Short note [Nota corta]

EVALUATION OF CATTLE OVARIES AND FOLLICLES BY HISTOLOGICAL ANALYSIS FOR POTENTIAL *IN VITRO* PRODUCTION OF EMBRYOS IN TROPICAL CONDITIONS †

[EVALUACIÓN DE OVARIOS Y FOLÍCULOS BOVINOS MEDIANTE ANÁLISIS HISTOLÓGICO PARA LA PRODUCCIÓN POTENCIAL *IN VITRO* DE EMBRIONES EN CONDICIONES TROPICALES]

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SUMMARY

Background. Reproduction is important in animal production, and follicle evaluation is a remarkable reproductive aspect by which ovaries can be accurately evaluated since ovarian follicles are the organ's functional units and each contain an oocyte and related somatic cells. **Objective.** The objective of this study was to evaluate the abattoir cattle ovaries and follicles for potential *in vitro* production of embryos. **Methodology.** Ovaries were collected and categorized based on the presence of corpus luteum (CL; with or without). Thereafter, ovaries were processed and follicles were counted through naked eye estimation as well histologically. Data were analyzed using the GLM procedure in SAS. Also, relationships between follicles in both types of ovaries were calculated using Pearson correlations. **Results.** From a total of sixteen cattle ovaries, 62.5% had absence of CL whereas 37.5% ovaries had CL. Regarding morphometric measurements, ovaries with CL were 48% higher ($P < 0.05$) in weight than those ovaries without CL. Correlations between length and weight in ovaries without CL were high ($r= 0.87$) and, diameter and weight in ovaries with CL were low ($r= 0.41$). Results exposed that the amount of follicles with 2-6 mm diameter were significantly ($P < 0.05$) higher (61% more) in ovaries without CL compared to those with CL. Primary and secondary follicles presence were higher ($P < 0.05$) in ovaries without CL (120% more) than in those with CL (89%). Greater ($P < 0.05$) numbers of normal and lower ($P < 0.05$) amount of degenerated follicles were found in ovaries without CL. **Implications.** Overall, our data suggest that ovaries without CL are a potential source for quality follicles and this study provides new insights that may serve as the baseline information to produce high-quality oocyte with potential use for *in vitro* production of tropical cattle. **Conclusions.** This study corroborated that CL influences growth and development of the follicles and cell degeneration. As a result, the developmental competence of oocytes and embryo production will be negatively affected by the ovaries with CL. Ovaries without CL will provide quality follicles and cumulus-oocyte complexes.

Keywords: Cattle; abattoir; ovary; corpus luteum; follicles.

RESUMEN

Antecedentes. La reproducción es importante en la producción animal, y la evaluación del folículo es un aspecto reproductivo notable por el cual los ovarios pueden evaluarse con precisión ya que los folículos ováricos son las unidades funcionales del órgano y cada uno contiene un ovocito y células somáticas relacionadas. **Objetivo.** El objetivo de este estudio fue evaluar los ovarios y folículos del ganado matadero para la producción potencial in vitro de embriones. **Metodología.** Los ovarios se recolectaron y clasificaron según la presencia de cuerpo lúteo (CL; con o sin). Posteriormente, se procesaron los ovarios y se contaron los folículos a través de la estimación a simple vista, también histológicamente. Los datos se analizaron utilizando el procedimiento GLM en SAS. Además, las relaciones entre los folículos en ambos tipos de ovarios se calcularon utilizando correlaciones de Pearson. **Resultados.** De un total de

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dieciséis ovarios de ganado, el 62.5% tenía ausencia de CL mientras que el 37.5% de los ovarios tenía CL. Con respecto a las mediciones morfométricas, los ovarios con CL fueron 48% más altos (P <0.05) en peso que los ovarios sin CL. Las correlaciones entre la longitud y el peso en los ovarios sin CL fueron altas (r = 0,87) y el diámetro y el peso en los ovarios con CL fueron bajos (r = 0,41). Los resultados expusieron que la cantidad de folículos con un diámetro de 2-6 mm fue significativamente (P <0.05) mayor (61% más) en los ovarios sin CL en comparación con aquellos con CL. La presencia de folículos primarios y secundarios fue mayor (P <0.05) en ovarios sin CL (120% más) que en aquellos con CL (89%). Se encontraron mayores (P <0.05) cantidades de folículos degenerados normales y menores (P <0.05) en los ovarios sin CL. **Implicaciones.** En general, nuestros datos sugieren que los ovarios sin CL son una fuente potencial de folículos de calidad y este estudio proporciona nuevas ideas que pueden servir como información de referencia para producir ovocitos de alta calidad con uso potencial para la producción in vitro de ganado tropical. **Conclusiones**. Este estudio indicó que CL influye en el crecimiento y desarrollo de los folículos y la degeneración celular. Como resultado, la competencia de desarrollo de los ovocitos y la producción de embriones se verá afectada negativamente por los ovarios con CL. Los ovarios sin CL proporcionarán folículos de calidad y complejos de cúmulos y ovocitos.

Palabras clave: ganado; matadero; ovario; cuerpo lúteo; folículos

INTRODUCTION

Reproduction is a vital trait for animal production and indispensable for the subsistence of animal species but in dairy production systems, reproductive efficiency is a critical factor that interferes with the economic viability of farms (Bettencourt *et al.,* 2015). The reproductive efficiency of bovine animals is gradually decreasing day by day in tropical countries such as Bangladesh (Kamal, 2010). Higher environmental temperatures, lower productivity and more incidences of reproductive problems (Lima *et al.,* 2013) generally characterize tropical countries.

There are many factors interfering with reproduction such as repeat breeding (Yaginuma, 2019), more numbers of small and lesser number of dominant follicles (Gimenes *et al.,* 2009), early embryonic mortality and prolonged inter calving duration (Sood *et al.,* 2017), higher circulating concentrations of estradiol, progesterone and insulin-like growth factor-I (Gimenes *et al.,* 2009). Hence, *in vitro* embryo production (IVEP), multiple ovulation and embryo transfer (MOET) techniques have already been adopted by many developed countries to overcome those limitations (Sreenivas *et al.,* 2014 and Deb *et al.,* 2016). Before adopting these technologies, clear and sharp knowledge is needed regarding the abattoir ovaries, follicles and oocyte quality.

Ovaries are the principal organs of the female reproductive tract where follicles are found around the ovarian surface (Adams *et al.,* 1989) and supply the female germ cells (Alam *et al.,* 2014). Follicles are fluid-filled, blister-like structures that have developing oocytes (Britt, 2008) and most of the follicles (>99 %) degenerate during their growth and maturation (Carroll *et al.,* 1990). Antral follicles surged by the follicle-stimulating and luteinizing hormone for ovulation and, the rest of that follicles change their histological structure through degeneration and make CL (Holesh and Lord, 2019). Follicles number and their types vary between mammal species, and within the same species (Batista *et al.,* 2014). Beside these, follicle size is also important because it interferes with the efficiency of oocyte collection (Montes-Quiroz *et al.,* 2019).

With regard to ovary characteristics, the, follicle counting method is a remarkable reproductive feature for determining ovary characteristics accurately. Besides these, ovarian types (with or without CL) and qualitative evaluation of ovaries might be a novel tool for increasing the IVEP efficiency (Penitente-Filho *et al.,* 2015). The most practical approach for IVEP is to use abattoir ovaries because these ovaries are the cheapest source of oocyte collection (Hufana-Duran *et al.,* 2005; Deb *et al.,* 2016). But quality oocytes are the major problem for doing IVEP as good quality oocytes depends on better normal follicles and ovaries. Mondal *et al.* (2008) reported that a higher number of worthy quality oocytes were recovered from ovaries without CL compared to those ovaries with CL with great potential on *in vitro* fertilization and maturation.

Very little research has been done regarding the ovarian types and naked-eye estimation of follicles (Khandoker *et al.,* 2016); *in vitro* production in bovine blastocyst with oocyte from abattoir ovary (Deb *et al.,* 2016). Also, research oocytes cattle ovaries and follicles evaluation by hystological analysis in tropical conditions is rare. Therefore, the main objective of this study is to elucidate the effects of CL on the number and quality of ovarian follicles and, the second objective is to identify normal follicles and make differences of ovarian cellular integrity based on the granulosa cell degeneration.

MATERIALS AND METHODS

Collection of reproductive organs

Sixteen cattle ovaries from crossbred females (Holstein-Friesian × Sahiwal) were randomly collected immediately after the slaughtering of animals from the abattoir of Mymensingh city in Bangladesh. The latitude and longitude of this city range over 24°

44' 36.4128" N and 90° 23' 54.1824" E, respectively and its climate zones include tropical monsoon. Collected ovaries were placed in a thermo flask (having 0.9% normal saline solution) at 25°C and were transported to the Reproductive Biotechnology Laboratory at the Department of Animal Breeding and Genetics from the Bangladesh Agricultural University. Slaughtered cattle age was determined by dentition technique and age varied from 2.5 to 3.0 years.

Processing and morphometric measurement of ovaries

Collected ovaries were separated from the reproductive organ and transferred to petri dishes with saline solution. Then, the ovaries were trimmed to remove the surrounding tissues and overlying bursa. Thereafter, ovaries were categorized based on the presence or absence of CL and subjected to morphometric measurements. The weights of the ovaries were measured with a digital balance (Precisa, XB-220A, Switzerland). Length (distance between anterior and posterior pole) and, diameter (distance between medial and lateral surface) of the ovaries and follicles were measured with slide calipers. Visible follicles numbers on the surface of ovaries (with or without CL) were counted by naked-eye and categorized into two diameters: 2-6 mm and more than 6 mm (Talukder et al., 2011).

Histology

Ovaries were cut with surgical blades (No. 10, Keisei Medical Industrial Co., Ltd., Tokyo) and forceps in order to obtain 5 parts of cross-section cuts and then, they were fixed into Bouin's solution (picric acid, 37-40% formalin and glacial acetic acid) for 24 hours. Dehydration of ovaries was done using graded (70%) alcohol (Changshu Hongsheng Fine Chemical Co., Ltd, China) and cleaned with xylene (Merck KGaA, Darmstadt, Germany). Then ovarian parts were left in an incubator at 60°C for 1 hour with 50% paraffin and 50% xylene and embedded with melted paraffin (Thermo Fisher Scientific India Pvt. Ltd.). Paraffin blocks were hardened by allowing them to rest at room temperature for a period of 24 hours and then blocks were trimmed with the help of a surgical blade (No. 10, Keisei Medical Industrial Co., Ltd., Tokyo) and forceps for further processing. Six µm-thick sections (every 10th section) were prepared by using a rotatory microtome (Thermo Fisher Scientific, Waltham, Massachusetts, USA), placed upon a glass slide, and dried at room temperature. The sections were stained with hematoxylin and eosin. Finally, the stained sections were permanently mounted with a coverslip using DPX mounting reagent (Merck Specialty Private Limited, Mumbai, India).

Microscopy

For each ovary, every tenth serial section was observed with the help of a light microscope (10 x 10X) for identification of follicles and granulosa cells. Counting start from the left upper corner to the right, and double counting was avoided. Follicles were categorized into four classes as described by Sarker *et al.* (2015): 1) primordial follicle has a single layer of flattened granulosa cells surrounding the oocyte; 2) primary follicle with a distinct layer of cuboidal granulosa cells; 3) secondary follicle have two or more granulosa cells layers but no antrum and; 4) antral cavity with numerous granulosa cells layers are present in antral follicle. When follicles contained oocytes with pyknosis, large vacuoles, condensed cytoplasm, disappearing of nuclear membranes, shrinkage of nucleus, swollen granulosa cells, or loss of granulosa cells the follicles are to be considered as degenerated. In principle, the granulosa cell layer was considered as key criteria for follicular degeneration. The follicles and the granulosa cell layer were counted using the Olympus light microscope (Olympus CX41; Olympus Industrial America Inc., Orangeburg, NY, USA) based on cell integrity of ovaries.

Statistical analysis

Data were analyzed using the GLM procedure in SAS (Statistical Analysis System Institute Inc. Cary, NC, USA) with the following model:

$$Y_{ijk} = \mu + S_i + T_j + e_{ijk}$$

Where Y_{ijk} is the observation/dependent variable, μ is the population mean, S_i is the effect of the ovary with corpus luteum (CL), T_j is the effect of the ovary without corpus luteum and e_{ijk} is the random error. In this study, independent variables were ovary with and without CL, and dependent variables were weight, length, diameter, primordial, primary, secondary and antral follicles. Data were presented as mean ± SEM (standard error of the mean) and differences at $P < 0.05$ were considered as statistically significant. Correlations between ovarian morphological features and follicles numbers with presence or absence of CL in ovaries were performed by Pearson's correlation.

RESULTS

Quantitative evaluation of ovaries

From a total of sixteen cattle ovaries, 62.5% had absence of CL whereas 37.5% ovaries had presence of CL.

Effect of CL on morphometric measurements and visible follicles number in ovaries

Morphometric parameters of bovine ovaries in relation to the presence or absence of CL is given in Table 1.

The weight of the ovaries with CL was significantly ($P < 0.05$) different (48% higher) than those without CL ovaries. Both the length and diameter of both types of ovaries were similar. Positive correlation exists between weight and diameter ($r = 0.41$) of ovaries with CL, but length and diameter were negatively correlated. Both the length and diameter of the ovaries without CL were significantly ($P < 0.01$) correlated with the weight of ovaries (Table 2). Follicles diameters were categorized into 2-6 mm and >6 mm (Table 1), and results revealed that weight of 2-6 mm follicles were significantly ($P < 0.05$) higher (61%) in ovaries without CL compared to those ovaries with CL.

Effect of CL on histologically counted follicles

Several types of follicles in both types of ovaries were determined histologically (Table 3). Results showed that primary, secondary and total follicles (primordial to antral) numbers were 89, 120 and 51%, respectively higher in ovaries without CL than those ovaries with CL. Both primordial (non-growing) and antral follicles were similar in ovaries with or without CL.

Table 1. Effect of CL on morphometric measurements and visible follicles number (Mean ± SEM) in cattle ovaries.

Ovarian Types	Morphometric parameters			Visible follicles number	
	Weight (g)	Length (cm)	Diameter (cm)	2-6 mm	> 6 mm
Ovary with CL (n=6)	2.93 ± 0.38	2.05 ± 0.12	1.22 ± 0.09	9.83 ± 2.23	0.50 ± 0.33
Ovary without CL (n=10)	1.52 ± 0.30	1.93 ± 0.09	1.14 ± 0.07	15.90 ± 1.73	1.00 ± 0.26
Level of sig.	*	NS	NS	*	NS

CL, corpus luteum. *Indicate significant at $P < 0.05$ and *NS* indicate non-significant ($P > 0.05$) effect on the parameters.

Table 2. Correlation between morphometric measurements of cattle ovaries.

	Ovary with CL		Ovary without CL	
	Weight (g)	Length (cm)	Weight (g)	Length (cm)
Length (cm)	0.29^{NS}		0.87**	
Diameter (cm)	0.41^{NS}	-0.07^{NS}	0.82**	0.51^{NS}

CL, corpus luteum. Correlation between follicles was expressed as r. **Significant at $P < 0.01$ and *NS* indicate non-significant
($P > 0.05$).

Table 3. Effect of CL on histologically counted follicles number (Mean ± SEM) in cattle ovaries.

Ovarian Types	Number of follicles per ovary				
	Primordial	Primary	Secondary	Antral	Total follicles
Ovary with CL (n = 6)	51.17 ± 11.47	17.50 ± 5.07	1.50 ± 0.55	12.83 ± 1.95	83.00 ± 13.19
Ovary without CL (n = 10)	73.50 ± 8.88	33.10 ± 3.93	3.30 ± 0.43	15.50 ± 1.51	125.40 ± 10.22
Level of sig.	NS	*	*	NS	*

CL, corpus luteum. Figures in the parenthesis indicate number of observation. *indicate significant at $P < 0.05$ and *NS* indicate non-significant ($P > 0.05$) effect on the parameters.

Table 4. Correlation between follicles number of cattle ovaries.

	Ovary with CL				Ovary without CL			
	Primordial	Primary	Secondary	Antral	Primordial	Primary	Secondary	Antral
Primary	0.15^{NS}				0.26^{NS}			
Secondary	-0.28^{NS}	-0.01^{NS}			-0.47^{NS}	-0.19^{NS}		
Antral	-0.32^{NS}	0.68^{NS}	-0.16^{NS}		-0.24^{NS}	-0.08^{NS}	0.74*	
Total follicles	0.89^{NS}	0.57^{NS}	-0.25^{NS}	0.07^{NS}	0.90**	0.60^{NS}	-0.30^{NS}	-0.03^{NS}

CL, corpus luteum. Correlation between follicles was expressed as r. ** Significant at $P < 0.01$, *significant at $P < 0.05$ and *NS*, non-significant ($P > 0.05$).

Table 5. Effect of CL on normal and degenerated follicles numbers (Mean ± SEM) in cattle ovaries.

Ovarian Types	Number of follicles per ovary	
	Normal follicles	Degenerated follicles
Ovary with CL (n = 6)	5.00 ± 1.51	9.33 ± 1.03
Ovary without CL (n = 10)	13.00 ± 1.17	5.70 ± 0.80
Level of sig.	*	*

CL, corpus luteum. Figures in the parenthesis indicate number of observation and *indicate significant at $P < 0.05$.

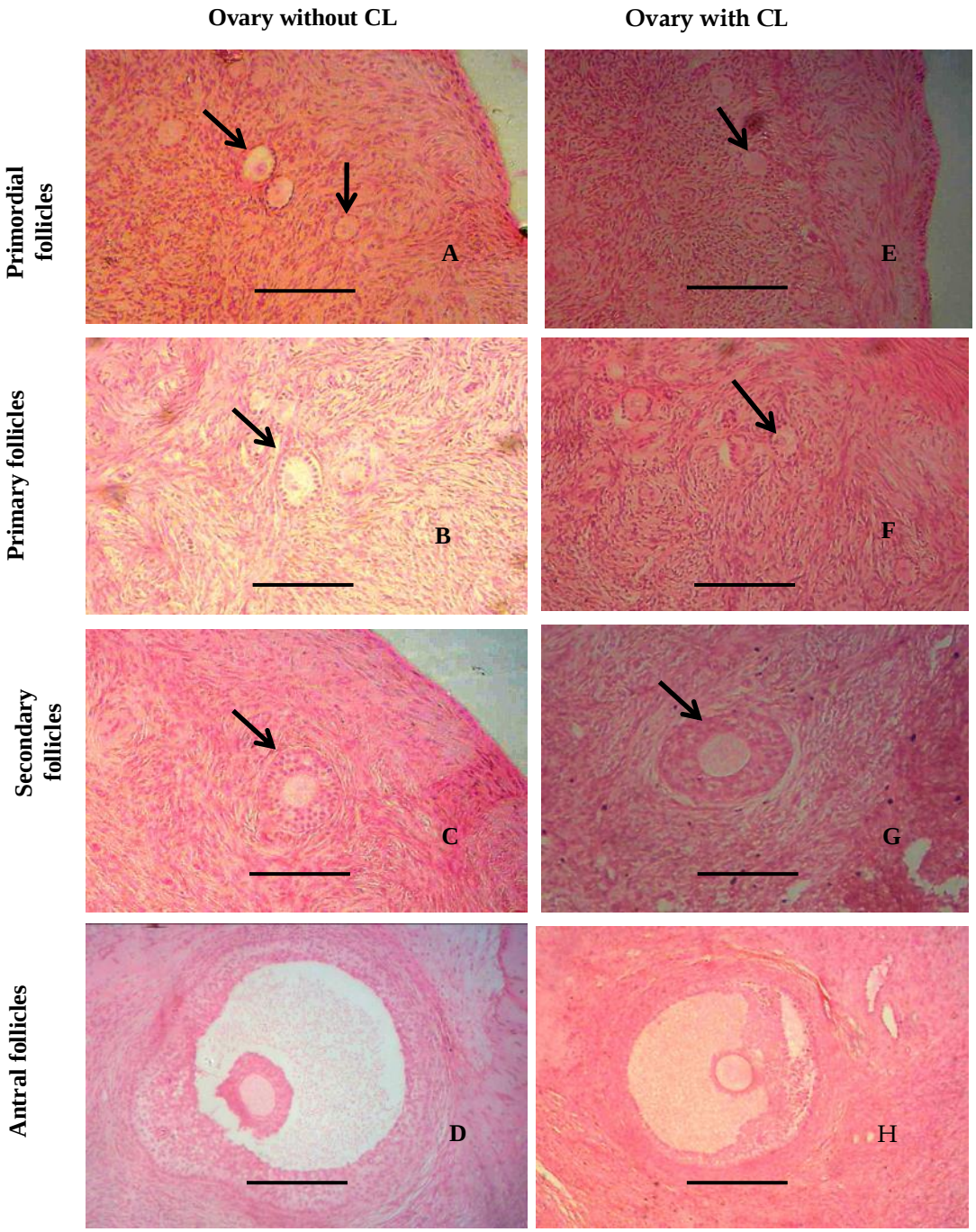

Figure 1. Histological section of ovaries with CL and without CL. ***A)*** Primordial follicles; ***B)*** Primary follicles; ***C)*** Secondary follicles; ***D)*** Antral follicle in ovary without CL and ***E)*** Primordial follicles; ***F)*** Primary follicles; ***G)*** Secondary follicles; ***H)*** Antral follicle in ovary with CL. Arrows indicate follicles and scale bars represent 100 µm.

A weak positive linear relationship subsisted between primordial and primary follicles number of both type ovaries. It was noted that the number of secondary follicles was negatively correlated with other types of follicles numbers in both ovaries. In CL absent ovaries, a positive linear relationship existed between primordial and total follicles numbers ($P < 0.01$); secondary follicles and antral follicles numbers ($P < 0.05$) (Table 4). The morphology of ovarian follicles (at 10 × 10X) is shown in Figure 1.

The mean normal and degenerated follicles numbers of ovaries with and without CL are given in Table 5. Higher ($P < 0.05$) numbers of normal follicles were found in ovaries without CL compared to those with CL ovaries. Degenerated follicles number also differed ($P < 0.05$) and it was about 63% higher in ovaries with CL compared to those without CL.

Follicle degeneration identification is difficult in primordial and primary follicles but degeneration can be better identified in both secondary and antral follicles using photomicrographs, which are depicted in Figure 2.

DISCUSSION

Quantitative evaluation of ovaries

Among the collected ovaries from abattoir, a lower number (n = 6) of ovaries had CL and the rest of the ovaries (n = 10) did not have CL. This pattern may be explained by the fact that the ovaries were obtained from animals that have been culled out due to their low reproductive performance. In this study, all the slaughtered animals' age varied from 2.5 to 3.0 years and cattle mostly came close to heat as well as conceived within this age. If female animals do not get pregnant after several natural or artificial inseminations, then farmers become worried about the reproductive performance of those animals and they often sell them to butchers. This explains why absent CL ovaries numbers were greater in slaughtered cattle ovaries than that of the CL present ovaries. This finding is similar to other studies using abattoir ovaries from cows (Khandoker *et al.,* 2016), buffaloes (Khandoker *et al.,* 2011) and goats (Asad *et al.,* 2016).

Morphometric measurements of ovaries and follicles

In this study, higher ovary weights ($P < 0.05$) were found in ovaries with CL (2.93 ± 0.38 g/ovary) compared to those without CL ovaries (1.52 ± 0.30 g/ovary). That may be due to the presence of CL in the ovaries. Jablonka-Sharif *et al.* (1993) reported that CL is an extracellular material within the ovary having a definite span of its growth, maintenance and regression that might be the cause of differences in length, diameter, and weight of the ovary containing it. In this study, there were no differences found between length and diameter of ovaries with or without CL. This finding partly agrees with Bhajoni *et al.* (2018), Asad *et al.* (2016) and Khandoker *et al.* (2011) who carried out experiments on cattle, goat and buffalo ovaries, and found significantly ($P < 0.05$) higher weight, length and diameter in ovaries with CL.

More follicles of 2-6 mm ($P < 0.05$) and >6 mm ($P > 0.05$) were found in ovaries without CL than those with CL. That may be due to lack of progesterone activity as CL secretes progesterone hormone and this hormone negatively interfere with the follicular development. That is why a lower number of follicles were found in ovaries with CL, which was similar to the findings of Hafez (1993) and Bhajoni *et al.* (2018). There is a strong relation exist between follicles diameter and oocyte maturation. Ideally, follicles diameter varies from 4-6 mm and 62% more 2-6 mm follicles found in absent CL ovaries than that of the CL present ovaries. This describes that maximum numbers of quality follicles and oocytes can be collected from without CL ovaries.

Histological analysis of follicles

Histological findings showed that primary, secondary and total follicles numbers were significantly higher in CL absent ovaries than the CL present ovaries. The causes of more follicles contained in CL absent ovaries than those of CL ovaries have been well understood as fits the endocrinological interpretation. It is well known that all female mammals are born with a large number of follicles that decline rapidly as puberty approaches. However, follicular variations depends on the presence or absence of CL in the ovaries because CL has an inhibitory effect on follicular development (Hafez, 1993) and this finding is in similar to Khandoker *et al.* (2016) who studied ovarian type effects and collection techniques on the number of follicles in bovines.

There was a great opportunity for collecting large number of oocytes from the follicles of ovaries without CL, which facilitates *in vitro* maturation, fertilization, and subsequent development. In CL absent ovaries, the number of primordial follicles was correlated with the total number of follicles ($r = 0.90$, $P < 0.01$) and secondary follicles were correlated with the antral follicles ($r = 0.74$, $P < 0.05$). This corroborated with those cows had greater pool of dormant follicles would consequently have more follicles moving into the growing pool, have a larger pool of tertiary follicles which will be suitable for better collection of cumulus-oocyte complex and *in vitro* embryo production. Similar findings were reported in bovine and caprine ovaries by Cushman *et al.* (1999), Silva-Santos *et al.* (2014), Khandoker *et al.* (2016) and Asad *et al.* (2016).

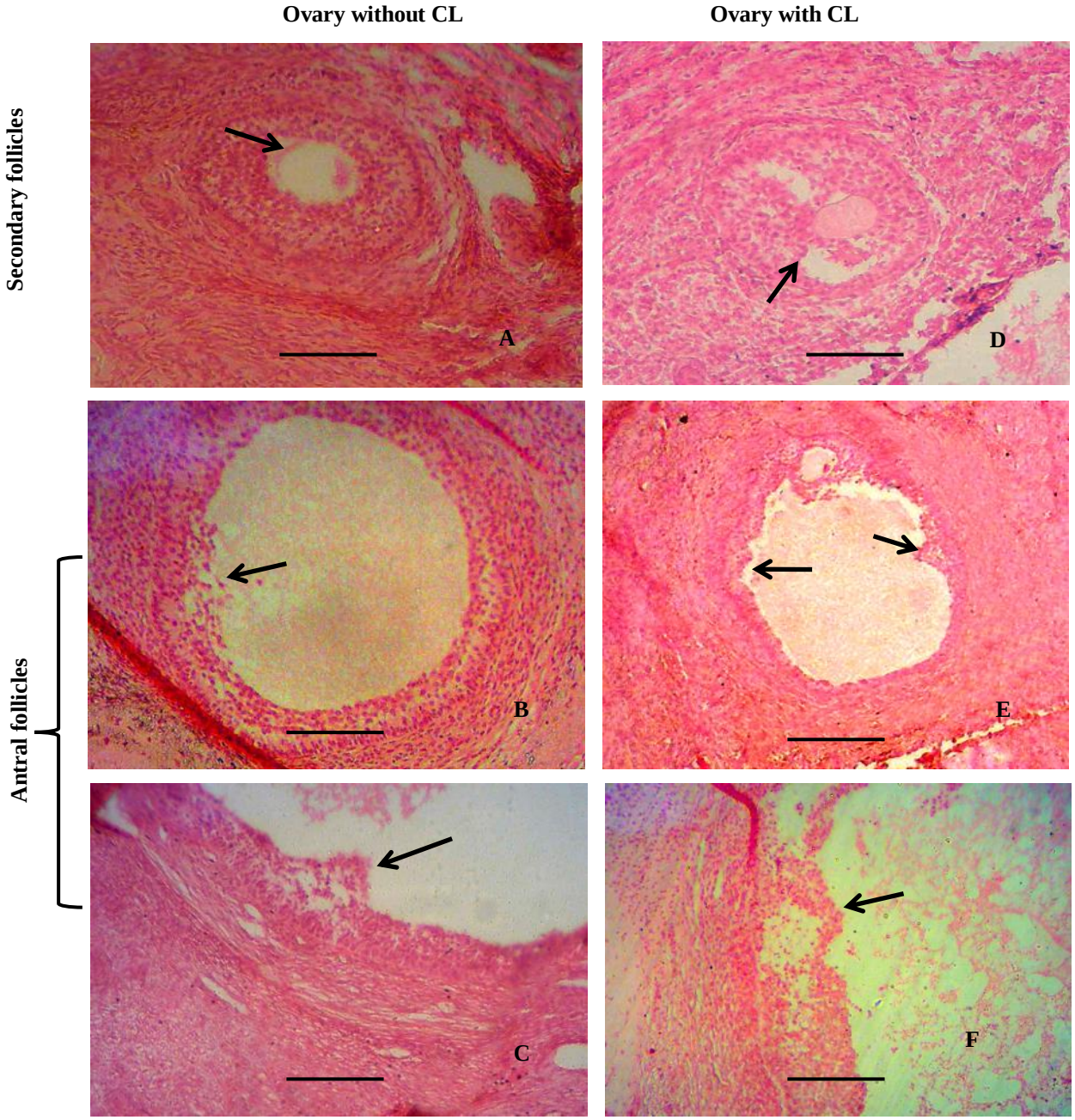

Figure 2. Histological section of ovaries with CL and without CL showing the degeneration of follicles (at 10 × 10X). ***A)*** Degeneration of secondary follicle; ***B)*** & ***C)*** Degeneration of antral follicle in ovary without CL. ***D)*** Degeneration of secondary follicle; ***E)*** & ***F)*** Degeneration of antral follicle in ovary with CL. Arrows indicate degeneration and scale bars represent 100 µm.

CONCLUSIONS

This study corroborated that corpus luteum influences growth and development of the follicles and cell degeneration. As a result, the developmental competence of oocytes and embryo production will be negatively affected by the ovaries with corpus luteum. However, those cattle ovaries retrieved from abattoir without corpus luteum are capable of producing a large number of available good-quality follicles and oocytes. Ovaries without corpus luteum will provide quality follicles and cumulus-oocyte complexes. Thus, the current study provides useful data for *in vitro* embryo production. Further immunohistochemistry studies are required for evaluation that is more accurate.

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Conflicts of Interest statement. The authors declare no conflicts of interest.

Compliance of ethical standards. This research complies with the ethical standard required for the research in Bangladesh in relation to the handling of biological material.

Data availability. Data are available with the first author (rumaahbau@gmail.com) upon reasonable request**.**

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