assisted Reproductive Genetic*, 28:679–688.
11. Samad, H. A., & Raza, A. (1999). Factors affecting recovery of buffalo follicular oocytes. *Pakistan Veterinary Journal*. 19: 56-59.
12. Bukar, M. M., Amin, J. D., Sivachelvan, M. N., & Ribadu, A. Y. (2006). Postnatal histological development of the ovaries and uterus and the attainment of puberty in female kid goats. *Small Ruminant Research*, 65:200-208.
13. Razzaque, W. A. A., Sahatpure, S. K., Pawshe, C. H., & Kuralkar, S. V. (2008). Biometry of ovaries and follicular count in cycling and non cycling Nagpuri buffaloes (Bubalus Bubalis). *Journal of Buffalo Bulletin*, 27(1):150-153.
14. Kouamo . J, Dawaye. S.M , Zoli. A.P. and Bah. G.S. (2014). Evaluation of bovine (Bos indicus) ovarian potential for in vitro embryo production in the Adamawa plateau (Cameroon), Open Veterinary Journal, 4(2): 128-136.
15. Acar, D. B., Birdane, M. K., Dogan, N., & Gurler, H. (2013). Effect of the stage of estrous cycle on follicular population, oocyte yield and quality, and biochemical composition of serum and follicular fluid in Anatolian water buffalo. *Animal reproduction science*, *137*(1-2), 8-14.
16. Alves, T., & Felton, D. (2014). TrustZone: Integrated Hardware and Software Security-Enabling Trusted Computing in Embedded Systems (July 2004).
17. Laine, L., Abid, S., & Albillos, A. (2011). Portal Hypertension V: Proceedings of the Fifth Baveno International Consensus Workshop.
18. De Loos, F., Van Beneden, T., Kruip, T. A. M., & Van Maurik, P. (1992). Structural aspects of bovine oocyte maturation in vitro. *Molecular reproduction and development*, *31*(3), 208-214.

19. Linder, P. W., Torrington, R. G., & Seemann, U. A. (1983). Formation constants for the complexes of levulinate and acetate with manganese (II), cobalt (II), nickel (II), copper (II), zinc (II) and hydrogen ions. *Talanta*, *30*(4), 295-298.
20. Cushman, R. A., Desouza, J. C., Hedgpeth, V. S., & Britt, J. H. (1999). Superovulatory response of one ovary is related to micro- and macroscopic population of follicles in contra lateral ovary of cow, *Biology of reproduction*, 60: 349-354.
21. Maya, M., Tsuyoshi, T., Hiromi, N., Sawa, Y., Seizo, H., & Masafumi, T. (2005). The relationship between ovarian weight and follicular population in heifers. *Journal of reproduction and development,* 51: 689-693.
22. Wani, A. N. (1995). In vitro maturation and in vitro fertilization of ovine oocytes. MVSc thesis, Faculty of postgraduate studies Sher-e –Kashmir University of Agricultural Science and Technology, Srinagar.
23. Das, G. K., Jain, G. C., Solanki, V. S., & Tripathi, V. N. (1996). Efficacy of various collection methods for oocyte retrieval in buffalo. *Theriogenology*, 46: 1403-1411.
24. Raza, A., Samad, H. A., Rehman, N. U., & Zia, E. U. H. (2001). Studies on in vitro maturation and fertilization of Nili-Ravi buffalo follicular oocyte. *International Journal of Agriculture and Biology*. 3: 503–506.
25. Kumar, G. (1997). Geology of Arunachal Pradesh. *GSI Publications*, *2*(1).
26. Nandi, S, G., Kumar, V., & Chauhan, M. S. (2006). In vitro production of bovine embryos: We need to stop or proceed - a review. *Agricultural. Review,* 27(2):122-129.
27. Greve, T., & Madison, V. (1991). In vitro fertilization in cattle: a review. *Reproduction Nutrition. Development*, 31: 147-157.
28. Drost, M. (2007). Bubaline versus bovine reproduction. *Theriogenology*, 68: 447-449.