Comparison of Two Different Schemes of Once-weekly Ovum Pick Up in Dairy Heifers

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ABSTRACT : To compare two different schemes, continuous scheme (CS) and discontinuous scheme (DCS), of once-weekly ovum pick up (OPU) with ultrasound-guided follicular puncture technique, Holstein heifers were randomly divided into two groups of five. After characterization of their two normal estrous cycles, the heifers were subjected to consecutive 20 weeks of once-weekly OPU under two schemes: the CS (one week interval between continuous OPU, total 100 OPU sessions performed) and the DCS (OPU fixed to the day 3 and day 10 of each estrus). Then, the status of ovaries and artificial insemination results were observed. On oocyte yield, the total number of punctured follicles using DCS was lower than that using CS, but the mean numbers of punctured follicles and recovered oocytes per session were higher in DCS than CS group. So the total number of recovered oocytes was similar in both groups. There were also no differences in the quality of recovered oocytes, nor in the developmental ability of oocytes fertilized *in vitro* between groups. The heifers in the DCS group showed regular estrous cycles with stable estrous signs through the periods of before, during, and after OPU, while those in CS group showed longer estrous cycles and less estrous signs during and/or after OPU compared with before period. Furthermore, the mean number of inseminations required for obtaining pregnancy after completion of the experiments was lower in DCS than CS group. The research demonstrates that similar quantity and quality oocytes can be achieved, and the side effects on donors are lower in DCS that needs fewer OPUs than CS group, and DCS is superior to CS. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 3 : 314-319)***

Key Words : Ovum Pick Up, Dairy Heifer, Oocyte, Follicle

INTRODUCTION

Transvaginal follicular aspiration under ultrasonic guidance for recovering bovine oocytes was originally established by Pieterse et al. (1988). Since then, the ultrasound-guided transvaginal ovum pick up (OPU) technique which overcomes some disadvantages of superovulation and use of ovaries obtained after slaughter, combined with *in vitro* embryo production (IVP), has become an intense field due to its potential commercial application. OPU is the most flexible and repeatable technique to produce embryos from nearly any given live donor. It can be used in cattle of high genetic value for obtaining valuable oocytes. The application of this technique to juvenile calves can further accelerate genetic gain by substantially decreasing generation interval. It also suits bovines with genital tract illness or low sensitivity to superovulation.

Several different schemes have been tried for OPU. Generally, they can be divided into once weekly or twice weekly according to the interval between punctures. During certain short periods, more oocytes can be obtained with twice-weekly puncture, but for long-term OPU, the most popular aspiration schedule is once weekly (Pieterse et al., 1991a; Looney et al., 1994). However, long time once-weekly punctures still cause certain side effects on the oocyte donors (Klossok et al., 1997). It is suggested that if the follicular aspiration is restricted to days 0-12 of the estrous cycle, the influence of the follicular puncture on subsequent ovarian dynamics could be either diminished or abolished (Takenouchi et al., 2001). The aim of the present study is to compare two different schemes of once-weekly OPU and estimate whether discontinuous long-term OPU can substitute for continuous long-term OPU for reducing the negative effects on donors.

MATERIALS AND METHODS

All chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

Animal

Ten dairy heifers (Holstein) between 12 and 13 months of age at the beginning of the research with a similar weight and health condition were used in the study. The heifers were barnhoused and fed a mixed ration consisting of hay and a commercial concentrate pellet. The heifers were provided by the Songjiang Experimental Animal Facility affiliated to the Shanghai Institute of Medical Genetics, China.

Experimental design

Experimental periods : Whole experiment was divided

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into three periods: i) Pre-OPU period, the heifers were observed during two consecutive estrous cycles; ii) OPU period, the heifers were subjected to OPU under two schemes for 20 consecutive weeks; iii) Post-OPU period, examinations of the ovaries were performed by palpation per rectum and ultrasonography after the last OPU session and persist in artificial insemination on all donors with same semen, starting from second estrus till pregnancy.

Experimental groups : Ten heifers were randomly divided into two groups of five: Continuous scheme group (CS), OPUs were continuously performed once a week; Discontinuous scheme group (DCS), OPUs were fixed to the day 3 (day 0=day of estrus) and day 10 of each estrus, and no OPU was performed during the late half of the estrous cycle.

Transvaginal follicular aspiration

Materials : Ovum pick up equipment consisted of a portable ultrasound unit (SSD-500, Aloka Co. Tokyo, Japan) with a sector scanner mounted on a properly designed vaginal support, equipped with a needle guide. The 60 cm long needle (18 gauge, FHK, Tokyo, Japan) was connected to a regulated vacuum pump (FHK, model 4) which draws the sample into a 50 ml sterile tube placed in a 39°C electric warmer.

OPU : OPU was performed as described by Petyim et al. (2000) with a minor modification. The standing heifer was restrained in an adjustable squeeze chute. After emptying the rectum and thoroughly cleaning the vulva and perineal area with water and iodine, the transducer was advanced into the external os of the cervix. The ovaries were manipulated per rectum and positioned over the transducer face so that the targeted follicle was transected by the built-in puncture line on the ultrasound monitor, which represented the projected needle path. Follicular fluid was aspirated using continuous negative pressure (about 95 mmHg). When the targeted follicles were stabilized on the puncture line, the needle was inserted in the guide and advanced through the vaginal wall and into the follicle antrum. Then the system was rinsed with Dulbecco's phosphate buffered saline (Gibco, Grand Island, NY; pH 7.4) medium containing 3% bovine serum albumin and 2IU/ml heparin. All follicles (>2 mm diameter) were aspirated.

Oocyte categorization : After recovery, the contents and flushing medium were filtered through a embryo filter. Cumulus-oocyte complexes (COCs) were searched under stereomicroscope. The COCs were classified into 3 categories (grade A, B and C) as described by Neglia et al (2003): A or B, uniform cytoplasmic appearance and enclosed within 3 (Grade B) or more (Grade A) layers of viable compact granulosa cells; C, less than three layers of granulosa cells or partially denuded.

Clinical observations

The heifers were observed three times daily for signs of estrus: excitement, vocalization, lowering of the back, vulvar oedema and redness and mucous discharge were noted. These parameters were scored and summarized to give an overall impression of estrous status: 0=not estrus; 1=uncertain; 2=weak estrous signs; 3=strong estrous signs.

***In vitro* maturation, fertilization and culture**

Oocytes underwent IVM, IVF and IVC using procedures of our lab as described by Huang et al. (2000, 2001) with slight modification. Briefly, The COCs were matured for 23-24 h in TCM-199 (Gibco, Grand Island, NY) consisted of 10% fetal calf serum (FCS), 10 μg/ml LH, 1 μg/ml E2 and 1 μg/ml FSH under humidified atmosphere of 5% CO_2 in air at 38.5°C, and fertilized by frozen-thawed semen from the same ejaculate of the same bull. (1×10^6 sperm/ml in BO medium with 1.95 mg/ml heparin) for 6 h. All the inseminated oocytes were cultured in B2 medium (Laboratoire C.C.D. France) microdrops with 10% FCS on a Vero cell monolayer under humidified atmosphere of 5% CO_2 in air at 38.5°C.

Statistical analysis

Data were analyzed with SAS version 6.12 by *t-test*, ANOVA and Chi-square (ttest, glm and freq procedures).

Table 1. Number and characteristics of aspirated follicles and recovered oocytes using two different schemes (mean±SE)

Characteristics	Continuous scheme	Discontinuous scheme
Number of aspiration session	100	70
Number of aspirated follicles	1,041	875
Mean number of follicles aspirated /heifer/session	10.4±0.6	12.5±0.7*
Categories of follicle at aspiration /heifer/session		
2-3 mm	4.7±0.3	6.9±0.5*
4-7 mm	4.6±0.3	4.9±0.3
>8 mm	1.2±0.1	0.6±0.1*
Total number of recovered oocytes	701	658
Recovery rate %	67.3	75.2*
Mean number of oocytes recovered /heifer/session	7.0±0.3	9.4±0.5*

* Values are significantly different compared with those in CS, $p<0.05$.

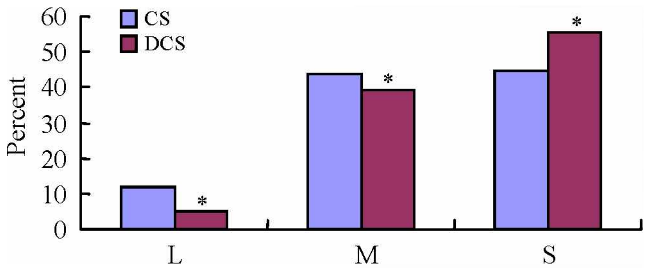

Figure 1. The proportion of punctured follicles using two schemes. L: large follicles; M: middle follicles; S: small follicles. * Values are significantly different compared with those in CS within each size of follicles (p<0.05).

Differences were considered as significant if P values were <0.05.

RESULTS

Follicle puncture and oocyte recovery

In total, 100 OPU sessions and 70 OPU sessions were performed for the CS and DCS respectively (Table 1). The mean numbers of punctured follicles and recovered oocytes per session were lower using the CS than using the DCS. Due to a lower recovery rate with CS, the number of recovered oocytes was similar to that of DCS (Table 1).

The CS had significantly more large size follicles and fewer small size follicles per session than the DCS, but there was similar number of middle size follicles between the two schemes (Table 1). However, although the proportion of the number of small size follicles was significantly ($p<0.05$) higher in DCS than CS, the reverse was observed in large and middle sizes of follicles (Figure 1).

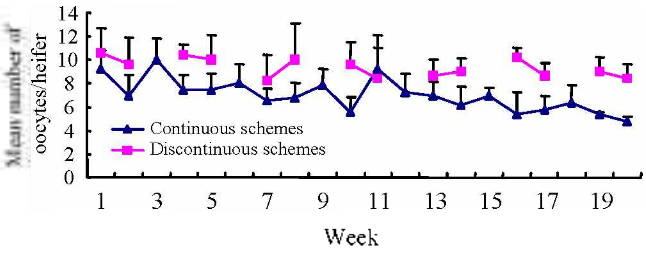

Figure 2. Mean number of oocytes recovered /heifer/week using two schemes of ovum pick up.

The numbers (/heifer/session) of punctured follicles and recovered oocytes with the CS during three periods of ovum pick up were significantly different (Table 2). Mean number (/heifer/week) of recovered oocytes in CS showed a decreasing trend (Figure 2).

Oocyte quality and developmental competence after IVF

No differences were observed in the proportion of oocyte categories of the recovered oocytes between the two schemes. Furthermore, after pooling all categories, there were no differences in cleavage rate at 48 h and blastocyst rate on 8th day after the start of IVF (Table 3).

Estrous cycle characteristics

During the pre-OPU period, all heifers showed regular and normal estrous cycle characteristics. During the OPU period the heifers in the CS showed a prolonged estrous cycle and weak signs of estrus ($p<0.05$), while in the DCS, the cycle characteristics did not differ from those of the pre-OPU period. During the post-OPU, all heifers still showed normal in the DCS. In the CS, the cycle length nearly recovered, but the signs of estrus were still weak ($p<0.05$) (Table 4).

Table 2. The numbers (/heifer/session) of punctured follicles and recovered oocytes using the two schemes during three periods of ovum pick up (mean±SE)

Schemes	Follicles			Oocytes		
	Early	Mid	Late	Early	Mid	Late
Continuous scheme	12.4±1.5[a]	10.0±0.7[ab]	9.1±0.6[b]	8.2±0.7[a]	7.2±0.6[ab]	5.9±0.4[b]
Discontinuous scheme	13.4±1.4	12.0±1.3	12.3±0.7	11.1±1.3	9.7±1.0	9.3±0.6

Within each category numbers sharing the different letter within each row are significantly different, p<0.05.
Early: early OPU period (1-6 weeks); Mid: middle OPU period (7-13 weeks); Late: late OPU period (14-20 weeks).

Table 3. The proportion of oocytes with different categories of quality, and cleaved and developed to blastocyst after IVF

Schemes	Oocyte category			Cleavage rate (%)	Blastocyst rate (%)
	A (%)	B (%)	C (%)		
Continuous scheme	23.1	36.9	39.9	62.1	23.6
Discontinuous scheme	24.3	36.9	38.8	60.7	24.7

Table 4. Estrous cycle characteristics during the pre-ovum pick up, ovum pick up and post- ovum pick up periods (mean±SE)

	Continuous scheme			Discontinuous scheme		
	pre-OPU	OPU	post-OPU	pre-OPU	OPU	post-OPU
Number of cycles	10	28	5	10	30	5
Cycle length	20.8±0.3[a]	23.8±0.3[b]	22.0±0.4[a]	20.8±0.2	21.7±0.3	21.0±0.3
Score of estrous signs	3.0±0.0[a]	2.3±0.1[b]	2.4±0.2[b]	3.0±0.0	2.7±0.1	3.0±0.0

Within each category numbers sharing the different letter within each row are significantly different, p<0.05.

Ovary status and insemination results

A thickening and hardening of 7 sides of 10 ovaries was evaluated in the CS, but in only 2 sides in the DCS.

The mean number of inseminations required to obtain pregnancy between the two schemes is different (1.2±0.2 and 2.2±0.4 for the DCS and CS, respectively) (Mean±SE, $p<0.05$).

DISCUSSION

At present, the continuous scheme is mainly used on once-weekly ovum pick up. To our knowledge, this is the first report on discontinuous once-weekly OPU. Our results showed that on quantity, the total number of follicles using CS was higher, but the mean number of punctured follicles per session was lower than those using DCS, and mean number (/heifer/week) of recovered oocytes appeared a decreasing trend, which was confirmed by Pieterse et al. (1991b). The reason may be the different follicular waves. The follicular waves in the CS were all initiated by follicular punctures which removed the inhibitory effects of estradiol and inhibin on FSH and recruited a new cohort of follicles (Adams et al., 1992). One of the follicular waves in the DCS was initiated by follicular punctures too, but the other was the first follicular wave of the estrous cycle and the time of OPU was Day 3 of each cycle, which has been stated to yield the highest number of follicles (Pieterse et al., 1991b). Furthermore, because of a long-term and continuous punctures, thickening and hardening of the ovarian tunia albuginea were seen in the CS, which decreased the number of follicles.

Although the total number of follicles was higher in CS than DCS, due to a lower recovery rate with CS than DCS, the number of recovered oocytes was similar in both groups. There are several reasons as follows:

The higher number of large follicles in the CS : On day 7 follicles stay in the dominant phase after passing recruitment, selection and growth, and have a larger size, while on day 3 follicles stay in the selection phase with smaller diameter (Merton et al., 2003). Large follicles make low recovery rate very likely due to the rapidly collapsing follicular wall that may trap the COC (Presicce et al., 2002).

The higher number of small follicles in the DCS : It has been stated that the total number of oocytes recovered was correlated with the number of small follicles and, the more small follicles there are, the higher recovery rate is (Manik et al., 2003).

Formation of CL-like structures (Corpus luteum-like structures) : CL-like structures were mainly developed from the puncture of large follicles. During OPUs, the development of CL-like structures caused practical difficulties since they are highly vascular. Passage through the CL-like structure resulted in blood entering the tubing system, which caused a risk for coagulation and difficulty in searching for oocytes (Petyim et al., 2000).

Local hardening of ovaries can be seen after multiple repeated penetrations : Healing was nearly complete after 10 days (Chastant-Maillard et al., 2003). So nearly all punctures in the CS were applied under incomplete healing, which made OPU more difficult.

Formation of blood-filled follicles : At puncture, the peri-follicular vessels might become perforated, resulting in bleeding into the central cavity. Histological examination of ovaries also confirmed the presence of blood-filled follicles (Chastant-Maillard et al., 2003). The entirely blood-filled follicles may lead to inaccuracy in identifying follicles suitable for puncture due to similar echogenic characteristics and equal thickness of the wall, implying an inadequate number of punctured follicles and increased risk for blood clots in the needle and tubing system (Petyim et al., 2000). In the DCS, blood-filled follicles may be reabsorbed during the late half of the estrous cycle when no follicular puncture was applied.

On quality, the recovered oocytes expressed in each category showed no significant difference between the two OPU schemes. Correspondingly, the *in vitro* competence did not differ comparing the DCS with the CS. Maybe their maturations were inferior to that of cows (Yeh et al., 2004), so their blastocyst rates were not high. The main difference between two schemes was that there were more follicles at the early stage of atresia in the CS. Follicles will enter dominant phase on day 6 when dominant follicles made other follicles atretic (Merton et al., 2003). Because the period from atresia to follicle's elimination may take 1-2 weeks (Merton et al., 2003), on day 7 when follicles were punctured, the atretic follicles stayed at the early stage of atresia. It has been indicated that remarkably early signs of atresia apparently have a positive effect on developmental competence and oocyte quality is only affected at the very late stage of atresia (Salamone et al., 1999; De Wit et al., 2000). This may partly be explained by the similarity in ultra-structure observed between oocytes undergoing prematuration and early atresia (Assey et al., 1994). Besides, Zeuner et al. (2003) showed that adding exogenous follicular cells from atretic follicles to good bovine COCs during IVM had no impact on fertilization, blastocyst formation or hatching after IVF. So the quality of the recovered oocytes did not differ between the two OPU schemes.

If a scheme is to be used in practice, it is important to understand not only its effectiveness but also how it affects oocyte donors. The heifers in the CS showed an irregular estrous cycle and weak signs of estrus, while those in the DCS showed regular and normal estrous cycle characteristics. The reason may be that the follicular punctures of DCS were restricted to day 0-10 and DCS had a long intermission. During this intermission time the corpus luteum will be degenerating and progesterone will be decreasing. When the corpus luteum degenerates completely, basic concentration of progesterone was maintained, and high frequency of LH pulses caused the final growth of the dominant follicle. High concentrations of estrodiol induced the LH and FSH surge that leads to ovulation and estrus. In the CS, more CL-like structures producing progesterone and continuous aspirations altering regular follicular wave may contribute to the irregular estrous cycle. In agreement with Petyim et al., 2001, some ovaries were harder and thicker in CS than DCS group. This demonstrated that some histological changes had happened, which influenced the function of ovaries and increased the difficulties of inseminations. So the mean number of inseminations required to obtain pregnancy between schemes is different.

In conclusion, for long-term OPU, there is no difference between the two schemes in oocyte quantity and quality, and the side effects on donors are lesser using DCS that needs fewer OPUs.

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