



Effect of ovarian types and collection techniques on the number of follicles and the quality of cumulus-oocyte-complexes in cow

Khandoker MAMY*, NF Atiqah and N Ariani

Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Beg Berkunci No. 3, 90509 Sandakan, Sabah, Malaysia

Abstract

This experiment was conducted for evaluation of bovine slaughterhouse ovary, follicles, and cumulus-oocyte-complexes (COCs) and to compare the effect of collection techniques on the recovery rate of COCs. The collected slaughterhouse ovaries was classified as corpus luteum present (CL+) and corpus luteum absent (CL-) groups. It was found that 62.5% of the ovaries collected were CL- type and 37.5% were CL+ type. For collection of COCs, blunt dissection and aspiration techniques were performed and number of follicles collected was recorded. The higher number of follicles were dissected and aspirated from CL - ovaries (11.2 ± 1.8 and 37.8 ± 14.9 , respectively) compared to CL+ ovaries 10.5 ± 1.5 and 28.3 ± 15.6 , respectively). The follicular materials collected from both techniques were observed under microscope to categorize the COCs as A (oocyte surrounded with cumulous cells homogenously), B (oocyte surrounded with cumulous cells partially), C (oocyte not surrounded at all by cumulous cells) and D (degeneration observed both in oocyte and cumulous cells). Grade A and grade B were classified as normal and grade C and grade D were considered as abnormal COCs. The result indicated that ovaries having no CL contributing more total number of COCs per ovary (6.8 ± 1.0) and also contributing higher normal COCs (5.7 ± 0.9) than that of ovaries with CL (6.0 ± 2.0 and 4.5 ± 1.5 , respectively) in blunt dissection technique. But same trend of result was not found in aspiration technique. Similarly, higher percentage of COCs recovery rate was also recorded in blunt dissection ($61.6 \pm 4.6\%$ vs $16.5 \pm 4.9\%$, on total basis) than aspiration ($48.6 \pm 2.9\%$ vs $11.7 \pm 4.1\%$, on normal basis) technique. So, ovaries without CL and blunt dissection technique found more suitable for harvesting the higher number and superior quality of COCs for extending the *in vitro* embryo production experiment.

Key words: slaughterhouse ovary, corpus luteum, blunt dissection, aspiration

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Introduction

The cow is usually produces a single ovum during the period of ovulation. If the ovum is fertilized *in vivo*, a cow only delivers a calf after an average of nine months of gestation (Hafez, 1993) and this situation causes slow genetic improvement. Hence, over a decade, there were a lot of researches done towards the implementation of embryo technologies to fasten the genetic manipulation of livestock which involves multiple ovulation and embryo transfer (MOET), *in vitro* embryo production (IVEP), cloning, and transgenesis (Asad, 2015; Sahaet *al.*, 2014; Sreenivaset *al.*, 2014; Freitas and Melo, 2010; Moadalet *al.*, 2008; Hyttelet *al.*, 1997; Wang *et al.*, 2007; Sianturi, 2001; Sirad and Blondin, 1996).

From those mentioned, IVEP has becoming more popular method of producing embryos from slaughter house derived ovaries (Hoqueet *al.*, 2011). The IVEP system involves at least four steps, namely (i) evaluation of ovaries, efficient collection and grading of oocytes; (ii) *in vitro* maturation (IVM) of these oocytes; (iii) *in vitro* fertilization (IVF) of the matured oocytes; and (iv) *in vitro* culture (IVC) of the resulting embryos (Freitas and Melo, 2010). Nowadays, IVEP is becoming a useful tool for maximizing the number of offspring from valuable cows, producing calves from infertile and slaughtered cows, and producing commercial beef cattle production program without brood cows (Sianturi, 2001; Hyttel *et al.*, 1997; Sirad and Blondin, 1996). In Malaysia, very limited research so far has been done on *in vitro* production of embryos

*Corresponding author: mamyahiak@ums.edu.my

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from slaughterhouse derived cattle oocytes (Sianturi, 2001).

To produce embryos by *in vitro* techniques, it is necessary to recover the oocytes and undergo maturation of oocytes, fertilize, and develop those developing zygotes to blastocyst stage so that they can be transferred to the recipient (Hoque *et al.*, 2012). COCs contains the oocytes and in recent years, the percentage of oocytes reaching the blastocyst stage by *in vitro* techniques still varies. In some cases, the low development of IVM oocytes is related to their quality at the beginning of maturation. Mondal (2008) has reported that higher average number of good quality oocytes was recovered from ovaries without corpus luteum compared to the ovaries with corpus luteum, which thus, can be effectively used for IVM and IVF. In addition, it has been shown that oocytes with at least four layers of cumulus cells have good result for IVM and IVF (Yang *et al.*, 1993).

There is a huge gap between the demand and supply of milk and meat in Malaysia (Jamaludin *et al.*, 2014) due to small cattle population and low productivity. In Malaysia, there is still no information so far has been found on evaluation of bovine slaughterhouse ovaries, follicles, and cumulus-oocyte-complexes (COCs) for IVEP. Moreover, in Sabah, it is really difficult to get remarkable number of slaughterhouse ovaries to collect COCs for initiating the embryo production experiment as rarely slaughtered cow in this area. So, for successful IVEP experiment in cow for the evaluation of ovaries it is important to find out the efficient collection techniques of COCs. Therefore, present study was performed as an initial work for planning and execution of future pragmatic research on *in vitro* production of bovine embryos to increase cattle productivity in Sabah as well as in Malaysia. The main purpose of this research is to determine the optimum type of ovary (corpus luteum present or absent) and COCs recovery technique that is most suitable to produce higher number of good quality COCs for IVEP experiment.

Materials and Methods

The experiment was conducted at the Anatomy and Physiology Laboratory, Faculty of Sustainable Agriculture, Universiti Malaysia Sabah (UMS)

Sandakan Campus from July 2015 to September 2015.

Collection, processing and evaluation of ovaries

Cow's ovaries with unknown reproductive history were collected from Hafiz Farm Slaughterhouse at Lahad Datu, Sabah and put in falcon tube containing 0.9% physiological saline that was warmed at 25-30°C and kept in a polystyrene box to maintain this temperature during transporting the ovaries (Mondalet *et al.*, 2008). The ovaries were transported to the laboratory within 4-5 hours of slaughter. The ovaries were then transferred to sterilized Petri dish and rinsed thoroughly by physiological saline at 25°C. Each ovary was trimmed to remove the surrounding tissues and overlying bursa. The ovaries were then observed and categorized as corpus luteum absent (CL-) and corpus luteum present (CL+) groups (Figure 1) and the number of both types of ovaries were recorded (Mondalet *et al.*, 2008). The follicles on the surface of the ovary for dissection or aspiration were counted and data was recorded.

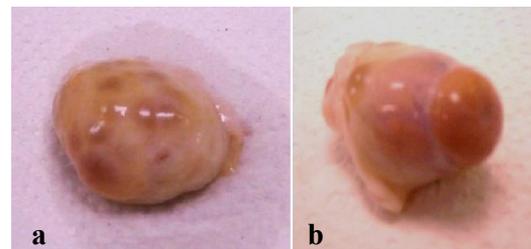


Figure 1. Ovaries without corpus luteum (a) and ovaries with corpus luteum (b)

Collection and grading of cumulus-oocyte-complexes (COCs)

Two collection techniques were applied in this study which was blunt dissection and aspiration techniques. For blunt dissection technique, individual follicles were dissected from the ovaries by using scissors and forceps {Figure 2 (a)} and the number was recorded. Then, the follicular material was harvested individually in the Petri dish. A small amount of physiological saline solution (0.9% NaCl) with 5% bovine serum albumin (BSA) was added on the slides with follicular materials collected, followed by observing under microscope to grade the COCs.

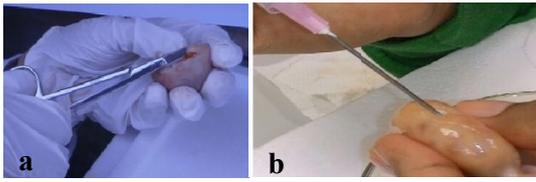


Figure 2. Representative photograph showing blunt dissection (a) and aspiration (b) techniques

For aspiration technique, 10-ml syringe was loaded with 0.9% physiological solution (1.0 to 1.5 ml), and 18G needle was put in the ovarian parenchyma near the vesicular follicles and all visible follicles in the ovarian surface were aspirated{Figure 2 (b)}. After that, the aspirated follicular materials were transferred slowly into a falcon tube to avoid damage of the cumulus cells and the numbers of follicles aspirated were also recorded.

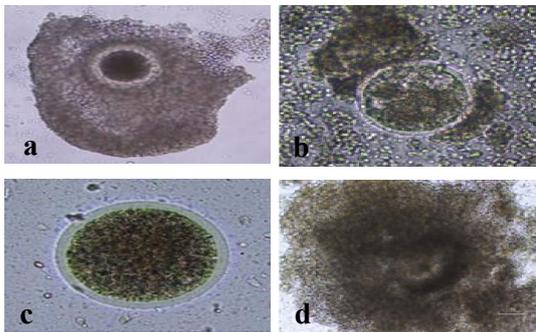


Figure 3. Grading of collected COCs; (a) Grade-A; (b) Grade-B; (c) Grade-C and (d) Grade-D

Statistical analysis

The data collected was compiled, tabulated and analysed in accordance with the objectives and descriptive statistics was performed with the help of Microsoft Office Excel 2007.

Table 1. Types of ovary and number of follicles and cumulus-oocyte-complexes per ovary using blunt dissection technique*

Ovarian type	Total number of dissectible follicle/ ovary (mean ± SE)	Follicle dissected/ ovary (mean ± SE)	Collected COCs per ovary (mean ± SE)		
			Normal	Abnormal	Total
Ovary without CL (6)	15.5±2.7 (93)	11.2±1.8 (67)	5.7±0.9 (34)	1.2±0.5 (7)	6.8±1.0 (41)
Ovary with CL (2)	11.0±2.0 (22)	10.5±1.5 (21)	4.5±1.5 (9)	1.5±0.5 (3)	6.0±2.0 (12)

*Parentheses indicates total numbers

Results

Quantitative evaluation of bovine slaughterhouse ovaries

Bovine ovaries were collected from local slaughterhouse and were classified into two groups which were with corpus luteum (CL+) and without corpus luteum (CL-). Among 16 ovaries collected, 10 were found as CL- ovaries and 6 as CL+. It was found that 62.5% of the ovaries collected were CL- type and 37.5% were CL+.

Quantitative and qualitative evaluation of follicles and COCs using blunt dissection and aspiration techniques

Blunt dissection technique

The results for number of dissectible follicles on the surface of the ovary, the number of follicles dissected and the number of COCs collected is summarized in Table 1. Total visible dissectible follicles recorded on the surface of both types of ovaries were 115. Among them 93 were recorded in 6 without corpus luteum ovaries and 22 in 2 with corpus luteum ovaries. On an average, 15.5±2.7 dissectible follicles per ovary were found in CL- group and 11.0±2.0 dissectible follicles per ovary were found in CL+ group. From Table 1, it shows that a total of 88 follicles were dissected from both types of ovaries and among them 67 obtained from 6 CL- ovaries and 21 from 2 CL+ ovaries. Based on the mean value, it is shown that number of follicles dissected from CL- was higher (11.2±1.8) than that of CL+ (10.5±1.5) ovaries. Similarly, the higher number of total COCs per ovary was collected from CL- group (6.8±1.0) than in CL+ group (6.0±2.0).

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Then, when the COCs were classified into normal and abnormal, higher number of normal (grade A and B) COCs were found in CL- group ovaries (5.7 ± 0.9) than that of CL+ group ovaries (4.5 ± 1.5) while reverse trend of result was found in abnormal with respective mean of 1.2 and 1.5 COCs per ovary (Table 1).

Aspiration technique

An alternative aspiration technique was also adopted in this experiment for collecting of COCs and the results are summarized in Table 2. In this technique, total aspirable follicles observed in both types of ovaries were 323 from 8 ovaries and among them 180 was found in 4 CL- group ovaries and 143 in 4 CL+ group ovaries. Again average of 45.0 ± 14.3 follicles per ovary was recorded in CL- group and 35.8 ± 14.4 follicles per ovary were recorded in CL+ group. Table 2 further shows that higher number of follicles aspirated per ovary in CL- group (37.8 ± 14.9) than that of CL+ group (28.3 ± 15.6). On the other hand, a little higher number of total COCs was collected from CL+ group (5.3 ± 2.3) than that of CL- group (4.5 ± 1.4). Similarly, higher number of normal COCs (4.8 ± 2.0) per ovary was obtained from CL+ ovaries than that of CL- ovaries (2.8 ± 1.1). Both findings are unusual and similar unusual result also found in abnormal COCs, with the mean of COCs per ovary is 0.5 in CL+ ovary and 1.8 in CL- ovary (Table 3).

Comparison of blunt dissection and aspiration techniques

To compare the blunt dissection and aspiration techniques, the results of COCs recovery rate was calculated according the formula of Abdel-Khalehet *al.* (2010) and the result is summarized

in Table 3. From Table 3, it is clear that among the total COCs recovery ($61.6 \pm 4.6\%$), $48.6 \pm 2.9\%$ normal COCs recovery rate were found higher in blunt dissection technique compared to aspiration technique ($16.5 \pm 4.9\%$ and $11.7 \pm 4.1\%$ total and normal, respectively). When comparing these two methods on the basis of abnormal COCs recovery rate, again higher recovery rate of COCs was recorded in blunt dissection technique (13.0 ± 3.4) than that of aspiration technique ($4.8 \pm 2.6\%$).

Discussion

Evaluation of bovine slaughterhouse ovaries

From the observation of this study, it was shown that among the 16 ovaries collected from slaughterhouse, 10 ovaries were obtained as without corpus luteum (62.5%) and 6 ovaries were obtained as with corpus luteum (37.5%). The numbers of ovaries having no corpus luteum usually obtaining from non-cyclic cows were slaughtered for economic reason. Commonly, less reproductive performing cows were slaughtered and cause high possibility to get more CL- ovaries from the slaughterhouse during sample collection.

The same trend of result was also reported in buffaloes by Khandoker *et al.* (2011) where among 136 ovaries, 93 were found without CL and the remaining 43 ovaries with CL. Similar results also reported in goat (Asad, 2015; Saha *et al.*, 2014; Mondalet *al.*, 2008 and Islam *et al.* (2007). Those findings further supported the statement that for economic reason, typically less reproductive performing cows are slaughtered in slaughterhouse and most of them might be non-

Table 2. Types of ovary and number of follicles and cumulus-oocyte-complexes per ovary using aspiration technique

Ovarian type	Total number of aspirable follicle (mean \pm SE)	Number of follicle aspirated (mean \pm SE)	Collected COCs per ovary (mean \pm SE)		
			Normal	Abnormal	Total
Ovary without CL (4)	45.0 ± 14.3 (180)	37.8 ± 14.9 (151)	2.8 ± 1.1 (11)	1.8 ± 1.4 (7)	4.5 ± 1.4 (18)
Ovary with CL (4)	35.8 ± 14.5 (143)	28.3 ± 15.6 (113)	4.8 ± 2.0 (19)	0.5 ± 0.5 (2)	5.3 ± 2.3 (21)

cyclic. Commonly, the older or finisher non-cyclic animals were brought for slaughtering in the slaughterhouse.

Quantitative evaluation of bovine ovarian follicles

The results obtained from this study shows that ovaries having no corpus luteum contributing larger number of follicles for dissection (15.5 ± 2.7) and aspiration (45.0 ± 14.3) than that of the ovaries with corpus luteum for dissection (11.0 ± 2.0) and aspiration (35.8 ± 14.5). The results further indicated that higher number of follicles were able to dissected and aspirated from ovaries without CL (11.2 ± 1.8 and 37.8 ± 14.9 , respectively) than that of ovaries with CL (10.5 ± 1.5 and 28.3 ± 15.6 , respectively) (Table 2 and 3). The higher number of follicles found in without CL ovaries than those of with CL ovaries fits with the endocrinological explanation. The presence of corpus luteum in cyclic ovary cause a higher level of progesterone hormone production in which 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 (Webb *et al.* 1999). In noncyclic female, the absence of corpus luteum cause no negative effect of progesterone on anterior pituitary and thus estrogen-progesterone levels remains balanced which allows the growth of follicles. Ginther *et al.*, (1996) stated that ovaries without CL, the decrease in progesterone leads to increase in GnRH which stimulates the release of follicle stimulating hormone (FSH) and this hormone causes the rapid growth of ovarian follicles.

The results strongly supported by the previous finding of Asad (2015), who reported that higher number of follicles aspirated per ovary in without CL group (2.92 ± 0.08) than those of the with CL group (2.52 ± 0.11) in goat. Similar findings also found in buffalo ovaries by Khandokeret *al.* (2011) where significantly higher number of follicles were collected in ovaries without CL (6.78 ± 0.18) than in CL containing ovaries (4.09 ± 0.26).

Quantitative and qualitative evaluation of COCs

Both the number and quality of the COCs are important to initiate the embryo culture experiment and the results of the present study clearly indicated that ovaries without corpus luteum contributing higher number of total COCs per ovary (6.8 ± 1.0) than that of with corpus luteum group ovaries (6.0 ± 2.0) at least in blunt dissection technique (Table 2).

From this study, it was also found that more normal (A and B grade) COCs was dissected from ovaries without corpus luteum (5.7 ± 0.9) compared to ovaries with corpus luteum (4.5 ± 1.5) (Table 2). Nandi *et al.* (2000) stated that when ovaries had a corpus luteum, the oocyte recovery rate decreases. This is because there will be restriction of follicular development as lutein cells occupy most of the ovary (Kumar *et al.*, 2004). Hafez (1993) mentioned that in the presence of CL in ovary, the growth of follicle is inhibited while atresia is increased. These statements can be the physiological explanation for lower number of COCs in the with CL ovaries compared to without CL ovaries. Our finding further supported by other researchers, they have done their research in goat (Asad, 2015; Khandokeret *al.*, 2011; Mondalet *al.*, 2008 and Islam *et al.*, 2007).

Similarly, the higher number of normal COCs in CL absent group ovaries may be due to the hormonal effect of CL. When CL is absent in the ovary, progesterone which has role in follicular degeneration could not be produced (Hafez, 1993). Thus, folliculogenesis can occur successfully and further there is more chance to produce high quality of COCs. Therefore, the types of ovary at the time of COCs collection have affected the quantity and quality of COCs recovered as well as usable oocytes in animals for use in IVEP program. A bit exception result, in respect of total COCs aspirated per ovary was found higher in CL+ group ovaries (5.3 ± 2.3) than that of CL- group of ovaries (4.8 ± 2.0) though the total number of aspirable follicles and follicles aspirated were recorded higher in CL- group ovaries (45.0 ± 14.3 vs 37.8 ± 14.9) than that of CL+ group of ovaries (35.8 ± 14.5 vs 28.3 ± 15.6). This finding further suggested that to collect COCs by aspiration technique, efficiency of the

technician is the prerequisite factor and also reflects the superiority of blunt dissection technique once more again.

Table 3. COCs recovery rate in aspiration and blunt dissection techniques*

Collection technique	Recovery rate of COCs (RR %) (mean \pm SE)		
	Normal	Abnormal	Total
Blunt dissection (8)	48.6 \pm 2.9	13.0 \pm 3.4	61.6 \pm 4.6
Aspiration (8)	11.7 \pm 4.1	4.8 \pm 2.6	16.5 \pm 4.9

*Parentheses indicates total numbers

Comparison of blunt dissection and aspiration techniques

Various techniques can be used to collect the oocytes from the ovaries and the number of oocytes recovered can be influenced by the technique used to harvest the oocytes. From the result obtained in this study, when comparing the techniques of collecting the COCs, blunt dissection technique was found more efficient than that of aspiration technique on the harvesting of higher number of COCs (61.6 \pm 4.6% vs 16.5 \pm 4.9%). Blunt dissection technique also recovered higher rate of normal quality (A and B grade) of COCs (48.6 \pm 2.9%) than that of aspiration technique (11.7 \pm 4.1%). The statements are in agreement with similar study done in goat by Singh *et al.* (2013) where the number of oocytes retrieved was significantly higher in dissection method (2.52 \pm 0.28) than aspiration (1.35 \pm 0.18). Less number of COCs retrieved by aspiration technique might be because aspiration disrupts cumulus cells or damages the whole oocytes (Nowshari, 2004). Shiraziet *al.* (2005) also reported high number of denuded oocytes recovered through aspiration.

It is observed that aspiration technique requiring less time on harvesting the COCs compared to blunt dissection technique. However, blunt dissection technique is found more efficient for COCs retrieval with higher number and better quality of recovered COCs. Blunt dissection technique seems to be more practical in a place like Sabah where slaughterhouse ovaries getting is really difficult. This is because when comparison of follicles collected and COCs recovery from each ovary is evaluated (recovery

rate of COCs), it was found that, in blunt dissection technique, the COCs recovery was averagely more than 50% than that of the aspiration technique, the COCs recovered was found not even one-third of follicles aspirated. Since more than half of the total follicles could contribute COCs, it shows that there is high possibility to get more number of COCs by blunt dissection technique although the number of sample is less. Besides that, from the results obtained, it can be seen that blunt dissection technique does not require expertise and needful skills compared to aspiration technique. Anyone can perform blunt dissection technique to collect COCs though the person is inexperienced in handling such process. Thus, by comparing the techniques, blunt dissection technique was found to be more efficient than that of the aspiration technique in terms of number as well as good quality of COCs collection.

Conclusion

From the results, it can be concluded that comparatively higher number of ovaries were found without corpus luteum compared to ovaries with corpus luteum showing that usually noncyclic cows are slaughtered in the slaughterhouse due to economic reason. Ovaries without CL contributing larger number of follicles dissected and aspirated per ovary compared to ovaries with CL. Furthermore, comparatively higher number of total COCs and superior quality of COCs (A and B grade) were possible to obtain from without CL ovaries, suggested to be suitable for collecting COCs for initiating *in vitro* embryo production experiment in cow. On the other hand, blunt dissection technique was found to be more efficient than aspiration technique for COCs collection and this technique seems to be more practical in the place where getting slaughterhouse ovaries is difficult.

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