Asian-Aust. J. Anim. Sci.
Vol. 20, No. 4 : 496 - 500
April 2007

www.ajas.info

Effects of Collection Methods on Recovery Efficiency, Maturation Rate and Subsequent Embryonic Developmental Competence of Oocytes in Holstein Cow*

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ABSTRACT : Holstein cow ovaries obtained at a slaughterhouse were used to study the influence of the oocyte collection methods (slicing, puncture, aspiration I and II) on recovery efficiency and subsequent *in vitro* maturation and embryonic development competence of immature oocytes recovered. In the slicing method, the whole ovarian was chopped into small pieces with a surgical blade. In the puncture method, the whole ovarian surface was punctured by 18-g needle. In other 2 aspiration methods, collected oocytes by aspirating from the visible follicles using an 18-g needle attached to a 5 ml syringe (aspiration I) or using a constant negetive pressure (-80 mmHg) with a vacuum pump (aspiration II). The oocytes were classified into 4 classes on the basis of the morphology of cumulus cells and cytoplasmic appearance of oocyte. Slicing (9.6±0.4) and puncture (9.7±0.4) yielded a larger number of oocytes per ovary than other two aspiration methods (aspiration I and II were 5.8±0.3 and 5.6±0.4, respectively) ($p<0.05$). The number of the highest quality oocytes (grade A) per ovary was significantly higher in slicing (4.2±0.2) and puncture (4.6±0.1) methods than in other methods (aspiration I and II were 1.2±0.2 and 1.4±0.2, respectively) ($p<0.05$). The rate of nuclear maturation of the highest and higher quality oocytes (grade A and grade B, respectively) was not affected by the oocytes collection methods. The oocytes collection methods also did not influence subsequent embryonic developmental competence after *in vitro* fertilization with M II stage oocytes. It is concluded that slicing and puncture methods of the ovaries can be used as an alternative techniques to aspiration by the syringe or vacuum pump. (**Key Words :** Collection Methods, Holstein Cow, Recovery Efficiency, Oocyte Maturation Rate, Embryonic Developmental Competence)

INTRODUCTION

Development and application of assisted-reproduction technologies like *in vitro* embryo production through *in vitro* maturation, fertilization and culture of oocytes, production of cloned and/or transgenic cattle, establishment of oocyte banks, etc. can be expected to bring about a significant increase in the population of superior genetic merit cattle (Manik et al., 2003). *In vitro* production embryo technology (IVP) has been successfully applied in a number of animal species with transferred embryos resulting in live offspring (Gordon, 2003). The availability of enough number of oocytes is the pre-requisite to any investigation for the development and optimization of reproduction techniques. Ovaries of the slaughtered animals are the cheapest and the most abundant source of primary oocytes for large scale production of embryos through *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) (Agrawal et al., 1995). However, the majorities of Holstein cows slaughtered are either old, unproductive and/or unhealthy, and have reproductive diseases. The development of an efficient oocyte collection procedure is, therefore, very significant for cows. An important aim of an oocyte recovery method is to maximize the total number of oocytes and the yield of high-quality oocytes (evenly granulated cytoplasm surrounded by several layers of compact cumulus cells) recovered per ovary, which can be used for IVM, IVF, and *in vitro* culture (IVC) (Shirazi et al., 2005). However, no enough reports evaluating the relative efficacy of the methods have been published in the Holstein cows. The present experiment was, therefore, aimed at recording the effects of collection techniques on the quantity and quality of oocytes recovered per ovary, *in vitro* maturation,

* Supported by Zhejiang Science and Technology Committee (Grant Agreement No: 2004C12037).
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Received March 20, 2006; Accepted June 20, 2006

in vitro fertilization, and subsequent embryonic development.

MATERIALS AND METHODS

Reagents and media

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. All reagents were cell culture-tested quality. M199 with L-glutamine (GIBCO, Carlsbad, CA, USA) , supplemented with 10% (v/v) fetal calf serum (FCS), 5 mM sodium lactate, 26 mM sodium bicarbonate, 1 IU/ml pFSH, 1 IU/ml hCG, 1 μg/ml 17 β-estradiol, 50 IU/ml penicillin, and 50 μg /ml streptomycin was used to oocyte maturation prior to IVF. The embryo culture medium consisted of modified synthetic oviductal fluid medium (mSOF) supplemented with 0.5 mM citrate and 1×essential and non-essential amino acids (GIBCO) (Keskintepe et al., 1995; Keskintepe and Brackett, 1996).

Oocytes recovery

Bovine ovaries were obtained from the local slaughterhouse and transported to the laboratory within 2 h in a 0.9% (w/v) NaCl solution containing 100,000 IU/L penicillin, 100 mg/L streptomycin, and 250 mg/L amphotericin B. At the laboratory, the ovaries were washed three times in saline. Each ovary was individually processed and harvested by one of the following techniques according to methods by Das et al. (1996). 1) In slicing method: Ovaries were placed in a graded plastic Petri dish containing saline and were chopped into small pieces with a surgical blade. The sliced stromal tissues were discarded. 2) In puncture method: Ovaries were stabilized in a Petri dish with forceps. Follicles, which were visible on the surface ranging from 2.0 to 6.0 mm in diameter, were submerged in saline and punctured with an 18-g needle. Follicular fluid was encouraged to escape with gentle pressure on the adjacent stroma of the punctured follicle. 3) In aspiration I method: Follicular fluid from surface follicles (2.0 to 6.0 mm in diameter) was aspirated through a sterile 18-g needle attached to a 5 ml syringe containing sterile saline. Aspirated contents were expelled into a fresh Petri dish containing saline. 4) In aspiration II method: Oocytes were aspirated by applying a constant negative pressure (–80 mmHg) with a vacuum pump (Cook Australia, Brisbane, Australia). The aspiration needle was connected through PVC tubing into a sterile 50 ml plastic tube (Falcon, Franklin Lakes, NJ, USA). During aspiration, a rotatory movement within the follicle was used to dislodge the cumulus-oocyte complex (COC), at times, adhered to the follicular wall. In all 4 techniques, the Petri dish was kept undisturbed for 5 min allowing oocytes to settle.

Experiment 1: Effects of collection methods on quantity and quality of oocytes

To evaluate effects of 4 collection methods on quantity and quality of oocytes recovered per ovary, the oocytes recovered by 4 techniques were examined under an inverted microscope and the numbers of oocytes harvested were counted. Accroding to Chauhan et al. (1998) and de Wit and Kruip (2001), the oocytes were classified as follows: Grade A: COCs with an unexpanded cumulus mass having at least 5 layers of cumulus cells, and with homogenous cytoplasm. Grade B: COCs with 2-4 layers of cumulus cells, and with homogenous cytoplasm. Grade C: Oocytes partially denuded of cumulus cells and/or with irregular shrunken cytoplasm. Grade D: Oocytes completely denuded of cumulus cells and/or with irregular shrunken cytoplasm. The number of 4 grades oocytes obtained was recorded for each ovary.

Experiment 2: Effect of collection methods on *in vitro* maturation of oocytes

In this experiment, a number of two grades COCs (grades A and B) in experiment 1 were washed 3 times with the IVM medium, matured in 50 μl droplets of the IVM medium, covered with paraffin oil in 35 mm Petri dish for 22 h in a CO_2 incubator (5% CO_2 in air) at 38.5°C. COCs evaluated as grade C and D were discarded, because of their lower *in vitro* embryonice development competence after IVF compared with those of other grades (Bilodeau-Goeseels and Panich, 2002). At the end of maturation period, COCs were removed from the maturation medium and denuded of cumulus cells mechanically as well as by treatment with 1 mg/ml hyaluranidase solution. The proportions of oocytes in metaphase-II stage (M II, oocytes with a visible polar body) were determined from the viable oocytes.

Experiment 3: Effect of collection methods on subsequent embryonic developmental competence

To study on subsequent embryonic developmental competence derived from the oocytes obtaining by 4 different collection methods, COCs classified into grade A and grade B were matured *in vitro* as same as in experiment 2, and were fertilized *in vitro* according to subsequent description. The COCs were washed twice in warm Hepes-buffered Tyrode's albumin lactate pyruvate solution (TALP, Bavister et al., 1983) and once in fertilization medium (a bicarbonate-buffered modified TALP, Parrish et al., 1986) and then placed in 50 μl droplets approximately (10 to 12 COCs per droplet) of fertilization medium containing 10 μg/ml of heparin. Moreover, 20.0 μM penicillamine, 10.0 μM hypotaurine, and 2.0 μM epinephrine were added into fertilization medium according to Miller et al. (1994). Frozen bull semen was thawed and prepared by a swim-up

Table 1. Effects of 4 different collection methods on the quantity and quality of oocytes in Holstein cows

Methods	Number of ovaries	Oocytes/ovary (Mean±SE)				
		Total	A	B	C	D
Slicing	75	9.6[a]±0.4	4.2[a]±0.2	2.0±0.3	1.8±0.3	1.6±0.1
Puncture	82	9.7[a]±0.4	4.6[a]±0.1	1.7±0.2	2.1±0.1	1.2±0.2
Aspiration I	80	5.8[b]±0.3	1.2[b]±0.2	2.4±0.1	1.0±0.1	1.1±0.2
Aspiration II	86	5.6[b]±0.2	1.4[b]±0.2	2.1±0.1	1.5±0.3	0.5±0.1

Mean values in the same column with different superscripts (a, b) differ significantly at $p<0.05$.

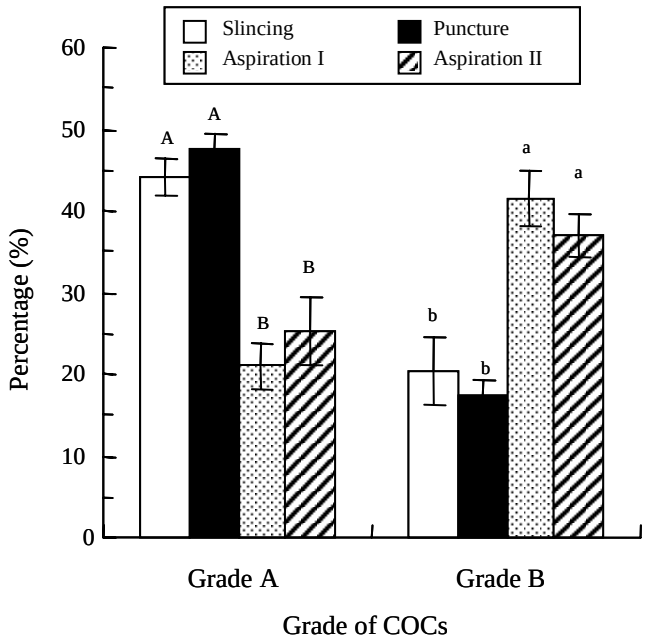

Figure 1. Percentage of COCs classified into grade A and B in total COCs recovered by 4 different collection methods.

procedure (Parrish et al., 1986). Sperm cells were added to the fertilization drops at a concentration of 2×10^6/ml. Twenty hours after insemination, cumulus cells were removed from the presumptive zygotes by vortexing for 2 min in 500 μl of hyaluronidase solution (300 μg/ml in Hepes-buffered Tyrode's solution; TLH). Presumptive zygotes were washed twice in TLH and once in mSOF medium. Twenty to 25 embryos were cultured in 50 μl drops under light mineral oil. Embryos were assessed for cleavage 48 h after the beginning of culture, and at 216 h for determination of the number of embryos reaching the blastocyst stage. Cleaved zygotes were transferred to fresh mSOF drops at 48 h.

Statistical analysis

All experiments were replicated 3 times. Data were expressed as mean±S.E. and were analyzed by analysis of variance (ANOVA) using the general line model procedures of the SAS software (1989). For all date, the model included the main effects of collection methods. Comparisons were considered significantly different if $p<0.05$.

Table 2. Effects of 4 different collection methods on *in vitro* maturation of oocytes using grade A and B COCs

Methods	Total number of oocytes		Maturation rate (%±SE)	
	Grade A	Grade B	Grade A	Grade B
Slicing	62	49	84.1±3.1	82.3±1.8
Puncture	71	74	82.9±4.7	80.1±2.1
Aspiration I	68	61	78.6±1.9	78.9±3.7
Aspiration II	59	64	81.7±2.5	80.2±2.4

RESULTS AND DISCUSSION

Experiment 1: Effects of collection methods on quantity and quality of oocytes

As show in Table 1, slicing (9.6±0.4) and puncture (9.7±0.4) of the ovaries yielded a higher ($p<0.05$) number of oocytes per ovary compared to the aspiration I and II (5.8 ±0.3 and 5.6±0.2, respectively). However, the numbers of oocytes harvested per ovary by slicing and puncture methods were not significantly different, and also aspiration I and II techniques yielded similar number of oocytes per ovary. The number of excellent oocytes in grade A per ovary collected by slicing (4.2±0.2) and puncture (4.6±0.1) method were evidently higher than those collected by aspiration I and II (1.2±0.2 and 1.4±0.2, respectively) ($p<0.05$). As shown in Figure 1, a significantly higher percentage of grade A COCs was found in puncture (47.5±1.9%) and slicing (44.1±2.4%) groups than those in aspiration I and II (21.1±2.8 and 25.2±4.2%, respectively) groups ($p<0.05$). Whereas the percentage of grade B COCs was higher in aspiration I and II (41.4±3.4 and 37.1± 2.6%, respectively) than other two methods (slicing 20.5±4.1%, puncture 17.4±1.7%) ($p<0.05$). No significant difference was found in percent of COCs classified into grade C and D for the different collection methods.

Three methods for collection of oocytes have been described in domestic animals: aspiration of the oocyte from follicles (Datta et al., 1993; Boediono et al., 1995), slicing the ovaries (Carolan et al., 1992; Mogas et al., 1992; Pawshe et al., 1994) and puncture of visible surface follicles (Wani et al., 1999; Shirazi et al., 2005), and these methods were used with varying degrees of success. In a study on buffalo (Das et al., 1996), the assessment of the efficacy of the 3 oocyte collection methods (slicing, puncture, and

Table 3. Effects of 4 different collection methods on embryonic developmental competence of oocytes using grade A and B COCs

Methods	Total number of oocytes		Cleavage rate (%±SE)		Blastocyst rate (%±SE)	
	Grade A	Grade B	Grade A	Grade B	Grade A	Grade B
Slicing	80	72	77.8±3.4	70.1±3.1	27.8±4.6	21.6±2.4
Puncture	93	81	74.2±3.1	71.8±2.8	25.1±1.5	23.8±2.2
Aspiration 1	80	81	81.1±3.3	75.1±4.1	28.4±2.6	23.3±3.1
Aspiration II	87	84	79.3±4.3	74.6±4.5	28.3±2.1	19.7±1.8

aspiration) on oocyte recovery (ovaries obtained at an abattoir) indicated that slicing technique yielded significantly ($p<0.05$) more oocytes per ovary (5.7) than follicle aspiration technique (1.7), which was in accordance with the results of the current study on Holstein cows. As much as 66 oocytes per ovary have been harvested in cattle (Carolon et al., 1992) and 93 oocytes per ovary in sheep (Mogas et al., 1992), using the slicing technique. The lower number of oocytes obtained by slicing in this study may due to the different slicing techniques. The slicing technique, chopped the ovary into small pieces with a surgical blade, was used in this study, while other researchers used blades to incise the follicles on the ovarian surface. A number of oocytes, therefore, were retained in ovary without recovery or were disintegrated due to injuring during chopping. This factor may contribute to the lower recovery of oocytes in the present study.

Experiment 2: Effect of collection methods on IVM of oocytes

The results of IVM using grade A and grade B COCs collected by 4 methods were summarized in Table 2. There was no statistically significant difference in the proportion of M II oocytes after IVM of grade A and B COCs harvested by 4 methods, although a slightly higher percentage of M II oocytes were found in grade A COCs obtained by slicing and puncture (84.1±3.1 and 82.9±4.7%, respectively) than those obtained by aspiration I and II (78.6±1.9 and 81.7±2.5%, respectively) methods. No significant difference was seen when comparing slicing to puncture methods as well as comparing aspiration I to aspiration II methods for the percentage of M II oocytes in grade A COCs.

The maturation rate of oocytes in the present experiment (grade A COCs 78.6±1.9 to 84.1±3.1%; grade B COCs 78.9 ±3.7 to 82.3±1.8%) was the similar with other (78.2±6.1 to 84.8±4.9%) (Sutton-McDowall et al., 2005). It was suggested in Table 2 that the grade A and B oocytes showed similar maturation rate in despite of collection methods, which in accordance with the results of recent research on camel (Nowshari, 2005). The rate of nuclear maturation of the oocytes was not affected by the oocytes collection methods.

Experiment 3: Effect of collection methods on subsequent embryonic developmental competence

As show in Table 3, no difference in the percentage of embryos at the cleavage stage using grade A COCs was noted among 4 groups. The percentages of blastocyst, which zygotes development *in vitro* after 216 h fertilization, were also no difference in grade A COCs obtained by different 4 methods. No significant difference in proportion of embryos at the cleavage stage and blastocysts stage using grade B COCs collected by 4 methods was shown. Grade A COCs had obtained higher embryo cleavage than grade B COCs, excluding COCs recovered by puncture technique. However, for blastocyst grade A COCs had obtained higher rate than grade B COCs regardless of collection methods.

No work has been reported on the effect of the oocytes collection methods on subsequent embryonic developmental competence. In this study it was observed that the oocytes recovered by 4 different collection methods had the similar cleavage rate and blastocyst yield of the embryos after IVF of the M II stage oocytes. Therefore the high quality oocytes showed high maturation rate and the proportion of embryos at the cleavage and blastocysts stage in despite of oocytes collection methods. In the recent research (Luo et al., 2006), 22.8% of blastocyst rate was obtained by culturing embryos in Synthetic Oviduct Fluid (SOF) supplemented with polyvinyl-alcohol (PVA)+5 ng/ml of Vascular Endothelial Growth Factor (VEGF), which in accordance with the results of the present study.

We concluded that the recovery of oocytes using the slicing and puncture techniques yielded more oocytes per ovary than other 2 aspiration methods. The rate of nuclear maturation of the oocytes was not affected by these different oocytes collection methods. The oocytes collection methods also showed no influence subsequent embryonic developmental competence after IVF using M II stage oocytes.

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