THE FOLLICLE CHARACTERISTIC AND IMMATURE OOCYTE QUALITY OBTAINED FROM REPEATED TRANSVAGINAL OOCYTE RETRIEVAL IN *Bos indicus* BEEF COWS

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ABSTRACT. The development and availability of follicles is an indicator to predict which of the follicle sizes are suitable to recover the oocytes assisted by means of ultrasonography of transvaginal oocyte retrieval (OPU). Thus, the study was done in order to characterize the follicular recruitment and distribution in response to the repeated removal of follicles, and thus to determine the availability of follicles and immature oocytes harvested repeatedly for two consecutive days of OPU in beef cows. Results indicated that 24-h OPU showed significantly greater numbers of medium and large follicles than small categories ($P<0.05$). However the 48-hr of OPU does not showed any differences of follicles categories ($P>0.05$). The mean total number of follicles and immature oocytes recovered were higher ($P<0.05$) in 24-hr OPU (13.76±1.2 and 7.38 ± 1.7) compared to 48-hr OPU (9.08 ± 1.5 and 3.54 ± 1.00) with the oocyte retrieval rate of 51.22% and 38.17%, respectively. The morphological classification indicated the 24-hr oocyte retrieval produced 62% of suitable immature oocytes that can be used for *in vitro* embryo production. In conclusion, the repeated removal of two consecutive days of OPU has averted the development of dominant follicle, and thus, gave an atmosphere to the subordinate follicles to continue growth relatively to an equal proportion of small, medium and large categories of follicles. Due to the reduction of follicle and recovery rate at 48-hr it is suggested that OPU be carried out later than 48 hour so that the follicle has more time to increase the diameter size.

Keywords: follicle development, immature oocytes, transvaginal oocyte retrieval (OPU), follicle stimulating hormone (FSH), oocyte quality, *in vitro* embryo production,

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INTRODUCTION

Ultrasound-guided technology has emerged to impale the small and preovulatory follicles in cattle (Pieterse *et al*., 1989; Pieterse *et al*., 1991)**.** This technology offers a new reproductive approach in harvesting oocytes from live cattle (Callesen *et al*., 1987; Pieterse *et al*., 1988: 1991; Kruip *et al*., 1991) which is so called transvaginal ultrasound-guided oocyte retrieval (OPU).

OPU is an alternative procedure to domestic females which are genetically valuable but unable to produce viable embryos through the natural procedures. The technology can also be used on oocytes harvested from older ovulating or nonovulating cows, or cows with physical injuries or having an abnormal cervix.

Oocytes from live donor cows are an important source of known quality genetic material for use in *in vitro* production of embryos repeatedly (Akshey *et al*., 2004; Velez *et al*., 2012). The *in vitro* embryo production is efficient in order to exploit the female gamete as steps to shorten the generation interval in a modern animal husbandry. Many studies have been done to understand the development of the follicles in order to predict the availability of follicle sizes which is suitable for oocyte harvesting assisted by OPU.

The cattle used for OPU can be either hormone stimulated or non-stimulated (Pieterse *et al*., 1989; Pieterse *et al*., 1992; Walton, 1993; Bungartz *et al*., 1995), clinically infertile animals (Looney *et al*., 1994) or from young prepubertal heifers and early postpartum cow. Thus, the technology is an aid of getting oocytes for *in vitro* embryo production and can also be used for germplasm preservation of endangered exotic species.

Oocytes from live donors are usually used in *in vitro* embryo production or for *in vitro* culture system. The oocytes retrieved could differ in their growth phase, cell morphology, age and the stage of atresia corresponding to the follicle population on ovary stroma. The size of follicles plays a role in OPU because it mirrors the activity of the follicles and only oocytes of befitting size could be impaled, and aspirated through OPU technique. The morphological classification of oocyte is associated with oocyte quality and the meiotic competence of the oocytes. Thus, it is beneficial to observe the relationship between the distribution of the follicles and the retrieval rate of immature oocytes in a two consecutive days of OPU in order to identify if OPU could be done repeatedly.

Therefore, the aim of this study was to characterize follicular recruitment and distribution in response to the repeated removal of follicles, and thus, to observe the availability and quality of immature oocyte retrieved repeatedly in two consecutive days of OPU in beef cows.

MATERIALS AND METHODS

Animal Selection And Management

A total of 13 Kedah-Kelantan crossbred beef cows were selected from a breeding

herd managed at MARDI Research Centre, Kluang, Johor. The cows ranged from 3 to 4 year in age and body weight from 250kg to 350 kg with an average Body Condition Score of 4.5 kg (scale 1: severely thin, 4: moderate, 8: obese) adapted with slight modification of the method described by Richard *et al*. (1986). The cows were assigned into two treatments: two consecutive; 24 (24-h) and 48 (48-h) of repeated OPU after removal of controlled internal drug releasing device (CIDR; Pfizer New Zealand Ltd).

During the study, the cows were managed in a semi-intensive system, whereby cows were released for grazing in the morning and maintained indoor for the rest of the day in individual pens and fed with cattle pellets and had free excess to water. The cattle pellets contained 15.0% crude protein and 17.6 megajoule calculated gross energy (GE) were fed to cows based on the maintenance requirement of beef cows (ARC, 1980) at a rate of 1 kg per 100 kg body weight per day in addition to the estimated intake of feed from grasses.

Oestrous Synchronisation And Hormonal Treatment

The cows were on feed adaptation period for 14 days before oestrous synchronization. All cows at random stages of oestrous cycle were inserted intravaginally with CIDR containing 1.38 g progesterone for 7 d, followed with an intramuscular injection of 500 µg cloprostenol of prostaglandin synthetic analogue ($PGF_{2\alpha}$; Estrumate Schering-Plough Animal Health, Australia) at day 5. Superovulation was done using a total of 17 ml follicle stimulating hormone (FSH; Ovagen®, InterAg, New Zealand) and was administered twice a day at decreasing doses for four consecutive days (3.5 ml x 2, 2.5 ml x2, 1.5 ml x 2, and 1 ml x 2), beginning on day 3 post CIDR insertion.

Ultrasonography Of Follicle Development Study

Both ovaries were scanned using a 5.0 MHz mechanical annular array transrectal (AAS) transducer (Pie Medical, Maastricht, Netherlands) attached to an ultrasonographic scanner (Pie Medical Scanner 250+, Maastricht, Netherlands). The transducer lubricated with an aseptic gel was inserted into the vagina. The cows were given 2 ml of an epidural anaesthesia injection of 2% lignocaine (Troy Laboratories, Limited, Australia) before the transducer insertion into the vagina.

The transducer was adhered along the wall of the vagina in order to visualize the follicles situated on the surface of ovary medulla, through an ultrasound monitor. The image of the ovary was frozen, and the diameter of follicles was measured using an electronic caliper of the ultrasound device. The diameter of a follicle of unequal shape was measured twice and the average was taken as the diameter of the follicle. Then, the diameter of follicles was categorized

into small (≤ 4 mm), medium (> 4.1 mm – 8.0 mm) and large (≥ 8.1 mm) follicles.

Preparation Of The Cows

The cows were administered a local anaesthesia of 3-5 ml of 2% lignocaine (Troy Laboratories, Limited, Australia) at the upper epidural region in order to prevent abdominal straining and give better manipulation of the ovaries during the palpation. The cow was restrained to the head crushed on the standing positioned. The perineum then was cleaned with hibiscrub before the guidance and probe insertion into the vagina.

Oocyte Retrieval And Selection

The OPU was conducted immediately after measuring the follicle's diameter. A guidance system was incorporated into the OPU device alongside the transducer of an ultrasonographic scanner (Pie Medical Scanner 250 plus, Maastricht, The Netherlands) in order to visualize the ovaries images. A 19 G 19G x 2" 1.1 × 50 mm luer type disposable, single lumen needle (Terumo Neolus, Belgium) was connected to a stainless steel connector for oocyte aspiration. The silicone tubing was attached to the stainless steel connector and passed through along the stainless steel tube. Then it was placed alongside a mechanical annular array sector (AAS) multi-angle dual frequency transducer (Pie Medical, Maastricht, The Netherlands) in a stainless steel casing with a holder with a diameter of 45 mm as described by Bols *et al.* (1995).

The transducer in a stainless steel casing was lubricated and inserted into the vagina through the perineum. During aspiration, the ovaries were pushed against the head of transducer and a needle in stainless steel casing was pushed through the vaginal wall and impaled into the follicle to be punctured. The aspiration was done by means of a footstep connected to a vacuum pump and collecting bottle by a silicone coated tubing that was controlled by the foot of the technician.

One follicle was impaled and aspirated each time. When the aspiration completed, the needle was withdrawn and flushed together with the suction set using complete flushing medium (Vigro®, AB Technology Inc., Pullman, USA) consisting of phosphate buffer saline (PBS) with 10% heparin, serum and antibiotic. The procedure was discontinued when follicle size ≥ 4 mm was not visible on the ultrasound monitor. The collecting bottle containing oocytes then was brought to the laboratory for oocyte examination and evaluation.

The collected fluid containing immature oocytes was poured into several 60 mm petri dishes. Subsequently all oocytes were examined and separated into three morphological classification under a stereomicroscope. The morphological classification of oocyte quality based on cumulus-oocyte complex (COC) followed the method described by Wurth and Kriup (1992), that is, "A" for presence of a clear

and compact cumulus and translucent ooplasm, "B" for dark and compact cumulus and dark ooplasm, and "C" for dark and expanded cumulus and dark ooplasm.

Statistical Analysis

Data were analysed using independent t-test of SPSS version 21. The three categories of follicles of each period of 24-h and 48-h were analysed with one-way analysis of variance (ANOVA), where as data of follicle numbers, follicle sizes categories, and oocyte quality in a 24-h and 48-h of two consecutive days of OPU were analysed using ANOVA for a repeated measure. A confidence level of 95% or more was considered to be statistically significant.

RESULTS AND DISCUSSION

The ultrasonography for follicle categories showed the mean of both medium and large follicles observed to be higher in 24 hours compared to 48 hours OPU ($p<0.05$) (Table 1). However, both groups did not show any difference in proportion of follicles of small diameter ($p>0.05$). In this study, more oocytes can be retrieved from medium and large follicles on 24-h and 48-h repeated OPU.

The retrieval rate of oocyte was 51.22% and 38.17% in 24-h and 48-h of repeated OPU, respectively (Figure 1). It shows the retrieval rate of oocytes that has been impaled in two consecutive days of OPU was 13% higher in 24-h compared to 48-h OPU. Similarly, the mean total number of follicles in 24-h OPU was approximately 34.01% higher ($P<0.05$) compared to 48-h OPU (Figure 1). Subsequently, the morphological quality classification of oocytes indicated that 24-h oocyte retrieval presented higher number of immature oocytes, which might be suitable candidates for *in vitro* embryo production (*in vitro* culture system: *in vitro* maturation, fertilization and culture) technologies.

Table 1. The number of small, medium and large follicles (mean ± SEM) following ultrasanography of two consecutive days of transvaginal oocyte retrieval (OPU) in beef cows

Period of oocyte retrieval[1]	Mean no. of follicles observed[2]	No. of follicles			Mean no. of oocytes retrieved[3]
		Small (≤ 4 mm)	Medium (>4 – 8 mm)	Large (≥ 8.1 mm)	
24-h	13.76±1.2 ^{x}	2.23±0.5 $^{a\,x}$	4.84±0.7 $^{b\,x}$	5.69±0.7 $^{b,\,x}$	7.38±1.69 ^{x}
48-h	9.08±1.5 ^{y}	1.62±0.6 $^{a\,x}$	2.15±0.5 $^{a\,y}$	3.31±0.$^{8\,a,\,y}$	3.54±1.0 ^{y}

1 Period of oocyte retrieval: 24-h = 24 h after CIDR removal; 48-h = 48 h after CIDR removal
2 Mean of follicle observed = Number of follicle/Number of cattle
3 Mean of oocyte retrieval = Number of oocyte recover/Number of animals
a,b Values within rows with different superscripts are significantly different at $p<0.05$
x,y Values within with columns different superscripts are significantly different ($p<0.05$)

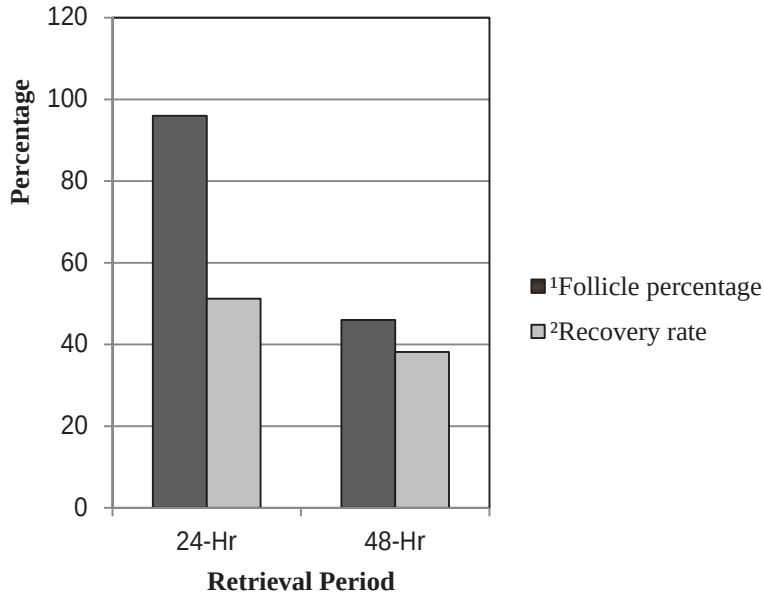

Figure 1. The rate at which follicles are available and oocytes are retrieved in two consecutive days of transvaginal oocyte retrieval (OPU).

1 Follicle number percentage = Total number of follicles observed (24 or 48-Hr) / Grand total number of follicles x 100

2 Retrieval rate = Number of oocyte retrieved / Total number of follicles x 100

The results showed that more immature oocytes could be selected as candidates for *in vitro* embryo production technologies. The study demonstrated that OPU technology is an appropriate tool that can be used to exploit female reproductive capacity although they are differing in oestrous cycle phases.

Oocytes from live donors are a good source in order to obtain quality and valuable genetic materials for *in vitro* embryo production technologies. Thus, the present study is a pioneering effort in getting fresh good quality oocytes from live donors that can be offered in large numbers. In the study, it was shown that the follicles developed in an equal proportion of small, medium and large sizes at 24-h OPU. Similarly, 48-h OPU gave equal proportions of small, medium and large sized follicles.

In cattle, antral follicles of 3-8 mm in diameter are the main suppliers of oocytes used for *in vitro* embryo production by OPU (Joel *et al.*, 2002). In a follicular development, the follicles develop in a wave of 4 to 12 follicles at a time, and a cow usually has two to four waves of these growing follicles in one oestrous cycle.

A follicle wave involved the synchronous development of a group of follicles from a pool of growing follicles (Driancourt, 1991). The largest follicle which is gonadotrophin-dependent

from this pool is then recruited into a population which grows continuously, and hence inhibits the other surrounding or subordinate follicles from growing and thus undergo atresia.

However, in OPU cattle, the retrieval opposes the development of dominant follicle and this gives a space for the subordinate follicle to grow and increase in size. Thus in this study, enhancing the availability of a relatively homogeneous and equal proportions of small, medium and large follicles allows the suitable harvesting of oocytes at 48-h OPU.

Although the OPU could be done in two consecutive days, the availability of mean number of follicles suitable for retrieval, and number of oocytes recovered on 48-h OPU reduced approximately 30% to 50% of actual total number of follicles available and oocyte retrieval of 24-h OPU. This could probably be due to the duration of OPU that was too short and the time lapse was insufficient to recruit the development of new follicles, and unable to increase the diameter size.

Although the two consecutive days of OPU increased the retrieval rate from 51.22% to 89.39% (Figure 1), it produced

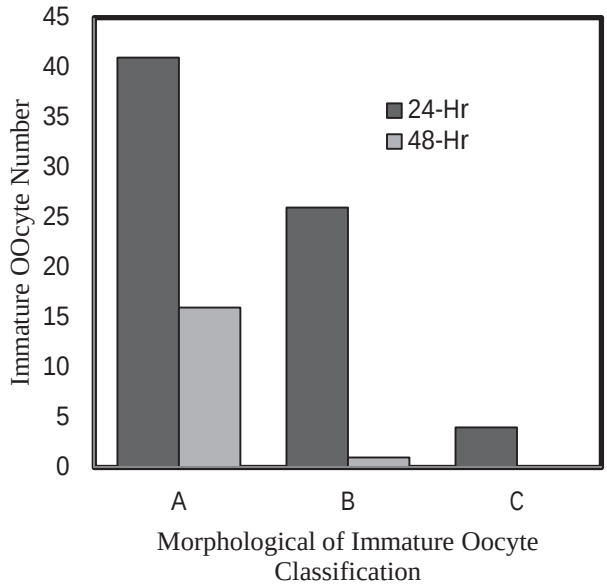

Figure 2. The morphological of immature oocyte classification (mean ± SEM) following ultrasonography in two consecutives days of transvaginal oocyte retrieval (OPU) in beef cattle. The morphological classification of oocyte was followed as described by Wurth and Kriup (1992); (A) presence of a clear and compact cumulus and translucent ooplasm, (B) dark and compact cumulus and dark ooplasm, and (C) dark and expanded cumulus and dark ooplasm.

an erroneous effect on animal behaviour. Therefore, the repeated OPU of two consecutive days of the present study was determined to be not suitable. It could be suggested to extend the time of oocyte retrieval from 48-h to 72-h or 96-h interval in order to conduct OPU.

The other factors that contributed to the lower number of oocyte retrieved during the 48-h OPU was the degree of calmness and stage of passiveness of the animals. This could probably be related to their experience during the 24-h OPU which led to the animals' discomfort and uneasiness, and difficulty in handling, even though they were treated with local anaesthesia. Thus, the condition led to the reduced retrieval rate of the immature oocytes. The two consecutive days of OPU would only showed a beneficial effect for 24-h OPU and increased the degree of discomfort and the animals tended to struggle in 48-h OPU.

The number of follicles developing within each ovary did not differ. The repeated OPU on two consecutive days did not increase further the total number of follicles. It only resulted in a decrease in number of medium and large follicles ($p<0.05$). The results of our investigation also demonstrated that 62% of the oocytes recovered from individual animals via OPU were suitable for use in *in vitro* embryo production (Figure 2).

The availability of follicles and immature oocytes is higher at 24-h than 48-h of repeated OPU. Due to the reduction in number of follicles and the recovery rate in 48-h OPU, it is suggested that OPU be carried out later than 48 hours so that the follicle has more time to increase in size. Further studies should be carried out to examine the ultra structure quality in order to determine the stages of meiosis of those immature oocytes aspirated from these animals for *in vitro* embryo production.

CONCLUSION

The retrieval rate obtained in the present study described an application of the technology under field conditions. Repeated retrieval at short intervals is possible but it may produce uncertainty in animal behaviour. However, the aspirated immature oocytes obtained are viable and could potentially be used for *in vitro* embryo production technology. In addition, the immature oocytes can be obtained from cows irrespective of the differences in oestrous phases.

Repeated removal for two consecutive days of OPU has altered the follicular recruitment and distribution by avoiding the development of dominant follicles. Thus, this condition gave an atmosphere to the subordinate follicles to continue to grow in an equal proportion of small, medium and large sized follicles. However, the lower percentage of oocytes that can be used in the *in vitro* culture system upon OPU suggests further research.

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