Reprod Dom Anim doi: 10.1111/j.1439-0531.2007.00873.x
ISSN 0936-6768

Effects of Ovum Pick-up Frequency and FSH Stimulation: A Retrospective Study on Seven Years of Beef Cattle *In Vitro* Embryo Production

R De Roover[1], JMN Feugang[2], PEJ Bols[3], G Genicot[1] and Ch Hanzen[1]

[1]*Faculté de Médecine Vétérinaire, Service d'Obstétrique et de Pathologie de la Reproduction des ruminants, équidés et porcs, Université de Liège, Sart Tilman, Boulevard de Colonster 20, 4000 Liège, Belgium;* [2]*Animal Genomics and Proteomics, Department of Animal and Dairy Sciences, Missisipi State University;* [3]*Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, Laboratory of Veterinary Physiology, University of Antwerp, Universiteitsplein 1, Gebouw U, Wilrijk, Belgium*

Contents

The aim of this retrospective study was to compare the number of follicles, cumulus oocyte complexes (COCs) and cultured *In Vitro* Produced (IVP) embryos obtained from 1396 non-stimulated Ovum Pick-up (OPU) sessions on 81 donor animals in a twice weekly OPU scheme. Results were obtained from 640 sessions following FSH-LH superstimulation, on 112 donors subjected to OPU once every 2 weeks. The stimulation protocol started with the insertion of an ear implant containing 3 mg norgestomet (Crestar, Intervet, Belgium) 8 days before puncture (day −8). The dominant follicle was ablated by ultrasound-guided follicle puncture on day −6. On day −3 and day −2, cows were injected with FSH (Ovagen, ICP) twice daily (8 AM to 8 PM), i.e. a total dose of 160 μg FSH and 40 μg LG per donor per stimulation cycle. Animals were punctured 48 h after the last FSH injection (day 0). Progesterone implants were removed the next day. Stimulated donor cows were treated with this protocol at 14-day intervals. Follicles were visualized with a Dynamic Imaging ultrasound scanner, equipped with a 6.5 MHz sectorial probe. Follicles were punctured with 55 cm long, 18 gauge needles at an aspiration pressure corresponding to a flow rate of 15 ml/min. Cumulus oocyte complexes were recovered and processed in a routine IVF set-up.

Results demonstrate that, expressed per session, FSH stimulation prior to OPU increases production efficiency with significantly more follicles punctured and oocytes retrieved. However, when overall results during comparable 2-week periods are considered (four non-stimulated sessions vs one stimulated), more follicles are punctured and more oocytes are retrieved using the non-stimulated protocol. No significant differences in the number of cultured embryos could be detected, indicating that FSH/LH stimulation prior to OPU might have a positive effect on *in vitro* oocyte developmental competence as more embryos are cultured with less, presumably better-quality, oocytes.

Introduction

For more than a decade, ultrasound-guided transvaginal oocyte retrieval (Ovum Pick-up, OPU) has been the tool of choice for oocyte retrieval in living donor cows. After a transvaginal technique had been developed, modified from human reproduction (Pieterse et al. 1988), numerous researchers started using OPU routinely in veterinary-assisted reproduction (Kruip et al. 1994). Apart from being a basic technique in both research and commercial programmes (Looney et al. 1994; Hasler 1998; Galli et al. 2001) the research goal of this highly repeatable, low-invasive procedure for the retrieval of high-quality fresh oocytes was invariably to increase the number and the quality of the retrieved oocytes (Bols et al. 1996; Hashimoto et al. 1999; Ward et al. 2000; Seneda et al. 2003). Biological and technical factors both have important effects on oocyte quality (Bols et al. 2004). The former include, amongst many other factors, hormonal stimulation prior to follicular puncture. In some donors, where ovarian activity is found to be too low, follicular growth needs to be stimulated. FSH/LH combinations, apart from Pregnant Mare Serum Gonandotrophin (PMSG), are well known and widely used hormones in classical ET. However, modifications in dose, administration regimens or timing of treatments are necessary, as the final aim of stimulation prior to OPU is to generate an acceptable amount of additional follicles instead of initiating ovulation as in classic ET. PMSG is a glycoprotein with both FSH and LH activity. Because of its long half-life of several days, it has to be administered only once, between days 10 and 12 of the oestrous cycle, causing an effect on the target glands for more than a week. Commercial FSH/LH preparations contain both FSH and LH, in a ratio typically effective for each product. These have a short half-life (30–60 min) and must be administered twice daily.

Pieterse et al. (1988) aspirated follicles from stimulated cows 2 days following treatment with 1000–3000 IU PMSG, when an active mid-cycle corpus luteum (CL) was present. In comparison with a group of non-stimulated donors, the highest recovery rates (RR) were obtained in the PMSG-treated groups, whereas the ovaries of stimulated donors were larger and produced more follicles with diameters of 5–10 mm, which were easier to puncture. Subsequently, they showed (Pieterse et al. 1992) that the mean total number of aspirated follicles per cycle differed significantly between non-stimulated and stimulated sessions, at 4.3 and 7.1, respectively. However, PMSG treatment had the opposite effect on the RR, which was significantly lower at 42%, compared to 60% without stimulation. Meintjens et al. (1995) compared RRs following OPU on pregnant donors, either treated with 20 or 40 mg FSH or non-stimulated donors. They found the highest percentage of viable oocytes in the group, which received 40 mg FSH. FSH treatment, at a dose rate of 4–5 mg twice daily for 3 days (Looney et al. 1994). Stimulated donors produced more grade 1–2 embryos per session (1.38) than non-treated animals (0.96), resulting in more transferable embryos. However, the increased number of follicles detected is often inconsistent when the effect of stimulation depended on the day of puncture, as

demonstrated by Paul et al. (1995). Stubbings and Walton (1995) found no differences in the mean number of follicles available for puncture each week between non-stimulated cows, punctured twice a week (14.2 ± 1.9) and FSH-stimulated cows, punctured only once (15.7 ± 3.3).

Although results from PMSG or FSH/LH stimulation of ovarian activity prior to OPU are not consistent, a positive effect on follicular growth is often observed. It should be noted, however, that treatments with FSH are reported to induce asynchrony between maturation of the oocyte and its surrounding follicle (de Loos et al. 1991) or between nuclear and cytoplasmic maturation (Bousquet et al. 1995), resulting in reduced developmental rates (Blondin and Sirard 1995). On the other hand, FSH has had positive effects on the proportion of follicles with a diameter > 6 mm (Lonergan et al. 1994), the quality of oocytes retrieved after slaughter (Lu et al. 1991), and the number of developing viable blastocysts (Van Soom et al. 1995). Goodhand et al. (2000) concluded that progestagen plus oestradiol-17β treatment did not affect follicle, oocyte or embryo production when oocyte donors were aspirated once per week. However, they do agree that FSH treatment significantly increased the number of aspirated follicles, and resulted in more best-quality oocytes.

Over the years we have accumulated a huge data set on OPU-IVF treatment results of Belgian Blue donor cows. The aim of this study was to compare the follicle, cumulus oocyte complex (COC) and embryo production results of OPU sessions once every 2 weeks with FSH stimulation, with the results of an OPU scheme without FSH and a puncture frequency of two times per week. Results will be compared per session and over 2-week periods. Special attention is given to animals or sessions, in which a bad response to FSH stimulation was observed.

Materials and Methods

Animals

A comparison was made between the period from 1996 to 1999, when OPU-IVF was performed on animals without previous ovarian stimulation (n = 1396 sessions) at an OPU frequency of two times per week, and the period from 1999 to 2003, when donors were stimulated with FSH prior to OPU (n = 640 sessions), which was performed once every 2 weeks. Donor animals were of the Belgian Blue Breed. All were privately owned and rolled into the assisted reproduction programme for various reasons. Most of them had a history of problems in classical ET programmes. All donors were housed in the veterinary faculty and fed a mixed ration consisting of hay and a commercial concentrate pellet, which were sufficient to maintain a constant body condition score throughout the duration of the study.

Stimulation protocol

All animals were superstimulated at 2-week intervals using the protocol as described by De Roover et al. (2005). An ear implant of 3 mg norgestomet (Crestar®) was inserted 8 days prior to OPU (day −8), without injecting norgestomet-estradiol-valeriate. Two days later (day −6), the dominant follicle was removed by ultrasound-guided transvaginal follicle aspiration. Starting on day −3, the cows received an FSH injection (2.5 ml of Ovagen ICP BIO, Bodinco, the Netherlands; containing 17.6 mg NIADDK-O-FSH-17) in the morning and in the evening for two consecutive days (8 AM to 8 PM, total of four injections). Animals were subjected to OPU 48 h after the last FSH injection (day 0). Ear implants were removed the next day (day + 1).

Transvaginal ultrasound-guided oocyte retrieval

Follicles were visualized using an ultrasound scanner, equipped with a 6.5 MHz sectorial probe (Dynamic Imaging model MCV) fitted in a custom made intravaginal OPU probe-holder. An 18-gauge disposable hypodermic needle (Braun, Germany) connected to a 50 ml conical tube (Becton Dickinson Gmbh, Heidelberg, Germany) via a Teflon tubing was used for follicular puncture. Negative pressure was applied using a vacuum aspiration pump (Labotect, Germany) with an aspiration vacuum adjusted to a flow rate of 15 ml of water per minute. The COC collection tube was kept in a warm water bath at 39°C. Oocytes were collected into TCM 199 medium (Sigma M4530, Bornem, Belgium) with HEPES buffer (Sigma H0763), 2% newborn calf serum (NBCS; Gibco 1610-167, Merelbeke, Belgium), antibiotics (Gibco 15140-106) and 2.2 IU/ml heparin (Sigma H9399). Cows received an epidural anaesthesia (4 ml lidocaine 2%, Kela Laboratories, Hoogstraten, Belgium) to prevent them from straining during the procedure. After emptying the rectum and cleaning the vulva and perineal area, the transducer was inserted into the vagina. The ovaries were manipulated per rectum and positioned in front of the probe, in order to obtain a clear image on the ultrasonographic monitor. Before, during and after OPU, the needle and Teflon tubing were thoroughly rinsed to prevent blood from clotting or oocytes from sticking to the Teflon tubing. Immediately following OPU, oocytes were searched for using a stereomicroscope, washed and transferred to maturation medium.

Oocyte maturation and fertilization

All retrieved COCs were subsequently submitted to routine *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) techniques, as described earlier (De Roover et al. 2005). In short, COCs were matured in TCM 199 (Sigma) supplemented with 10% oestrous cow serum (ECS; home-made and tested for embryo production), 0.5 mg/l FSH (FMV Ulg, Liège, Belgium), 5 mg/l (FMV Ulg), 3μg/ml pyruvate (P-3662, Sigma) and 10 μg/ml penicillin-streptomycin (Gibco 15140-148). Oocyte maturation took place in 1 ml of medium for 24 h in an atmosphere of 20% O_2 and 5% CO_2 at 39°C and a relative humidity of 100%. Matured oocytes were then fertilized in Tyrode supplemented with 6 g/l albumin (Roche, Brussels, Belgium), 1460 μl/l lactate (L-4263, Sigma), 220 μl/l pyruvate (P-3662, Sigma) and 2 mg/l heparin (H8514, Sigma). Oocytes were

fertilized in 100 μl drops (final volume) with Percoll-treated, frozen-thawed semen at an insemination dosage of 2×10^6 spermatozoa/ml for 18–20 h under the same culture conditions. Following fertilization, presumptive zygotes were freed of cumulus by vigorous pipetting, and of excessive sperm cells, and transferred to culture medium.

Embryo culture conditions

Two different culture media were used subsequently. The first (1999–2001) was a bovine oviduct epithelium co-culture (BOEC) in Ménézo B2 (50μl drops; Laboratoires CCD, Paris, France). The second (2001–2003) was a synthetic oviduct fluid (SOF) medium-based system (400 μl drops; Minitübe, Tiefenbach, Germany) without co-culture.

BOEC-Ménézo B2

Oviducts carrying a large CL were collected in a slaughterhouse the day before fertilization and transported to the laboratory on ice. Oviducts were washed in physiological serum, cleaned with alcohol (70%) and washed again. Tubal cells were recovered by squeezing the oviduct between two microscopic slides, after which they were washed five times with phosphate-buffered serum (PBS, Bio-Whittacker, Heidelberg, Germany), 10% Fetal Calf Serum (FCS; Sigma), 100 000 IU/l penicillin (Invitrogen, Merelbeke, Belgium), 100 mg/l kanamycine (Invitrogen) and 1 ml/l amphotericin (Invitrogen). Cells were subsequently put in culture for 24 h in 3 ml TCM 199 supplemented with 20% ECS, 100 000 IU/l penicillin (Invitrogen), 100 mg/l streptomycin (Invitrogen), 1 ml/l amphotericin (Invitrogen) and 30 mg/l sodium pyruvate (Sigma). The next day, cells were concentrated by sedimentation and 20 μl of the concentrated cell solution was added to 1 ml of Ménézo B2, amino acids (20 μl/ml BME, 10 μl/ml MEM; Sigma), 20% ECS, 100 000 IU penicillin (Invitrogen), 100 mg/l streptomycin (Invitrogen) and 30 mg/l Na-pyruvate (Sigma). The cell solution was used to prepare the culture drops (50 μl). The culture drops, covered with mineral oil, were used to culture zygotes starting the next day. On day 4 of culture, the cleaved embryos were put in new medium consisting of Ménézo B2 and cells. The number of embryos at the 58-cell stage was recorded. This *new* medium was prepared on the same day and in the same way as described above. During the first 4 days, the medium with cells but without zygotes was kept in an incubator. The culture medium was Ménézo B2 with 20% ECS and amino acids (20 μl/ml BME, 10 μl/ml MEM). The volume was 400 μl covered with 400 μl of mineral oil, in an atmosphere of 20% O_2 and 5% CO_2 at 39°C and 100% humidity. On day 7 of culture, embryos were graded using IETS criteria.

SOF-ECS

Zygotes were placed in 400 μl of SOF culture medium (Minitub®, Tiefenbach, Germany) covered with mineral oil (maximum 15 embryos/400 μl) and supplemented with 5% ECS and amino acids BME 20 μl/ml (Sigma B6766) and MEM 10 μl/ml (Sigma M7145). After 3 days of culture (day 4 pi), embryos were transferred to new medium, 400 μl SOF under mineral oil as described above, in an atmosphere of 5% O_2 and 5% CO_2 and 90% N_2 at 39°C and 100% humidity. On day 7 of culture, embryos were graded using IETS criteria.

Statistical analysis

ANOVA was used for comparisons of mean values. A chi-squared test was used for proportions. Values of $p < 0.05$ were considered statistically significant (STATISTICA 6.0, Statsoft Inc., North Melbourne, Germany).

Results

Table 1 gives an overall view of the generated databases with details of oocyte retrieval and embryo culture conditions. As different oocyte collection frequencies were used throughout the period studied, final comparisons were made at the level of the number of individual OPU sessions and of the outcome of the 2-week periods, as one of the puncture schemes consisted of only one puncture session every 2 weeks (see Table 2).

Table 3 compares subsequent results of two different *in vitro* embryo production systems. These were obtained after OPU and IVP of embryos during stimulated and non-stimulated sessions. Results of the non-stimulated sessions are expressed per session and recalculated per 2-week period, as a basis for comparison with the stimulated sessions, which were held only once every 2 weeks. Data in Table 3 show a higher RR

Table 1. Overview of generated databases with details of oocyte retrieval and embryo culture conditions over time

	OPU-IVF sessions between 1996 and 1999: non-stimulated	OPU-IVF sessions between 1999 and 2003: FSH stimulated
Number of donor animals (N)	81	112
Age of donors (years, minimum–maximum)	1.5–15	1.5–11
Number of OPU sessions (N)	1396	640
OPU frequency	2 sessions/week	1 session every 2 weeks
IVM conditions	TCM 199, FSH/LH, ECS	
IVF conditions	TALP, heparin, frozen-thawed semen	
IVC conditions	Ménézo B2, BOEC	Ménézo B2, BOEC (259 sessions) SOF, ECS (381 sessions)

OPU, Ovum Pick-up; BOEC, bovine oviduct epithelium co-culture; IVM, *in vitro* maturation; IVF, *in vitro* fertilization; IVC, *in vitro* culture; ECS, oestrous cow serum; SOF, synthetic oviduct fluid.

Table 2. Basis of comparison between databases expressed per individual OPU sessions and per 2-week periods

OPU-IVF sessions between 1996 and 1999: non-stimulated two sessions a week		OPU-IVF sessions between 1999 and 2003: FSH stimulated one sessions every 2 weeks
Expressed per session 1396 sessions	Expressed per 2-week periods 310 periods[a]	Expressed per session (per 2-week periods) 640 sessions (periods)

[a]In non-stimulated sessions, donors were punctured four times for each period of 2 weeks. However, 1396/4 does not correspond to 310 periods. This difference is caused by session numbers, which were not always multiples of 4. For example, if an animal was punctured 10 times (which equals $2 \times 4 + 2$ additional sessions), only 2×4 sessions were taken into account (corresponding to two periods of 2 weeks).
OPU, Ovum Pick-up; IVF, *in vitro* fertilization.

Table 3. OPU-IVF results following non-stimulated and stimulated OPU-IVF sessions

	Non-stimulated sessions (1996–1999)		Stimulated sessions (1999–2003)
	Per session (two sessions/week)	Calculated per 2-week period *	Per session = per 2-week period (one session every 2 weeks)
Animals	81	71	112
Number of sessions or periods	1396	310	640
Number of follicles	9968	8981	9531
Mean number of follicles	7.1 ± 3.9[a]	29 ± 11.6[b]	14.9 ± 9.9[c]
Number of oocytes	5713	5135	7536
Mean number of oocytes	4.1 ± 3.1[a]	17.0 ± 8.6[b]	11.8 ± 8.2[c]
Recovery rate (%)	57[a]	57[a]	79[b]
Number of embryos at day 7	997	927	2190
Mean number of embryos	0.7 ± 1.2[a]	3.0 ± 2.9[b]	3.4 ± 3.9[b]
Development (%)	17[a]	18[a]	29[b]

[a]Different superscripts amongst columns indicate statistically significant differences, $p < 0.05$ (ANOVA for comparison of mean values, chi-square for proportions).
*In those cases in which data in two full weeks (four sessions) were available.
OPU, Ovum Pick-up; IVF, *in vitro* fertilization.

Table 4. Results of OPU-IVF following non-stimulated sessions, expressed per session and calculated per 2-week period, and FSH stimulated sessions, expressed per session (held once every 2 weeks), for a subpopulation of the five best and the five worst producing animals

	OPU-IVF sessions between 1996 and 1999: non-stimulated		
	Expressed per session*	Expressed per period of 2 weeks* (=4 sessions)	OPU-IVF sessions between 1999 and 2003: FSH stimulated[†]
Best producing animals (n = 5)	n = 160	n = 34	n = 49
Follicles (mean ± SD)	10.6 ± 4.6[a]	42.9 ± 12.7[b]	24.9 ± 18.4[c]
Oocytes (mean ± SD)	6.1 ± 3.8[a]	24.9 ± 8.5[b]	21.7 ± 14.9[c]
Recovery rate (%)	58[a]	58[a]	87[b]
Embryos (mean ± SD)	1.6 ± 1.7[a]	6.9 ± 3.7[b]	6.4 ± 6.4[b]
Embryos (%)	26	28	29
Worst producing animals (n = 5)	n = 147	n = 32	n = 69
Follicles (mean ± SD)	4.5 ± 2.6[a]	18.5 ± 7.1[b]	9.4 ± 4.9[c]
Oocytes (mean ± SD)	1.9 ± 1.7[a]	7.7 ± 4.3[b]	6.7 ± 4.2[b]
Recovery rate (%)	42[a]	42[a]	71[b]
Embryos (mean ± SD)	0.2 ± 0.5[a]	0.9 ± 1.2[b]	1.7 ± 2[c]
Embryos (%)	11[a]	12[a]	25[b]

*In non-stimulated sessions, donors were punctured four times for each period of 2 weeks.
[†]In stimulated sessions, donors were punctured only once every 2 weeks.
[a,b,c]Different superscripts between columns indicate statistical significant differences (ANOVA for comparison of mean values, chi-square for proportions, $p < 0.05$).
OPU, Ovum Pick-up; IVF, *in vitro* fertilization.

for stimulated animals than non-stimulated animals, at 79% vs 57%, respectively. In addition, the oocytes of stimulated animals gave a higher percentage of blastocyst development. As no differences were found between developmental percentages with the B2 medium (259 sessions) and the SOF medium (381 sessions), these results were pooled.

Individual production results were tabulated in Table 4, which compares production data of the five best-producing donor animals with data of the five worst producers. The results were generated by donors that underwent at least 2 months of OPU, corresponding to 16 non-stimulated sessions (four periods of 2 weeks, with two sessions per week) or four stimulated sessions (one session every 2 weeks).

Discussion

The most important findings of this retrospective study can be summarized as follows: (i) expressed per session,

the RR, mean numbers of punctured follicles, COCs and embryos following ovarian stimulation are significantly higher than the mean production results obtained without ovarian stimulation; (ii) expressed per period, the RR without ovarian stimulation is lower than with ovarian stimulation; mean numbers of punctured follicles, COCs and embryos without ovarian stimulation are significantly higher than the mean production results obtained with ovarian stimulation; and the mean number of embryos without ovarian stimulation equals the mean number with ovarian stimulation; (iii) Although largely different in absolute numbers, production results show similar tendencies when individual data of good and bad producers are considered.

Several remarkable differences between the data sets on non-stimulated and stimulated OPU sessions can be noted. Multiple changes were introduced in the OPU-IVF system over the years, which all attributed to the improvement of the blastocyst production in this semi-commercial environment. First, the difference in RR (number of COC retrieved/number of follicles punctured, expressed as a percentage) between stimulated and non-stimulated sessions should be considered. Whereas others (Pieterse et al. 1991; Walton et al. 1993) have shown better RRs in non-stimulated animals, we repeatedly found better RRs during stimulated sessions than during non-stimulated sessions, with RRs of 79% and 57%, respectively. Although the increasing experience of the OPU team certainly contributed to higher RRs, the role of FSH stimulation cannot be excluded.

Secondly, FSH stimulation clearly resulted in an increase in blastocyst development rate, at 18% on average without stimulation, and 29% with FSH stimulation prior to OPU. The stimulation protocol used was similar to the one used by Sirard et al. (1999), who showed the importance of a coasting period to provide COCs in an ideal follicular environment, in which developmental competence could be acquired so as to improve blastocyst development rates. Finally, a new culture technique without co-culture was introduced and used for a large part of the stimulated OPU sessions. Commercial embryo production is usually complex because oocytes from different donors need to be cultured separately, often in small groups. When the co-culture system was abandoned, culture conditions became apparently less favourable for a lower number of oocytes (4 or less), resulting in fewer blastocysts as described by Ferry et al. (1994). In other words, if the co-culture method would be used throughout the whole study, the number of stimulated sessions producing zero embryos would probably be slightly lower. In this particular case, the increase in the number of COCs per OPU session after FSH stimulation has permitted the use of an embryo culture method without co-culture, as larger groups of oocytes can be cultured together. An additional advantage is the substantial improvement in embryo quality from a sanitary point of view, eliminating pathogen contamination due to the co-culture system.

Considering the increase in OPU experience and the changing culture conditions throughout the period studied, the most reliable parameter for comparison is probably the number of follicles, generated with or without stimulation. An OPU protocol without stimulation at a puncture rate of two times per week, and an OPU session with FSH stimulation once in 2 weeks, address two different follicle populations (Merton et al. 2003). While follicles increase in size after stimulation, it has been established that large follicles contain oocytes that are more capable of developing to the blastocyst stage than oocytes enclosed in smaller follicles (Lonergan et al. 1994; Machatkova et al. 2004). In terms of follicular waves, follicle puncture done only once every 2 weeks after FSH stimulation permits aspiration of follicles that are somewhat older and more atretic, especially due to the coasting period (interval between the last FSH injection and OPU). Such follicles provide COC with an ideal environment, in which developmental competence can be acquired (Sirard et al. 1999). Follicles punctured two times per week are smaller and belong to the growth phase of the developing wave.

The results of the study show that large differences can be observed in terms of the average number of follicles and oocytes at the individual animal level, both for non-stimulated and stimulated sessions. Some donors consistently produced a low amount of oocytes per session, when compared with others that did not show low-outcome sessions. Large variability in the reaction to FSH stimulation protocols amongst donors has already been reported in MOET and OPU programmes in adult (Hasler et al. 1983; Lerner et al. 1986) and pre-pubertal bovine donors (Taneja et al. 2000). This is probably caused by several environmental factors such as heat and level of nutrition, as well as animal factors such as the stage of the oestrous cycle, donor age differences, genetics (Ptak et al. 2003), number of FSH-responsive follicles present in the ovary at the initiation of superovulatory treatment (Monniaux et al. 1983) and size of the primordial and tertiary follicle pool (Cushman et al. 1999). Animals showing a lower superovulatory response had fewer primordial and tertiary follicles.

Production characteristics, as well as cost efficiency, determine the feasibility of embryo production technologies in commercial breeding programmes. OPU-IVF following FSH stimulation, at a puncture rate of one session every 2 weeks, implies an important reduction in workload, with less OPU sessions and lower IVM, IVF and embryo culture work and costs. Results are satisfactory for most of the animals when this OPU protocol is used. Based on the presented results, more embryos can be obtained with increased OPU frequency and without stimulation, on the condition that the RR improves. However, this also implies that the embryo culture method needs to be adapted accordingly, as our current method does not yield satisfactory results for group culture of low numbers of COCs. On the other hand, reducing the number of steps in manipulating oocytes and embryos, simplifying labour-consuming methods and increasing the number of oocyte batches per production step are some issues that may lead to reduced production costs per embryo. Whether the number of oocytes per time period or per session has to be increased is an open question. Often, a balanced choice needs to be made. On the one hand, if the

objective was to produce embryos from high valuable problem donors, stimulation may have to be abandoned in favour of non-stimulated OPU at a frequency of two times per week. On the other hand, if the objective was to produce a high number of embryos with minimal effort and costs, OPU following FSH stimulation may be a good approach, although this does not apply to all animals. In addition, some animals will need a customized stimulation protocol as reported earlier (De Roover et al. 2005).

Local Belgian cattle breeding conditions should certainly be considered. Hardly any farmer has a large herd of recipient animals or labour force. Synchronizing recipients, observing heat, culturing, processing and transferring embryos two times per week, in comparison with once every 2 weeks, makes a big difference in economic terms. Therefore, the use of the FSH stimulation protocol increases efficiency, both for the cattle owner and the ET practitioner, and most probably leads to reduced costs.

In conclusion, findings of this retrospective study indicate that embryo production in non-stimulated OPU sessions, recalculated per 2-week period, is lower than that in one stimulated OPU session every 2 weeks. Whereas the average follicle and COC production per 2-week period are lower in stimulated than in non-stimulated sessions, the percentage of blastocyst development is higher per stimulated session. FSH stimulation prior to OPU can be a good approach for *in vitro* embryo production on the condition that animals react to the stimulation.

Acknowledgement

The authors thank Fiona de Ocampo and Tim Johnson for editing and carefully reading the manuscript.

References

Blondin P, Sirard MA, 1995: Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Mol Reprod Dev **41,** 54–62.

Bols PEJ, Van Soom A, Ysebaert MT, Vandenheede JMM, de Kruif A, 1996: Effects of aspiration vacuum and needle diameter on cumulus oocyte complex morphology and developmental capacity of bovine oocytes. Theriogenology **45,** 1001–1014.

Bols PEJ, Leroy JLMR, Vanholder T, Van Soom A, 2004: A comparison of a mechanical sector and a linear array transducer for ultrasound-guided transvaginal oocyte retrieval (OPU) in the cow. Theriogenology **62,** 906–914.

Bousquet D, Milovanov C, Bell JC, Durocher J, Smith LC, 1995: Nuclear and cytoplasmic maturation of oocytes aspirated from large follicles in superovulated heifers. Theriogenology **43,** 172.

Cushman RA, DeSouza JC, Hedgpeth VS, Britt JH, 1999: Superovulatory response of one ovary is related to the micro- and macroscopic population of follicles in the contralateral ovary of the cow. Biol Reprod **60,** 349–354.

De Roover R, Genicot G, Leonard S, Bols P, Dessy F, 2005: Ovum pick up and in vitro embryo production in cows superstimulated with an individually adapted superstimulation protocol. Anim Reprod Sci **86,** 13–25.

Ferry L, Mermillod P, Massip A, Dessy F, 1994: Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage. Theriogenology **42,** 445–453.

Galli C, Crotti G, Notari C, Turini P, Duchi R, Lazzari G, 2001: Embryoproduction by ovum pick-up from live donors. Theriogenology **55,** 1341–1357.

Goodhand KL, Staines ME, Hutchinson JSM, Broadbent PJ, 2000: In vivo oocyte recovery and in vitro embryo production from bovine oocyte donors treated with progestagen, oestradiol and FSH. Anim Reprod Sci **63,** 145–158.

Hashimoto S, Takakura R, Minami N, Yamada M, 1999: Ultrasound-guided follicle aspiration: effect of the frequency of a linear transvaginal probe on the collection of bovine oocytes. Theriogenology **52,** 131–138.

Hasler JF, 1998: The current status of oocyte recovery, in vitro embryo production, and embryo transfer in domestic animals, with an emphasis on the bovine. J Anim Sci **76** (Suppl. 3)**,** 52–74.

Hasler JF, McCauley AD, Schermerhorn EC, Foote RH, 1983: Superovulatory responses of Holstein cows. Theriogenology **19,** 83–99.

Kruip ThAM, Boni R, Wurth YA, Roelofsen MWM, Pieterse MC, 1994: Potential use of ovum pick-up for embryo production and breeding in cattle. Theriogenology **42,** 675–684.

Lerner SP, Thayne WV, Baker RD, Hensche T, Meredith S, Inskeep EK, Dailey RA, Lewis PE, Butcher RL, 1986: Age, dose of FSH and other factors affecting superovulation in Holstein cows. J Anim Sci **63,** 176–183.

Lonergan P, Monaghan P, Rizos D, Boland MP, Gordon I, 1994: Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization and culture in vitro. Mol Reprod Dev **37,** 48–53.

Looney CR, Lindsey BR, Gonseth CL, Johnson DL, 1994: Commercial aspects of oocyte retrieval and in vitro fertilization (IVF) for embryo production in problem cows. Theriogenology **41,** 67–72.

de Loos FAM, Bevers MM, Dieleman SJ, Kruip ThAM, 1991: Morphology of preovulatory bovine follicles as related to oocyte maturation. Theriogenology **35,** 527–535.

Lu KH, Shi DS, Jiang HS, Goulding D, Boland MP, Roche JF, 1991: Comparison of the developmental capacity of bovine oocytes from superovulated and non stimulated heifers. Theriogenology **35,** 234.

Machatkova M, Krausova K, Jokesova E, Tomanek M, 2004: Developmental competence of bovine oocytes: effects of follicle size and the phase of follicular wave on in vitro embryo production. Theriogenology **61,** 329–335.

Meintjens M, Bellow MS, Broussard JR, Paul JB, Godke RA, 1995: Transvaginal aspiration of oocytes from hormone-treated pregnant beef cattle for in vitro fertilization. J Anim Sci **73,** 967–974.

Merton JS, de Roos APW, Mullaart E, de Ruigh L, Kaal L, Vos PLAM, Dieleman SJ, 2003: Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology **59,** 651–674.

Monniaux D, Chupin D, Saumande J, 1983: Superovulatory responses of cattle. Theriogenology **19,** 55–81.

Paul JB, Looney CR, Lindsay BR, Godke RA, 1995: Gonadotropin stimulation of cattle donors at estrus for transvaginal oocyte collection. Theriogenology **43,** 294.

Pieterse MC, Kappen KA, Kruip ThAM, Taverne MAM, 1988: Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. Theriogenology **30,** 751–762.

Pieterse MC, Vos PLAM, Kruip ThAM, Wurth YA, Van Beneden ThH, Willemse AH, Taverne MAM, 1991: Transvaginal ultrasound guided follicular aspiration of bovine oocytes. Theriogenology **35,** 857–862.

Pieterse MC, Vos PLAM, Kruip ThAM, Wurth YA, Van Beneden ThH, Willemse AH, Taverne MAM, 1992: Repeated transvaginal ultrasound-guided ovum pick-up in ECG-treated cows. Theriogenology **37,** 273.

Ptak G, Tischner M, Bernabò N, Loi P, 2003: Donor-dependent developmental competence of oocytes from lambs subjected to repeated hormonal stimulation. Biol Reprod **69,** 278–285.

Seneda MM, Esper CR, Garcia JM, Andrade ER, Binelli M, Oliveira JA, Nascimento AB, 2003: Efficacy of linear and convex transducers for ultrasound-guided transvaginal follicle aspiration. Theriogenology **59,** 1435–1440.

Sirard MA, Picard L, Dery M, Coenen K, Blondin P, 1999: The time interval between FSH administration and ovarian aspiration influences the development of cattle oocytes. Theriogenology **51,** 699–708.

Stubbings RB, Walton JS, 1995: Effect of ultrasonically-guided follicle aspiration on estrous cycle and follicular dynamics in Holstein cows. Theriogenology **43,** 705–712.

Taneja M, Bols PEJ, Van de Velde A, Ju JC, Schreiber D, Tripp MW, Levine H, Echelard Y, Riesen J, Yang X, 2000: Developmental competence of juvenile calf oocytes in vitro and in vivo: influence of donor animal variation and repeated gonadotropin stimulation. Biol Reprod **62,** 206–213.

Van Soom A, Bols PEJ, de Kruif A, 1995: Improved results in IVF-treatment of sterility patients by careful selection of bulls and by stimulation of cows with FSH before slaughter (28. TAGUNG PHYSIOLOGIE UND PATHOLOGIE DER FORTPFLANZUNG). Reprod Dom Anim **29** (Suppl. 3)**,** 1–16.

Walton JS, Christie KA, Stubbings RB, 1993: Evaluation of frequency of ultrasonically guided follicle aspiration on bovine ovarian dynamics. Theriogenology **39,** 336.

Ward FA, Lonergan P, Enright BP, Boland MP, 2000: Factors affecting recovery and quality of oocytes for bovine embryo production in vitro using ovum pick-up technology. Theriogenology **54,** 433–446.

Submitted: 19.02.2007

Author's address (for correspondence): R De Roover, Faculté de Médecine Vétérinaire, Service d'Obstétrique et de Pathologie de la Reproduction des ruminants, équidés et porcs, Université de Liège, B42 Sart Tilman, B-4000 Liège, Belgium. E-mail: rderoover@msn.com