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Effect of Ovarian Types on Retrieval of Follicles and Culturable Cumulus Oocyte Complexes in Bovine

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ABSTRACT

The experiment was conducted for evaluation of bovine slaughter house ovary, follicles and recovery rate of cumulus oocyte-complexes (COCs) and to compare different COCs. The collected slaughter house ovaries were classified as without corpus luteum (CL-) (type-I) and with corpus luteum (CL+) (type-II). It was found that 53.13% of the ovaries collected were without CL and 46.87% were with corpus luteum. For collection of COCs, aspiration technique was performed and accordingly number of follicles collected was recorded. The mean number of follicles recovered per type-I ovary were recorded as 5.30 which was significantly higher ($P<0.01$) than the corresponding values 3.27 for type-II ovary. The mean recovery of cumulus oocyte complexes were recorded as 3.41 and 1.67 per type-I and type-II ovaries respectively. The follicular materials collected from both the techniques were observed under microscope to categorize the COCs as grade A (oocyte surrounded with more than 4-5 layers of cumulus cells homogenously), grade B (oocyte surrounded with 2-4 layers of cumulus cells partially), grade C (oocyte surrounded with 1-2 layers cumulus cells) and grade D (denuded) oocytes with degeneration observed both in oocyte and cumulus cells). Grade A and grade B were classified as culturable and grade C and grade D were considered as non-culturable. Ovaries having no CL contributed more numbers of culturable COCs per ovary (3.41) than that of ovaries with CL (1.67) in aspiration technique.

HIGHLIGHTS

- The numbers of follicles recovered per ovary (without CL) were more than the ovaries (with CL).
- Ovaries without CL contributed more numbers of culturable oocytes than the ovaries with CL.

Keywords: Slaughterhouse ovary, corpus luteum, type-I, type-II, cumulus oocyte complexes

Harvesting single ovum is a prime factor of ovulating cows and upon fertilization *in vivo*, produces a calf with 9 months gestation period (Sarwar *et al.*, 2020). Plethora of researches was executed to maximize the *in-vitro* embryo production and implementation of multiple ovulations and embryo transfer (MOET) in assisted reproductive technologies (ART) has opened up the area of research in the field of female reproduction (Asad, 2015). The

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production of viable embryos *in-vitro* (IVP) is highly variable and selecting the good quality embryo appears as one of the challenging steps in ART technique. In the same context, (Camargo *et al.*, 2018) reported more numbers of the viable embryos produced in inseminated oocytes. The production of low-quality embryos might be due to some factors such as oocyte source, slaughter house ovaries with highly heterogeneous oocyte population, media composition, environment and embryo genotype (Khandoker *et al.*, 2016). Generally, cows with poor reproductive performance are slaughtered for meat purpose (Bhajoni *et al.,* 2018), with more active CL in cattle and buffalo ovaries (Rajesh *et al.*, 2018). Therefore, to predict accurately and noninvasively, which oocytes have the ability to develop fully to term after fertilization would provide a useful tool in successful *in vitro* embryo production. So, there should be some noninvasive selection criteria for oocytes that are to be used for *in-vitro* embryo production system based on morphology (developmental competence) starting from ovarian morphology including presence of CL, total antral follicular count, maturation signaling to quality blastocyst formation. Ahmed *et al.*, (2015) had reported that higher good quality oocytes were recovered from ovaries without corpus luteum compared to the ovaries with corpus luteum, which thus, can be effectively used for IVM and IVF. Therefore, for successful IVEP experiment in cow for the evaluation of ovaries, it is important to recover higher number of culturable oocytes. Therefore, present experiment was conducted in order to determine the effect of ovarian status (type-I and type-II) on follicular population and retrieval of culturable oocytes for IVEP experiment.

MATERIALS AND METHODS

Location of work

The experiment was conducted in the Department of Veterinary Physiology and Central Instrumentation Facility, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam, India. The ovaries were collected early in the morning immediately after slaughter in normal saline containing antibiotics (Penicillin G: 0.06 g/ml) maintaining 37^{o}C in a thermos flask and then the ovaries were transported to the laboratory within 2-3 hours of slaughter in normal saline containing antiobiotic (Penecillin G: 0.06 g/ml), maintaining 37^{o}C in a thermos flask.

Collection of ovaries and classification of follicles

The extraneous tissues from the ovaries were removed and after that ovaries were washed 3-5 times with normal saline solution containing antibiotic prior to further processing. After that, the ovaries were observed, categorized as type-I (without corpus luteum) and type-II (with corpus luteum) and the number of both types were recorded in the present experiment followed by washing in the transportation medium. From each ovary, visible follicles were counted and follicular diameters were measured using Divider. The follicles were classified based on diameter into three categories, Small (S: <3 mm), Medium (M: 3 - 6 mm) and Large (L: >6 - <10 mm). The oocytes were collected from all the three categories of follicles by aspiration technique. One ml of sterile aspiration medium was taken in a 10 ml disposable syringe attached to 18 gauge needle for medium and large follicles and 20 gauge needle for small follicles. After aspiration of all the follicles, the content of the syringe containing follicular fluid and oocytes were placed in a search dish and examined under a stereozoom microscope. The COCs were separated from the debris and picked individually and then were kept in another petridish with washing medium followed by grading according to Mahesh *et al.* (2015). Only Grade A and Grade B COCs were selected for *in vitro* maturation.

STATISTICAL ANALYSIS

The statistical analysis of the experimental data were carried out by Chi-square and Z test (SAS 9.3 version).

RESULTS AND DISCUSSION

Follicular population in relation to status of ovaries collected from slaughter house

The numbers of follicles in relation to slaughter house ovarian status are presented in Table 1 and Fig. 1. A total of 224 bovine ovaries were collected from local slaughter house. Out of 224 ovaries, 119 (53.13%) were without corpus luteum (Type-I) and 105 (46.87%) were with corpus luteum (Type-II**).** Overall, 974 follicles were observed in both types of ovaries. Among them, 631 follicles were

recorded in type-I ovary (from 119 ovaries) without corpus luteum and 343 in type-II ovary (from 105 ovaries) with corpus luteum (Table 1 and Fig 1). Based on the mean value percentage and 95% Confidence Interval (CI), it was observed that number of follicles recovered per ovary in type-I ovary (without CL) was 5.30 (4.56-6.33) which was significantly higher (P<0.01) than corresponding values 3.27 (2.82-3.89) for type-II ovary (with CL).

Table 1: Follicular population in relation to slaughter house ovarian status

Ovarian status	Total no. of ovaries	Total no. of follicles	Follicle recovered per ovary Mean (95% CI)
Type-I (CL-)	119	631	5.3[a] (4.56-6.33)
Type-II (CL+)	105	343	3.27[b] (2.82-3.89)
Total	**224**	**974**	

[a, b] Means bearing different superscripts in a column differ significantly (P<0.01).

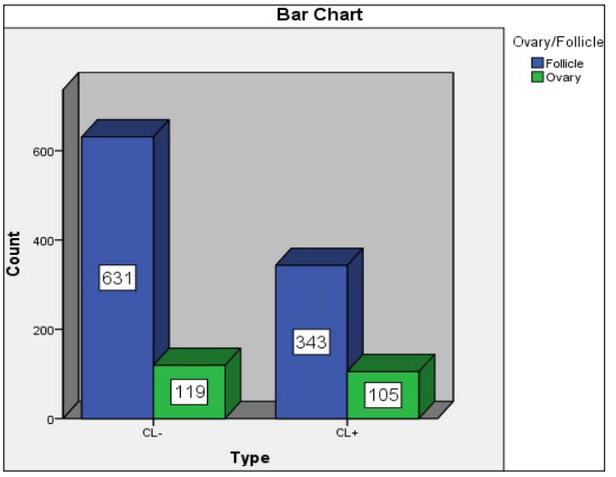

Fig. 1: Follicular dimension in Type-I and Type- II ovaries

From the experiment it revealed that follicles recovered per ovary in both type-I and type-II ovaries differed significantly (P<0.01) showing strong correlation, in which type-I ovary (5.3 nos. follicles per ovary) had 1.62 times higher chances to occur than that of follicle of type-II (3.2 nos. follicles per ovary). The ovaries having no corpus luteum usually obtaining from non-cyclic cows were slaughtered for economic reason. Commonly, poor reproductive perform cows were slaughtered and therefore higher chance to get more ovaries (without CL) from the slaughter house. Similar results were also reported in cattle (Bhajoni *et al*., 2018) and buffalo (Mahesh *et al*., 2014; Rajesh *et al*., 2018). On the other hand, the findings of the present experiment related to higher numbers of visible follicles on the surface of ovary without corpus luteum were in close agreement with the earlier findings (Ahmed *et al.*, 2015), who reported higher number of follicles aspirated per ovary without CL as compared to ovary with CL. Besides, higher numbers of follicles observed in ovaries without CL than ovaries with CL showed correlation with the endocrinological aspects. The presence of corpus luteum in cyclic ovaries causes increase progesterone hormone production giving a negative response to anterior pituitary gland for the restriction of gonadotrophin secretion and leads to follicular degeneration and inhibition of the development of large follicles (Khandoker *et al.*, 2016). In non cyclic female, the absence of corpus luteum causes no negative effect of progesterone on anterior pituitary and thus estrogen-progesterone level remains balanced which allows the growth of follicles.

Different sizes of follicles (<3 mm, Small), (>3 mm-6 mm, Medium) and (>6 mm - <10 mm, Large) in type- I and type- II ovaries observed in the present experiment are presented in Table 2 and Fig. 2.

Table 2: Effect of ovarian status (Type-1 and Type-II) on follicular diameter % (Mean ± SE)

Ovarian Status	No of different sizes of follicles			Total
	Small, <3mm	Medium (>3-6 mm)	Large (> 6mm<10)	
Type-I (CL-)	193 (56.27[a]±1.83)	297 (67.96[a]±1.99)	141 (72.68[a]±1.98)	631
Type-II (CL+)	150 (43.73[b]±2.23)	140 (32.04[b]+2.29)	53 (27.32[b]+2.10)	343
Total	**343**	**437**	**194**	**974**

[a, b] Means bearing different superscripts in a column differ significantly (P<0.01).

The mean percentages (Mean ± SE) of small, medium and large size follicles in type-I ovary were recorded as 56.27 ± 1.83, 67.96 ± 1.99 and 72.68 ± 1.98 respectively which was significantly higher (P<0.01) than the values recorded

in type-II ovary. From the present experiment it revealed that both types of ovaries (type-I and type-II) differed significantly (P<0.01) in respect of diameter of follicles and the number of small, medium and large size follicles.

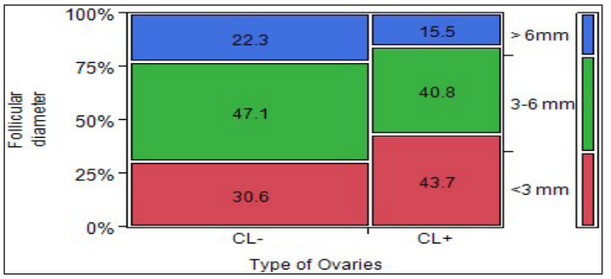

Fig. 2: Recovery of cumulus of oocyte complexes (COCS)

Generally, corpus luteum acts as an extra cellular material within the ovary having a definite span of its growth, maintenance and regression. The more numbers of follicles in the ovary without CL might be an indicative of higher level of gonadotropins and steroid activity in it (Asad *et al*., 2016). Basically, CL containing ovaries have lesser number of follicles which may be attributed to the negative effect of CL on follicular growth (Khandoker *et al.*, 2016). Similarly, Bhajoni *et al.* (2018) also reported highest (6.32 ± 0.75 *vs* 3.33 ± 0.18) number of medium size follicles in type-I ovary as compared to type-II ovary.

Recovery of different cumulus oocyte complexes (COCs) from Type-I and Type-II ovaries by aspiration method

The numbers of cumulus oocyte complexes recovered from both type-I and type-II ovaries by aspiration method are presented in Table 3. A total of 406 and 175 cumulus oocyte complexes (COCs) were aspirated out from 119 (type-I) and 105 (type-II) nos. ovaries. The COCs recovered per ovary were 3.41 (95% CI: 2.96-4.02) and 1.66 (95% CI: 1.40-1.90) respectively in type- I and type-II ovaries. However, from the present experiment, it was observed that the presence of CL on ovary adversely affected the total oocytes yield when compared with ovaries without CL in aspiration. Cumulus oocyte complexes per ovary varied significantly (P<0.01) with respect to type of ovary. The recovery rates of COCs in case of type-I ovary (3.41) (95% CI: 2.96-4.02) were significantly higher (P<0.01) as compared to type-II ovary (1.67) (95% CI: 1.49-1.90). Non luteal phase without CL yielded significantly higher (P<0.01) numbers of oocytes when compared to luteal phase (with CL).

Table 3: Recovery of different cumulus oocyte complexes (COCs) from bovine ovaries with or without corpus luteum by aspiration method

Ovarian status	Total nos. of ovaries	Total nos. of COCs recovered	COCs recovered per ovary (95% CI)
Type-I	119	406	3 .41[a] (2.96-4.02)
Type-II	105	175	1.66[b] (1.49-1.90)

[a, b] Means bearing different superscripts in a column differ significantly (P<0.01).

Similarly, Rajesh *et al.* (2018) recorded 1.78 and 3.63 as mean oocyte recovery rate per ovary whereas 1.35 from ovaries with CL as compared to 1.97 from the ovaries without CL. Their findings reflected pronounced effect of the ovarian status (presence or absence of CL) on oocyte recovery rate indicating a negative effect of CL on oocyte recovery rate in bovine. However, the sublimed reasons for such differences attributed to the reproductive status of the donor animal from which the oocytes were recovered, season of recovery along with the procedure applied, environmental effect, number of ovaries processed and the method of selecting ovaries from the slaughter house (Ahmed *et al.*, 2015; Khandoker *et al.*, 2016). The decreased recovery rate of bovine oocytes in the presence of CL due to restricted follicular development as the luteal cells of CL occupy most of the portion of the ovary (Asad, 2015). Similarly, Mahesh *et al.* (2014) also reported that the functional structure on the ovaries such as CL and follicular size affects the recovery rate of oocytes in buffaloes. On the contrary, Naby *et al.* (2013) recorded higher rate of bovine oocyte from the ovaries with CL as compared to ovaries without CL. However, the present findings showed that non-luteal phase (without CL) ovaries yielded significantly (P<0.01) higher numbers of oocytes when compared to luteal phase (with CL) ovaries. Although the effect of presence or absence of CL was reflected in respect of numbers of oocytes recovered but no significant differences were observed in *in vitro* maturation of culturable oocytes in type-I and type-II

Table 4: Effect of ovarian status on recovery of different grades of oocytes

Ovary	Oocytes Grade % (Mean ±SE)								Total
	Grade A		Grade B		Grade C		Grade D		
	No.	%	No.	%	No.	%	No.	%	
Type-I (n=119)	107	26.35^{NS} ±.2.91	116	28.57^{NS} ±2.24	101	24.88^{NS} ±2.15	82	20.20^{NS} ±1.99	406
Type-II (n=105)	49	28.00^{NS} ±3.39	65	37.14^{NS} ±3.65	33	18.86^{NS} ±2.96	28	16.00^{NS} ±2.77	175
	156		181		134		110		581

^{NS}Non-significant.

ovaries which might be due to the fact that progesterone secreted by the luteal cells of the CL inhibited estrus and gave the negative feedback on the anterior pituitary to secret follicle stimulating hormone (FSH). As a result, the growing follicles regressed and became atretic and lead to lower oocyte recovery from CL containing ovaries.

The numbers and mean percentages of different grades of oocytes recovered in type-I and type-II ovaries are presented in Table 4. The numbers and mean percentages of Grade A, B, C and D oocytes recovered in type-I ovary were 107 (26.35 ± 2.19), 116 (28.57 ± 2.24), 101 (24.88 ± 2.15) and 82 (20.20 ± 1.99) respectively. Similarly, the corresponding values for type-II ovary were also recorded as 49 (28.00 ± 3.39), 65 (37.14 ± 3.65), 33 (18.86 ± 2.96) and 28 (16.00 ± 2.77) respectively.

The recovery percentages of Grade A and Grade B oocytes (culturable oocytes) were found to be higher than Grade C and Grade D oocytes in both type-I and type-II ovaries (223 culturable oocytes in type-I ovary out of 119 nos. ovaries and 114 culturable oocytes in type II ovary out of 105 nos. ovaries) and different grades of oocytes A, B, C, D were insignificantly associated with types of ovaries (without and with CL). However, the proportions of different grades of oocytes were similar in both types of ovaries (without and with CL). From the experiment, it was also evident that different grades of COCs (A, B, C, D) associated non-significantly with types of ovary. The discrepancy in the recovery rate of different types of oocytes by aspiration techniques might be due to presence of CL, size of ovary, status of reproductive cycle and season of the study (Ahmed *et al.*, 2015; Kang *et al.*, 2019).

CONCLUSION

The numbers of follicles recovered per type-I ovary was 5.30 (4.56-6.33) which was significantly higher (P<0.01) than corresponding values 3.27 (2.82-3.89) of type-II ovary. The numbers and mean percentages of A, B, C and D grades of the oocytes were recovered as 107 (26.35 ± 2.19), 116 (28.57 ± 2.24), 101 (24.88 ± 2.15) and 82 (20.20 ± 1.99) respectively in type-I ovary. Similarly, for type-II ovary, corresponding values were recorded as 49 (28.00 ± 3.39), 65 (37.14 ± 3.65), 33 (18.86 ± 2.96) and 28 (16.00 ± 2.77) respectively. Generally higher numbers of ovaries were found without corpus luteum compared to ovaries with corpus luteum showing that non-cyclic cows are slaughtered in the slaughterhouse due to economic reason. Ovaries without CL contributed more numbers of follicles as compared to ovaries with CL by aspiration method. Moreover, comparatively higher numbers of total COCs and superior quality of COCs (A and B grades) were obtained from ovaries without CL, indicated to be suitable for collecting COCs for initiating *in vitro* embryo production experiment in cow. In the same way, aspiration was found to be the best efficient technique than other techniques for COCs collection and therefore, this technique seems to be more beneficial as less tissue debris come out. From the present experiment, it can be inferred that the numbers of follicles were significantly higher (P<0.01) in the ovaries without CL as compared to the ovaries with CL.

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