Effects of superstimulation with FSH on follicular population and recovery rate of oocytes in the growing phase of the first and second follicular wave

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ABSTRACT: Effectiveness of *in vitro* production of embryos (IVP) is limited among other factors by the recovery rate of oocytes. Gonadotropin superstimulation can improve the recovery rate of oocytes. The effect of FSH treatment on follicular population and recovery rate of oocytes at ovum pick-up (OPU) in the growing phase of the 1st as well as the 2nd follicular wave after superstimulation was the object of our experiment. Twelve unpregnant milking cows (15–20 kg milk per day) housed on a dairy farm were used in the experiment. The cows bearing corpus luteum were synchronized by $PGF_{2\alpha}$ (day 0) and they were treated by FSH (Folicotropin inj. sicc. ad us vet., Spofa Prague, Czech Republic, single doses 80, 80, 80, 80, 40 and 40 UI) in 12 h intervals on days 12, 13 and 14. Transvaginal ultrasonographic puncture of oocytes in cows bearing a new corpus luteum was performed on day 7 (OPU 1, various phase of the follicular wave, removal of the dominant follicle) and it was repeated on days 10 (OPU 2, growing phase of the follicular wave – control), 16 (OPU 3, growing phase of the first follicular wave after superstimulation) and 20 (OPU 4, growing phase of the second follicular wave after superstimulation). All follicles > 2 mm were punctured. The ovarian follicles (ultrasonographically) and numbers and qualities of obtained oocytes (microscopically) were evaluated during and immediately after OPU. Follicular population was divided to small (FS, 2–5 mm), medium (FM, 5–9 mm) and large (FL, > 9 mm) follicles. Oocytes were classified as 1st (intact cumulus, > 3 layers of cumulus cells), 2nd (complete 1–3 layers of cumulus cells), 3rd (incomplete layers of cumulus cells, expanded cumulus mass) and 4th (absence of corona cells, degenerated oocytes) classes. Although we found the least of FS (x = 1.0) during OPU 3, significantly more FM (x = 24.7) and FL (x = 3.1) follicles were found at this procedure in comparison with others. Likewise a significantly higher number of oocytes (x = 8.1) was obtained at OPU 3 in comparison with OPU 1 and OPU 2. Significantly higher number of FM (x = 6.1) was found and non-significantly higher number of oocytes was obtained at OPU 4 in comparison with OPU 1 and 2. The results show that administration of FSH increases the number of follicles and the number of collected oocytes in the growing phase of the 1st follicular wave after superstimulation, nevertheless a higher number of follicles and a higher recovery rate of oocytes can be expected in the growing phase of the 2nd follicular wave after superstimulation as well.

Keywords: ovum pick-up; superstimulation; cattle

Recently safe and repeatable techniques of *in vitro* embryo production have been developed. The source of oocytes were ovaries of slaughtered donors for early *in vitro* procedures (Galli *et al.*, 1996). Laparotomy and laparoscopy were used in the first experiments to obtain oocytes from live donors. Now the oocytes are more frequently recovered from live animals by transvaginal ultrasound-guided oocyte collection (Galli *et al.*, 2001). Ultrasound-guided oocyte aspiration (ovum pick-up, OPU) combined with *in vitro* embryo production represents replacing procedures of traditional embryo transfer technologies in cattle (Kruip *et al.,* 1991). Transvaginal follicular aspiration was performed in adult animals (Pieterse *et al.*, 1988; Van der Shans *et al.,* 1991), prepubertal heifers older than two months (Lohuis, 1995; Looney *et al.,* 1995; Steeves *et al.,* 1999; de Paz *et al.,* 2001) and pregnant animals (Meintjes *et al.,* 1995; Guyader-Joly *et al.,* 1997) as well. OPU procedure can be repeated without serious damage of ovaries (Petyim *et al.,* 2000; Kurykin, 2001) and even allows normal cyclicity of donors (Båge *et al.,* 2001). Different schedules can be used for oocyte recovery.

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Collection once a week results in the recovery of a smaller number of oocytes due to the presence of a dominant follicle at each successive collection that causes the regression and degeneration of subordinate follicles (Galli *et al.,* 2001). Collection twice a week allows the maximum recovery of oocytes in a given time interval because the aspiration of follicles present at the ovaries induces and synchronizes follicular waves. A subsequent puncture 3 or 4 days later allows the aspiration of a newly recruited pull of follicles before the establishment of follicular dominance and regression of subordinate follicles (Garcia and Salaheddine, 1998).

Some authors studied whether OPU after gonadotropin pre-stimulation resulted in superior total embryo yield after *in vitro* fertilization. The effects of exogenous gonadotropins were investigated in oocyte collection from slaughterhouse ovaries (Blondin *et al.,* 1996, 1997) and in OPU (Bousquet *et al.,* 2000; de Ruigh *et al.,* 2000; Goodhand *et al.,* 2000) as well. Aspirations were performed in one-week (Goodhand *et al.,* 2000) or two-week (Sirard *et al.,* 1999; de Ruigh *et al.,* 2000) intervals. The average yield of oocytes and embryos per OPU after stimulation was higher compared to the twice per week OPU scheme without stimulation. However, the total number after a longer period of time was lower in comparison with the collection performed twice a week (de Ruigh *et al.,* 2000; Galli *et al.,* 2001).

The objective of our study was to determine the effects of FSH superstimulation (Folicotropin inj. sicc. ad us. vet., Spofa, Prague, Czech Republic) on the number of aspirated follicles and collected oocytes in dairy cows during two consequent follicular waves after treatment.

MATERIAL AND METHODS

Animals and stimulation

Twelve Holstein dairy cows were used as oocyte donors. Cows were housed on a commercial dairy farm, average milk yield was 15–20 kg milk per day.

All cows bearing corpus luteum were synchronized (day 0, D0) using cloprostenol (Oestrophan inj. ad us. vet., Léčiva, Prague, 500 μg *i.m. pro toto*). Further examination was performed seven days later (D7). Ovarian structures (new corpus luteum and follicles) were examined by ultrasonography and follicles were aspirated (ultrasound-guided follicle aspiration, OPU 1). Aspiration was repeated 3 days later (OPU 2, D10). The cows were superstimulated using FSH (Foli-cotropin inj. sicc. ad us. vet., Spofa, Prague) at 6 decreasing doses (80, 80, 80, 80, 40, 40 I.U.) on days 12, 13 and 14. Further aspirations were performed on days 16 and 20 (OPU 3, 4) (Table 1).

Ultrasound-guided transvaginal follicular aspiration

A real-time B-mode ultrasound scanner (Aloka SSD-500) equipped with a 5 MHz convex transducer (Aloka UST-974-5) was used during the transvaginal ultrasound-guided follicular aspiration. The transducer was mounted in a plastic handle (Eickemeyer 303922) with stainless needle guide. A needle (V-OPAA-1760, Cook) was connected via silastic tubing (V-OPAL-1700-L, Cook) to a 50 ml plastic tube (Falcon). Follicular fluid was aspirated using continuous negative pressure 10 kPa (75 mm Hg) applied with a suction pump (Storz).

All follicles classified as small (FS, 2–5 mm), medium (FM, 5–9 mm) and large (FL, > 9 mm) were punctured. Collected cumulus oocyte complexes (COCs) were classified into 4 categories according to the presence of cumulus cells surrounding the oocyte:

1. intact COCs with 3 or more cumulus cells layers
2. COCs with 1–3 layers of cumulus cells
3. incomplete layer of cumulus cells or expanded cumulus cells
4. completely denuded or degenerated oocytes

Statistical analysis

The results were analysed by Wilcoxon's test.

RESULTS AND DISCUSSION

The number of small (FS), medium (FM) and large (FL) follicles and recovery rate of oocytes (O) are presented in Table 2.

Table 1. Diagram of superstimulation and OPU in experimental cows

D0	D3	D7	D10	D12	D13	D14	D16	D20
PG	oestrus	OPU 1	OPU 2	FSH stimulation			OPU 3	OPU 4

Table 2. Mean number of FS, FM, FL, O and statistical differences between OPU 1–4

	OPU 1 (D7)	OPU 2 (D10)	OPU 3 (D16)	OPU 4 (D20)
FS	7.9			
FM	2.2			
FL	0.5			
O	3.5			
FS	–	5.8		
FM	–	3.6		
FL	–	0.2		
O	–	4.4		
FS	*	**	1.0	
FM	**	**	24.7	
FL	**	**	3.1	
O	**	**	8.1	
FS	–	–	**	6.1
FM	**	*	**	6.1
FL	–	–	**	0.1
O	*	–	–	6.2

* α <0.05; ** α <0.01)

The lowest number of small follicles was observed on D16 (OPU 3), the differences on the other days were not significant. The highest number of medium follicles was observed on D16 (OPU 3), number of FM on D20 (OPU 4) was higher than number of FM on D7 and D10 (OPU 1 and OPU 2). The highest number of large follicles was found on D16, the differences on the other days were not significant.

The number of collected oocytes was higher on D16 than on D7 and D10, but it did not differ from D20. The number of collected oocytes on D20 was higher than on D7 and did not differ from D10 and D16.

The groups of collected oocytes are presented in Table 3.

The number of oocytes of the 3rd group was higher on D16 than on D7 and D20. The number of oocytes of the 4th group was higher on D16 than on D7 and D10, respectively. No other differences were found.

The scheme of superstimulation and aspirations used in the experiments enabled to compare OPU results without previous stimulation and those observed at the 1st and 2nd follicular wave after stimulation. Each aspiration was performed in the growing phase of a new follicular wave (Garcia *et al.,* 1998) except for the OPU 1 on D7, which was used with the aim to remove all large follicles and to start a new follicular wave.

Table 3. The distribution of oocytes among groups at OPU 1–4

	OPU 1 (D7)	OPU 2 (D10)	OPU 3 (D16)	OPU 4 (D20)
Group 1	0.42a	1.08a	1.08a	1.50a
Group 2	0.92a	1.00a	1.58a	1.75a
Group 3	0.75a	1.42a,b	2.42b	1.00a
Group 4	1.33a	1.17a	3.00b	1.92a,b

Mean values with different superscripts differ between OPU 1–4 (α <0.01)

OPU 2 represented the unstimulated aspiration for the comparison with stimulated aspiration (OPU 3) after FSH treatment. OPU 4 was performed during the growing phase of the second follicular wave after stimulation, which has not been described yet.

The OPU 1 and OPU 2 results were not significantly different, however, the number of FS tended to be lower and the number of FM and collected oocytes tended to be higher at OPU 2. OPU 3 clearly proved the stimulation effect of FSH contained in Folicotropin inj. sicc. ad us. vet. These results confirmed the data of other authors (Sirard et al., 1999, deRuigh et al., 2000; Goodhand et al., 2000), who used different medicaments.

The lowest number of small follicles and the highest number of medium and large follicles during OPU 3 were significantly different from other aspirations. This demonstrated that small follicles present in ovaries during FSH treatment responded positively to the stimulation and during subsequent aspiration they already were in higher follicle categories. It indirectly supported the data about a new follicular wave emergence after follicular aspiration (Garcia *et al.,* 1998) although detailed observations of follicular dynamics were not the objective of the study. The number of oocytes collected during OPU 3 was significantly higher than during OPU 1 and OPU 2, however it only tended to be higher compared with OPU 4.

Changes were also observed in the proportions of oocyte quality classes. Stimulation before OPU 3 yielded a significantly higher number of group 3 oocytes than those at D7 (OPU 1) and D20 (OPU 4) and of group 4 oocytes than those at D7 and D10 (OPU 2), but no increase in the number of group 1 and group 2 oocytes was apparent. This increase in the proportion of lower-quality oocytes is difficult to explain and contradicts the finding of Sirard *et al.* (1999) who obtained higher-quality oocytes using a similar superstimulation scheme.

It is evident from the results presented here that oocytes can be collected also at D20 (OPU 4), i.e. four days after stimulated puncture (OPU 3). The number of middle-sized follicles collected by OPU 4 was significantly higher than those obtained by OPU 1 or OPU 2, but no such difference was found in the number of small and large follicles. Although the cows were no more stimulated prior to OPU 4 and their ovaries were subjected to repeated punctures (OPU 1, 2 and 3) a short time before, the population of follicles present in ovaries at D20 was sufficient for puncture. The number of oocytes obtained by OPU 4 lay between the values of OPU 2 and OPU 3, but the differences OPU 4 vs. OPU 2 and OPU 4 vs. OPU 3 were insignificant.

Because of the limited number of cows used in our experiment and lack of relevant information sources, the suggested OPU scheme requires verification. Continuing studies should also concentrate on the developmental competence of oocytes which was outside the scope of this clinical study. Studies of dynamics of follicular development after stimulated puncture could also contribute to a draft of new OPU-associated donor stimulation systems.

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