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Characterisation of low, medium and high responders following FSH stimulation prior to ultrasound-guided transvaginal oocyte retrieval in cows

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Abstract

In human IVF, the concept of 'low responders' is well known and generally defined as women with poor-response to gonadotrophin stimulation in a previous induction cycle. The objective of this retrospective study is to describe and characterise the concepts of 'low-, medium-, and high-response' and 'low, medium, and high responders' in bovine-assisted reproduction by analysing the OPU–IVF results obtained following 665 gonadotrophin-stimulated sessions conducted in 112 animals, nearly all of which were previously unsuccessful in traditional multiple ovulation and embryo transfer (MOET) programs.

They were submitted to OPU and IVP between 1999 and 2003. In reference to these 665 OPU sessions, categories of response were defined based on the overall mean ± S.D. follicles aspirated and COC obtained i.e., for follicles 14.7 ± 9.8 and for COCs 11.7 ± 8.1. So arbitrary cut-off values to define the categories of sessions were for follicles 5 and 25, and for COC 4 and 20. The three categories for follicles punctured in one session were therefore follicle low-response (FLR) ≤ 5 follicles, follicle medium-response (FMR) 6–24 follicles or follicle high-response (FHR) ≥ 25 follicles and for COCs recovered in one session COC low-response (CLR) ≤ 4 COC, COC medium-response (CMR) 5–19 COC or COC high-response (CHR) ≥ 20 COC. In addition, four

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categories of animals were also defined: (1) a low responder animal (LRA) had at least one OPU session in which FLR and CLR were observed (genuine low-response, see Section 3); these animals did not have any high-response sessions, (2) a medium responder animal (MeRA) had only medium-responses, (3) a high responder animal (HRA) had at least one OPU session in which FHR and CHR were observed; these animals did not have any low-response sessions, and (4) mixed responder animals (MiRA) had both low and high-responses. Finally, we distinguished biological (animals) and technical (recovery rate and ultrasound resolution) causes of response differences.

In 'low, high, medium and mixed reponders,' different results were obtained ($p < 0.05$): mean follicle numbers (8.8 ± 4.8^{a}, 22.4 ± 10.5^{c}, 13.2 ± 5.2^{b},15.1 ± 10.2^{d}), COC numbers (6.3 ± 3.9^{a}, 18.5 ± 8.2^{c}, 10.4 ± 4^{b}, 12.0 ± 8.3^{d}), embryo numbers (1.8 ± 2.1^{a}, 5.6 ± 4.9^{c}, 2.5 ± 2.7^{b}, 3.5 ± 3.8^{d}) and also for recovery rate (72%[a], 83%[b],79%, 79%) and percentage embryo development (29%, 30%[a], 24%[b], 29%).

In conclusion, the results of this study demonstrate that variability in OPU results has technical (ultrasound resolution and recovery rate) as well as biological (animal) aspects. Selection of animals with extreme (high or low) follicle and COC production results allows us to distinguish three populations: 'low, medium, and high responders' to FSH stimulation.

Keywords: Ovum pick-up; Assisted reproduction; Cow; FSH; 'Low responders'; 'Medium responders'; 'High responders'

1. Introduction

There are multiple constraints on the implementation of advanced assisted reproductive technologies in the dairy and beef industry. One of the most obvious is the inability to achieve a high and consistent superstimulatory response following hormonal treatment in cattle [1–6]. One of the possible explanations for low and inconsistent embryo yields is the small number of follicles that entered the growing pool of follicles. Cushman et al. [7] distinguished three populations of superovulated animals: 'low, medium, and high responders'. They were able to link these animal populations to the preantral pool of follicles. Animals with fewer preantral follicles had a lower response to gonadotrophin treatment than those with a larger reserve of preantral follicles.

Wide ranges in superstimulatory response have been observed in multiple ovulation and embryo transfer (MOET) as well as OPU [8,9]. Methods to control follicular wave dynamics, such as dominant follicle ablation or treatment of animals with oestradiol and progesterone, have reduced the variability [8] resulting from the puncture of cows at different stages of follicular development. At the same time, such methods have improved response by taking advantage of endogenous recruitment and selection mechanisms [8]. Whereas superovulation used in MOET programs maximise the number of ovulations, stimulation treatments prior to OPU aims at increasing the number of follicles suited for puncture [9]. However, high variable results are obtained with regard to the number of follicles punctured and oocytes retrieved in both stimulated and non-stimulated animals [10–12].

In human IVF, the concept of 'low responders' is well known and generally defined as women with poor-response to gonadotrophin stimulation in a previous induction cycle.

First described in 1981 by Garcia et al. [13], it is now a common clinical problem in IVF practice, affecting up to a quarter of the cycles started (reviews [14,15]). Despite the widespread use of such terminology as 'poor-response to gonadotrophin stimulation,' no standard definition of this condition exists (reviews [14,15]). In general, 'poor-response' implies a failure to achieve a certain number of follicles or a particular serum oestradiol level. 'Poor' and 'good' responders have been found to differ in age, the time necessary for follicles to achieve maturity, the numbers of COCs collected, the fertilisation rate, the rate of cell division, LH receptor expression on the granulosa cells and early menopause [16]. Conversely, no relation has been found with FSH receptor expression on granulosa cells [17,18]. It has also been found that low-response to gonadotrophin treatment is associated with accelerated luteinisation of mature follicles [19]. Numerous factors can influence the occurence of a 'low-response' such as advancing age, reduced total ovarian mass, environment, or specific conditions, such as ovarian cysts. Other factors concern individual differences in drug pharmacokinetics or bioactivity between different batches of stimulating hormones. In many cases, however, suboptimal or low-responses remain unexplained, reflecting the complexity of the regulation of ovarian function [14].

Diagnosis of poor-response in human-assisted reproduction can be made maintaining an IVF cycle and achieving a poor stimulation outcome. The problem can be also determined through ovarian reserve testing, including basal FSH levels, a clomiphene challenge test, and inhibin B concentration measurement [14,15]. In addition, ultrasonographic assessment of the ovarian function and determination of the resting or antral follicular populations allow the prediction of a poor-response to stimulation. However, it is difficult to conclude on the sole basis of a normal test result that the quantity and quality of the eggs produced will be good [14]. To the best of our knowledge, the usefulness of such a test has not been systematically investigated in cattle, although the problem of low responders to gonadotrophin treatment is reported in classical MOET programs [8].

The objective of this retrospective study is to describe and characterise the concepts of 'low, medium, and high-response' and 'low, medium, and high responders' in bovine-assisted reproduction by analysing the OPU–IVF results obtained following 665 gonadotrophin-stimulated OPU sessions conducted on 112 donor cows, all problem animals in MOET programs.

2. Materials and methods

Over a 4-year period (1999–2003), 112 cows were submitted to transvaginal oocyte retrieval (OPU) and in vitro embryo production (IVP). All work was done by one OPU team, one veterinarian and two technicians (one cow-side and one in the laboratory). The cows were fed a mixed ration consisting of hay and a commercial concentrate pellet, and housed in free stalls at the Faculty of Veterinary Medicine in Liège, Belgium. Ninety-nine percent of the animals were enrolled in the OPU–IVF program following disappointing results (no or very few transferable embryos were obtained) in classical MOET programs, with the hope of producing additional offspring by in vitro production (IVP).

The cows were enrolled in the following protocol. On day 8 before OPU (day 0), a norgestomet ear implant without the prescribed oestrogen injection (Crestar, Intervet,

Belgium) was inserted. Two days later (d-6), the dominant follicle was punctured (DFR: dominant follicle removal, follicle >8 mm). Starting on day 3, the cows received an FSH injection with 50 μg FSH/LH (Ovagen ICP BIO, Bodinco, The Netherlands, containing 17.6 mg NIADDK-O-FSH-17) in the morning and evening for 2 days in a row (8 a.m.–8 p.m., total of four injections). The animals were submitted to OPU 48 h following the last FSH injection (day 0). All visible follicles were aspirated. The norgestomet implant was removed the next day (day 1).

This treatment was repeated at 14-day intervals, e.g. with 14 day intervals between OPU sessions. Prior to OPU, the cows received epidural anaesthesia (5 cc procaine 2%) to prevent them from straining. Follicles were visualized using an ultrasound scanner, equipped with a 7.5 MHz mechanical sector transducer (Dynamic Imaging, Agritronics, Belgium) and mounted in a custom-made OPU handle. These were punctured and aspirated using 55 cm long, hypodermic, 18 g disposable needles (refer 304622, Becton Dickinson, Belgium) at a vacuum aspiration pressure corresponding to 15 mL water/min. During the procedure, the ovary was gently massaged while the needle was being inserted. The follicular fluid was collected, together with the oocytes, in a 50 mL plastic tube (BD352070, Becton-Dickinson, Belgium) filled with DPBS (Bio Whittacker, 04-479Q Belgium) containing 0.1‰ phenol red, 0.05‰ NaOH, 0.1‰ BSA-Fraction-V, and kept at 39 °C in a warm water bath. Oocytes were searched for using a binocular microscope.

All retrieved cumulus oocyte complexes (COCs) were subsequently submitted to routine [20] (in vitro maturation (IVM), fertilisation (IVF) and culture (IVC) techniques. COCs of individual cows were treated separately throughout the procedure. COCs were matured in TCM 199 (Sigma, Bornem, Belgium) with 10% estrous cow serum (ECS) (home-made and tested for embryo production), 0.5 mg/L FSH (Ulg FMV PhR, Liège, Belgium), 5 mg/L LH (Ulg FMV PhR, Liège, Belgium), 3 μg/mL pyruvate (P-3662, Sigma, Bornem, Belgium), and 10 μl/mL penicillin-streptomycin (Gibco 15140-148, Merelbeke, Belgium) added. Oocyte maturation took place in 1 mL of medium for 24 h in an environment with 20% O_2 and 5% CO_2, at 39 °C and a relative humidity of 100%. Matured oocytes were then fertilised in Tyrode supplemented with 6g/L albumine (Roche, Brussels, Belgium), 1460 μl/L lactate (L-4263, Sigma, Bornem, Belgium), 220 μl/L pyruvate (P-3662, Sigma, Bornem, Belgium) and 2 mg/L heparin (H-8514, Sigma, Bornem, Belgium). Oocytes were fertilised in 100 μl drops (final volume) with Percoll-treated, frozen-thawed semen at an insemination dosage of 2×10^6 spermatozoa/mL under the same culture conditions for 18–20 h. After fertilisation, presumptive zygotes were freed of cumulus (vigorous pipetting) and excessive sperm cells and put in culture. Two different culture media were used subsequently, the first (from 1999 to 2001, 259 OPU sessions) a bovine oviduct epithelium co-culture (BOEC) in Ménézo B2 (50 μl drops) (Laboratoire CCD, Paris, France), the second (from 2001 to 2002, 306 OPU sessions) an SOF-based medium system (400 μl) (Minitübe, Tiefenbach, Germany) without co-culture.

The total number of punctured follicles (all sizes), the total number of COCs retrieved, and the final total number of embryos produced were assessed for each OPU session. With reference to the 665 OPU sessions, categories of responses were defined based on the overall mean ± S.D. (mean per session) of the number of follicles obtained (all sizes) and the number of COCs retrieved i.e. for follicles 14.7 ± 9.8 and for COCs 11.7 ± 8.1. Low- and high-response cut-off values were 5 and 25 for follicles, and 4 and 20 for COC. The

Table 1
Definition of the different response categories (based on follicle and COC numbers) and animal categories (based on responses)

Follicle and COC numbers		
FLR	Follicle low-response	5 or less follicles punctured
FMR	Follicle medium-response	Between 6 and 24 follicles punctured
FHR	Follicle high-response	25 or more follicles punctured
CLR	COC low-response	4 or less COC collected
CMR	COC medium-response	Between 5 and 19 COC collected
CHR	COC high-response	20 or more COC collected
Donor animal categories		
LRA	Low responder animal	At least one session with FLR AND CLR
MeRA	Medium responder animal	Only medium-responses
HRA	High responder animal	At least one session with FHR AND CHR
MiRA	Mixed responder animal	Both high- and low-responses

four categories for follicles and COCs are as shown in Table 1; with these data four animal categories were defined (Table 1).

2.1. Statistical analysis

To compare means between groups (percentages were analysed after arcsin transformation), variances were tested: if variances were equal, a Student's *t*-test was used for comparison of means; if variances were different, a Welch test was used. The Pearson's correlation coefficient was used to evaluate correlations (Statistica 6.0).

3. Results

The overall results are derived from a database of 665 OPU sessions performed on 112 donor animals. The number of OPU sessions per donor varied between 1 and 25 (mean 5.9 ± 3.1). Overall, 9806 follicles were punctured (mean per session: 14.7 ± 9.8) resulting in a retrieval of 7764 COC obtained (mean per session: 11.7 ± 8.1), corresponding to a recovery rate of 79%. All obtained COC were matured and produced 2231 blastocysts (mean per session: 3.4 ± 3.8), resulting in an overall percentage of development of 29%.

3.1. Results per session

Results are shown in Table 2.

A high-response for both follicles (FHR) and COC (CHR) occurred in 9% of OPU sessions (59/665). Conversely, a low-response for both follicles (FLR) and COC (CLR) also occurred in 9% of OPU sessions (61/665).

A more detailed analysis within the low follicle (77 sessions) and COC (94 sessions) response data subset is presented in Table 3. Considering the cut-off values of five follicles and four COC, four categories can be distinguished.

Table 2
Occurrence of low (FLR), medium (FMR) and high (FHR) follicle response, and low (CLR), medium (CMR) and high (CHR) COC response

	FLR (follicle ≤ 5)	FMR (follicle > 5 and <25)	FHR (follicle ≥ 25)	*N*	%
CLR (COC $n \leq 4$)	61	33	0	94	14
CMR (COC > 4 and <20)	16	443	25	484	73
CHR (COC ≥ 20)	0	28	59	87	13
N	77	504	84	665	100
%	11.5	76	12.5		100

Table 3
Detailed analysis of the low-response sub-dataset: different categories and frequencies of occurrence of FLR and CLR

	FLR follicles, $n \leq 5$	FMR and FHR follicles, $n > 5$	Total
CLR (COC $n \leq 4$)	Genuine low-response, $n = 61$ (9%)	Recovery rate problem, $n = 33$ (5%)	94
CMR and CHR (COC > 4)	Observational problem, $n = 16$ (2%)	Medium or high-response, $n = 555$ (84%)	571
Total	77	588	665

Nine percent of the cases showed a low number of follicles and COCs (genuine low-response) while 5% was characterised by a medium or high number of follicles but a small number of COCs. In 2% of the cases, a low number of follicles but a medium or high number of COC was found.

Data analysis according to categories of animals is shown in Table 5.

Twenty-four animals (21%) out of 112 were responsible for at least one 'genuine LR' session and thus were categorized as 'low responders'. However, not all OPU sessions that were performed on these animals matched the LR definitions. These 'low responders' underwent 175 OPU sessions, corresponding to 26% of all OPU sessions (175/665), of which 35% (61/175) could be categorized as genuine LR, namely, that both the number of follicles punctured and the number of oocytes retrieved were too low ($\leq$cut-off values). This is 9% (61 out of 665 sessions) expressed relative to the total number of OPU sessions performed (Table 4).

Twenty-one animals (19%) out of 112 were responsible for at least one 'genuine HR' session and thus were categorized as 'high responders' (HRA). However, not all OPU sessions that were performed on these animals matched the HR definitions. These 'high responders' underwent 141 OPU sessions, corresponding to 21% of all OPU sessions (141/665), of which 42% (59/141) could be categorized as HR, namely, that the number of follicles punctured and the number of oocytes retrieved was high. This is 9% (59 out of 665 sessions) expressed relative to the total number of OPU sessions performed.

Twenty-nine animals (26%) showed neither high nor low-response, and thus were classified as 'medium responders' (MeRA).

Table 4
Comparison of OPU and embryo culture results of the four animal categories: low (LRA), medium (MeRA), mixed (MiRA), high (HRA) responders (total number and mean ± S.D., percentage)

	Animals	OPU	OPU/ animal	Follicles	COC	RR (%)	Blasto	Development (%)
LRA	24 21%	175 26%	7.3	1533 8.8 ± 4.8^{a}	1105 6.3 ± 3.9^{a}	73 ± 30^{a}	316 1.8 ± 2.1^{a}	29
MeRA	29 26%	86 13%	3.0	1137 13.2 ± 5.2^{b}	893 10.4 ± 4^{b}	$82 \pm 25^{b,d}$	212 2.5 ± 2.7^{b}	24^{a}
HRA	21 19%	141 21%	6.7	3162 22.4 ± 10.5^{c}	2611 18.5 ± 8.2^{c}	$86 \pm 22^{b,c}$	787 5.6 ± 4.9^{c}	30^{b}
MiRA	38 34%	263 40%	6.9	3975 15.1 ± 10.2^{d}	3155 12.0 ± 8.3^{d}	$82 \pm 31^{b,d}$	916 3.5 ± 3.8^{d}	29
Total	112 100%	665 100%	5.9	9806 14.7 ± 9.8	7764 11.7 ± 8.1	81 ± 29	2231 3.4 ± 3.8	29

Within a column, different superscripts: $p < 0.05$.

Thirty-eight animals (34%) showed a high-response and a low-response at least once, and thus were classified as 'mixed responders' (MiRA).

As we feared that the results of analysis of this database might have been affected by the unequal number of OPU sessions among the different animal categories, the same analysis was repeated on a subpopulation of animals, all of which underwent at least four OPU sessions (see Tables 5 and 6).

A high-response for both follicles (FHR) and COC (CHR) occurred in 7% of OPU sessions (17/252). Conversely, a low-response for both follicles (FLR) and COC (CLR) occurred in 17% of OPU sessions (43/252).

Finally, after analysis of the 'at least four OPU' subpopulation, we came to the same conclusions as after analysis of the entire population, namely, that the selection of animals with extreme (high or low) production results allows us to distinguish three animal populations: 'low, medium, and high responders'. Animals showing both high and low production results were classified in the 'mixed' category.

Of the 23 low responders, 21 were classified as low responders after one OPU session and all 23 after two sessions. Within the mixed responder class, two animals would have

Table 5
Occurrence of low (FLR), medium (FMR), and high (FHR) follicle responses, and low (CLR), medium (CMR), and high (CHR) COC responses in animals that were submitted to OPU at least four times

	FLR (follicle ≤ 5)	FMR (follicle > 5 and <25)	FHR (follicle ≥ 25)	*N*	%
CLR (COC $n \leq 4$)	43	21	0	64	25.9
CMR (COC > 4 and <20)	10	152	5	167	66.3
CHR (COC ≥ 20)	0	4	17	21	8.3
N	53	177	22	252	100
%	21	70	9	100	

Table 6
Comparison of OPU and embryo culture results of the four animal categories: low (LRA), medium (MeRA), mixed (MiRA), and high (HRA) responders (total number and mean ± S.D.) responses in animals that were submitted to OPU at least four times

	Animals	OPU	Follicles	COC	RR (%)	Blasto	Development (%)
LRA	23 (37%)	92 (37%)	564 6.1 ± 4.0^{a}	359 3.9 ± 3.1^{a}	74 ± 34^{a}	81 0.9 ± 1.5^{a}	23^{a}
MeRA	16 (25%)	64 (25%)	840 13.1 ± 4.4^{b}	663 10.4 ± 3.6^{b}	82 ± 26^{bd}	183 2.9 ± 3.1^{b}	28
HRA	8 (13%)	32 (13%)	780 24.4 ± 10.9^{c}	633 19.8 ± 6.6^{c}	87 ± 27^{bc}	174 5.4 ± 4.7^{c}	27
MiRA	16 (25%)	64 (25%)	834 13 ± 6.8^{b}	627 9.8 ± 4.9^{b}	81 ± 34^{bd}	187 2.9 ± 3.1^{b}	30^{b}
Total	63	252	3018 12 ± 8.3	2282 9.1 ± 6.6	77 ± 32	625 2.5 ± 3.2s	27

Within a column, different superscripts: $p < 0.05$.

been misclassified in the low responders whereas afterwards they appeared to adhere to the mixed responder group.

For the 8 high responders, more sessions were necessary: 4 of 8 high responder animals were identified after 2 sessions, 5 of 8 after 3 sessions and all 8 after 4 sessions.

Correlations were also calculated: first with all the data (63 animals, 4 × 63 sessions): for follicles punctured, between-sessions (session 1–2, 1–3, 1–4, 2–3, 2–4, and 3–4; 63 animals) correlations ranged from 0.54 (between punctured follicles in sessions 1 and 4) to 0.73 (between punctured follicles in sessions 1 and 2), and were all significant.

For COC collected, between-sessions (session 1–2, 1–3, 1–4, 2–3, 2–4, and 3–4; 63 animals) correlations ranged from 0.79 (between collected COC in sessions 1 and 4) to 0.93 (between collected COC in sessions 3 and 4), and were all significant.

Correlations between follicles punctured and COC (63 animals) collected were: for sessions 1–4: 0.84, 0.87, 0.84, and 0.86 (all significant).

Second, within response groups, correlations between punctured follicles and collected COC were as follows: HR group (8 animals, 32 sessions) 0.8, LR group (23 animals, 92 sessions) 0.74, MeR group (16 animals, 64 sessions) 0.66, MiR group (16 animals, 64 sessions) 0.78, overall (63 animals, 252 sessions) 0.88, (all significant).

Within response groups, correlations for follicles between-sessions (session 1–2, 1–3, 1–4, 2–3, 2–4, 3–4) were not always significant (HR group: 0.76 session 1–2; LR group: 0.42, session 1–2; MeR group: 0.50 session 2–3, 0.51 session 2–4, 0.6 session 3–4; MiR group: 0.57 session 1–4, 0.57 session 3–4).

Within response groups, correlations for COC between-sessions (sessions 1–2, 1–3, 1–4, 2–3, 2–4, 3–4) were not always significant (HR group: 0.71 session 1–3, 0.9 session 2–3, 0.75 session 3–4; LR group: 0.7 session 1–2, 0.66 session 2–3, 0.55 session 3–4; MeR group: 0.76 session 1–2, 0.64 session 1–3, 0.77 session 2–3, 0.66 session 3–4; MiR group: 0.63 session 1–2, 0.72 session 2–3, 0.61 session 2–4, 0.89 session 3–4).

4. Discussion

Optimization of oocyte production has been an important goal throughout OPU research during the past decade due to the constant need for more oocytes of good quality [21]. When highly valuable individual donor animals are used, the number of oocytes retrieved and the number of embryos cultured can be very low (in our case, under cut-off values). Oocyte recovery rates are influenced by many factors, which can be divided into biological and technical factors [23]. On the biological side, stimulation with FSH/LH can be used to increase the number of follicles and retrieved oocytes when used prior to OPU (for reviews [3,24]). However, as some animals show a 'low-response,' the application of FSH/LH stimulation is not always successful. In other words,ovarian stimulation does not constantly result in an increase in the number of follicles or retrieved oocytes.

We defined the 'low-response' status based on both follicle and COC numbers, in order to distinguish technical and biological cases of 'low-response'.

Ultrasound technology offers tremendous possibilities for the study of ovarian follicular development in vivo, but the method has its limitations ([25–27], our own observations). Counting follicles with a diameter of less than 6 mm, and specifically those with a diameter of 3 mm, by ultrasound visualization can be very inaccurate. This can clarify the fact that, in some cases, follicles are outnumbered by the amount of retrieved oocytes. Overall, the recovery rate in this study (expressed as the number of retrieved oocytes per 100 follicles punctured) was particularly good, although low recovery rates occasionally occurred. Both the ultrasound follicle observation and the low recovery rates can be regarded as purely technical problems and should be distinguished from the biological or genuine low-response problem, which is mainly determined by the donor animal itself.

The differences between the groups are three-fold: (1) recovery rates, (2) differences in percentage blastocyst development and (3) the number of follicles that appeared on the ovaries following superstimulation, which greatly influenced all other parameters and divided the population in distinct subpopulations. A poor-response session identifies a group of animals, which are less likely to respond well in subsequent stimulation attempts, as demonstrated in human reproduction [14]. On the other hand, a high-response session identifies a group of animals that will probably respond well in subsequent stimulation attempts. Indeed as we showed in the Section 3, one or a few OPU sessions are enough to classify animals as 'low or high responders'. These results confirm the results obtained by other authors (OPU, [22] and SOV [6]).

In human medicine [28], distinction is made between 'unexpected poor-responses' and 'expected poor-responses', with one LR session being indicative for further LR in later sessions whereas this was not the case for 'unexpected low-responses'. To be able to make such distinction in bovine OPU, more study is needed.

It has been documented that, among many other factors such as nutrition and physiological status [29], the stimulation protocol has a profound influence on the occurrence of low-response cases. The use of gonadotrophin-releasing hormone agonist (GnRHa) for the stimulation of human ovaries dramatically reduces the percentage of LR [30–32]. In addition, it has been reported in cows that systematic ablation of the dominant follicle also reduces the variability in superstimulatory responses [8]. Part of the solution to the problem of 'low responders' may be achieved by using alternative stimulation

treatments. Although it is tempting to conclude that the problem of poor ovarian response may be solved by simply increasing the dose of gonadotrophins used, it has been shown in human reproduction that this is not the case [14]. We found in our previous OPU experiments that an increased FSH dose influenced only the follicle diameters instead of the follicle numbers [33]. Nevertheless, an improvement of our current protocol could be a better timing of the start of the FSH treatment relative to the time of wave emergence, Nasser et al. [34] found a better result of a superovulatory treatment when FSH injections started at the time of wave emergence compared with the results of later treatments.

In the study of the LR problem in human reproduction, in which menopause has been linked to the exhaustion of the primordial follicle reserve [35], serious indications suggest that the pool of growing follicles should be considered. Pre-antral follicular growth seems to be largely independent of gonadotrophins, although there are indications that gonadotrophins may have a permissive function in pre-antral follicular development (for an extensive review [36]). It has been shown that, in rats, recruitment of follicles in the growing pool is influenced by the total size of the silent follicle pool [37]. After 2 days of in vitro culture of bovine ovarian cortical explants, Wandji et al. [38] demonstrated a sharp decline in the number of primordial follicles, in favour of an increase in the number of primary follicles. These findings suggest that the aetiology of LR is not only restricted to growing pool follicle dynamics but may also be associated with numbers of non-growing (preantral) follicles. In humans, 'low responders' tend to be older women with diminishing primordial follicle reserve. It is not clear at the moment whether or not this is the case with bovine LR animals. The presence of animals of the mixed category is intriguing therefore and merits further investigation.

All these factors can have a profound influence on the design of new stimulation protocols. Follicular development is a long process starting with primordial follicles, recruited in the growing pool of preantral follicles in a continuous manner. They then become FSH-dependent and are recruited in a cohort of antral follicles. The combination of stimulating the follicle growing pool (GH, long-term application) with down-regulation of the natural FSH concentrations by estrogens prior to stimulation treatment is one of the possibilities [39] for further research. Several authors also suggested taking ovarian tissue biopsy to evaluate the pool of preantral follicles [7,40]. Others have examined the possibility of selection of animals by ultrasound [6]. Still another possibility is to influence follicular development through nutrition. A review by Scaramuzzi and Murray [29] dealt with the long-term and short-term effects of nutrition on folliculogenesis. Long-term effects may involve stimulation of the earliest stages of folliculogenesis, in which activated follicles emerge from the primordial follicle pool; short-term nutritional effects on the other hand are likely to involve stimulation of the terminal phase of follicle growth, probably by the paracrine and autocrine processes involved in follicle selection and atresia.

In conclusion, the results of this study demonstrate that variability in OPU results has technical (ultrasound resolution, recovery rate) as well as biological (animal) aspects. Selection of animals with extreme (high or low) follicle and COC production results allows us to distinguish three populations: 'low, medium, and high responders' to FSH stimulation. Further research will be conducted on the differences between 'high and low responders'.

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