

Effect of Collection Technique on Yield of Bovine Oocytes and The Development Potential of Oocytes from Different Grades of Oocytes

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ABSTRAK

SIANTURI, R.G., M.THEIN, H. WAHID dan Y. ROSNINA. 2002. Pengaruh teknik koleksi oosit sapi terhadap hasil panen oosit dan potensi perkembangan oosit dari kualitas oosit yang berbeda. *JITV* 7(3): 188-193.

Teknik koleksi oosit penting dalam menghasilkan jumlah oosit yang optimal yang dapat digunakan dalam produksi embrio secara *in vitro*. Dalam penelitian ini, oosit sapi yang didapat dari ovaria yang diperoleh dari RPH dikoleksi dengan teknik aspirasi dari folikel-folikel berukuran 2-6 mm dan teknik pengirisan. Setelah dikoleksi, kualitas oosit-ooisit diklasifikasikan dalam 4 kategori (A, B, C dan D) berdasarkan keberadaan lapisan sel-sel kumulus yang mengelilingi oosit. Oosit-ooisit dari tiap kategori dimatangkan secara *in vitro* dalam inkubator CO₂ selama 22-24 jam dan persentase ekspansi sel-sel kumulus dan pematangan oosit diobservasi. Jumlah total oosit (group A+B+C+D) dan jumlah oosit kualitas baik (hanya group A dan B) yang terkoleksi per ovarium dengan teknik aspirasi adalah 12,02 dan 8,21 dan dengan teknik pengirisan adalah 29,38 dan 19,65 (P<0,01). Persentase total ekspansi dari sel-sel kumulus dari oosit A, B, C dan D adalah 97,1%, 88,3%, 6,0% dan 20,6% berturut-turut. Persentase pematangan dari oosit-ooisit A, B dan C adalah 91,4%, 82,3% dan 35,0% dimana tidak ditemukan oosit yang matang pada oosit group D. Persentase pematangan berbeda nyata di antara oosit group A dan C dan juga di antara group B dan C (P<0,05). Dapat disimpulkan, bahwa koleksi oosit dengan teknik pengirisan menghasilkan 2,4 kali lebih banyak oosit per ovarium dibandingkan dengan teknik aspirasi. Persentase pematangan oosit tertinggi didapat dari oosit group A yang dilapisi lebih dari 3 lapisan sel-sel kumulus. Oosit dengan kategori A dan B dapat diklasifikasikan sebagai oosit kualitas baik yang dapat digunakan dalam produksi embrio sapi secara *in vitro*.

Kata kunci: Koleksi oosit, aspirasi, pengirisan, kualitas oosit, pematangan

ABSTRACT

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Oocyte collection technique is important to obtain a maximum number of oocytes to be employed on *in vitro* production of embryos. In this study, immature bovine oocytes were collected from slaughterhouse ovaries by two techniques: aspiration of 2- to 6-mm follicles and slicing. Following collection, oocyte qualities were classified into four categories (A, B, C, and D) on the basis of cumulus attachment. Oocytes of each category were matured *in vitro* in CO₂ incubator for 22-24 hours and cumulus expansion and maturation rates were observed. The total number of oocytes (group A+B+C+D) and yield of good quality oocytes (only group A and B) recovered per ovary by aspiration were 12.02 and 8.21, and by slicing were 29.38 and 19.65 (P<0.01), respectively. The total cumulus cells expansion rates of A, B, C and D oocytes were 97.1%, 88.3%, 6.0% and 20.6% respectively. Maturation rates for A, B and C categories of oocytes were 91.4%, 82.3% and 35.0% respectively while no matured oocyte was observed for group D oocytes. Maturation rates were significantly different between group A and C and also between B and C but not between A and B (P<0.05). In conclusion, slicing technique recovered more oocytes per ovary (2.4 times) than that of aspiration and the best maturation rate was observed from category A oocytes which surrounded by more than 3 layers of cumulus cells. However oocytes of category A and B can be considered as good quality oocytes.

Key words: Collection technique, aspiration, slicing, oocyte quality, maturation

INTRODUCTION

The process of *in vitro* embryo production begins with harvesting oocytes from donor's ovaries either of live animals or slaughtered animals. To date, the most common source of oocytes is ovaries collected from slaughterhouses. Ovaries obtained from the slaughterhouse constitute an economical source of

oocytes. This allows for large scale and economical production of embryos. However, the quality of these oocytes is highly variable (GANDOLFI *et al.*, 1997).

The quantity and quality of oocytes which can be retrieved from a given number of ovaries are therefore important factors. Methods to obtain immature oocytes from ovaries of slaughtered cows are aspiration, dissection and slicing (CAROLAN *et al.*, 1994).

Recovery of oocytes by aspiration of vesicular follicles, using an appropriate syringe and needle, has been the most commonly employed in terms of speed of operation. In comparison, it is possible to recover oocytes by follicular dissection, but this method is three times slower than by aspiration. However, the slicing or dissecting method is reported to yield a better oocyte recovery rate than aspiration. It has been reported in bovine (CAROLAN *et al.*, 1994), sheep (WAHID, 1993) and goat (MARTINO *et al.*, 1994) that slicing of ovaries followed by washing with oocytes collection medium were effective in obtaining a higher number of immature oocytes.

The ability to identify good quality oocytes is of considerable importance. The appearance of an oocyte and its cumulus cells investment have been used to estimate or assess the developmental potential of the oocyte, i.e. the ability of an oocyte to undergo normal maturation, fertilization and development to the blastocyst stage. Many reports have indicated that classification of bovine oocytes, based on visual assessment of the compactness and morphology of the cumulus, can be used to select immature oocytes most capable of maturation, fertilization and cleavage *in vitro*. Oocytes surrounded by a tight and complete multi-layered cumulus investment and containing an ooplasm with a sandy appearance are most likely to be developmentally competent (YOUNIS *et al.*, 1991). When immature oocytes were classified according to the number of layers of cumulus left around the oocytes following aspiration, the thicker the cumulus the better is the chance for development (LONERGAN, 1992). The number of quality grades has varied from 3 (MADISON *et al.*, 1992), or 5 (LONERGAN, 1992) to as high as 9 (HAZELEGER and STUBBINGS, 1992).

The aims of this study were 1) to compare the efficiency of aspiration and slicing methods in terms of the availability of oocytes quantity for each category and the total number of oocytes obtained per ovary, and 2) to determine the maturation potential of each category of oocytes.

MATERIALS AND METHODS

Collection of ovaries

Ovaries were collected from the cows at the local slaughterhouse. The ovaries were trimmed free of fatty and hial tissues, and pooled in a thermos flask containing phosphate buffered saline (PBS) supplemented with penicillin-streptomycin (penicillin 100 IU/ml and streptomycin 100 µg/ml; Gibco, Grand Island, USA) at 33 to 35°C. The ovaries were collected regardless of any phase of oestrous cycle and pregnancy and transported to the laboratory within 3 hours of slaughter. At laboratory, the ovaries were cleaned to

remove blood, rinsed three times in warm PBS and immediately processed.

Aspiration

Ovaries were placed in a beaker containing warm PBS at 30 to 35°C. The ovary was rolled on the sterile paper towel to dry the surface and to remove the attached blood. Ovarian follicles of 2- to 6-mm in diameter were aspirated with a 10-ml disposable syringe and a 18-gauge needle primed with 1.5 ml of warm TALP Hepes (BAVISTER *et al.*, 1983) supplemented with bovine serum albumin 3 mg/ml (BSA, A-8022, Sigma) and gentamicin 50 µg/ml (G-1264, Sigma). After all visible follicles were threaded and aspirated, the contents in the syringe were slowly poured into a conical tube and kept in the incubator and the oocytes were allowed to settle down for a few minutes. The sediment was taken transferred into a 90-mm petri dish and added with TALP Hepes. The entire process was undertaken in a sterile environment in a laminar flow cabinet in a warm room at 29-30°C. After searching under 10-40X stereo microscope, the oocytes were pooled into a 35-mm petri dish and washed three times in TALP Hepes.

Slicing

The corpora lutea were excised and large follicles (greater than 6 mm in diameter) were ruptured to prevent gelling of the follicular contents. The ovary was placed in a 90-mm petri dish containing 8-10 ml of warm TALP Hepes. The ovary was firmly held using dissecting forceps, cut into two halves starting from the stalk and laid flat cut surface facing downwards. Then the ovarian surface was sliced with a surgical blade to recover the oocytes from follicles. When slicing had been completed, the sliced ovary was thoroughly rinsed with warm TALP Hepes. The ovarian contents were allowed to settle down and then oocytes were searched under a stereomicroscope.

In vitro maturation

After 3 washes with TALP Hepes, categorized oocytes were washed again 2 times in a 35-mm dish containing maturation medium under mineral oil (M-8410, Sigma) and transferred into a 4-well dish (Nunc) containing 500 µl of maturation medium under mineral oil. The maturation medium consisted of M199 (Gibco), 10% FBS (Gibco), 0.02 unit/ml ovine FSH (Embryo S, Jurox, NSW, Australia), 1 µg/ml oestradiol (β-oestradiol, Sigma), 0.2 mM sodium pyruvate (Sigma) and 50 µg/ml gentamicin sulphate (Sigma). The oocytes were incubated in 5% CO₂ in air with maximum humidity at 39°C for 22-24 hours.

Evaluation of oocyte maturation

Oocyte maturation was determined by cumulus expansion and nuclear stage.

Evaluation of cumulus expansion

Following *in vitro* maturation, the oocytes were examined for evidence of cumulus mass expansion before being processed and evaluated to determine the stage of nuclear maturation. Cumulus mass expansion was assessed and recorded according to the classification scheme of HUNTER and MOOR (1987).

Evaluation of oocyte nuclear stage

At the end of maturation, oocytes were transferred into a 1.5-ml microcentrifuge tube containing 100 μ l of 3% sodium citrate solution. The oocytes were freed from cumulus cells by high-speed vortexing for 5 minutes. The cumulus-free oocytes were fixed in a 35-mm dish containing aceto-ethanol solution. The dish was sealed with a piece of paraffin film and stored at 5°C for 24 h. For staining, 5 to 10 of fixed oocytes were transferred onto a clean glass slide with 4 spots of wax-vaseline mixture at the periphery to fix the four corners of the cover-slip. The oocytes were stained by passing through 1% aceto-lacmoid stain from one end of the cover-slip and blot-dried at the opposite end with a tissue paper. When the staining was completed, the nuclear materials of the oocytes were examined under a phase-contrast microscope (Leitz Wetzlar, Germany) at 400X magnification. Expulsion of the first polar body and second metaphase (MII) chromosomal arrangement were taken as evidence of nuclear maturation. Maturation rate was calculated as the percentage of metaphase II-oocytes.

Experimental design

Experiment 1: Effect of collection techniques on yield of bovine oocytes

The efficacy of two oocyte collection methods, aspiration and slicing, was compared in terms of the availability of good quality oocytes and total number of oocytes obtained per ovary. A total of 180 ovaries were used for aspiration in 14 replicates, and 33 ovaries were used for slicing in 8 replicates. All oocytes recovered

were classified into 4 categories (A, B, C and D) on the basis of their cumulus cells attachment (SIANTURI, 2001). The number of oocytes per each category and the total number of oocytes recovered per ovary were compared between the two collection methods.

Experiment 2: Effect of oocyte quality on maturation rate *in vitro*

The aim of this experiment was to determine the maturation potential of each category of oocytes classified on the basis of cumulus attachments. In three replicates, a total of 536 oocytes were classified into four categories (A, B, C and D) using the classification as mentioned in experiment 1. Each group of oocytes was matured in IVM 22-24 h at 39°C in humidified air and 5% CO₂. At the end of IVM, the cumulus cells expansion of each oocyte from all categories was assessed as full, moderate, slight or no expansion.

To evaluate the nuclear maturation, only 100 out of the total *in vitro* matured oocytes (A=37; B=20; C=23; D=20) were freed from cumulus cells, fixed with aceto-ethanol for 24 h and stained with 1% aceto-lacmoid. The nuclear stage of oocytes was examined under a phase-contrast microscope. The maturation rates of oocytes of all categories were recorded and analysed.

Statistical analysis

Statistical analyses were conducted by means of Student's t-test and Chi-square analysis, as appropriate (STEEL and TORRIE, 1980).

RESULT AND DISCUSSION

Experiment 1

The number and the proportion in each category of oocytes per ovary, which were obtained by aspiration and slicing techniques are presented in Table 1. Significantly, more oocytes were recovered per ovary by slicing technique than that of by aspiration (29.38 vs 12.02 oocytes/ovary; $P < 0.01$). Proportion of oocytes (shown in brackets) in each category was not significantly different except for category D ($P > 0.01$; 7.58% vs 14.22% of total oocytes/ovary for aspiration and slicing respectively).

Table 1. Effect of different collection methods on the recovery rate and type of bovine follicular oocytes

Method of collection	Total no. of ovaries	Mean of oocytes/ovary \pm SEM (%)				Mean total no. of oocytes/ovary \pm SEM (%)
		A	B	C	D	
Aspiration	180	3.34 \pm 0.47 ^a (27.71)	4.87 \pm 0.53 ^a (40.66)	2.86 \pm 0.37 ^a (24.05)	0.89 \pm 0.11 ^a (7.58)	12.02 \pm 0.85 ^a (100)
Slicing	33	9.82 \pm 1.71 ^b (33.68)	9.83 \pm 1.35 ^b (34.02)	5.40 \pm 0.96 ^b (18.08)	4.32 \pm 0.94 ^b (14.22)	29.38 \pm 4.68 ^b (100)

A,B,C,D : Categories of A, B, C and D oocytes

Values in the same column with different superscripts differ significantly at $P < 0.01$

Percentage of acceptable oocytes (A and B oocytes) of total oocytes, obtained from aspiration (27.71% and 40.66%) and slicing (33.8% and 34.02%) were not significantly different.

The number of high quality oocytes recovered per ovary is an important consideration in the *in vitro* production of embryos. Therefore, to obtain a larger number of good quality oocytes, it is necessary to choose the appropriate recovery method under certain conditions. The recovery rate of bovine oocytes per ovary was significantly higher by slicing than by aspiration and this finding was in agreement with earlier studies (LONERGAN, 1992). One reason for the high recovery rate of oocytes by slicing is the harvest of oocytes from small diameter follicles.

Although the percentages of A, B and C categories of oocytes collected did not differ significantly between the two collection methods, a slightly lower percentage of group A oocytes and higher percentage of groups B and C oocytes were obtained by aspiration. This might be because aspiration of the follicles causes a partial loss of cumulus cells due to excessive pressure exerted by a syringe (BOLS *et al.*, 1997). MARTINO *et al.* (1994) stated that the integrity of the cumulus remained intact by slicing than by aspiration, but a large number of degenerated and small diameter oocytes were harvested. Likewise, the percentage of D group oocytes was significantly higher by slicing than by aspiration in this study.

Normally, half the number of total oocytes is considered as acceptable oocytes (A plus B oocytes) (HAMANO and KUWAYAMA, 1993). In our study, the percentages of good quality of oocytes (68.37% and 67.70%) are higher than which norm but in agreement with some other reports (XU *et al.*, 1992). However, if

the oocytes with heterogeneous ooplasm from A and B oocytes were discarded as in the scheme suggested by YOUNIS *et al.* (1991), the percentages found in this study might decrease.

Experiment 2

As shown in Table 2, the total cumulus expansion rates of A, B, C and D oocytes were 97.1%, 88.3%, 6.0% and 20.6% respectively. There was no significant difference on the total of cumulus expansion rates between groups A and B oocytes, but there was significantly higher rate ($P<0.05$) on the full cumulus expansion of oocytes than in B oocytes (85.4% vs 37.2%).

Out of 100 oocytes (A=37; B=20; C=23; D=20) stained, only 85 oocytes (A=35; B=17; C=20; D=13) were available for assessment of maturation. Some oocytes were lost during processing and some were unidentifiable. Group A had the highest maturation rate (91.4%) and followed by group B (82.3%) but this was not statistically significant.

Highest maturation rate (91.4%) was obtained from group A with maximum full expansion. Total cumulus expansion rates were not different significantly between group A and B oocytes but full expansion rates between them differed significantly ($P<0.05$). However, maturation rates did not differ significantly between A and B ($P<0.05$). Thirty five percent of group C oocytes had second metaphase chromosome plate but oocytes from group D had no evidence of second metaphase plate. Group D oocytes had been already expanded before *in vitro* maturation so that expansion score was not applicable and not recorded.

Table 2. Cumulus expansion and maturation rates of oocytes recovered in the four different categories

Category of oocytes	Cumulus expanded oocytes n (%)					Matured oocytes n (%)	
	No. oocytes cultured	Full	Moderate	Slight	Total expanded	No. oocytes stained	Matured n (%)
A	103	88 (85.4) ^a	10 (9.8)	2 (1.9)	100 (97.1) ^a	35	32 (91.4) ^a
B	231	86 (37.2) ^b	51 (22.1)	67 (29.0)	204 (88.3) ^a	17	14 (82.3) ^a
C	134	1 (0.8) ^c	2 (1.5)	5 (3.7)	8 (6.0) ^b	20	7 (35.0) ^b
D	68	7 (10.3) ^d	4 (5.9)	3 (4.4)	14 (20.6) ^c	13	0 (0) ^c

Data were pooled from 3 replicates

Values with different superscripts in the same column are significantly different ($P<0.05$; Chi-square)

Proper oocyte selection in the laboratory is crucial for successful embryo production *in vitro*. The presence of an intact complement of cumulus cells surrounding the oocytes and a homogenous-appearing ooplasm are the best indicators of the ability of immature oocytes to undergo maturation and thus embryonic development (YOUNIS *et al.*, 1991; DE LOOS *et al.*, 1991). In the present study, the classification of oocytes was based on the investment of the cumulus cells surrounding the oocyte. The maturation rates of A and B category of oocytes were 91.4% and 82.3% respectively and there was no significant difference between them. The results were similar to the studies of SHIOYA *et al.* (1988) and KONISHI *et al.* (1996) who found no significant difference on the maturation rate among >5, 3-4 and 1-2 layers of cumulus cells. In this study, significantly better maturation rates were obtained from cumulus oocyte complexes (A and B oocytes) than denuded or partially denuded (C oocytes). The incidence of nuclear maturation was reduced in cumulus-free oocytes. Therefore, at least a layer of cumulus cells is required to promote normal nuclear maturation of IVM oocytes. Since no maturation was noted in D category, the oocytes in D may be considered as degenerated oocytes.

CONCLUSION

The slicing method recovered more acceptable oocytes (2.4 times) and more total number of oocytes (2.4 times) per ovary than aspiration. The proportion of acceptable oocytes (A and B oocytes) from the two recovery methods were not much different. Slicing technique should be employed in *in vitro* production of embryos in the circumstances where the availability of bovine oocytes is limited and when the genetically valuable animal expires.

The competence of maturation of A and B oocytes were much higher than C oocytes and no maturation was observed in D oocytes. This suggests that only oocytes from groups A and B can be used for IVP of embryos to achieve good results.

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