COMPARISON OF QUALITATIVE AND QUANTITATIVE CHARACTERISTICS OF OOCYTES BETWEEN CATTLE AND BUFFALO

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CERTIFICATE

*This is to certify that thesis entitled "**COMPARISON OF QUALITATIVE AND QUANTITATIVE CHARACTERISTICS OF OOCYTES BETWEEN CATTLE AND BUFFALO**" submitted to the Department of Animal Production and Management, Faculty of Animal Science and Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE in ANIMAL SCIENCE**, embodies the result of a piece of bona fide research work carried out by **ZANNATUL NAIM, Registration No. 14-05810**, under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.*

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Dedicated To
My Beloved Parents

LIST OF ABBREVIATIONS

Full Word	Abbreviation
Artificial Insemination	AI
Analysis of Variance	ANOVA
Assisted Reproductive Technologies	ARTs
Bovine Serum Albumin	BSA
Control	C
Degree Centigrade	°C
Corpus Luteum	CL
Corpus Luteum Present	CL+
Corpus Luteum Absent	CL-
Centimeter	cm
Cumulus Oocyte Complexes	COCs
Carbon Di Oxide	CO_2
Completely Randomized Design	CRD
Department of Livestock Services	DLS
Dulbecco's Phosphate Buffer Solution	D-PBS
Export Promotion Bureau	EPB
And Others	*et al.*
Food and Agriculture Organization	FAO
Fetal Bovine Serum	FBS
Food and Mouth Disease	FMD
Follicular Stimulating Hormone	FSH
Fiscal Year	FY
Gross Domestic Product	GDP
Gram	g
Hour/Hours	hr./hrs.
In Vitro Culture	IVC
In Vitro Fertilization	IVF
In Vitro Maturation	IVM
In Vitro Production	IVP
Kilo Gram	kg

Full Word	Abbreviation
Luteinizing Hormone	LH
Left Ovary	LO
Milligram	mg
Metaphase I	MI
Metaphase II	MII
Milliliter	ml
Millimeter	mm
Multiple Ovulation and Embryo Transfer	MOET
Metric Tones	MT
Number of Sample	n
Nano Gram	ng
Number	No./ no.
Oestrous Cow Serum	OCS
Probability	p
Phosphate Buffer Solution	PBS
Correlation Coefficient	r
Reproductive Disorders	RDs
Right Ovary	RO
Super ovulated Cow Serum	SCS
Standard Deviation	SD
Synthetic Oviductal Fluid	SOF
Tissue Culture Media	TCM
United States of America	USA
Volume	v
Video Cassette Recorder	VCR
Versus	vs.
Weight	w
Microliter	µl
Micrometer	µm

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The Author

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ABSTRACT

Cattle and buffalo play a vital role in the livestock production sector in Bangladesh, but their production is problematic due to some reproductive difficulties. To address this issue, *In Vitro* Embryo Production (IVP) would be the appropriate option of animal production. Thus, in this study, the qualitative and quantitative features of the ovary and oocytes of these two species were compared in order to gain a full understanding of them and to pick the optimal oocytes for IVP. The ovaries were collected from the local slaughter houses, aspirated the follicular fluid, and classified the oocytes. The nuclear diameter of oocytes was measured to know the change occurred due to maturation. The ovary of cow and buffalo differed significantly ($p<0.01$) in weight (1.75 vs. 4.83 g), length (1.53 vs. 2.29 cm), width (0.89 vs. 1.57 cm), and depth (0.63 vs. 1.03 cm). Normal oocytes per ovary were 1.19 in cattle and 1.50 in Buffalo, while abnormal oocytes were 0.96 in cattle and 1.13 in Buffalo. Also, a significant difference ($p<0.05$) was observed between the oocytes' nuclear diameter (cattle 85.64 ± 8.16 µm vs. buffalo 99.92±8.65 µm) and before & after maturation of oocytes within the same species. It may be concluded that, the buffalo ovary was more prominent in size and heavier in weight than the cattle ovary. Besides, any type of ovaries - irrespective of the ovary's position and CL status - can be a good source of potential oocytes.

Keywords: cattle, buffalo, diameter, *in vitro* maturation

CHAPTER: I
INTRODUCTION

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INTRODUCTION

In Bangladesh, livestock plays an important role in GDP. Animal farming took place in 1.43% of the total GDP while the growth rate is 3.04% in the FY2019-2020 (DLS, 2020). Among the livestock species, the cattle and buffalo are the major contributor in production of meat, milk and earning foreign exchange (MoF, 2020; FAO and AGAL, 2005). Cattle, the most popular species of livestock, remains almost 243.91 lakh in number whereas the buffalo's population is about 14.93 lakh (DLS, 2020). According to FAO and AGAL (2005), Bangladesh's cattle population contributes 1.79 percent to the global cattle population and 5.47 percent to the Asian cattle population. A survey showed that the number of cattle in Bangladesh rose by 0.3 percent compared to 0.4 percent worldwide (Kamal, 2010). The main features of zebu cattle are their smaller size and slower growth rate in comparison to exotic breeds; yet, their adaptation to disease, hot-humid environmental stress, and unfavorable nutritional conditions are quite high (Islam *et al*., 2018). Still, in nearly every year, a considerable percentage of cattle remain barren due to low reproductive performance such as long intervals in calving. In addition, the breeding and production disorders of cross-bred dairy livestock dramatically lower their output and they are of major concern to dairy producers around the world since most breeding disturbances have unfavorable effects on future fertility (Khair *et al*., 2014). Reproductive disorders (RDs) such as abortion, anoestral illnesses, metritis,

recurrent breeders, retained placenta and one production disease (cross-bred dairy bovine clinical mastitis) are the most prevalent diseases in the cow production system (Khair *et al*., 2014).

On the other hand, buffalo plays important role in production of proteins namely, meat and milk. It also indispensable in use of draught and transportation. According to DLS (2020), only 1.4 million of indigenous buffalo is present and no high productive breed is available in Bangladesh. While the swamp and river types are prevalent across the country, they have lower yields of production than the Indian subcontinent breeds like Murrah. The general share of buffalo milk production is approximately 51.2% in India, 59.5% in Pakistan, 66.6% in Nepal, 18.0% in Sri Lanka; however, it is only 1.4% in Bangladesh (Samad, 2020). Additionally, one of the primary impediments to buffalo reproduction is illness prevalence. Buffalo are found to be susceptible to brucellosis, TB, leptospirosis, bovine viral diarrhea, fascioliasis, FMD, and protozoan infection (Samad, 2020).

The animal origin products play a great role for the development of a country's economy. They are a big source of earning the foreign currency also contribute in a country's GDP (MoF, 2020). According to DLS (2020), the demand of milk is 250 ml/day/head whereas the availability is 175.63 (ml/day/head). We know that milk is an ideal type of food and crucial for human brain development, unfortunately, a clear deficit is present in our country. Meat and milk output decreased because to the long vacation period for COVID-19 outbreak in 2019-2020 (MoF, 2020).

On the other hand, public demand of animal products is anticipated to increase. The price of beef and buffen are climbing up day by day because the availability of these meat is not fulfilling the demand of our population.

Behind that there are various factors. Firstly, the race's poor genetic potential. Secondly, the sickness of reproduction. Thirdly, various reproductive difficulties, such as inherent reproductive issues, including weak/silent oestrous signals, seasonal anoestrus, a lengthy post-partum anoestrus period, delayed adolescence, and poor conception rates (Nandi *et al*., 2002).

Therefore, the most welcoming solution of this problem is application of assisted reproductive technologies (ARTs) for improving the production of animal (Singha *et al*., 2015). Several types of ARTs are present, such as *in vitro* embryo cultivation, embryo transfer, estrus synchronization and MOET (Multiple Ovulation and Embryo Transfer). The *In Vitro* Production (IVP) of embryo is getting more popularity and support day by day because it limits the concern of animal welfare for experimenting in the laboratories (Khandoker *et al*., 2012). The implementation of IVP can be projected to significantly increase the number of genetically advanced animals (Wang *et al*., 2008). *In vitro* fertilization technology (IVF) is a means of producing embryos for genetic alteration and embryo transfer (Nandi *et al*., 2002). These strategies are also used to preserve races and species that are on the endangered list (Kakkassery *et al*., 2010).

However, the efficiency of IVP technologies evaluated as the proportion of immature oocytes reaching the blastocyst stage seldom exceeds the threshold of 30%-40% in animal species, including bovine, equine and porcine (Rizos *et al*., 2008). This suggests that the percentage of oocytes that do not develop after *in vitro* maturation, fertilization and culture is very high (Aguila *et al*., 2020). Moreover, since slaughterhouse-derived animals are the most common source of ovaries, many significant aspects affecting the

quality of the oocyte such as age of the donor, estrous cycle stage, nutritional state, genetic potential, reproductive distress and other characteristics are often unknown (Lonergan and Fair, 2016).

The IVP raw material is the lowest and most numerous sources of oocytes and ovaries of slaughtered animals for the large-scale generation of embryos (Sağirkaya *et al*., 2004; Sharma *et al*., 1996). In fact, the recovery of total and used quality oocytes from ovaries in the slaughterhouse is still poor and the fertilization rate is 40%–55%. In buffalo species, about 10-15%, the output of blastocysts, the efficiency of IVP is substantially lower than that of cattle. (Palta and Chauhan, 1998).

In-vitro maturation is perhaps the most essential stage of the entire in-vitro embryo development process. Ruminant oocytes are normally developed in a humidified atmosphere at 39°C, below 5% CO_2. The optimum ripening time (more than 90% of the metaphase II oocytes) is 22-24hr.

However, very few information is available on *in vitro* maturation and fertilization of buffalo oocytes. When compared to cattle, preliminary results reported by several workers were quite poor (Hegab *et al*., 2009; Totey *et al*., 1992). At the best of my knowledge, there are no available research works on comparative study on the basis of qualitative and quantitative characteristics of ovaries and oocytes between cattle and buffalo.

Therefore, in this research, the comparative studies of cattle and buffalo ovaries and oocytes were discussed through the output of the experiments carried out. The change of the diameter of the oocytes due to maturation were also compared so that it could be helped to understand more about the oocytes structure and developmental competence. It has been believed that

through this study, we will gain more knowledge of oocyte's quality and diameter change due to maturation. Additionally, these procedures will reveal and keep improving, resulting in increased oocyte quality and embryo development percentages.

Considering the above discussion, the present research program was undertaken with the following objectives.

- To compare the qualitative and quantitative ovarian parameters between cattle and buffalo
- To evaluate the oocytes before and after maturation
- To assess the potential qualities of oocytes recovered from ovaries of cattle and buffalo

CHAPTER: II

REVIEW OF LITERATURE

CHAPTER: II

REVIEW OF LITERATURE

Several works have been conducted on characteristics assessment of ovary and oocytes throughout the world on different animal species. The most relevant studies on cattle and buffalo's ovary and oocyte were summarized here. To the best of my knowledge, no works were found on comparative analyses based on the characteristics of ovaries and oocytes between cattle and buffalo. So, the individual studies on the two species were discussed, and the works of literature were arranged according to the descending order of the published year. Firstly, the studies performed in Bangladesh were explained; after that, the studies of the whole world were summarized.

Ovaries of indigenous river buffalo and collected from slaughterhouses within a thermo-flask containing 0.9% normal saline for transportation. COCs with oocyte of 120-125 μm in diameter were selected for maturation. The maturation medium was prepared with TCM-199 supplemented with 0.1 mg/ml sodium pyruvate, 0.08 mg/ml gentamycin sulfate, 5% (v/v) fetal bovine serum (FBS), and 100 ng/ml follicle-stimulating hormone (FSH). To investigate the 17β-estradiol, the maturation medium was supplemented with 0, 0.5, 1.0, or 1.5 μg/ml of 17β-estradiol. Cumulus cells surrounding the oocytes were mechanically removed with the help of 0.1% (w/v) hyaluronidase and using a small-bore pipette. Denudate oocytes were fixed in aceto-ethanol (acetic acid: ethanol = 1:3) for 48 hours and stained with 1% (w/v) aceto-orcein to examine the stage of oocyte maturation. The

oocytes were measured using an ocular micrometer attached to an inverted microscope (Labomed, Inc). In buffaloes, supplementation of *in vitro* maturation medium with 17β-estradiol significantly increased cumulus expansion rate. The estradiol is referred as a promoter of oocyte nuclear maturation in buffaloes and goats (Maksura *et al*., 2021).

Mahzabin *et al*. (2020) experimented with cattle ovaries to determine the potentiality of slaughterhouse ovaries in terms of IVF and IVP. A total of 16 ovaries were collected and classified according to CL status. The data was analyzed by Pearson t-test to co-relate different parameters of ovaries. A positive correlation ($r = 0.41$) between weight and diameter of ovaries was found, whereas a negative pattern is observed in-between length and diameter of ovaries with CL. In CL absent ovaries, the length and diameter of ovaries are significantly related ($p<0.01$) to weight. Moreover, the results disclosed that the volume of follicles with a diameter of 2-6 mm was considerably greater ($p<0.05$) (61% more) in CL-free ovaries compared to CL. The primary and secondary follicles were more common ($p<0.05$) in CL-free ovaries (120% greater) than in CL ovaries (89 percent). Major ($p<0.05$) normal and lower ($p<0.05$) follicle degeneration numbers have been detected in ovaries without CL. Therefore, it was concluded that this negatively affects the developmental ability of oocytes and the generation of embryos with CL in the ovaries. Ovaries without CL offer excellent follicles and complex cumulus oocytes (Mahzabin *et al*., 2020).

Islam *et al*. (2018a) reported a biometrical study of four species with their different breed and type of animal that are cattle (local, Local × Holstein Friesian, and local × Sahiwal), buffalo (swamp and river type), goat (Black

Bengal, Jamunapari cross, and Shirohi cross) and sheep (Local Sheep and Carole). The weight of the ovaries was measured with electrical weighing balance and length with slide calipers. After that, a baseline for morphometric characteristics was established of the ovary. This study revealed that the right ovary has a higher mean value than the left one. Local × Holstein Friesian has significantly higher values ($p<0.05$) than the rest of the breed. River buffalo have higher values ($p<0.05$) on most criteria than swamp buffalo. The Jamunapari goat has greater values ($p<0.05$) than Shirohi and Black Bengal. In addition, Carole sheep had higher values ($p<0.05$) than local sheep on most criteria (Islam *et al*., 2018a).

Rahman *et al*. (2015) conducted a study on buffalo oocytes that showed that oocyte yield per ovary was higher in ovaries without corpus luteum. Normal grade COCs (Grade A and Grade B) were suitable for *in vitro* production (IVP) of buffalo embryos. A total of 134 buffalo ovaries were obtained from the slaughterhouse and categorized into two groups based on the presence (n=49) or absence (n=85) of corpus luteum. Eight hundred twenty-eight follicles (828) were counted at the ovarian surface, 608 being from CL absent and 220 from CL containing ovaries. A significantly higher ($p<0.05$) number of follicles were obtained in the cl missing ovary than in the CL present ovary. Mainly, more normal COCs were found in CL absent ovaries than in CL containing ovaries. In this study, the total number of follicles (7.15±0.16), aspirated follicles (5.24±0.15), normal COCs (1.84±0.08), and the total number of COCs (2.76±0.10) per ovary were significantly higher ($p<0.05$) in ovaries without CL. TCM-199 media was used for maturation as control, where 41 oocytes were used. Of those 41 oocytes, 24 and 7 oocytes were reached at the MII and MI stages, respectively. Germinal vesicle

breakdown has occurred in 3 oocytes while seven oocytes have remained with germinal vesicles. Therefore, it has been showed that 5% BSA supplementation could improve the maturation and fertilization of buffalo oocytes (Rahman *et al*., 2015).

Singha *et al*. (2015) conducted a study to determine an effective media for oocytes maturation in local cattle. Different types of supplements were used with the primary media TCM-199. The findings showed that the oocyte recovery rate per ovary was 3.35, and the overall rate of IVM was 74.6%. The maturation rate is 75.5±3.9% in TCM and 62.2±20.2% in SOF medium. It is concluded that TCM 199 and SOF supplemented with either FBS or OCS and FSH may be used as a medium for IVM of indigenous zebu oocytes in Bangladesh (Singha *et al*., 2015).

Khandoker *et al*. (2011) conducted a study on buffalo ovaries and oocytes to evaluate the slaughterhouse ovaries, follicles, and COCs with the view of IVP of embryos. Among the 136 ovaries (68 left and 68 right), 93 ovaries contained CL, whereas 43 were absent. The length (cm) of the right ovary (2.32±0.06) was significantly ($p<0.05$) higher than the left ones (2.14±0.05). However, significant differences were absent between the width and weight of ovaries. On the other hand, the length, width, and weight were significantly ($p<0.05$) higher in CL than without CL containing ovaries. COCs were collected from left ovaries at a higher rate (2.42 per ovary) than from right ovaries at a lower rate (2.32 per ovary), and the COCs were categorized into normal and abnormal categories. The left ovaries contained a more significant number of normal COCs than the right ovaries. The number of aspirated follicles and COCs was considerably greater in CL-free

ovaries than in CL-containing ovaries. Additionally, normal COCs were more prevalent in ovaries with CL than in ovaries without CL. It was concluded that ovaries lacking the corpus luteum have a greater quantity and grade of cumulus-oocyte complexes than those with the corpus luteum (Khandoker *et al*., 2011).

Goswami, (2004) examined the ovarian status in terms of the existence or absence of corpus luteum and the morphological quality of COCs, and the association between follicular diameter and COC quality. According to CL condition, he classified the ovary into three types. Type I has a functional CL where type II has a regressed CL followed by type III is absent of CL. The number of follicles was more significant ($p<0.01$) in type III ovaries. On the other hand, the COCs were classified as Grade A, B, C, and D according to the COCs density. The follicles with 2-6 mm diameter were substantially greater ($p<0.01$) in type III ovaries than in type II or type I ovaries. Additionally, the number of grade A and grade B COCs was substantially higher ($p<0.01$) in follicles with a diameter of 2-6 mm. So, he referred to 2-6 diameter follicles of CL absent ovaries may be a valuable source of oocytes for IVM and IVF (Goswami, 2004)

Argudo *et al*., (2020) assessed the influence of the bovine CL on oocyte morphometric and functional characteristics and embryo development. The ovaries were classified in 2 parameters - 1) from cows having a CL in one ovary (CL+) and no CL in the contralateral ovary (CL-), and 2) from cows with no CL in either ovary as a control group ©. It was found that oocytes from CL ovaries were more extensive, with thinner zona pellucida, and shown lower activity of glucose-6-phosphate dehydrogenase than ovaries

lacking a CL. It is also found that the corpus luteum did not influence nuclear maturation rate, oocyte diameter, and zona pellucid thickness after maturation. The diameter of the zona pellucida and the width of the oocyte (minus the zona pellucida) were determined using software (AmScope V.3.7) that was explicitly created and calibrated for microscopic measurements. It was reported that the oocytes' diameters were 126.6, 124.2, and 123.6 (µm) for CL+, CL- and C, whereas the diameter after maturation was 126, 126.7, and 127 (µm), respectively. Therefore, it was concluded that oocytes collected from CL+ ovaries were larger and metabolically more prepared to continue maturation than those CL- ovaries (Argudo *et al*., 2020).

A study conducted by Guimarães *et al*. (2020) examined *in vitro* cow embryo development from zebu and taurine donors. Ovum pick-up was used to obtain COCs from 167 *Bos taurus* and 161 *Bos indicus* donors. The oocytes were transferred to 60-mm Corning slides, washed with the maturation medium two to three times, and then transferred to 35-mm Cornin slides containing the maturation medium (containing 9.0 ml Earle's Salts tissue culture medium (TCM) 199 (Sigma Aldrich, USA), 1.0 ml fetal bovine serum (FBS). The oocytes were then grown in an incubator (38.7°C, 99.9% humidity, 5% CO2) for 22–24 days and fertilized for 18 to 22 hr. Pregnancy was detected between 30 and 45 days after transfer. *Bos indicus* donors (n = 2556) produced more oocytes than *Bos taurus* donors (n = 1903) (p = 0.008). In conclusion, the *Bos indicus* showed better results than the *Bos taurus* in higher oocyte recovery, several viable oocytes, and embryos (Guimarães *et al*., 2020).

Mahes *et al*. (2014) conducted a study to determine the effect of three oocyte harvesting procedures (aspiration, puncture, and slicing) on the recovery efficiency and subsequent maturation and parthenogenetic development of immature oocytes retrieved from buffalo ovaries. The ovaries were collected from a local slaughterhouse, aspirated them with mentioned three techniques, matured the oocytes with several supplements based on TCM-199, and then cultured the matured oocytes to evaluate the *in vitro* developmental competency. It was found that the total average number of oocytes retrieved per ovary using slicing (7.88±0.54) was higher than either aspiration (2.50±11) or piercing (3.59±18). It was found that slicing (5.40±29) yielded more culture-grade oocytes per ovary than either puncture (2.45±12) or aspiration (1.94±11). The aspiration approach had a higher percentage of culture-grade oocytes (77.67%) than the puncture (68.24%) or slicing (68.51%) procedures. Oocyte recovery was lower ($p<0.05$) in ovaries with CL than in ovaries without CL (3.01vs.5.17). Thus, it can be said that CL did not influence the oocytes' capacity to achieve MII (73.07 percent vs.75.77 percent) (Mahesh *et al*., 2014).

Leal *et al*. (2013) conducted a study to explore the morphometric parameters of buffalo and cow in different reproductive conditions. The ovaries were collected from Murrah x Mediterranean buffaloes and Brazilian Zebu cows. It was found that a higher value of weight, length, width, depth, and surface follicles for cattle (7.7 ± 2.5 g, 30.9 ± 5.0 mm, 21.7 ± 3.3 mm, 15.4 ± 2.1 mm and, 35.6 ± 18.5 per ovary, respectively) than buffalo (4.0 ± 2.2g, 24.4 ± 4.0 mm, 17.1 ± 2.8 mm, 13.1 ± 2.2 mm, and 21.5 ± 14.1 per ovary, respectively). The data was analyzed by Student’s t-test and found a highly significant difference ($p<0.001$) between these two species for above

mentioned parameters. It was also observed a negative correlation of the number of surface follicles for buffalo in case of pregnant animal. At last, it was found and stated that the bubaline species had the lowest values for all variables tested and the ovary of the bubaline was smaller, lighter, and had fewer surface follicles than the ovary of the bovine (Leal *et al*., 2013).

A study on African Zebu Cattle was conducted by Bello *et al*. (2012). The morphology of reproductive tract was analyzed giving preference on heifer and adult cow. A statistical significance difference ($p<0.05$) was found between heifer and adult cow in terms of ovarian weight. A total of 0.43 ± 0.03 kg and 0.79 ± 0.02 kg of genitalia were found in the heifers and cows studied. No significant difference was found between heifer and cow in case of oviduct diameter and between the right and left length of oviduct. The mean ovarian weights of the heifers were 3.80 ± 0.12g and 4.88 ± 0.04g, respectively; those of the cows were 3.53 ± 0.10g and 5.48 ± 0.04g. From left to right, the mean length of the cow oviduct was 30.04 ± 0.08 cm and 30.21 ± 0.07 cm, respectively; that of the heifer was 21.68 ± 0.18 cm and 22.14 ± 0.16 cm (Bello *et al*., 2012).

Jaji *et al*. (2012) was looked for pregnancy effect on biometrical changes of ovary and uterus for Red Bororo Cattle. The sample was taken of 30 pregnant and 10 non – pregnant cows. The uteri and ovary showed an increasing trend of size changes ($p<0.05$ to $p<0.001$) at third trimester of pregnancy. The left ovary was measured 3.52 cm in length, 2.21 cm in diameter, 1.25 cm in thickness, and 3.03 g in weight in non-pregnant Red Bororo cows, while the right ovary were measured 4.33 cm in length, 3.01

cm in diameter, 1.35 cm in thickness, and 4.89 g in weight. It was found that a larger size right ovary than left and a larger right uterine horn than left one in his analysis. The cervix revealed significant length reductions ($p < 0.05$ to $p < 0.01$) and increases in first trimester to third trimester entire term, indicating the sphincter muscle contraction cervix closing during pregnancy (Jaji *et al*., 2012).

Kakkassery *et al*. (2010) researched with bovine oocytes where they used three retrieval methods, namely aspiration, slicing, and puncture. Here different breed ovaries were used from slaughterhouses such as South Indian breeds like Kangayam, Khillari, Hallikar, and crossbred cattle of Kerala. For each method, they classified the oocytes from A to D according to the COCs quality. It was found that for every technique, grade A oocytes showed a higher maturation rate, and group D demonstrated no maturation. In comparison, grades B and C showed 53 to 68% and 35 to 44% COCs expansion, respectively. Moreover, each grade of oocytes obtained through different retrieval methods were incubated at 38.5°C with 5% carbon dioxide tension and maximum humidity for 24 h in TCM-199 medium supplemented with LH, FSH, estradiol, pyruvate, and fetal calf serum. A zoom stereo microscope (Leica MZ-6, Leica microsystems, Germany) was used for magnifying of oocytes and identifying the germinal vesicle breakdown. Before observation, the oocytes were stained with aceto-orcein stain and denuded them with vortexing, and examined for extruded polar bodies. In the end, it was stated that the shape of the cumulus-oocyte complex has a significant influence on the IVM of bovine oocytes. Oocytes with several layers of cumulus cells (three or more layers) matured more

rapidly than oocytes that were denuded or had a lower number of cumulus cells (Kakkassery *et al*., 2010).

Jamil *et al*. (2008) worked on Riverian buffalo's follicular oocytes, and the purpose of this study was to compare the efficacy of oocytes on the effect of collecting procedures, season (low and high breeding seasons), and ovarian condition (presence or absence of corpus luteum). The result revealed that the dissection method provides more oocytes than the aspiration and puncture method. Maximum oocytes recovered in peak breeding season (845) than a low breeding season (602) and from with CL (275) than without CL (421) ovaries. In the end, it was concluded that the method of oocyte collection, the season, and the ovarian condition of the buffalo at the time of oocyte harvest all have a substantial effect on the retrieval of viable oocytes for use in IVF procedures (Jamil *et al*., 2008).

Leal *et al*. (2007) carried an experiment with the objective of the ovarian morphometric study and *in vitro* maturation of oocytes from slaughterhouse buffalo ovaries. The study reveals that the ovary's mean weight, length, width, and height were 3.83 g, 2.27 cm, 1.08 cm, and 1.56 cm. The mean sizes of the corpus luteum and dominant follicle were 1.40 cm and 7.77 mm, respectively. Another study on *in vitro* maturation were carried using TCM-199 medium supplemented with 10% fetal bovine serum, sodium pyruvate, LH, FSH, estradiol, and gentamicin. After IVM, the oocytes were taken from the medium and placed in TCM 199 medium with type v hyaluronidase, where the granulosa cells were collected with a glass micropipette. This was done using an inverted fluorescent microscope. With denuded oocytes transferred to 10µl Hoescht (33342) in glass slides. The

oocytes' percentage that progressed to metaphase II was 36.68 percent (Leal *et al*., 2007).

Ali *et al*. (2006) investigated pathological lesions and studied biometry of the genital organs of Zebu cows. Several pathological lesions were found in the tract such as ovario-bursal adhesions (7.27%), cystic ovary (2.72%), cystic corpus luteum (0.9%), parovarian cysts (1.81%), teratomas, pyometra (6.36%), fibrosity of cervix, tortuosity of cervix (1.81%) and double cervices (1.81 %). A similar mean value (weight, length, width, thickness) were found between right and left ovaries as well as right and left horns (20.69 ±0.59 and 19.76 ± 0.58 cm, respectively). So, it may be stated that the zebu cow's biometric characteristics and pathological circumstances are comparable to those of other cattle breeds worldwide (Ali *et al*., 2006).

Khammas *et al*. (2005) presented the biometry of genital organs of female Iraqi buffalo. Almost same results were found for right and left ovaries but the number and weight of functional CL was higher in right than left one. The right horns represented more caruncles than the left horns but the length and diameter of those are almost similar. So, it can be concluded that the genital organs of female buffalo are smaller than that of cows and right ovary is more functional than the left ones (Khammas *et al*., 2005).

An experiment conducted by Kunbhar *et al*. (2003) with Thari cows had been found that the right ovary is heavier and bigger in size than the left one. The mean length, width, and thickness of the right ovary were 2.56, 1.33,

and 1.46 cm, respectively, while the left ovary was 2.50, 1.3, and 1.4 cm. So, it was stated that right ovary is more active than the left. It was also observed that the measurement of uterine horn, vagina, cervix, uteri, etc. from 50 non-gravid reproductive tract. The vulva, vagina, cervix, and corpus uterus all had mean lengths of 10.24, 20.67, 7.80, and 1.7 cm, respectively. The widths were 8.94, 5.08, 2.72, and 2.17 cm, respectively. The mean length of the right uterine horn was 21.63 cm, while the mean length of the left uterine horn was 20.90 cm. Through this study it was showed that the anatomical structures of genital tract of Thari cow that may help to diagnose diseases and give rapid treatment to the animal (Kunbhar *et al*., 2003).

Raghu *et al*. (2002) experimented on follicular size and oocytes diameter of buffalo ovaries. It was found that the larger the follicles and oocytes, the larger the *in vitro* developing capacity of buffalo oocytes. In this study, COCs were sucked from tiny (3 mm), medium (3–8 mm), and large (>8 mm) follicles of normal ovaries and cystic ovarian follicles using a 20-gauge needle linked to a 5-ml plastic syringe. A medium diameter investigated for oocytes of varied size follicles and their influence on *in vitro* maturation, cleavage, and embryo yield was explored — i) <126 μm; ii) 127–144 μm; iii) 145–162 μm; and (iv) >163 μm. The oocyte diameter without COCs was determined using a precalibrated ocular micrometer at a magnification of 110x. The aspirated COCs were classified according to Chauhan *et al.* (1998) classification scheme (Raghu *et al*., 2002).

Singh *et al.* (2001) conducted a study based on buffalo oocytes. It was studied on the effect of corpus luteum on recovery of oocytes and *in vitro*

maturation and fertilization. A 943 and 1136 pieces of ovaries were taken with and without CL, respectively. In addition, on average, 0.41 and 0.67 oocytes per ovary were recovered from ovaries with and without corpus luteum, respectively. The COCs were classified into good and fair quality. The excellent quality and fair quality oocytes maturation rates were 74.48% and 37.35%, respectively. 392 and 768 no. of oocytes were harvested, and 38.40% and 40% were matured from CL present and absent oocytes, respectively (Singh *et al*., 2001).

Otoi *et al.* (1997) studied on the relationship between the diameter of bovine oocytes and developmental competence concluded that the nuclear maturation rates of oocytes with diameters larger than 115 µm were significantly higher than those of oocytes with diameters less than 115 µm. The oocytes were classified into six groups based on their diameters: 110 µm, 110 to 115 µm, 115 to 120 µm, 120 to 125 µm, 125 to 130 µm, and 130 µm. The mean oocyte diameter was 114.0 4.8 µm. Oocytes with a diameter greater than 110 µm to 120 µm were considerably inferior developmental competency than oocytes with a diameter of 120 µm to 130 µm. The diameter of the oocytes was determined using a video micrometer (Olympus VM-30, Tokyo, Japan) coupled to an inverted microscope's VCR camera. These findings indicated that bovine oocytes have gained full meiotic competence but not yet complete developmental competence to blastocysts at a diameter of 115 µm. Oocytes attained complete reproductive success at a diameter of 120 µm (Otoi *et al*., 1997).

Fair *et al.* (1995) found a relationship between oocytes diameter and follicle and their developmental competence. The correlation coefficient between

them is $p<0.0001$. Oocytes were recovered by aspiration and/or slicing methods and classified into four groups based on diameter. The groups are < 100 µm, 100 to <110 µm, 110 to < 120 µm and >120µm which *in vitro* developmental rate to metaphase II were 21.2%, 42.3%, 75.9%, and 80.7%, respectively. The diameter of oocytes was measured with a micrometer fitted to an inverted microscope (Fair *et al*., 1995).

Based on the above discussion, it may be said that cattle and buffalo had different values for ovarian weight, length, width, depth, and surface follicle numbers. Different values were shown between right and left ovaries & CL present, and CL absent ovaries. Various methods of follicular fluid collection were mentioned, which had a significant difference in oocytes recovery among those methods. Several studies had been shown the relation between follicular diameter and the number of oocytes recovery. Different types of classification of oocyte's grading had been discussed as well. Also, many measured the nuclear diameter with different methods and microscopes. Some effects of various media on oocytes maturation had also been illustrated. In conclusion, this section provides information about most of the relevant literature works on the qualitative and quantitative characteristics of ovary and oocytes, which help understand the overall research scenario in this sector.

CHAPTER: III

MATERIALS AND METHODS

CHAPTER: III

MATERIALS AND METHODS

3.1 List of Required Materials

- Microscope
- CO_2 incubator (NUAIR)
- Autoclave
- Laminar airflow
- Balance
- 18-gauge needle (TERUMO, Beijing, China)
- 10 ml disposable plastic syringe
- 30 ml Petridish (Greiner bio-one, Frickenhausen, Germany)
- Rubber-tube
- Watch-glass
- Glass Pasteur pipette with long/medium tip
- Spirit
- Rubber-tube with pipette
- Coverslip
- Culture plate
- Spirit lamp
- Slides
- Micropipette
- Dropper
- 0.9% saline solution
- D-PBS
- 1% aceto-orcein stain
- Glacial acetic acid
- Ethanol
- TCM-199/EBSS (HyClone)
- Paraffin oil

3.2 Location of the study

The experiment was conducted at the laboratories of Animal Production and Management & Animal Genetics and Breeding departments of Sher-e-Bangla Agricultural University from July, 2020 to June, 2021.

3.3 Collection of the ovaries

Ovaries of cattle and buffalo without knowing the reproductive characteristics were collected from local slaughterhouses - situated at Townhall and Krishimarket, Mohammadpur, Dhaka. Ovaries were collected as soon as possible after slaughtering of animals and transported to the laboratory within a thermo-flask containing 0.9% physiological saline solution at 25-30 °C within 2 to 3hrs after slaughtering of animals as described previously (Maksura *et al*., 2021; Karmaker *et al*., 2020; Hoque *et al*., 2016) (Plate: 1).

3.4 Preparation for the experiment

Personal protective equipment such as apron, mask, and gloves were used to maintain hygienic conditions. The utensils were washed with soap and rinsed with tap water. Then these were disinfected with 100% alcohol. 0.9% saline solution and D-PBS were made before starting the main experiment.
Necessary equipment such as the microscope, the weight balance, the autoclave machine was placed in the appropriate position and connected to the electricity. On the other hand, the CO_2 incubator, the laminar airflow was checked so that they could run properly.

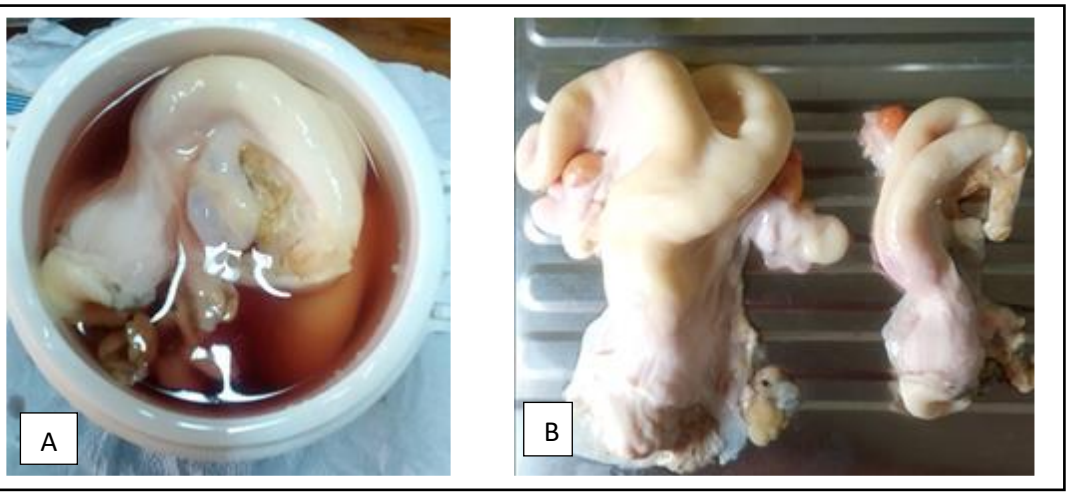


Plate 1: Female reproductive tract of cattle & buffalo. Female reproductive tract within thermo-flask containing 0.9% normal saline (A). Comparison of the uterine horn of female reproductive tract between cattle (right) and buffalo (left) (B).

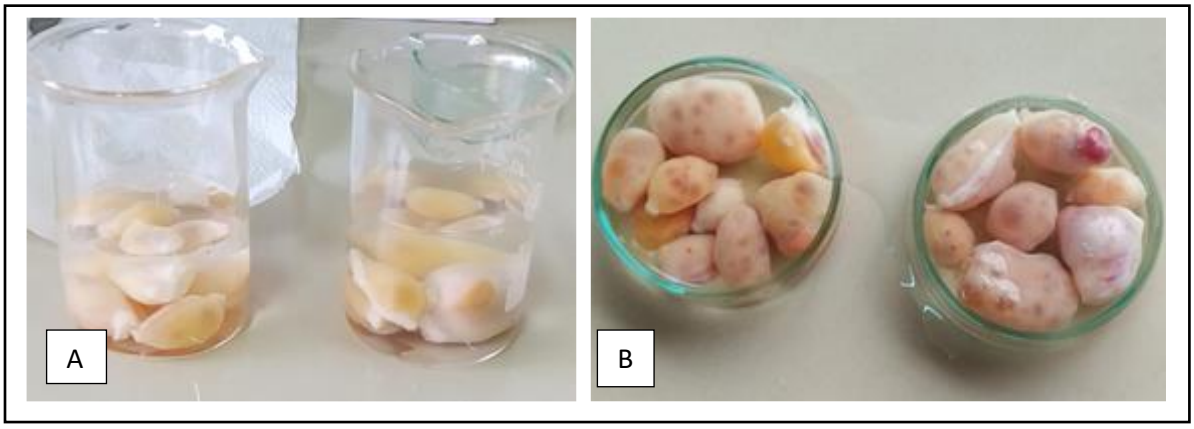


Plate: 2. Trimmed ovaries. Ovaries after trimming unnecessary parts dipped into 0.9% physiological saline solution in Beaker or in Petridish. Separated by position of ovary in the animal (right and left).

3.5 Processing of ovaries

A total of 40 cattle and 20 buffalo ovaries were collected. The ovaries were separated from the reproductive tracts and trimmed the unnecessary parts like flesh, fat tissues, veins, etc. (Plate: 2). Then according to the position of the ovary in the reproductive tract, they were separated into the two beakers within 0.9% saline solution. The beakers were marked as left and right previously. The left ovaries were placed in the left named beaker, and the right ovaries were placed in the right marked beaker. Further, the ovaries of each beaker were observed closely and classified according to the present and absence of corpus luteum as corpus luteum (CL) present (Plate: 3A & 3C) and corpus luteum (CL) absent (Plate: 3B & 3D), respectively.

3.6 Assessment of morphology

After trimming the extra materials from the ovary, it was rinsed with saline solution several times. After that, the following measurements were taken with different equipment such as balance, slide calipers etc. as described earlier by (Md. Reazul *et al*., 2018; Hoque *et al*., 2016).

i) Length, width, and depth: The ovary was placed over the dry tray, and the measurements were assessed by slide calipers (Plate: 4). The unit was recorded as a centimeter (cm).

ii) Weight: The weight of the ovary was recorded by digital electronic balance and counted the unit as gram (g).

iii) Number of visible follicles: The ovary was observed closely, and the visible follicles numbers were counted and recorded.

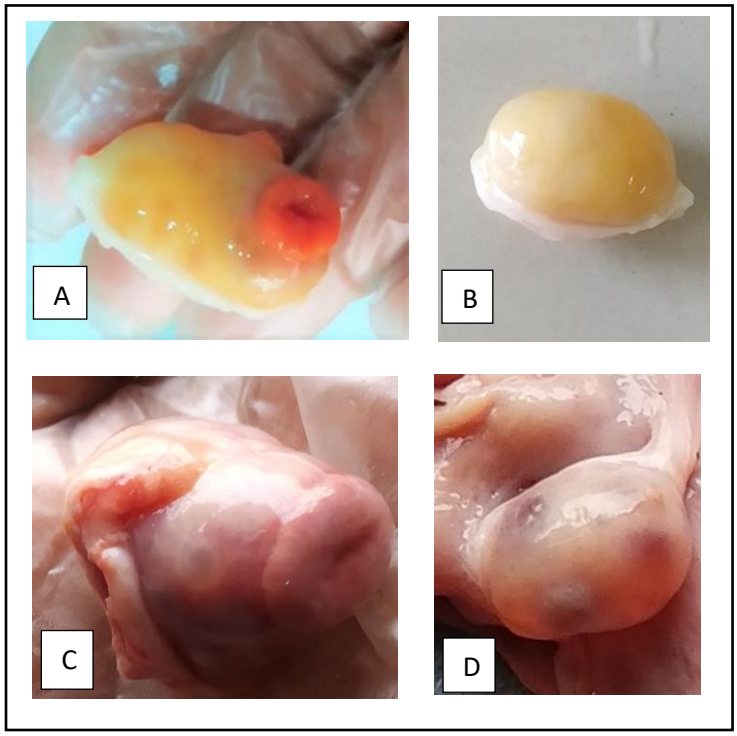


Plate: 3 Ovaries. Ovary of cattle: with CL (A), without CL (B). Ovary of buffalo: with CL (C), without CL (D).

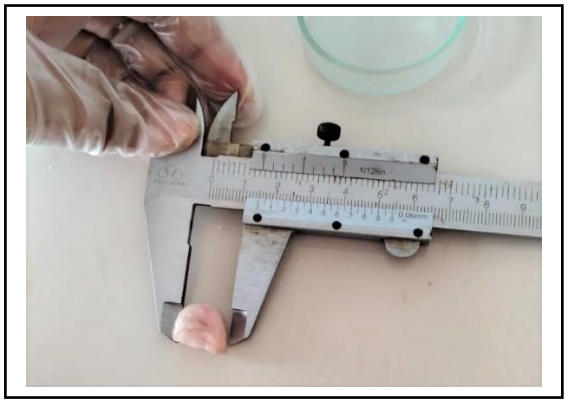


Plate: 4. Measuring the length, width and depth of ovaries by slide calipers

3.7 Collection of follicular fluid

The procedure of follicular fluid collection was followed by the previous experiment conducted by Singha *et al*. (2015). The follicular fluid was collected by the Aspiration method. A sum of 193 cattle and 132 buffalo ovarian follicles were observed. The ovary was held in the left hand (Plate: 5A) while the syringe with the needle was grabbed in the right hand. Then the needle was punctured beneath the follicle carefully so that the follicle would not rupture. The fluid was pushed up into the syringe by the plunger to collect the fluid within the barrel. The fluid was put down to the Petridish slowly so that the oocytes would not be broken. This procedure was repeated for each follicle to collect all of the fluid. Then, a small amount of previously made sterilized standard saline solution was put in the follicular fluid as if the oocytes did not adhere to the bottom of the Petridish (Plate: 5C) or watch glass (Plate: 5B).

3.8 Oocytes pick-up

3.8.1 Rubber-tube with pipette preparation

The procedure was followed according to the instructions as described previously by Islam *et al*. (2007). At first, one glass Pasteur pipette was taken to give heat to the joint of the body and the tip with the spirit lamp (Plate: 6A). When the glass became softened, it was held with a cloth and broken at the point of joint gently. The tip was not overheated so that it could have melted quickly. Then, approximately 1 foot of rubber- tube and

the direction of the pipette were taken. To add the tip with the rubber tube, one end of the tip was placed within the edge of the rubber tube.

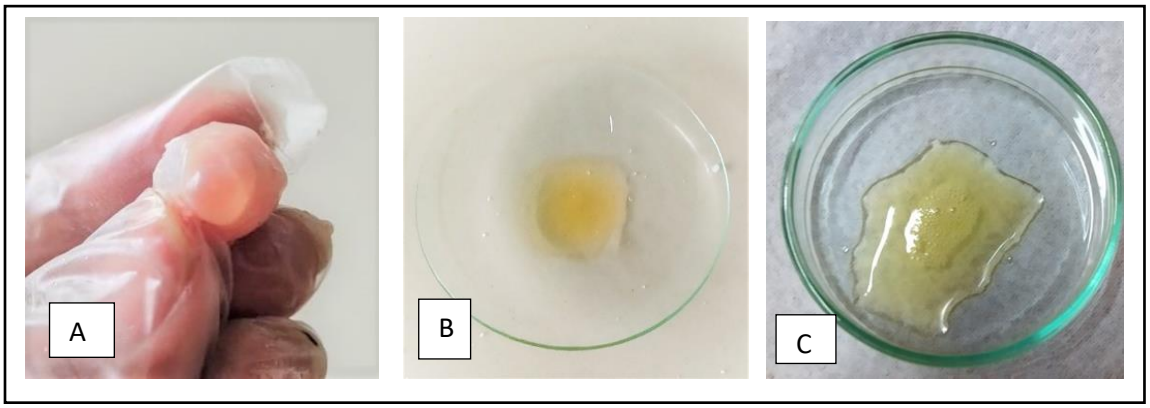


Plate: 5 Collection of follicular fluid**.** Follicle (A). Follicular fluid in watch-glass (B). Follicular fluid in Petridish (C).

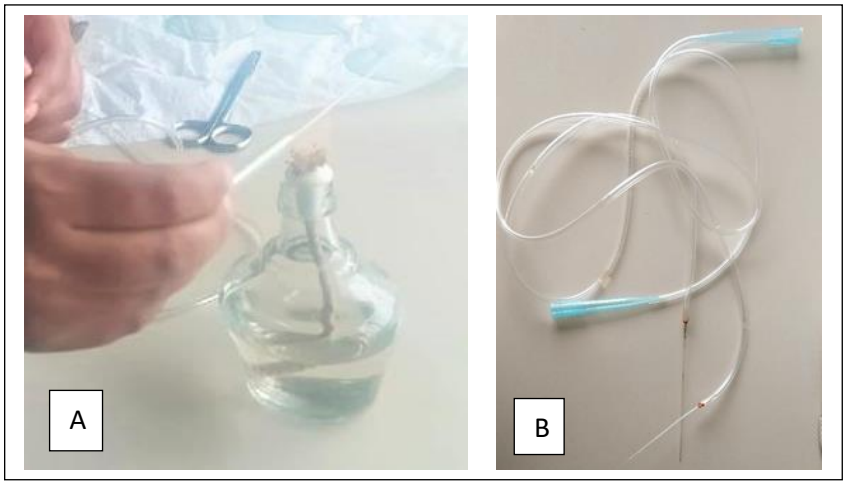


Plate: 6 Preparation of Pasteur pipette. Preparing rubber-tube with Pasteur pipette. Heating the Pasteur pipette with spirit lamp (A). Prepared rubber-tube with Pasteur pipette (B).

Further, heat was applied to it to melt the rubber and got adhere to the tip of the pipette. At the same time, a thick cloth was used to press the melted tube gently to avoid burning the hand. After that, to be more functional, the tip was needed to be the finest. For this reason, the open end of the tip was heated for few seconds and pulled by covering it with the cloth quickly to make it the finest as much as possible (Plate: 6B).

3.9 Grading of oocytes

The COCs were graded according to the presence of cumulus cell attachment as described by Khandoker *et al.* (2001). There were four grades of COCs.

Grade A: oocytes surrounded by cumulus cells;
Grade B: oocytes partially surrounded by cumulus cells;
Grade C: oocytes not surrounded by cumulus cells and
Grade D: degeneration observed both in oocytes and cumulus cells

Grade A and B were considered as normal, and grades C and D as abnormal COCs. The number of different grades of COCs in each category was recorded.

According to Aguila *et al.* (2020), in general, the best quality COC has a complete cumulus cover with several compact cell layers; the average quality has a partial cumulus cover and/or a slightly expanded cumulus with fewer than five cell layers; but the worst quality has a darker cytoplasm and

the existence of dark spots with expanded cumulus, all of which are indicative of follicular atresia.

The follicular fluids were collected in the watch-glasses (Plate: 5B), and 90mm Petridish (Plate: 5C) was left for 5min to settle down oocytes. A total number of 172 cattle and 84 buffalo oocytes were aspirated while normal oocytes (grade A+B) were 95 and 48 and abnormal oocytes (grade C+D) were 77 and 36, respectively. Observation of watch-glasses was done under the microscope at 4x to count the total number of oocytes. The 10x magnification was used to observe the oocytes. Then, the oocytes were counted and recorded according to the above classification (3.9), and images were taken for further diameter measurement.

3.10 Measurement of diameter of Oocytes

After taking photos of the oocytes, the images were measured with the help of Micro-measure (Scalar Corp.; version 1.0.0.1) software. Here a total of thirty (30) cattle and twenty (20) buffalo oocytes' diameter were noted. The plates were placed under the 4x lens of the microscope and searched for the matured oocytes. The diameter of oocytes without COCs were recorded as micrometer (µm) unit (Plate: 8). The oocytes' diameter was measured before and after maturation for determining the difference of diameter change of oocytes in between two species.

3.11 *In vitro* maturation of oocytes

Firstly, 10% of ovaries were placed aside for maturation purposes, and follicular fluid was collected using the abovementioned method (3.7). Before

starting the primary maturation process, the glass utensils such as the culture plate, beaker were autoclaved to disinfect for 15-20 min in the autoclaved machine. Precaution was taken before opening the hot lid and bringing out the hot utensils. 100% alcohol mixing with water was used to disinfect other materials like droppers, plastic culture plates, etc.

The maturation media (TCM-199), paraffin oil, micropipette, dropper, and culture plate were brought into the laminar airflow. Wearing hand gloves, 100µm medium had been pulled on from the bottle with the help of a micropipette (Plate: 7A). Then, it was poured gently into the culture plate so that it could not spread on it. One drop into the small (30mm) and four/ five drops into the large (75mm) culture plate were taken. The solution was left into there to bring the temperature into normal that is into the room temperature.

Meanwhile, a microscope was plugged in, and oocytes were observed under the 4x lens. When the solution was cold, it was bright red color. While the medium turned to room temperature, its color changed to pink. By observing its color, the culture plates were brought on a tray close to the microscope. After that, grade A, B, and healthy-looking grade C oocytes were picked up with a pipette and carefully put into the medium drops. Four to five oocytes were put off into each drop (100µl) of medium (Plate: 7B & 7C). Then, the culture plates were again brought into the laminar airflow to pour the paraffin oil on them (Karmaker *et al*., 2020).

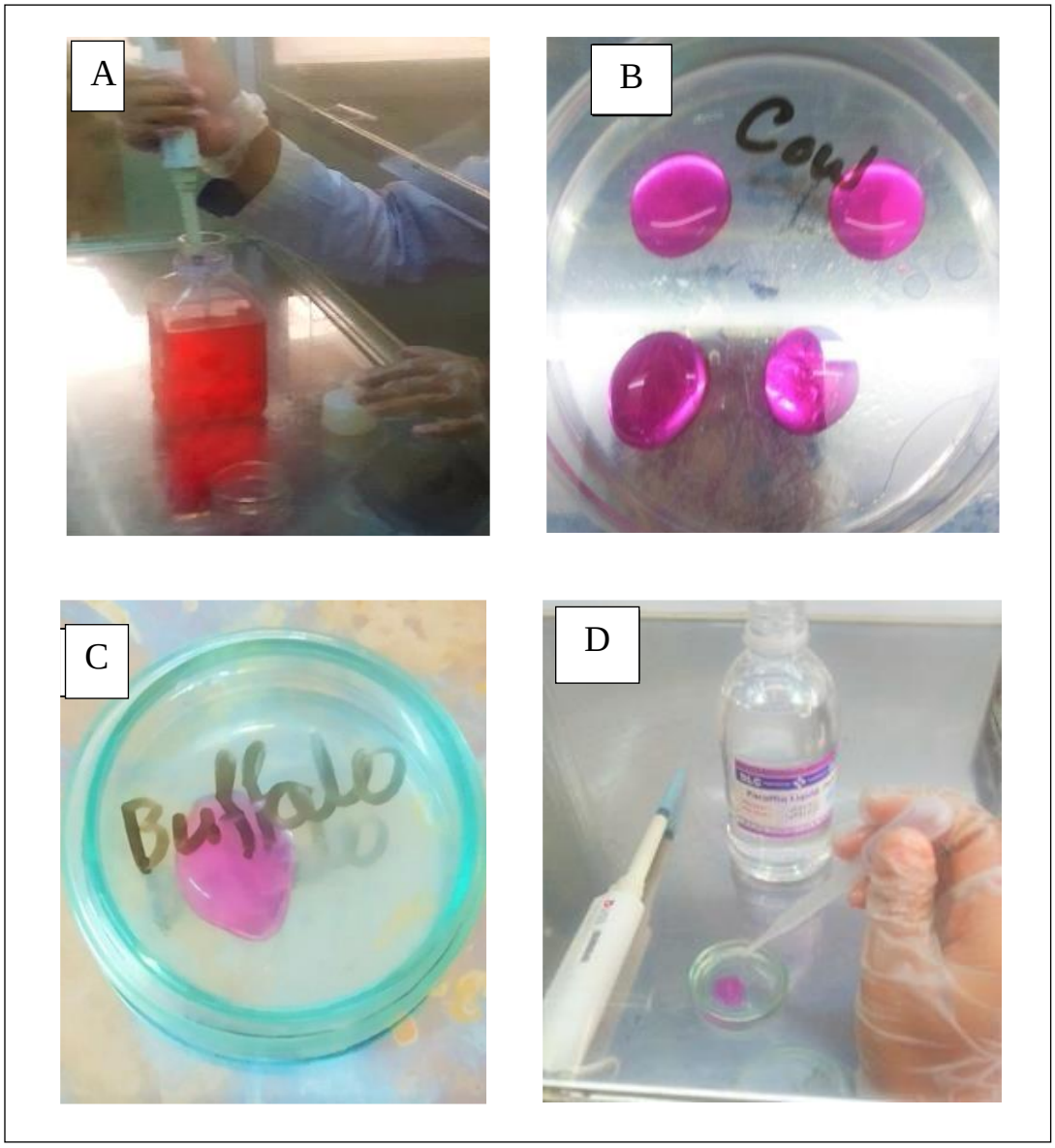


Plate: 7 IVM Procedure.100µl media was taken with micro-pipette (A). Culture plate with TCM droplet and oocytes (B & C). Adding paraffin oil in the culture plate (D).

The reasons were paraffin oil helps the oocytes preventing moisture loss and infection from contaminants. So, the oil was pulled by the dropper and

placed on the culture plates until it covered the drops of TCM (Plate: 7D). The culture plates were placed on the incubator, covering the plate with a lid and marking with a marker pen according to the species' name. The incubator was heated previously and observed as if it would run properly. Then, counting the time, it was left for overnight (24hrs.) in a 5% CO_2 (Singha *et al*., 2015) at 38.5°c in humidified air (Maksura *et al*., 2021).

After 24 hrs. the culture plates were brought out cautiously from the incubator (Singha *et al*., 2015). A sum of twenty (20) cattle and fifteen (15) buffalo oocyte's nuclear diameter were measured after the maturation of oocytes according to the previous procedure described at 3.10. After staining, multiple oocytes were placed in one watch-glass to capture the image jointly.

Before leaving the lab, some points were kept in mind: the equipment switches were turned off or not and whether these were placed in the appropriate position. Whether or not utensils such as the Petridish, culture plate, beaker, and other materials used to collect the sample and smear it with dirt were cleaned. The leftover chemicals in the beaker were covered with foil paper, and finally, the perishable samples or goods were dumped into the dustbin.

3.12 Morphological study of oocytes

At first, staining was conducted to study the morphology of oocytes. The staining procedure was followed by the previous experiment of Kumar *et al.* (2018) with some modifications. Firstly, the COCs were washed with phosphate buffer solution (PBS) several times to remove the debris from the

oocytes. For this purpose, follicular fluid within the watch-glass was taken under the microscope. Then, with the help of a rubber tube (Plate: 6B), COCs were picked up and transferred to another watch-glass containing PBS. This procedure was repeated to remove the cumulus cells as much as possible.

Secondly, the washed oocyte was taken on the slide and placed a coverslip gently on it. The coverslip edges were added adhesive agents like nail polish to adhere with the slide, and the cells under the coverslip could not flow out. However, one side of the cover slide was remained open so that the following solutions could get into it.

Thirdly, a Petridish was filled with the fixative solution (ethanol: acetic acid in a 3:1 ratio), and the slides were then dipped in it overnight. The entire procedure, from washing to place for fixing, took between twenty to thirty minutes. Finally, following an overnight fixation, the slides were merged with 1% aceto-orcein in 45% glacial acetic acid for 30 minutes, followed by a distaining solution wash – PBS (Maksura *et al.*, 2021; Kumar *et al*., 2018). This aceto-orcein staining was carried to observe the oocytes clearly (Kakkassery *et al*., 2010). Then, the slides were observed under the microscope.

3.13 Statistical Analysis

Data was analyzed by an independent t-test (IBM, SPSS, statistics, version 25) to compare the data with various parameters. (Raghu *et al*., 2002). The difference between groups was considered significant when P-value was <0.05. All values were expressed as mean ± SD (Standard Deviation).

CHAPTER: IV
RESULT AND DISCUSSION

CHAPTER: IV

RESULT AND DISCUSSION

4.1 Comparison between cattle and buffalo ovary

The morphometric characteristics of ovaries have been compared in between the cattle and buffalo (Table 1). The weight, length, width, and depth of ovaries between the two species showed a significant difference ($p<0.01$) which agreed with the study of Leal *et al.* (2013). In this study, the buffalo's ovary demonstrated a higher value than the cattle ovary. In contrary, the number of follicles per ovary present in the ovaries' surface had no statistical difference between the two species (Table 1). An average 4.83±4.39 number of follicles were found per ovary in cattle which agreed to Islam *et al.* (2007) with goat experiment.

Moreover, the results of bubaline ovarian weight (4.83 ± 2.16 g) support the findings of Vale and Ribeiro (2005), and Waheed (2011). However, it does not support the mean value of buffalo ovarian weight of Khandoker *et al.* (2012) and Leal *et al.* (2007). In addition, Bello *et al.* (2012), Monteiro *et al.* (2008), and Leal *et al.* (2013) highly contradict with the bovine ovarian weight (1.75 ± 1.2 g) of this study.

The reasons of this difference may be due to the lower body weight and lower body conditioned breeds which were the source of ovaries in the present study. A positive association between genital system weight and body size & body weight has been found by Danell (1987) and Dobson and

Kamonpatana (1986) which proved that lower body weight animal has smaller size of ovary than the higher body weight animal. Further, it may be described that the population of animals had heterogeneity and individual physiological characteristics, which may differ among studies (Leal *et al*., 2013).

However, the differentiation between two species of the present study may be caused by the species difference (Leal *et al*., 2013), age difference *(Bello et al*., 2012), and pregnancy status of the animal (Jaji *et al*., 2012). Further, follicular concentration in the ovaries which reflects the ovary's propensity for antral follicles (Murasawa *et al*., 2005), various amount of circulating hormones with hormone receptors presence in ovary (Mohammadpour, 2007) and presence of more CL (corpus luteum) containing ovaries in the buffalo sample might be the factors of differentiation.

4.2 Comparison of oocyte quality between cattle and buffalo

In Table 2, the comparison of oocytes' number has been showed between the species. No significant difference between cattle and buffalo oocytes' number was found though a higher mean value was showed for buffalo (Table 2).

The number of buffalo oocytes per ovary (1.31±1.03) supported the result of Das *et al*. (1996) and Islam *et al*. (2007) in the case of goat. This data disagreed with Leal *et al*. (2007) and Mahesh *et al*. (2014), who found a higher number of oocytes, 4.24 and 4.58 per ovary, respectively. These forms of fluctuation or a lesser number of COCs per ovary observed in this

study can be explained by the animal's non-cyclicity. Generally, animals with poor reproductive performance are slaughtered, and the majority of them are non-cyclic. Thus, the slaughterhouse may provide most of the non-cyclic ovaries (Islam *et al.*, 2007) which may provide lower number of oocytes.

Table: 1. Comparison between morphometric parameters of the ovaries of cattle and buffalo

Parameters of ovaries	Species of Animal		Level of significance
	Cattle	Buffalo	
Weight (g)	$1.75^{b} \pm 1.2$	$4.83^{a} \pm 2.16$	**
Length (cm)	$1.53^{b} \pm 0.43$	$2.29^{a} \pm 0.28$	**
Width (cm)	$0.89^{b} \pm 0.29$	$1.57^{a} \pm 0.52$	**
Depth (cm)	$0.63^{b} \pm 0.22$	$1.03^{a} \pm 0.23$	**
Number of visible follicles per ovary	4.83±4.39	8.25±5.17	NS

*The different superscripts indicated significant difference between two values in the same row; **, p<0.01 (significant); NS, Non-Significant*

On the other hand, the diameter of oocytes differed significantly ($p<0.05$) that was 85.64±8.16 µm in cattle and 99.92±8.65 µm in buffalo (Table 2). This data (Table 2) agreed with the statement of Raghu *et al*. (2002). It was stated that "the diameters of oocytes in buffalo seem to be greater than in cattle". The mean value of oocytes' diameter of the present study was lower than the results of other studies (Barkawi *et al*., 2007; Raghu *et al*., 2002;

Wit and Kruip, 2001) for both cattle and buffalo. However, Gupta *et al*. (2000) found a wide range of diameters for buffalo oocytes (45 – 270 µm).

The difference of oocytes' diameter among the mentioned studies may be explained that the sample of the present study had more CL absent ovaries than the CL present ovaries because the CL present ovaries contained larger diameter oocytes than the CL absent ovaries (Argudo *et al*., 2020). Also, the sample size, the different techniques of measurement of oocytes, variations in follicle size (primordial, preantral, and antral) and location of oocytes (cortical and peripheral), as well as oocytes' growth stage (immature, in vivo matured) could be the major caused for this difference (Raghu *et al*., 2002).

Besides, oocytes diameter is related to morphology of the COC (Wit and Kruip, 2001), follicular diameter (Arlotto *et al.,* 1996; Carolan *et al.,* 1994; Fair *et al*., 1995; Morbeck *et al*., 1992), follicular atresia (Blondin and Sirard, 1995; Wit *et al*., 2000), ability to continue and complete meiosis process (Fair *et al.*, 1995) and developmental competence (Arlotto *et al*., 1996; Eppig *et al.*, 1992).

4.3 Comparison of oocytes nuclear diameter before and after maturation

The nuclear diameter of oocytes excluding zona pellucida in pre and post maturation are presented in Table 3. The diameter of oocytes indicated a value of the significant difference ($p<0.05$) that showed an increasing trend of nuclear diameter changes after maturation of oocytes. The rise in oocyte diameter during 24 hr. of culturing is consistent with the findings of Barkawi

et al. (2007), Arlotto *et al.* (1996) and Osaki *et al.* (1997), and could be a result of RNA production during oocyte growth (Lucas *et al.*, 2002). This result is contradictory to the findings of Argudo *et al*. (2020) who did not find any significant difference between the two diameters of oocytes and Barkawi *et al.* (2007) who found the larger diameter of oocytes in both pre and post maturation. This contrast might be made due to the different methods of diameter measurement, different maturation media and time and sample size differences among the mentioned studies. However, a few information is present that is related to the nuclear diameter changes of the oocytes due to maturation. So, further studies are required to reveal the consequences of maturation on the diameter of oocytes.

Table: 2. Comparison between oocyte's quality of cattle and buffalo

Parameters		Species of Animal		Level of significance
		Cattle	Buffalo	
Number of COCs per ovary	**Normal oocytes (Grade A+B)**	1.19±0.81	1.50±0.89	NS
	Abnormal oocytes (Grade C+D)	0.96±0.85	1.13±1.15	NS
	Average	1.08±0.83	1.31±1.03	NS
Diameter of nucleus per oocytes (µm)		$85.64^{b} \pm 8.16$	$99.92^{a} \pm 8.65$	*

*The different superscripts indicated significant difference between two values in the same row; *, p<0.05 (significant); NS, Non-Significant*

T**able: 3. Comparison between nuclear diameter of oocytes before and after maturation**

Species of animal	Diameter of nucleus per oocytes (µm)		Level of significance
	Before maturation	After maturation	
Cattle	$85.64^{b} \pm 8.16$	$94.09^{a} \pm 11.23$	*
Buffalo	$99.92^{b} \pm 8.65$	$109.2^{a} \pm 8.65$	*

*The different superscripts indicated significant difference between two values in the same row; *, p<0.05 (significant); NS, Non-Significant*

4.4 Comparative assessment of ovaries and oocytes

4.4.1 Qualitative and quantitative assessment according to the position of the ovary in animal

The present study found that the right ovary of cattle had a higher quantitative value than that of left ovary but no significance difference was present between them (Table 4) which agreed with the previous studies of Talukder *et al*. (2011) and Islam *et al*. (2018) for sheep and goat, accordingly. According to Islam *et al*. (2018a) the right and left ovaries showed a larger size and shaped than the present study (Table 4). However, the total number of COCs differed significantly where the right ovary showed a greater value than the left one.

Moreover, the parameters of oocytes quality demonstrated that the right ovary had more normal grade (A+B) and abnormal grade (C+D) oocytes per ovary than the left one. The number of follicles per ovary was 5.90±5.09 vs. 3.75±3.33 (right vs. left) and number of oocytes per ovary was 1.34±0.82 vs. 0.81±0.76 (right vs. left) (Table 4). This result demonstrated that the right ovary is more active than the left one, which support the findings of Kunbhar *et al*. (2003), Khammas *et al*. (2005) and Abdul-Hamed (1998). Further, the conventional physiological explanation for ovarian activity (Singh, 1974) agrees with this results (Table 4).

On the other hand, no significance difference was present between right and left ovaries of buffalo in aspect of all the parameters (Table 5). Here the Weight, Length, Width, Depth of right ovary were 6.17±1.86g, 2.41±0.23cm, 1.85±0.54cm, 1.16±0.23cm, respectively whereas the left ovary was 3.50±1.62g, 2.17±0.29cm, 1.30±0.38cm, 0.59±0.27cm, respectively. The number of oocytes per ovary was 1.13±0.88 in right and 1.50±1.15 in left (Table 5). Exceptionally, all of the grades of left ovary oocytes showed a higher number than the right ovary oocytes which was revealed the study of Khandoker *et al.* (2012).

4.4.2 Qualitative and quantitative assessment according to the corpus luteum status in the ovary

In Table 6, a statistically significant difference was found between with CL and without CL ovaries in the case of weight, width, and depth while the CL present ovaries demonstrated an upper mean value (2.91±1.66, 1.08±0.29, and 0.80±0.2, respectively) than the CL absent ovaries (1.47±0.85,

0.85±0.28, and 0.59±0.20, respectively). The present study revealed that CL present ovaries were heavier in weight and larger in shape because CL is an extracellular, fleshy materials which generated from tertiary follicles after ovulation (Mahzabin *et al*., 2020; Sarker, 1993; Singh *et al*., 1974). On the other hand, Mahzabin *et al*. (2020) found a significant difference in length of ovaries which is contradictory to the present study (Table 6).

The variation in the ovary weight and size may be attributed to the presence of CL (Jablonka-Shariff *et al*., 1993,) and the number of primordial follicles, which are dependent on the cyclicity of the animal (Danell, 1987), and implantation of zygote in the uterus of animal (Abdoon and Kandil, 2001; Boediono *et al*., 1995). Jablonka-Shariff *et al*. (1993) reported that fresh weight and DNA content of CL rose linearly from days 2-12 of the estrous cycle. The ovine CL grows hyperplasia which may create a higher weight.

Table: 4. Quantitative assessment of cattle ovaries and oocytes according to the position of ovary in animal

Parameters		Position of the ovary in the animal		Level of significance
		Right	Left	
Weight (g)		1.98±1.4	1.54±0.92	NS
Length (cm)		1.57±0.45	1.50±0.42	NS
Width (cm)		0.91±0.25	0.88±0.33	NS
Depth (cm)		0.67±0.14	0.59±0.27	NS
Number of visible follicles per ovary		5.90±5.09	3.75±3.33	NS
Number of COCs per ovary	**Grade A**	1.30±0.86	1.30±0.68	NS
	Grade B	1.40^{a}±0.82	0.75^{b}±0.78	*
	Grade C	1.15^{a}±0.81	0.55^{b}±0.60	*
	Grade D	1.50^{a}±0.82	0.65^{b}±0.81	*
	Average	1.34^{a}±0.82	0.81^{b}±0.76	*

*The different superscripts indicated significant difference between two values in the same row; *, p<0.05(significant); NS, p>0.05 (non-significant).*

However, the follicles number and oocytes numbers per ovary were showed no significant relation between two CL status though the mean values were greater in CL bearing ovaries (Table 6). Boediono *et al*. (1995) and Islam *et*

al. (2018) observed almost similar result for different species of animals. This study proved the statement of the previous studies (Abdoon and Kandil, 2001; Boediono *et al.,* 1995; Moreno *et al.,* 1993) that was CL present ovaries produce more good quality oocytes due to the higher progesterone level in the blood and the constant follicular change due to the follicular degradation in ovary.

In case of buffalo, only depth of ovaries (1.23±0.23 vs. 0.91±0.14) were found a higher p value ($p<0.05$) between with and without CL ovaries while others were statistically non-significant (Table 7). A higher number of follicles/ovaries in CL-bearing vs. non-CL-bearing ovaries was demonstrated for buffalo that were 11.00 vs. 6.60. The number of follicles highly depends on the estrous cycle, progesterone production, and CL status of animal. The number of big follicles in the ovaries of early pregnant buffalo cows is dramatically reduced. While, the developing follicles are smaller in size and have a lower chance of continuing into the later stages (Abdoon and Kandil, 2001; Boediono *et al.*, 1995).

On the other hand, according to Das *et al*. (1996) and Jamil *et al*. (2008), CL dramatically decreased the number of ovarian follicles in buffaloes and produced less oocytes which contradict to this study (Table 7). These scientists attributed the contradiction to the breed or genotypic differences in ovarian function between the river and the swamp-type buffaloes.

However, almost all of the qualitative and quantitative parameters of ovary had a higher mean value in CL present than CL absent ovaries for both species (Table 6 & 7).

Table: 5. Quantitative assessment of buffalo ovaries and oocytes according to the position of ovary in animal

Parameters		Position of the ovary in the animal		Level of significance
		Right	Left	
Weight (g)		6.17±1.86	3.50±1.62	NS
Length (cm)		2.41±0.23	2.17±0.29	NS
Width (cm)		1.85±0.54	1.30±0.38	NS
Depth (cm)		1.16±0.23	0.59±0.27	NS
Number of visible follicles per ovary		9.75±6.18	6.75±4.27	NS
Number of COCs per ovary	**Grade A**	1.50±0.57	2.00±0.81	NS
	Grade B	1.00±0.81	1.50±1.29	NS
	Grade C	1.00±0.81	1.25±0.50	NS
	Grade D	1.00±1.41	1.25±1.89	NS
	Average	1.13±0.88	1.50±1.15	NS

NS= p>0.05 (non-significant)

Table: 6. Quantitative assessment of cattle ovaries and oocytes according to corpus luteum status

Parameters		Corpus luteum status in the ovary		Level of significance
		Present	Absent	
Weight (g)		2.91^{a}±1.66	1.47^{b}±0.85	*
Length (cm)		1.78±0.43	1.47±0.41	NS
Width (cm)		1.08^{a}±0.29	0.85^{b}±0.28	*
Depth (cm)		0.80^{a}±0.21	0.59^{b}±0.20	*
Number of visible follicles per ovary		7.38±3.66	4.19±4.37	NS
Number of COCs per ovary	**Grade A**	1.38±0.74	1.28±0.77	NS
	Grade B	1.67±0.57	1.00±0.70	NS
	Grade C	1.33±0.57	1.40±0.54	NS
	Grade D	1.00±1.00	1.80±0.83	NS
	Average	1.13±0.75	1.06±0.85	NS

*The different superscripts indicated significant difference between two values in the same row; *, $p<0.05$(significant); NS, $p>0.05$ (non-significant)*

Table: 7. Quantitative assessment of buffalo ovaries and oocytes according to corpus luteum status

Parameters		Corpus luteum status in the ovary		Level of significance
		Present	Absent	
Weight (g)		5.96±2.23	4.16±2.04	NS
Length (cm)		2.33±0.20	2.27±0.34	NS
Width (cm)		1.83±0.6	1.42±0.42	NS
Depth (cm)		1.23^{a}±0.23	0.91^{b}±0.14	*
Number of visible follicles per ovary		11.00±6.92	6.60±3.7	NS
Number of COCs per ovary	**Grade A**	1.67±0.57	1.80±0.83	NS
	Grade B	1.00±1.00	1.40±1.14	NS
	Grade C	1.33±0.57	1.00±0.70	NS
	Grade D	1.33±1.5	1.00±1.73	NS
	Average	1.33±0.88	1.30±1.12	NS

*The different superscripts indicated significant difference between two values in the same row; *, p<0.05(significant); NS, p>0.05 (non-significant)*

The qualitative and quantitative parameters of cattle and buffalo ovaries have been compared according to the ovary's position (Table 8) and corpus luteum status (Table 9). Here, the data of twenty (20) left, and twenty (20) right ovaries for cattle, and ten (10) left, and ten (10) right ovaries for buffalo are presented (Table 8).

Table 8 illustrated a statistically significant difference ($p<0.05$) between cattle and buffalo ovaries - right vs. right and left vs. left - in the aspect of quantitative characteristics but the significant difference was absent in terms of oocyte's quality. Also, it showed that the right ovary was heavier and more prominent in size than the left ovary in both species, which corresponded with the studies of Kunbhar *et al*. (2003) and Khammas *et al*. (2005) for buffalo. According to Islam *et al*. (2018a), the biometrical values of ovary agree with the present study but the ovarian weight showed a greater value for both cattle and buffalo species than this study.

The data (Table 8) disagreed with Ali *et al*. (2006) and Chacur *et al*. (2009) for Zebu cattle in the case of ovarian weight, length, width, and thickness that were higher than the finding. This might be happened due to the different breed, body condition, reproductive status, and regional differences in animal rearing among these studies. On the contrary, the average number of different graded oocytes per ovary did not differ significantly between the two species (Table 8).

Among the 40 cattle ovaries, eight (8) ovaries had CL, while thirty-two (32) ovaries had no CL. In addition, among the twenty (20) buffalo ovaries, eight (8) ovaries were recorded as a CL present, whereas twelve (12) as a CL

absent ovary. A statistically significant difference ($p < 0.05$) was found between CL present ovaries and between CL absent ovaries in terms of weight, length, width, and depth for cattle and buffalo (Table 9).

The findings of cattle (Table 9) were related to the ovarian weight and length of Mahzabin *et al*. (2020) and ovarian length, follicle and COCs number of Islam *et al.* (2007) with goat experiment. A higher mean value of surface follicles was found in the buffalo than the cattle ovary (Table 9), which can be explained by the review of Vale and Ribeiro (2005). It was described that bubaline and bovine has a massive difference in primordial follicles from 60 to 100 thousand in cows and 12 to 20 thousand in buffaloes.

However, the parameters for the quality of oocytes showed no significant relationship between those two CL statuses (Table 9).

Table: 8. Comparison of qualitative and quantitative characteristics of ovaries and oocytes between cattle and buffalo according to the ovary's position

Parameters		Right		Level of sig.	Left		Level of sig.
		Cattle	Buffalo		Cattle	Buffalo	
Weight (g)		1.98^{b}±1.4	6.17^{a}±1.86	*	1.54^{b}±0.92	3.50^{a}±1.62	*
Length (cm)		1.57^{b}±0.45	2.41^{a}±0.23	*	1.50^{b}±0.42	2.17^{a}±0.29	*
Width (cm)		0.91^{b}±0.25	1.85^{a}±0.54	*	0.88^{b}±0.33	1.30^{a}±0.38	*
Depth (cm)		0.67^{b}±0.14	1.16^{a}±0.23	*	0.59^{b}±0.27	0.90^{a}±0.16	*
Number of visible follicles per ovary		5.90±5.09	9.75±6.18	NS	3.75±3.33	6.75±4.27	NS
No. of COCs per ovary	**Grade A**	1.30±0.86	1.50±0.57	NS	1.30±0.68	2.00±0.81	NS
	Grade B	1.40±0.82	1.00±0.81	NS	0.75±0.78	1.50±1.29	NS
	Grade C	1.15±0.81	1.00±0.81	NS	0.55^{a}±0.60	1.25^{b}±0.50	*
	Grade D	1.50±0.82	1.00±1.41	NS	0.65±0.81	1.25±1.89	NS
	Average	1.34±0.82	1.13±0.88	NS	0.81±0.76	1.50±1.15	NS

*The different superscripts indicated significant difference between two values in the same row (cattle RO vs. buffalo RO and cattle LO vs. buffalo LO); *, $p<0.05$(significant); NS, $p>0.05$ (non-significant)*

Table: 9 Comparison of qualitative and quantitative characteristics of ovaries and oocytes between cattle and buffalo according to corpus luteum status

Parameters		CL Present		Level of sig.	CL Absent		Level of sig.
		Cattle	Buffalo		Cattle	Buffalo	
Weight (g)		2.91^{b}±1.66	5.96^{a}±2.23	*	1.47^{b}±0.85	4.16^{a}±2.04	*
Length (cm)		1.78^{b}±0.43	2.33^{a}±0.20	*	1.47^{b}±0.41	2.27^{a}±0.34	*
Width (cm)		1.08^{b}±0.29	1.83^{a}±0.6	*	0.85^{b}±0.28	1.42^{a}±0.42	*
Depth (cm)		0.80^{b}±0.21	1.23^{a}±0.23	*	0.59^{b}±0.20	0.91^{a}±0.14	*
No. of visible follicles per ovary		7.38±3.66	11.00±6.92	NS	4.19±4.37	6.60±3.70	NS
No. of COCs per ovary	**Grade A**	1.38±0.74	1.67±0.57	NS	1.28±0.77	1.80±0.83	NS
	Grade B	1.67±0.57	1.00±1.00	NS	1.00±0.70	1.40±1.14	NS
	Grade C	1.33±0.57	1.33±0.57	NS	1.40±0.54	1.00±0.70	NS
	Grade D	1.00±1.00	1.33±1.5	NS	1.80±0.83	1.00±1.73	NS
	Average	1.13±0.75	1.33±0.88	NS	1.06±0.85	1.30±1.12	NS

*The different superscripts indicated significant difference between two values in the same row (cattle CL+ vs. buffalo CL+ and cattle CL - vs. buffalo CL -); *, p<0.05(significant); NS, p>0.05 (non-significant)*

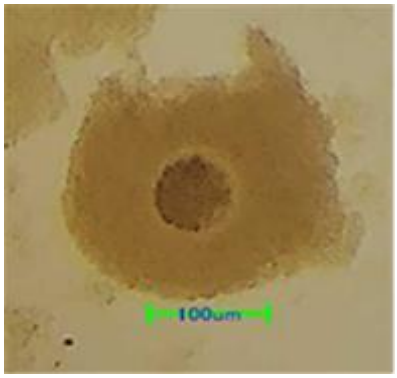
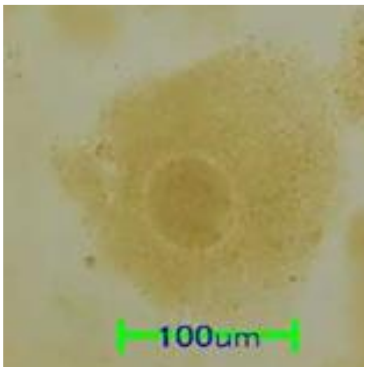
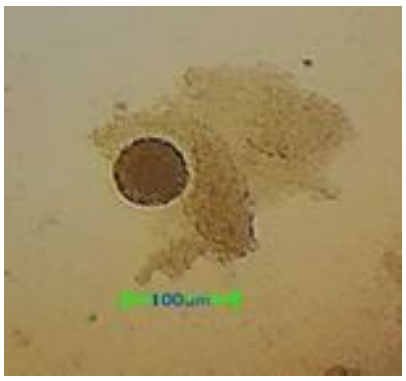
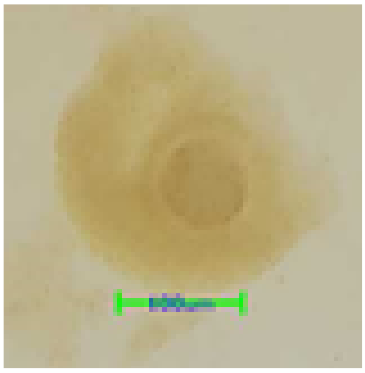
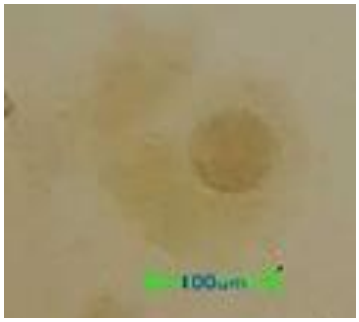
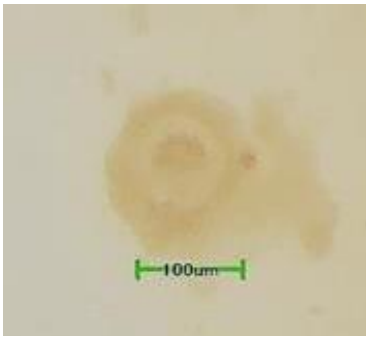
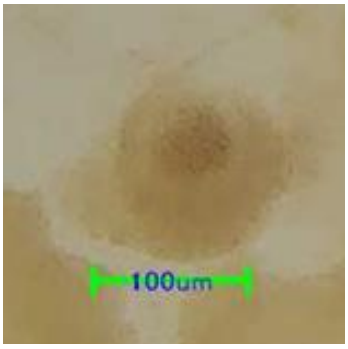
Grade of oocytes	Cattle	Buffalo
Grade A		
Grade B		
Grade C		
Grade D		

Plate: 8 Grading of oocytes. The bar represents 100µm.

4.5 Morphological study of oocytes between cattle and buffalo

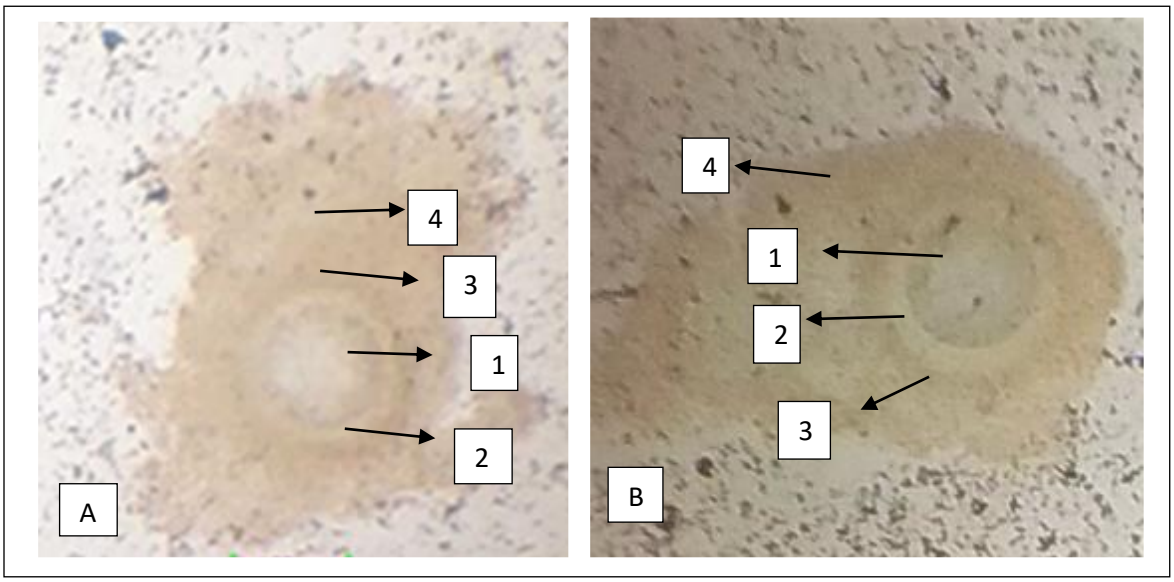


Plate: 9 Structure of oocytes. Cattle (A) and Buffalo (B). Nucleus (1), Zona pellucida (2), Corona radiata (3), and Cumulus complexes (4).

The staining was used to observe the oocytes' structures closely. There was no structural differences found between cattle and buffalo oocytes. Here, the images of oocytes were showed the following parts (Plate 9).

Oocytes' nucleus were circular in shape and densely packed with cytoplasm. It contained yolk granules that nourish the developing cell (Germinal Vesicle, 2019). The zona pellucida was surrounded the nucleus of oocytes. It is a glycoprotein layer that surrounds the plasma membrane (Gilbert, 2013). The corona radiata was the innermost layer of the cumulus oophorus cells and located directly beneath the zona pellucida, the ovum's inner protective glycoprotein layer (Pansky, 1982). Cumulus oophorus was the cells that surround and connect the corona radiata to the follicular antrum. The

cumulus complex expanded that is known as cumulus expansion after maturation. There were several layers of cells present surrounding the corona radiata. Numerous studies demonstrate that cumulus growth is required for oocyte maturation, as the cumulus complex serves as the oocyte's direct interface with the growing follicular environment (Yokoo and Sato, 2004).

CHAPTER: V

SUMMARY & CONCLUSION

CHAPTER: V
SUMMARY & CONCLUSION

Cattle and buffalo are the two important species of livestock which have the great contribution to GDP in our country. Though the production of meat and milk of these two species are popular among the people of our country, their production are not satisfactory. The main hindrances of their production are the reproductive difficulties and different diseases. Therefore, IVP can open a new door for mitigating those problem. The ovary is the raw material for this technique and the success rate of IVP mostly depend on the quality of oocytes. Successful embryo development requires proper oocyte recovery and laboratory selection. It is believed that oocytes with a full complement of cumulus cells surrounding them and uniform ooplasm are the most likely to mature and develop. Therefore, the present study was conducted to investigate the comparison between the qualitative and quantitative parameters of ovary and oocytes of these two species (cattle and buffalo) to know about them thoroughly to select the right oocytes for IVP. Also, we want to improve the IVP technique for these two species of animals. In this study, the ovaries were collected from local slaughterhouses and the follicular fluid were aspirated by aspiration method. The COCs were graded according to cumulus cell attachment to the oocyte. TCM-199 medium was used for oocyte maturation and determined the diameter of oocytes before and after maturation by the Micro-measure software. 1% aceto-orcein stain was used to stain the oocytes and observed closely.

The results showed that buffalo ovaries' weight, length, width, and depth were higher than the cattle ovaries, whereas no statistical difference was

observed between oocyte numbers. The buffalo oocytes were comparatively bigger than the cattle oocytes - in terms of weight (1.75±1.2 vs. 4.83±2.16 g), length (1.53±0.43 vs. 2.29±0.28 cm), width (0.89±0.29 vs. 1.57±0.52 cm), and depth (0.63±0.22 vs. 1.03±0.23 cm) of oocytes, respectively. The right ovary weights 1.98±1.4 vs. 6.17±1.86 g, length 1.57±0.4 vs. 2.41±0.23 cm, a width 0.91±0.25 vs. 1.85±0.54 cm and depth 0.67±0.14 vs. 1.16±0.23 cm, number of follicles per ovary 5.90±5.09 vs. 9.75±6.18, and number of COCs per ovary 1.34±0.82 vs. 1.13±0.88, in cattle vs. buffalo ovary, respectively. On the other hand, the left ovary weights 1.54±0.92 vs. 3.50±1.62 g, length 1.50±0.42 vs. 2.17±0.29 cm, width 0.88±0.33 vs. 1.30±0.38 cm, depth 0.59±0.27 vs. 0.90±0.16 cm, the number of follicles per ovary 3.75±3.33 vs. 6.75±4.27, and number of COCs per ovary 0.81±0.76 vs. 1.50±1.15 in cattle vs. buffalo ovary, respectively.

Also, the diameter of oocytes had increased after maturation for both species (85.64 ± 8.16 vs. 94.09 ± 11.23 μm for cattle and 99.92 ± 8.65 vs. 109.2 ± 8.65 for buffalo oocytes). No difference was found between right and left ovaries parameters; on the contrary, CL present ovaries had a higher value in specific parameters. CL containing ovaries had 2.91±1.66 vs. 5.96±2.23 g of weight, 1.78±0.43 vs. 2.33±0.20 cm of length, 1.08±0.29 vs. 1.83±0.6 cm of width, 0.80±0.21 vs. 1.23±0.23 cm of depth, number of follicles per ovary 7.38±3.66 vs. 11.00±6.92, and number of COCs per ovary 1.13±0.75 vs. 1.33±0.88 in cattle vs. buffalo, respectively. On the contrary, CL absent ovaries had 1.47±0.85 vs. 4.16±2.04 g of weight, 1.47±0.41 vs. 2.27±0.34 cm of length, 0.85±0.28 vs. 1.42±0.42 cm of width, 0.59±0.20 vs. 0.91±0.14 cm of depth, number of follicles per ovary 4.19±4.37 vs. 6.60±3.7, and number of COCs per ovary 1.06±0.85 vs. 1.30±1.12 in cattle vs. buffalo, respectively. The number of normal oocytes was 1.19±0.81 in cattle and

1.50±0.89 in buffalo per ovary, whereas the abnormal oocytes number was 0.96±0.85 in cattle and 1.13±1.15 in buffalo per ovary.

There are some limitations of this experiment. Firstly, the reproductive tract of buffalo was unavailable because this study was conducted at the time of the COVID-19 outbreak when the supply of buffalo was limited than the normal situation. So, the sample size – the ovary – was small (n=20) for buffalo. Secondly, for measuring the diameter of oocytes, the sample size was less. There were thirty (30) cattle and twenty (20) buffalo and twenty (20) cattle, and fifteen (15) buffalo oocytes measured before and after maturation, respectively, which could be increased.

Overall, the buffalo ovary was more prominent in size and heavier in weight than the cattle ovary, and any type of ovaries, irrespective of the ovary's position and CL status, can be a good source of good quality oocytes. The diameter of the ovarian nucleus can be changed because of maturation. Further study should be conducted to investigate the change of nuclear diameter of oocytes after maturation because a bit of information is available on diameter change on oocytes. The achieved knowledge of this study should be implemented in IVP, genetic engineering, embryo transfer, transgenic animal production and so on.

CHAPTER: VI

REFERENCES

CHAPTER: VI

REFERENCES

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